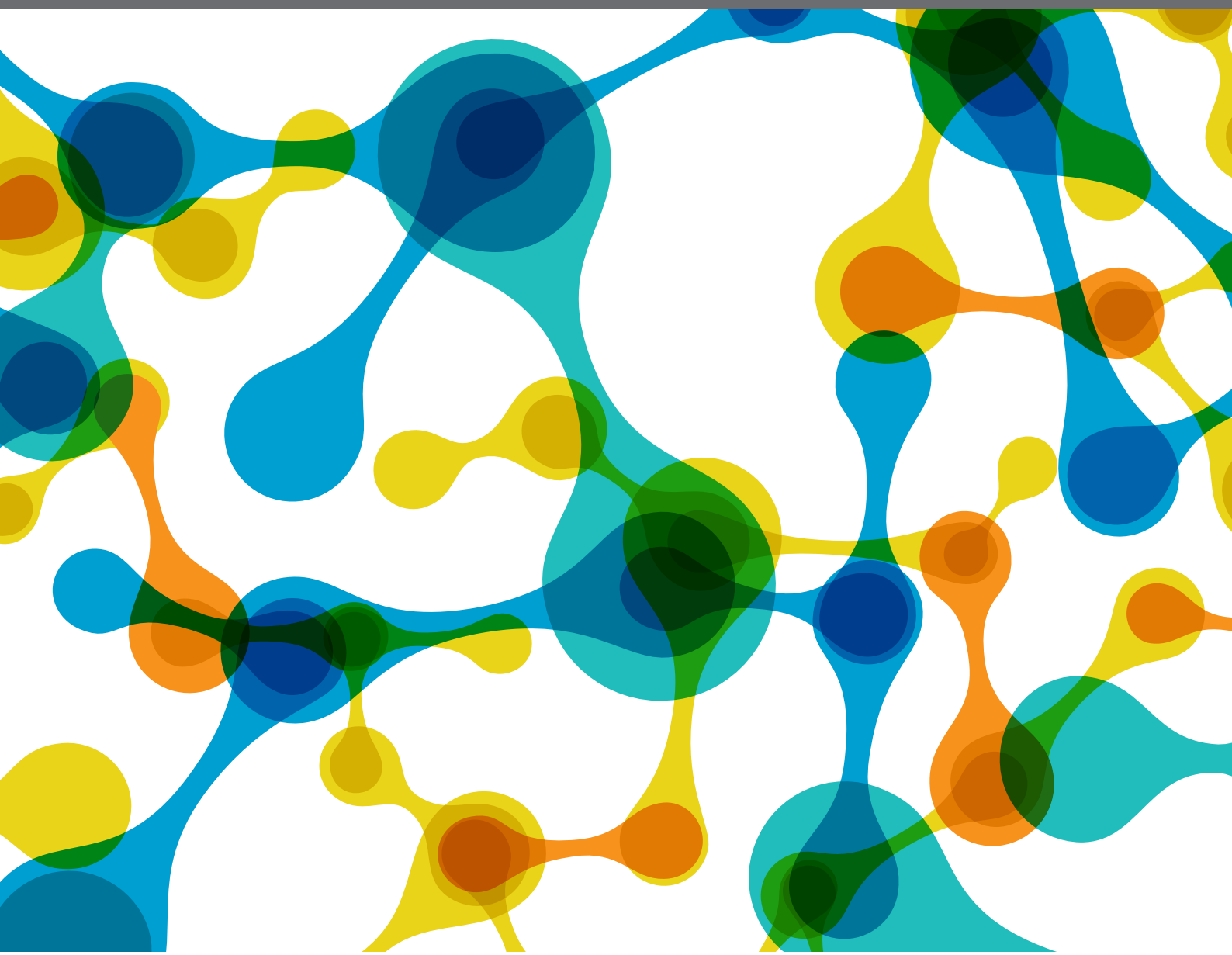
CEPHALOPOD RESEARCH ACROSS SCALES - MOLECULES TO ECOSYSTEMS, 2nd Edition

EDITED BY: Erica A. G. Vidal, Rui Rosa and Graziano Fiorito
PUBLISHED IN: Frontiers in Physiology



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ISSN 1664-8714
ISBN 978-2-88971-978-5
DOI 10.3389/978-2-88971-978-5

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CEPHALOPOD RESEARCH ACROSS SCALES - MOLECULES TO ECOSYSTEMS,
2nd Edition

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Publisher's note: In this 2nd edition, the following article has been added: Vidal EAG, Rosa R and Fiorito G (2021) Editorial: Cephalopod Research Across Scales - Molecules to Ecosystems. *Front. Physiol.* 12:752075. doi: 10.3389/fphys.2021.752075

Citation: Vidal, E. A. G., Rosa, R., Fiorito, G., eds. (2021). Cephalopod Research Across Scales - Molecules to Ecosystems, 2nd Edition. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-978-5

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frontiers in Physiology

EDITORIAL
published: 04 October 2021
doi: 10.3389/fphys.2021.752075

Editorial: Cephalopod Research Across Scales - Molecules to Ecosystems

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Keywords: behavior, cephalopods, muscle physiology and contractility, ocean acidification, paralarvae, reproductive tactics, sexual selection, tentacles

Editorial on the Research Topic

Cephalopod Research Across Scales - Molecules to Ecosystems

This Research Topic aims to draw a picture on recent advances in cephalopod research inspired by selected papers from the Cephalopod International Advisory Council Conference—CIAC 2018 held in Saint Petersburg, Florida, USA, during 12–16 November, 2018, but also includes papers on topics encompassing the theme of the conference, "*Cephalopod Research Across Scales - Molecules to Ecosystems.*" Along with this Research Topic there are two other companion special issues in Bulletin of Marine Science (Judkins et al., 2020) and in Fisheries Research (González and Pierce, 2021) that include papers from the CIAC 2018 Conference. The conference was preceded by five workshops lasting over two days (10 and 11 November) on themes of particular interest by the scientific community, as a tradition during CIAC conferences and a brief overview of the goals and outcomes of the workshops are also introduced here. Other accounts of the CIAC 2018 Conference and workshops can be found at the CIAC web site (https://cephalopod.wordpress.com).

Overall, this Research Topic consists of 12 contributions. Nine of the papers are primary research articles, one of them is a hypothesis theory driven paper and, there are two review articles. The overarching topics that are being addressed by the contributions in this Research Topic reflect several key issues that provide a comprehensive and integrative view on cephalopod physiology and related subthemes, including sexual selection, behavior, climate change, feeding and metabolism, and welfare.

Intra-sexual selection includes processes such as sperm competition and cryptic female choice, and one of the consequences of intra-sexual selection is that male reproductive traits tend to evolve and diverge at high rates. Ibáñez et al. tested this hypothesis by studying the evolution and diversification of several reproductive traits (e.g., hectocotylized arm length, ligula, and spermatophore lengths) across benthic octopuses (including 87 species), employing phylogenetic analytical methods. The results point out that male reproductive traits have evolved in a correlated way with body size and exhibited accelerated rates of evolution (at least in several Antarctic and deep-water lineages) presumably due to sexual selection. Morse and Huffard presented a riveted and comprehensive review summarizing the current knowledge of reproductive behavior within the Cephalopoda. The authors have combined existing information on pre- and post-copulatory behaviors to build novel insights, relating to cephalopod mate choice, sensory ecology, and the proliferation of polyandry among the class. They have concluded that sperm competition and possibly cryptic female choice are likely to be critical determinants of which individuals' alleles get transferred to subsequent generations in cephalopod mating systems. Gaps within the current

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Edited and reviewed by:
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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *02 August 2021*
Accepted: *20 August 2021*
Published: *04 October 2021*

Citation:
Vidal EAG, Rosa R and Fiorito G (2021) Editorial: Cephalopod Research Across Scales - Molecules to Ecosystems. Front. Physiol. 12:752075. doi: 10.3389/fphys.2021.752075

knowledge of how sexual selection operates are also uncovered and highlighted, valuably indicating areas of interest for new research on the elaborated mating systems of cephalopods.

Further exploring the sexual selection topic, but addressing the perspective of squids, Marian et al. provide a rich review on male alternative reproductive tactics in loliginids. The authors argue that loliginid squid provide unique models to explore sexual selection as they have two distinct fertilization environments in the female body that differ in aspects such as fertilization timing and success and, are associated with male alternative reproductive tactics, namely "sneaker" and "consort." Large consort males fight other males to gain access to females and deposit spermatophores within the female mantle cavity, while small sneaker males, engage in furtive mating and deposit spermatophores near the female buccal seminal receptacle. These results lead to the conclusion that several ejaculate traits (e.g., spermatophore and sperm morphology and functioning) have unique features that may have evolved in response to the fertilization environment faced by each temporary or permanent male morph.

Apostólico and Marian contribution deals with an unexplored sexual selection topic in cephalopods: the underlying mechanisms responsible for the expression of male alternative reproductive phenotypes. Based on observations made in captivity they described, first-time, males of the loliginid squid *Dorytheuthis pleii* of intermediate size and age displaying behaviors of both sneakers and consorts males simultaneously, leading to the hypothesis of them being a transitional stage between these both phenotypes. The result corroborates the ontogenetic hypothesis, indicating that small young males adopt sneaker tactics to mate instead of competing with large consort males for females, but as they grow, they modify the morphology of their ejaculates and mating behavior, going through an "intermediate" stage, before becoming large consort males.

Evaluation of the ontogenetic underlying factors responsible for the expression of a particular behavior provide valuable insights into developmental constraints (Boletzky, 1997) and ultimately, the way behavioral adaptations have evolved. Vidal and Salvador documented the ontogeny of predatory behaviors in *Doryteuthis opalescens* looking closely at the specialized tentacular strike behavior of squids. Loliginid paralarvae do not capture prey as adults, they use progressive predatory behaviors in which the tentacles function as arms, because important morphological and structural features are not yet formed, namely, clubs, stalks, and muscle fibers. By examining the relationship between overall morphology and predatory behaviors, the authors were able to uncover the interconnected morphological and behavior traits that enabled squid to perform the tentacular strike behavior (i.e., clubs, stalks and arm crown development, and schooling behavior), leading to the conclusion that the expression of the tentacular strike involves different levels of development and thus represents a major developmental milestone.

Cephalopods are highly diverse and thrive in a wide range of marine habitats, from coastal waters to the deep-sea, reflecting their successful adaptations to distinct habitats and motivate the need to learn more about the interrelationships between their life-cycles and environmental conditions (Rodhouse et al., 2014; Schickele et al., 2021). One of the major threats to life in the oceans is ocean acidification (OA). Presently, there is an increasing evidence of detrimental OA effects on the behavioral ecology of certain marine taxa, including cephalopods (e.g., Gutowska et al., 2010). Of particular interest are the sensitivity of cephalopod early life stages—particularly embryos and paralarvae—to environmental stressors related to OA, and two of the papers in this Research Topic have addressed this issue. By studying the developmental and behavioral ecology (namely shelter-seeking, hunting, and response to a visual alarm cue) of the common cuttlefish, *Sepia officinalis* early life stages, Moura et al. argue that the degree of phenotypic plasticity shown by the early ontogenetic stages to OA is linked to: (i) the great adaptability of cuttlefish to highly dynamic coastal and estuarine zones, and (ii) the fact that the embryos already face harsh conditions (hypoxia and hypercapnia) inside their eggs during early development. Zakroff and Mooney report the results of their experimental study exposing *Doryteuthis pealeii* embryos and paralarvae to the combined effects of OA and warming. They found that when reared under severe, chronic acidification and warming these early stages show a range of responses from sensitive to resistant. For example, time to hatching, which increased with acidification, decreased drastically under warming and, further, removed delays caused by acidification driven by between clutch differences. Noticeably, their study strongly aligns with the theme of this Research Topic, showing how global processes (OA and warming) interact across biological (individual, egg capsule, and clutch) and temporal (across the breeding season) scales to affect squid development.

Abiotic conditions are known to affect processes of growth and sexual development, and biochemical composition of cephalopods. Pascual et al. showed significant changes in biochemical composition (e.g., protein, glucose, cholesterol, acylglycerides, glycogen) of the gonad, hepatopancreas and muscle of *Octopus maya* relative to sex and season. Such findings may serve as reference values for several physiological indicators in *O. maya*, useful for programs monitoring wild populations, as well as to design diets and management protocols to produce octopus under controlled conditions. Regarding cephalopod growth patterns, it is known that young juvenile *Sepia officinalis* can grow up to 12% body weight per day, but how the metabolic costs associated with such substantial growth rate impacts muscle performance is unknown. Lamarre et al. integrated several aspects of contractility, protein synthesis, and energy metabolism in mantle of juvenile cuttlefish to provide a comprehensive insight into these biochemical processes.

Considering that cephalopods high metabolism is fueled by a protein-based diet in a muscular body, muscle physiology studies (e.g., Kier, 2016) may bring into the light important facts to improve our understanding of the adaptability of cephalopods to environmental conditions. One major pathway involved in muscle metabolism is the evolutionary conserved mTOR-signaling cascade, which regulates cell growth and homeostasis in response to a wide range of factors. Maiole et al. tackle this topic by characterizing *Octopus vulgaris* mTOR functional domains through designing and testing an *in vitro* protocol of

resistance exercise and inducing fatigue in arm samples. They found a high level of homology with vertebrates' mTOR and revealed the activation of the mTORC1 pathway in the exercise paradigm, which suggests that mTORC1 activity can be used as a marker to assess cephalopod response to changes in physiological and ecological conditions and, thus improve their maintenance and welfare.

Cephalopod welfare has become a timely topic that attracts great interest recently (Fiorito et al., 2015) due to the increasing importance of cephalopods in scientific and commercial activities requiring a clear understanding of their physiological and behavioral responses to anesthetic agents. Roumbedakis et al., hit the target with a study that evaluated short and long-term effects of anesthesia using different agents (magnesium chloride, ethanol, and clove oil) and cold seawater (11 and 13°C) on growth and mortality of *Octopus maya* juveniles. Based on the adopted criteria, metabolic (oxygen consumption) and behavioral (prey capture) responses to anesthesia were evaluated. Exposure to all concentrations tested of magnesium chloride, cold seawater (13°C), and clove oil reduced or inhibited the incidence of attacks to prey after recovery from anesthesia. Only clove oil proved to have a long-term effect on growth. Based on the results, the authors propose that anesthesia can be suppressed during short-term handling (<180 s), when no pain, distress, or suffering are involved. But when handling requires an anesthetized animal, the use of ethanol (3.0%) or cold sea water (11°C) were recommended to improve octopus welfare.

Processes of vomiting or regurgitation have been described in several invertebrate marine species, which prompt consideration of whether cephalopods have also this capability. In a stimulating "hypothesis and theory" article, Sykes et al. provide several different rationales to support the idea that cephalopods can vomit. Among the different lines of thought, the authors describe anecdotal reports of regurgitation-like behavior in several species, including *Sepia officinalis*, *Sepioteuthis sepioidea*, *O. vulgaris*, and *Enteroctopus dofleini*.

In compiling this Research Topic, we hope to provide an overview toward timely, significant and innovative cephalopod research, while also framing areas for future work, usefully highlighted in several of the papers shared here, to encourage wide readership in this exciting field of research. Our special thanks go to the 56 authors, representing 13 countries and, also to the reviewers for their valuable assistance. Finally, we thank the chair of the CIAC 2018, Heather Judkins. CIAC conferences are the opportune and stimulating occasion for outstanding scientific content encouraged by a warm atmosphere! We look forward for the next CIAC conference that will be held during 2–8 April, 2022 in Sesimbra, Portugal, with the theme "*Cephalopods in the Anthropocene: multiple challenges in a changing ocean.*"

WORKSHOPS

The workshop "*Paralarval and juvenile cephalopods: an updated identification guide,*" led by Erica A. G. Vidal, Liz Shea, and Heather Judkins focused on disseminating knowledge on the identification of cephalopod early life stages and on gathering a team of researchers to compile existing taxonomic information to create an updated book for the identification of cephalopod early life stages. During the workshop, the 27 participants from 13 countries, adopted a "hands on" approach and worked in groups on identifying cephalopod early life stages, sharing expertise on particular families and creating an effective interaction atmosphere between students and experts in the field. The output from this workshop will be the book "*Cephalopod Early-life stages: An Identification Handbook*" that is being prepared to be published by Springer Nature in 2022.

The workshop "*The biogeochemical role of cephalopods in the world's oceans,*" led by Henk-Jan Hoving, aimed to gather the available knowledge on the role of cephalopods in the oceans' biogeochemistry and energy transfer (e.g., migration, consumption, respiration and excretion, terminal spawning) in marine food webs, with the ultimate goal to provide a comprehensive and integrative review paper on the topic.

The workshop "*Hard structures of cephalopods and their application in your field of study*" was led by Alexander Arkhipkin, Catalina Perales-Raya, and Fedor Lishchenko, with the main goal to have the most recent update on use of recording structures in studies of cephalopod taxonomy, age, growth, and population structure. The workshop was attended by 23 participants (scientists as well as students) from eight countries around the Globe. Theoretical sessions included general introduction on the recording structures of cephalopods, specific methodologies dedicated to processing of statoliths, cephalopod beaks, and gladii, as well as their shape analysis. Additionally, Kathleen Ritterbush gave a talk on the shell hydrodynamics of ammonoid cephalopods. Practical sessions took place with participants having first-hand experience in extraction of recording structures (statoliths, beaks, and stylets of octopuses) from cephalopod bodies, their sectioning and examination. At the final part of the workshop a broad discussion on the key knowledge gaps and promising fields for future studies took place. The most important subjects for future studies included (a) Terminology-related issues; (b) Physiology of increment deposition; (c) Problems with aging accuracy and precision; (d) Validation methods and associated problems; (e) The best aging methods for each group of species; (f) Species in which hard structures provide reliable tools for age estimation and, (g) Species for future research.

The workshop: "*Genetic Tools and Live Imaging in Cephalopods*" led by Eric Edsinger had a hands on approach with computer software interaction to cover several practical issues on implementing genetic and live imaging tools for cephalopods, namely: (1) Identifying genes of interest in annotated genomes, (2) Designing transgenic reporters, biosensors, and CRISPR-Cas9 guide RNAs, (3) Injection of mRNA/constructs/CRISPR-Cas9, (4) Live imaging approaches for light-based genetic tools and, (5) Applications of genomic resources and genetic tools to cephalopod biology, emerging genetic models, among others future prospects. During the workshop, embryos, and hatchlings were produced and used for expressing injected mRNAs, transgenic constructs, or genome edited genes, allowing a practical, interactive and also first-hand experience among the participants.

The workshop "*Cephalopod Science: the direction of future research and the relevance of new policies*" led by Giovanna Ponte, Ian Gleadall, and Graziano Fiorito provide the ground for a brainstorming session to identify likely avenues for novel ground-breaking cephalopod research areas and their potential effects on and benefits to human society. A discussion on the changes in policy for experimentation and fisheries of cephalopods occurring in different regions of the world and the potential effects of these changes on cephalopod research in both global and local contexts were also addressed, with the aim to prepare a white paper to summarize and report on these topics.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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frontiers in Physiology

ORIGINAL RESEARCH
published: 26 July 2019
doi: 10.3389/fphys.2019.00975

Cuttlefish Early Development and Behavior Under Future High CO_2 Conditions

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The oceanic uptake of carbon dioxide (CO_2) is increasing and changing the seawater chemistry, a phenomenon known as ocean acidification (OA). Besides the expected physiological impairments, there is an increasing evidence of detrimental OA effects on the behavioral ecology of certain marine taxa, including cephalopods. Within this context, the main goal of this study was to investigate, for the first time, the OA effects (∼1000 μatm; ΔpH = 0.4) in the development and behavioral ecology (namely shelter-seeking, hunting and response to a visual alarm cue) of the common cuttlefish (*Sepia officinalis*) early life stages, throughout the entire embryogenesis until 20 days after hatching. There was no evidence that OA conditions compromised the cuttlefish embryogenesis – namely development time, hatching success, survival rate and biometric data (length, weight and Fulton's condition index) of newly hatched cuttlefish were similar between the normocapnic and hypercapnic treatments. The present findings also suggest a certain behavioral resilience of the cuttlefish hatchlings toward near-future OA conditions. Shelter-seeking, hunting and response to a visual alarm cue did not show significant differences between treatments. Thus, we argue that cuttlefishes' nekton-benthic (and active) lifestyle, their adaptability to highly dynamic coastal and estuarine zones, and the already harsh conditions (hypoxia and hypercapnia) inside their eggs provide a degree of phenotypic plasticity that may favor the odds of the recruits in a future acidified ocean. Nonetheless, the interacting effects of multiple stressors should be further addressed, to accurately predict the resilience of this ecologically and economically important species in the oceans of tomorrow.

Keywords: ocean acidification, cuttlefish, early life stages, embryogenesis, behavior

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *29 April 2019*
Accepted: *11 July 2019*
Published: *26 July 2019*

Citation:
Moura É, Pimentel M, Santos CP, Sampaio E, Pegado MR, Lopes VM and Rosa R (2019) Cuttlefish Early Development and Behavior Under Future High CO_2 Conditions. Front. Physiol. 10:975. doi: 10.3389/fphys.2019.00975

INTRODUCTION

Over the past centuries, atmospheric carbon dioxide (CO_2) concentration has been increasing, with a current value of ∼ 415 ppm (NOAA, 2019), being the highest registered in the past 800,000 years. However, due to human dependence on fossil fuels combustion, it is expected to continue rising until 950–1000 ppm (in the higher-emissions scenario – RCP8.5) by 2100 (Rhein et al., 2013).

Oceans absorb about 30% of the CO_2 present in the atmosphere. This natural process of CO_2 absorption is driven by the physico-chemical balance between the differences in partial pressure of CO_2 between the air and the sea surface (Ciais et al., 2013). However, when a higher amount of CO_2 reacts with seawater, it increases the formation of carbonic acid (H_2CO_3), increases the amount of bicarbonate ions (HCO_3^-) and reduces the availability of carbonate ions (CO_3^{2-}) (Rhein et al., 2013). These changes cause an increase in the production of hydrogen ions (H^+) and a subsequent reduction of seawater pH. When this process happens for an extended period it is known as ocean acidification (OA). As CO_2 levels continue to rise, forecasts indicate that, by the end of this century, the average ocean surface pH will be 0.2–0.4 pH units lower than present values (Rhein et al., 2013). Therefore, this pH drop may represent a serious threat to the health of the world's ocean ecosystems (Cubasch et al., 2013).

Calcifying organisms, as well as those with minimal physiological buffering capacities (e.g., calcareous sponges, corals and most echinoderms) (Knoll et al., 2007) are expected to be particularly affected by OA. On the other hand, higher resilience is expected from organisms equipped with a more powerful capability to maintain their homeostasis and to compensate for extra and intracellular pH perturbations, such as teleosts and cephalopods (Hu et al., 2015). Nonetheless, an increasing number of studies have been reporting a myriad of OA-related impacts over these mollusks (Rosa et al., 2013; Pimentel et al., 2015, 2016; Spady et al., 2018).

Several studies have already demonstrated, for certain marine taxa, including cephalopods, the detrimental consequences of OA at different behavioral levels, e.g., foraging (Dixson et al., 2015; Pimentel et al., 2016), hunting (Dixson et al., 2015; Pistevos et al., 2017; Spady et al., 2018), predation vulnerability (Dixson et al., 2010; Munday et al., 2014, 2016) and response to olfactory cues (Munday et al., 2009; Dixson et al., 2010). One of the mechanisms pointed for some behavioral disruptions when individuals are exposed to OA is the GABA-A receptor. The GABA-A receptor is the primary inhibitory neurotransmitter in the vertebrate and invertebrate's brain and, when connected with GABA (γ-aminobutyric acid) in OA conditions, suffers an outflow of Cl^- (chloride ions) and/or HCO_3^- from neurons, resulting in depolarization and excitation, i.e., abnormal behaviors (Nilsson et al., 2012; Tresguerres and Hamilton, 2017). However, more research is still needed to better understand how this is underpinned by disruption in brain functions.

Cephalopods are the most neural-developed invertebrates and are considered to have vertebrate-like cognition, underpinned by a well-developed brain, a complex nervous system and sophisticated sensory organs (Fiorito et al., 2015). All cephalopods' hatchlings have a close-to-optimal central nervous system and most of them are strikingly similar to adults, both in morphology and basic behaviors, such as signaling and camouflage (Boyle, 1987). Additionally, cephalopods present a high level of plasticity to environmental changes (Fiorito et al., 2015; Doubleday et al., 2016), with very effective regulatory and excretory systems to that allow them to tolerate high CO_2 concentrations over long exposure times (Hu et al., 2015). However, there are differences within the cephalopods' species, since active pelagic squids show higher sensitivity to elevated pCO_2 when compared with cuttlefish and octopods, which have a nekton-benthic and benthic lifestyles, respectively (Hu et al., 2014). Such differences suggest that different lifestyles and energetic limitations can be a key feature in the ability to mobilize energy resources to fuel acid-base compensatory processes (Hu et al., 2014).

The common cuttlefish (*Sepia officinalis*) is a nekton-benthic species (Reid et al., 2005; Guerra, 2006) with its major activity during the night, spending the day camouflaged on the sand to avoid predators (Boyle, 1987; Reid et al., 2005). Its well-developed and wide visual field (Mäthger et al., 2013) is highly important to its defensive (Chichery and Chanelet, 1976; Boyle, 1987; Reid et al., 2005) and predatory behaviors (Wells, 1958; Messenger, 1968), taking into account that these animals are active predators (Wells, 1958; Messenger, 1968). Besides this, this species has an active lifestyle with high metabolic rates, it is naturally exposed to hypercapnia during its embryonic development (Melzner et al., 2009) and has an elevated tolerance for different environments (Reid et al., 2005; Guerra, 2006).

Previous studies on the impact of OA on cuttlefish have mainly focused on impacts on the cuttlebone (Gutowska et al., 2008, 2010; Dorey et al., 2013; Sigwart et al., 2015), embryonic development (Lacoue-Labarthe et al., 2009; Hu et al., 2011; Dorey et al., 2013; Rosa et al., 2013; Sigwart et al., 2015) and development of newly hatched/juvenile individuals (Gutowska et al., 2008; Lacoue-Labarthe et al., 2009; Hu et al., 2011; Dorey et al., 2013; Sigwart et al., 2015). Regarding the cuttlebone mineralization process, all studies pointed out hypercalcification under high CO_2 levels (Gutowska et al., 2008; Dorey et al., 2013; Sigwart et al., 2015), but with an irregular $CaCO_3$ deposition (Gutowska et al., 2010). The other studies also suggest: (i) an increase in the frequency of premature hatching, (ii) an increase of pre-hatching critical partial pressure (P_{crit} – the point at which the rate of oxygen consumption was no longer maintained independently of ambient pO_2), (iii) a decrease in pre-hatching routine metabolic rate (Rosa et al., 2013), and (iv) decrease of perivitelline fluid pH (Hu et al., 2011). Studies assessing OA impacts on cephalopods' behavior have so far focused solely on squids (Spady et al., 2014, 2018), while the effects over other cephalopod groups, including cuttlefishes, remain largely unaddressed (Maneja et al., 2011).

In this context, the general objective of the present study was to evaluate, for the first time, a set of developmental and behavioral responses in early developmental stages, by exposing embryos and soon-after hatchlings to OA (ΔpH = 0.4, ~1000 μatm). To this end, some features as embryonic development time, hatching success, survival rate and biometrics (weight, length and Fulton's condition index) throughout the embryogenesis and 20 days after hatching (DAH) were analyzed. Moreover, an array of behavioral trials were performed in cuttlefish 15 DAH, to scrutinize the influence of acidification on critical behaviors for early life stages, namely: (1) shelter-seeking – to evaluate preference for a darker or lighter areas; (2) hunting behavior – to assess the amount of prey captured, reaction and successful catch time latency, and capture

effectiveness; (3) response to visual alarm cues (ink) – to evaluate the reaction to a visual conspecific-related stimulus (ink), usually used as an alarm cue.

MATERIALS AND METHODS

Exposure of Embryos and Hatchlings to Ocean Acidification

Sepia officinalis eggs, at stage I-VI [initial stage of embryogenesis, Naef (1928)] were hand collected during low tide in Mitrena area located in Sado estuary, on the west coast of Portugal (38°47′25.81″ N; 8°79′49.34″ W). The eggs were transported to Laboratório Marítimo da Guia (MARE-ULisboa) and immediately transferred to an isolated tank for an acclimatization period of 6 days. It is worth noting that the main feature of the Portuguese western coast is the occurrence of coastal upwelling in response to the intensification of northerly winds. Therefore, cuttlefish inhabiting this region are exposed to seasonal variability of seawater carbonate parameters due to the emergence of deepwater masses (Álvarez-Salgado et al., 1997; Borges and Frankignoulle, 2002), and such variability is observed in **Table 1**.

After the acclimatization period, the eggs were incubated under two pCO_2 treatments (3 replicates each treatment; $N = 85$ eggs per replicate) for ∼65 days (∼45 days during the embryonic development plus 20 days after hatching). The pCO_2 scenarios were chosen to reflect: (a) the annual present pH conditions (pCO_2 ∼ 400 ppm; pH = 8.1) and (b) the near-future expected pCO_2 (pCO_2 ∼1000 ppm; ΔpH = 0.4; based on RCP8.5 projections). The acclimation period took place in a total of six independent experimental life support systems (each tank with 22 L of volume) supplied by natural seawater filtered down to 0.35 μm and through UV radiation, under a semi-closed system. To further assure seawater quality, the experimental tanks were also equipped with mechanical (glass wool) and biological (bioballs matured with nitrifying bacteria) filtration. Temperature was set to 18°C, the average temperature of the spawning season of *S. officinalis* in the western coast of Portugal, and controlled by placing the experimental tanks in water baths connected to chillers (Hailea, Guangdong, China). Room illumination was provided through overhead fluorescent lighting (MASTER TL-D Super 80, 4000 K, 3350 lumen), under a photoperiod of 12 h light: 12 h dark.

During exposure, and on a daily basis, pH, salinity and temperature were monitored manually, as well as hatching and mortality rates. The total alkalinity (TA) was calculated weekly, using the absorbance of water samples measured with an UV spectrophotometer (UV-1800 spectrophotometer, Shimadzu, North America) (Sarazin et al., 1999). Seawater carbonate system speciation was also calculated weekly from salinity, temperature, TA, and pH (total scale) measurements, using CO2SYS software (Lewis et al., 1998) with dissociation constants from Mehrbach et al. (1973) as refitted by Dickson and Millero (1987) (see **Table 1**). pH was quantified manually with Metrohm pH meter (826 pH mobile, Metrohm, Filderstadt, Germany) connected to a glass electrode (±0.001; Schott IoLine, SI analytics, Mainz, Germany) and calibrated against the seawater buffers Tris–HCl (Tris) and 2-aminopyridine-HCl (AMP) (Mare, Liège, Belgium) according to Dickson et al. (2007). The experimental pH levels were adjusted automatically, via solenoid valves controlled by an automated system (Profilux 3, Kaiserslautern, Germany) connected to individual pH probes (Blueline 25 pH, SCHOTT Instruments, Mainz, Germany). Profilux pH hysteresis was set at ±0.05 margins (lower limit of the system), to minimize the degree of pCO_2 variation inherent to simulated hypercapnic treatments (as observed in **Table 1**) and the respective repercussions (see Jarrold and Munday, 2019 and references therein). The pH of natural seawater was reduced by the injection of a certified CO_2 gas mixture (Air Liquide, Miraflores, Algés, Portugal), via air stones, and balanced positively through aeration with CO_2-filtered air (using soda lime, Sigma-Aldrich, St. Louis, MO, United States). Salinity was measured using a refractometer (V2Refractometer, TMC, Iberia, Portugal) and maintained (∼35) by adding more seawater. Ammonia, nitrites and nitrates levels were monitored using colorimetric tests (Tropical Marine Centre, United Kingdom) and maintained below detectable levels, lower than 0.5, 0.2, and 80 mg/L (Fiorito et al., 2015), respectively. The cuttlefish hatchlings were fed *ad libitum* with frozen brine shrimp enriched with spirulina and the uneaten food was removed at the end of each day.

TABLE 1 | Seawater carbonate chemistry during the exposure of *Sepia officinalis* to different pH conditions (three replicates for each treatment).

Parameters	Control	Acidification
Temperature (°C)	17.8 ± 0.9	17.9 ± 0.8
Salinity	36 ± 1	36 ± 1
pH	8.05 ± 0.06	7.73 ± 0.07
TA (μmol/kgSW)	2584.7 ± 267.2	2522.0 ± 248.5
pCO_2 (μatm)	461.6 ± 85.1	1016.3 ± 180.7
TCO_2 (mmol/kgSW)	2316.7 ± 214.2	2406.4 ± 230.7
HCO_3^- (mmol/kgSW)	2101.1 ± 176.8	2266.9 ± 213.6

Values for pCO_2 were calculated weekly from salinity, temperature, pH total scale (pH) and total alkalinity (TA), using CO2SYS software (Lewis et al., 1998). Values are represented as mean ± standard deviation.

Hatchlings' Biometrics

Dorsal mantle length (DML), total body length (TBL) and total body weight (TBW) were measured in all individuals with 20 DAH. Both mantle and total length were measured through image analysis and the TBW was registered with an analytical scale. Fulton's Condition Index (K) was calculated according to Fiorito et al. (2015), as follows:

$$K = \frac{TBW}{DML^3} \times 100$$

These measurements were performed in two different groups, due to the difference in development time upon collection. Hatching and mortalities were monitored daily to further calculate development time, hatching success and survival rate. At the end of the experiment, individuals were anesthetized and euthanized, according to the Guidelines for the Care and Welfare

of Cephalopods in Research (Fiorito et al., 2015), and preserved for future biochemical analysis.

Behavioral Analysis

The behavioral tests performed were: (1) shelter-seeking, (2) hunting behavior and (3) visual detection of a conspecific-related stimulus. All tests were performed with hatchlings 15–20 DAH. Individuals participating in the first two behavioral tests were trialed in different days. Individual cuttlefish were carefully transferred from its holding tanks to the arena of each test and all arenas were designed according with the specific needs of each test. All performed tests, described below, were performed following a 10 min acclimatization period (Darmaillacq et al., 2004; O'Brien et al., 2016, 2017) and recorded with a Canon LEGRIA HF R56 camera. At the end of each test, individuals were returned to their holding tanks and testing arenas were cleaned between trials.

Shelter-Seeking

Shelter-seeking tests ran in a rectangular arena (area = 145 cm^2; volume = 4000 mL) with dark walls and a transparent bottom. Half of the arena was topped with a dark and opaque cover (14.5 cm Black + 14.5 cm Opaque), as to provide a fully shaded area, while a light and semi-translucid cover was placed over the other half as to produce an area uniformly illuminated by a diffuse light, placed circa 50 cm above the arena. In this arena, a neutral area (4 cm) was assigned and adjusted according to Jutfelt et al. (2017). Each cuttlefish was randomly and gently introduced through a small entryway in the middle of the upper zone. Following acclimatization period, activity was recorded during 15 min. using a camera placed underneath the arena. An individual was assumed to have a light or dark preference only if it left the neutral area and spent ≥70% of the total test time (≥630 s) in one of the chosen areas. If both these criteria were not met, the individual was assumed to have no preference for either light or dark conditions. The sides corresponding to the lighter and darker areas were switched randomly between trials to prevent lateralization-associated bias.

Hunting

An opaque arena (area = 80 cm^2; volume = 500 mL) with a small sand shelter, to minimize stress, was used to observe the hatchlings hunting behavior. A single random cuttlefish was gently placed in the arena and the test was performed for 10 min, after the acclimatization period and after the introduction of the prey. To each cuttlefish 5 prey items (*Gammarus* sp.) were introduced through a tube present at a corner of the tank and available to hunt during the test, since a preliminary test revealed that 5 was the maximum number of prey that one individual could consume within the test period. The attack latency time was accounted between the reaction to the prey and its catch (successful attack). A lamp was placed 30 cm above the arena to ensure enough and equally distributed lighting and the cuttlefish activity was recorded with a camera at the same high. A total of 28 cuttlefish and 140 prey were used for this test.

Visual Detection of Conspecific Visual Stimulus – Ink

The purpose of this test was to evaluate the response to the visual component of an ink stimulus, used as a defense mechanism and perceived as an alarm cue in cephalopod species (Gilly and Lucero, 1992; Boal and Golden, 1999; Bush and Robison, 2007; Wood et al., 2008; Mezrai et al., 2018). This was tested using a round glass arena (area = 100 cm^2, volume = 500 mL), with a central glass compartment that allowed the visual display of the cue (commercial cuttlefish ink) whilst blocking the chemically mediated cue component. The test started with the introduction of the cue, that took place from above through an opaque tube fixed above the cue-compartment. To avoid disturbance to the test, both the perimeter and the top of the arena were fully covered in an opaque overlay from which the cue-introducing tube was placed. Additionally, a sham-test, in which clear seawater was introduced instead of the ink, was performed after the acclimatization and 5 min. prior to the stimulus introduction, in order to control for the presence of other factors. A lamp was placed above the arena and a camera was placed below to record the cuttlefish's activity, after the acclimatization period, including the sham-test and the reaction to the ink. Here, a total of 28 individuals was used and their reactions were divided into four classes (0 = no perceived reaction; 1 = increase of ventilation and/or branchial movements; 2 = color changes and/or cessation of swimming; 3 = escape, attack and/or dorsal arms raised) adapted from Wood et al. (2008).

Data Analysis

All image and video footage were analyzed with specific programs, i.e., ImageJ was used to obtain biometric data (length measures) from photography and BORIS software (Behavioral Observation Research Interactive Software v.6.0.5 – 2018-01-29) was used to analyze all video data. While using BORIS software, specific commands were defined considering the specificities of each test. For the shelter-seeking test, four commands were defined to register the time spent in each area: (a) start of test, (b) entrance into the lighted area, (c) entrance into the shaded area, and (d) entrance into the neutral area. For the hunting behavior test, four commands were defined to acquire the timings and attack effectiveness of the individuals: (a) prey introduction, (b) reaction to the prey, (c) attack, and (d) catch. For the visual detection test, five commands were defined: (a) reaction, (b) no perceived reaction, (c) reaction 1 (increase of ventilation and/or branchial movements), (d) reaction 2 (color changes and/or cessation of swimming) and (e) reaction 3 (escape, attack and/or dorsal arms raised).

After data visualization, statistical analyses of the defined variables were performed with RStudio Software (Version 1.1.456 – © 2009–2018 RStudio, Inc.). All Generalized Linear Models (GLM) were performed with pH as factor. For all the variables analyzed, replicates and hatching date (considers the difference in development time upon collection) were first included in generalized linear mixed models (GLMM) as random effect, to account for potential variability in the experimental design and for the dependency within these factors. Random effects were removed from the models whenever the amount

of variation explained was lower than 5%. The best model for each output was chosen according to the calculation of Akaike Information Criterion (AIC), i.e., the best model was the one that featured the smallest AIC. The GLMM with Gaussian family was used to analyze weight (with hatching date as a random factor) and DML (with replicates as a random factor). The same model with Gamma family and log link function was used to analyze the Fulton's index (with replicates and hatching date as random factors). The GLM with Gaussian family was performed to analyze TBL.

The reaction and catch time in the hunting behavior test were analyzed with the Gamma family and inverse link function. The Binomial family of distribution was used to analyze the shelter-seeking test (choice/no choice and black/white) and the visual detection test for reaction variable. Count data were analyzed with the Poisson family, i.e., development time, hatching success, hatchlings survival (within identity as a link function) and the successful attacks observed in the hunting behavior test. To analyze the type of reaction in the visual detection test, a multinomial logistic regression model was performed with the four classes in test, mentioned above. All statistical differences were considered when p-value $< \alpha$ with $\alpha = 0.05$.

RESULTS

Development Time, Survival and Hatchlings' Biometrics

Development time was similar in both treatments ($\sim$ 59 $\pm$ 9 days), i.e., there was no significant effect under OA (**Figure 1**; $p > 0.05$; GLM, Poisson family, more details in **Supplementary Table 1**). Likewise, neither hatching success (73.33 $\pm$ 1.80% under normocapnia and 70.20 $\pm$ 1.36% under hypercapnia) nor survival rate after 20 DAH (66.86 $\pm$ 1.10% and 69.30 $\pm$ 1.99%, under normocapnia and hypercapnia, respectively) were significantly affected by high CO_2 treatment (**Figure 1**; $p > 0.05$; GLM, Poisson family, see more statistical details in **Supplementary Table 1**).

Ocean acidification effects on the DML (**Figure 2**; $p > 0.05$; GLMM, Gaussian family, analysis in **Supplementary Table 2**.), TBL (**Figure 2**; $p > 0.05$; GLM, Gaussian family, analysis in **Supplementary Table 3**), TBW (**Figure 2**; $p > 0.05$; GLMM, Gaussian family, analysis in **Supplementary Table 2**) and Fulton's index (K) (**Figure 2**; $p > 0.05$; GLMM, Gamma family, analysis in **Supplementary Table 4**) were also not statistically significant.

Behavioral Responses

No significant differences were found between treatments in the choice rate of shelter, nor in the light/shade preference in the shelter-seeking test (**Figure 3**; $p > 0.05$; GLM, Binomial family, analysis in **Supplementary Table 1**).

Likewise, no significant differences were observed regarding to their hunting behavior (namely in the reaction to prey and in the attack duration) between control and OA treatments (**Figure 4**; $p > 0.05$; GLM, Gamma family, analysis in **Supplementary Table 3**), as well as for predatory success rate (**Figure 4**; $p > 0.05$; GLM, Poisson family, more details in **Supplementary Table 1**).

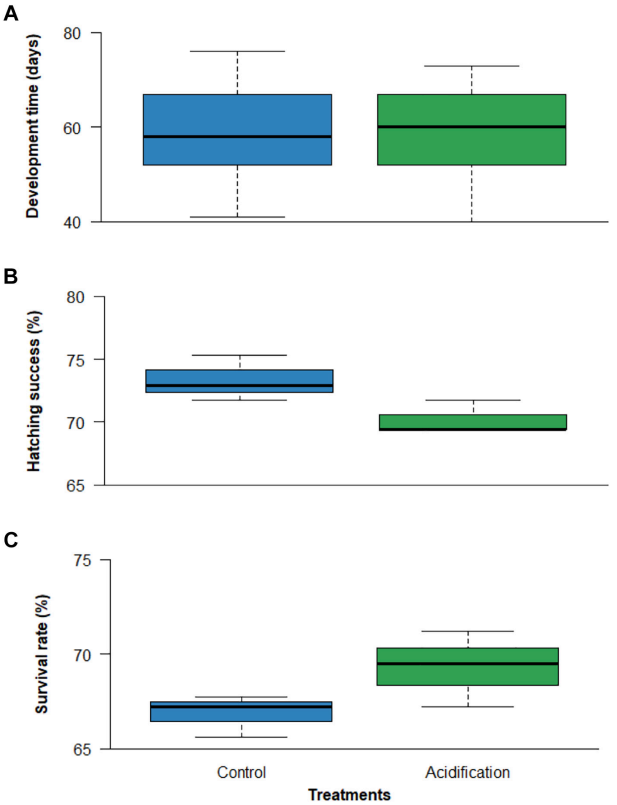

FIGURE 1 | Effect of ocean acidification (ΔpH = 0.4) on: **(A)** development time, **(B)** hatching success and **(C)** survival rate [20 days after hatching] of the common cuttlefish *Sepia officinalis*. Boxplots illustrate median, upper and lower quartile, and inter-quartile range.

Similarly, the visual detection of conspecific visual stimulus (ink) was also not significantly affected by the experimental CO_2 treatments (**Figure 5**; $p > 0.05$; GLM, Binomial family for the reaction/no perceived reaction and multinomial logistic regression for the type of reaction, both analyses can be found in **Supplementary Table 1**). A large proportion of individuals had no perceived reaction to the stimulus, 68.75% under normocapnia and 58.33% under hypercapnia. Noteworthy, 8.33% of the individuals exposed to hypercapnia presented the more severe type of reaction – type 3 (escape, attack and/or dorsal arms raised), whereas no individuals under normocapnia showed this type.

DISCUSSION

The effects of OA in cephalopod early stages are still fairly unknown. However, some studies suggest that OA does not impair normal embryonic development (Dorey et al., 2013; Rosa et al., 2013), survival rates (Dorey et al., 2013) or body size (Lacoue-Labarthe et al., 2009; Hu et al., 2011; Dorey et al., 2013; Rosa et al., 2013; Sigwart et al., 2015). Accordingly, the results here presented suggest that near-future CO_2 did not elicit major impacts on the development time, survival rates

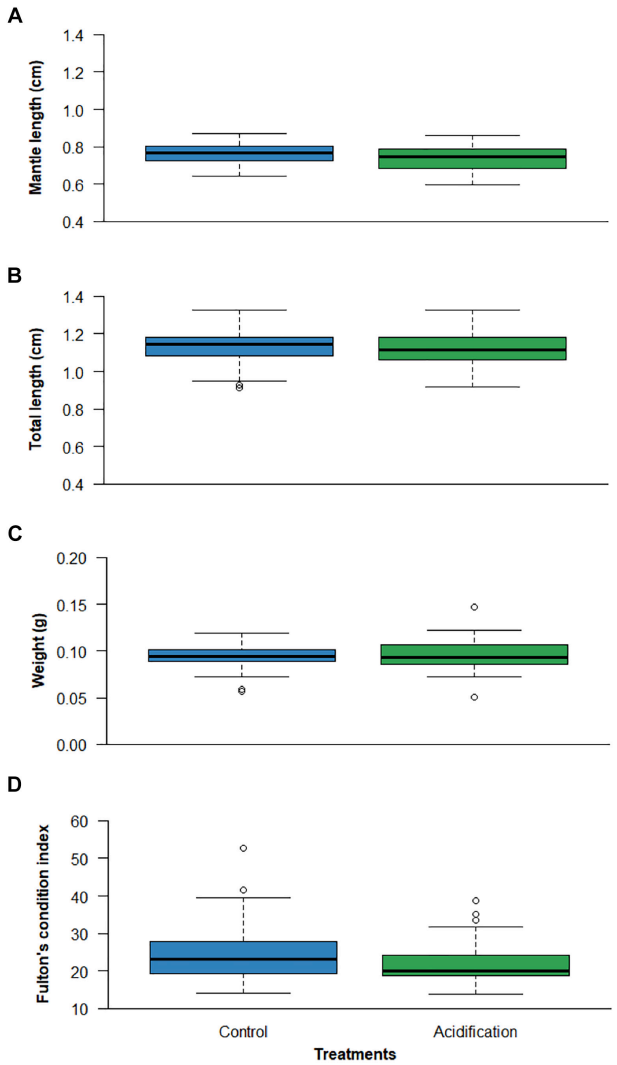

FIGURE 2 | Effect of ocean acidification (ΔpH = 0.4) on: **(A)** dorsal mantle length (DML), **(B)** total body length (TBL), **(C)** total body weight (TBW) and **(D)** Fulton's condition index (*K*) of the common cuttlefish *Sepia officinalis*. Boxplots illustrate median, upper and lower quartile, and inter-quartile range. Circles indicate individual outliers outside the inter-quartile range.

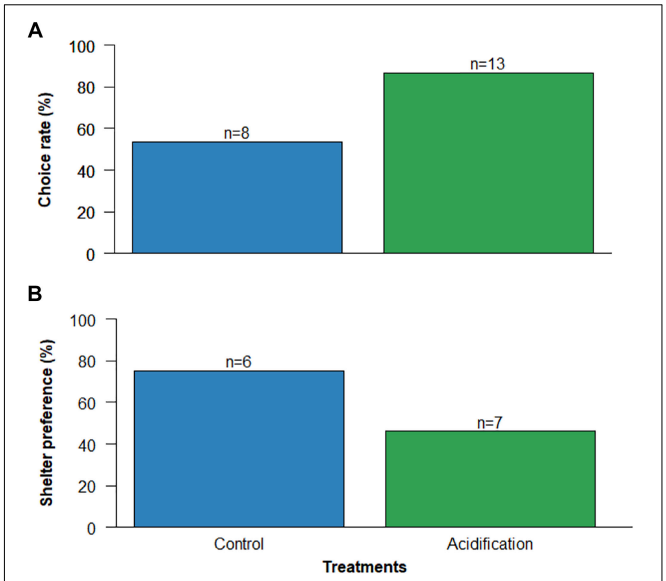

FIGURE 3 | Effect of ocean acidification (ΔpH = 0.4) on the shelter-seeking behavior of the common cuttlefish *Sepia officinalis* with 15–20 DAH: **(A)** capacity to make a choice and **(B)** preference for a darker shelter. Values represent the number of individuals in each treatment who made the respective choice.

and size of *S. officinalis* early stages. The abiotic conditions inside cuttlefish eggs have already been characterized as stressful conditions - with high levels of pCO_2 and HCO_3^-, and low pH (Melzner et al., 2009; Rosa et al., 2013), a consequence of increasing energy expenditure during egg development and swelling (Lacoue-Labarthe et al., 2009; Dorey et al., 2013). Ocean acidification will amplify these already hypercapnic conditions inside the cuttlefish eggs (Hu et al., 2011; Dorey et al., 2013; Rosa et al., 2013), as the water from the outside environment enters (in this case water with high levels of pCO_2) into the hypertonic perivitelline fluid eggs (Hu et al., 2011; Dorey et al., 2013). Giving these embryonic conditions, cuttlefish hatchlings may be consequently more adapted to develop in the future ocean pH conditions.

The common cuttlefish is a species that usually stays hidden in the sand during daytime, to avoid predation (Boyle, 1987; Reid et al., 2005), registering higher activity during the night (Guerra, 2006). Thus, it would be expected that a higher percentage of individuals would choose the shadow side of the shelter arena under control conditions, because of their preference of dark over light environment (Guerra, 2006). However, a high percentage of animals did not make a choice related with shelter dark/bright under control conditions (46.67%). This may be explained by the fact that these organisms use camouflage as their primary line of defense (Boyle, 1987) and, thus, potentially do not prioritize choosing between different light intensity. Another potential explanation can be the lack of enough lightening contrast, which may have made these animals "comfortable" on the neutral area and in the bright side, removing the necessity to move to a safer option. Further studies focusing on camouflage success should be addressed to better understand these results. These mollusks are active predators (Wells, 1958; Messenger, 1968), which was demonstrated by the short time between the first reaction to prey and the time to effectively catch it (control ∼17 s and acidification ∼12 s). The present findings support those obtained by Maneja et al. (2011), who also showed that cuttlefish early stages do not seem to be affected by near-future OA. However, Spady et al. (2018) found the opposite results in squids, with a decrease of hunting behavior (increase of attack latency in both species – bigfin reef squid and pygmy squid – and reduction in the proportion of individuals who attacked their prey in pygmy squid), in the animals exposed to an acidified environment. These findings support the claim of differences insensitivity to elevated pCO_2 between pelagic (squids) and

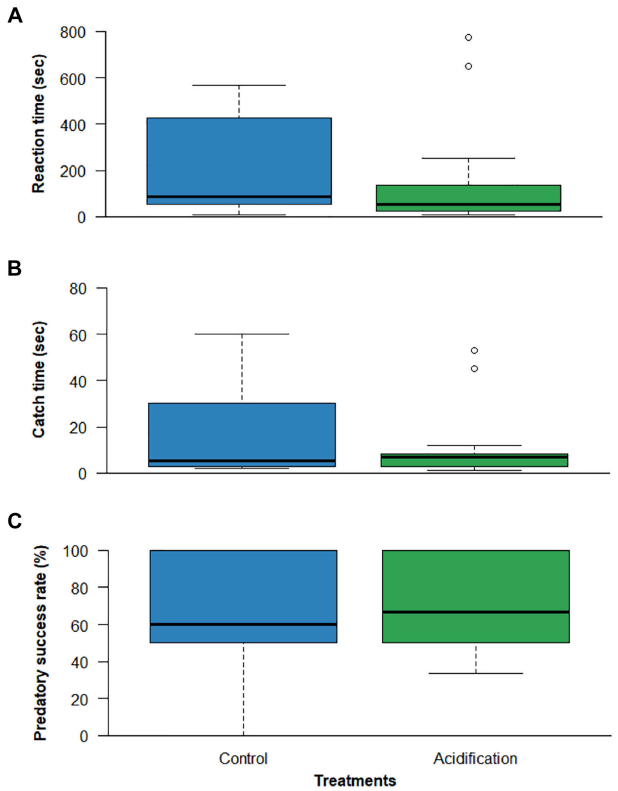

FIGURE 4 | Effect of ocean acidification (ΔpH = 0.4) on: **(A)** time of reaction to the prey, **(B)** time to catch the prey, and **(C)** predatory success of the common cuttlefish *Sepia officinalis* with 15–20 DAH. Boxplots illustrate median, upper and lower quartile, and inter-quartile range. Circles indicate individual outliers outside the inter-quartile range.

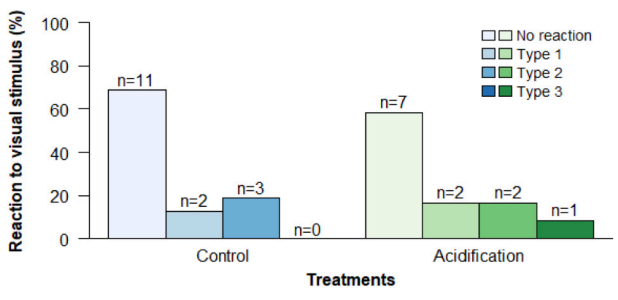

FIGURE 5 | Effect of ocean acidification (ΔpH = 0.4) on the reaction to a conspecific visual stimulus of the common cuttlefish *Sepia officinalis* with 15–20 DAH. Values represent the number of individuals in each treatment who made the respective choice.

nekton-benthic/benthic species (cuttlefish and octopods). When compared with cuttlefishes, squids have more difficulty in reallocating energy toward compensatory processes since they have a lifestyle at the edge of energetic limits due to their high locomotory costs (O'dor and Webber, 1986). Therefore, squids showed more sensitivity when exposed to changing environments (Rosa and Seibel, 2008; Hu et al., 2014) than the nekton-benthic/benthic species that have a slower lifestyle and a better pH buffering/regulatory mechanisms and thus, more resilient (Gutowska et al., 2008; Dorey et al., 2013; Hu et al., 2014). In the visual stimulus test, responses with a higher severity level, e.g., reaction type 3, in the animals from control scenario were expected, as it was observed by Wood et al. (2008) with Caribbean reef squid. However, a high percentage of individuals had no perceivable reactions to the ink stimulus, either in control and acidification treatments. Usually, more severe behaviors are also more visible reactions, which may have a higher effective role in warning their conspecifics about the danger nearby. The different results obtained in this study and those obtained by Wood et al. (2008) may be related with the different lifestyles (pelagic and nekton-benthic/benthic species) and with the life-stage of the animals used in these studies, thus early stage animals may prefer to direct their energetic reserves to grow, instead of defensive behaviors without effective acting, i.e., the ink eject effect is smaller in youngers (smaller size) than in adults (bigger size, more ink). Thus, younglings may opt for defensive behaviors that save more energy. Behavioral freeze is known as cuttlefish response toward certain threats (Bedore et al., 2015), which could account for at least some of non-perceived reacting animals. In this context, a more specialized approach focusing on this response would be necessary. Yet, further research must be conducted, especially at the neurological level, to corroborate the lack of behavioral responses reported here, and to understand how changes in other behaviors are underpinned by OA-related disruption in brain functions.

In general, OA affects survival, fitness and behavioral patterns in many marine organisms (Kaplan et al., 2013; Pimentel et al., 2014, 2016; Dixson et al., 2015). Nevertheless, the present study showed that future CO_2 levels might not elicit significant changes on the developmental and behavioral responses during the early ontogeny (embryos and hatchlings) of the common cuttlefish *S. officinalis*. Such findings are likely related with their nekton-benthic (and active) lifestyle, their adaptability to highly dynamic coastal and estuarine zones, and the already harsh conditions (hypoxia and hypercapnia) inside their eggs, which may favor the odds of the common cuttlefish recruits to endure the future acidified ocean through a possible pre-adaptation to these adverse conditions (Melzner et al., 2009; Hu, 2016). Gutowska et al. (2008) also supported this prediction, showing that *S. officinalis* did not exhibit sensitivity to elevated CO_2 levels within the range of concentrations that elicits a negative response in most other invertebrates (e.g., corals and bivalves). One of the greatest issues caused by OA is the dissolution of calcium carbonate minerals, affecting some taxa with $CaCO_3$ structures [e.g., (Fabry et al., 2008; Stocker et al., 2013)], such as corals [e.g., (Erez et al., 2011; Camp et al., 2017)] and mollusks [e.g., (Kurihara et al., 2007; Talmage and Gobler, 2010; Maneja et al., 2011; Kaplan et al., 2013)]. On the other hand, there are some species that under OA are able to maintain or even increase their calcifying structures, like the cephalopod *S. officinalis* (Gutowska et al., 2008, 2010; Dorey et al., 2013). In some calcifying organisms, this hypercalcification phenomenon appears to be an

energy demanding process and the associated energetic trade-offs under acidification affect the organism's normal growth rates (Stumpp et al., 2011). Yet, it is still not fully understood what the consequences (e.g., metabolic and behavioral level) of this phenomenon on these organisms are.

However, this species showed to be quite resilient to near-future OA. Additionally to the characteristics already mentioned, its short life cycle (1–2 years) may enhance the chance for evolutionary adaptation (Hu, 2016) and detrimental consequences may only turn visible in a very high CO_2 environment or in combination with other climate change factors such as temperature (Lacoue-Labarthe et al., 2009; Rosa et al., 2013), hypoxia (Rosa et al., 2013), and/or with exposition to contaminants. Nevertheless, the shallow-water environments that *S. officinalis* occupies are particularly susceptible to anthropogenic pressures and climate-change stressors, which makes the study of cumulative effects (i.e., of multiple stressors) of paramount importance to accurately predict how this ecologically and economically important species will fare in the future.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

Research was conducted under approval of Faculdade de Ciências da Universidade de Lisboa animal welfare body (ORBEA) and Direção-Geral de Alimentação e Veterinária (DGAV) in accordance with the requirements imposed by the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

AUTHOR CONTRIBUTIONS

ÉM, RR, MP, CS, ES, and VL designed the experiment. ÉM, MP, CS, and MRP performed the experiment. ÉM, RR, MP, CS, and ES analyzed the data. All authors contributed to the writing of the manuscript.

FUNDING

This study was funded by the Portuguese Foundation for Science and Technology (FCT) through the strategic project UID/MAR/04292/2013 grant to MARE, doctoral grant to CS (SFRH/BD/117890/2016), ES (SFRH/BD/131771/2017), MRP (SFRH/BD/111691/2015), post-doctoral grant of MP (SFRH/BPD/117533/2016), VL (PTDC/BIA-BMA/28317/2017) and Investigador FCT Consolidation Grant to RR, and Program MAR2020 through the project VALPRAD (MAR-01.04.02-FEAMP-0007).

ACKNOWLEDGMENTS

The authors would like to acknowledge Cláudia Pereira for all the support during this study.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00975/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

REVIEW
published: 21 August 2019
doi: 10.3389/fphys.2019.01035

Tactical Tentacles: New Insights on the Processes of Sexual Selection Among the Cephalopoda

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The cephalopods (Mollusca: Cephalopoda) are an exceptional class among the invertebrates, characterised by the advanced development of their conditional learning abilities, long-term memories, capacity for rapid colour change and extremely adaptable hydrostatic skeletons. These traits enable cephalopods to occupy diverse marine ecological niches, become successful predators, employ sophisticated predator avoidance behaviours and have complex intraspecific interactions. Where studied, observations of cephalopod mating systems have revealed detailed insights to the life histories and behavioural ecologies of these animals. The reproductive biology of cephalopods is typified by high levels of both male and female promiscuity, alternative mating tactics, long-term sperm storage prior to spawning, and the capacity for intricate visual displays and/or use of a distinct sensory ecology. This review summarises the current understanding of cephalopod reproductive biology, and where investigated, how both pre-copulatory behaviours and post-copulatory fertilisation patterns can influence the processes of sexual selection. Overall, it is concluded that sperm competition and possibly cryptic female choice are likely to be critical determinants of which individuals' alleles get transferred to subsequent generations in cephalopod mating systems. Additionally, it is emphasised that the optimisation of offspring quality and/or fertilisation bias to genetically compatible males are necessary drivers for the proliferation of polyandry observed among cephalopods, and potential methods for testing these hypotheses are proposed within the conclusion of this review. Further gaps within the current knowledge of how sexual selection operates in this group are also highlighted, in the hopes of prompting new directions for research of the distinctive mating systems in this unique lineage.

Keywords: cryptic female choice, cuttlefish, mate choice, octopus, polyandry, sperm competition, squid, reproduction

OPEN ACCESS

Edited by:
Graziano Fiorito, Stazione Zoologica Anton Dohrn, Italy

Reviewed by:
Andrea Tarallo, Department of Sciences and Technologies, University of Sannio, Italy
Gustavo Bueno Rivas, Texas A&M University, United States

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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *25 March 2019*
Accepted: *29 July 2019*
Published: *21 August 2019*

Citation:
Morse P and Huffard CL (2019) Tactical Tentacles: New Insights on the Processes of Sexual Selection Among the Cephalopoda. Front. Physiol. 10:1035. doi: 10.3389/fphys.2019.01035

1. INTRODUCTION

Sexual selection is the competition within one or both sexes of a species toward optimising individual reproductive success (Darwin, 1906; Bateson, 1983). The resulting disparity in reproductive outcomes among individuals in a species can lead to the development of specific behaviours and/or phenotypic traits that can enable individuals who display them to

increase their genetic contribution to subsequent generations (West-Eberhard, 1983; Andersson and Simmons, 2006). Anisogamy, which is the differential investment between males and females toward their gametes in most animal mating systems (Kodric-Brown and Brown, 1987), typically results in conflicting strategies for enhancing reproductive output between males and females of the same species (Chapman et al., 2003). Females, which have a relatively higher investment toward gametes, generally have reproductive capacities that are resource-limited (Bateson, 1983; Kodric-Brown and Brown, 1987). Meanwhile males, which are usually less limited by their gamete production, are primarily limited by the number of female gametes they can successfully fertilise (Kodric-Brown and Brown, 1987). Therefore, where anisogamy exists sexual selection can impose females to evolve mechanisms by which they can obtain more resources to create higher numbers of healthy viable eggs, and/or to fertilise these eggs with sperm from higher quality and/or genetically compatible males (Kirkpatrick, 1982; Kodric-Brown and Brown, 1987; Tregenza and Wedell, 2000; Kokko et al., 2003). Dissimilarly, sexual selection will often drive males of a species to develop traits or behaviours that enable them to achieve copulations with a higher number of females, to mate with healthier more fecund females and to attain greater fertilisation success with the females they mate with (Parker, 1970; Kodric-Brown and Brown, 1987; Reinhold et al., 2002).

The cephalopods (Mollusca: Cephalopoda) are a class of invertebrates that might provide a different type of model for studying the mechanisms and impacts of sexual selection. Spermatozoa of male cephalopods are encased in a finite number of discrete spermatophores that are transferred to the female, in some cases individually (Mann et al., 1970). Depending on species, males may or may not be able to regenerate spermatophores (Anderson et al., 2002). In species where males can regenerate spermatophores, the time and energy needed to do so can potentially limit male mating frequency during a spawning period (Mann et al., 1970; Anderson et al., 2002). The constraint of having a fixed or limited male reproductive capacity might lead to a higher investment by males toward their gametes, and could present a system where both male and female mate selection might be important to the reproductive success of individuals within species (e.g., Huffard et al., 2008, 2010). Additionally, several other cephalopod characteristics make this a unique and interesting class of animals for studying the processes of sexual selection. Male and female promiscuity are reported across this class (Hall and Hanlon, 2002; Hanlon et al., 2002; Huffard et al., 2008; Arnold, 2010; Squires et al., 2013; Morse et al., 2015). Males are known to employ size-conditional mating strategies (Hanlon et al., 1997; Hall and Hanlon, 2002; Huffard et al., 2008; Lin et al., 2018). Females in some species are known to be selective of mates (Hall and Hanlon, 2002; Wada et al., 2005a), can store sperm (Perez et al., 1990; Hanlon et al., 1999; Morse, 2008; Hoving et al., 2010; Bush et al., 2012; Cuccu et al., 2014) and can potentially be selective about which sperm they use (Naud et al., 2004; Shaw and Sauer, 2004; Buresch et al., 2009; Sato et al., 2013). While females of the southern bottletail squid (*Sepiadarium austrinum*) can gain nutritional and likely fecundity benefits through the consumption of spermatophores (Wegener et al., 2013), in most cases females receive no identifiable resources or parental care from the males they mate with. This suggests that male quality and/or genomic compatibility might be important factors in female mate selection, as observed in other animals (Jennions and Petrie, 1997; Tregenza and Wedell, 2000; Kokko et al., 2003). Furthermore, cephalopods' capacity for complex behavioural and visual displays can enable unique modes of courtship and/or discretion of potential mates (Corner and Moore, 1981; Hanlon et al., 1994; Huffard, 2007; Mäthger and Hanlon, 2007; Mäthger et al., 2009).

This review is divided into four broad sections following the introduction. The first of these briefly summarises the present knowledge of how reproduction takes place within each of the nine currently recognised extant cephalopod orders (Allcock et al., 2015; Sanchez et al., 2018). The following section focuses in greater detail on pre-copulatory behaviour observed in three coastal cephalopod families, the loliginid squid (Myopsida: Loliginidae), cuttlefish (Sepiida: Sepiidae) and octopuses (Octopoda: Octopodidae), and specifically addresses the mechanisms and behaviours that might lead to differential copulatory rates within these more thoroughly studied mating systems. The third section summarises recent advances in understanding the post-copulatory processes that might lead to differential male fertilisation success among the five cephalopod families where this topic has been investigated. This review concludes with a final section highlighting some of the gaps in the current knowledge of cephalopod mating systems, and which might serve as feasible and productive topics for investigation in the near future. Biases in coverage by this review reflect the skew of existing behavioural research toward more accessible, abundant, and day-active species instead of offshore, nocturnal, and rarer forms.

2. REPRODUCTIVE BIOLOGY OF CEPHALOPODS

2.1. General Reproductive Strategies and Life History Traits of the Cephalopoda

Shallow-water coastal cephalopods are typically known for having relatively fast growth rates and short life-cycles ended with a terminal spawning season (Joll, 1976; Le Goff and Daguzan, 1991; Jackson, 2004; Sato et al., 2008). By contrast, more protracted spawning periods have been observed in deep-sea and cold-water pelagic taxa which might have longer life spans, and pygmy species which can increase lifetime fecundity through release of multiple clutches (Boletzky, 1986; Rocha et al., 2001). Difficulty in finding mates, small egg-clutches due to limitation of resources and low offspring survival, as well as stable environmental conditions with reduced predation of adults have each been hypothesised as selective pressures toward increased parental investment and multiple spawning events for cephalopod taxa occupying deep-sea habitats (Rocha et al., 2001; Hoving et al., 2015). Some life history characteristics of deep-sea and oceanic cephalopods include relatively longer life-cycles, prolonged embryonic development within larger eggs, maternal care of egg masses, intermittent or continuous spawning over a

terminal breeding season and/or iteroparity throughout the adult phase (Villanueva, 1992; Seibel et al., 2000; Rocha et al., 2001; Hoving et al., 2004, 2008, 2015; Barratt et al., 2007; Laptikhovsky et al., 2007, 2008; Arnold, 2010; Bush et al., 2012; **Table 1**).

This latter mode of life history is exemplified by the nautilids (Nautiloidea: Nautilida), which are expected to live more than 20 years and spawn seasonally each year once sexually mature (Mikami and Okutani, 1977; Saunders, 1984; Arnold, 2010; Dunstan et al., 2011). Nautilids are taxonomically distinct from other cephalopods in that they are the only extant representatives of ectocochleate, or externally shelled cephalopods (Cephalopoda: Nautiloidea; Voss, 1977; Sanchez et al., 2018). However, several coleoid taxa (Cephalopoda: Coleoidea) are also reported to spawn over multiple seasons. These taxa include: several oegopsid squids (Decapodiformes: Oegopsida; Harman et al., 1989; Hoving et al., 2004), *Vampyroteuthis infernalis* (Octopoda: Vampyroteuthidae; Hoving et al., 2015), *Opisthoteuthis* spp. (Octopoda: Opisthoteuthidae; Villanueva, 1992), *Graneledone* spp. (Octopoda: Megaleledonidae; Bello, 2006; Guerra et al., 2012), *Octopus chierchiae* (Octopoda: Octopodidae; Rodaniche, 1984) and the currently undescribed larger Pacific striped octopus ("LPSO"; Octopoda: Octopodidae) which has a continuous spawning phase (Caldwell et al., 2015). These taxa, with the exceptions of *O. chierchiae* and LPSO are all either deep-sea or pelagic cephalopods. Some of the larger oegopsid squid, *V. infernalis* and the giant Pacific octopus (*Enteroctopus dofleini*) have relatively slower growth rates and are estimated to live for 2–8 years (Hartwick, 1983; Hoving et al., 2015; Hoving and Robison, 2017). However, the rest of the coleoid cephalopods are thought to have life-spans of only several months to 2 years (**Table 1**), and in the case of terminal spawners, die shortly after breeding (McGowan, 1954; Roper, 1965; Joll, 1976).

2.2. Reproductive Biology in Nautilida

The order Nautilida (**Figure 1**) contains only two genera, *Allonautilus* and *Nautilus*, and as mentioned above these taxa are the only extant representatives of the cephalopod subclass: Nautiloidea. Correspondingly, their life histories are relatively unique among cephalopods in that they have opportunities to breed continuously throughout their extended life spans (Mikami and Okutani, 1977; Saunders, 1984; Arnold, 2010). Distributional data have indicated that nautilid populations, where sampled, always have an operational sex ratio (OSR) biased toward males (1:3 in Saunders and Ward, 1987). Additionally, Haven (1977) who sampled a population of *Nautilus pompilius* year round to depths of 340 m, found an increase in female catch rates between January and May. These data suggest there might be a seasonal migration of females in this species, possibly related to an annual breeding, feeding, or spawning season.

Relatively very little is known about the reproductive habits of nautilids in the wild. Aquarium observations have provided a basic understanding of copulatory behaviour in captive individuals (*N. macromphalus* in Mikami and Okutani, 1977; *N. pompilius* in Arnold, 2010). Successful copulations takes place by the male grasping the female with his tentacles and drawing the pair's mantle apertures together. The male then uses an enlarged labial tentacle, called a spadix, to push the female's buccal tentacles to the side and transfer one long spermatophore (∼30 cm) to the female's organ of Valenciennes (Mikami and Okutani, 1977), an analogue to the seminal receptacle in some coleoid cephalods except that the spermatophores appear to remain intact within the organ of Valenciennes until the time of fertilisation (Arnold, 2010). The exact method of fertilisation is still not understood in nautilids. However, it has been hypothesised that the spermatophore(s) break during egg-laying and the spermatozoa migrate independently toward the oocyte micropyle(s) (Arnold, 2010).

Copulations have been reported to last as long as 30 h (Mikami and Okutani, 1977), and females have been observed as passive throughout the process. Males are frequently observed to bite the females on the mantle and shell during copulation (Arnold, 2010), the reason for which is still not understood. Bites were observed to leave marks on the shell, suggesting that these could theoretically be used as an indication of a female's mating and/or possibly egg-laying history (Arnold, 2010). However, males' response to and/or preference for females with different numbers of bite marks have not been assessed.

Arnold (2010) additionally indicated that male copulation attempts are extended to any object that shares a similar shape and size of another nautilid, and that male/male copulation attempts are common. This suggests that nautilids may have difficulty recognising conspecifics and/or discriminating between sexes. This aspect of social naiveté might be related to living in the pelagic environment where the ability to find mates could be limiting to reproductive success. In this context, it might to be less costly for males to waste time and/or energy attempting unviable copulations, than to risk missing an opportunity to transfer gametes to a suitable mate, as has been hypothesised for other cephalopod species (Hoving et al., 2012; Morse et al., 2015).

In captivity, nautilids deposit eggs both singly and in small clusters on aquarium floors over an extended annual period, and to do so over multiple years (Carlson et al., 1992; Arnold, 2010). The eggs' exteriors are tough, flexible and opaque white in colour (Mikami and Okutani, 1977). Embryonic development in nautilids takes from 9 months to over a year (Arnold, 2010), and there have been no observations of maternal egg care. Upon hatching, juveniles appear like miniature adults and are immediately capable of actively swimming and feeding on cut-up pieces of prawn (Carlson et al., 1992).

2.3. Reproductive Biology in Oegopsida

Depending on species, male oegopsid, or "open-eye" squids (Decapodiformes: Oegopsida; **Figure 2**), are thought to use either a hectocotylised arm or an elongated terminal organ to deliver sperm to the female (Nesis, 1996; Hoving et al., 2004). However, the method and placement of sperm transfer can take place in a variety of ways depending on the taxon. In *Lycoteuthis lorigera*, females can store spermatophores in dorsal pouches located on the neck (Hoving et al., 2007). *Illex* spp. (Oegopsida: Ommastrephidae) are not known to have any seminal receptacle, and sperm are stored only inside spermatophore casings within females' mantle cavities (Durward et al., 1980; Arkhipkin and Laptikhovsky, 1994). External spermatophore placement is

TABLE 1 | The life history characteristics pertaining to reproductive biology are summarised below for the nine extant orders of Cephalopoda.

Order	Approx. no. of species	Size range	Lifespan	Method of sperm transfer	Female sperm storage organ	Site of fertilisation	Reproductive cycle[32]	Fecundity	Maternal care	Hatchling type
Nautilida	7[1]	Shell up to 229 mm in diameter[1]	>20 years[5]	Spadix[15]	Organ of Valenciennes[15]	Oocyte micropyles (hypothesised[28])	PS[28]	10–20 eggs/year[40,41]	Not reported	Direct developing[50]
Oegopsida	236[2]	20 to at least 2,000 mm ML[3]	Up to at least 2 years[6]	Hectocotylus or elongated terminal organ[16]	Dorsal pouches[18]; inside MC[19]; or external[16]	Thought to be external[16]	MS[33]; or ITS[16]	Up to 6 million[16]	Extended egg care in two species[45,46]	Planktonic larvae[51]
Myopsida	50[2]	20–900 mm ML[3]	1–2 years[7]	Hectocotylus[3]	Sperm receptacle near BA; or inside MC[20]	External[29]	ITS[29]	~2,000–55,000[42]	ANG[47]	Planktonic larvae[51]
Idiosepiida	6[2]	<25 mm ML[1]	80–151 days[8,9]	Hectocotylus[1]	Sperm receptacle near BA[21]	External[30]	CS[34]	53–922[34]	Not reported	Direct developing[34]
Sepiolida	70[2]	Up to 80 mm ML[1]	5 months to reports of 2 years[10]	Hectocotylus[1]	Internal spermatheca[22]; or external[23]	External[22]; or "confined external"[31]	ITS[22]; or CS[35]	Up to 931[43]	ANG[48]	Direct developing[51]
Sepiida	120[2]	60–510 mm ML[1]	1–2 years[11]	Hectocotylus[1]	Paired sperm receptacles near BA; or external[24]	External[22]	ITS[36]	Up to 8,000[36]	ANG[49]	Direct developing[51]
Spirulida	1[2]	~45 mm ML[1]	18–20 months[1]	Hectocotylus[1]	Sperm receptacle near BA[1]	Unknown	Unknown	Unknown	Not reported	Unknown
Octopoda	300[2]	15 mm ML (~1 g) to over 600 mm ML (>180 kg)[4]	~7 months[12] to 5 years[13]	Hectocotylus in Incirrata[4]; Unknown in Cirrata	Oviduccal glands[25]; ovaries[26]; or inside dismembered hectocotyli within MC[27]	Oviduccal glands[25]; or inside ovaries[26]	STS[37]; MS[38]; or CS[39]	30[44]-700,000[37]	Extended egg care in Incirrata[12]; not reported in Cirrata	Planktonic larvae[37]; or direct developing[12]
Vampyromorphida	1[2]	Up to 130 mm ML[4]	Predicted >8 years[14]	Funnel (hypothesised[17])	Infraorbital pits[17]	Unknown	Suggested to be PS[14]	Up to 20,711[14]	Not reported	Unknown

ANG, accessory nidamental gland; BA, buccal area; CS, continuous spawning; ITS, intermittent terminal spawning; MC, mantle cavity; ML, mantle length; MS, multiple spawning; PS, polycyclic spawning; STS, simultaneous terminal spawning. [1](Jereb and Roper, 2005); [2](Allcock et al., 2015); [3](Jereb and Roper, 2010); [4](Jereb et al., 2014); [5](Dunstan et al., 2011); [6](Hoving and Robison, 2017); [7](Jackson, 2004); [8](Tracey et al., 2003); [9](Sato et al., 2008); [10](Marine Biological Laboratory, 2019); [11](Gabr et al., 1998); [12](Tranter and Augustine, 1973); [13](Hartwick, 1983); [14](Hoving et al., 2015); [15](Mikami and Okutani, 1977); [16](Hoving et al., 2004); [17](Pickford, 1949b); [18](Hoving et al., 2007); [19](Durward et al., 1980); [20](Hanlon et al., 1997); [21](Sato et al., 2010); [22](Squires et al., 2013); [23](Hoving et al., 2009); [24](Naud et al., 2005); [25](Froesch and Marthy, 1975); [26](Perez et al., 1990); [27](Laptikhovsky and Salman, 2003); [28](Arnold, 2010); [29](Hanlon et al., 2004); [30](Sato et al., 2013); [31](Hoving et al., 2008); [32](Rocha et al., 2001); [33](Nesis, 1996); [34](Nishiguchi et al., 2014); [35](Laptikhovsky et al., 2008); [36](Laptikhovsky et al., 2003); [37](Joll, 1976); [38](Rodaniche, 1984); [39](Caldwell et al., 2015); [40](Okubo et al., 1995); [41](Uchiyama and Tanabe, 1999); [42](Hixon, 1980); [43](Salman and Önsoy, 2010); [44](O'Dor and Malacaster, 1983); [45](Seibel et al., 2000); [46](Bush et al., 2012); [47](Barbieri et al., 1996); [48](Collins et al., 2012); [49](Richard et al., 1979); [50](Carlson et al., 1992); [51](Boletzky, 1987). Taxonomic orders and the sequence they are presented in are based on phylogenies described in Allcock et al. (2015).

FIGURE 1 | The Palau nautilus, *Nautilus belauensis* (Nautiloidea: Nautilida). Photograph taken by Manuae, and downloaded via Wikimedia under licence: [CC BY-SA 3.0 (https://creativecommons.org/licenses/by-sa/3.0)].

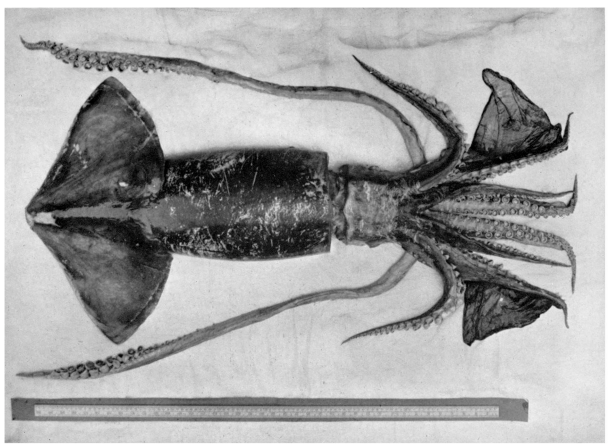

FIGURE 2 | A dead specimen of the neon flying squid, *Ommastrephes bartamii* (Decapodiformes: Oegopsida). Photograph taken by the British Museum of Natural History, and downloaded from the public domain via Wikimedia.

also common in several species of deep-sea squids, including *Architeuthis* sp. (Hoving et al., 2004), *Octopoteuthis deletron* (Hoving et al., 2012), *Taningia danaei* (Hoving et al., 2010) and *Moroteuthis ingens* (Hoving and Laptikhovsky, 2007). This method of spermatophore placement has been suggested as a consequence of size dimorphism between the sexes, in that smaller males need to be able to mate quickly and escape from larger and potentially cannibalistic females (Hoving et al., 2004). Where studied, externally placed spermatophores in oegopsid species enter through females' skin autonomously to achieve implantation and storage (Hoving and Laptikhovsky, 2007).

The oegopsids have the highest fecundity among the cephalopod class. Oocyte counts have led to estimations of fecundity reaching as high as 1–6 million in *Dosidicus gigas* (Ehrhardt et al., 1983) and 3- 6 million in *Architeuthis* sp. (Hoving et al., 2004). Both of these deep-water species have extended or multiple spawning events, enabling higher fecundity than their counterpart taxa in coastal or shallow-water habitats (Rocha et al., 2001). Egg deposition and care are variable among the oegopsids. In deep-sea habitats where there are typically few hard surfaces enabling egg attachment, where known, most oegopsids lay eggs in neutrally buoyant egg masses that they let go into the water column (Guerra et al., 2002; O'Shea et al., 2004; Staaf et al., 2008). By contrast, females in the family Enoploteuthidae lay single, buoyant egg-capsules (Young and Harman, 1985). Maternal egg care has been reported in *Bathyteuthis berryi* and *Gonatus onyx.* These species have been known to carry their egg masses in their arms and guard them from predators and parasites throughout their development (Seibel et al., 2000; Bush et al., 2012). In the case of *G. onyx*, embryonic development is estimated to take up to 9 months, and females will drop their two feeding tentacles after egg deposition, presumably to better hold the egg mass with their eight arms (Seibel et al., 2000). Larval morphology is highly variable among oegopsids, however all taxa studied to date are planktonic upon hatching (Boletzky, 1987).

2.4. Reproductive Biology in Myopsida

The myopsid, or "closed-eye" squids (Decapodiformes: Myopsida; **Figure 3**) are an order of neritic squids that typically live for only 1–2 years (Jackson, 2004), spawn in large assemblages (Hanlon, 1998; Hanlon et al., 2002; Jantzen and Havenhand, 2003) and are thought to reproduce over only one breeding season (McGowan, 1954; Roper, 1965). Males can transfer sperm in at least three ways within myopsid taxa. Typically, pairs can mate in either a head to head or parallel position, and males use their hectocotylised arms to place spermatophores in either the seminal receptacles near the females' buccal mass or inside females' mantle cavities near the distal ends of their oviducts (Hanlon et al., 1997; Jantzen and Havenhand, 2003). Female *Lolliguncula brevis* have specialised pads on the inside of their mantle walls where males place spermatophores during parallel mating (Hanlon et al., 1983). A third method of spermatophore placement has been observed during sneaker copulations (described in section 3.1.1) in *Loligo vulgaris.* Sneaker males in this species have been observed to opportunistically place spermatophores directly into females' arms either on or near eggs that are about to be deposited onto an egg mass (Hanlon et al., 2002). Fertilisation in myopsids is external, and takes place at the time of egg deposition (Hanlon et al., 2004; Shaw and Sauer, 2004; Naud et al., 2016).

Where studied, spawned egg counts in Myopsida have ranged from 2,024 in *L. brevis* to 55,308 in *Doryteuthis pealei* (Hixon, 1980). Myopsids deposit eggs on the substrate, either in single clutches or in communal egg masses (Hanlon et al., 1997; Jantzen and Havenhand, 2003). Female myopsids possess an accessory nidamental gland (ANG) and it is has been hypothesised that bacterial communities, cultured here and passed to egg capsules, might help to protect myopsid eggs from fouling or harmful microbes (Barbieri et al., 1996). All myopsids are intermittent terminal spawners (Roper, 1965; Hanlon et al., 2004), meaning the females deposit eggs in multiple batches over a single

FIGURE 3 | The bigfin reef squid, *Sepioteuthis lessoniana* (Decapodiformes: Myopsida) from Komodo National Park. Photograph by P. Morse.

FIGURE 4 | The two-toned pygmy squid, *Idiosepius pygmaeus* (Decapodiformes: Idiosepiida). Photograph taken by krokodiver and downloaded via Flickr under licence: [CC BY-SA 2.0 (https://creativecommons.org/licenses/by/2.0/)].

spawning period and die shortly after (Rocha et al., 2001). One species, *D. opalescens*, was previously reported to have simultaneous terminal spawning (McGowan, 1954). However, females of this species have since been observed depositing eggs in multiple batches, and to re-join the shoal between discrete egg-laying events (Hanlon et al., 2004). No maternal egg care has been reported within this order (apart from the protective properties imparted by the ANG), and all myopsid larvae are planktonic upon hatching (Boletzky, 1987).

2.5. Reproductive Biology in Idiosepiida

Pygmy squids (Decapodiformes: Idiosepiida) are an order of small, short-lived (80–150 days: Tracey et al., 2003; Sato et al., 2008), continuous spawning, shallow-water, coastal cephalopods comprising the single genus, *Idiosepius* (Nishiguchi et al., 2014; **Figure 4**). Copulations in this group take place in a head to head position where the male attaches spermatangia to the base of the female's arms (Kasugai, 2000; Sato et al., 2013). It is thought that the spermatozoa might then actively swim from the spermatangia to the female's seminal receptacle located near the buccal membrane (Sato et al., 2010). Like in myopsids, egg fertilisation in idiosepiids is external and takes place at the time of egg deposition (Sato et al., 2013). In captivity, female *I. pygmaeus* have been observed to lay a total of 53–922 eggs over up to eight egg clutches (Nishiguchi et al., 2014). Eggs are deposited individually into an egg capsule which is attached to the substrate (Natsukari, 1970) and are reported to hatch after approximately 7–40 days of development depending on species (Nabhitabhata et al., 2004; Kasugai and Segawa, 2005). Idiosepiids are direct developing, however all hatchlings are planktonic during their juvenile stage (Nishiguchi et al., 2014).

2.6. Reproductive Biology in Sepiolida

Similar to other decapod cephalopods (Cephalopoda: Decapodiformes), fertilisation in most bobtail squids (Decapodiformes: Sepiolida) takes place externally (Rodrigues et al., 2009; Squires et al., 2013; Wegener et al., 2013). However in at least one species, the pelagic *Heteroteuthis dispar*, fertilisation has been reported to take place either in the female oviducts or visceropericardio coelom through what the authors refer to as "confined external fertilisation" (Hoving et al., 2008). During copulation, sepiolid males usually use their arms to latch onto females' necks (Rodrigues et al., 2009; Squires et al., 2013; **Figure 5**), or in the case of *Rossia pacifica* grasp females from a parallel position (Brocco, 1971). In most cases males then use their hectocotylised first left arms to transfer spermatangia to inside females' mantle cavities where sperm is stored in posterior pouch-like receptacles (Hoving et al., 2008; Rodrigues et al., 2009; Squires et al., 2013). *Rossia moelleri* is an interesting exception. Males of this species are known to implant spermatangia into females' external mantle tissue (Hoving et al., 2009). These authors suggest that a combination of mechanical and chemical processes aid the spermatangia to enter through females' skin autonomously to the oviducts where it is hypothesised that fertilisation might occur in this species.

Sepiolids tend to lay comparatively fewer and larger eggs than most other coleoid cephalopods (Rocha et al., 2001; Laptikhovsky et al., 2008; Squires et al., 2013). Fecundity in sepiolids has been recorded up to 646 eggs in captive southern dumpling squid, *Euprymna tasmanica* (Squires et al., 2013), and up to 931 eggs in *R. macrosoma* (Salman and Önsoy, 2010). Maternal care has not been reported among sepiolid taxa, but many female sepiolids are known to disguise their eggs with opaque egg casings, ink or sand (Arnold et al., 1972; Rodrigues et al., 2009; Squires et al., 2013), and to inoculate their eggs through the ANG (Collins et al., 2012). Sepiolids resemble their adult forms upon hatching and lack a planktonic phase (Boletzky, 1987).

2.7. Reproductive Biology in Sepiida

In sepiids (Decapodiformes: Sepiida; **Figure 6**), fertilisation, where studied, is always external (Naud and Havenhand, 2006). Eggs are fertilised by sperm either stored in females' seminal receptacles, located ventral to their buccal membrane, or from

recently deposited spermatophores on females arms and/or buccal areas (Naud et al., 2005). Reproductive behaviour has been recorded in great detail for *Sepia officinalis* and *S. apama* (Hanlon et al., 1999; Naud et al., 2004). Copulations in these species take place in the head to head position. Pairs face each other, intertwine arms and males use their hectocotylised fourth left arms to transfer spermatophores from their funnel to females' seminal receptacles and/or directly onto females' buccal areas. Males then use the hectocotylus to break open spermatophores, and possibly to manipulate their placement on the female. The female then uses either the stored or externally placed sperm to fertilise her eggs individually at the time of deposition (Naud et al., 2005).

FIGURE 5 | A male (left) southern dumpling squid, *Euprymna tasmanica* (Decapodiformes: Sepolida) grasps the female (right) around the lower mantle during mating. Photograph taken by Zoe Squires, downloaded via Wikimedia and cropped under licence: [CC BY 4.0 (https://creativecommons.org/licenses/by/4.0)].

Large sepiids are estimated to live from 1–2 years (Le Goff and Daguzan, 1991; Gabr et al., 1998) and are predicted to lay up to 8,000 eggs over intermittent spawning periods (Laptikhovsky et al., 2003). Female sepiids are not known to physically guard their eggs, but similar to sepiolids, several mechanisms for hiding eggs are employed across these taxa. Where observed, female sepiids always attach their eggs to the substrate or a hard object (Adamo et al., 2000; Hall and Hanlon, 2002). *Sepia officinalis* lay opaque eggs, darkened with ink to minimise detection by predators (Boletzky et al., 2006). Female *S. esculenta* achieves the same result by attaching sand and rubble to their eggs with a sticky exterior (Natsukari and Tashiro, 1991). Both *S. latimanus* and *S. pharaonis* hide their eggs in coral crevices, possibly to help guard them against predatory fish (Corner and Moore, 1981; Gutsall, 1989). The flamboyant cuttlefish (*Metasepia* spp.) have been observed to lay their eggs in live rock and coconut shells (in captivity: Grasse, 2014; in the wild: C.L. Huffard, personal observations). As with myopsids and sepiolids, female sepiids possess an ANG which is thought to aid in inoculating their eggs against harmful pathogens (Richard et al., 1979). Similar to sepiolids, sepiids are direct-developing and spend their entire life histories on or near the seafloor (Boletzky, 1987), suggesting that dispersal might be more limited in these orders than in most other cephalopod taxa.

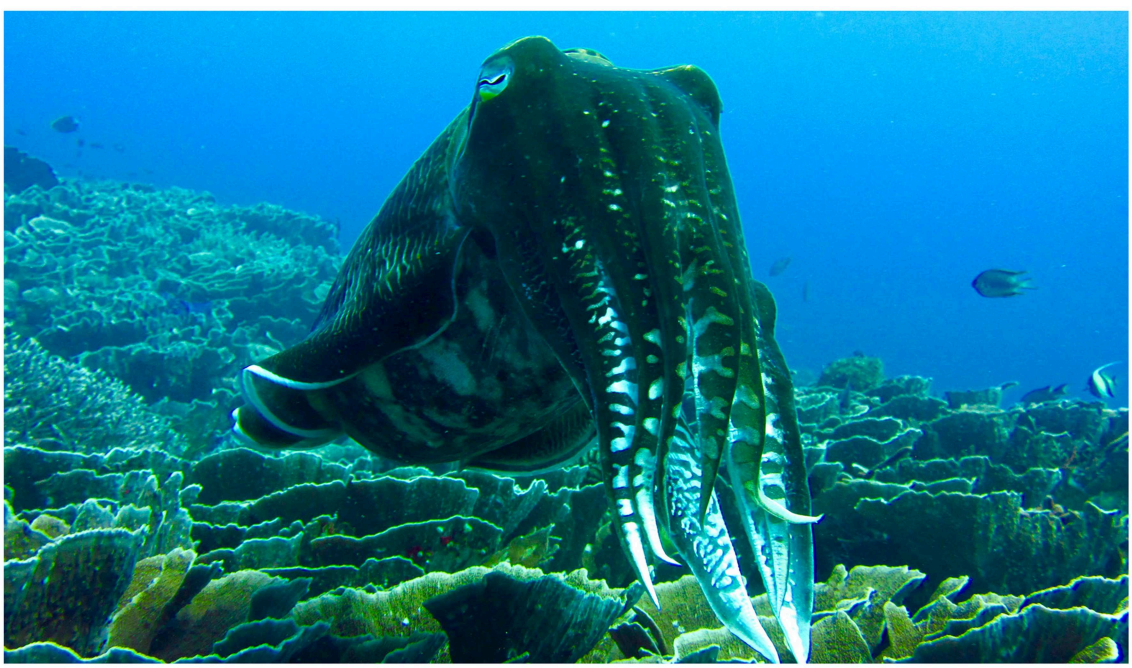

FIGURE 6 | The broadclub cuttlefish, *Sepia latimanus* (Decapoformes: Sepiida) from Komodo National Park. Photograph by P. Morse.

2.8. Reproductive Biology in Spirulida

Spirulida (Decapodiformes: Spirulida) is a monotypic order comprising the Ram's horn squid (*Spirula spirula*; **Figure 7**). Very little is know about the life history or behaviour of this mesopelagic squid, but size profiling of dead specimens caught at different depths and different times of year has led to some insights (Bruun, 1943; Clarke, 1970). This small (up to 45 mm mantle length) and elusive cephalopod is predicted to have an 18–20 month lifespan and is thought to reach sexual maturity at 12–15 months of growth (Jereb and Roper, 2005). Due to observations that the smallest individuals have been caught at the greatest depths (1,000–1,750 m), it has been suggested that females might deposit eggs at the bottom of continental slopes (Jereb and Roper, 2005).

2.9. Reproductive Biology in Octopoda

The octopods (Octopodiformes: Octopoda) can be broadly divided into two suborders: the incirrate octopods (Octopoda: Incirrata; **Figure 8**), and the cirrate octopods (Octopoda: Cirrata). Egg fertilisation in the incirrate octopods is always internal (Froesch and Marthy, 1975). The male hectocotylus, which is usually the third right arm (Robson, 1929), terminates in a specialised organ called a ligula (Wells and Wells, 1972). The ligula is composed of erectile tissue in some species (Thompson and Voight, 2003), and it is thought that this structure aids in spermatophore placement and/or removal of competing spermatophores (Voight, 1991; Cigliano, 1995). Males are hypothesised to use the ligula to reach inside the female's mantle aperture and presumably locate one of the two oviducts (Wells and Wells, 1972; **Supplementary Video 1**). Spermatophores are then passed from the male's terminal organ, which is inside the mantle, through the funnel and into the base of the hectocotylised arm (Wells and Wells, 1972). The spermatophores are carried through a ventral groove in the hectocotylus to the ligula using a wave of contractions along the arm (Wodinsky, 2008). The male then uses the ligula to place each spermatophore at one of the openings to the female's paired oviducts (Wells and Wells, 1972). This process can happen while the male is mounting the female's mantle (e.g., *Eledone* spp. in Orelli, 1962; and *Hapalochlaena* spp. in Tranter and Augustine, 1973; Overath and Boletzky, 1974; **Figure 9A**), by the male reaching over to the female with the hectocotylus from a distance (e.g., *O. digueti* in Voight, 1991; and algae octopus, *Abdopus aculeatus* in Huffard et al., 2008; **Figure 9B**), or in a beak to beak mating position with the female at times enveloping the male in her web (LPSO in Caldwell et al., 2015; and occasionally

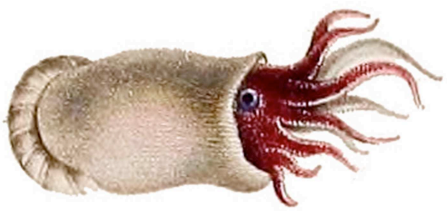

FIGURE 7 | An artist's rendition of the ram's horn squid, *Spirula spirula* (Decapodiformes: Spirulida). This image was drawn by Lesueur in 1807, and was downloaded from the public domain via Wikimedia.

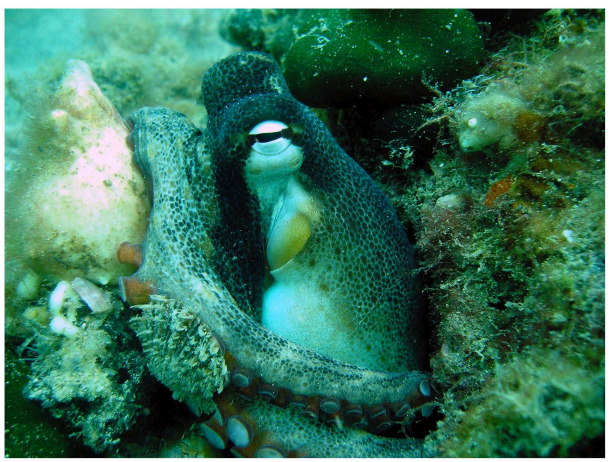

FIGURE 8 | The gloomy octopus, *Octopus tetricus* (Octopodiformes: Octopoda) from Fremantle, Western Australia. Photograph by P. Morse.

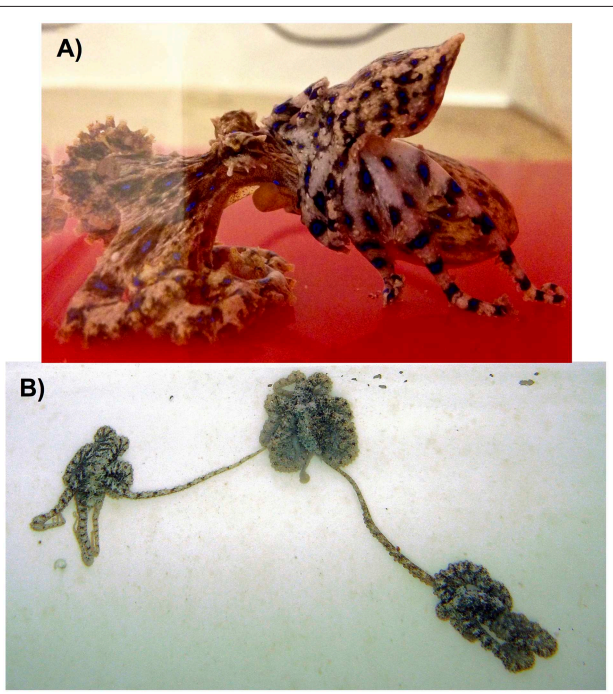

FIGURE 9 | Examples are shown of both the mount **(A)** and reach **(B)** copulation postures displayed by incirrate octopuses. **(A)** A male southern blue-ringed octopus (*Hapalochlaena maculosa*) mounts the female's mantle as he uses his hectocotylised third right arm to transfer spermatophores to the female's distal oviducts. Photograph taken under laboratory conditions by P. Morse; **(B)** Two male algae octopus (*Abdopus aculeatus*) simultaneously mate with a female (centre) by reaching with their hectocotylised third right arms to transfer spermatophores to her distal oviducts. Photograph taken by C. Huffard at Lizard Island, Australia.

O. oliveri in Ylitalo et al., 2019). Males of some species have been observed to use both mounting and reach strategies (*O. cyanea* in van Heukelem, 1966; and *O. tetricus* in Huffard and Godfrey-Smith, 2010), and might use the mounting position more often with females that are unreceptive (P. Morse personal observations with *O. tetricus*).

Osmotic pressure from exposure to seawater (Hanson et al., 1973), and possibly mechanical rupture from the ligula, break open spermatophores and sperm is usually stored as spermatozoa inside the spermathecae of female's oviducal glands (Froesch and Marthy, 1975). However, in several deep-water octopuses (e.g., *Eledone* spp., *G. macrotyla* and *Vulcanoctopus hydothermalis*) spermatangia migrate to the female's ovaries, where fertilisation occurs (Orelli, 1962; Perez et al., 1990; González et al., 2008; Guerra et al., 2012). In the pelagic environment, where the likelihood of encountering a conspecific of the opposite sex may be the relatively low, males in three genera of incirrate octopods, *Argonauta, Tremoctopus* and *Ocythoe*, have hectocotyli that fill with sperm, get broken off and left inside the female's mantle cavity (Laptikhovsky and Salman, 2003). Males of the cirrate octopods do not have a ligula (Villanueva, 1992), and it has not yet been established how copulation occurs.

Fecundity is highly variable among incirrate octopods, and egg count estimates have ranged from approximately 30 in *Bathypolypus arcticus* (O'Dor and Malacaster, 1983) up to 700,000 in *O. cyanea* and *O. tetricus* (van Heukelem, 1966; Joll, 1976). Fecundity within the cirrate octopods has so far only been assessed for *O. grimaldii,* and the maximum fecundity estimated in this species was 3,202 based on follicular sheath and remaining egg counts (Boyle and Daly, 2000). *Opisthoteuthis* spp. lay single eggs continuously throughout their adult life cycle, and there is no indication of parental care within these octopods (Villanueva, 1992; Daly et al., 1998; Boyle and Daly, 2000). Where studied, all incirrate octopods display some form of extended egg care (Joubin, 1933; Tranter and Augustine, 1973; Hanlon et al., 1985; Voight and Grehan, 2000; Huffard and Hochberg, 2005; Miske and Kirchhauser, 2006). Most female incirrate octopods attach eggs to hard substrates, usually inside dens or shelters, where they guard and clean the eggs until hatching (Overath and Boletzky, 1974; Joll, 1976). This maternal behaviour has also been reported in two species of deep-sea octopuses, *Graneledone* sp. and *Benthoctopus* sp. during ROV observations (Voight and Grehan, 2000). These authors suggest, that in an environment with limited substrate, these octopuses aggregate around deep-sea rock outcrops as they begin their brooding phase.

Several other octopod species have ways of carrying their developing eggs with them. *Amphioctopus* spp., *Macrotritopus defilippi, H. maculosa* and *Wonderpus photogenicus*, which all live in sand or silt habitats, carry their eggs in the ventral aboral web, in line of water expelled from the funnel (Tranter and Augustine, 1973; Hanlon et al., 1985; Huffard and Hochberg, 2005; Miske and Kirchhauser, 2006). The pelagic *Boliataena microtyla* carries its eggs and reportedly also their larvae within their arms (Young, 1972). *Tremoctopus* spp. carry their eggs using a calcified material that they secrete from their web and attach to their dorsal arms (Naef, 1928). *Vitreledonella richardi* carries its developing eggs and possibly newly hatched larvae within the female's mantle cavity (Joubin, 1933). The argonauts (Incirrata: Argonautidae) carry their eggs within their shell (Laptikhovsky and Salman, 2003). *Ocythoe* spp. have long winding oviducts, where embryos develop as they pass through (Naef, 1928), making the species of this genus the only known ovoviviparous cephalopods. Upon hatching, octopod larvae are either benthic and resemble their adult forms (e.g., members of the subfamily Bathypolypodinae, Boletzky, 1987; and *H. maculosa*, Tranter and Augustine, 1973) or are planktonic (e.g., many *Octopus* spp., Boletzky, 1987).

2.10. Reproductive Biology in Vampyromorphida

The order Vampyromorphida fits phylogenetically within the superorder Octopodiformes (Allcock et al., 2015), and is represented by only a single extant species, *V. infernalis* (Young et al., 1998; **Figure 10**). This midwater species occupies the mesopelagic to bathypelagic zones (500–3,000 m, Seibel et al., 1997), and ROV footage has never captured them mating. Therefore, knowledge of reproduction in *V. infernalis* is limited to observations made from dead specimens. *V. infernalis* males lack a hectocotylised appendage, and it is thought that they use their funnel to transfer spermatophores into females' spermathecae, which in this species are two sperm storage pits located beneath females' eyes (Pickford, 1949b). Single *V. infernalis* eggs have been found drifting freely in open waters, suggesting that females might deposit eggs singly into the water column (Pickford, 1949a). Examination of oocyte development and numbers in dead specimens indicate that female *V. infernalis* have multiple spawning events throughout their lifetime, and can have potential fecundity up to 20,711 (Hoving et al., 2015). The paralarvae of *V. infernalis* resemble adults except for that they have a set of oblique fins, which later get reabsorbed as the adult fins grow in (Young and Vecchione, 1999). The paralarvae can swim freely in deep water habitats, however it is not known whether hatchlings have a free-drifting phase before

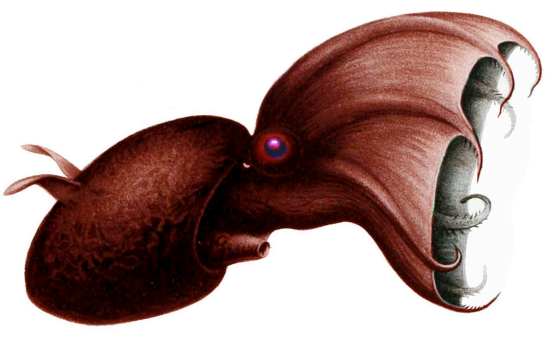

FIGURE 10 | An artist's rendition of the vampire squid, *Vampyroteuthis infernalis* (Octopodiformes: Vampyromorphida). This image was originally designed by Carl Chun in 1910, and was downloaded from the public domain via Wikimedia.

metamorphosing into the described paralarval form (Young and Vecchione, 1999).

3. PRE-COPULATORY BEHAVIOUR IN COASTAL CEPHALOPODS

3.1. Female Choice and Male/Male Competition

3.1.1. Loliginidae

Loliginid squid are among the most social of the cephalopods, in that they hunt in shoals and all species mate in large spawning aggregations (Hanlon, 1998). These spawning aggregations are consistently observed to have male-biased OSRs (1.4:1 in Hanlon et al., 2002; 1:1–3:1 in Jantzen and Havenhand, 2003), where there is a high turnover of mates for both males and females and intense male/male aggression over females takes place (Hanlon et al., 1997, 2002; Jantzen and Havenhand, 2003). Currently, females of only one loliginid species are known to be selective of male partners. Wada et al. (2005a) observed *Sepioteuthis lessoniana* in the laboratory and reported that females rejected more than half of copulations attempted by small subordinate males, but rather chose to copulate in 95% of attempts by larger, more dominant males. Hanlon et al. (1994) provided an excellent account of different body patterning and postures employed by male *Loligo vulgaris* within spawning assemblages. These authors suggested that males of this species use courtship in the forms of both chromatophore patterning and by displaying enlarged testes, which are visible in this species through the mantle, to females. However, additional field and laboratory observations of mating behaviour in *Loligo* spp. have suggested that females are likely to accept copulations with all attempting males (Hanlon et al., 2002; Shaw and Sauer, 2004), which questions the need for courtship behaviour. It is possible that, rather than for courtship, these body patterns and testis displays are used for sex identification within loliginid mating systems.

In both *Loligo* and *Sepioteuthis,* male/male aggression and dominance hierarchy greatly influence copulatory success among males (Hanlon et al., 1994, 1997, 2002; Jantzen and Havenhand, 2003; Wada et al., 2005a). Females of these genera usually arrive at spawning grounds already with a paired consort male, and lone large males that are already waiting at egg-laying sites, frequently challenge paired males for consort status (Hanlon et al., 1997, 2002; Jantzen and Havenhand, 2003). These challenges take the form of intense visual signalling and occasionally fin beating. Both Hanlon et al. (2002) and Jantzen and Havenhand (2003) report high turnovers of consorts in *L. vulgaris* and *S. australis,* respectively. Additionally, smaller "sneaker" males attempt sneaker copulations with already paired females, by quickly moving in between females and consort males and attempting to mate with females in a head to head position (Sauer et al., 1997; Hanlon et al., 2002). Sneaker males time their attempts for when females are about to deposit an egg capsule, and place spermatophores either onto females' arms or directly on egg capsules (Hanlon et al., 2002). Jantzen and Havenhand (2003) observed some *S. australis* sneaker males to mimic female body patterns in order to obtain sneaker copulations without prompting aggression from consort males.

Consort males in both genera place spermatophores internally, close to the opening of females oviducts, however this happens in a "male parallel" position in *Loligo* spp. (Hanlon, 1998), while *S. australis* are observed to most often do this in an upturned position (Jantzen and Havenhand, 2003). Hanlon et al. (2002) report that *L. vulgaris* females arrive at spawning sites already having sperm in their receptacles from what are thought to be from previous head to head copulations. It is likely that females of both genera will copulate with males opportunistically outside of spawning aggregations, and store sperm until future egg depositions. Although females of most loliginid species have not been observed to be selective about which males they copulate with (Hanlon et al., 1997, 2002; van Camp et al., 2004; cf. Wada et al., 2005a), the high frequency of multiple copulations between egg laying intervals (Hanlon et al., 2002; Jantzen and Havenhand, 2003), differential male mating strategies with different methods of sperm placement (Hanlon et al., 1997, 2002), females' capacity to store sperm and to possibly be selective about which sperm they use during external fertilisation (Hanlon et al., 2002; Shaw and Sauer, 2004) all suggest that sperm competition, and conceivably post-copulatory female choice could greatly influence male reproductive success in loliginid mating systems (addressed in section 4.2).

3.1.2. Sepiidae

Cuttlefish have highly promiscuous mating systems, and as mentioned in the previous section females spawn multiple times over one or two breeding seasons (Le Goff and Daguzan, 1991; Gabr et al., 1998). Both *Sepia apama* and *S. latimanus* are known to have spawning aggregations in which males congregate around egg-laying sites in order to attempt copulations with spawning females (up to six individuals in *S. latimanus*, Corner and Moore, 1981; up to 1000s of individuals in *S. apama,* Hall and Hanlon, 2002). Field observations at these spawning sites have revealed detailed accounts of natural reproductive behaviour in these species.

As with loliginid squid, the OSR observed at wild cuttlefish spawning assemblages are always male biased (4:1–11:1 in *S. apama*, Hall and Hanlon, 2002; ∼3:1–4:1 in *S. latimanus*, Corner and Moore, 1981), consistent with both field and aquarium observations of females copulating with multiple males between egg-laying intervals (aquarium observation of *S. officinalis,* Adamo et al., 2000; and *Sepiella japonica*, Wada et al., 2006; field observations of *S. latimanus,* Corner and Moore, 1981; and *S. apama,* Hall and Hanlon, 2002) and intense male/male aggression over females (Corner and Moore, 1981; Adamo et al., 2000; Schnell et al., 2015). However in contrast to loliginid squid, female cuttlefish are reported to frequently reject male copulation attempts (Corner and Moore, 1981; Adamo et al., 2000; Hall and Hanlon, 2002; Schnell et al., 2015). Notably, wild *S. apama* females have been observed to reject 70% of male copulation attempts and only 3% of all copulations were forced (Hall and Hanlon, 2002), emphasising that pre-copulatory female discretion of males plays an important role in this mating system.

Within *S. apama* spawning sites, larger males typically compete with each other, using moderate physical contact and occasional biting, to gain consort status with females, to which they then transfer multiple spermatophores and mate guard (Hall and Hanlon, 2002). Meanwhile, smaller, lone males either try to locate unguarded females or attempt sneaker copulations with already-paired females (Norman et al., 1999; Hall and Hanlon, 2002). Sneaker copulations in cuttlefish mating systems can be defined in three ways: (i) the sneaker male either overtly follows a guarded female and copulates with her while the consort male is distracted; (ii) the sneaker male can copulate with the guarded female while concealed from the consort male's view, such as by hiding under rocks where females lay eggs; or (iii) similar to *S. australis,* sneaker males can mimic female chromatophore patterning while approaching and mating with the female, to avoid aggression from the consort males (Norman et al., 1999; Hall and Hanlon, 2002). Male *S. plangon*, which do not spawn in aggregations, have also been observed to display female patterning on one half of the body that is exposed to a nearby male, while showing typical male patterning to a female with their other half of the body (Brown et al., 2012).

Field observations of *S. apama* have indicated that females can accept or reject copulations with males regardless of size or mating strategy. Hall and Hanlon (2002) reported that females often rejected large males to later accept copulations with relatively smaller males. Consort males gain more copulations with the females they guard (Hall and Hanlon, 2002; Naud et al., 2004), and this is intuitively an advantageous strategy as males compete intensely for this role. However, small males might still achieve a competitive copulatory success overall, by investing less time per female and thereby being able to copulate with more females. A variety of chromatophore and postural displays are very common during pre-copulatory behaviour in cuttlefish (Corner and Moore, 1981; Boal, 1997; Norman et al., 1999; Adamo et al., 2000; Hall and Hanlon, 2002; Brown et al., 2012; Schnell et al., 2015; Kubodera et al., 2018). In some cases these displays have been suggested as means of courtship (Corner and Moore, 1981; Kubodera et al., 2018). However, there is no direct evidence of females preferentially mating with particular males that display different intensities of these displays, leading some authors to suggest that visual displays, observed during cuttlefish mating interactions, might be used for signalling agonistic intent and in sex recognition (Boal, 1997; Hall and Hanlon, 2002; Schnell et al., 2015).

During laboratory choice trials, *S. officinalis* females also showed no preference for male size or social hierarchy. However, females interestingly spent more time with males that had copulated more recently (Boal, 1997). This finding probably suggests one of two things: either (a) That females show preference for a male trait or behaviour based on chemical or visual cues that have not yet been measured (see section 3.3); or (b) That females can discern males' mating history, possibly based on chemical cues, and are attracted to males that have already established a high copulatory success. If the latter case applies, then there could be a selective advantage for females to prefer males with higher copulatory success, because this is likely to result in them having sons which also have higher copulatory success than competing males. In this way a female preference for male promiscuity could be reinforced through achieving more grandchildren, and this would lead to a *Fisherian* run-away process (Fisher, 1930).

Overall, the fact that there is intense male competition for females during spawning and that female cuttlefish frequently reject male copulation attempts, presumably based on cues other than size or hierarchy, support that female choice plays an important role in the differential reproductive success of male cuttlefish. However, it is still not certain what criteria females might use to discern between potential mates. Also, the details of female sperm storage, external fertilisation and vigilant mate guarding by consort males leading up to egg deposition all suggest that the timing and order of sperm placement are likely to influence the resulting fertilisation patterns (see section 4.5).

3.1.3. Octopodidae

Octopuses are different from cuttlefish and squid in that they are mostly solitary animals, with little to no social interactions outside of agonistic disputes over den space or mates (Cigliano, 1993; Huffard et al., 2008), cannibalism (Hanlon and Forsythe, 2008) and predominantly opportunistic copulations (Young, 1962; Anderson et al., 2003; cf. Huffard et al., 2008). Within mating systems that have been observed in the field, OSR is generally more balanced than is common in decapods (1:1–3.5:1 in *Abdopus aculeatus,* Huffard, 2005; 0.34:1–1.8:1 in *Octopus hubbsorum,* Lopez-Uriarte and Rios-Jara, 2009). This might suggest that pre-copulatory choice could be important for both male and female mate selection within this family, and is consistent with observations that females of at least three species can initiate copulations with males (*O. cyanea*, Wells and Wells, 1972; *Hapalochlaena lunulata*, Cheng and Caldwell, 2000; and *H. maculosa*, Morse et al., 2015). As mentioned previously, all members of the Octopodidae family are terminal spawners with the exceptions of *O. chierchiae* (Rodaniche, 1984) and LPSO (Caldwell et al., 2015).

There is limited evidence for sex recognition and courtship in octopuses. Cheng and Caldwell (2000) observed *H. lunulata* males to attempt copulations with other males as often as with females, suggesting no form of sex recognition prior to copulation in this species. However, *O. bimaculoides* are able to discriminate between different sexes of conspecifics based on odour cues (Walderon et al., 2011). It has been suggested that some octopuses use behavioural cues for sex recognition and possibly courtship. Packard (1961) suggested that male *O. vulgaris* might display their proximal suckers, which are sexually dimorphic and bigger on males in this species, to signal their sex and obtain copulations with females. However, in a follow-up study, males of this species were not observed to display their enlarged suckers to females during laboratory copulations, and therefore there would have been no opportunity for females to assess this trait (Wells and Wells, 1972). Voight (1991) has suggested that the ligula might be used in courtship and influential to male copulatory success. Ligulae have species-specific morphology among octopuses, and Voight (1991) reported male *O. digueti* to display their ligulae to females and make contact with females using their ligulae prior

to copulation. However, this study stated that no evidence of true courtship was found. A tactile phase leading up to copulation has also been noted within in pairs of *O. vulgaris, O. cyanea* and *O. tetricus* during laboratory observations (Wells and Wells, 1972; Morse, 2008), and it is possible that this behaviour enables female assessment of males' ligulae. Both *A. aculeatus* and *Amphioctopus marginatus* males have been observed in the field to display different chromatophore patterns to females before copulation (Huffard, 2007; Huffard and Godfrey-Smith, 2010), and in the case of *A. aculeatus*, males approached conspecifics differently depending on the colour pattern displayed by the approached individual. In addition to chromatophore displays, *A. aculeatus* pairs have been observed to synchronously perform a mantle bounce behaviour and females of this species have been observed to change postures to what is called a "DACT display" prior to copulation (Huffard, 2007).

It is possible that some of these behaviours are means of sex and/or species recognition but it is not clear if they are methods of courtship. Females of at least five species of octopus are known to frequently resist and/or reject male copulation attempts in laboratory conditions (*O. cyanea*, Wells and Wells, 1972; *O. digueti*, Voight, 1991; *O. tetricus*, Morse, 2008; *O. bimaculoides* Mohanty et al., 2014; and *H. maculosa* Morse et al., 2015). However, no investigations have yet successfully compared female receptivity to varying forms or intensities of the above traits and behaviours. Additionally, most observations of octopus reproductive behaviour have taken place in the laboratory, where artificial measures of OSR, and confined spaces that limit females' ability to reject copulations, make it difficult to accurately assess potential female preferences and/or which males will achieve higher copulatory success within natural mating systems.

Octopuses known to aggregate in the wild share certain behavioural characteristics in common. *A. aculeatus* (Huffard et al., 2008), LPSO (Caldwell et al., 2015) and *O. tetricus* (Scheel et al., 2016) show specific chomatophore and postural displays to conspecifics, and males and females occupy adjacent dens which facilitates repeated copulations among pairs. Of these three species, male *A. aculeatus* also exhibit high levels of aggression to compete for mate guarding status, denning proximity, and repeated copulations with larger females (Huffard, 2007; Huffard et al., 2008, 2010). Like in loliginid squids, both male and female *A. aculeatus* engaged in opportunistic copulations while foraging away from their dens, and smaller males attempted to gain sneaker copulations with guarded females by camouflaging themselves or hiding behind rocks to not instigate aggression from the guarding males (Huffard et al., 2008). In this species, females were observed to accept copulations with nearly all males. However, due to competition among males, large mate guarding males obtained higher copulation rates within the studied populations (Huffard et al., 2008). Like in loliginid squid and cuttlefish, the high levels of female promiscuity, sperm storage and mate guarding all suggest, that in addition to differential copulatory rates, sperm competition most likely plays an influential role in male reproductive success within shallow-water octopus mating systems (see section 4.6).

3.2. Differential Copulatory Success in Females

Currently, male preference of females and differential female copulatory rates have not been extensively noted within loliginid squid or cuttlefish taxa. One study reported that younger *S. australis* females laid more eggs than older females during 1 month of observations in aquaria (van Camp et al., 2005). These authors have suggested that this might signify male preference in this species toward younger females. However, females' capacity for sperm storage and intermittent egg laying among loliginid squid (Shaw and Sauer, 2004; Buresch et al., 2009) means that many of the females might not have finished laying eggs during the duration of this study, and so this limits direct evidence to support the theory of male choice in this species. Among the cuttlefish, male *S. apama* have been observed to preferentially attempt copulations with unfamiliar females (Schnell et al., 2015). However, this observation may have been indicative of the males withholding spermatophores from females with which they had already mated, and might not necessarily result in differential copulatory rates among females within this mating system.

There are however some indications of male choice for females in octopuses. Field observations of *A. aculeatus* have observed males to preferentially mate guard and copulate more with larger females, which are likely to have a higher egg-laying capacity than smaller females (Huffard et al., 2008). Males of this species were also observed to have longer bouts of male/male aggression over larger females, however were more likely to engage in competitive bouts over medium sized females which are less likely to soon be usurped by other larger males (Huffard et al., 2010). Similar observations have been made of *O. bimaculoides* in the laboratory, where higher levels of male-male aggression were reported in the presence of immature females (Mohanty et al., 2014). These authors have hypothesised that a first-male sperm precedence in fertilisation patterns could lead to a greater male investment toward mating with smaller or younger females in some *Octopus* mating systems. However, this hypothesis has yet to be verified through analyses of brood paternities.

Observations of male preference and differential female copulatory success in female octopuses, but not necessarily in the decapods are likely related to differences in OSR among these mating systems (**Table 2**). In loliginid squid and cuttlefish where OSR is more often to be heavily male biased (Hall and Hanlon, 2002; Jantzen and Havenhand, 2003), it is more likely that males might attempt copulations with every possible female they have access to. In shallow-water octopuses where the OSR is more balanced (Huffard, 2005; Lopez-Uriarte and Rios-Jara, 2009), male selection of females might be an important factor to female reproductive success.

3.3. The Roles of Signalling and Sensory Ecology in Precopulatory Mate Choice

3.3.1. Visual Signalling

Cephalopods possess a unique system of neurally controlled chromatophores, leucophores, iridiophores and dermal muscles that allow them to rapidly change the colour, tone, pattern and texture of their skin (Packard and Hochberg, 1977; Mäthger

TABLE 2 | The precopulatory behaviours of the Loliginidae, Sepiidae and Octopodidae families are summarised below.

Family	Spawning assemblages	OSR	Pair forming	Conditional mating strategies	Male agonistic behaviour	Mate guarding	Courtship	Female rejections	Female preference	Male preference
Loliginidae	Yes[1,2]	1:1–3:1[2]	Temporary (consort males)[1,2]	Yes[1,2]	Yes[1,2]	Yes[1,2]	Suggested in *L. vulgaris*[12]	In *S. lessoniana*[17]; not reported in other species	For large dominant males in *S. lessoniana*[17]	Not reported
Sepiidae	In some species[3,4]	3:1[3]–11:1[4]	Temporary (consort males)[4]	Yes[4]	Yes[4]	Yes[4]	Suggested in two species[3,13]	Yes (frequent)[4]	Unclear. Toward males that have mated more recently in *S. officinalis*[21]	Possibly toward novel females in *S. apama*[22]
Octopodidae	Not reported	0.34:1[5]–3.5:1[6]	Male and female pairs observed to occupy adjacent dens in four species[7,8,9,10]	Observed in *A. aculeatus*[8]	In at least two species[8,11]	Observed in *A. aculeatus*[8]	Suggested, but not confirmed in many species. Possible courtship through ligula contact[14]; and/or chromatophore displays[15,16]	In at least five species[18,14,19,11,20]	Unclear	Toward larger females in *A. aculeatus*[8]; and toward younger females in *O. bimaculoides*[11]

[1](Hanlon et al., 2002); [2](Jantzen and Havenhand, 2003); [3](Corner and Moore, 1981); [4](Hall and Hanlon, 2002); [5](Lopez-Uriarte and Rios-Jara, 2009); [6](Huffard, 2005); [7](Yarnall, 1969); [8](Huffard et al., 2008); [9](Caldwell et al., 2015); [10](Scheel et al., 2016); [11](Mohanty et al., 2014); [12](Hanlon et al., 1994); [13](Kubodera et al., 2018); [14](Voight, 1991); [15](Huffard, 2007); [16](Huffard and Godfrey-Smith, 2010); [17](Wada et al., 2005a); [18](Wells and Wells, 1972); [19](Morse, 2008); [20](Morse et al., 2015); [21](Boal, 1997); [22](Schnell et al., 2015). Taxonomic families and the sequence they are presented in are based on phylogenies described in Allcock et al. (2015).

and Hanlon, 2007). This ability enables cephalopods to employ impressive crypsis behaviours for defence against potential predators (Huffard, 2006; Krajewski et al., 2009; Staudinger et al., 2011). Additionally, several studies have identified cephalopods to use these pattern-changing abilities as a means of intra-specific signalling (Hanlon et al., 1994; Boal et al., 2004; Palmer et al., 2006). As mentioned above, visual displays using various chromatophore patterns have been observed in spawning assemblages of loliginid squid and cuttlefish, as well as in pre-copulatory behaviours of octopuses (Corner and Moore, 1981; Hanlon et al., 1994; Hall and Hanlon, 2002; Huffard, 2007; Huffard and Godfrey-Smith, 2010; Schnell et al., 2015). It is so far postulated that visual signals might aid in sex and species recognition, and for displaying agonistic intent between con-specifics (Boal, 2006; Scheel et al., 2016). However, no studies so far have shown a conclusive response of opposite sex receivers to these signals, which leaves the role of visual signalling in courtship unclear.

An important aspect of visual signalling in cephalopods is that most studied taxa are not able to discriminate between different wavelengths of light like in human colour vision (Messenger et al., 1973; Mäthger et al., 2009), but rather are sensitive to the angles in which light is travelling (Moody and Parriss, 1961; Saidel et al., 1983; Shashar et al., 1996). This is termed polarisation-sensitivity, and is common amongst invertebrates and has also been reported in some birds and fish (Cronin et al., 2003). Polarisation-sensitivity is considered especially useful in deep-water environments where the wavelength spectrum of light decreases with depth but properties of polarised light remain intact (Shashar and Cronin, 1996). The ability to discriminate polarised light properties likely helps cephalopods with both navigation and in locating crustacean prey-items that have highly polarised exoskeletons (Shashar and Cronin, 1996). However, cephalopods are also able to change the polarised patterns reflected from their skin using their chromatophores and iridiophores (Shashar et al., 1996; Boal et al., 2004). Because cephalopods use skin patterning for visual signalling (Palmer et al., 2006), are polarisation-sensitive (Moody and Parriss, 1961) and have the ability to alter the polarised patterns reflected from their skin (Shashar et al., 1996), this presents the likely possibility that that cephalopods might have the capacity to use polarised signalling as a means of intra-specific communication, imperceptible to the human eye (Mäthger et al., 2009).

Evidence for use of polarised signalling as a communication channel in cephalopods is still very limited. In the laboratory, *S. officinalis* responded differently to their own mirror image depending on whether or not the mirror distorted the reflectance of polarised light (Shashar et al., 1996), and female *S. officinalis* have been observed to display more polarised patterns than males (Boal et al., 2004). However, neither the quantity nor the nature of these displays differed in response to the number or sex of conspecifics viewed by the displaying female (Boal et al., 2004), making it unclear what type of information might be sent or received through polarised signals and what benefit these signals might have for the signaler or receiver. At present, no studies have yet incorporated imaging polarimetry within a context of investigating mate choice or potential

courtship. As visual signalling has been observed as an important component of pre-copulatory behaviour in studied cephalopods, the further integration of imaging polarimetry within field or laboratory mate choice studies might be an interesting topic to explore and could reveal substantially more information about cephalopod communication.

3.3.2. Chemoreception

Cuttlefish, squid, and octopus can sense chemical stimuli both from a distance using olfactory organs close to the eyes, and upon contact with objects using chemoreceptor cells located on the lips and suckers (Budelmann, 1996). Distance chemoreception between conspecifics has not yet been investigated within the Loliginidae, however at least some species have the capacity to obtain information from chemical stimuli in the water (Lucero et al., 1992). Tactile chemoreception of conspecific eggs has been investigated within *D. pealei,* and it is suggested that a pheromone present in egg capsules of this species triggers males to engage in male/male agonistic behaviour to compete over females (Buresch et al., 2003; King et al., 2003). This mechanism may be partially responsible for the synchronised spawning assemblages within loliginid taxa (Buresch et al., 2003; King et al., 2003). Distance chemoreception has been investigated in slightly more detail among the cuttlefish. *Sepia officinalis* increase ventilation rates when exposed to seawater containing odour from conspecifics, suggesting that this species can detect other members of its species based on chemical stimuli from a distance (Boal and Marsh, 1998). However, *S. officinalis* does not display any change in approach behaviour based solely on odours from conspecifics of different sex or mating history (Boal and Golden, 1999). Therefore, it is currently not supported that distance chemoreception would play a role in sex identification or mate choice in this species. However, it has not yet been assessed whether chemical cues might influence female receptivity to approaching males.

Distance chemoreception could play a role in the mating system of at least two octopus species. Laboratory trials with *O. bimaculoides* revealed that this species can detect conspecifics based on odour cues, and that ventilation rates of individuals were different depending on the sex of conspecifics that were detected (Walderon et al., 2011). Similar studies with *H. maculosa* found that the change in female ventilation rates in response to male odours correlated with agonistic behaviour and the probability that the female would reject a copulation attempt from the detected male (Morse et al., 2017). Therefore, distance chemoreception might enable some octopuses to determine the sex of conspecifics, and possibly to locate and/or discriminate between potential mates. Octopuses also possess many more chemoreceptors per sucker than decapods (10,000 cells per sucker in octopuses compared to ∼100 cells in cuttlefish suckers, Budelmann, 1996). This is likely related to the way in which octopuses reach into holes and crevices while foraging for food (Budelmann, 1996). However, a neurological study in *O. vulgaris* has also identified that the olfactory lobes, responsible for processing the sensory of chemical stimuli, are integrated with parts of the brain that regulate signal molecules involved in reproductive behaviours as well as feeding (Polese et al., 2015). As mentioned above, a tactile phase prior to copulation has been observed in *O. vulgaris* and *O. cyanea* (Wells and Wells, 1972), *O. digueti* (Voight, 1991) and *O. tetricus* (Morse, 2008). It is feasible that because octopuses have well-developed tactile chemoreception, that this could be used by some species to identify species, sex or possibly relatedness and/or quality or potential mates. As yet, the role of tactile chemoreception in mate choice has not been investigated within any cephalopod mating system.

4. POST-COPULATORY SEXUAL SELECTION IN COASTAL CEPHALOPODS

4.1. The Role of Sperm Competition in Sexual Selection

The aspects of female promiscuity and sperm storage strongly suggest that post-copulatory processes take an influential role in sexual selection within cephalopod mating systems. The previous section addressed how different traits or behaviours can lead to differential copulatory rates within species. However, reproductive success is based on the quantity of alleles passed on to future generations, and in highly promiscuous mating systems where females store sperm from multiple males in between egg laying intervals, copulatory rates alone will not necessarily determine the reproductive success of individuals. The differential fertilisation success between males that have copulated with the same female is referred to as sperm competition (Parker, 1970). Sperm competition can impact the relative reproductive success of males if certain morphological traits or behaviours can help some males to achieve increased fertilisation success (Parker, 1970). Sperm competition can also affect the reproductive success of females if fertilisation can be biased toward males that are more genetically compatible (Zeh and Zeh, 1996, 1997; Tregenza and Wedell, 2000; Mays and Hill, 2004), or if whichever trait or behaviour used by males to achieve higher fertilisation success can be inherited by their sons (Yasui, 1997; Kokko et al., 2003).

A multitude of factors can affect sperm competition in animal mating systems. Several of these include: The numbers of males contributing sperm to a female (Parker, 1990), the relative contributions of sperm provided by each male (Parker, 1990), removal of previous males' sperm by subsequent male partners (Birkhead and Hunter, 1990), preferential locations for sperm placement (Naud et al., 2005), differential sperm motility (Birkhead et al., 1999), cryptic female choice (CFC) of sperm (Eberhard, 1996), and differential longevity of sperm and/or stratification of sperm within sperm storage receptacles that can lead to differences in fertilisation success based on the order of copulation by competing males (Birkhead and Hunter, 1990; Naud and Havenhand, 2006; Squires et al., 2015; Hirohashi et al., 2016). The current understanding of how sperm competition might impact cephalopod mating systems is still in its infancy. However all of the above mechanisms could potentially influence the relative fertilisation success of male cephalopods (**Table 3**). The following will summarise the current knowledge of sperm competition in cephalopods based

TABLE 3 | Sperm competitive behaviours and evidence for cryptic female choice among five studied families of Cephalopoda are summarised below.

Family	Sperm loading	Sperm removal	Differential sperm placement	Dimorphic sperm	Evidence for separation of sperm in female storage organ(s)	Evidence for cryptic female choice	Known Predictors of paternity
Loliginidae	Yes[1,2]	Not reported	Yes[1,2]	Yes[12]	Distinct switch in embryo paternity along *L. reynaidiii* egg strings[13]; mating plugs in *D. plei*[14]	Female *D. pealei* can eject unwanted spermatophores, and possibly influence paternity by delaying egg deposition[2]	Higher copulatory rates[1,2]; internal spermatophore placement[1]; CFC[2]; and interval between copulation and egg deposition[2]
Idiosepiidae	Yes[3]	Not reported	Not reported	Not reported	Not reported	Female *I. paradoxus* observed using their buccal mass to remove recently transferred spermatophores[3]	Not yet investigated. However, mating chronology[3] and duration[19] are suggested to influence paternity
Sepiolidae	Not reported	Suggested[7]	Not reported	Not reported	Suggested due to invaginations of the spermatheca[8]	Hypothesised[16]	Last male paternity bias[16]
Sepiidae	Yes[4,5]	Yes[8,4,9]	Yes[11]	Not reported	Different sperm compositions observed in paired receptacles of *S. apama*. Females also have access to separate spermatangia externally placed by males[11]	Preferential sperm use from externally placed spermatangia in *S. apama*[2]	External placement of spermatophores[11]; CFC[11]; and suggestion of bias to recently mated males[20]
Octopodidae	Hypothesised[6]	Suggested[10]	Not reported	Not reported	Evidence suggests that sperm is mixed in the oviduccal glands[15]	Female *O. vulgaris* can control release of peptides that influence chemotaxis of sperm[17]; possible removal of unwanted spermatophores in *O. oliveri*[18]	Paternity has been anecdotally observed to correlate with copulation duration[21]; mating chronology[15,18]; terminating member of copulation[15]; male size[18] and male relatedness to the female[15]

[1](Iwata et al., 2005); [2](Buresch et al., 2009); [3](Sato et al., 2016); [4](Hall and Hanlon, 2002); [5](Wada et al., 2006); [6](Morse et al., 2015); [9](Squires et al., 2013); [8](Hanlon et al., 1999); [9](Wada et al., 2010); [10](Cigliano, 1995); [11](Naud et al., 2005); [12](Iwata et al., 2018); [13](Naud et al., 2016); [14](Saad et al., 2018); [15](Morse et al., 2018a); [16](Squires et al., 2015); [17](De Lisa et al., 2013); [18](Ylitalo et al., 2019); [19](Sato et al., 2016); [20](Hanlon et al., 2005); [21](Morse, 2008). Taxonomic families and the sequence they are presented in are based on phylogenies described in Allcock et al. (2015).

on observations of sperm loading, sperm removal, female choice of sperm, sperm morphology and relative paternity patterns.

4.2. Sperm Competition in Loliginidae

Sperm competition has been investigated relatively more thoroughly in loliginid squid than in other cephalopod taxa. The current literature suggests that males employ sperm loading (Hanlon et al., 1997, 2002; Jantzen and Havenhand, 2003), but that also sperm placement (Iwata et al., 2005), the interval between copulation and egg deposition (Buresch et al., 2009; Hirohashi et al., 2016), spermatophore morphology (Iwata et al., 2018) and possibly CFC of stored sperm (Shaw and Sauer, 2004; Buresch et al., 2009) can all influence fertilisation patterns. Loliginid males compete for consort status with females that they copulate with repetitively and guard from rival males (Hanlon et al., 1997, 2002; Jantzen and Havenhand, 2003). This suggests that sperm loading may be important for male fertilisation success. Sperm loading has been confirmed in laboratory paternity experiments with *Heterololigo bleekeri* (Iwata et al., 2005) and *Doryteuthis pealei* (Buresch et al., 2009) where higher copulatory rates, and presumably more spermatophores transferred, resulted in higher male fertilisation success. These studies also found that paternities were biased to males that copulated with females in a parallel position that enabled internal placement of spermatophores (Iwata et al., 2005; Buresch et al., 2009), however that sperm from sneaker males, placed in the seminal receptacles, had greater longevity (Hirohashi et al., 2016).

A recent investigation of spermatophore morphology in the chokka squid, *Loligo reynaudii* revealed that large and small males, which typically employ consort and sneaker mating strategies, respectively, have distinct types of spermatophores specialised for their mating strategy and method of sperm transfer (Iwata et al., 2018). However, genotyping of *L. reynaudii* egg strings obtained from wild spawning assemblages have confirmed that paternity was typically biased to the male observed guarding the female at the time of collection (Naud et al., 2016). Interestingly, microscopy analyses of female seminal receptacles in *D. plei* have revealed that everted spermatophores can act as a mating plug, limiting females' ability to access stored sperm from competing males the female had previously mated with for up to 48 h (Saad et al., 2018). These authors hypothesised that this could be a sperm competitive strategy, advantageous for sneaker males that can intercept females between copulations with consort males and egg deposition. Overall, paternity bias to higher copulatory rates, the parallel mating strategy and mate guarding males suggest that consort males will generally achieve higher fertilisation success within the mating systems of loliginid squids, and this further explains both why males compete vigorously for this mating strategy (Hanlon et al., 1997, 2002; Jantzen and Havenhand, 2003) and why sneaker males have to metabolically invest so heavily into producing competitive sperm (Hirohashi et al., 2016; Iwata et al., 2018; Saad et al., 2018).

Female loliginid squid may also have the capacity to influence the paternities of their egg capsules post-copulation. A female *D. pealei* has been observed to eject spermatophores from her mantle after a forced copulation (Buresch et al., 2009), and these authors also identified that the interval between copulation and egg deposition greatly affects egg capsule paternity. When females of this species laid egg capsules within 40 min of copulation, the egg capsules were fertilised mostly by older sperm from previous male partners. However, after 140 min, paternity of egg capsules was biased to the most recent male to have copulated with the female. Additionally, Naud et al. (2016) observed a distinct switch in embryo paternity along *L. reynaudii* egg strings, suggesting that females of this species might have been using different males' sperm for egg fertilisation in non-random patterns. If females can reject spermatophores and presumably can choose when to lay egg capsules (Buresch et al., 2009), then these observations combined with females' capacity to be selective of stored sperm use during external fertilisation (Shaw and Sauer, 2004; Naud et al., 2016), suggest that female loliginids might be capable of controlling which males' sperm fertilise their egg capsules.

4.3. Sperm Competition in Idiosepiidae

Owing to their ease of husbandry in captivity (Nishiguchi et al., 2014) there is a currently growing literature investigating the mating system of idiosepiids. Relatively recent laboratory investigations have revealed novel insights to processes of post-copulatory mate choice within the Japanese pygmy squid (*Idiosepius paradoxus*), suggesting that both sperm competition and CFC might be prevalent within the mating system of this taxon (Sato et al., 2013, 2016). These studies indicated that both larger males and males who copulated for longer with females, externally transferred more spermatophores to the base of females' arms during mating (Sato et al., 2016). However, females of this species were observed to use their buccal masses to remove spermatophores from larger males, favouring the retention of spermatophores by males who copulated with them for longer durations (Sato et al., 2016). Additionally, these females were more likely to be selective of transferred spermatophores during subsequent copulations, suggesting possible female post-copulatory trade-up behaviour (see Pitcher et al., 2003) in this species (Sato et al., 2013). Such findings emphasise that both mating chronology and mating duration may be of critical importance to paternal success within this idiosepiid, but interestingly analysis of brood paternity in *I. paradoxus* did not reveal any fertilisation bias to recently mated males (Sato et al., 2016).

4.4. Sperm Competition in Sepiolidae

Similar to the idiosepiids, the amenability of some sepiolids to the laboratory environment has prompted recent and novel investigations of sperm competition within this family. Squires et al. (2013) observed that male *Euprymna tasmanica* repeatedly contract their mantle "pumping" while copulating with females. Interestingly, this study found that the frequency of this male pumping behaviour increased with number of copulations the female recently had, and these authors hypothesised that this pumping behaviour might indicate male removal of accessory seminal fluids left behind by competing males. This correlation of pumping behaviour and female mating history implies that male *E. tasmanica* were able to assess the amount of sperm stored

by females. However, in this study the males also increased the frequency of their pumps even when they had recently mated with the same female, suggesting that they might not be able to recognise their mates or whether the stored sperm was their own (Squires et al., 2013).

Analysis of brood paternities in *E. tasmanica* revealed that multiple paternity is very common (Squires et al., 2014) but that paternity is frequently biased to the last males to mate with the female (Squires et al., 2015). The spermatheca of female *E. tasmanica,* the site of sperm storage, has been described as "invaginated" and "highly pocketed" (Norman and Lu, 1997; Squires et al., 2013), suggesting that females may be able to partition different males' sperm and use it selectively to fertilise their eggs through CFC. Despite indications of a last-male sperm bias and the potential capacity of females to partition male sperm, evidence of female choice either in the forms of pre-copulatory trade-up behaviour or CFC have not yet been observed in this family.

4.5. Sperm Competition in Sepiidae

Sperm competition behaviours have been relatively well-documented within cuttlefish taxa during observations in both the laboratory and field. Cuttlefish males perform sperm removal (Hanlon et al., 1999; Wada et al., 2005b, 2006, 2010), some degree of sperm loading (Hall and Hanlon, 2002; Wada et al., 2006) and non-random patterns of fertilisation have been observed within females' egg masses (Naud et al., 2005). As mentioned in section 3.1.2, cuttlefish males compete for consort status with females whom they guard from rival males and occasionally pass more than one spermatophore (Corner and Moore, 1981; Adamo et al., 2000; Hall and Hanlon, 2002). Copulating multiple times with the same female and mate guarding suggests that relative sperm contributions are likely important for fertilisation success within these mating systems. Additionally, Hanlon et al. (1999) observed three stages of copulation in *Sepia officinalis*. The first stage, which is the longest, is spent using the siphon to flush water over females' buccal areas, likely attempting to remove sperm from either the seminal receptacles or from spermatangia left on females' exterior. The second stage consisted of males transferring new spermatophores to females' buccal membranes, and the third stage was spent breaking open the newly placed spermatophores and ensuring spermatangia attachment.

Male sperm removal has also been indicated in *S. apama* (Hall and Hanlon, 2002; Naud et al., 2004). However, Naud et al. (2005) found that water flushing did not reduce the counts of spermatangia found on females' buccal areas in *S. apama*, suggesting that males of at least this species possibly aim to remove sperm specifically from within seminal receptacles. Male *S. lycidas* use arm III to scrape old sperm masses from females' buccal areas, and spend more time doing this if they are not the last male to have copulated with the female (Wada et al., 2010). This same study identified that larger males of this species will also spend more time than smaller males removing sperm. These authors suggest that smaller males might choose to pass spermatophores sooner if copulation might be likely to get interrupted by a larger male. *Sepiella japonica* has also been observed to briefly remove previous males' spermatangia using arm IV (Wada et al., 2006). However, these authors suggest that male *Sepiella* spp. might invest more time toward sperm loading than removal compared to *Sepia* spp. In this study, *S. japonica* males were observed to display intense mate guarding and in most cases would transfer more than one spermatophore to guarded females.

Currently, cuttlefish fertilisation patterns have been investigated only within wild populations of *S. apama.* Naud et al. (2004) found that males of all sizes and mating strategies had equal fertilisation success among eggs sampled from spawning areas. However, paternity comparisons within individual females' egg clutches were biased to spermatangia left on females' mantles and buccal areas in Naud et al. (2005). This suggests that it might be advantageous for males to copulate with females shortly before egg deposition and to place sperm externally on females rather that in the seminal receptacle. This pattern is supported in a study by Hanlon et al. (2005), in which a female-mimicking sneaker male that achieved a copulation with a female directly prior to egg deposition, was observed to fertilise that egg. If there is a last-male paternity bias to egg fertilisation in *S. apama* this would emphasise the importance of male sperm removal behaviour, and the monopolisation of access to females by consort males near and at egg-laying sites (Hall and Hanlon, 2002).

It is also noteworthy that Naud and Havenhand (2006) discovered that sperm, stored within intact spermatophores, can have longevities up to 2 months. As *Sepia* spp. are intermittent terminal spawners (Rocha et al., 2001), this suggests that females might be able to use stored sperm for future egg fertilisations, and might possibly do so outside of spawning aggregations. Future studies investigating which males' sperm are stored in seminal receptacles versus placed externally as spermatangia might yield further information about sperm competition in this species. Also, the combination of female pre-copulatory choice of males (section 3.1.2) with the suggestion of a last-male paternity bias, presents a question of whether females might assess potential male partners differently based on the types of males they have recently copulated with. Future studies observing female receptivity to sequential males, either in the field or laboratory, might elucidate whether female trade-up behaviour occurs in cuttlefish mating systems.

4.6. Sperm Competition in Octopodidae

The mechanisms of sperm competition are much less understood within octopus mating systems. It is probable that males of several species perform sperm loading and sperm removal. However, this has only been formally addressed within one laboratory study (Cigliano, 1995), and currently very few controlled paternity experiments have allowed fertilisation success to be compared among different males (cf. Morse, 2008; Morse et al., 2018a; Ylitalo et al., 2019). Copulation durations are generally much longer in octopuses than in decapods (Joll, 1976; Morse et al., 2018a). Copulations have been observed to last more than an hour in most studied taxa, with the longest copulation being reported as 360 min in laboratory observations of *Octopus tetricus* (Joll, 1976).

Field observations of *Abdopus aculeatus* also report males to guard and copulate repeatedly with certain females (Huffard et al., 2008), and laboratory studies with *Hapalochlaena maculosa* have observed males of this species to mate for longer with both unfamiliar females and females that had recently mated with another competing male (Morse et al., 2015). Prolonged copulation durations, mate guarding and multiple copulations with the same females suggest that sperm loading might be an important factor for male fertilisation success. However, it is currently not known whether longer copulation times allow males to pass more spermatophores to females, and/or also might allow males to remove more sperm from previous males, or whether increased copulation time might also be a form of mate guarding whereby the males could be monopolising female opportunities to mate with competing males.

One study, assessing sperm transfer in an unidentified pygmy octopus, found that this species had three phases of copulation, similar to sepiids (Cigliano, 1995). This author suggested that males might use their ligulae to remove competing sperm from females' oviducts during an initial phase of copulation, prior to transferring new spermatophores. Males were also observed to spend more time with the ligula inserted in the female's mantle cavity prior to spermatophore transfer if the female had recently copulated with a different male. However, males spent less time doing this if they were the last males to copulate with the same female. Males could apparently assess females' recent mating history based on the presence or absence of sperm in either the distal portion of females' oviducts or the oviducal glands (Cigliano, 1995). However, it is impressive that males were able to determine if that sperm was their own, as the mechanism enabling them to do this is currently unknown and evidence for mate recognition among octopuses is very limited (Boal, 2006; but cf. possible cases in Caldwell et al., 2015; and Morse et al., 2015).

Four molecular studies within the Octopodidae have so far confirmed multiple paternities within egg clutches of *O. tetricus* (Morse, 2008), *O. vulgaris* (Quinteiro et al., 2011), *H. maculosa* (Morse et al., 2018a) and *O. oliveri* (Ylitalo et al., 2019). It has so far been postulated that female octopuses might benefit from polyandry due to increased genetic diversity of their offspring (Quinteiro et al., 2011) and the likelihood of reducing fertilisation to related males (Morse et al., 2018a). However, these hypotheses have not yet been empirically tested. As copulation durations are markedly longer in octopuses than decapods (Joll, 1976; Morse et al., 2018a), it remains an interesting question whether female octopuses might be able to influence male fertilisation success by controlling the duration of copulations with different males. Studies of two octopus species have observed females to consistently be the terminating member of copulations, suggesting that they might have control over the length of their copulation time (*O. digueti*, Voight, 1991; and *H. lunulata*, Cheng and Caldwell, 2000). Additionally, a combination of male mating order and whether or not a copulation was ended by the female had a strong effect on paternal success in *H. maculosa* (Morse et al., 2018a). Therefore, female control of copulation time could be a possible form of intra-copulatory mate choice in some species. However, where studied, there is mixed support for the correlation of copulation duration and male fertilisation success (Morse, 2008; Morse et al., 2018a).

Although not yet empirically demonstrated, the reproductive system of female octopuses suggests that CFC may also occur in this family. Female octopuses possess paired, muscular and innervated oviducal glands (Froesch and Marthy, 1975), which they could theoretically use to selectively pump, or block access of sperm to the egg during fertilisation. Additionally, chemoattractant peptides have been found in egg capsules of *O. vulgaris*, that can influence the chemotaxis of male sperm (De Lisa et al., 2013). This suggests that both mechanical and chemical processes might potentially be used by some female octopuses in manipulating the storage or fertilisation success of different males' sperm in their oviducal glands. However, at present there is very little evidence that female octopuses do this (Morse et al., 2018a).

5. CONCLUSIONS AND NEW DIRECTIONS FOR RESEARCH

5.1. A Summary of Pre- and Post-copulatory Behaviours Warranting Further Investigation Among Well-Studied, Coastal Cephalopods

Currently, the mechanisms of sexual selection are more thoroughly understood within some decapod mating systems than in those of octopuses. The coastal spawning aggregations of *Sepia apama* and loliginid squid have enabled much more detailed investigations within natural settings. Within loliginid mating systems, females of most studied species appear receptive to copulations with every attempting male (cf. *Sepioteuthis lessoniana,* Wada et al., 2005a). However it is strongly suggested that females might be selective of which sperm they use to fertilise egg capsules (Naud et al., 2016). As copulations are usually very quick in these taxa (2–39 s in Hanlon et al., 2002), it might be more parsimonious for females to avoid potential male aggression and the time or energy spent on rejecting males, by being receptive to every copulation and then to control egg capsule paternities post-copulation. Continued observations in the field might be able to further identify the context of both spermatophore rejections and varying intervals between copulation and egg deposition. If females eject spermatophores more or less often with and/or can adjust the timing of egg capsule deposition after copulating with different males that have varying displays, mating strategies or morphologies, then females might use these mechanisms as a form of CFC to bias paternity to genetically fitter and/or more compatible males (Eberhard, 1996; Tregenza and Wedell, 2000).

Within cuttlefish mating systems, females appear highly selective of male partners. It is presently unknown what cues females might use to discriminate between potential males, whether certain males get preferential spermatophore placement in females' seminal receptacles or buccal areas, whether the suggestion of a last-male paternity bias is accurate and whether

this consistently leads to increased female selectivity with successive males. It is suggested here that further studies of cuttlefish taxa, either in the field or in large aquaria with male-biased OSR, might provide this information if they can asses the context of different spermatophore placements, compare egg paternities to the order of copulation with genotyped males and compare female-male rejection rates between the first and subsequent males that attempt to copulate with females within egg-laying intervals.

There is still much that can be learned about the processes of mate choice and sperm competition among the octopuses. Further observational studies and/or laboratory choice trials in species where visual courtship displays and female-male rejection are common might unveil whether cues such as ligula morphology or visual displays influence pre-copulatory mate choice in these taxa. Additionally, further paternity comparisons with genotyped candidate fathers (e.g., Morse et al., 2018a) across additional taxa could reveal whether certain types of males gain higher fertilisation success within octopus mating systems, and also whether female brood paternities might be biased toward longer copulation durations, indicating sperm loading, or toward recent males, suggesting the influence of sperm-removal. If sperm loading is identified as an important factor in male fertilisation success, then it will be worthwhile investigating differential copulation durations in species where copulations are frequently terminated by females. This might determine whether females can influence their brood paternities by adjusting copulation times with males that display different morphology or behaviour.

As females of at least two octopus species are suggested to be capable of conspecific sex recognition based on odour cues (Walderon et al., 2011; Morse et al., 2017), it is worthwhile continuing to investigate the role of both distance- and tactile chemoreception within octopus mating systems. Two interesting follow-up questions that could be investigated within laboratory experiments are a) Whether males respond differently to touch or odours from sexually mature vs. immature females; and b) Whether either sex responds differently to touch or odours from novel vs. familiar conspecifics. Answering these questions could help to define the role of chemosensory in octopus social recognition and mate choice behaviours. Additionally, as mentioned in section 3.3.1, visual displays have been reported as part of pre-copulatory behaviour of all loliginids, cuttlefish and octopuses studied in the field (Corner and Moore, 1981; Hanlon et al., 1994; Hall and Hanlon, 2002; Huffard, 2007; Huffard and Godfrey-Smith, 2010; Schnell et al., 2015). However, in order to make sense of these behaviours it is necessary to interpret how these displays are perceived by receiving conspecifics. As most cephalopods are polarisation-sensitive (Moody and Parriss, 1961), yet colour-blind (Mäthger et al., 2009), the further integration of imaging polarimetry into field studies and laboratory mate choice trials is suggested to reveal valuable information about the way cephalopods might communicate within spawning assemblages or in a context of sex identification and/or courtship (**Table 4**).

5.2. Addressing Widespread Polyandry Among the Cephalopod Class

A common theme amongst all studied cephalopod mating systems is the extremely high level of both male and female promiscuity (Hall and Hanlon, 2002; Hanlon et al., 2002; Huffard et al., 2008; Arnold, 2010; Squires et al., 2013; Morse et al., 2015). Male promiscuity is common within animal mating systems, and can develop easily as an evolutionarily stable strategy (see Smith, 1982) because promiscuity directly increases male reproductive success (Bateson, 1983). Female promiscuity is less commonly reported among species where females do not receive material resources or parental care from the males they mate with, because females have a finite number of eggs they can lay in a lifetime and therefore their reproductive success is typically not limited by the numbers of males they can copulate with (Kodric-Brown and Brown, 1987). Additionally, copulating with lots of different males can be potentially quite costly to females due to the increased risk of injury during copulations (Adamo et al., 2000; Hoving et al., 2010), decreased foraging time (Huffard et al., 2008), increased risk of disease transfer (Thrall et al., 2000), increased energy expenditure (Franklin et al., 2012) and decreased life expectancy (Franklin and Stuart-Fox, 2017). As polyandry appears to be an evolutionarily stable strategy among cephalopods, it is inferred that promiscuous females must achieve some type of selective advantage over non-promiscuous females in order to offset the inherent costs of multiple mating described above.

So far, polyandry in cephalopods has been suggested to benefit females by either helping to overcome potential sperm-limitation (van Camp et al., 2004), increasing the genetic diversity of females' offspring (Quinteiro et al., 2011), and/or optimising offspring quality (Squires et al., 2012; Naud et al., 2016; Morse et al., 2018a). Sperm limitation might be an important factor to female reproductive success in species that have high egg-laying capacities and that might have infrequent encounters with opposite sex conspecifics (e.g., *Architeuthis* spp., Hoving et al., 2004). However sperm limitation probably cannot explain polyandrous behaviour in female cephalopods that have smaller fecundities and that would have the capacity to fertilise all their offspring to one male (e.g., Sepiolidae or *Hapalochlaena* spp.). Offspring diversity probably does increase the fitness of promiscuous females. However, this mechanism alone being the drive for cephalopod polyandry is not consistent with observations of female-male rejections in many taxa, or with observed paternities consistently biased toward particular males (Iwata et al., 2005; Naud et al., 2005, 2016; Morse, 2008; Buresch et al., 2009; Squires et al., 2015; Morse et al., 2018a; Ylitalo et al., 2019) rather than shared more equally between candidate fathers as would be expected in a bet-hedging strategy.

The optimisation of offspring quality appears to be a robust hypothesis for the evolution of polyandry in cephalopod mating systems (Squires et al., 2012; Naud et al., 2016). However, the exact processes for how female promiscuity might lead to enhanced offspring quality still remain unclear. It has been previously hypothesised that nutritional benefits provided from

TABLE 4 | New directions for the research of sexual selection in cephalopods are summarised below. Ten questions warranting further investigation in the near future are presented alongside summaries of potential methodology for addressing them.

Theme	10 questions worth continued investigation	Examples of methodology
Precopulatory Behaviour	How do females of some cuttlefish and octopus species discriminate among male visual displays?	Compare rates of female rejection/receptivity to varying intensities of display. Ideally incorporate imaging polarimetry to quantify and simulate how displays are perceived by the female.
	Do female cuttlefish perform trade-up[1] mate choice behaviour?	Compare rates of female-male rejection among controlled sequential laboratory pairings. Additionally, confirm prevalence of last male paternity using genotyped candidate fathers.
	What are the roles of chemoreception in social recognition and mate choice?	In spp. that cannot visually recognise individuals, assess whether subjects can recognise individuals through distance or tactile chemoreception. Compare ventilatory and/or retreat response to odours and/or touch of familiar vs. novel conspecifics.
	How do males of some octopus species recognise if they were the last male to mate with a female?	Assess whether male octopuses can distinguish the spermatophores/spermatozoa of other males from their own using tactile chemoreception.
	Is sexual selection for sophisticated reproductive behaviours partially responsible for the evolution of complex cognition among cephalopods?	Make a comparative study examining performance on tasks assessing cognitive attributes such as object permanence, working memory, and theory or mind among cephalopod taxa with a variety of reproductive strategies. Use principal component analyses to identify whether particular reproductive dynamics, such as spawning in assemblages, is a predictor of cognitive performance.
Postcopulatory Processes	What criteria influences CFC in cephalopods with external fertilisation?	In controlled laboratory conditions, further identify what factors and/or context (e.g., male phenotype or mating order) lead to higher rates of spermatophore removal and/or delay in egg deposition in spp. where CFC is easily observable (e.g., *D. pealei*).
	How might sperm-attractant peptides influence fertilisation patterns of octopuses?	Use laboratory pairings of genotyped candidate parents, and compare (A) resulting paternity; (B) allelic signatures of sperm remaining in oviducal glands after egg deposition; and (C) concentrations of sperm-attractant peptides in the female reproductive tract at different intervals between copulation with each male and egg deposition.
	Is CFC more common in species where either female-male rejections are rare, or copulations are often forced by males?	Use a meta-analysis to compare presence of CFC behaviour with rates of female rejection and forced copulations among studied cephalopod species.
	Can a "good sperm" hypothesis[2] help to explain widespread polyandry among cephalopods?	Identify whether copulatory rates and/or fertilisation success are correlated among fathers and sons (e.g., heritable) within each laboratory-amenable family.
	Does polyandry help to facilitate inbreeding avoidance?	In spp. with limited dispersal (e.g., *Euprymna* spp. or *H. maculosa*) compare paternal success among genotyped candidate parents having variable but known relatedness to the female.

[1] *(Pitcher et al., 2003)*; [2] *(Yasui, 1997)*.

accessory seminal fluids, obtained either through spermatophore consumption or absorption within the female reproductive tract, can help to increase metabolic resources females have available toward producing healthy offspring (Squires et al., 2012; Wegener et al., 2013). A controlled study of *Euprymna tasmanica* has indicated that females of this species, which mated multiply, laid eggs with a higher hatchling to egg mass ratio than females that were only allowed to mate once (Squires et al., 2012). These authors suggested that the added nutritional benefit of receiving extra spermatophores might enable females of some species, particularly ones with internal sperm storage, to maximise their reproductive output relative to maternal investment. However, Squires et al. (2012) also advocated that nutritional benefits likely coincide with indirect, genetic benefits of female promiscuity to provide selective advantages for the widespread polyandry observed among cephalopod taxa.

Postcopulatory fertilisation bias to either reproductively successful males or genetically compatible males are two indirect mechanisms that could also lead to selective advantages for polyandry (Zeh and Zeh, 1996, 1997; Yasui, 1997). However, at present neither scenario has yet been investigated within a cephalopod mating system. Postcopulatory mechanisms might be especially applicable if females either cannot accurately assess male fitness or relatedness during pre-copulatory choice, and/or have limited control of which males they copulate with. In these contexts, polyandrous females could theoretically benefit from accepting sperm from multiple males if differential sperm fertilisation ability, or CFC consistently bias brood paternities to either the fittest or least related males. In the former scenario, if females' offspring are disproportionately sired to males that are innately capable of obtaining a higher fertilisation success, then promiscuous females are also likely to have sons with higher fertilisation success and therefore

more grandchildren than non-promiscuous females (Yasui, 1997). This mechanism could potentially be investigated within laboratory paternity comparisons over several generations, and might be supported if copulatory rates and/or fertilisation success are correlated between fathers and their sons. In the case of post-copulatory mechanisms biasing paternity to genetically compatible males, it is possible that female promiscuity is a form of ensuring inbreeding avoidance (see Tregenza and Wedell, 2002). A recent molecular study assessing the relatedness of populations in a holobenthic octopus with limited dispersal revealed high frequencies of close relatives within spawning sites (up to 78% half-half sibling pairs in *H. maculosa*, Morse et al., 2018b). Genomic studies within wild populations across additional cephalopod taxa, and paternity comparisons with known relatedness between mothers and candidate fathers could explain whether inbreeding avoidance might be one of the evolutionary drives for promiscuous behaviour in cephalopods (**Table 4**).

5.3. The Mating Behaviour of Most Cephalopods Is Still Unknown

Finally, it is worth noting again that the bulk of current knowledge for cephalopod sexual selection is still confined to the five families: Loliginidae, Idiosepiidae, Sepiidae, Sepiolidae and Octopodidae. The extreme depths, pelagic environments and specialised nutritional requirements of pelagic and/or deep-sea cephalopod taxa make it difficult to observe them in their natural habitats or maintain them for robust laboratory studies (Hoving et al., 2013). However, at least nautilids appear amenable to aquarium settings (Mikami and Okutani, 1977; Arnold, 2010), and hopefully methods will become available in the future for maintaining other deep-sea or pelagic cephalopod species successfully in the laboratory. Investigating pre-copulatory behaviour and fertilisation patterns of additional cephalopod taxa, either through laboratory rearing or ROV voyages, can likely provide valuable context to the current understanding of sexual selection and behavioural ecology in this unique class of animals.

AUTHOR CONTRIBUTIONS

PM compiled the literature and was the primary author. CH provided an expert review of content and revisions to the original manuscript.

ACKNOWLEDGMENTS

We would like to thank the James Cook University School of Research for helping to fund the stipend and lab space during part of the writing of this manuscript. We would also like to thank Kyall Zenger, Mark McCormick, Mark Meekan, Graziano Fiorito, and Annie Lindgren for their helpful feedback on an earlier version of this manuscript. Thank you also to three reviewers, whose insights assisted in strengthening this manuscript. Finally, I would like to thank Joe Rosin for his assistance with the title of section 5.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01035/full#supplementary-material

Supplementary Video1 | A short copulation in a pair of southern blue-ringed octopus (*Hapalochlaena maculosa*). The male inserts both right arm 2 and his hectocotylus (right arm 3) into the female's right mantle aperture at sec 21, and can be seen removing them at sec 26. This copulation was likely interrupted by the presence of the camera. Video by P. Morse.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 23 August 2019
doi: 10.3389/fphys.2019.01051

Interrelationship Between Contractility, Protein Synthesis and Metabolism in Mantle of Juvenile Cuttlefish (*Sepia officinalis*)

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *26 April 2019*
Accepted: *31 July 2019*
Published: *23 August 2019*

Citation:
Lamarre SG, MacCormack TJ, Bourloutski É, Callaghan NI, Pinto VD, Andrade JP, Sykes AV and Driedzic WR (2019) Interrelationship Between Contractility, Protein Synthesis and Metabolism in Mantle of Juvenile Cuttlefish (Sepia officinalis). Front. Physiol. 10:1051. doi: 10.3389/fphys.2019.01051

Young juvenile cuttlefish (*Sepia officinalis*) can grow at rates as high as 12% body weight per day. How the metabolic demands of such a massive growth rate impacts muscle performance that competes for ATP is unknown. Here, we integrate aspects of contractility, protein synthesis, and energy metabolism in mantle of specimens weighing 1.1 g to lend insight into the processes. Isolated mantle muscle preparations were electrically stimulated and isometric force development monitored. Preparations were forced to contract at 3 Hz for 30 s to simulate a jetting event. We then measured oxygen consumption, glucose uptake and protein synthesis in the hour following the stimulation. Protein synthesis was inhibited with cycloheximide and glycolysis was inhibited with iodoacetic acid in a subset of samples. Inhibition of protein synthesis impaired contractility and decreased oxygen consumption. An intact protein synthesis is required to maintain contractility possibly due to rapidly turning over proteins. At least, 41% of whole animal $\dot{M}O_2$ is used to support protein synthesis in mantle, while the cost of protein synthesis (50 μmol O_2 mg protein^{-1}) in mantle was in the range reported for other aquatic ectotherms. A single jetting challenge stimulated protein synthesis by approximately 25% (2.51–3.12% day^{-1}) over a 1 h post contractile period, a similar response to that which occurs in mammalian skeletal muscle. Aerobic metabolism was not supported by extracellular glucose leading to the contention that at this life stage either glycogen or amino acids are catabolized. Regardless, an intact glycolysis is required to support contractile performance and protein synthesis in resting muscle. It is proposed that glycolysis is needed to maintain intracellular ionic gradients. Intracellular glucose at approximately 3 mmol L^{-1} was higher than the 1 mmol L^{-1} glucose in the bathing medium suggesting an active glucose transport mechanism. Octopine did not accumulate during a single physiologically relevant jetting challenge; however, octopine accumulation increased following a stress that is sufficient to lower Arg-P and increase free arginine.

Keywords: anaerobic metabolism, cycloheximide, glucose, iodoacetic acid, jetting, octopine

INTRODUCTION

Cephalopods have a short life span and a semelparous reproductive pattern which gives them the reputation of living in the "fast lane." Early juvenile cuttlefish (*Sepia officinalis*) grow at a rate of up to 12% body mass per day (Sykes et al., 2006, 2014). To support this rapid growth rate, cuttlefish mantle muscle has an unusually high fractional rate of protein synthesis compared to most fish and other invertebrate species (Lamarre et al., 2016). The cost of protein synthesis accounts for a major proportion of overall metabolic rate in marine organisms and is typically estimated between 11 and 42% of total oxygen consumption (Fraser and Rogers, 2007). To our knowledge, the cost of protein synthesis has never been measured in cephalopods but both protein synthesis and metabolic rates are highly sensitive to fasting in cuttlefish (Lamarre et al., 2016); this observation suggests that protein synthesis accounts for a substantial proportion of basal oxygen consumption in cephalopods as well.

Cuttlefish and other cephalopods utilize jet propulsion as a means of locomotion to avoid predators and other disturbances. Jet propulsion is energetically inefficient relative to the undulatory swimming used by fish and perhaps the best demonstration of this is provided by O'Dor and Webber (1986), who determined that the net cost of transport (J Kg^{-1} m^{-1}) is 3–4.5 times higher in squids compared to trout. This means that in order to travel the same distance, cephalopod mantle muscles have to work much harder than fish swimming muscles. For squids, the magnitude of this differential is not equal for all body sizes as jetting efficiency scales allometrically, with juveniles being more efficient than adults (O'Dor and Hoar, 2000; Bartol et al., 2008). In mammalian skeletal muscles, secondary metabolic demands are also imposed on the tissue during recovery from intense exercise in the form of an increase in rates of protein synthesis (Wong and Booth, 1990a,b; Biolo et al., 1995; Phillips et al., 1997). To our knowledge, the occurrence of this phenomenon has never been examined in cephalopods. If rates of protein synthesis are already maximized to support growth in juvenile cuttlefish, frequent jetting events may require a redistribution of resources away from growth and toward recovery, prolonging the time to maturity. If they do maintain scope to further increase rates of protein synthesis in the mantle muscle following a jetting event, it may impose yet another energetic demand on their already highly solicited aerobic metabolism.

Jetting events result from powerful contractions of the mantle muscle. In squid and cuttlefish forced to exercise to exhaustion, activity is supported by anaerobic means involving the transphosphorylation of arginine-phosphate and the breakdown of glycogen to generate ATP, with octopine generated as an end-product (Storey and Storey, 1979; Pörtner et al., 1993). In contrast, in scallops forced to swim to exhaustion or exposed to acute hypoxia, little octopine accumulates during the challenge; the main increase in octopine concentration is instead observed during the subsequent recovery period (Gäde et al., 1978; Grieshaber, 1978). This delayed increase in octopine production may be caused by the low affinity of octopine dehydrogenase (ODH) for pyruvate, which does not reach a sufficient concentration to activate the enzyme until anaerobic glycolysis has been upregulated (van Os et al., 2012). If protein synthesis is activated to facilitate recovery from exercise, the additional energetic demands it imposes must be supported by either aerobic or anaerobic metabolism. Characterizing which specific means of generating ATP are utilized will allow a better understanding of growth and development of cuttlefish and inform aquaculture practice with respect to dietary requirements.

Here, we tested the hypothesis that the protein synthesis is stimulated following muscle contractions simulating a jetting event in the mantle of early juvenile cuttlefish. We examined electrically stimulated mantle muscle strip preparations and integrated information from different levels of organization to develop a better understanding of the energetics in mantle of young animals. Preparations were forced to contract at a frequency observed under normal jetting activity (Trueman, 1980) and were treated with cycloheximide (CHX), which interferes with the translocation step in protein synthesis, and iodoacetic acid (IAA), that inhibits glycolysis at the level of glyceraldehyde-3-phosphate dehydrogenase. The contractile protocol was subsequently applied to determine rates of tissue oxygen consumption ($\dot{M}O_2$), rates of protein synthesis, glucose utilization, and octopine production. The data set allowed a calculation of a protein energy budget and provides new insights into the subtle importance of glucose trafficking in mantle muscle.

MATERIALS AND METHODS

Ethics Statement

All the procedures were approved by the CCMAR Animal Welfare Committee (ORBEA CCMAR-CBMR) and the Direcção-Geral de Alimentação e Veterinária (DGAV) of the Portuguese Government, according to National (Decreto-Lei 113/2013) and the EU legislation (Directive 2010/63/EU) on the protection of animals used for scientific purposes. In addition, protocols were approved by institutional Animal Care Committees at Université de Moncton (UdeM-18-02) and the Memorial University of Newfoundland. Procedures were only applied to live animals by authorized users.

Animal Husbandry and Euthanasia

Experiments were done during May 2018, at CCMAR's Ramalhete Aquaculture Station (Ria Formosa, Portugal – 37°00′22.39″N; 7°58′02.69″W). Cuttlefish (*S. officinalis*) were reared from hatching to the juvenile stage, in a 1500 L round fiberglass black tank in an open seawater system, according to the latest culture technology described in Sykes et al. (2014). All procedures were applied to juveniles of a F2 captive stock. Animals were 3–8 weeks post hatch (final part of the hatchling stage and entering the juvenile stage) with a mass of 1.1 ± 0.04 (SEM) g (N = 41). Temperature, salinity, and dissolved oxygen saturation (DO_2) were measured daily, at 9 h 30 min, in the stock tank. Both temperature and DO_2 were measured with a VWR DO220 probe, while salinity was measured with a

VWR EC300 salinity meter. Water temperature was 20.8 ± 1.14 (SD) °C, salinity was 34.6 ± 0.71 (SD) g L^{-1} and DO_2 was 101.0 ± 1.60 (SD)%. Cuttlefish were fed thawed grass shrimp (*Palaemonetes varians*) *ad libitum* on a daily basis during the experimental period.

In terminal experiments, animals were euthanized in seawater containing 10% ethanol (Sykes et al., 2012). Time to cessation of ventilation was ≈2 min and, afterward, assurance of death was achieved by ventral bisection of the brain and severing of the ventral nerve and optical lobes (Lewbart and Mosley, 2012). Specimens were splayed open to expose two wings of mantle tissue which was subsequently sampled and carefully skinned.

Whole Animal Oxygen Consumption

Resting $\dot{M}O_2$ was assessed using an automated intermittent flow respirometry system (Q-Box AQUA, Qubit Systems, Kingston, ON, Canada). Animals were removed from their holding tank, weighed, and quickly transferred to a 215 mL cylindrical respirometry chamber (4 cm diameter). The chamber was housed in a darkened 10 L reservoir of continuously aerated seawater at 20°C. Animals were transferred into the respirometry chamber at ≈16:00 h, and $\dot{M}O_2$ was monitored overnight. Oxygen levels were recorded at 15 min intervals throughout the experiment: 5 min with the system in closed loop followed by a 10 min flush cycle between each reading. Animals were housed in the respirometer for 8–16 h, and $\dot{M}O_2$ returned to baseline levels within the first 2–3 h after transfer into the system. The resting $\dot{M}O_2$ values reported here represent the mean of at least measurements taken in the final 2 h of the experiment. $\dot{M}O_2$ was corrected for any background level.

Experimental Design and Incubation Media

All experiments, other than whole animal $\dot{M}O_2$ measurements, involved isolated preparations of either mantle strips (oxygen consumption and contractile studies) or mantle sheets (protein synthesis and metabolite levels), which were prepared as described below. The overall experimental design is shown in **Figure 1** and discussed in detail in the appropriate sections that follow. Basic incubation medium consisted of 0.22 μm filtered sea water supplemented with 1 mmol L^{-1} glucose. The one exception to this was an experiment to test the immediate impact of high frequency stimulation on metabolite levels in which the bathing medium did not contain glucose. Medium was further supplemented with either 25 μmol L^{-1} CHX to impair protein synthesis, with the other preparation receiving an equivalent volume of DMSO as a vehicle control, or 1 mmol L^{-1} IAA to impair glycolysis.

Contractile Performance of Mantle Strips

Contractile performance of mantle preparations was measured to assess the impact of inhibition of protein synthesis and glycolysis and to set the experimental parameters for metabolic studies. Animals were euthanized as described above and paired circumferential sections (i.e., perpendicular to the long axis of the animal) of ≈8 mm long by 1 mm wide were cut from the widest (funnel-side) side of the ventral mantle. The skin was gently removed by blunt dissection and a 6-0 silk suture was tied around one end of each muscle strip. The opposite end of the muscle strip was then clamped between two platinum electrodes in a double-walled 20 mL Plexiglas chamber and the suture thread fastened to a calibrated isometric force transducer (Harvard Apparatus, South Natick, MA, United States). The chambers contained basic incubation medium. The medium was continuously aerated and maintained at 20.0 ± 0.01°C using a recirculating water bath. Strips were stimulated to contract via field stimulation across the platinum electrodes using a Grass SD9 stimulator (Grass Technologies Inc., Warwick, RI, United States) and were gently stretched to optimal peak tension development prior to commencing each experiment, as in previous studies (Milligan et al., 1997). Force transducers were interfaced to a PowerLab 4/26 (ADInstruments, Colorado Springs, CO, United States) data acquisition system and data were recorded and analyzed using associated LabChart 8 software.

After mounting, preparations were allowed to equilibrate without stimulation for 10 min before being triggered to contract six times, as a measure of basal performance, at a frequency of 0.2 Hz using 100 V (nominal), 5 ms duration square wave pulses. One of the paired preparations was then treated with either CHX or IAA. Preparations were incubated for an additional 10 min, stimulated six times at 0.2 Hz to assess treatment effects on basal contractility, and then stimulated at 3 Hz for 30 s for a total of 90 contractions to simulate a maximum jetting event. The latter stimulation frequency corresponds to the reported maximum jetting frequency of *S. officinalis* (Trueman, 1980) and the 30 s duration of the challenge reflects the approximate time to 50% loss of maximum force production assessed in preliminary preparations. Following the maximum jetting protocol, muscles were left unstimulated for 1 h to recover before being forced again to contract six times at 0.2 Hz.

Tension measurements were normalized to absolute stress using the nominal cross-sectional area of the preparation and an assumed muscle density of 1.06 g cm^{-3} (Layland and Altringham, 1995), as previously described (Shiels et al., 2010). Each data point represents the mean of at least three representative contractions. Muscle performance was calculated as the change in peak tension (F_{max}) compared to tension developed under the initial six contraction response that served as the hallmark. F_{max} following the simulated jetting protocol was quantified from the final three contractions in the 30 s stimulation train.

Oxygen Consumption, Protein Synthesis, and Metabolite Levels During Recovery From Jetting

Rates of oxygen consumption and protein synthesis were determined during the recovery period and metabolite levels after the recovery period following the simulated maximum jetting challenge. The same mantle sheet preparations provided enough tissue mass for measurements of protein synthesis and metabolites; whereas, $\dot{M}O_2$ studies were from mantle strips prepared as above.

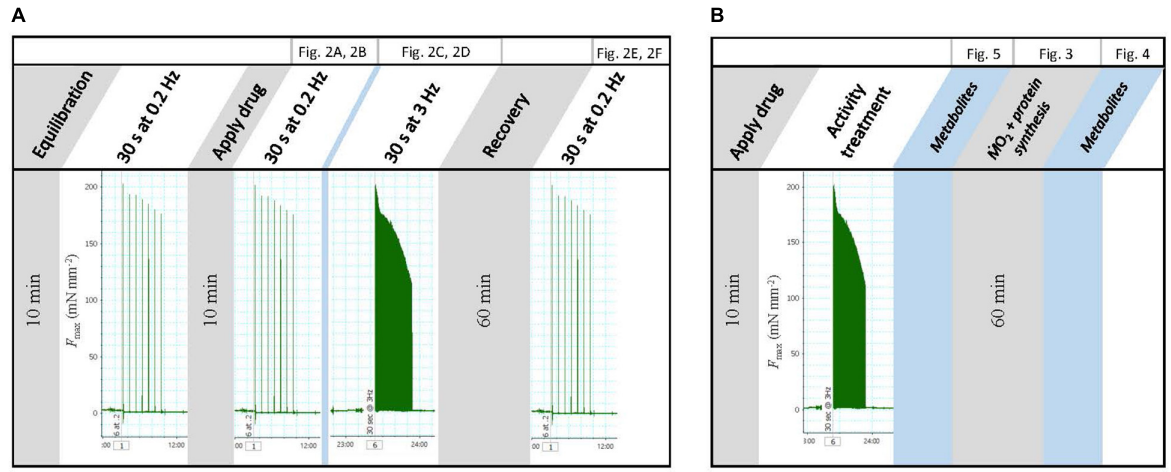

FIGURE 1 | Schematic of experimental design. **(A)** Contractility study. **(B)** $\dot{M}O_2$, protein synthesis, and metabolite levels.

Oxygen Consumption

The rate of oxygen consumption was measured in paired preparations of mantle strips with a mass of 14.96 ± 3.43 mg. Following isolation, strips were maintained for 10 min in either basic medium (N = 5) or medium containing CHX (N = 4) or IAA (N = 4). Thereafter, one strip was immediately placed in the respiration chamber. The second strip was induced to contract at 3 Hz for 30 s (90 contractions); field stimulation was again applied with 100 V square wave pulses of 20 ms duration with a switch in polarity at 15 s. Following contraction, the strip was immediately placed in the respirometry chamber equilibrated with the same incubation medium. The system consisted of a pair of 1 mL respiration chambers fitted with freshly calibrated Clark type polarographic electrodes and magnetic stirrers (OX1LP, Qubit Systems Inc., Kingston, ON, Canada). The temperature was maintained at 20.0 ± 0.01°C using a recirculating water bath. Oxygen concentration was recorded at a rate of 1 Hz using Logger Pro 3.12 (Vernier Inc., Beaverton, OR, United States). Once tissue O_2 consumption decreased the O_2 concentration below 6.8 mg L^{-1}, the medium was aerated for approximately 20 s, until it reached saturation (oxygen saturation was considered to equal 7.2 mg L^{-1} in 35 g L^{-1} seawater at 20.0°C). O_2 concentration was recorded this way for up to 60 min. Between 6 and 12 $\dot{M}O_2$ measurements per mantle strip were obtained. After correcting for the background O_2 consumption (average of 35.9 ± 15.1% of total O_2 consumption) $\dot{M}O_2$ is reported in nmol O_2 g^{-1} min^{-1}. The relationship between $\dot{M}O_2$ and time was then fitted using an exponential decay function. This function was then used to integrate the total O_2 consumption for a period of 60 min which we report as μmoles O_2 g^{-1} of mantle.

Protein Synthesis

For measurements of protein synthesis and metabolite levels, mantle sheets were prepared by isolating mantle as described above, and dividing it into two pieces with a mass of 112.00 ± 3.00 mg. Each sheet preparation was then immediately placed into basic incubation medium. Following a 10 min incubation in media with glucose alone or supplemented with CHX or IAA, one of the paired mantle preparations was induced to contract as described above (N = 6 for all conditions). The two mantle sheets were then immediately transferred into two 15 mL centrifuge tubes each containing 2 mL of fresh incubation medium for 1 h. The incubation medium was supplemented with 0.75 mmol L^{-1} phenylalanine and 0.75 mmol L^{-1} deuterated phenylalanine (ring-D5-phenylalanine, Cambridge Isotope Laboratories Inc., Tewksbury, MA, United States). Oxygen concentration was maintained constant by gently bubbling the medium with air. Following the incubation period, the mantle sheets were delicately blotted dry to remove excess incubation medium and flash frozen in liquid nitrogen. An aliquot of the incubation medium was frozen for later glucose and octopine analysis.

The rate of protein synthesis (ks) was determined by measuring the incorporation of deuterated phenylalanine into protein using gas chromatography and mass spectrometry (GC-MS) as previously described (Lamarre et al., 2015, 2016). Briefly, ≈10 mg of mantle sheet was homogenized in 0.2 mol L^{-1} perchloric acid (PCA) using a sonicating homogenizer (Q55 Sonicator, Qsonica Inc., Newtown, CT, United States). The homogenate was centrifuged at 10,000 × g for 5 min at 4°C. The supernatant was then transferred into a clean tube and frozen as it contained the free phenylalanine pool. The protein pellet was washed three times by resuspending it in 0.2 mol L^{-1} PCA and centrifuging as above. The protein pellet was then hydrolyzed in 6 mol L^{-1} HCl at 110°C for 18 h. Phenylalanine was extracted from the free amino acid pool and the protein-bound pool using solid phase extraction (Bond Elut C18, Agilent Inc., Santa Clara, CA, United States). The final eluate was evaporated to dryness and stored at 4°C until GC-MS analysis. The extracted amino acids were derivatized using pentafluorobenzyl bromide as an alkylating agent and analyzed in the GC-MS as described in Lamarre et al. (2015). The system was composed of an Agilent gas chromatograph (model 7890B) interfaced with a single quadrupole mass selective detector (MSD 5977B). Peak

detection and integration were performed using MassHunter (Version B07.01 SP2, Agilent). *ks* (% day^{-1}) was calculated using $k_s = 100 \times \frac{S_b}{S_a} \times \frac{1440}{t}$, where S_b and S_a are the deuterated phenylalanine enrichment of the protein-bound and free pools, respectively; t is the incorporation time in min and 1440 is the conversion from min to day.

Metabolite Analysis

Tissue preparation for analysis of octopine, arginine, and glucose followed standard protocols previously used with *S. officinalis* mantle (Storey and Storey, 1979; Capaz et al., 2017). Mantle was homogenized in 6% cold PCA. Following centrifugation at 10,000 × *g* for 10 min, the supernatant was neutralized with 2 mol L^{-1} $KHCO_3$ and recentrifuged prior to analysis. Octopine and glucose were also determined in incubation media of isolated mantle preparations. All assays were conducted using a microplate reader. Octopine and arginine concentration were determined based on the mmol L^{-1} extinction coefficient of 6.22 for NADH at 340 nm and the calculated pathlength (Capaz et al., 2017). For assay purposes, ODH, that catalyzes the conversion of pyruvate + L-arginine + NADH + H^+ to octopine + NAD^+ + H_2O was purified from scallop muscle as described in Capaz et al. (2017). Octopine concentration was assessed in 100 mmol L^{-1} TRIS, 10 mmol L^{-1} NAD+, pH 9.3. Following addition of excess ODH the reaction was monitored for 2 h to ensure it ran to completion. Mantle extracts from a large *S. officinalis* (≈500 g) were stimulated to tetanus to accumulate a high amount of octopine; serving in this way as a positive control and ensuring that the assay conditions were not limiting. Arginine was measured in media containing 50 mmol L^{-1} imidazole, 4 mmol L^{-1} pyruvate, 0.4 mmol L^{-1} NADH, and excess ODH, pH 7. Tissue arginine content was calculated from standard curves. An attempt was made to determine the level of arginine phosphate following acid hydrolysis (Langer et al., 1976) as successfully applied to jumbo squid (Seibel et al., 2014). In our hands the procedure yielded only qualitative information. Glucose was determined with an assay kit: D-Glucose GOD-POD (NZYTech, Lisbon, Portugal).

Impact of Jetting on Metabolite Levels

A limited experiment was also conducted to assess the immediate effect of high frequency electrical stimulation on mantle muscle metabolite levels. Paired mantle sheets were prepared as above. In this instance, the mantle sheets were incubated in 0.22 μm filtered seawater without any further additions or in media containing glucose and IAA. The experimental design was based on noted high tissue levels of glucose in the major study and was intended to assess if glucose was called upon during the fast contraction challenge. A second pair of preparations was treated with IAA to inhibit glycolysis and to potentially gain further insight into the role of intracellular glucose during contraction.

Data Analysis and Statistics

Data presented in text and figures are expressed as mean ± standard error. Difference in contractile performance between control and either CHX or IAA treated preparations was assessed with an unpaired *t*-test. For mantle O_2 consumption, rate of protein synthesis, and metabolite levels, two-way ANOVAs with repeated measures were used to assess differences between treatments. Rate of glucose and octopine uptake or release into the incubation media was assessed with a one sample *t*-test versus a theoretical value of zero. GraphPad Prism was used for statistical analysis and statistical significance was set at $p < 0.05$ in all cases.

RESULTS

Mantle muscle accounted for 28.2 ± 1.16% and head 33.6 ± 1.44% of total body mass (N = 8). Whole animal $\dot{M}O_2$ was 5.47 ± 0.46 μmol g^{-1} h^{-1} (N = 6).

Contractility of Mantle Muscle

Isometrically contracting mantle muscle strips remained functionally viable beyond the duration of the experimental challenges reported here. Average peak tension (F_{max}) development following the initial 10 min equilibration period was 104 ± 18 mN mm^{-2} (N = 22) and in concurrent studies, a number of preparations exhibited F_{max} values in excess of 300 mN mm^{-2} (data not shown). As such, the preparation is considered to be robust and a suitable model for both contractile and metabolic studies.

Tension development in the second wave of six contractions increased following the 10 min unstimulated incubation period in control preparations by approximately 35 mN mm^{-2} (**Figures 2A,B**; open bars), suggesting the initial 10 min acclimation may not have been sufficient for full recovery. The post rest potentiation was significantly reduced in strips exposed to IAA relative to controls (p = 0.036; **Figure 2B**). A similar pattern of F_{max} inhibition was noted in muscles treated with CHX, but the effect was not statistically significant (p = 0.113) (**Figure 2A**).

All muscles were able to pace at 3 Hz with no increase in resting tension or evidence of tetanus. Most preparations were able to sustain full F_{max} for the first ≈45 contractions in the simulated maximum jetting protocol (3 Hz for 30 s) before steadily decaying to values 10–75% of initial after 90 contractions. No significant effects of CHX or IAA treatment relative to controls were noted during this challenge (**Figures 2C,D**).

Control preparations more than fully recovered from the jetting protocol after a 1 h rest period, exhibiting F_{max} ≈ 50% higher than hallmark initial levels. Preparations treated with CHX to block protein synthesis failed to recover to the same extent as paired control muscles (p = 0.027), although F_{max} levels did return to pre-exercise levels (**Figure 2E**). Inhibiting glycolysis with IAA abolished this potentiation of F_{max} (p = 0.044) and preparations failed to fully recover from the challenge (**Figure 2F**).

Oxygen Consumption During Recovery From Simulated Jetting

$\dot{M}O_2$ was determined for isolated mantle strips. One of two paired mantle preparations was subjected to stimulation

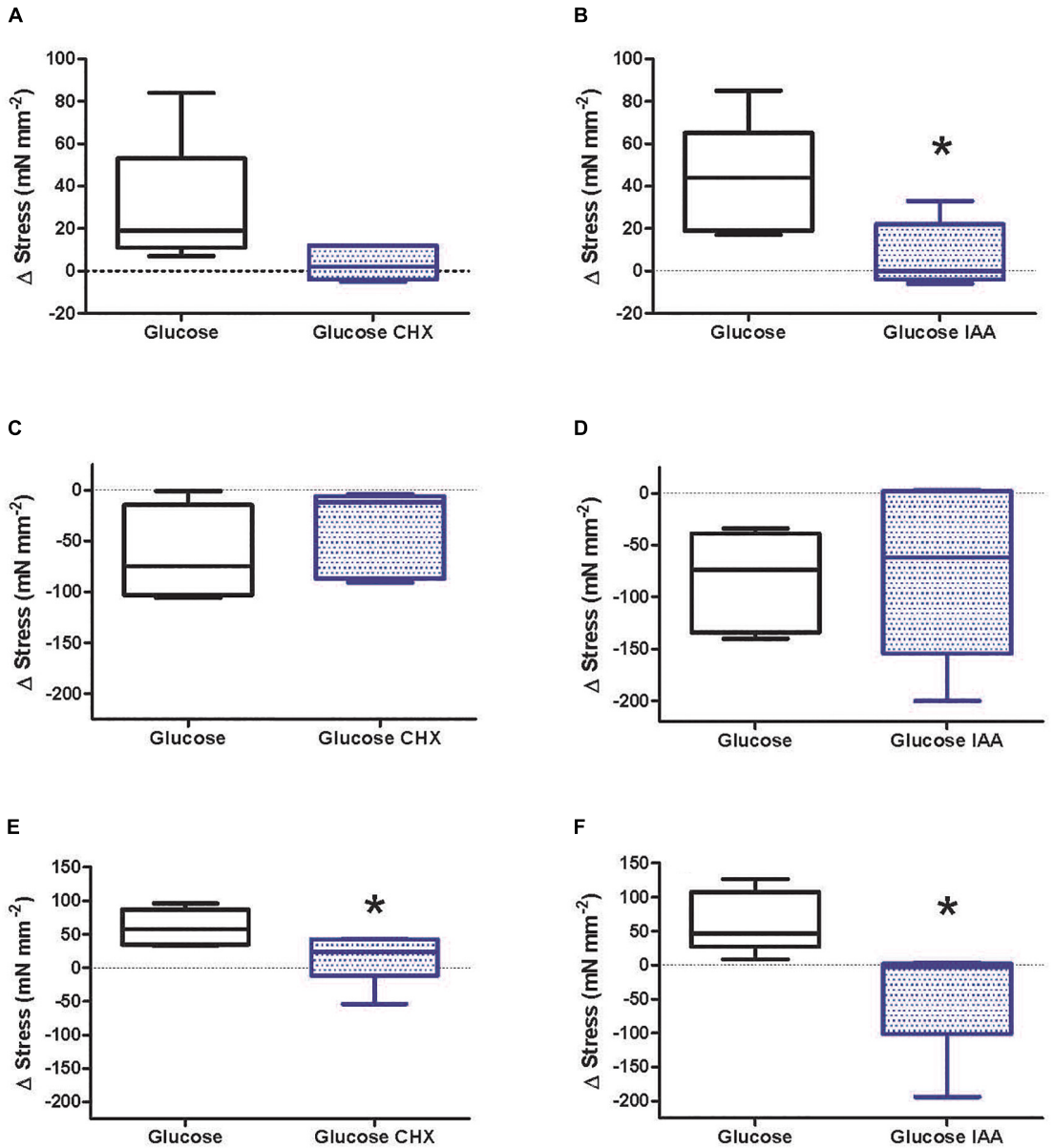

FIGURE 2 | Relative peak stress development by isometrically contracting mantle muscle strips from cuttlefish (*Sepia officinalis*). Preparations were initially stimulated to contract six times at 0.2 Hz. Stress developed during this period served as the level at which all further challenges were related. All experiments involved paired preparations. Bathing media contained glucose (open bars) plus additional 25 μM CHX (stippled bars; left panels) or additional 1 mM IAA (stippled bars; right panels). Panels **(A,B)** – Δ stress development following a 10 min rest followed by a second wave of six contractions at 0.2 Hz. Panels **(C,D)** – Δ stress development following contractions for 30 s at 30 Hz [immediately following **(A,B)**]. Panels **(E,F)** – Δ stress development following 1 h rest [following **(C,D)**]. *Significant difference in response of matched preparation in media containing glucose alone relative to preparations in media with either additional CHX or IAA. *N* = 6 for CHX experiments and *N* = 5 for IAA experiments. Box and whisker plots show the range and median value.

and thereafter incubated for 1 h in various media. This challenge was similar to that reported in **Figures 2E,F** in the contractile study. **Figure 3A** shows a representative response for an individual preparation incubated in basic medium. All preparations showed initial elevations in $\dot{M}O_2$ presumably in part due to oxygen limiting conditions and/or elevated energy demand at some point in setting up the tissue from dissection through the pre-incubation and stimulation periods to measurements of $\dot{M}O_2$ of the incubates. The total oxygen consumed during the incubation period, normalized to 1 h, is presented in **Figure 3B**. The total oxygen consumed was significantly higher in preparations incubated with glucose alone than with either CHX or IAA included in the media. This was the case for analysis within unstimulated and stimulated preparations. Total oxygen consumed was significantly (30%) higher in preparations that had been

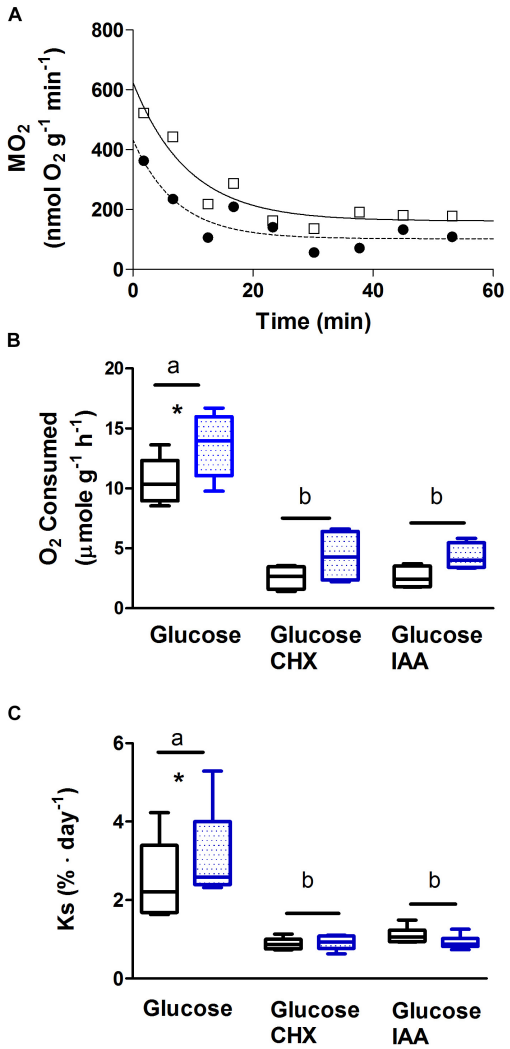

FIGURE 3 | Rate of oxygen consumption and protein synthesis by isolated mantle muscle strips from cuttlefish (*Sepia officinalis*). One of two paired mantle strips was stimulated to contract for 30 s at 3 Hz simulating jetting behavior. Oxygen consumption and protein synthesis was then measured during the recovery period. **(A)** Representative trace of rate of oxygen consumption. The curve with squares and solid line represents oxygen consumption by a mantle preparation that had been forced to contract; the dashed line with circles is the matched unstimulated paired preparation. **(B)** Average rate of oxygen consumed over a 40–60 min period following contraction. **(C)** Rate of protein synthesis measured over a 1 h period following contraction. Open bars represent unstimulated preparations; stippled bars indicate preparations that were stimulated. Text below *x*-axis shows additions to media. For CHX and IAA additions, preparations were incubated for 10 min prior to stimulation, during stimulation, and throughout the post contraction incubation period. For $\dot{M}O_2$ measurements $N = 7$ for glucose; $N = 4$ for CHX and IAA. For protein synthesis $N = 6$ in all cases. Values with different letters are significantly different. *Significant difference between unstimulated and stimulated preparations. Box and whisker plots show the range and median value.

stimulated (13.6 ± 1.2 µmol g^{-1} h^{-1}) to contract prior to the incubation period than unstimulated (10.5 ± 0.9 µmol g^{-1} h^{-1}) preparations when incubated in medium containing glucose alone. This trend was also observed when CHX or IAA was included in the media.

Protein Synthesis During Recovery From Simulated Jetting

One of two paired mantle sheets was subjected to stimulation as above and thereafter incubated for 1 h in various media. **Figure 3C** shows the mean rate of protein synthesis in the hour following the stimulation. The rate of protein synthesis in preparations incubated with glucose alone in the medium was significantly higher than in preparations receiving either CHX or IAA. This occurred for both unstimulated and stimulated preparations. When incubated in media containing glucose alone, preparations that had been stimulated ($3.1 \pm 0.5\%$ day^{-1}) showed a significant increase in the rate of protein synthesis by 24% over control unstimulated preparations ($2.5 \pm 0.4\%$ day^{-1}). There was no difference in the rate of protein synthesis between unstimulated and stimulated preparations when the media contained either CHX or IAA.

Metabolite Levels During Recovery From Simulated Jetting

Metabolite levels in mantle following the 1 h recovery period are shown in **Figure 4**. Glucose content of mantle sheets was approximately 3 µmol g^{-1} under all conditions (**Figure 4A**). There was no difference due to either stimulation or incubation condition in glucose content of mantle sheets. Mantle sheets were incubated in media containing 1 mmol L^{-1} glucose. There was no change in glucose concentration in the medium for preparations incubated with medium containing only glucose or in those containing glucose and CHX (**Figure 4B**). In preparations incubated with IAA in the medium there was a net increase in glucose in the medium (i.e., release from tissue) at a rate of approximately 3.3 µmol g^{-1} h^{-1}. Eleven IAA-treated preparations ($N = 5$ unstimulated; $N = 6$ stimulated) showed an increase in glucose in the medium. Stimulation prior to the incubation period had no effect on glucose uptake or release.

Following 1 h of incubation, the mean level of mantle muscle octopine ranged from 1.43 to 2.99 µmol g^{-1}. There was no significant difference in octopine content as a function of media composition or between unstimulated and stimulated preparations under any condition (**Figure 4C**). Octopine levels increased in the media during the incubation period under all conditions at mean rates of between 0.04 and 0.4 µmol g^{-1} h^{-1} (**Figure 4D**). There was no difference in the rate of octopine production as a function of stimulation or additions to the incubation media.

Following 1 h of incubation the mean level of arginine ranged from 12 to 16 µmol g^{-1} (**Figure 4E**). There was no significant difference in arginine content as a function of media composition. Also, stimulation had no effect on arginine level in paired preparations regardless of experimental condition. It was not possible to quantitate arginine phosphate with the method utilized; however, 28 of 36 preparations from the incubated preparations showed detectable arginine phosphate with a typical content of 3 µmol g^{-1}. This value must be viewed with

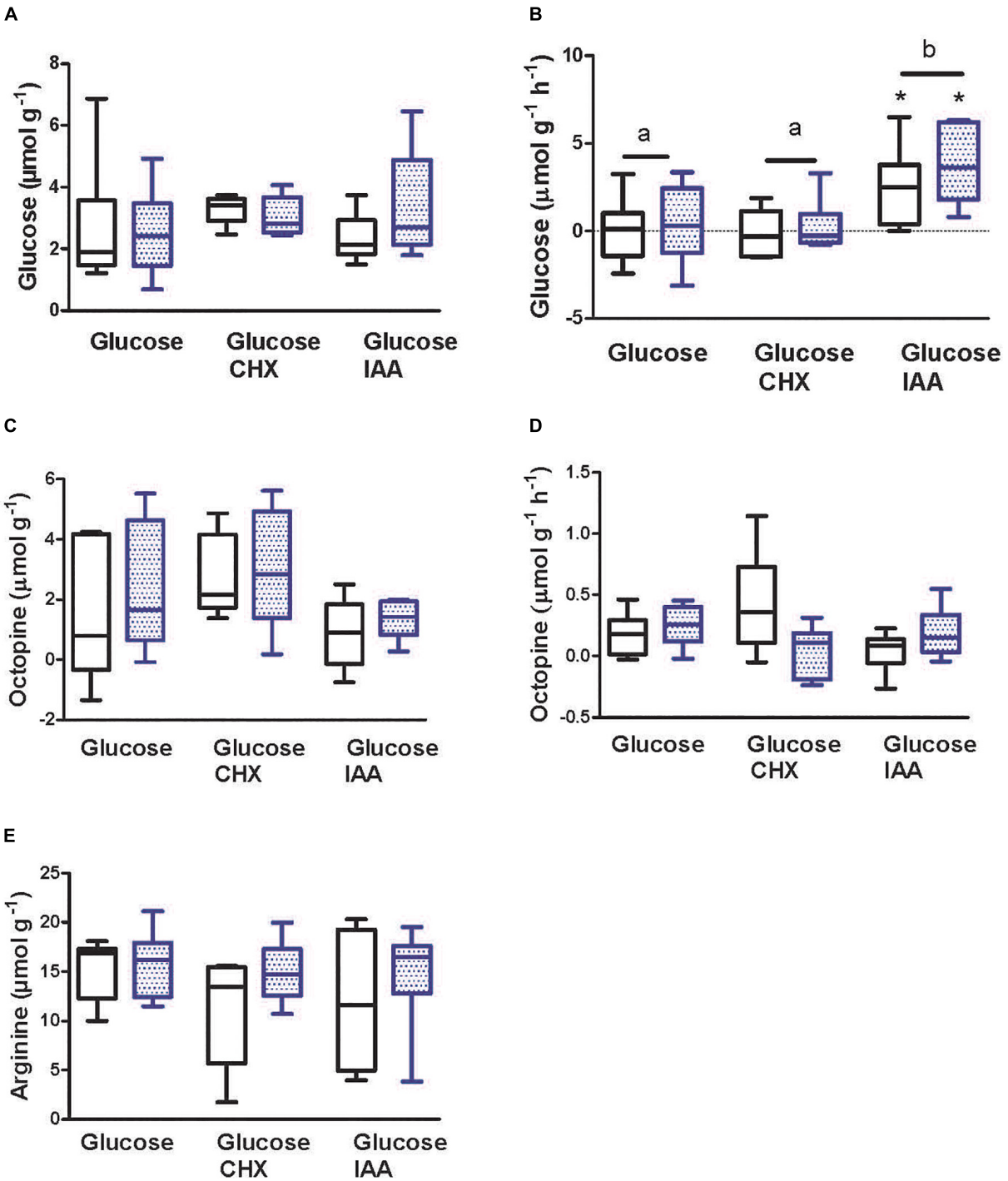

FIGURE 4 | Glucose, octopine, and arginine levels following 1 h recovery from intense contractility. One of two paired mantle muscle sheets from cuttlefish (*Sepia officinalis*) was stimulated to contract for 30 s at 3 Hz simulating jetting behavior. **(A,C,E)** tissue concentrations after a 1 h period following contraction. **(B,D)** rate of uptake or release of metabolite into the bathing medium during the 1 h period following contraction. Open bars represent unstimulated preparations; stippled bars indicate preparations that were stimulated. Text below *x*-axis shows additions to media. For CHX and IAA additions, preparations were incubated for 10 min prior to stimulation, during stimulation, and throughout the post contraction incubation period. *N* = 6 under all conditions except tissue glucose in stimulated preparations incubated with CHX, tissue octopine in unstimulated preparations incubated with CHX, and media glucose in unstimulated preparations incubated with IAA where *N* = 5. Values with different letters are significantly different. *Significant difference from zero (one sample *t*-test) for rate of glucose appearance in bathing medium. Box and whisker plots show the range and median value.

caution given the qualitative nature of the analysis and is likely an underestimate.

Metabolite Levels Immediately Following Simulated Jetting

Metabolites were assessed immediately following the simulated maximum jetting contractions. The contractile challenge was similar to that in **Figures 2C,D**. One pair of mantle sheets was maintained in medium without additional glucose, the other pair received glucose plus IAA in the medium. Glucose content was approximately 3 μmol g^{-1} and did not change in different media or due to contraction (**Figure 5A**). Octopine content ranged from 0.21 to 0.82 μmol g^{-1} and did not change under any condition (**Figure 5B**). Tissue arginine content ranged from 17 to 24 μmol g^{-1} and similarly did not change under any condition (**Figure 5C**). Only 1 of 24 preparations gave a positive signal for arginine phosphate.

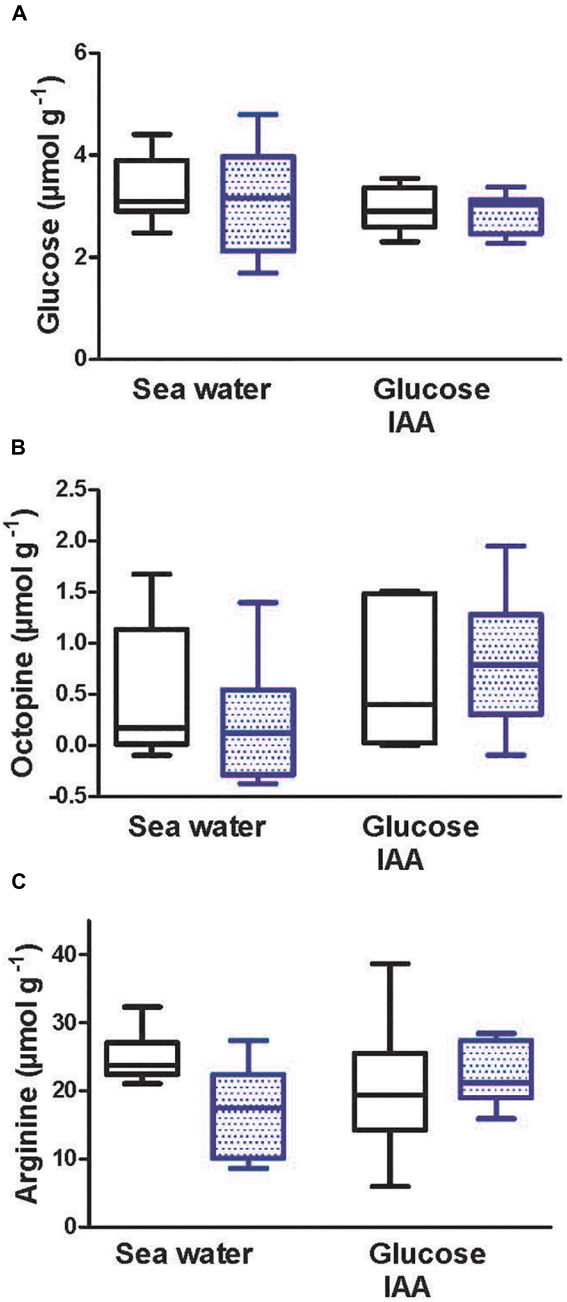

FIGURE 5 | Glucose **(A)**, octopine **(B)**, and arginine **(C)** levels in mantle muscle sheets immediately following intense contractility. One of two paired mantle sheets from cuttlefish (*Sepia officinalis*) was stimulated to contract for 30 s at 3 Hz simulating jetting behavior and thereafter analyzed. Open bars represent unstimulated preparations; stippled bars indicate preparations that were stimulated. Text below *x*-axis shows additions to media. For IAA additions, preparations were incubated for 10 min prior to stimulation. *N* = 6 under all conditions. Box and whisker plots show the range and median value.

DISCUSSION

Blockage of Protein Synthesis or Glycolysis Decreases Force Development Under Basal Conditions but Not Simulated Jetting Activity

The contractility experiment was designed to lend insight into mantle muscle physiology at both basal (i.e., low but not minimal) and high energy demand states. Force development during the initial hallmark challenge averaged a stress of 104 mN mm^{-2}, in keeping with a previous study that reported peak contractile stress during tetanus of 226 mN mm^{-2} in similar preparations from *S. officinalis* of unknown but likely larger body mass, as animals were trawled off Plymouth, United Kingdom (Milligan et al., 1997). In concurrent assays, a number of preparations exhibited F_{max} values in excess of 300 mN mm^{-2} (data not shown). The current experiments were terminated after approximately 70 min but in parallel experiments force development could be sustained for at least a further hour. The preparation thus proved to be robust and suitable for metabolic studies.

Inclusion of CHX in the bathing medium resulted in a significant decrease in force following a 1 h recovery period after a simulated jetting event. One possible explanation for the loss in force is an impairment of synthesis of one or more proteins that have a high turnover rate and are necessary to support some aspect of contractility or provision of energy. Although the turnover rate of any specific protein, to our knowledge, has not been reported for any mollusk, a high rate of protein degradation may occur in *S. officinalis* mantle given its high calpain activity (Lamarre et al., 2012, 2016). Our interpretation is consistent with findings from a more well-studied muscle system, the perfused rabbit heart; hearts perfused with a cardioplegic medium prior to global ischemia show better contractile performance during normoxic reperfusion. The inclusion of CHX in the cardioplegic medium decreased that protective response and the protective effect of the cardioplegic medium is associated with a significant upregulation of a number of genes and proteins associated with contractility and energy metabolism (McCully et al., 2009). There was no significant decrease in stress development between

control and CHX treated preparations either following the first 10 min rest period (during which time CHX was applied) or immediately after the high energy jetting challenge. It is possible that the time for CHX to take effect was too short to induce differences in protein turnover.

Inclusion of IAA in the bathing medium, under basal conditions (initial 10 min rest and 1 h rest following simulated jetting), resulted in a decrease in stress development relative to controls. Here, we accept that the primary impact of IAA is on glycolysis through inhibition of glyceraldehyde 3-P dehydrogenase although other effects independent of glycolysis are possible due to the non-specific mechanism of IAA on cysteine residues in proteins. With this caveat, we propose that an intact glycolysis during the recovery period is required to maintain subsequent contractile integrity. The impairment of contractility by IAA has been recognized in a variety of muscle types from different vertebrate species (e.g., frogs, Sandow and Karczmar, 1950; fish, Driedzic and Hart, 1985; rats, Ruff and Weissman, 1991). The relationship between glycolysis and contractility is discussed below. Glycolytic impairment however, had no impact on stress development following the 30 sec, simulated jetting challenge, suggesting that glucose metabolism is not a determinate of stress development during the high energy demand period.

Overall, the findings show that both an intact protein synthesis and glycolysis are required to maintain functional integrity during a 1 h recovery period following intense contractility. Under stimulated jetting conditions even control preparations lose stress but impairment of glycolysis does not exacerbate the response.

Relationship Between Oxygen Consumption and Protein Synthesis Under Basal Conditions

$\dot{M}O_2$, determined for whole animals was 25–50% of the values previously reported for *S. officinalis* from the same geographic location but with a body mass of approximately 50 g (Lamarre et al., 2016; Capaz et al., 2017). A higher $\dot{M}O_2$ was anticipated based on the allometric equation for animals ranging in mass from 15 to 494 g (Melzner et al., 2007) however, weak allometric scaling between $\dot{M}O_2$ and mass was previously reported in smaller cuttlefish (Johansen et al., 1982). Differences in respirometry methods or size-dependent stress levels such as the body size/chamber volume may be critical, but it seems likely that the scaling relationship does not apply to small animals (Johansen et al., 1982). Indeed, there are other situations in cephalopods, such as in the Loliginidae family, that show little allometric scaling and $\dot{M}O_2$ is proportional to body mass (Seibel, 2007).

Regardless of the above comments, the $\dot{M}O_2$ reported here should be acceptable for further analysis of relationships within this particular experiment. The average rate of protein synthesis of isolated, unstimulated mantle was 2.51% day^{-1}. This rate is the same as that found in the mantle of whole animals with a body mass of 50 g (Lamarre et al., 2016). Again higher rates of protein synthesis were expected in the smaller animals used in this study based on the general premise of allometric scaling of protein synthesis observed in dumpling squid (*Euprymna tasmanica*) (Carter et al., 2009; Moltschaniwskyj and Carter, 2010). Once more, lower than anticipated rates of oxygen consumption and protein synthesis are self-consistent within this study and suggest a lack of strong allometric scaling of both in small cuttlefish.

Unstimulated mantle had a $\dot{M}O_2$ of 10.58 μmol g^{-1} h^{-1} and a 1 g cuttlefish has a total mantle mass of 0.28 g. Mantle tissue in a 1 g animal could therefore account for a $\dot{M}O_2$ of 2.96 μmol h^{-1}. Whole animal $\dot{M}O_2$ was 5.47 μmol g^{-1} h^{-1}. Therefore, in a 1 g animal, mantle could account for 54% of the basal level of oxygen consumption. Both $\dot{M}O_2$ and rate of protein synthesis were decreased by the presence of CHX. In unstimulated mantle $\dot{M}O_2$ was decreased by 76% while protein synthesis was decreased by a comparable level of 65%. The parallel findings provide strong support for the concept that the decrease in $\dot{M}O_2$ by CHX is primarily due to an inhibition of protein synthesis but we cannot rule out secondary responses. If mantle accounts for 54% of the whole animal $\dot{M}O_2$ and 76% of mantle $\dot{M}O_2$ is due to protein synthesis, then a minimum of 41% of the whole animal oxygen consumption is being used to fuel protein synthesis in mantle under basal conditions. The 41% may be an underestimate given that CHX treatment did not block protein synthesis entirely.

The cost of protein synthesis may be estimated from $\dot{M}O_2$ and the fractional rate of protein synthesis. The total protein content of *S. officinalis* mantle is 166 mg g^{-1} (Sykes et al., 2009). Based on a fractional rate of protein synthesis of 2.51% day^{-1}, this equates to 0.17 mg protein g^{-1} h^{-1}. The CHX sensitive $\dot{M}O_2$ was 8 μmol g^{-1} h^{-1} (i.e., 10.58–2.57). Therefore, the cost of protein synthesis was 50 μmol O_2 mg $protein^{-1}$. Values for marine invertebrates using the approach of inhibition with CHX range from 14 to 148 μmol O_2 mg $protein^{-1}$ (Fraser and Rogers, 2007). Our calculated value sits in the middle of this range lending credibility to the findings and indicating that the protein synthetic machinery in *S. officinalis* is similar to other marine invertebrates.

Protein Synthesis Is Stimulated Following Contractility

In preparations incubated with just glucose in the medium, $\dot{M}O_2$ was 29% higher following recovery from contractility than in unstimulated mantle. Similarly, protein synthesis was 24% higher following contraction than in unstimulated preparations. The essentially 1–1 correlation suggests that, the increase in oxygen consumption was due primarily to increases in protein synthesis and not a repayment of an anaerobic energy debt. The post exercise increase in protein synthesis in *S. officinalis* mantle muscle is similar to that which occurs in mammalian muscle (e.g., Biolo et al., 1995; Phillips et al., 1997). In mammalian, skeletal muscle activity may be associated with damage to contractile fibers (Gibala et al., 1995). We speculate that following activity, in cuttlefish, aimed at escaping predators or conspecifics, mechanisms are in place to enhance protein synthesis in mantle perhaps to repair damaged muscle fibers.

In preparations incubated with CHX there was a tendency for $\dot{M}O_2$ to be higher in stimulated than control preparations but

with equivalent rates of protein synthesis. The marginally higher $\dot{M}O_2$ may represent an oxygen debt associated with repayment of anaerobic energy metabolism (i.e., Arg-P and/or ATP).

Necessity for Low Rates of Glycolysis Under Basal Conditions

Inhibition of glycolysis with IAA during the non-contractile incubation periods resulted in a decrease in stress development, $\dot{M}O_2$, and protein synthesis. This suggests that glucose is required to fuel NADH production for oxidative phosphorylation, which in turn would drive ATP synthesis to support contractility and protein synthesis. Extracellular glucose was not called upon under any of the incubation conditions, implying that glycogen reserves are utilized. Unfortunately, due to an analytical error, glycogen levels are not available. More recent studies show that with unstimulated preparations, incubated in media containing glucose plus taurine, the final glycogen level was 1.56 ± 0.7 μmol glucosyl units/g (N = 9) (Driedzic et al., unpublished). The difference in $\dot{M}O_2$ between preparations incubated with glucose alone versus those with glucose plus IAA is 8–9 μmol g^{-1} h^{-1}. Based on the standard relationship of glucose + 6 O_2 yielding 6 CO_2 + 6 H_2O this would require a rate of glycolysis of 1.33–1.5 μmol g^{-1} h^{-1}. As such, even if the initial glycogen level was only 3 μmol glucosyl units/g there would be sufficient starting levels to fuel aerobic metabolism and a low level of octopine production. In addition to direct provision of ATP to the contractile apparatus, a further nuance of the relationship between glycolytic inhibition and stress development should be considered. There is abundant evidence, from well-studied mammalian heart and skeletal muscle systems, that glycolysis under aerobic conditions leads to preferential provision of ATP generated in the cytosol to fuel membrane bound enzymes to support Na^+ and Ca^{2+} transients (Nakamura et al., 1993; Xu et al., 1995; Dizon et al., 1998; Losito et al., 1998; Okamoto et al., 2001; Sepp et al., 2014). We propose that, as in mammalian systems, glycolytic inhibition over a number of minutes could impair Na^+–K^+ ATPase resulting in a derangement of intracellular Na^+ and secondarily Ca^{2+} balance or could act directly on Ca^{2+} regulation at either the sarcoplasmic reticulum or the sarcolemma and in doing so lead to contractile failure.

Conundrum of Glucose Gradients and Transport

A further nuance to observations on glucose levels deserves consideration. Glucose measured in tissue prior to and following a 1 h incubation was approximately 3 μmol g^{-1} wet weight. If all of the tissue free glucose is considered to be in the intracellular water and if the water content is 80% (Sykes et al., 2009) then the concentration of glucose in intracellular space is approximately 4 mmol L^{-1}. But *in vivo* glucose levels in hemolymph never exceeds 2 mmol L^{-1} (Lamarre et al., 2012). Furthermore, mantle sheets were bathed in media containing 1 mmol L^{-1} glucose. The concentration of intracellular glucose was thus higher both prior to and following tissue incubation. There is also the finding that IAA treatment resulted in an increase in media glucose. The questions are "how can such a high intracellular to extracellular glucose gradient be maintained" and "where is the increased glucose in the media coming from." There is preliminary evidence for an active glucose uptake by mantle of the Japanese oyster (*Crassostrea gigas*) (Bamford and Gingles, 1974) and a Na^+ coupled, active transport of glucose, occurs in frog skeletal muscle (Kitasato and Marunaka, 1985). Given that glucose uptake has been little studied in marine invertebrates (Martínez-Quintana and Yepiz-Plascencia, 2012), the possibility of active glucose uptake by mantle of *S. officinalis* should be considered. Impairment of glycolysis with IAA could lead to a negative cascade whereby membrane based Na^+–K^+ ATPase would be compromised, and in turn impairing the ability to maintain an elevated intracellular glucose level leading to a movement of glucose down its concentration gradient and an increase in media glucose. The question of the source of glucose appearing in the media with IAA treatment is more difficult to resolve. Glucose could be released from glycogen but this requires an active glucose 6-phosphase that was undetectable in mantle from older cuttlefish (Speers-Roesch et al., 2016) but present in low activities in white muscle of numerous finfish (Short and Driedzic, 2018). It may be that the enzyme is active in younger *S. officinalis*, as well.

Anaerobic Octopine Production Occurs During Recovery Not Activity

Loss of stress development during simulated jetting was similar with or without IAA in the medium, whereas, glycolytic blockage impaired restoration of stress development during the ensuing 1 h rest period (**Figure 2**). In preparations sampled immediately after the stimulated jetting event there was maintenance of high levels of intracellular glucose and no increase in octopine in either control preparations or those with glycolytic blockage (**Figure 5**), revealing that anaerobic glycolysis is not activated during the 30 s intense contractile challenge. Further tentative insights are gained by a general comparison of information obtained from all preparations sampled immediately after intense activity and following the subsequent rest period. Excluding preparations treated with IAA, octopine content significantly increased from 0.34 ± 0.19 (N = 12) (**Figure 4C**, sum of open bars) to 2.36 ± 0.41 μmol g^{-1} (N = 23) (**Figure 5B**, sum of open bars) (p = 0.0016; t-test) during a 1 h incubation period without contractile stimulation.

The current results show that in early juvenile *S. officinalis* a single physiologically relevant burst of mantle activity does not result in the anaerobic production of octopine. This finding differs from the substantial increase in mantle octopine to 8.6 μmol g^{-1} noted in larger cuttlefish (75 – 135 g) that had been chased to exhaustion (Storey and Storey, 1979). At this point we do not know if increases in mantle octopine require greater energy demand than that applied here, such as repeated jetting events, or if other factors such as age/body size or blood borne signals are determinants of octopine production. Our findings with isolated mantle of *S. officinalis* are consistent with the response in adductor muscle of scallop (*Pecten maximus*); an activity response within the normal physiological window did

not result in an increase in octopine production but octopine did accumulate when animals were forced to swim until exhaustion. Octopine further accumulated while in recovery for the first 10–20 min but returned to minimal levels after 12 h (Gäde et al., 1978). A similar pattern of octopine change was noted in another species of scallop (*Chlamys opercularis*) (Grieshaber, 1978).

Intense activity did not result in a change in arginine levels (**Figure 5C**). Again, based on an exercise challenge, it was anticipated that arginine levels would increase in association with a decrease in Arg-P (Storey and Storey, 1979). There was no change in arginine content between unstimulated and stimulated preparations following the rest period (**Figure 4E**). During the 1 h rest period, the pattern of arginine content was the mirror image of octopine levels; the rest period lead to a decrease in arginine content (again excluding IAA preparations) from 20.9 ± 1.94 (N = 12) (**Figure 5C**, sum of open bars) to 14.3 ± 0.8 μmol g^{-1} (N = 24) (**Figure 4E**, sum of open bars) (p = 0.0008; t-test). The method utilized here to detect Arg-P was only qualitative; that withstanding, we failed to detect Arg-P in preparations immediately following intense activity but following the rest period Arg-P was detected in most preparations at approximately 3 μmol g^{-1}. Overall, the data suggest that Arg-P had discharged in all preparations, including unstimulated controls, prior to the *in vitro* challenges. This was presumably a response that occurred during capture, anesthesia, and dissection of mantle even though animals appeared to be unstressed and did not release ink. During the 1 h recovery period, Arg-P pools began to recover, in association with a decrease in free arginine. This is a common pattern observed in squid, cuttlefish and scallop during recovery from exhaustive activity (Gäde et al., 1978; Grieshaber, 1978; Storey and Storey, 1979; Pörtner et al., 1993).

CONCLUSION

By measuring contractility, $\dot{M}O_2$, protein synthesis, and metabolite levels we are able to bring new insights into the integrative physiology of mantle muscle in early juvenile (≈1 g) *S. officinalis*. At this stage of the life cycle cuttlefish grow at a rate of 12% per day (Sykes et al., 2006, 2014) that is high even by cephalopod standards (Forsythe, 1993). The rapid growth rate is supported by the current finding that 41% of whole animal $\dot{M}O_2$ is used to support protein synthesis in mantle. The cost of protein synthesis in mantle is in the range reported for other aquatic animals, thus this component of growth is not elevated in *S. officinalis*. Under basal conditions an intact protein synthesis is required to maintain contractility possibly due to rapidly turning over proteins consistent with high calpain activity in mantle. A single jetting challenge stimulates protein synthesis by approximately 25% over a 1 h post contractile period. This response is similar to that which occurs in mammalian skeletal muscle. It may be that proteins are degraded during contractility perhaps to supply amino acids for ATP production, and that during recovery there is a compensatory protein synthesis. This compensated rate of protein synthesis appears to be mainly supported by a 30% increase of $\dot{M}O_2$.

Stress development, $\dot{M}O_2$, and protein synthesis are inferred to be supported by glycogenolysis in the isolated preparations but the use of endogenous amino acids should not be ruled out as the enzymatic profile of *S. officinalis* mantle reveals high levels of proteolytic activity, transaminases, and glutamate dehydrogenase (Speers-Roesch et al., 2016). Intracellular glucose is higher than can be accounted for by facilitated diffusion of glucose suggesting an active transport mechanism. Glycolysis is not activated and octopine does not accumulate during a single physiologically relevant exercise challenge; however, we cannot rule out that multiple challenges may activate octopine production.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

SL, TM, NC, JA, AS, and WD participated in all aspects of the study. ÉB and VP performed the experiments and conducted the data analysis. WD wrote the initial draft of the manuscript that was revised by other authors. VP and AS were responsible for the animal husbandry.

FUNDING

TM, SL, and WD were supported by the Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grants. NC was the recipient of a Vanier Graduate Scholarship from the NSERC and a travel grant from the CPB section of the Canadian Society of Zoologists. AS was supported by Fundação para a Ciência e a Tecnologia (FCT) through Programa Investigador FCT 2014 (IF/00576/2014). This study was supported by Portuguese national funds from Programa Operacional Mar2020 (Portugal2020/FEAMP) – Project SEPIACUL (Project Number 16-02-01-FMP-53), from FCT through Plurennial funding to CCMAR (UID/Multi/04326/2019), and from operational programs CRESC Algarve 2020 and COMPETE 2020 through project EMBRC.PT (ALG-01-0145-FEDER-022121). This study was also support by the Portuguese node of EMBRC-ERIC, specifically EMBRC.PT (ALG-01-0145-FEDER-022121).

ACKNOWLEDGMENTS

We thank Mr. Juan C. Capaz and Mr. João Reis at the Ramalhete Station for valuable input and logistical support with the study.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

BRIEF RESEARCH REPORT
published: 11 September 2019
doi: 10.3389/fphys.2019.01161

mTOR as a Marker of Exercise and Fatigue in *Octopus vulgaris* Arm

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Cephalopods are highly evolved marine invertebrates that colonized almost all the oceans of the world at all depths. This imposed the occurrence of several modifications of their brain and body whose muscle component represents the major constituent. Hence, studying their muscle physiology may give important hints in the context of animal biology and environmental adaptability. One major pathway involved in muscle metabolism in vertebrates is the evolutionary conserved mTOR-signaling cascade; however, its role in cephalopods has never been elucidated. mTOR is regulating cell growth and homeostasis in response to a wide range of cues such as nutrient availability, body temperature and locomotion. It forms two functionally heteromeric complexes, mTORC1 and mTORC2. mTORC1 regulates protein synthesis and degradation and, in skeletal muscles, its activation upon exercise induces muscle growth. In this work, we characterized Octopus vulgaris mTOR full sequence and functional domains; we found a high level of homology with vertebrates' mTOR and the conservation of Ser^{2448} phosphorylation site required for mTORC1 activation. We then designed and tested an *in vitro* protocol of resistance exercise (RE) inducing fatigue in arm samples. We showed that, upon the establishment of fatigue, a transient increase in mTORC1 phosphorylation reaching a pick 30 min after exercise was induced. Our data indicate the activation of mTORC1 pathway in exercise paradigm and possibly in the regulation of energy homeostasis in octopus and suggest that mTORC1 activity can be used to monitor animal response to changes in physiological and ecological conditions and, more in general, the animal welfare.

Keywords: mTOR, cephalopods, muscle, metabolism, welfare, growth, exercise

OPEN ACCESS

Edited by:
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Stazione Zoologica Anton Dohrn, Italy

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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *29 May 2019*
Accepted: *28 August 2019*
Published: *11 September 2019*

Citation:
Maiole F, Giachero S, Fossati SM, Rocchi A and Zullo L (2019) mTOR as a Marker of Exercise and Fatigue in Octopus vulgaris Arm. Front. Physiol. 10:1161. doi: 10.3389/fphys.2019.01161

INTRODUCTION

Cephalopods are an important component of marine ecosystems, they count around 800 living marine species, and reached over time unprecedented environmental adaptability. They are not only abundant but also ecologically and economically important (Boyle and Rodhouse, 2005). Their incredible evolutionary success and high adaptability to extreme environments such as the polar and tropical climates and even the deep ocean have been possible through modifications at several levels of their biological organization, from genome to nervous system and body organization (Garrett and Rosenthal, 2012; Nakajima et al., 2018). Nonetheless, a high degree of conservation of regulatory genes has been recently assessed among cephalopods, vertebrates, insects, and other marine invertebrates (Albertin et al., 2012).

Cephalopods have large, well-developed brains, and their brain-to-body-mass ratio exceeds that of all the other invertebrates. Muscles represent their major body constituent and, octopuses in particular, have a rapid growth rate reaching up to 5% body weight per day and manifest a high feed conversion rate, with 30–60% of ingested food being incorporated in their own weight (Iglesias et al., 2000; Aguado and García García, 2002; García García and Cerezo Valverde, 2006). Based on these aspects, studying muscle metabolism in this species can answer important questions relative to the animal biology of growth and welfare.

Several investigations have been carried out so far to elucidate the bases of their muscle metabolism and energy consumption and its relation to environmental condition (Seibel and Carlini, 2001; Villanueva et al., 2017). Yet, little is known about specific metabolic pathways involved in processes such as growth, aging, and environmental adaptability.

Similar to vertebrates, muscles of their body are typically striated and for several functional aspects, they can be assimilated to skeletal muscle fibers (Zullo et al., 2017). In vertebrates, the skeletal muscle mass is regulated by a fine equilibrium between anabolism and catabolism, which determines the rate of protein synthesis and degradation as well as muscle fiber size (Schiaffino et al., 2013). An unbalance between anabolic and catabolic pathways leads to atrophy of muscle fibers, when protein degradation exceeds synthesis rate, and to hypertrophy when new protein synthesis is induced.

Several signaling pathways, including the evolutionary conserved mTOR signaling cascade, maintain this process. mTOR is serine/threonine protein kinase that exists in two functionally distinct protein complexes, the rapamycin-sensitive mTOR complex 1 (mTORC1) and the rapamycin-insensitive mTOR complex 2 (mTORC2), that are defined by the presence of Raptor (regulatory protein associated with mTOR) and Rictor (rapamycin-insensitive companion of mTOR) (Saxton and Sabatini, 2017).

mTORC1 plays a critical role in muscle homeostasis because it senses and integrates a broad range of cellular signals from growth factors, hormones, cytokines, amino-acid availability, cellular energy levels and muscle activity (Zoncu et al., 2011). Genetic and pharmacological studies have shown that mTORC1 is required to maintain muscle mass through the regulation of protein translation and autophagy process (Yoon, 2017).

A number of studies have also reported that resistance exercise (RE)-mediated hypertrophy requires mTORC1 activation, mainly through the phosphorylation of mTOR at serine 2448 (Ser^{2448}) (Nave et al., 1999; Chiang and Abraham, 2005; Copp et al., 2009). The resulting increase in myofibrillar protein synthesis and muscle mass takes place by the integration of multiple signaling pathways that are able to control and modulate transcription factors and in turn protein synthesis and degradation.

In this work, for the first time in a cephalopod, we investigated the conservation of mTOR and its role during a particular class of training such as the RE inducing muscle fatigue. Muscle fatigue is defined as the inability of muscles to maintain the required power output. Upon establishment of fatigue, muscle contractile force shows a rapid decline reversible by rest. We tested samples of arm with a specifically designed RE and showed the induction of a fatigue state. We next assessed mTOR pathway following tetanization and show a sharp increase in its activation state and its time dependency.

We believe that studying mTOR pathway may be important to understand the ability of muscles, over time, to adapt to various physiological and ecological conditions such as exercise response, metabolic stress and nutrient availability and may prompt to the development of advanced methods for monitoring cephalopod welfare.

MATERIALS AND METHODS

Animals Treatment

Specimens of *O. vulgaris* were collected from local anglers of the Ligurian coast. Adult animals of both sexes ($n = 9$) ranging between 150 and 250 g and not showing signs of damage and/or regeneration of the arms employed (L2 or L3) were selected for this study.

Following captures, the animals were placed in 80 × 50 × 45 cm marine aquarium tanks filled with artificially prepared sea water (SW, Tropic Marine) and kept at a temperature of ∼18°C at 12 h light/dark cycle. Octopus environment was enriched with sand substrate and clay pot dens. Water cleaning and oxygenation were assured by a pump-filter and aeration system which continuously circulated the water through biological filters; all relevant chemo/physical water parameters were checked daily to prevent unhealthy or stressful conditions for the animals. Animals were left to adapt to captivity for at least 10 days before experimentation. Octopuses were inspected daily and fed with shrimps 3 times per week. Particular attention was paid to housing, animal care, and health monitoring. All our research conformed to the ethical principles of the three Rs (replacement, reduction and refinement) and of minimizing animal suffering, following the Directive 2010/63/EU (Italian D. Lgs. n. 26/2014) and the guidelines from Fiorito et al. (2015).

For molecular biology experiments, 3 animals were anesthetized in ethanol 2% (v/v) in SW. Brain and arm samples (from L2 or L3), devoid of skin and suckers, were collected, frozen in liquid nitrogen and immediately stored at −80°C. Brain samples (from the supraoesophageal mass) were employed as an additional control in western blotting experiments.

For biomechanical experiments a total of 6 animals were anesthetized in 3.5% $MgCl_2$ in SW, since ethanol exposure induced muscle stiffness. After anesthesia, a single segment (∼4–5 cm) per animal was cut from the middle-end of the L2 or L3 arm. Arm samples were moved to ∼18°C oxygenated artificial sea water (ASW) (pH 7.6) containing: NaCl, 460 mM; KCl, l10 mM; $MgCl_2$, 55 mM; $CaCl_2$,

11 mM; Hepes, 10 mM; glucose 10 mM. This temperature was the same as that of the aquarium where the animals were maintained and was within the temperature range of the Mediterranean sea.

Given the large portion of arm excised and/or the dissection of the brain, the animals underwent terminal anesthesia in order to prevent animal suffering or distress and following the Guidelines for the Care and Welfare of Cephalopods in Research published by Fiorito et al. (Fiorito et al., 2014, 2015).

Ethics Statement

This study was carried out in accordance with the recommendations of Fiorito et al. (Fiorito et al., 2014, 2015). The protocol was approved by the Institutional Review Board and by the Italian Ministry of Health (authorization no. 465/2017-PR).

RNA Preparation and Sequencing

Total RNA has been extracted from octopus arm segments (n = 3) using RNeasy Microarray Tissue Mini Kit (Qiagen) and contaminating DNA has been degraded by treating each sample with RNase-Free DNase Set (Qiagen). The purity of total RNA extracted has been estimated measuring 260/280 and 260/230 absorbance ratios. For each sample, 1 μg of total RNA extracted have been retrotranscribed with ImProm-II(TM) Reverse Transcription System (Promega) following the manufacturer's instructions. mTOR gene has been divided into small fragments up to 1.5 kb and the correspondent primers were designed (**Supplementary Table S1**). 2 μL of Octopus cDNA was used as a template for PCR. Fragments obtained from PCR have been purified and sequenced using the automated Sanger method (Applied Biosystems 3130 DNA Analyzer). The identity of segments has been verified and analyzed by BLASTX and BLASTP programs. Consensus sequences were obtained from fragment overlap. Nucleotide and protein sequences were aligned with the human mTOR using Clustal Omega and protein similarities have been calculated using BLASTP at NCBI Genbank. Protein domain annotation and conservation level were assessed by NCBI Conserved Domain Database. Molecular weight was predicted from the amino acid sequence using the ExPASy Compute pI/Mw tool.

Resistance Exercise Protocol

Muscle fatigue was investigated *in vitro* on total arm segments (n = 6) using a Dual-Mode Lever Arm System (Aurora Scientific – 300C-LR) mounted on a muscle test chamber with integrated stimulating electrodes (Aurora Scientific – 801C). Field stimulation was delivered through a current-voltage bi-phase stimulator (Aurora Scientific – 701B). Data were acquired at a sampling frequency of 10 kHz, bandwidth filtered at 10 Hz - 3.3 kHz and further analyzed with a LabVIEW based Data Acquisition and Analysis System (Aurora Scientific – 604A and 605A). Test chamber was bath filled with temperature-regulated ASW continuously circulating at ∼18°C. Arm samples were placed in the chamber, tied tightly at the force transducer system and allowed to rest for about 5 min before starting the protocol. Recordings were made in isometric condition, thus enabling measurements of the force developed over time upon electrical stimulation of the sample. Muscle length was adjusted such that a transient passive force could be visualized (Milligan et al., 1997; Thompson et al., 2014). Samples were tested for their response to a RE protocol consisting of three tetanic stimulation (pulse width: 0.2 ms negative; pulse frequency: 100 Hz; train duration: 3 s) interspersed with 4 s rest each, repeated 7 times with a rest of 120 s between each repetition. After tetanization, samples were prepared for western blotting. In details each sample, following removal of skin and sucker, was cut in 4 equal segments and maintained in oxygenated ASW at ∼18°C for four different time points (0, 30, 60, and 120 min). A small arm sample, not undergoing RE, was also cut from each arm and used as control (−/−) of western blotting. All samples were then frozen in liquid nitrogen and immediately stored at −80°C.

Protein Extraction and Western Blotting Analysis

To obtain total lysate, 10–20 mg of brain and arm tissue (n = 4 for each experimental group), have been homogenized with TissueLyser (Qiagen) in 600 μL of extraction buffer (1% NP40 – 1% SDS – 50 mM Tris-HCl pH 7,6 – 150 mM NaCl) with protease inhibitor (Complete EDTA-free protease inhibitors, Roche) and phosphatase inhibitor cocktails (Sigma-Aldrich). After incubation for 5 min at 100°C, samples have been sonicated and centrifuged at 4°C for 15 min at 13000 × rpm to remove cell debris. The soluble fraction was collected, and protein concentration was determined using the bicinchoninic acid (BCA) assay method (Thermo Scientific). For Western blotting, 80 μg of tissue lysates were boiled in 5X sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.05% bromophenol blue, 5% β-mercaptoethanol, deionized water), resolved by SDS-polyacrylamide gels (SDS-PAGE) and transferred onto nitrocellulose membranes (GE Healthcare BioSciences; 0.20 μm pore size). Mouse gastrocnemius muscle lysates were used as control samples (Rocchi et al., 2016). The following antibodies were used: polyclonal β-actin (A2066, Sigma-Aldrich – 1:50000) and phospho- Ser 2448-mTOR (2971, Cell Signaling Technology – 1:5000). Antibodies were chosen on the basis of the sequence similarity with respective rabbit and human epitopes used to develop the antibodies. Incubation in ECL substrate chemiluminescent detection reagent (GE Healthcare BioSciences) was performed for 1 min at room temperature. The chemiluminescent blots were imaged with the ChemiDoc MP Imaging System (GE Healthcare BioSciences). The Band Analysis tools of ImageLab software version 5.2.1 (Bio-Rad) were used to select and determine the density of the bands in all the blots.

Statistical Analysis

The software SigmaPlot 13.0 (Systat Software, Inc.) was used for statistical analysis. Normality of the dataset was first assessed with a normality test (Shapiro Wilk). All the dataset analyzed were not normally distributed hence non-parametric test were employed

in further analysis. In particular, non-parametric Mann-Whitney Rank Sum Test was used to compare two data sets. Multiple comparisons were performed with Kruskal-Wallis One Way Analysis of Variance (Holm-Sidak correction). *P*-values <0.05 were considered significant.

RESULTS

Sequencing of *Octopus vulgaris* mTOR

We sequenced arm samples (n = 3) and obtained a single *O. vulgaris* mTOR full-length cDNA sequence consisting of 7629 bp that includes 117 bp of the 5′UTR and an open reading frame (ORF) of 7512 bp encoding 2503 amino acid residues (**Figure 1**) with a predicted molecular weight of 284.5 kDa. The sequence has been submitted to NCBI under the GenBank Accession No. KY774846. mTOR analysis revealed it as a large multidomain protein and a member of the phosphatidylinositol kinase family. All vertebrate mTOR domains were found to be conserved (**Figure 1**). HEAT domain at the N-terminus, which mediates protein-protein interaction, FAT domain that binds DEPTOR, an inhibitor of mTOR, FKBP12-rapamycin binding (FRB) which is the target for rapamycin, a macrolide that inhibits mTOR signaling pathway. The catalytic function is mediated by PI3Kinase domain which contains the phosphorylation sites that activate the protein and FAT C-terminal (FATC) domain, at the COOH-terminus. Protein BLAST revealed that S2448 and S2481 residues, whose phosphorylation is respectively involved in mTORC1 and mTORC2 activation (Chiang and Abraham, 2005; Copp et al., 2009), are conserved. Note that S2448 position of human and mouse correspond to S2386 residues in *O. vulgaris* mTOR protein sequence.

Induction of Muscle Fatigue

Arm samples (n = 6) were tested for their ability to develop peak tetanic tension under the designed RE protocol. As a control, arms that did not undergo RE were also tested for their ability to produce peak tetanic tension and no difference was observed over the experimental time scale. Typical traces of samples undergoing RE are shown in **Figure 2A** where a clear reduction of the peak tetanic tension is observed already at the third repetition of the protocol. Peak forces developed during the first tetanic stimulation of each repetition were measured and normalized to the maximum peak tetanic tension developed by each sample. A gradual and significant decline from the first to the last experimental session can be clearly noticed (Kruskal-Wallis One Way Analysis of Variance, $P < 0.05$). In details, the force was reduced by ∼50% at the third repetition and by to ∼70% from the fourth repetition, maintaining a plateau until last stimulation session (**Figure 2**). This decline in peak isometric tetanic tension is a typical indication of the establishment of muscle fatigue (Enoka and Duchateau, 2008; Fitts, 2012).

Effect of Fatigue on mTOR Phosphorylation

To assess the level of mTORC1 activation, we monitored Ser^{2386} mTOR phosphorylation in control and tetanized arms at different time points after the induction of fatigue (0, 30, 60, and 120 min). For Western blotting analysis, we used a polyclonal antibody against residues surrounding Ser^{2448} of human mTOR protein. The antibody specificity for the phosphorylated form of mTOR has been largely confirmed in human, rat and mouse tissues. Based on the extent of protein sequence similarity (see **Figure 1**), we expected significant cross-reactivity with the homologous proteins in *Octopus vulgaris*. Indeed, the phospho Ser^{2448} mTOR antibody strongly detected a single band at ∼250 kDa in control samples from octopus brain (supraoesophageal mass), octopus arm and mouse muscle (**Figure 3A**) thus confirming the expression of mTOR in octopus samples. We next performed western blotting analysis on control and RE arm samples (**Figure 3B**).

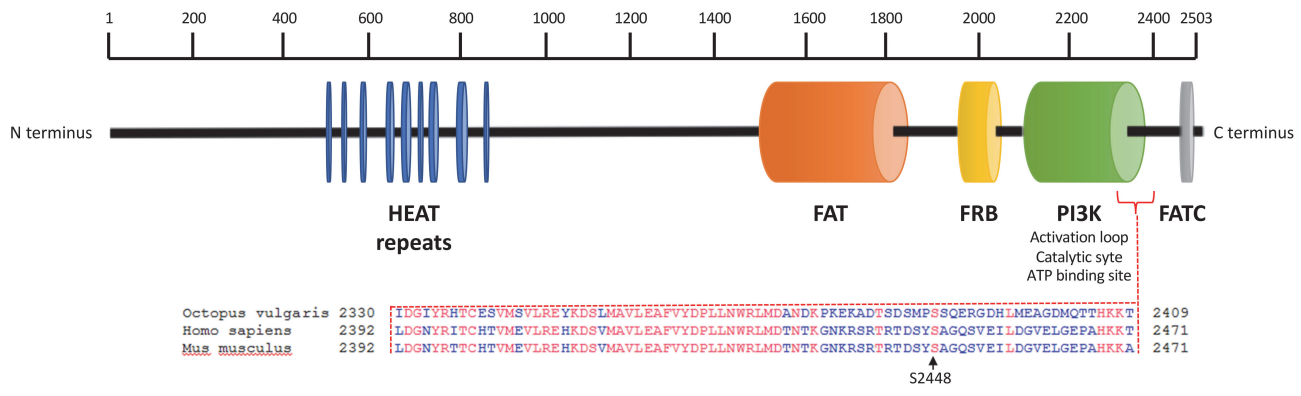

FIGURE 1 | *Octopus vulgaris* mTOR domain architecture and protein alignment in the region of the conserved human and mouse phosphorylation site at Ser2448. *Octopus vulgaris* mTOR full sequence contains about 2503 amino acids; the N-terminus comprises 9 HEAT repeat motifs (blue) followed by a FAT domain (orange). Downstream, mTOR includes a FKPB12-rapamycin binding (FRB) domain (yellow). The C-terminal contains a catalytic domain PI3K (green), with the mTORC1 activation site at Ser^{2386} (conserved between octopus, human and mouse genomes), and a FATC domain (gray).

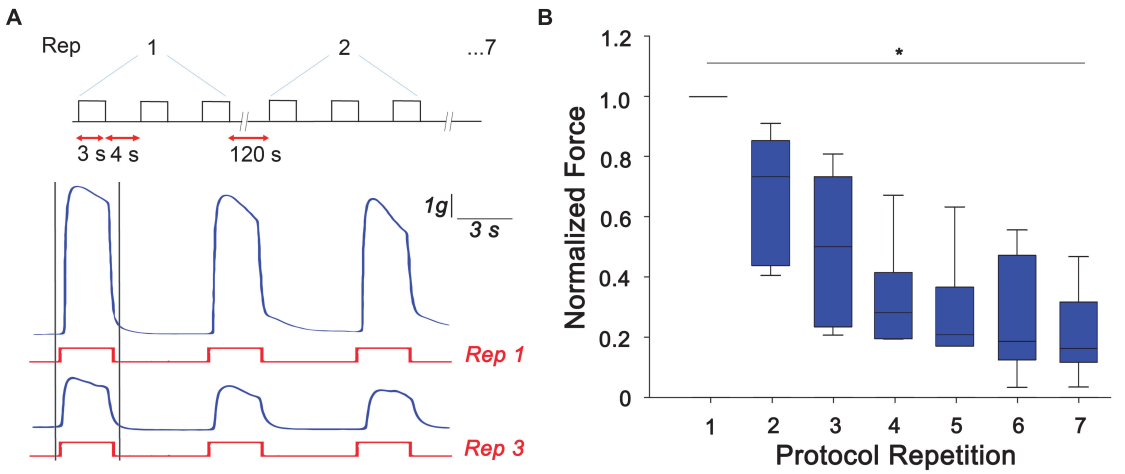

FIGURE 2 | Muscle fatigue induction. **(A)** Resistance exercise (RE) protocol designed to induce muscle fatigue response consisting of three tetanic stimulation (pulse width: 0.2 ms negative; pulse frequency: 100 Hz; train duration: 3 s), 4 s rest each, repeated 7 times with a rest of 120 s. Exemplary traces of force produced (in blue) at the first (Rep 1) and third (Rep 3) stimulus (in red). Peak tetanic force has been analyzed for the first tetanus of each repetition (highlighted with gray bars). **(B)** Peak tetanic force, normalized to the maximum peak tetanic force developed by each sample, recorded during the first tetanus of each repetition (Kruskal-Wallis One Way Analysis of Variance, $*P < 0.05$; $n = 6$).

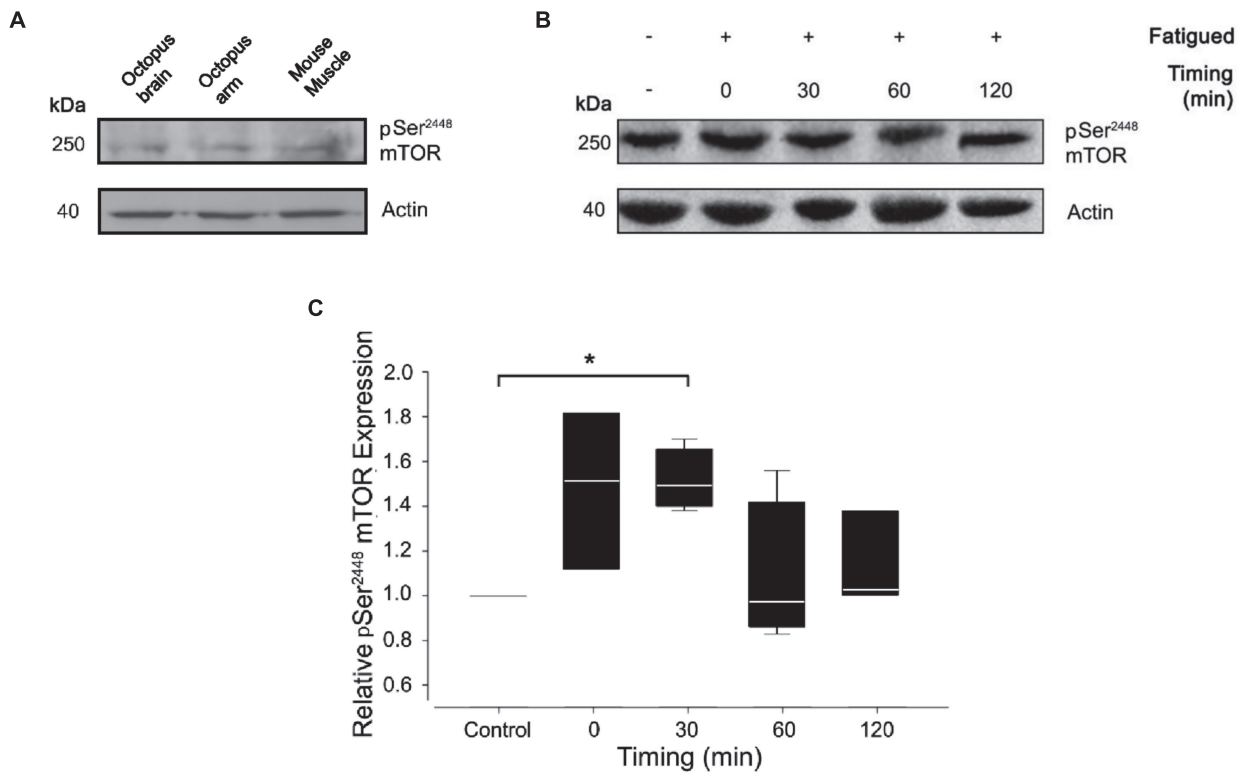

FIGURE 3 | Effect of muscle fatigue induction on mTOR activation. **(A)** pSer^{2448} mTOR and actin antibody validation. Resulting bands were obtained at 250 kDa for pSer^{2448} mTOR and at 42 kDa for actin in lysates from octopus brain, octopus arm and mouse muscle. **(B,C)** Representative experiment **(B)** and quantification **(C)** of western blotting analysis of pSer^{2448} mTOR expression in control and fatigued samples at different timing post RE. Note that the antibody against pSer^{2448} mTOR recognize S2386 in octopus mTOR protein sequence. Data were expressed relative to the control and normalized to β-actin (Kruskal-Wallis One Way Analysis of Variance, Holm-Sidak method, $*P < 0.05$ versus control; $n = 4$ for each experimental group).

Quantification analysis revealed a gradual increase of mTOR phosphorylation reaching a pick 30 minutes from the end of the RE and returning to pre-fatigued values within 60 min (**Figure 3C**; Mann-Whitney Rank Sum Test; $P < 0.05$ versus control). These variations are the result of a cumulative effect of fatigue in the entire sample composed by muscle and nerve cord. A contribution of nerve cord on the phosphorylation state of mTOR cannot be ruled out; however, given the small volume occupied by the nerve cord (and therefore its contribution to the total lysate), this is considered to be minor.

DISCUSSION

Cephalopods range greatly in body size and morphology and these differences are displayed in the environment they occupy and in their behavioral pattern. Both habitats and phylogeny have been demonstrated to affect also the metabolism of individual species (Seibel and Carlini, 2001; Seibel, 2007). Moreover, a large body of evidence shows that diet and feeding strategies changes during development, growing, and senescence (Villanueva et al., 2017) and, consequently, the metabolic requirements of a cephalopod are finely regulated alongside each animal life stage and in response to ecosystem fluctuations.

Given the great diversity of cephalopod species, the identification of genes and molecular markers for health and diseases is currently one of the targets of cephalopod European aquaculture.

As muscles represent the major body constituent, studying metabolic pathways may provide important information on animal biology, environmental adaptability and, consequently, on its welfare.

Cephalopod body muscles are mainly striated and composed of uninucleated cells. Although presenting several features typical of cardiac muscles they can be functionally compared to vertebrate skeletal muscles (Zullo et al., 2017) Skeletal muscles play a central role in the maintenance of the body function and integrity, including the generation of movements and the regulation whole-body metabolism (Leone et al., 2005; Izumiya et al., 2008). The control of muscle mass is crucial for mobility, disease resistance, and more in general for the animal wellness. Skeletal muscles have intrinsic adaptability to a wide range of ecological and physiological stimuli such as acute and chronic exercise paradigms (Tsika, 2012). This plasticity is dependent on the ability of muscles to quickly react to external cues, including nutrients, neural activity, growth factors, hormones, and mechanical loading (Bodine, 2006; Frost and Lang, 2007; Sandri, 2008). Indeed, animals commonly experience throughout their life profound changes in muscle metabolism that can bring to muscle hypertrophy (an increase in the size of muscle cells manifested during growing and exercise) or conversely to atrophy (a general decrease in muscle size occurring during aging, systemic disease or injury) (Adams and Bamman, 2012). Cephalopods are similar in those aspects and, furthermore, they manifest two interesting phenomena, namely regeneration and senescence, where relatively rapid but profound modifications of their body occur (Nodl et al., 2015; Imperadore and Fiorito, 2018; Zullo et al., 2018, 2019; Roumbedakis and Guerra, 2019). Therefore, the comprehension of the molecular pathways responsible for muscle mass regulation in this species is strictly important.

The evolutionary conserved mTOR signaling cascade is one major pathway involved in muscle metabolism in vertebrates. mTOR is a highly conserved protein sensing cellular nutrition and energy status and its molecular pathway is related to the muscle resting/stress/exercise state. A number of studies have reported that acute and chronic mechanical loading is sufficient to promote mTORC1 activation, thus regulating skeletal muscle mass. In addition, mTORC1 kinase activity is required for muscle fiber hypertrophy program (Bodine et al., 2001; Rommel et al., 2001; Frost and Lang, 2007; Bentzinger et al., 2008; Sandri, 2008; Risson et al., 2009). mTORC1 has been identified as a master regulator of mRNA translation and protein synthesis and its activation through phosphorylation leads to the inhibition of autophagy-mediated protein degradation (Kim et al., 2011; Sandri, 2013).

Several lines of evidence have shown that mTORC1 signaling have also important functions, both adaptive and maladaptive, in cardiac cells in response to upstream signals, such as IGF-1 cascade, pressure overload and β-adrenergic stimulation.

mTOR expression is required for a correct development of the cardiovascular system in embryo and postnatal life and in the adaptation to stress conditions as shown in animal models of systemic or cardiomyocyte-specific inactivation of mTORC1 (Zhang et al., 2010; Shende et al., 2011).

As said above, octopus muscle cells shared properties typical of cardiac mammalian cells, thus making the octopus arm also extremely interesting in a comparative perspective with cardiac cells.

In fact, it is known that both mechanical stress and workload greatly affects mammalian heart function and efficiency. mTORC1 activation in the heart during chronic stress has also been shown to have detrimental effects, including the induction of pathological hypertrophy and oxidative stress. This is induced by the activation of several hypertrophic signaling cascades and determine an increase in protein synthesis eventually leading to cardiac hypertrophy (Frey et al., 2004). Accordingly, regulated inhibition of mTORC1 activity was shown to reduce cardiovascular damage in response to pressure overload, metabolic cardiomyopathies and aging (Flynn et al., 2013; Wu et al., 2013).

Mechanotransduction is known to be also a mechanism associated with cardiomyopathy through the involvement of sarcomeric Z-disc proteins such as the MLP Family (Frey et al., 2004).

Interestingly, the uninucleated octopus arm muscles are associated with the extracellular membrane through dense body know as peripheral couplings. These are located at the level of the Z line at corresponding locations in adjacent muscle cells and in association with the subsarcolemma cisternae (the

octopus muscle sarcoplasmic reticulum) (reviewed in Zullo et al., 2017). Peripheral couplings appears as finger-like processes connecting muscle cells to a collagen matrix (Feinstein et al., 2011).

The nature and function of this structure has not yet been clarified but it seems possible that they participate in muscle coordination and in mechanisms of cell signaling and stretch sensing (Zullo et al., 2017) similarly to what proposed for vertebrates sarcomeres (Luther, 2009).

In addition, vertebrate cardiac and cephalopod striated muscle cells share some physiological properties with cardiac cells (as the presence of Ca^{2+} currents are at the base of spike generation) and few of the genes involved in muscle formation such as NK4, a gene essential for cardiac muscle formation in a number of metazoans, were found to be expressed in cephalopod locomotory muscles (e.g., arm, funnel, mantle; Navet et al., 2008; Bonnaud-Ponticelli and Bassaglia, 2014). Hence, the possibility of comparing both functional and molecular events occurring at the level of single muscle cells in octopus muscles and cardiac cells is of a great advantage.

In this study, we recognized *Octopus vulgaris* mTOR as a member of the phosphatidylinositol kinase family, present in both the brain and muscles as a large multidomain protein. We show that all vertebrate mTOR domains important for the protein activation and function were maintained (for domain annotation analysis see **Figure 1**). Several conserved phosphorylation sites had been identified in vertebrates: Ser^{2448}, Ser^{2481}, Thr^{2446}, Ser^{1261} (Nave et al., 1999; Peterson et al., 2000; Cheng et al., 2004; Acosta-Jaquez et al., 2009). Among them, Ser^{2448} is specifically involved in the activation of the mTORC1 (Chiang and Abraham, 2005; Copp et al., 2009) whose catalytic function is mediated by the PI3Kinase domain. Interestingly, octopus Ser^{2386} mTOR (corresponding to human and mouse Ser^{2448} mTOR) was found at a conserved location close to the catalytic domain PI3K and FAT C-terminal (FATC) domain, at the COOH-terminus thus suggesting a possible conservation of function of octopus mTORC1 in the control of muscle response to mechanical loading.

To further assess this point we tested a RE protocol on samples of octopus arms and studied the response in term of fatigue induction and phosphorylation at Ser^{2386} mTOR. Octopus arms are mostly muscular and present a peripheral nervous system running in a central position controlling the simultaneous contraction of a relatively large group of muscles (Fossati et al., 2011; Zullo et al., 2011, 2019). We showed that arms undergoing RE rapidly reach and maintain a fatigue state manifested by a reduction of their force at maximal isometric contraction up to ∼70%. Muscle fatigue was accompanied by a gradual, but transient, increase of mTOR phosphorylation reaching a pick 30 min after fatigue induction.

It is conceivable to think that the activation of the mTORC1 pathway here observed is upstream to a cascade of events that may further induce muscle mass increase. Indeed, acute mechanical loading is known to be a major regulator of skeletal muscle mass through mTORC1 signaling cascade, with an increase in mechanical loading resulting in muscle hypertrophy and a decrease resulting in muscle atrophy.

In vertebrates, both skeletal and cardiac muscle are able to adapt to work loads (Russell et al., 2000). Skeletal muscle cells substantially differ in many aspects such as their speed of contraction, intracellular calcium handling, fatigue resistance, as many others. These differences often account for their classification as fast and slow fibers. Relevant to fatigue, this characteristic is known to be greater in fast compared to slow muscles due to factors intrinsic to the motor unit including the pattern of innervation and the set of metabolic changes occurring in the muscle fibers (Allen et al., 2008). Octopus arms are composed by three main muscle types (longitudinal, transverse and oblique muscles) differently arranged within the arm bulk. A detailed study on the fiber identity of each muscle type is not yet available but, based on the current knowledge, cephalopod body muscles seems not to functionally differentiate in slow and fast type as they all express the same types of myosin heavy chains (MHC) (Shaffer and Kier, 2016). Moreover, in the present study all arm muscles were simultaneously stimulated during RE thus all contributing to force and fatigue to various extents. Thus, no direct comparison can be made between the fatigue induction typical of slow and fast mammalian skeletal fibers.

In addition to its involvement during muscle exercise, mTORC1 has also been recently shown to be a key component in the regulation of muscle development and contributes to the formation of new individual muscle cells, a process referred to as hyperplasia (Rion et al., 2019).

This aspect is particularly relevant for the following three considerations: (1) muscle cells in cephalopod body mass are uninucleated, (2) cephalopods have a virtually indeterminate growth, and (3) they manifest high regeneration abilities (Zullo et al., 2017, 2019; Imperadore and Fiorito, 2018). Any increase in body mass may therefore be due to a combination of hypertrophy and hyperplasia, both controlled by mTORC1 signaling pathway. It is noteworthy that cephalopod lifelong increase in body mass cease upon the first mating event. This is accompanied, in both sexes, by the onset of senescence, a short stage of the animal life soon followed by its death (Anderson et al., 2002; Roumbedakis and Guerra, 2019). Hence, we can further suggest mTOR as an indicator of age-related events likewise the occurrence of senescence.

In conclusion, we believe that mTOR can become a versatile tool for measuring the animal health and predictive of diseases in both wild and captive animals. Its use as a marker of wellness may further provide hints for the improvement of animal maintenance procedures of both aquaculture and experimental animals.

DATA AVAILABILITY

The datasets generated for this study can be found in the GenBank Accession No. KY774846.

ETHICS STATEMENT

The animal study was reviewed and approved by the Local Ethical Committee [OPBA (Organismo Preposto al Benessere degli Animali) of the IRCCS (Istituto di Ricovero e Cura a Carattere Scientifico) Ospedale Policlinico San Martino, Genoa, Italy] and by the Italian Ministry of Health (authorization no. 1111/2016-PR).

AUTHOR CONTRIBUTIONS

LZ and AR conceived and designed the research, and interpreted the experimental results. SF performed the first experiments. FM and SG performed the experiments and analyzed the data. LZ carried out the statistical tests. LZ, AR, and FM drafted the manuscript. All authors revised and approved the final version of the manuscript.

FUNDING

This work has been supported by the COST ACTION FA1301.

ACKNOWLEDGMENTS

We thank local anglers from the Italian coast for animal collection from the wild and Riccardo Navone of the animal facility for animal care and maintenance.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01161/full#supplementary-material

TABLE S1 | List of primers (forward and reverse) used for octopus mTOR fragments amplification with the relative amplicon lengths.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

BRIEF RESEARCH REPORT
published: 13 September 2019
doi: 10.3389/fphys.2019.01180

Behavior of "Intermediate" Males of the Dimorphic Squid *Doryteuthis pleii* Supports an Ontogenetic Expression of Alternative Phenotypes

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The expression of alternative reproductive tactics (ARTs) by different-sized males of loliginid squids has been extensively investigated. In loliginids, alternative phenotypes are characterized by discontinuous differences in behavior, body size, sperm deposition site, and morphology and functioning of ejaculates. Large consort males guard females, display agonistic behaviors toward rival consort males, and mate with females in the male-parallel (MP) position. Small sneaker males avoid fighting contests and instead adopt furtive behaviors to access females guarded by consort males, mating with females in the head-to-head (HH) posture. Recently, the reappraisal of preserved material from the loliginid squid *Doryteuthis pleii* showed that intermediate-sized males (so-called "intermediate" males) had both sneaker- and consort-like ejaculates, leading to the hypothesis of them being a transitional stage between both phenotypes. Here, we describe observations made in captivity showing that intermediate males can display agonistic behaviors toward consort males and mate with females in both mating positions, depending on the male's current reproductive context, i.e., generally in HH, but switching to MP when the female is laying eggs. Such unusual findings of intermediate males simultaneously displaying behaviors of both sneaker and consort males comprise additional evidence corroborating the ontogenetic hypothesis for phenotypic expression of ARTs in this species. Taken together, our results indicate that (1) instead of competing with large consort males for female access and monopolization, small/young males adopt sneaker tactics to obtain mating opportunities, and (2) as they continue to grow, they gradually modify the morphology of their ejaculates and their mating behavior, going through an "intermediate" stage, before becoming large consort males.

Keywords: alternative reproductive tactics, intrasexual male dimorphism, male–male competition, ontogeny, reproductive success

OPEN ACCESS

Edited by:
Erica A. G. Vidal,
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Reviewed by:
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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *23 May 2019*
Accepted: *02 September 2019*
Published: *13 September 2019*

Citation:
Apostólico LH and Marian JEAR (2019) Behavior of "Intermediate" Males of the Dimorphic Squid Doryteuthis pleii Supports an Ontogenetic Expression of Alternative Phenotypes. Front. Physiol. 10:1180. doi: 10.3389/fphys.2019.01180

INTRODUCTION

Mating behavior of loliginid squids (Loliginidae, Cephalopoda) is notable not only because they exhibit several complex behaviors, including agonistic contests and mate guarding (Hanlon and Messenger, 2018), but also because, for several species, males within the same population can express alternative reproductive tactics (ARTs) when pursuing the fertilization of females' ova

(**Supplementary Figure S1**) (e.g., Hanlon, 1996; Hanlon et al., 2002; Iwata et al., 2005). Large (consort) males pair with females, protecting them from the harassment of other consort males. Female guarding occurs before and after mating and also while the female is laying eggs (Hanlon et al., 1997; Hanlon, 1998). It consists of a series of agonistic exhibitions toward rival males, including the expression of stereotyped body patterns (e.g., the exhibition of red stripes laterally along the body, referred as "lateral flames") (**Supplementary Figure S1D**) and even the engagement in physical bouts with other consort males (DiMarco and Hanlon, 1997). During mating, they place themselves below the females and mate with them in a position called "male-parallel" (MP), inserting their spermatophores inside the female's body and implanting them near her oviduct opening (**Supplementary Figures S1A,E,F**). Small (sneaker) males, however, do not display any agonistic behaviors toward other males. Instead, they make attempts of mating stealthily with females guarded by consort males (Hanlon et al., 2002). During mating, they place themselves in front of the female and mate with her in a position known as "head-to-head" (HH), usually without any resistance from consort males, placing their spermatophores near the seminal receptacle of the female, located close to her mouth region (**Supplementary Figures S1A–C**) (e.g., Hanlon, 1996; Hanlon et al., 1997).

In addition to contrasting behavioral strategies, different mating positions and spermatophore attachment sites (**Supplementary Figure S1**), male alternative phenotypes in loliginid squids also have singularities concerning morphological and physiological traits, particularly related to their body size ranges and to attributes of their ejaculates (e.g., Iwata and Sakurai, 2007; Iwata et al., 2015, 2018; Apostólico and Marian, 2017, 2018a). The overall morphology of spermatophores and spermatangia (i.e., everted spermatophores, implanted in the female during mating) of different male morphs is clearly distinct (**Supplementary Figures S1C,F,H,I**) (e.g., Iwata et al., 2015; Apostólico and Marian, 2017). They may also diverge, for example, in terms of spermatophore size and sperm size, volume, and swimming behavior (e.g., Iwata et al., 2011; Hirohashi and Iwata, 2014; Apostólico and Marian, 2017, 2018a). Sneaker males, for instance, produce smaller spermatophores, with less but longer sperm, which also have the unique ability to swarm after release, a feature not observed so far in any consort sperm (e.g., Iwata and Sakurai, 2007; Hirohashi et al., 2013, 2016; Apostólico and Marian, 2017, 2018a).

Expressing one or the other male phenotype can either represent an immutable pathway, meaning that individuals will always play one or the other mating tactic throughout their entire life, or it can be a reversible pathway, meaning that males may shift from one tactic to another once or even interchange between them multiple times in life (Taborsky et al., 2008). Although limited to a few number of studies, several mechanisms responsible for the expression of ARTs have been proposed for different loliginid species. While studying the mating behavior of *Doryteuthis pealeii* both in captivity and on natural spawning grounds, Hanlon et al. (1997) proposed that male phenotypic expression should correspond to flexible tactics, as males seemed to be able to switch between sneaker and consort behaviors according to the mating context to which they were momentarily exposed to (e.g., male size compared to those of the rival males around). For males of *Heterololigo bleekeri*, on the other hand, Iwata and Sakurai (2007) suggested that the expression of male phenotypes should represent permanent tactics. And finally, Mather (2016) observed that early adult males of *Sepioteuthis sepioidea* adopted sneaker tactics on natural environments, changing to consort tactics later in life as they grow.

Congruently with the last hypothesis, a detailed study on this matter focusing on *Doryteuthis pleii* also advocated the plausibility of an ontogenetic shift between divergent male morphs in the species (Apostólico and Marian, 2018b). The hypothesis was based on the description of males of intermediate size and age within the population, which were then hypothesized as a transitional phase from the sneaker to the consort morph. The so-called "intermediate" males diverged from typical sneaker and consort males due to the presence of sneaker- and consort-like spermatophores – along with peculiar spermatophores that shared intermediate characteristics between both types – in their reproductive system (**Supplementary Figures S1H,I**; Apostólico and Marian, 2018b).

The distribution of different spermatophore types within the reproductive organ of the same individual suggested the occurrence of a transition from sneaker- to consort-like spermatophores (Apostólico and Marian, 2018b). Therefore, intermediate males of *D. pleii* were considered as a potentially transitional phenotype during a progression from sneaker to consort. However, considering that all information was obtained after revisiting preserved material (Apostólico and Marian, 2018b), any conjecture on their behavior was not possible at that moment. Therefore, in order to continue exploring the unusual aspects of these intermediate males of *D. pleii*, the present study aimed at describing the behavior of these males in captivity, when in the presence of females and rival males. Such information could shed light on the ontogenetic hypothesis for phenotypic expression of ARTs in this species, e.g., if intermediate males indeed comprise a transitional phenotype, we expect they would display a transition from typical sneaker- to typical consort males' behaviors.

MATERIALS AND METHODS

Mature specimens of *D. pleii* were collected along the summer months (January–March) of 2018 and 2019, off São Sebastião Island (between 23°43′56″S, 45°17′21″W and 23°48′26″S, 45°14′27″W, São Paulo state, southeastern Brazil). Total experimentation period lasted for 10 weeks (5 weeks in each year). Daily expeditions to the collection site ($N = 17$) lasted about 4 h each day, often from 08:00 to 12:00. At the site, animals were sampled individually by hand-jigging and maintained alive inside the vessel in tanks of 250 L with a continuous flow of fresh seawater. On average, 20 individuals were collected each day, and mortality rate inside the vessel was about 30%. From all collected animals (in both years), a total of 117 females and 124 males survived and were taken to the Center of Marine Biology of University of São Paulo (CEBIMar-USP). There, the animals were

placed in tanks of 250–1000 L, with an open seawater system and water temperature from 26 to 29°C, and fed with fresh shrimps *ad libitum*. Males and females were maintained in separate tanks for 24 h before the beginning of the experiments. Only 71 out of 117 females and 66 out of 124 males were maintained for the trials, as the remaining animals either died in the tank or were used for tissue sampling for different approaches in other ongoing studies conducted by the authors.

Prior to the experiments, all males were measured and classified as either sneaker or consort males based on their body size, according to Apostólico and Marian (2018a). Males smaller than 169 mm of mantle length (ML) were assigned as sneaker males, whereas those larger than 169 mm ML were categorized as consort males. Sneaker and consort males were then maintained in different tanks to avoid physical attacks or cannibalism on the smallest individuals. For the trials, sneaker and consort males were assorted randomly from the respective tanks.

Experimental manipulations (N = 52) consisted in placing one random female with either (i) one consort male, (ii) one sneaker male, (iii) one sneaker and one consort male, (iv) two consort males, or (v) two sneaker males, and recording body pattern displays and mating positions adopted by each male. Observations were performed everyday, preferably between 07:30 and 18:30 h (i.e., during daylight) and interrupted during the night. Whenever possible, behaviors were recorded using a GoPro HERO + LCD (ca. 1.2 h of recorded behavior). Behavioral terminology followed Hanlon et al. (1994) and DiMarco and Hanlon (1997).

Each female was used only once, but some males were used in more than one trial. In 15 of the 52 conducted trials, there were no relevant interactions between individuals (i.e., neither agonistic behaviors, mating, or spawning) and therefore they were excluded from the results. Experiments lasted from 3 h up to 4 days. They were discontinued either when one of the animals died or when the female spawned. After spawning, females were anesthetized (in a 7.5% solution of $MgCl_2$ diluted in seawater) and dissected. The oviduct membranes and the seminal receptacle regions were then inspected under the stereomicroscope for the presence of implanted sneaker-, intermediate-, and/or consort-like spermatangia. Females that died before spawning were not processed and data on which spermatangia types were transferred during mating are lacking.

As intermediate males are characterized by intermediate body size between those of sneaker and consort males, it was not possible to know *a priori* which males were indeed "intermediate males" based solely on ML measurements. As an accurate identification of these individuals requires the analysis of their spermatophores and spermatangia, they were first classified as either "sneaker males" or "consort males" based on their body size for the experiments, with male morph appropriate identification being confirmed later, only after anesthesia and analysis of their ejaculates' morphology under stereomicroscope (**Supplementary Figures S1H,I**) (see Apostólico and Marian, 2017, 2018b).

The same method above was also used to confirm male morph identity of sneaker and consort males. Thus, males of small size (ML < 169 mm), which mated only in HH, showed no agonistic behavior, and had only sneaker-like spermatophores/spermatangia (**Supplementary Figures S1C,H,I**) were confidently assigned (and hereafter called) as sneaker males, whereas those of large size (ML > 169 mm), which adopted only MP mating posture, were aggressive toward rival males, and had only consort-like ejaculates (**Supplementary Figures S1F,H,I**) were classified (and hereafter called) as consort males. In turn, males of intermediate size (around 169 mm ML), which showed agonistic displays (see the section "Male Agonistic Behavior" in the section "Results"), adopted both MP and HH (see the section "Mating Posture" in the section "Results"), and had different spermatophore/spermatangia morphologies (**Supplementary Figures S1H,I**) (sneaker-, consort-, intermediate types – see the section "Spermatophore Morphology" in the section "Results") were labeled (and hereafter called as) intermediate males. Thus, based on body size, mating posture and behavior, and ejaculates' morphology, our total sample of 66 males was revealed to be composed of 34 consort (178–315 mm ML), 20 sneaker (102–169 mm ML), and 12 intermediate males (132–178 mm ML). The experimental sample (i.e., males selected from the total sample to participate in the trials) was composed of 19 consort males (178–285 mm ML), 6 sneaker males (102–156 mm ML), and 10 intermediate males (132–178 mm ML).

In Brazil, ethics approval is still not required for experimentation with cephalopods by the "Conselho Nacional de Controle de Experimentação Animal" (CONCEA). However, this study has been carried out in accordance with international protocols for the welfare of cephalopods to minimize animal suffering (following Moltschaniwskyj et al., 2007; Butler-Struben et al., 2018).

RESULTS

Results from the 37 trials are summarized in **Table 1**. Raw data from each experiment, including information on spermatophore and spermatangia morphology obtained from dissected males (i.e., from their storage organs) and females (i.e., from their oviduct membranes and seminal receptacles), respectively, are presented in **Supplementary Tables S1–S3**.

Male Agonistic Behavior

From all the interactions recorded in captivity, the display of agonistic behaviors between males was certainly the most common during the trials. Aggressive demonstrations advertised by males often started with the exhibition of typical skin colorations, such as the pattern known as "all dark" and the display of "lateral flames" and "mid-ventral ridge" along the body (**Supplementary Figure S1D** and **Figures 1A–C**), usually progressing to actual physical disputes, such as "fin-beating" (i.e., males eagerly colliding their fins against each other; **Figure 1D**). Occasionally, males even tried to attack each other, using their arms in attempts to hold the opponents and hurt them with their beaks. When two males were placed in the same tank, the consort male always displayed such agonistic behavior toward another consort male (**Table 1**, **Figures 1A,D**, and trials 14–18 in **Supplementary Table S2**), and less frequently toward a

TABLE 1 | Summary of the 37 trials performed with the squid *Doryteuthis pleii* in captivity.

Trial	*N*	Spawning	Agonistic behavior	Mating posture
♀ + ♂$_{CO}$	4	Yes	–	MP (03–04 h)
♀ + ♂$_{SN}$	3	No	–	HH (–)
	3	Yes	–	HH (03–26 h)
♀ + ♂$_{IN}$	6	No	–	HH (–)
	1	Yes	–	HH (03–16 h)
♀ + ♂$_{SN}$ + ♂$_{CO}$	2	No	♂$_{SN}$: No (100%), ♂$_{CO}$: No (100%)	♂$_{SN}$: HH (–), ♂$_{CO}$: ?
♀ + ♂$_{SN1}$ + ♂$_{SN2}$	1	Yes	♂$_{SN1}$: No (100%), ♂$_{SN2}$: No (100%)	♂$_{SN1}$: HH (24 h), ♂$_{SN2}$: HH (during spawning)
♀ + ♂$_{CO1}$ + ♂$_{CO2}$	5	No	♂$_{CO1}$: Yes (100%), ♂$_{CO2}$: Yes (100%)	♂$_{CO1}$: ?, ♂$_{CO2}$: ?
♀ + ♂$_{IN}$ + ♂$_{CO}$	3	No	♂$_{IN}$: Yes (67%), ♂$_{CO}$: Yes (100%)	♀$_{INT}$: HH (–), ♂$_{CO}$: ?
	5	Yes	♂$_{INT}$: Yes (20%), ♂$_{CO}$: Yes (20%)	♂$_{IN}$: HH (0h30–48 h) + MP (during spawning), ♂$_{CO}$: MP (during spawning-3 h)
♀ + ♂$_{IN}$ + ♂$_{SN}$	4	Yes	♂$_{IN}$: No (100%), ♂$_{SN}$: No (100%)	♂$_{INT}$: HH (4–32 h) + MP (during spawning), ♂$_{SN}$: HH (7–34 h)

The following criteria were analyzed: (1) "Spawning," if the female laid eggs after mating (Yes or No). (2) "Agonistic behavior," if the male showed any aggressive display toward the other male in the tank (Yes or No). Percentage value in parentheses represents the number of trials (from total number) in which agonistic behavior was observed. (3) "Mating posture," mating position adopted by each male (HH, head-to-head; MP, male-parallel). In parentheses, interval (in hours) between mating and spawning events. See text for a full description of each trial type. Raw data for each experiment are presented in ***Supplementary Tables S1–S3****.* ♀*, female;* ♂$_{CO}$*, consort male;* ♂$_{IN}$*, intermediate male;* ♂$_{SN}$*, sneaker male; –, not applicable; ?, unknown.*

smaller, intermediate male (**Table 1**, **Figures 1B,C**, and trials 26–29 in **Supplementary Table S3**). On the other hand, none of the sneaker males showed such behaviors toward either smaller or larger males (**Table 1** and trials 11–13 and 34–37 in **Supplementary Tables S2**, **S3**, respectively). Contrastingly, intermediate males occasionally showed agonistic behaviors, e.g., lateral-flames and fin-beating, toward larger consort males when they were placed together in the same tank (**Table 1**, **Figure 1C**, and trials 27–29 in **Supplementary Table S3**). At that time, these observations were disconcerting, as these males were mostly classified as sneaker males based on their size, but their behaviors were contrasting to those of typical sneaker males.

Mating Posture

All sneaker males mated only in HH (**Table 1** and **Figure 1E**), despite the mating context, i.e., whether alone with the female or in the presence of another male, and despite female status, i.e., if the female was in the imminence or not of spawning (**Table 1** and **Supplementary Tables S1–S3**). Sneaker males typically swam alone in the tank, i.e., not paired with females, suddenly approaching and grabbing the female with their arms to mate in HH. Also, when the females were present, they were constantly harassing them by mating attempts. Consort males, in turn, usually paired with a chosen female and swam alongside her up to hours and days before mating. They only mated in MP (**Table 1**, **Figure 1F**, and **Supplementary Tables S1–S3**), and mating occurred only close to (i.e., <4 h before) or during spawning (**Table 1** and **Supplementary Tables S1–S3**). So, instead of constantly trying to mate with females before spawning, consort males spend most of their time in protecting the female from other males in the tank.

Similarly to sneaker males, intermediate males did not pair with females, and instead tried constantly to abruptly grasp the female to mate in HH. Differently from sneaker males, though, the mating behavior of intermediate males seemed to depend on their current context (i.e., female status). They frequently performed HH (**Table 1**, **Figure 1G**, and **Supplementary Table S3**), regardless of whether another male was present or absent (either a sneaker or consort male), if spawning by the female has not occurred. However, they were able to switch to consort tactics, i.e., pairing with the female and mating in MP, when the female started laying eggs (**Table 1**, **Figure 1H**, and trials 33 and 37 in **Supplementary Table S3**).

Female Behavior

Although females were typically passive, they showed hostile behaviors toward sneaker and intermediate males at times along the trials. During constant harassment by these males, females often rejected their mating attempts by displaying the "all dark" body pattern or by escaping through rapid jet-propulsion movements. Rarely, females even tried to bite sneaker and intermediate males swimming around. However, none of these rejection behaviors was shown toward consort males during the experiments.

Spermatophore Morphology

A conclusive classification of male morph was only possible after dissection of individuals and inspection of their spermatophores and spermatangia (presented in **Supplementary Tables S1–S3**). Almost all males with typical consort body size (i.e., ML > 169 mm) had only consort-like ejaculates (**Supplementary Tables S1–S3**), except for the male on trial 19 (**Supplementary Table S3**), which was revealed to be an intermediate male with a ML of 178 mm. Interestingly though, not all males with typical sneaker body size were in fact sneaker males, being later classified as intermediate males based on their spermatophore morphology (**Supplementary Table S3**). Among these males, some had only sneaker-like and intermediate spermatophores, whereas others had only intermediate and consort-like ones, or even all three types altogether (**Supplementary Table S3**).

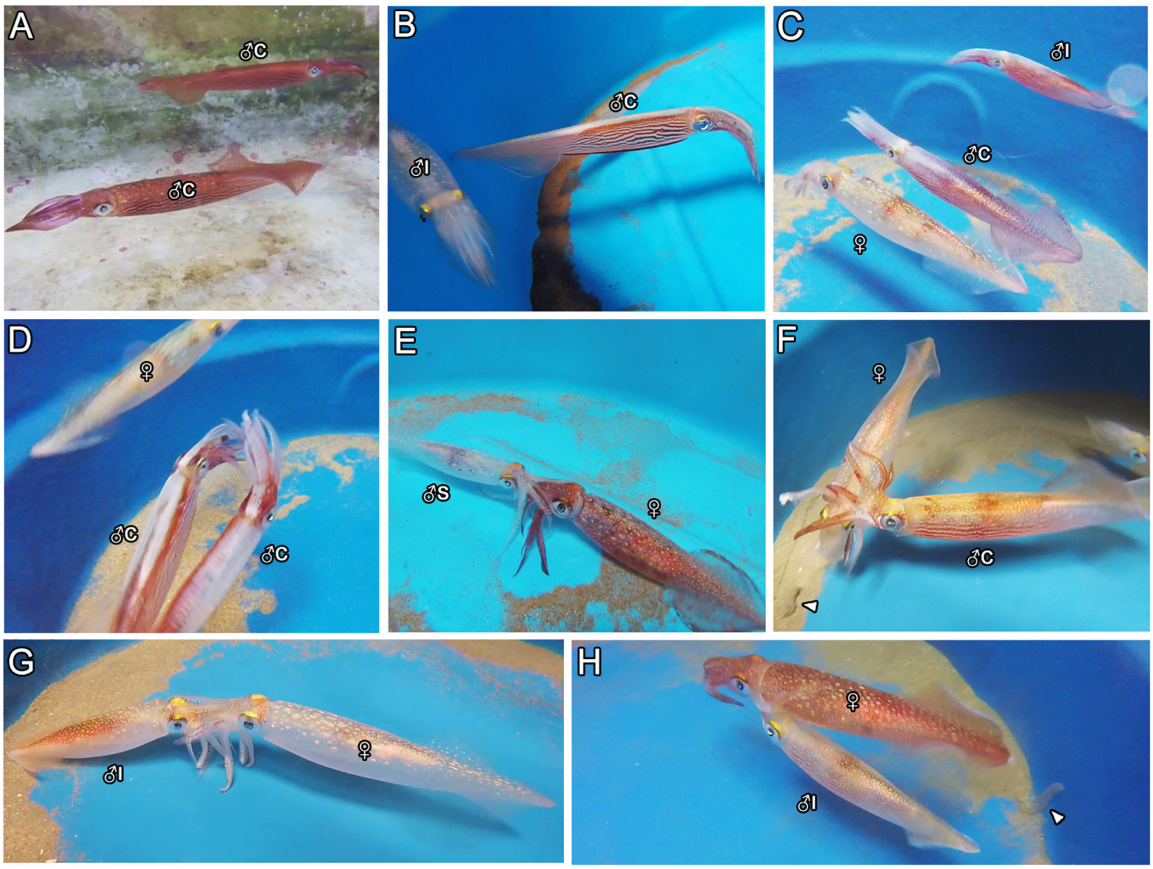

FIGURE 1 | Examples of male agonistic behaviors **(A–D)** and mating positions **(E–H)** observed during the trials. **(A)** Two consort males exhibiting the "all dark" body pattern to each other. **(B)** A consort male displaying "lateral flames" and "mid-ventral ridge" along the body toward a small (intermediate) male. **(C)** An intermediate male trying to fight a large consort male, which is paired with a female. **(D)** Two consort males fighting each other ("fin-beating"). **(E)** A sneaker male mating in head-to-head. **(F)** A consort male mating in male-parallel. **(G)** An intermediate male mating in head-to-head. **(H)** An intermediate male mating in male-parallel. Arrowheads indicate egg strings in panels **(F)** and **(H).** ♀ = female; ♂C = consort; ♂S = sneaker; ♂I, intermediate male.

Also, the peculiar males of intermediate size that had an unusual behavioral combination of agonistic displays (trials 27–29 in **Supplementary Table S3**) and mating postures (trials 33 and 37 in **Supplementary Table S3**) were confirmed to be intermediate males. While four of them had only intermediate and consort-like spermatophores, the other one had all three types (sneaker-, consort-like, and intermediate spermatophores) inside its storage organ (**Supplementary Table S3**).

Spermatangia Morphology

Inspection of the seminal receptacle and oviduct membranes of females (after spawning) were performed to confirm which spermatangia types (sneaker-like, intermediate, or consort-like; **Supplementary Figure S1I**) intermediate males transferred during mating in HH and MP, respectively (shown in **Supplementary Table S3**). Almost all intermediate males that mated only in HH (trials 25, 30–32, and 34–36 in **Supplementary Table S3**) transferred only sneaker-like spermatophores to the females' seminal receptacle (**Supplementary Table S3**). In trial 29, however, an intermediate male mated in HH and transferred intermediate spermatophores to the female (**Supplementary Table S3**).

Interestingly, intermediate males from trials 33 and 37 transferred only one type of spermatophore, even though they adopted both mating postures (**Supplementary Table S3**). While in trial 33 the male transferred only intermediate spermatophores on both HH and MP (as intermediate spermatangia were found both in the seminal receptacle and in the oviduct membranes, respectively), the male in trial 37 transferred only sneaker-like spermatophores. In this last trial, however, spermatangia were found exclusively near the seminal receptacle, but not attached to the oviduct membranes. As discussed below, small club-like spermatangia transferred during MP mating could have been already flushed from the female's mantle cavity by the time females were dissected.

DISCUSSION

This is the first description of the mating behavior of intermediate males of *D. pleii* in captivity. In this species, so-called

"intermediate males" have been previously characterized by intermediate size and age when compared to those of typical sneaker and consort males and by their internal morphology, particularly related to the presence of sneaker-, consort-like, and intermediate ejaculates and sperm with different behavior (i.e., aggregation vs. diffusion) simultaneously within a single individual (Apostólico and Marian, 2018b). Here, it has been shown that these males also manifest a combination of both male morphs' behaviors in terms of agonistic and mating displays. While typical sneaker males were never aggressive toward other males, independent of their relative size, and typical consort males were always aggressive toward other consort males (and even toward intermediate males sometimes), intermediate males were, in some trials, hostile when in the presence of large consort males. Also, regarding their mating posture, intermediate males preferably mated in HH (ca. 91% of times), resembling sneaker males, but were able to switch to MP, consistent with consort tactics.

Evidence on intermediate males of *D. pleii* simultaneously displaying behaviors of both sneaker and consort males comprise additional evidence supporting that these males may be indeed a transitional stage between both male morphs. According to the ontogenetic hypothesis for phenotypic expression of ARTs in this species (Apostólico and Marian, 2018b), young males adopt furtive tactics as sneaker males while small in size, instead of competing with large consort males for female monopolization. Therefore, they can guarantee mating opportunities and offspring paternity. However, as they continue to grow, they gradually modify the morphology of their ejaculates and their behavior from a sneaker- to a consort phenotype, going through an "intermediate" (morphological and behavioral) stage, before reaching a certain body size, at which they would benefit from adopting dominant tactics as large consort males (see Apostólico and Marian, 2018b). This is also congruent with a previous study on the loliginid squid *S. sepioidea*, in which early adult males play sneaker tactics, but later change to consort tactics as they grow (Mather, 2016).

In the loliginid squid *S. lessoniana*, small males consistently adopt sneaking tactics (Wada et al., 2005; Lin and Chiao, 2018). However, it seems that individuals are able to perform both mating tactics, with male "choice" on the expression of one tactic over the other resulting from visual signals manifested by the female (Lin and Chiao, 2018). When small males try to mate in MP, females consistently reject them through visual body patterns in their skin. However, when these males change to the male-upturned tactic (a sneaking tactic), their success rate is higher, as female rejection is lower (Lin and Chiao, 2018). Here, females of *D. pleii* rejected both sneaker and intermediate males during some of the trials, expressed by quick changes in body color, rapid jet-propulsion movements away from the male, and even aggressive displays. However, no rejection toward consort males was observed. This could be due to behavioral differences between males of different size. While small males (sneaker and intermediate ones) constantly harass the female by recurrently trying to copulate, consort males usually pair with the female but do not engage in mating attempts until the female is close to laying the eggs. However, none of the sneaker males even attempted to mate in MP during the trials, so it is unlikely that they use HH mating because of female rejection signaling in *D. pleii*.

The male behavior of adopting one or another tactic due to female rejection in *S. lessoniana* raises the hypothesis that male squids' mating strategy is context dependent (Lin and Chiao, 2018). Such male behavioral flexibility has been previously proposed for another loliginid, *D. pealeii*, as small males in this species often play sneaking tactics while in the presence of larger rivals, but switch to MP when the large male is withdrawn from the spawning ground, possibly because fertilization success is higher when adopting MP posture (Hanlon et al., 1997), as explained below. In the present study, however, sneaker and consort males adopted only HH and MP, respectively, and none of them ever attempted to switch between tactics, independently of mating context, i.e., presence or absence of other males (of same or different size). Also, although mating attempts by consort males seem to depend on female status (i.e., in the imminence or not of egg-laying), as they preferably mate with females close to or during spawning, the behavior of sneaker males does not, as they insistently mated only in HH, despite female status (**Table 1**). These observations challenge the idea that males perform both tactics or "choose" between them depending on female rejection signaling or on presence and size of opponent males, for example, as it may happen in other loliginid squids. Therefore, it seems that, at least in this population of *D. pleii*, legit sneaker and consort males do not shift willingly between divergent strategies depending on their current mating context.

In contrast, intermediate males of *D. pleii*, unlike typical sneaker and consort males, seem able to interchange between mating tactics depending on the context. Although they typically mated in HH (ca. 91% of times), they were able to switch to MP when the female started laying her eggs in two trials (**Table 1** and **Supplementary Table S3**). Such behavioral changes were observed in different scenarios, and they did not depend on size of the rival male. On trial 37, the intermediate male (ML 157 mm) was placed with a sneaker male (ML 110 mm) and a female (ML 146 mm). Before spawning, the intermediate male mated only in HH, but, as the female started laying egg strings on the substrate, it switched between tactics, pairing with the female and mating in MP (**Table 1** and **Supplementary Table S3**). Contrastingly, on trial 33, the intermediate male (ML 135 mm) cohabited the tank with a female (ML 145 mm) and a consort male (ML 193 mm). Before spawning, it mated in HH. Yet, even in the presence of a larger male, the intermediate male still switched to MP when the female started laying the eggs (**Table 1** and **Supplementary Table S3**). Therefore, it looks like mating context can influence the behavior of intermediate males, but it is apparently related to female status instead of size of rival males.

Interestingly, males of *H. bleekeri* may opt for HH when the female is far from spawning (from ca. 50 to 1 h before egg-laying), changing to MP when the female is about to lay eggs (from 15 min until egg-laying) (Iwata et al., 2005). Although sample size was small in that study (i.e., only three out of the six observed males performed such a behavior; Iwata et al., 2005), these results indicate flexible mating behaviors in that species. Male dimorphism was later demonstrated for *H. bleekeri*,

with a switch-point of ca. 220–230 mm in ML (Iwata and Sakurai, 2007; Iwata et al., 2015). From the three males displaying the aforementioned flexible mating strategy, just one had an intermediate size (ca. 220 mm), the other two being much larger than the switch-point (ca. 270 and 300 mm). Also, ARTs are suspected to be permanent in *H. bleekeri* (Iwata and Sakurai, 2007; Iwata et al., 2015). Therefore, the behavior observed in *H. bleekeri* should correspond to a distinct strategy, not related to a transition between male morphs, as observed in *D. pleii*.

Female status (as defined above) seems like a suitable candidate to explain the adoption of different tactics by intermediate males of *D. pleii*, as male fertilization success in loliginid squids is thought to be highly associated with sperm deposition site, due to temporal (i.e., timing between mating and fertilization) and spatial differences between both locations (i.e., near the oviduct vs. near the seminal receptacle) (e.g., Apostólico and Marian, 2017, 2018a). Due to the oocyte pathway during the egg-laying process, consort males are believed to be responsible for higher offspring paternity rates than do sneaker males, as sperm attached near the oviduct opening during MP is expected to contact the unfertilized eggs before those attached near the female's buccal membrane by HH (**Supplementary Figure S1G**) (e.g., Buresch et al., 2009; Shashar and Hanlon, 2013). Thus, if an intermediate male is able to visually detect that the female is about to or is already laying eggs, it may opt for mating in MP and depositing spermatophores near the female's oviduct opening, where the sperm will be promptly used for fertilization and has a higher chance of fertilizing the eggs, even though it has to compete with possible rival consort males. Yet, if the female is not ready to lay eggs, it may be preferable to mate in HH and place the spermatophores close to the seminal receptacle, where the released sperm could be maintained viable for an extended time (e.g., Hanlon, 1996; Hanlon et al., 2002).

If such a flexible strategy is advantageous to intermediate males of *D. pleii*, why do sneaker and consort males not interchange between tactics, too? Two hypotheses can be proposed, based on limitations of either male size or ejaculate type. First, body size of sneaker males may be too small for both MP mating – in which the male must hold the female body and insert spermatophores inside her mantle cavity – and for fighting consort males. Maybe after reaching a certain body mass, males are able to both perform MP and at least try competing with consort males. So, body size constraints could hamper consort behaviors by sneaker males. However, this hypothesis alone does not explain why consort males do not attempt HH copulations when the female is far from spawning.

Another plausible explanation resides in ejaculate morphology and functioning. Dimorphic ejaculates show several adaptations to each sperm deposition site, possibly associated with differences in the interval between mating and fertilization, presence of a sperm storage organ, and egg availability between the sites (Apostólico and Marian, 2017, 2018a,b). For example, sneaker and consort ejaculates show differences in morphology (short and club-like vs. elongate and hook-like spermatangia), sperm release mode (slow vs. fast), and sperm swimming behavior (aggregation vs. diffusion), respectively, that are presumably related to the distinct fertilization environments provided by the buccal membrane and mantle cavity. Therefore, theoretically, both types of ejaculates would be functionally suboptimal if sneaker and consort males interchanged between tactics. For example, the consort spermatangium would be too elongate for the buccal membrane, releasing sperm far from the seminal receptacle, and sperm release would be too fast, both characteristics possibly hindering sperm storage in the seminal receptacle (Apostólico and Marian, 2017).

If dimorphic ejaculates are specifically adapted to each deposition site, then how to explain the flexibility in mating behavior reported herein for intermediate males? One explanation could be that the reported flexibility in behavior is just the result of a physiological transition from sneaker to consort phenotype – then, spermatangia transferred during this time window could be suboptimal depending on their type and site of attachment. Suboptimal attachment could explain, for example, why no club-like spermatangia were found in the oviduct membranes of the female in trial 37, after MP mating with an intermediate male (**Supplementary Table S3**). It is possible that the club-like spermatangium cannot firmly attach itself to the oviduct membranes. Due to their small size, sneaker-like spermatangia could presumptively have less anchorage and attachment potential than consort-like ones, given their smaller ejaculatory apparatus and cement body (e.g., Marian, 2012a,b; Marian et al., 2012). If this is the case, then they could be more easily flushed from the female's mantle cavity. In trials 29 and 33, an inadequate attachment could also have happened due to intermediate spermatophores implanted near the seminal receptacle of the female (**Supplementary Table S3**). Intermediate spermatangia are disproportionally larger than the typical club-like ones found in that particular site. Further investigations accessing paternity rates of these intermediate males could help us understand if suboptimal attachment of spermatangia in unconventional sites may hamper the fertilization success of intermediate males.

Another explanation for the flexibility in mating behavior in intermediate males involves the fact that these males have a transition from sneaker- to consort-like ejaculates in their reproductive system (Apostólico and Marian, 2018b). Within this spermatangia gradient, the intermediate spermatangium type has consort-like morphology – i.e., elongate and hook-like (**Supplementary Figure S1I**) – but sneaker-like sperm – i.e., with self-swarming (Apostólico and Marian, 2018b). This indicates that the transition in spermatangium morphology happens earlier than in sperm swimming behavior (Apostólico and Marian, 2018b). Thus, when attempting HH copulations long before spawning, intermediate males may still have sneaker sperm in their spermatangia. In turn, when attempting MP mating, although sperm would still be sneaker-like, the hook-like spermatangium (from everted intermediate spermatophores) would be functionally suited for the mantle cavity site. Therefore, intermediate males could benefit from interchanging tactics during the transition from sneaker to consort phenotype. Present data on spermatangia type in each female site seem to corroborate this hypothesis, as intermediate males can transfer intermediate spermatophores to both female

sites (**Supplementary Table S3**). Also, although most of the intermediate males investigated herein transferred only sneaker-like spermatophores during mating (**Supplementary Table S3**), the hypothesis is not invalidated, as these males are likely "early" intermediate males that still had sneaker-like spermatophores in their storage organ (see below). With time, they would start transferring intermediate ejaculates to both female sites when mating in HH and MP. However, to further address this hypothesis, more data on whether intermediate spermatophores are interchangeably placed in both female sites is required. Moreover, additional investigations accessing not only the type of spermatangia and sperm transferred to females during each type of mating, but also analyzing long-term behavior of intermediate males are necessary.

When Apostólico and Marian (2018b) first discovered a few (and rare – but see discussion below) males with sneaker-, intermediate-, and consort-like spermatophores simultaneously in their storage organ (**Supplementary Figure S1H**), the authors promptly treated them as probable abnormalities. Only after further analyses, e.g., when the authors realized that these spermatophores were spatially separated inside the organ, they proposed that these rare males were in fact an "intermediate" stage in the ontogenetic transition from sneaker to consort phenotypes. According to their spatial distribution in the organ, males must first use all of their sneaker-like spermatophores (the oldest ones, located more anteriorly in the organ), the intermediate ones, and only then start transferring consort-like ejaculates (the newest ones, located more posteriorly in the organ). As aforementioned, most intermediate males investigated in the present study transferred only sneaker-like structures, despite the mating posture adopted. Also, although a few of them transferred intermediate spermatophores, none of them transferred consort-like ejaculates to females (**Supplementary Table S3**). These findings are congruent with (and provide new evidence on) a transition in ejaculate production proposed by Apostólico and Marian (2018b). Within the ontogenetic hypothesis, "early" intermediate males may have already started their phenotypic transition (thus being able to mate in both HH and MP), but still transfer the remaining sneaker-like ejaculates before transferring intermediate spermatophores. We hypothesize that, with time, intermediate males would eventually use all of their intermediate spermatophores, cease HH, and perform only MP matings, as legit consorts.

At last, it is important to highlight that ca. 18% (12 out of 66) of all males sampled herein were classified as intermediate males after spermatophore and spermatangia analyses, possibly indicating that intermediate males of *D. pleii* may not be as rare as previously believed (5 out of a total sample of 287 males in Apostólico and Marian, 2018b). The former study argued that intermediate males should correspond to a very rapid transitional stage during ontogeny of dimorphic males, thus explaining their rarity. Here, behavioral results have shown that intermediate males typically behave as sneaker males, i.e., they continuously pursue mating with (new or the same) females. So, these observations might be additional evidence that, if intermediate males do not stop mating and transferring spermatophores, evidence of intermediate ejaculates could be underestimated.

An alternative explanation for the numerical difference in intermediate males sampling could be related to the time of year in which each study was conducted. While samples were obtained mostly from late-spring to early-summer in the former study (Apostólico and Marian, 2018b), the present one was carried out from mid- to late-summer (i.e., until the end of the reproductive peak). Within the ontogenetic hypothesis proposed by Apostólico and Marian (2018b), some males benefit from maturing at smaller size and age and acting as sneaker males along the beginning of their reproductive phase, later changing to a consort morph as they reach a certain body size. If the behavioral and morphological transition is triggered along the ongoing reproductive peak of the population, one could expect finding not only an increasing number of intermediate males toward the end of the reproductive peak, but also a lot of "early" intermediate males. This last hypothesis seems plausible in face of present data, as not only more intermediate males were sampled during mid- and late-summer, but most of them still transferred sneaker-like spermatophores to females.

CONCLUDING REMARKS

Although sneakers and consorts of *D. pleii* do not interchange between tactics, intermediate males, in turn, may show a context-dependent tactic expression, as they can play consort tactics during the female egg-laying period. Therefore, when the female is not ready to lay eggs, they may adopt sneaking tactics and place their sperm in a more secure location, i.e., in the female's seminal receptacle, when it must survive for longer until the female is ready to lay her eggs. However, performing MP could still be more advantageous than HH during spawning, even if the male does not have a complete consort-like ejaculate, given that the male would gain more fertilizations due to the proximity to the site of egg release. Moreover, considering that the intermediate males which performed MP during the trials had previously transferred spermatangia to the buccal membrane of the same female, this combined strategy of playing both mating tactics may guarantee additional fertilizations for intermediate males.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because in Brazil, ethics approval is still not required for experimentation with cephalopods by the CONCEA, but this study has been carried out in accordance with international

procedures for the welfare of cephalopods recommended in the literature.

AUTHOR CONTRIBUTIONS

JM and LA designed the study, analyzed the data, and wrote and reviewed the manuscript. LA collected the animals, performed the experiments, and obtained the data on morphology.

FUNDING

The authors appreciate the financial support and grants provided by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP; Proc. 2017/16182-1), Coordination for the Improvement of Higher Education Personnel (CAPES - Financial Code 001), National Council for Scientific and Technological Development (CNPq; Proc. 142170/2017-8), and CAPES/PROEX.

ACKNOWLEDGMENTS

This study was conducted as part of the first author's Ph.D. thesis in the Graduate Program in Zoology of the Department of Zoology, at the University of São Paulo (USP). The authors thank the reviewers JV and WS, the editor EV, and an anonymous reviewer for the comments that helped to improve the quality of the manuscript. The authors are also grateful for the logistic support for animal collection and maintenance at the Center of Marine Biology of the University of São Paulo (CEBIMar-USP), and especially for the laboratory technicians who assisted in squid sampling. This is a contribution of NP-BioMar (Research Center for Marine Biodiversity – USP).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01180/full#supplementary-material

FIGURE S1 | Mating system of the loliginid squid *Doryteuthis pleii*. **(A)** Sneaker males (mantle length, ML < 169 mm) adopt head-to-head (HH) mating posture, whereas consort males (ML > 169 mm) adopt male-parallel (MP) mating posture to mate with females. **(B)** Frontal view of the female mouth region, showing the location of the seminal receptacle (blue star). **(C)** Sagittal section of the female's seminal receptacle, showing club-like spermatangia from sneaker males, attached during HH mating. **(D)** Consort males express stereotyped body patterns (e.g., the exhibition of red stripes along the body, known as "lateral flames") in agonistic contests with rival males for female monopolization. **(E,F)** During MP mating, consort males attach hook-like spermatangia to the oviduct membranes of females (green star), inside the mantle cavity. **(G)** During egg capsule formation, consort sperm contacts the eggs first, near the oviduct opening (green star), whereas sneaker sperm contacts the eggs when the egg mass travels near the mouth region of the female (blue star), before being deposited on the substrate. **(H)** Spermatophores from intermediate males, showing a possible transition from the sneaker-like (top) to the consort-like morphology (bottom). While sneaker and consort males have one type of spermatophore (the one on the top and on the bottom, respectively), intermediate males may store both types and intermediate spermatophores (middle) altogether. **(I)** Spermatangia from intermediate males. Club-like spermatangium (typical of sneaker males, left), intermediate spermatangium (center), and hook-like spermatangium (typical of consort males, right). All figures originally published in Apostólico and Marian (2018b) and reproduced here with permission.

TABLE S1 | Trials performed using one female with either (i) one consort (trials 01–04, *n* = 4) or (ii) one sneaker male (trials 05–10, *n* = 6) of *Doryteuthis pleii* in captivity.

TABLE S2 | Trials performed using one female with either (i) one consort and one sneaker male (trials 11–12, *n* = 2), (ii) two sneaker males (trial 13, *n* = 1), or (iii) two consort males (trials 14–18, *n* = 5) of *Doryteuthis pleii* in captivity.

TABLE S3 | Trials performed using one female with either (i) one intermediate male (trials 19–25, *n* = 7), (ii) one intermediate and one consort male (trials 26–33, *n* = 8), or (iii) one intermediate and one sneaker male (trials 34–37, *n* = 4) of *Doryteuthis pleii* in captivity.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared committee membership, though no other collaboration, with one of the authors, JM, in the Cephalopod International Advisory Council.

frontiers in Physiology

ORIGINAL RESEARCH
published: 09 October 2019
doi: 10.3389/fphys.2019.01238

Sexual Selection and the Evolution of Male Reproductive Traits in Benthic Octopuses

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Competition between same-sex organisms, or intra-sexual selection, can occur before and after mating, and include processes such as sperm competition and cryptic female choice. One of the consequences of intra-sexual selection is that male reproductive traits tend to evolve and diverge at high rates. In benthic octopuses, females often mate with more than one male in a single reproductive event, opening the venue for intra-sexual selection at multiple levels. For instance, males transfer spermatophores through hectocotylus, and can remove the spermatophores left by other males. Considering the limited evidence on post-copula competition in benthic octopuses, and the potential to affect the evolution of reproductive traits within octopodids, we put this hypothesis to a test employing a phylogenetic comparative approach. We combined data on hectocotylized arm length (HAL), ligula length (LL), spermatophore length (SL) with a Bayesian molecular phylogeny of 87 species, to analyze how reproductive traits have diverged across lineages and covary with body size (mantle length; ML). First, additionally to ML, we estimated the phylogenetic signal (λ) and mode of evolution (κ) in each reproductive trait. Second, we performed phylogenetic regressions to quantify the association among reproductive traits and their co-variation with ML. This analysis allowed us to estimate the phenotypic change along a branch into the phylogeny, and whether selection may have played a role in the evolution and diversification of specific clades. Estimations of λ were always high (>0.75), indicating concordance between the traits and the topology of the phylogenetic tree. Low values of κ (<1.0) suggested that evolution depends on branch lengths. All reproductive traits exhibiting a positive relation with ML ($\beta > 0.5$ in all cases). Overall, evolutionary rate models applied to the SL-ML regression suggested that octopuses of the family Megaleledonidae have evolved larger spermatophores than expected for their size. The regression HAL-ML indicated that HAL was more variable in Megaleledonidae than in the remaining clades, suggesting that the high divergence across species within this group might partially reflect intra-sexual selection. These results support the hypothesis that, at least in some lineages, sexual selection may account for the divergence in reproductive traits of male octopuses.

Keywords: Octopodoidea, sexual selection, sperm competition, cryptic choice, spermatophores, hectocotylus, ligula, phylogeny

OPEN ACCESS

Edited by:
Rui Rosa,
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Reviewed by:
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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *18 April 2019*
Accepted: *10 September 2019*
Published: *09 October 2019*

Citation:
Ibáñez CM, Pérez-Álvarez J, Catalán J, Carrasco SA, Pardo-Gandarillas MC and Rezende EL (2019) Sexual Selection and the Evolution of Male Reproductive Traits in Benthic Octopuses. Front. Physiol. 10:1238. doi: 10.3389/fphys.2019.01238

INTRODUCTION

The evolution of mate choice and mating competition has been a major component of Darwin's theory of sexual selection (Darwin, 1871). Since then, it has been generally accepted that different selective pressures acting on male and female attributes may give rise to sexually dimorphic traits, which are often interpreted as evidence of direct competition for mates within a given sex, differential success to attract potential mates from the opposite sex, or gametic competition (see Basolo, 1990; Birkhead, 1992; Evans and Sherman, 2013). At the gametic level, competition can occur before and after mating through sperm competition or cryptic female choice. Whereas sperm competition involves strategies by the male to either remove, displace or inhibit the sperm of other males, cryptic female choice constitutes female-biased selection through the use/removal of sperm to fertilize their eggs. Competition at the gametic level has been described in organisms from several phyla, such as insects, molluscs, birds and mammals (Mann, 1984), and is generally enhanced in polyandric species where females can mate with multiple males in a single reproductive episode (Birkhead, 1998; Gomendio, 2002). Because of its relevance to males' reproductive success (Eberhard, 1998; Snook, 2005), multiple responses have evolved to outcompete rival males, including: (i) the production of spermatophores or packages of sperm (Mann, 1984; Nigmatullin et al., 2003) that, when transferred to females, may occupy considerable space within the storage organs preventing other spermatophores from being stored (Thornhill and Alcock, 1983), (ii) the removal of other males' spermatophores during copulation (Cigliano, 1995), or (iii) the production of sperm with inhibitory effects on the rival males' sperm function (Snook, 2005). Because of this evolutionary arms race, the genitalia of several organisms exhibit extreme differences in size and shape across closely related species and are presumed to evolve faster than other traits (Eberhard, 1985; Genevcius et al., 2017).

Polyandry, sexual dimorphism and sexual selection have been described in several lineages of cephalopod molluscs and is widespread in this group (Mann, 1984; Hanlon and Messenger, 1996; Squires et al., 2012). Polyandrous behavior has been observed in female octopuses, potentially increasing post-mating sexual selection and driving the evolution of a myriad of sperm transfer strategies (e.g., male mounting female; **Figure 1A**; for other examples see Hanlon and Messenger, 1996; Cheng and Caldwell, 2000; Huffard et al., 2008; Gutierrez et al., 2012; Morse and Huffard, 2019). In all cases, male octopuses pack their sperm into spermatophores and transfer them to females by using a modified arm called hectocotylus. This specialized arm employed to deposit the sperm packages into the females' pallial cavity is characterized by two well defined segments, the calamus and the ligula (see Hanlon and Messenger, 1996; Wodinsky, 2008; Marian, 2015), and by a considerable inter-specific morphological variation (**Figure 1B**). This variation has been associated with the successful transference of spermatophores during mating (Robson, 1926); however, direct behavioral evidence on their role in removing or breaking down spermatophores from rival males remain speculative (Cigliano, 1995; Hanlon and Messenger, 1996; Norman et al., 2004), providing an important framework for evaluating untested hypotheses on sexual selection (see Voight, 2009). Furthermore, spermatophores also exhibit considerable inter-specific differences in size (e.g., ranging from 7 to 1130 mm in length), even after accounting for size effects (Voight, 2009). This is likely because individuals with larger spermatophores have greater sperm reservoirs and consequently much more sperm to fertilize females' eggs (Voight, 2001). Nonetheless, in spite of the high levels of morphological variation in these traits, the evolution of hectocotyli and spermatophores across benthic octopuses remains poorly understood, as previous comparative studies have not accounted for the evolutionary history of the lineages involved (see Voight, 2001, 2002, 2009).

Here, we study the evolution and diversification of these reproductive traits across benthic octopuses, employing phylogenetic analytical methods that take into consideration patterns of relatedness between different lineages. Phylogenetic methods are currently indispensable to understand patterns of phenotypic diversification and their underlying processes, as well as the direction and magnitude of inferred evolutionary changes (Felsenstein, 1985; Harvey and Pagel, 1991; Rezende and Diniz-Filho, 2012). Accordingly, recent phylogenetic comparative studies have been quite successful in reconstructing the evolution of life-history strategies in cephalopods in response to different environmental pressures (see Lindgren et al., 2012; Ibáñez et al., 2014, 2018; Pardo-Gandarillas et al., 2018). In the present work, we used a phylogenetic approach to: (a) reconstruct how hectocotyli and spermatophores have evolved along a molecular phylogeny including 87 species of benthic octopuses, (b) explore the correlated evolution between these traits and body size, and (c) employ variable-rates phylogenetic regression to determine which clades exhibit abnormally high rates of phenotypic evolution in response to selection. Even though results could be interpreted as putative evidence of strong post-copulatory sexual selection, we also discuss alternative adaptive scenarios that might have given rise to the observed differences across lineages.

MATERIALS AND METHODS

Dataset and Phylogeny

We performed an extensive literature review to obtain information on the variability of reproductive traits across different lineages of benthic octopuses. For our analyses, we selected descriptive measures that have been extensively studied with relatively standardized protocols and that could, therefore, be readily compared across different studies (see **Table 1**): mantle length (ML), arm length (AL), ligula length (LL), hectocotylized arm length (HAL), and spermatophores length (SL). Subsequently, we combined this information with a new phylogenetic hypothesis of benthic octopuses encompassing a total of 97 species, including outgroups (Mendeley Datasets: doi: 10.17632/5vkm46hm49.1), that are based on three mitochondrial genes (16S ribosomal RNA, Cytochrome oxidase I, Cytochrome oxidase III) and one nuclear gene (Rhodopsin). A detailed explanation regarding the analyses underlying the phylogenetic reconstruction, estimation of uncertainty and validation of our working phylogeny has been

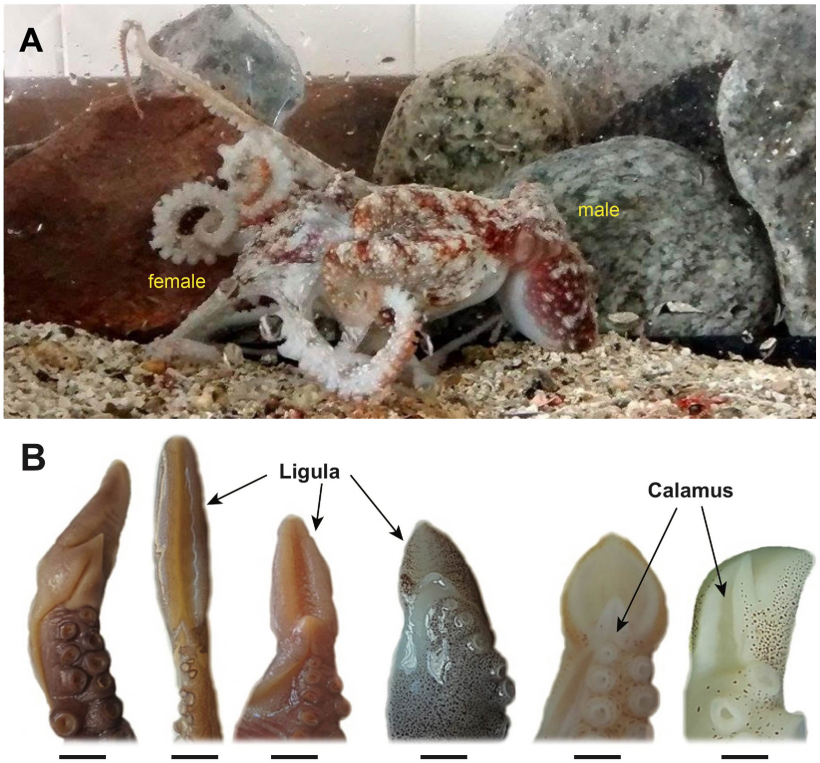

FIGURE 1 | Mating behavior and reproductive organs in benthic octopuses. **(A)** Mounting during the copula stage, in the small-sized benthic octopus *Robsonella fontaniana*, where male transfers spermatophores into the females' mantle cavity using the modified arm called hectocotylus. **(B)** Morphological diversity of benthic octopus hectocotyli, showing the differentiated ligula and calamus. From left to right: *Muusoctopus tangaroa*, *Pinnoctopus cordiformis*, *Muusoctopus longibrachus*, *Graneledone taniwha*, *Octopus huttoni*, *Octopus mernoo* (Photo credits: SC and CI, respectively).

provided elsewhere (Ibáñez et al., 2018). Briefly, phylogenetic relationships were inferred from a partitioned matrix (16S, COI + COIII, RHO) with a different substitution model for each gene. This matrix was composed of 97 species, including 88 species from the superfamily Octopodoidea, and two species from the superfamily Argonautoidea, six cirrates and the vampire squid *Vampyroteuthis infernalis* as outgroups. Bayesian analyses were conducted using MrBayes 3.2 with four chains, each with ten million generations, sampled every 1,000 generations. The first 1,000 trees of each run were discarded as burn-in, and a consensus of the remaining trees was calculated. For simplicity, we have excluded the outgroups, providing the reconstructed relationships for the 87 benthic species in the dataset (excluding *Vitreledonella richardi*, **Figure 2A**).

Statistical Analyses

We performed three complementary analyses to determine how the variation in reproductive traits is related to phylogenetic history. First, we performed univariate analyses to estimate the amount of phylogenetic signal (λ) and the mode of evolution (κ) of each trait (ML, HAL, LL, and SL) employing BayesTraits v3 (Meade and Pagel, 2017). Second, we ran three separate variable-rates regression models (Baker et al., 2016) to determine how HAL, LL, and SL vary as a function of body size (ML), and also diagnose in which lineages these structures have diverged more than expected after controlling for size effects. Third, we performed a phylogenetic principal component analysis (PCA) to determine the degree of covariation between reproductive traits and study correlated evolution between them after removing body size effects.

Phylogenetic signal in our univariate analyses was estimated employing Pagel's λ, which quantifies the tendency of closely related lineages to resemble each other in comparison to a Brownian motion model of evolution (Pagel, 1999, 2002): $\lambda = 1$ indicates that the distribution of the phenotypic traits along the tips of the phylogeny closely resemble the expectation based on Brownian motion (i.e., high phylogenetic signal), whereas $\lambda = 0$ shows that patterns of phenotypic resemblance due to shared phylogenetic history is negligible (i.e., low phylogenetic signal). The mode of phenotypic evolution was estimated using Pagel's κ, which scales the branch lengths between their original values and a single constant, mimicking gradual evolution when $\kappa = 1$, and a punctuated model of evolution when $\kappa = 0$ (Pagel, 1999, 2002). The posterior distribution of all parameters was visualized in the software Tracer V1.6 (Rambaut et al., 2013). To test if λ and κ estimated values were different from pre-established values, we first estimated the higher posterior distribution of these parameters for each trait in BayesTraits V3, forcing each parameter to have a value of 0 for λ (i.e., no signal) and

TABLE 1 | Summary of the studied species, with information on their body size (ML, maximum mantle length) and reproductive traits (SL, maximum spermatophore length; LL, maximum ligula length; HAL, maximum hectocotylized arm length; AL, maximum arm length; EL, maximum egg length).

Species	ML (mm)	SL (mm)	LL (mm)	HAL (mm)	AL (mm)	EL (mm)	References
Abdopus aculeatus	70	21	1.40	441	490	3	Voight, 2009; Jereb et al., 2014
Adelieledone piatkowski	73	38	11.46	182.21	189.8	16	Allcock et al., 2003; Jereb et al., 2014
Adelieledone polymorpha	105	41	16.49	207.27	274.6	16	Allcock et al., 2003; Barratt et al., 2008
Ameloctopus litoralis	30	6.40	3.60	60.30	270	10	Toll and Voss, 1998; Voight, 2009; Jereb et al., 2014
Amphioctopus aegina	100	27	6	240	300	2.4	Huffard and Hochberg, 2005; Voight, 2009; Jereb et al., 2014
Amphioctopus kagoshimensis	87	160	6.96	234.90	261	2	Toll and Voss, 1998; Jereb et al., 2014
Amphioctopus marginatus	80	80	2.80	192	240	3	Huffard and Hochberg, 2005; Jereb et al., 2014
Bathypolypus arcticus	65	64	14.95	84.50	130	18	Muus, 2002; Jereb et al., 2014
Bathypolypus sponsalis	70	34	15.40	140	210	15	Muus, 2002; Voight, 2009
Callistoctopus luteus	130	87	5.20	468	780	4	Toll and Voss, 1998; Jereb et al., 2014
Callistoctopus minor	29	23	6.64	72.50	145	22	Toll and Voss, 1998; Ibáñez et al., 2018
Callistoctopus ornatus	130	51	8.97	728	1040	3.5	Toll and Voss, 1998; Voight, 2009; Jereb et al., 2014
Cistopus chinensis	100	40	2.40	360	400	15	Jereb et al., 2014
Cistopus indicus	90	30	2.70	405	540	4.5	Toll and Voss, 1998; Voight, 2009; Jereb et al., 2014
Cistopus taiwanicus	140	33	0.70	525	700	7	Jereb et al., 2014
Eledone cirrhosa	150	54	6	360	450	9	Voight, 2009; Jereb et al., 2014
Enteroctopus dofleini	600	1130	144	2400	3000	8	Toll and Voss, 1998; Voight, 2009; Jereb et al., 2014
Enteroctopus megalocyathus	280	370	61.60	1260	1400	15	Ortiz et al., 2011; Jereb et al., 2014
Enteroctopus zealandicus	272	316	53.86	764.32	2067.2	12.5	O'Shea, 1999
Graneledone antarctica	45	8	4.50	145	165	–	Voss, 1976; O'Shea, 1999
Graneledone boreopacifica	145	131	8.55	1047.48	1218	16	Voss and Pearcy, 1990; Hochberg, 1998; Voight, 2009
Graneledone challengeri	145	–	8.55	356.84	433.5	19.5	O'Shea, 1999
Graneledone taniwha kubodera	154.5	–	10	44	99	22	O'Shea, 1999
Graneledone taniwha taniwha	170	118	12.92	381.99	418.2	24	O'Shea, 1999
Graneledone verrucosa	110	100	5.06	325.60	385	17	Allcock et al., 2003; Jereb et al., 2014
Grimpella thaumastocheir	50	30	3.50	157.50	225	15	Jereb et al., 2014
Hapalochlaena fasciata	50	16	6	112.50	150	9	Voight, 2009; Jereb et al., 2014
Hapalochlaena lunulata	50	–	5	80	100	–	Kaneko et al., 2011; Jereb et al., 2014
Hapalochlaena maculosa	57	67	7.41	134.52	177.3	9	Toll and Voss, 1998; Jereb et al., 2014
Megaleledone setebos	234	100	9.83	702	744.6	41.5	Allcock et al., 2003; Jereb et al., 2014
Muusoctopus eicomar	95	79	7.03	281.01	347.7	24	Ibáñez and Cifuentes-Bustamante, 2016; Ibáñez Pardo-Gandarillas, 2016
Muusoctopus eureka	110	75	8.80	176.22	587.4	19	Gleadall et al., 2010; Ibáñez Pardo-Gandarillas, 2016
Muusoctopus januarii	63	85	5.67	227.99	342.1	19	Allcock et al., 2006; Jereb et al., 2014; Ibáñez Pardo-Gandarillas, 2016
Muusoctopus johnsonianus	113	104	9.49	235.04	413.3	–	Allcock et al., 2006; Ibáñez Pardo-Gandarillas, 2016
Muusoctopus levis	50	–	4.40	100	142.4	–	Ibáñez Pardo-Gandarillas, 2016
Muusoctopus longibrachus	115	70	9.54	296.70	732.5	20	Ibáñez et al., 2006; Ibáñez Pardo-Gandarillas, 2016; Gleadall et al., 2010
Muusoctopus oregonensis	93	62	6.23	370.79	420.9	26	Voss and Pearcy, 1990; Ibáñez Pardo-Gandarillas, 2016
Muusoctopus profundorum	67	–	2.33	201	268	–	Gleadall et al., 2010; Ibáñez Pardo-Gandarillas, 2016
Muusoctopus rigbyae	105	104	16.80	315	420	24	Vecchione et al., 2009
Muusoctopus tangaroa	100	120	18.50	202	494	23	O'Shea, 1999
Muusoctopus thielei	65	–	8.45	143	175.5	–	Ibáñez Pardo-Gandarillas, 2016

(Continued)

TABLE 1 | Continued

Species	ML (mm)	SL (mm)	LL (mm)	HAL (mm)	AL (mm)	EL (mm)	References
Muusoctopus yaquinae	83	136.1	9.79	198.54	258.2	12	Voss and Pearcy, 1990; Ibáñez Pardo-Gandarillas, 2016
Octopus bimaculatus	200	35	1.40	700	1000	4	Jereb et al., 2014
Octopus bimaculoides	120	33	2.76	336	420	18	Jereb et al., 2014
Octopus californicus	140	70	30.80	396.90	490	17	Hochberg, 1998; Jereb et al., 2014
Octopus campbelli	36	–	6.60	78	104	1.7	O'Shea, 1999
Octopus conispadiceus	166	80	33.20	398.40	498	28	Toll and Voss, 1998; Jereb et al., 2014
Octopus cyanea	172	48	3.44	928.80	1032	3	Toll and Voss, 1998; Voight, 2009; Jereb et al., 2014
Octopus fitchi	29	19	–	–	–	–	Jereb et al., 2014
Octopus hongkongensis	200	200	28	644	164	–	Toll and Voss, 1998; Ibáñez et al., 2018
Octopus huttoni	57	39	9.80	129	180	3.1	O'Shea, 1999
Octopus insularis	120	35	2.04	453.60	504	1.5	Leite et al., 2008; Jereb et al., 2014
Octopus kaurna	85	88	6.80	416.50	595	11	Toll and Voss, 1998; Jereb et al., 2014
Octopus laqueus	24	22	0.62	57.36	106.8	2.6	Kaneko and Kubodera, 2005; Voight, 2009
Octopus maya	250	56	4.75	870	1125	17	Voss and Toll, 1998; Jereb et al., 2014
Octopus mernoo	85	–	15.47	105.40	168.1	23.5	O'Shea, 1999
Octopus mimus	155	58	2.79	716.10	930	3.2	Voight, 2009; Jereb et al., 2014
Octopus oliveri	69	34	1.52	224.25	273.1	7.5	Toll and Voss, 1998; O'Shea, 1999
Octopus pallidus	150	173	24	355.05	394.5	13	Toll and Voss, 1998; Jereb et al., 2014
Octopus parvus	40	12	2	120	280	1.8	Toll and Voss, 1998; Ibáñez et al., 2018
Octopus rubescens	100	60	11	405	450	4	Hochberg, 1998; Jereb et al., 2014
Octopus salutii	125	74	20.63	953.75	1148.7	6	Mangold, 1998; Toll and Voss, 1998; Voight, 2009
Octopus tehuelchus	60	57	2.70	180	240	15	Ré, 1998; Toll and Voss, 1998; Alves and Haimovici, 2011; Jereb et al., 2014
Octopus tetricus	135.5	24	4.10	418	547	3	O'Shea, 1999; Ibáñez et al., 2018
Octopus vulgaris	250	65	5.25	180.50	1375	2.7	Mangold, 1998; Toll and Voss, 1998; Voight, 2009; Jereb et al., 2014
Octopus wolfi	15	8.7	1.50	–	99	–	Toll and Voss, 1998
Pareledone aequipapillae	63	94	5.54	90.97	133.2	20	Allcock, 2005
Pareledone albimaculata	38	46	3.80	59.01	81.4	10	Allcock, 2005
Pareledone aurata	49	55	5.54	61.98	81.3	11	Allcock, 2005
Pareledone charcoti	70	61	7.98	66.99	128.1	13	Kubodera and Okutani, 1994; Allcock, 2005
Pareledone cornuta	60	71	5.58	64.02	108.8	20	Allcock, 2005
Pareledone felix	42	70	4.75	66.99	69.5	22	Allcock et al., 2007; Voight, 2009
Pareledone panchroma	41	37	4.26	50.02	56.8	14	Allcock, 2005
Pareledone serperastrata	36	52	3.06	59.00	62.1	7	Allcock, 2005
Pareledone subtilis	44	42	4.4	49.98	67.1	14	Allcock, 2005
Pareledone turqueti	60	72	8.5	–	250	19.8	Daly and Rodhouse, 1994; Allcock et al., 2007; Barratt et al., 2008
Paroctopus digueti	42	22	3.36	107.10	126	10	Jereb et al., 2014
Pinnoctopus cordiformis	310	228	15.50	686.34	261	7	Voight, 2009; Jereb et al., 2014
Praealtus paralbida	65	120	3.05	220.02	221	–	Allcock et al., 2004; Jereb et al., 2014
Robsonella fontaniana	69	50	6.90	258.75	345	5	Toll and Voss, 1998; Ibáñez et al., 2008; Jereb et al., 2014
Scaeurgus unicirrhus	90	84	9.90	56.34	405	3	Mangold, 1998; Toll and Voss, 1998; Voight, 2009; Jereb et al., 2014
Thaumeledone gunteri	50	50	8.45	85	100	10	Allcock et al., 2004; Jereb et al., 2014
Thaumeledone peninsulae	48	45	4.70	51.02	70.1	13	Allcock et al., 2004
Thaumeledone rotunda	62	74.40	14	87	127	16	Allcock et al., 2004
Thaumeledone zeiss	55	–	9.35	58.08	79.5	9.3	O'Shea, 1999
Velodona togata	180	174	16.20	688.50	810	19	Jereb et al., 2014
Vulcanoctopus hydrothermalis	60	54	5.70	168	240	5.5	González et al., 1998, 2002

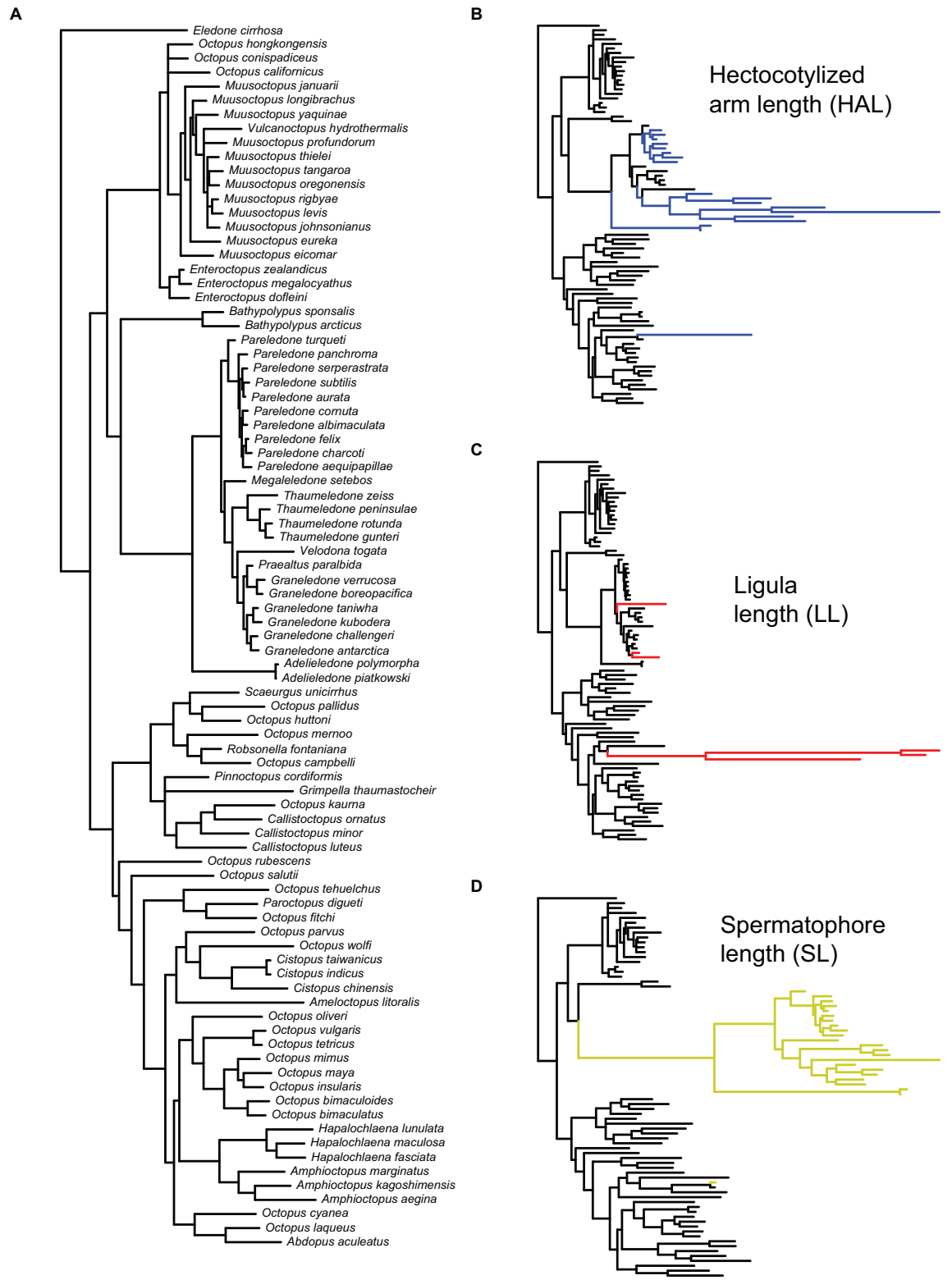

FIGURE 2 | Phylogeny of benthic octopuses and reconstructed phenotypic evolution of reproductive traits. **(A)** Phylogenetic hypothesis employed in this study (n = 87 spp.), with branch length proportional to the DNA sequence divergence (see section "Materials and Methods"). Results of the variable-rates regression model for **(B)** hectocotylized arm length (n = 84 spp.), **(C)** ligula length (n = 86 spp.), and **(D)** spermatophore length (n = 78 spp.). Branch lengths in these panels are scaled to estimated phenotypic change, and colored branches indicate regions in which positive selection is detected ($\Delta_V/\Delta_B > 2.0$).

0 and 1 for κ (i.e., perfectly punctuated or perfectly gradual evolution, respectively) and compared the fit of estimated vs. forced models with $\log_{10}$ Bayes Factor (BF). The larger the BF value, the better the fit of the estimated model in comparison against the forced one, with BF > 0.5 being generally interpreted as strong support for the estimated model and BF > 1 being considered decisive (Kass and Raftery, 1995). Because these univariate analyses include scaling effects, we expect HAL, LL, and SL to exhibit less signal (i.e., a lower λ) than ML if they evolve faster than this trait due to an evolutionary arms race (i.e., signal is expected to decrease if traits diverge fast in response to selection).

To estimate phenotypic selection on reproductive traits after removing potential scaling effects, we performed three separate variable-rates regressions with $\log_{10}$-transformed HAL, LL, and SL as a function of ML. This regression model was recently developed by Baker et al. (2016) and allows the rate of change to vary through the phylogenetic branches and identifies areas of the tree where the rate of evolution departs significantly from background levels (Venditti et al., 2011). With this purpose, this regression method estimates a branch-specific metric Δ_V/Δ_B that contrasts the expected phenotypic variance Δ_V along the branch due to changes in evolutionary rates (i.e., acceleration or deceleration) vs. the expectation attributable to the background evolutionary rate (Δ_B). Branches in which the amount of estimated phenotypic change doubles the background rate ($\Delta_V/\Delta_B > 2$) constitute regions of the phylogeny that were likely under positive selection (Baker et al., 2016). We implemented the variable-rates regression model with the phylogenetic independent contrast regression module in BayesTraits, employing the reversible-jump Markov chain Monte Carlo (RJMCMC) to determine whether $\Delta_V/\Delta_B > 2$ results were observed in more than 95% of the posterior distribution. In all Bayesian analyses described above, we ran 20,000,000 iterations via the MCMC method. Parameters were sampled every 1,000 iterations, excluding the first 25% of iterations. The 95% highest posterior density (95% HPD) for each parameter was calculated in Tracer, and all analyses performed in BayesTraits; outgroup and sister groups were excluded.

To determine to what extent the different reproductive traits studied here evolve in tandem, we performed a phylogenetic PCA (Revell, 2009) including $\log_{10}$-transformed ML, HAL, LL, and SL. To account for phylogenetic signal, λ was estimated concomitantly with parameters from the PCA. Because we were primarily interested in identifying potentially contrasting evolutionary strategies between lineages, we focused on the second and third principal components (PC2 and PC3) that provide information on differences in morphology or shape after removing size effects embedded in the first principal component.

Finally, to explore the association response to selection on other traits, the correlation between reproductive traits [spermatophore length (SL) and egg length (EL)] and morphological traits [arm length (AL) and HAL] were analyzed using phylogenetic generalized least squares (PGLS) regressions (Pagel, 1999). To account for phylogenetic signal, λ was estimated concomitantly with parameters from the regression model (Pagel, 2002). After reviewing different sources, egg length data was obtained only for 72 species (**Table 1**).

RESULTS

Among the species in our dataset, ML exhibited a 40-fold variation, with values ranging between 15 mm in *Octopus wolfi* to 600 mm in *Enteroctopus dofleini*. By contrast, reproductive traits tended to exhibit a higher variation between extremes, from 54-fold variation in HAL (i.e., 44–2,400 mm), 176-fold in SL (i.e., 6.4– 1,130 mm), and 257-fold in LL (0.56 – 144 mm) (see **Table 1**).

All traits exhibited a high phylogenetic signal, with λ > 0.75 statistically different from λ = 0, as indicated by BF > 11 in all traits (**Table 2**). As hypothesized (see section "Materials and Methods"), λ estimated for reproductive traits were generally lower than values for ML, agreeing with the observation that these traits exhibited more variation across species than ML. Regarding the mode of evolution inferred by κ, calculated in combination with λ in the univariate analyses, estimates for ML, HAL, LL, and SL were intermediate between κ = 0 and 1 and statistically different from those values according to BF estimates (BF > 1.39 for all comparisons) (**Table 2**). This implies that all traits evaluated tended to evolve slower than predicted (i.e., evolutionary stasis) in longer branches when compared to shorter branches (Pagel, 1999, 2002).

Phylogenetic regressions of reproductive traits as a function of ML indicated that all variables scaled positively with body size, with scaling exponents corresponding to 1.18 for HAL (95% HDP between 0.99 – 1.36), 0.73 (0.53 – 0.95) for LL, and 0.90 (0.71 – 1.10) for SL (**Figure 3**). Consequently, HAL tends to become disproportionally larger as size increases, whereas SL scales roughly isometrically, and LL is relatively shorter in larger lineages. According to variable-rates phylogenetic regressions controlling for these scaling effects, several regions of the phylogeny exhibited accelerated rates of phenotypic evolution, and met the criterion of $\Delta_V/\Delta_B > 2$ proposed as evidence of positive selection (**Figure 2**). This was particularly true for HAL and SL, for which we detected selection in a total of 33 and 44 branches, respectively, or roughly 20 to 30% of all branches (**Table 3**). Interestingly, separate analyses for both traits gave rise to similar qualitative results, suggesting accelerated rates of phenotypic divergence for these traits in Antarctic, and deep-sea octopuses from the family Megaleledonidae (**Figures 2B,D**). In contrast, evidence of positive selection in LL was limited to only 8 branches, or 4.9% of the total (**Table 3**), most of them involving the *Cistopus* clade (**Figure 2C**).

The phylogenetic PCA including $\log_{10}$-transformed ML, HAL, LL, and SL strongly supported correlated evolution between these reproductive traits (**Figure 4**). As expected, PC1 accounted for a substantial fraction of the variance in the original data (70.8%), which could be attributed to a variation associated with body size, whereas the remaining PCs involve phenotypic variation that is independent of size (i.e., "shape" for simplicity; **Figure 4**). Accordingly, ML loadings in the remaining PCs were very low because most variation in this trait was explained by PC1. After removing the effects of size, PC2, and PC3 combined accounted

TABLE 2 | Phylogenetic signal (λ) and evolutionary mode (κ) obtained in univariate analyses.

	Lambda (λ)	BF (λ > 0)	Kappa (κ)	BF (κ > 0)	BF (κ < 1)
ML	0.91 (0.77–0.98)	12.05	0.55 (0.29–0.82)	5.76	4.25
HAL	0.86 (0.67–0.97)	11.58	0.52 (0.24–0.83)	3.23	3.20
LL	0.87 (0.75–0.96)	28.23	0.22 (0.01–0.45)	11.92	13.47
SL	0.75 (0.45–0.96)	11.15	0.61 (0.31–0.93)	1.39	1.41

Numbers in parentheses correspond to the highest posterior density (95% HDP) and represent the 95% credibility confidence estimated with a Bayesian approach, whereas the Bayes factor (BF) provides the weight of the evidence.

for 91.1% of the variance in shape observed across lineages, with loadings indicating that most of the variance in HAL is explained by PC2 and in SL by PC3, with LL falling somewhere in between (**Figure 4**). Contrasting these results against the outcome of the variable-rates regressions, we can identify two distinct groups (**Figure 4**), one exhibiting reduced hectocotyli and large spermatophores (low HAL and high SL), and the other with relatively large hectocotyli with small ligulae (high HAL and low LL). As clearly illustrated in **Figure 4**, results from variable-rates regressions performed separately for each reproductive trait provided very consistent results and complementary evidence of positive selection across the same phylogenetic lineages.

Spermatophore length did not correlate with egg length (n = 72 species, r = 0.050, 95% HPD between 0.0013 and 0.1082, λ = 0.85, **Supplementary Figure S1**). Interestingly, the correlation between arm length and hectocotilized arm length was higher than zero (n = 85 species, r = 0.8124, 95% HPD between 0.7946 and 0.8372, λ = 0.75, **Supplementary Figure S2**).

DISCUSSION

The present results support our original hypothesis on reproductive traits in male benthic octopuses, evidencing that spermatophores, and hectocotyli (arm and ligula) exhibited accelerated rates of evolution, at least in several Antarctic and deep-water lineages (Megaleledonidae and *Cistopus*), presumably due to sexual selection. All reproductive traits showed a fold-range of morphological variation that is substantially larger than expected based solely on differences in body size (i.e., ML), and variable-rates regression analyses clearly indicated that several lineages tended to deviate substantially from allometric expectations. While our analyses focussed primarily on the variation in reproductive traits after statistically removing the effects of size (effectively using levels of ML divergence between lineages as a standard of comparison), body size may have also been under selection in some lineages within Octopodoidea, as observed in other clades exhibiting sexual dimorphism in size (e.g., Amphitretidae and Tremoctopodidae Jereb et al., 2014). Unfortunately, this possibility cannot be directly tested with our phylogenetic comparative approach in the absence of precise information on most aspects of reproductive behavior of the analyzed species, including competition for mates, mate choice and mating position, as well as their intraspecific body size variation. Additionally, other environmental variables and selective pressures may have contributed to body size evolution of many of these lineages. Nonetheless, it is important to consider that body size may also evolve in response to sexual selection and gametic competition. For instance, we detected a clear positive association between ML and SL, indicating that larger species – and presumably larger individuals within a species – have bigger spermatophores and consequently more sperm to transfer to females. Accordingly, this same trend was previously described by Voight (2009); therefore, we do not only provide support for such finding within a strict phylogenetic context, but we also detected which groups and lineages deviated from allometric expectations (see below).

Admittedly, while our analyses provided strong evidence of selection in several regions of the phylogeny, some limitations must be highlighted. First, note that our phylogenetic analysis detects regions of the phylogeny with extraordinary rates of evolution in comparison to background rates inferred from the same dataset, which is inherently conservative and can only detect selection in restricted regions of the phylogeny. Therefore, it is possible that we might have missed other evolutionary clades whose phenotypic diversification might be partly explained by selection (while decreasing the $\Delta_V/\Delta_B > 2$ might partly circumvent this problem, it would also increase the type I error). Second, our analyses do not inform specifically on the mechanisms that underlie these results. Consequently, alternative adaptive scenarios must be taken into account to determine the likelihood that observed patterns emerge from sexual selection. In this context, we believe that two possibilities are worth considering: (1) results reflected adaptation to environmental conditions, and/or (2) they partly reflected selection on correlated traits. With regard to the first scenario, the high evolutionary rates (i.e., $\Delta_V/\Delta_B > 2$) in HAL and SL involved primarily lineages of Antarctic and deep-sea octopuses from the family Megaleledonidae. Because ectotherm organisms inhabiting cold waters tend to exhibit lower fecundity, slower growth rates, and larger life-spans (van Voorhies, 1996; Ibáñez et al., 2018), it is plausible to expect that cold-adapted lineages may evolve larger spermatophores compared to warm-water species (Voight, 2009). Moreover, among deep-sea organisms that live in low densities the probability of a mating encounter is reduced (see Hoving et al., 2012), and therefore, selection may favor a high reproductive investment per mating. Consequently, we suggest it is possible that the colonization of cold-waters and deep-sea habitats might partly explain the high evolutionary rates detected in this clade (Megaleledonidae) and their larger spermatophores. Alternatively, it is also possible that some of the patterns detected reflect correlated responses to selection on other traits, which might be particularly true for HAL given its close association with AL (r = 0.81). All lineages detected in the variable-rates

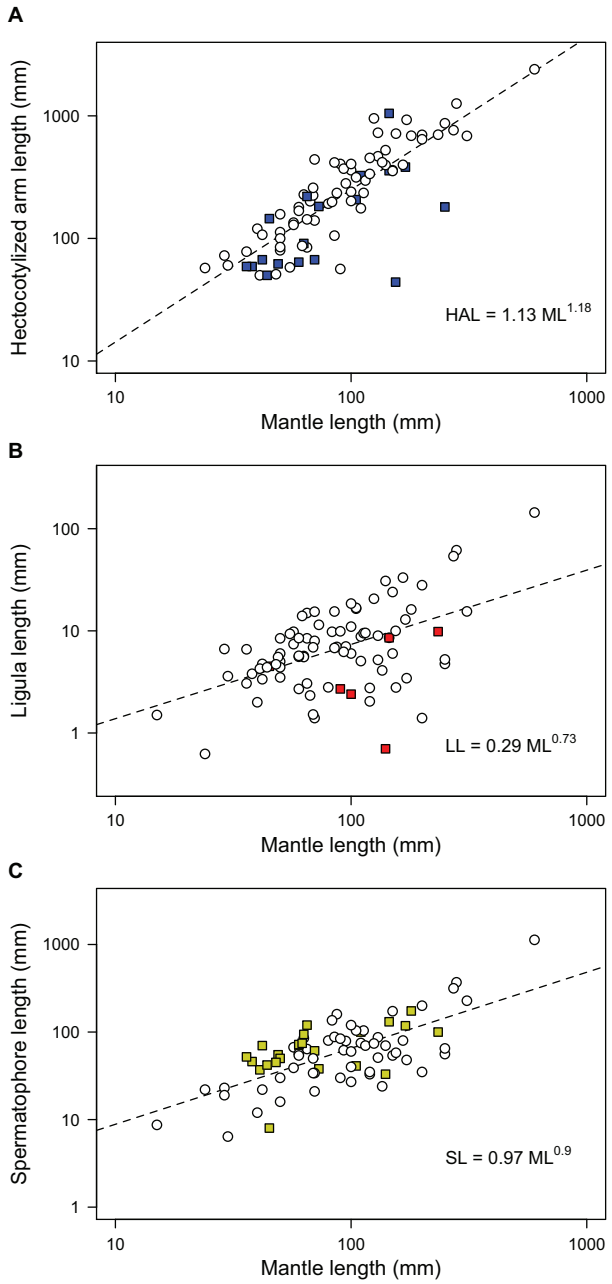

FIGURE 3 | Scaling of morpho-functional reproductive traits in benthic octopuses. Mantle length is plotted against **(A)** hectocotylized arm length, **(B)** ligula length, and **(C)** spermatophore length. Colored symbols correspond to the lineages where we detected positive selection – i.e., more divergence than expected based on background rates of phenotypic evolution – according to a variable-rates regression model. Colors as in **Figure 2**.

TABLE 3 | Results of the variable-rates model for positive selection over three reproductive traits of benthic octopuses.

	Total branches	Mean Δ_V/Δ_B (±SD)	Branches under selection	Mean $\Delta_V/\Delta_B > 2$ (±SD)
HAL ~ ML	160	3.04 ± 5.33	33	10.39 ± 8.43
LL ~ ML	164	1.47 ± 2.16	8	9.20 ± 6.04
SL ~ ML	150	2.03 ± 1.21	44	3.84 ± 0.52

regression for HAL exhibited smaller arms than predicted from allometry, though it is not clear exactly which selective pressures might favor smaller arms.

Importantly, while these alternative evolutionary scenarios might justify why members of the family Megaleledonidae differed from other groups, they failed to explain the extremely high diversity within this clade and its degree of phenotypic variation (i.e., HAL). Within Antarctic octopods, the family Megaleledonidae is the most diverse, with new species still being discovered (Xavier et al., 2018), and here we show that this highly speciose group also exhibited extremely elevated levels of phenotypic divergence in male reproductive traits (i.e., SL). We contend that the speciation rates observed in this clade in conjunction with the extremely high rates of phenotypic evolution cannot be explained by niche diversification, and likely reflect sexual selection (i.e., "runaway" sexual selection), where the coevolution of female mating preferences and male sexual characters promotes reproductive isolation and foments speciation (see Lande, 1982). The process of sperm competition has been well described in benthic octopuses, highlighting the role of cephalopod behavior in mediating intra-sexual competition (e.g., several males attempting to mate with a female simultaneously; reviewed by Hanlon and Messenger, 1996). Similarly, the occurrence of multipaternity has also been described in some species of octopuses, suggesting that females are able to fertilize eggs with the sperm of multiple males, decreasing the probability of fertilizing high number of eggs with the sperm of single male, as reported for *Graneledone boreopacifica* (Voight and Feldheim, 2009), *Octopus vulgaris* (Quinteiro et al., 2011), *E. dofleini* (Larson et al., 2015), *O. minor* (Bo et al., 2016), *Hapalochlaena maculosa* (Morse et al., 2018), and *O. oliveri* (Ylitalo et al., 2019). Additionally, it has been proposed (but not verified by other authors, nor by our own data) that species with large-sized ligula do not only use this structure for spermatophores transfer, but also to breakdown or modify the position of spermatophores from rival males (Cigliano, 1995; Hanlon and Messenger, 1996; Norman et al., 2004). Nonetheless, this behavior has been described in other taxa (such as insects) that use their copulatory organs to extract the sperm left by other males as a mechanism to counteract sperm competition (Birkhead and Moller, 1999). Finally, cryptic female choice favoring larger spermatophores has been reported in the sepiolid squid *Idiosepious paradoxus* through postcopulatory behavior (see Sato et al., 2013), and may therefore be taking place in closely related octopods.

While these studies leave no doubt that sexual selection is potentially an important factor shaping the evolution of benthic octopuses, the fact that this seems to be particularly the case for members of the family Megaleledonidae remains an open question. It is possible that this group has evolved mating strategies and reproductive habits that exacerbate sperm competition via, for instance, territoriality or female

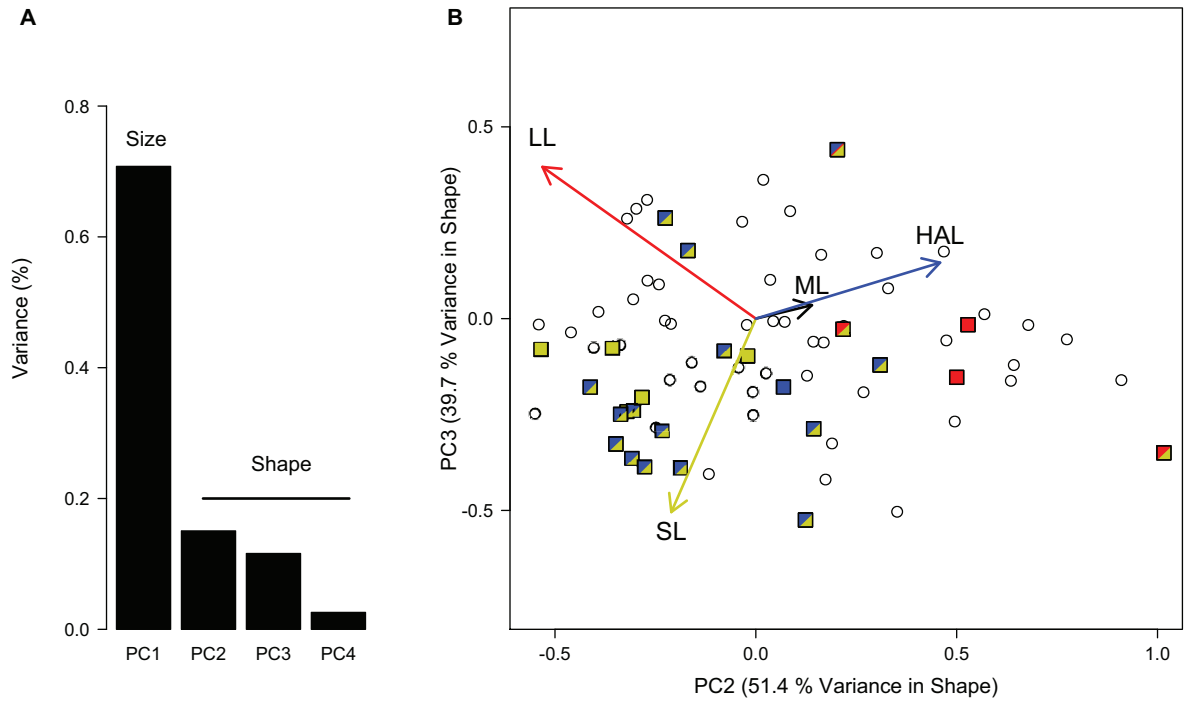

FIGURE 4 | Phylogenetic principal component analysis (PCA) to study the correlated evolution between reproductive traits. **(A)** We included $\log_{10}$-transformed ML, HAL, LL and SL, removed PC1 that encompassed primarily scaling effects, and worked with the remaining components that indicate differences in "shape" across lineages. **(B)** Species under positive selection according to variable-rates regression are shown in colors, as in **Figures 2**, **3**. Note that selection was detected in more than a single trait in several lineages.

postcopulatory selection. Interestingly, Rocha et al. (2001) speculated that the wide range of sizes of maturing ova described in the megaleledonids *Pareledone charcoti* and *Adelieledone polymorpha* could indicate repeated spawning in these species, contrasting with the majority of octopods that are considered terminal spawners. Another potential explanation that is not mutually exclusive corresponds to that, due to the environmental conditions encountered by these Antarctic deep-sea species (i.e., temperature and environmental stability; see Ibáñez et al., 2018), the impact of sexual selection become disproportionally important in this group in comparison to other benthic octopuses. Indeed, the lack of correlation between SL and EL suggest the absence of environmental selection on SL at the poles or at deep water environments.

In other words, we speculate that other factors shaping phenotypic evolution, such as predation, interspecific competition or environmental heterogeneity, may be relatively less important in the Antarctic deep-sea species. Perhaps the combined action of these two phenomena, namely the evolution of exclusive reproductive strategies in this clade in response to specific environmental pressures, may ultimately explain the very strong signal of selection and phenotypic divergence detected across males of this family. Overall, our phylogenetic approach provides some evidence of sexual selection within benthic octopuses, particularly for Megaleledonidae, and a potentially relevant role in their diversification. Detailed studies on different mating behaviors and how they relate with morphological and life-history traits are still necessary to better understand the adaptation of different cephalopod lineages to highly contrasting environments worldwide.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: https://data.mendeley.com/datasets/5vkm46hm49/1.

AUTHOR CONTRIBUTIONS

CI, ER, and MP-G conceived the idea, designed the study, analyzed the data, and led the writing of the manuscript. SC, JP-Á, and JC collaborated in literature review, writing, and provided the editorial advice. All authors have read and commented on the manuscript.

FUNDING

This work was partially funded by the FONDECYT research grants 1181153, 11170617, and 1170017 awarded to CI, SC, and ER, respectively. The additional support from the INACH research grant RG 50-18 awarded to MP-G and the CONICYT PIA/BASAL FB0002 to ER are also appreciated.

ACKNOWLEDGMENTS

We thank Prof. Elie Poulin and Prof. Marco Méndez from Universidad de Chile for their review of an early version of the manuscript. The comments of reviewers are greatly appreciated.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01238/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

PERSPECTIVE
published: 15 October 2019
doi: 10.3389/fphys.2019.01281

Male Alternative Reproductive Tactics and Associated Evolution of Anatomical Characteristics in Loliginid Squid

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Loliginid squids provide a unique model system to explore male alternative reproductive tactics (ARTs) and their linkage to size, behavioral decision making, and possibly age. Large individuals fight one another and the winners form temporary consortships with females, while smaller individuals do not engage in male-male agonistic bouts but use various sneaker tactics to obtain matings, each with varying mating and fertilization success. There is substantial behavioral flexibility in most species, as smaller males can facultatively switch to the alternative consort behaviors as the behavioral context changes. These forms of ARTs can involve different: mating posture; site of spermatophore deposition; fertilization success; and sperm traits. Most of the traits of male dimorphism (both anatomical and behavioral) are consistent with traditional sexual selection theory, while others have unique features that may have evolved in response to the fertilization environment faced by each temporary or permanent male morph.

Keywords: sexual selection, alternative phenotypes, ARTs, male dimorphism, consort, sneaker, Cephalopoda, Loliginidae

OPEN ACCESS

Edited by:
Erica A. G. Vidal,
Federal University of Paraná, Brazil

Reviewed by:
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The California State University, United States
Wen-Sung Chung,
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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *12 June 2019*
Accepted: *24 September 2019*
Published: *15 October 2019*

Citation:
Marian JEAR, Apostólico LH, Chiao C-C, Hanlon RT, Hirohashi N, Iwata Y, Mather J, Sato N and Shaw PW (2019) Male Alternative Reproductive Tactics and Associated Evolution of Anatomical Characteristics in Loliginid Squid. Front. Physiol. 10:1281. doi: 10.3389/fphys.2019.01281

INTRODUCTION

Since its formal conception nearly 150 years ago (Darwin, 1871), sexual selection has been an active field of evolutionary biology, particularly since the 1970's [reviewed in Birkhead and Møller (1998), Birkhead and Pizzari (2002), Birkhead (2010), Parker and Pizzari (2015)]. We now understand that this powerful selective force operates through both intrasexual and intersexual mechanisms, as well as before and after mating. Pre-copulatory processes generally include male-male competition to access females, and female choice of males based on their assessment of male "quality." Post-copulatory processes in polyandrous mating systems include sperm competition and cryptic female choice. Sperm competition, the contest between sperm from different males to access a female's ova (Parker, 1970), leads to male adaptations (both anatomical and behavioral) that maximize fertilization success, including ejaculate traits (sperm placement, number, size, and performance).

Cryptic female choice involves female control over male fertilization success (e.g., ejecting sperm or influencing their access to ova) (Eberhard, 1996).

Sexual selection drives the evolution of alternative reproductive tactics (ARTs). ARTs refer to discontinuous behavioral and other traits selected to maximize fitness in two or more alternative ways in the context of intraspecific and intrasexual reproductive competition (Oliveira et al., 2008). ARTs evolve when conspecific, intrasexual competitors find different solutions to reproductive competition. The concept of ARTs refers to alternative ways to obtain fertilizations (not just matings, since DNA studies in numerous taxa have shown that mating success does not predict fertilization success; e.g., Birkhead, 2010).

Males under intense sexual selection pressures may adopt one of two or more alternative patterns (Taborsky et al., 2008). If males physically compete for access to females and larger individuals win, small males may adopt an alternative way to achieve fertilizations through surreptitious mating (i.e., sneaker tactics). Male ARTs are found in numerous taxa and often involve a dominant tactic (guarder, territorial, bourgeois or consort) and an alternative way to obtain fertilizations (extra-pair, opportunistic, parasitic, sneaker or satellite) [reviewed in Oliveira et al. (2008)].

Sperm competition may be asymmetrical between males employing ARTs: dominant males gain priority access for their sperm, whereas extra-pair males may compensate by producing larger quantity or higher quality of sperm (Parker, 1990). In this context, the dominant male should invest in large body size and fighting behaviors, and the smaller in gonadal size and larger sperm size, speed or longevity (Taborsky, 1998). However, it is also important to consider spatial and temporal dynamics of fertilization when estimating the influence of sperm competition on the evolution of sperm traits. If the fertilization environment faced by each tactic is different, a dominant tactic may deviate from the classic role and produce larger, faster and more long-lasting sperm (Taborsky et al., 2018).

In this wider context of evolution of mating tactics, loliginid squid (Mollusca: Cephalopoda: Loliginidae) provide some unique behavioral and anatomical features with which to explore male sexual selection. Males of many species compete to guard and copulate with females. Unlike most animals, however, in some loliginid species there are two well-separated sites of spermatophore deposition and storage on the female body (Drew, 1911): on the buccal region near a sperm storage organ (seminal receptacle) or within the mantle cavity near the oviduct opening, associated with the two male mating tactics (**Figure 1**; "sneaker" vs. "consort," respectively – Shashar and Hanlon, 2013; Iwata et al., 2015). In both cases, the spermatophores evert and attach themselves autonomously at the deposition site when transferred to the female, by means of a combination of mechanical and chemical processes – the sperm are then released from the distal tip of the attached "spermatangia" (i.e., everted spermatophores) and slowly disperse (**Figure 1**; Drew, 1919; Marian, 2012a,b, 2015). As fertilization occurs during egg-laying, deposition in the mantle cavity is likely a more successful tactic due to the proximity to the site of egg string extrusion (**Figure 1**; Iwata et al., 2005; Naud et al., 2016), but deposition in the buccal region from sneakers can also lead to fertilization as the egg string is held by the female in this region after extrusion but prior to placing it on the seabed (**Figure 1**; Buresch et al., 2009).

Much progress has been made in the last 25 years in understanding the squid mating system with the application of approaches including extensive *in situ* (e.g., Sauer et al., 1997; Hanlon et al., 2002, 2004; Shashar and Hanlon, 2013; Mather, 2016) and *ex situ* behavioral studies (e.g., Lin and Chiao, 2018), experimental manipulations of captive specimens (e.g., Iwata et al., 2005; Buresch et al., 2009; Saad et al., 2018), paternity analyses (from *ex situ* samples: Iwata et al., 2005; Buresch et al., 2009; from *in situ* samples: Shaw and Boyle, 1997; Shaw and Sauer, 2004; Naud et al., 2016), *in vitro* experimentation of the functioning of spermatophores (e.g., Iwata et al., 2015; Apostólico and Marian, 2017), spermatology (e.g., Iwata et al., 2011; Hirohashi and Iwata, 2013; Hirohashi et al., 2013, 2016a,b; Iida et al., 2017), gonadal/ejaculate expenditure (e.g., Iwata and Sakurai, 2007; Apostólico and Marian, 2018a; Iwata et al., 2018), and age and development (Apostólico and Marian, 2018b).

Here, we present a fresh perspective on male squid ARTs, and we reconstruct the evolution of reproductive characters and loliginid ARTs, discussing the interplay between sperm competition and fertilization environment in the evolution of ejaculate adaptations.

MALE ALTERNATIVE REPRODUCTIVE TACTICS IN LOLIGINIDS: CA. 25 YEARS OF RESEARCH

In the genus *Sepioteuthis*, which might be regarded as basal within the family, the behavioral and morphological specializations mentioned above are somewhat different. In *Sepioteuthis sepioidea*, both consort and sneaker tactics are present. Consort males will pair with a female for a day or so and mate in the "male-parallel" position and deposit spermatophores inside the mantle cavity (like *Loligo* and *Doryteuthis*), while small males (perhaps sneakers although they are seen paired with females for hours with no other male present) will pair briefly with females and will "slap" a spermatophore externally at or around the base of the arms. The female will grab the spermatophores and either spit them away (rejection) or place them near the seminal receptacle near the mouth (acceptance) (Moynihan and Rodaniche, 1982; Hanlon and Messenger, 2018). Interestingly, Mather (2016) reported that both sneakers and consorts mate similar to the second method explained above, and that consorts only mate in "male parallel" immediately before egg deposition. Mather (2016) also noted that the female may take spermatangia deposited on her dorsal arm bases into her mantle cavity, and that males shift from sneaker to consort tactics as they grow larger. *Sepioteuthis sepioidea* groups do not gather at spawning sites, and females reject consorts and sneakers often, even after a few successful spermatophore transfers (Mather, 2016). Males of this species conduct elaborate agonistic bouts and the winners pair with the female temporarily; presumed sneakers, however, do not engage in agonistic bouts (Moynihan and Rodaniche, 1982;

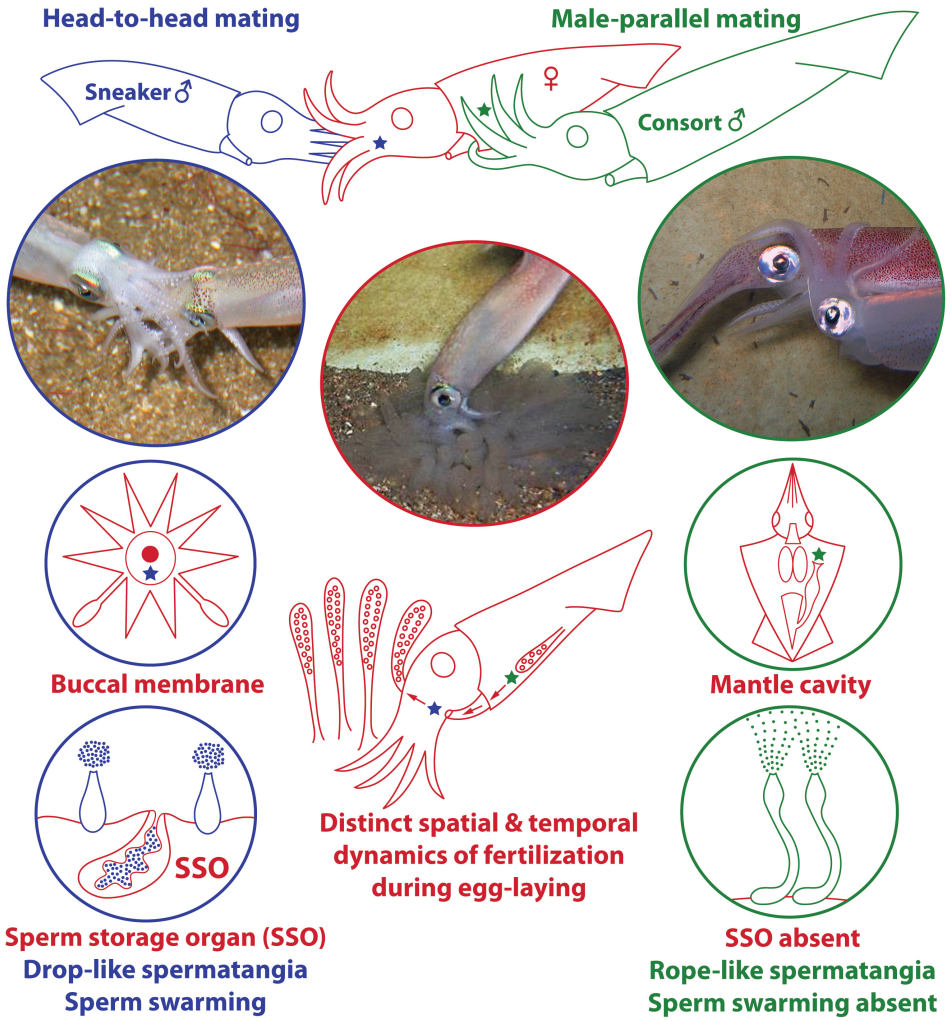

FIGURE 1 | Summary of male ARTs and associated evolution of anatomical characteristics in loliginid squid. In several loliginids, large consort males (green) fight other males to gain access to females (red) and deposit spermatophores within the female mantle cavity near the oviduct opening (green star) through "male-parallel" mating. Small sneaker males (blue) usually employ furtive mating and deposit spermatophores near the female buccal seminal receptacle (blue star) through "head-to-head" mating; a sperm storage organ (SSO) is present at the female buccal membrane. Each site offers unique fertilization environments that differ, for example, in fertilization timing and success, which possibly led to the evolution of unique ejaculate traits by each male morph. The photographs and drawings used in this plate were adapted from figures originally published in Marian (2012b) and Apostólico and Marian (2018b); they are reproduced here with permission.

Mather, 2016; Hanlon and Messenger, 2018). In *S. australis* observed at spawning sites (Jantzen and Havenhand, 2003), sneakers and consorts use postures and deposition sites that are basically similar to *S. sepioidea*, but female rejection of sneakers led to 67% of consorts and 33% of sneakers successfully transferring sperm. In captive *S. lessoniana* (Wada et al., 2005; Lin and Chiao, 2018) consort and sneaker males use different postures and different deposition sites, but smaller individuals who were rejected by females if they used the "consort" tactic could switch both posture and placement, demonstrating the facultative nature of switching tactics rapidly, depending on the behavioral context. *Sepioteuthis* spp. have complex mating systems that, unlike other loliginids, include distinctive courtship body patterns and behaviors by females and males (Hanlon and Messenger, 2018).

The tactics of male *Doryteuthis* and *Loligo* are somewhat different. Field and laboratory studies on three *Doryteuthis* species (*D. pealeii*, *D. pleii* and *D. opalescens*) and *Loligo reynaudii* during the 1990s and early 2000s revealed complex mating systems, which include communal spawning beds and at least two mating postures and sperm deposition sites, and a high degree of behavioral plasticity in both males and females (Hanlon, 1996, 1998; DiMarco and Hanlon, 1997; Hanlon et al., 1997, 2002, 2004; Sauer et al., 1997; Maxwell et al., 1998; Maxwell and Hanlon, 2000; Wada et al., 2005; Zeidberg, 2009). These studies provided substantial details of male ARTs of these species (**Table 1**). As in *Sepioteuthis*, the consort tactic consists of recurrent attempts to pair with females and repeated agonistic contests with other males. Consorts use a "male-parallel" mating posture and deposit their spermatophores within the female

TABLE 1 | Summary of male alternative reproductive tactics and related traits across Loliginidae.

Species	ARTs	Mating postures	Sperm storage sites	Spermatangia dimorphism	Sperm size dimorphism	Sperm swimming behavior dimorphism
Doryteuthis pealeii	SN/CO (1)	HH/MP (1)	BM/MC (2)	+(2, 3)[A]	?	?
Doryteuthis pleii	SN/CO (4)	HH/MP (4)	BM/MC (4)	+(4, 5)	+(4)	+(5)
Doryteuthis opalescens	SN/CO (6, 7)	HH/MP (6, 7)	BM/MC (7, 8)	?	?	?
Heterololigo bleekeri	SN/CO (9)	HH/MP (10)	BM/MC (10)	+(11)	+(11)	+(12)
Loligo reynaudii	SN/CO (13)	HH/MP (13)	BM/MC (14)	+(14)	?	+(15)
Sepioteuthis australis	SN/CO (16)	HH/MU/MP (16)	BM/HAR/MC (16)	?	?	?
Sepioteuthis lessoniana	SN/CO (17)	HH/MU/MP (18, 19)	BM/MC (17)	+(17)[B]	–(17)	?
Sepioteuthis sepioidea	SN/CO (20, 21)	HAR[C]/MP (20, 21)	BM/HAR/MC (20, 21)	?	?	?
Uroteuthis edulis	SN/CO (15)	?	BM/MC (15)	+(15)	+(15)	+(15)

[A]*Drew (1911: p. 359) illustration of the sagittal section of the female buccal membrane in D. pealeii is suggestive of the presence of "drop-like" spermatangia near the seminal receptacle, and Drew (1919: p. 424) spermatangium illustration clearly depicts a "rope-like" type;* [B]*Lin et al. (2019) showed that sneaker and consort males of S. lessoniana exhibit different oral extremity of the cement body in spermatophores, suggestive of spermatangia dimorphism;* [C]*Males and females of S. sepioidea rock together in parallel and then the male darts around and places spermatangia on her dorsal arm bases, from which she may take them to her buccal membrane (Hanlon and Messenger, 2018) or into the mantle cavity (Mather, 2016). References: (1) Shashar and Hanlon (2013); (2) Drew (1911); (3) Drew (1919); (4) Apostólico and Marian (2018a); (5) Apostólico and Marian (2017); (6) Hanlon et al. (2004); (7) Zeidberg (2009); (8) Fields (1965); (9) Iwata and Sakurai (2007); (10) Iwata et al. (2005); (11) Iwata et al. (2011); (12) Hirohashi et al. (2013); (13) Hanlon et al. (2002); (14) Iwata et al. (2018); (15) Hirohashi et al. (2016a); (16) Jantzen and Havenhand (2003); (17) Lin et al. (2019); (18) Wada et al. (2005); (19) Lin and Chiao (2018); (20) Mather, 2016; and (21) Hanlon and Messenger (2018). Abbreviations: ARTs, alternative reproductive tactics; BM, buccal membrane; CO, consort; HH, head-to-head; HAR, head/arm region; MC, mantle cavity; MP, male-parallel; MU, male-upturned; SN, sneaker; +, present; –, absent; ?, unknown.*

mantle cavity near the oviduct opening (**Figure 1**). Smaller sneaker males do not fight, but rather try quick extra-pair copulations in the "head-to-head" mating posture, placing their spermatophores near the female's seminal receptacle located on the buccal membrane (**Figure 1**). *Doryteuthis pealeii* males have four mating tactics: consort, lone large male, surreptitious sneaker, and bold sneaker. The first two are large males, the last two are small males. None of these are separate genetic morphs, but rather facultative alternative behaviors depending on the size of the males and the combinations of large and small males that are present in different groupings within the mating arena (Shashar and Hanlon, 2013).

Courtship and postcopulatory mate guarding vary substantially in loliginids. Courtship is known only in *S. sepioidea*, and has been seen in both sneakers and consorts. There are only hints of courtship in males of *Loligo* or *Doryteuthis*, and it consists only of synchronized swimming next to the female; no specific body patterns have been reported. With the exception of *S. sepioidea*, sneakers neither court nor mate guard in loliginids (as known thus far). Mate guarding by consorts after copulation is common in the few species studied (Hanlon and Messenger, 2018), but it is generally temporary (but see Mather, 2016), and females can and do continue to fertilize and lay eggs without male accompaniment. The tactics decided by consort males at this stage of the mating system are unknown.

DNA fingerprinting has been used to study paternity (i.e., fertilization success of competing males) of wild or captive *D. pealeii*, *L. reynaudii*, *L. forbesii*, and *Heterololigo bleekeri*, confirming multiple paternity among the offspring in at least some broods in all species (Shaw and Boyle, 1997; Buresch et al., 2001, 2009; Shaw and Sauer, 2004; Iwata et al., 2005; Naud et al., 2016). Consorts achieve much higher reproductive success than sneakers in all cases, from ∼70% of overall fertilization in *L. reynaudii* (Naud et al., 2016) to ∼90% in *H. bleekeri* (Iwata et al., 2005). Moreover, the reproductive success of consort males is influenced by the interval between mating and egg-laying (Buresch et al., 2009). Also, non-random patterns of paternity within single egg strings may indicate cryptic female choice (Shaw and Sauer, 2004; Naud et al., 2016).

Male ARTs always involve behavior, but also generally include distinct sets of morphological and physiological attributes (Taborsky et al., 2008). Accordingly, male dimorphism is present in the four loliginids (*H. bleekeri*, *D. pleii*, *L. reynaudii*, and *Uroteuthis edulis*) studied in detail to date, expressed as dimorphic ejaculates (Iwata and Sakurai, 2007; Iwata et al., 2015, 2018; Apostólico and Marian, 2017, 2018a,b; **Table 1**). Large and small males generally follow consort and sneaker tactics, with sneaker males developing small "drop-like" spermatangia, while consort males produce larger and elongate "rope-like" spermatangia (**Figure 1**; Iwata and Sakurai, 2007; Iwata et al., 2015, 2018; Apostólico and Marian, 2017, 2018a,b; **Table 1**). This tactic-associated dimorphism even extends to differences in sperm size, with sneakers producing consistently larger sperm (Iwata et al., 2011; **Table 1**).

Besides differences in spermatophore and sperm size (**Table 1**), some of the most intriguing adaptations of each ejaculate type are the duration of sperm release from the spermatangium, being much longer (ca. 5 h) in sneaker than consort spermatangia (ca. 2 h; Apostólico and Marian, 2017), and sperm swimming behavior after release, showing an aggregative behavior – swarming – in sneaker sperm (**Figure 1**; Iwata et al., 2011; Hirohashi and Iwata, 2013; Hirohashi et al., 2013, 2016a; Apostólico and Marian, 2017; **Table 1**). Physiological investigations of *H. bleekeri* demonstrated that swarming occurs because sneaker sperm migrate toward acidic environments (pH-taxis; Hirohashi et al., 2013).

Although there are mating postures and behaviors typical of each male tactic, with head-to-head (or male-upturned) mating typical for sneakers and male-parallel mating and agonistic behavior typical for consorts, they are not always exclusive. Mating behavior may vary according to the behavioral context in the mating arena. For example, consort males may copulate in the head-to-head position if the female is far from spawning (*H. bleekeri*; Iwata et al., 2005; *S. lessoniana*; Wada et al., 2005). In *D. pealeii*, sneakers can immediately switch their behavior when a consort male is removed and perform male-parallel mating, and then just as rapidly switch back to sneaker tactics and head-to-head mating when a consort male appears and pairs temporarily with the female (Hanlon et al., 1997; Shashar and Hanlon, 2013). Female choice (*S. lessoniana*; Lin and Chiao, 2018) or relative size of males (*S. lessoniana*; Wada et al., 2005) can also influence mating postures and behaviors.

In other animal taxa, ARTs can be either fixed or plastic, and in the latter case either simultaneous or sequential (Taborsky et al., 2008). In *H. bleekeri* ARTs are apparently fixed, with spermatophore length discontinuous across body size suggesting two distinct morphs (Iwata and Sakurai, 2007). However, in some loliginids the expression of ARTs can be sequential (e.g., *S. sepioidea*), and this can be observed only through long-term behavioral observations (Moynihan and Rodaniche, 1982; Mather, 2016). Males of *D. pleii* of intermediate size and age show a transition of sneaker to consort-like ejaculates inside the reproductive tract (Apostólico and Marian, 2018b), as well as a transition in mating behavior (Apostólico and Marian, 2019), so male dimorphism may be sequential in this species also (but see alternative hypotheses in Apostólico and Marian, 2018b). As intermediate-sized males of *L. reynaudii* (Iwata et al., 2018), *D. pealeii* (Shashar and Hanlon, 2013), and *S. lessoniana* (Lin et al., 2019) may display flexible tactics, their male ARTs could be sequential, too, but further studies are required.

DIMORPHIC MALE ADAPTATIONS: THE INTERPLAY BETWEEN SPERM COMPETITION AND FERTILIZATION ENVIRONMENT

Fertilization in loliginids generally occurs during egg-laying, first near the oviduct opening within the mantle, and then on the buccal membrane when the egg string is held within the female's arms (**Figure 1**; e.g., Drew, 1911; Sauer et al., 1997; Hanlon et al., 2002; Buresch et al., 2009; Iwata et al., 2015; Naud et al., 2016). This "confined external fertilization," occurring potentially in two different sites, creates a useful model to investigate the interplay between the pressures of sperm competition and fertilization environment. Some male adaptations follow the predictions of sperm competition theory, which predicts that if sneakers face a behavioral disadvantage they should show higher gonadal investment (Parker, 1990). In *D. pleii* sneakers have higher gonadal expenditure than consorts and the latter invest more in somatic growth (Apostólico and Marian, 2018a). Other adaptations are influenced by the respective sperm storage sites, which comprise a unique fertilization environment, differing in:

(1) Sources of spermatozoa: in the mantle cavity the only source is the attached spermatangia, but the buccal membrane has two distinct sources, the attached spermatangia and the seminal receptacle (**Figure 1**). Attached spermatangia of the mantle cavity and the buccal membrane provide sperm from recent matings, but the seminal receptacle possibly stores sperm from much earlier mating events (e.g., Hanlon, 1996; Hanlon et al., 2002);
(2) Fertilization success: due to proximity to the oviduct opening, fertilization success is higher for deposition in the mantle cavity site (**Figure 1**; e.g., Iwata et al., 2005; Naud et al., 2016);
(3) Interval between mating and fertilization: it can be longer for the buccal membrane as it has a sperm storage organ that may store sperm for a considerable period of time (**Figure 1**; e.g., Hanlon, 1996; Hanlon et al., 2002). Head-to-head mating may occur hours before spawning, while male-parallel usually occurs near or during spawning (Iwata et al., 2005; Wada et al., 2005);
(4) Physical features of each site (see Iwata et al., 2011): there may be differences in the risk of sperm dilution (e.g., while the buccal membrane is a more external site, the mantle cavity may exhibit considerable turbulence due to constant mantle contractions), as well as differences in pH, viscosity and salinity. Because fertilization occurs during egg-laying, there may be changes in viscosity of the jelly matrix or expansion (e.g., swelling) of the egg string as it is extruded from the mantle cavity (Boletzky, 1986) then exposed to seawater as it is moved to the buccal membrane.

In this context, differences in sperm size and spermatangia shape and function (**Figure 1**) are possibly associated with physical constraints specific to each deposition site (Iwata et al., 2011; Apostólico and Marian, 2017). Also, the slower sperm release (Apostólico and Marian, 2017) and longer sperm viability (Hirohashi et al., 2016b) in sneakers may be associated with the longer interval between mating and fertilization for spermatangia attached on the buccal membrane. Additionally, the smaller sneaker spermatangium is filled with fewer sperm (Iwata and Sakurai, 2007; Iwata et al., 2011; Apostólico and Marian, 2018a): if many oocytes are fertilized by sperm in the mantle cavity before reaching the buccal membrane, then sneakers may use fewer sperm per mating and invest in more mating events (Apostólico and Marian, 2018a).

Sperm size evolution has been a central issue in postcopulatory sexual selection theory. If a longer flagellum results in higher swimming speed, a classic prediction is that intense sperm competition could lead to larger sperm sizes (e.g., Snook, 2005). Also, due to the asymmetry in sperm competition (Parker, 1990), sneakers may expend more in gametic traits (e.g., sperm size). Interestingly, sneaker spermatozoa of *H. bleekeri* and *D. pleii* are 50% (Iwata et al., 2011) and 15% (Apostólico and Marian, 2018a) longer than consort sperm, respectively. However, at least for *H. bleekeri*, this difference in size does not result in higher

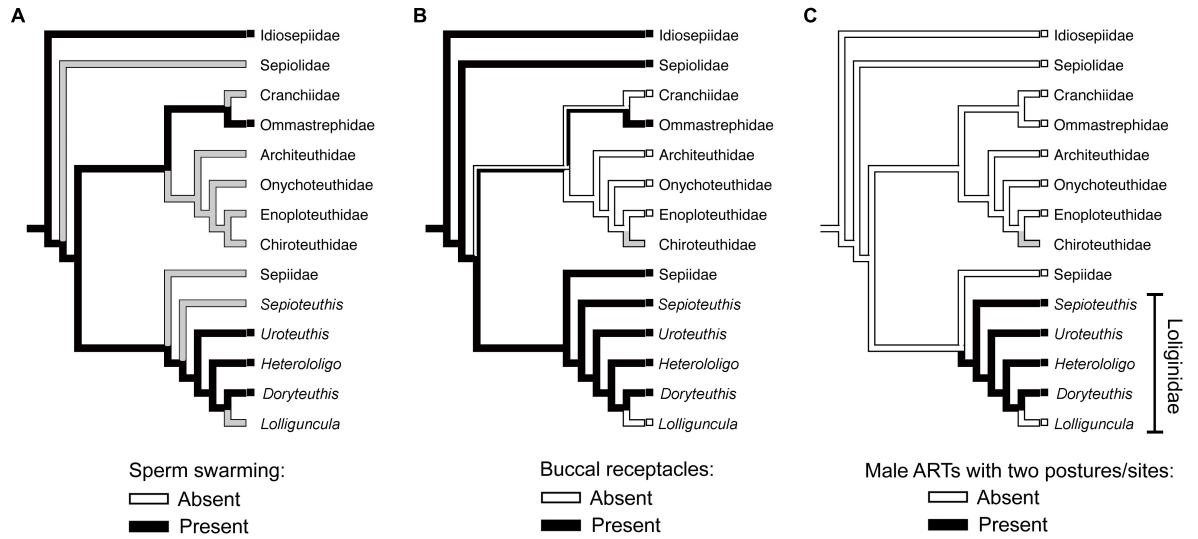

FIGURE 2 | Parsimonious ancestral state reconstructions (ASR) of reproductive characters based on the topology for Decapodiformes of Lindgren and Anderson (2018). Character states are traced and indicated by colored branches (i.e., ancestral reconstruction) and colored boxes to the left of taxon names (i.e., the observed character state in the respective taxon). White and black colors indicate absence and presence, respectively. Presence of more than one color in branches indicates equivocal (i.e., uncertain) reconstruction, and gray color indicates equivocal reconstruction owing to missing data. **(A)** ASR of sperm swarming. **(B)** ASR of buccal seminal receptacles. **(C)** ASR of alternative reproductive tactics involving two mating postures, two sperm deposition sites (buccal membrane and mantle cavity) and ejaculate dimorphism. The methodology used in the analyses is detailed in the **Supplementary Material**.

swimming speed and is not related to competition for space within the seminal receptacle (Iwata et al., 2011). Therefore, sperm competition alone does not explain sperm dimorphism in this species (Iwata et al., 2011).

Differences in sperm behavior could be associated with the spermatophore deposition site. The self-swarming trait of sneaker sperm (**Figure 1**) could be linked to collective sperm migration toward the seminal receptacle on the buccal membrane (Hirohashi and Iwata, 2013), and to slowing down sperm release from the spermatangium (i.e., by retaining sperm near its tip; Apostólico and Marian, 2017). Hirohashi et al. (2016a) hypothesized that sperm swarming was a primitive ejaculate attribute conserved in sneakers but lost in consort males who use mantle cavity deposition. We investigated this hypothesis with the available literature, using parsimonious ancestral state reconstructions based on a recent phylogenetic hypothesis for Decapodiformes, a large clade including squids, cuttlefishes, bobtail squids and ram's horn squid (**Figure 2**). The analyses indicate that sperm swarming and buccal receptacles were present in the decapodiform ancestor, but that ARTs typical of some loliginids (i.e., two mating postures, two sperm deposition sites and ejaculate dimorphism) evolved later (**Figure 2**), tending to support (Hirohashi et al., 2016a).

If sperm swarming and buccal seminal receptacles, but not male ARTs, are plesiomorphic within Loliginidae, swarming would not be an adaptation of loliginid sneaker males but could be related to sperm storage in buccal receptacles (Hirohashi and Iwata, 2013). In contrast, consort sperm that diffuse after release from the spermatangium (**Figure 1**) presumably evolved associated with the changes in male ARTs within Loliginidae. The loss of swarming in consort sperm, maybe through the evolution of shorter sperm tails (see Iida et al., 2017), could be the result of relaxed selection due to the absence of a sperm storage organ in the mantle cavity, or increased selection due to sperm competition. Parallel mating is usually performed near or during spawning (Iwata et al., 2005; Wada et al., 2005; Buresch et al., 2009), and consorts are frequently replaced in some species (Hanlon et al., 2002; Shashar and Hanlon, 2013), so intense sperm release should guarantee a higher number of fertilizations for the consort male as more oocytes leaving the oviduct would be fertilized. If sperm swarming could delay sperm release from the sneaker spermatangium (Apostólico and Marian, 2017), then the loss of swarming in consorts is likely an adaptation to sperm competition when the physical constraints of external fertilization are relaxed.

FUTURE DIRECTIONS

As described herein, loliginid squids provide a unique model group to study sexual selection due to the presence of two sperm deposition sites within the female body, each offering distinct fertilization environments for male gametes (**Figure 1**). However, some basic mechanisms operating in the squid mating system are still obscure, hindering its wide adoption as a model system to test sexual selection theory. Although all research teams involved with the present paper will continue in their respective areas to fill major gaps in our knowledge, we urge interdisciplinary approaches. A combination of tests of behavioral, genetic, reproductive biology and functional morphology data are

needed to unravel the complexities of the loliginid squid mating system. We need:

(1) *In situ* behavioral studies (e.g., Shashar and Hanlon, 2013; Mather, 2016; Naud et al., 2016): because they reveal the full range of male ARTs and female choice under natural conditions that cannot all be duplicated in lab studies. Long-term field observations could clarify changes in ARTs across ontogeny since small (younger) squids tend to be sneakers and larger (older) squids consorts.
(2) Female roles: male tactics and dimorphism are likely to be affected by female choice. Apart from *S. sepioidea*, in which the females actively manipulate spermatangia received during mating (Moynihan and Rodaniche, 1982; Mather, 2016; Hanlon and Messenger, 2018), we do not know all of the tactics that females use to exert choice. Manipulation of egg-string extrusion and position within the arms and sperm release from the seminal receptacle could bias fertilization success toward particular males (Naud et al., 2016), and pumping the mantle cavity after male-parallel mating could eject consort spermatangia (Buresch et al., 2009).
(3) Spawning context: some loliginids form dense spawning aggregations and large open spawning beds, providing opportunities both for male and female promiscuity with both pre- and post-copulatory sexual selection (Hanlon and Messenger, 2018). Others (e.g., *S. sepioidea* and *H. bleekeri*) do not form large aggregations and use hard substrates for egg attachment (Jereb and Roper, 2010; Mather, 2016; Hanlon and Messenger, 2018). Do these differences in mating/spawning conditions affect the male ARTs?
(4) Fertilization dynamics: how exactly are loliginid eggs fertilized in each site? The egg string is initially formed within the mantle cavity (Boletzky, 1986), but is sperm penetration more difficult by the time the string reaches the buccal membrane, where the string is fully expanded? Sperm stored in the seminal receptacle or from spermatangia recently placed there by sneaker males would only have access to fertilization at this time. Knowledge of the structure and formation of the egg case (Iwata et al., 2019) is necessary to understand this.
(5) Sperm competition: we lack understanding of inter- and intra-tactic sperm competition, the interplay between it and the fertilization environment, and how they have influenced adaptations at both individual and gametic levels. The role of mate guarding in sperm competition is unknown but of probable importance to greater fertilization success by the guarding male. The extent of sperm swarming throughout the Cephalopoda is largely unknown (see **Figure 2A** and **Table 1**) and requires investigation.
(6) Behavioral plasticity: How diverse is this plasticity with respect to consort vs. multiple forms of sneaking in different species? Does plasticity in mating behaviors affect male dimorphism, especially in structural and physiological differences in ejaculates?
(7) Expression of ARTs: how do genetic and environmental factors underlie the expression of male ARTs, especially in the physiological transition between dimorphic males?
(8) Evolution of male ARTs: there are ten genera in the Loliginidae, but at present we have information about either behavior or physiology for only four genera, and not the whole spectrum of information for any species. Cross-disciplinary and comparative studies are needed across the range of loliginid species.

Nevertheless, a substantial increase in knowledge of loliginid male ARTs has been achieved within a relatively short period (1996–2019), and we look forward to the next 25 years of research on this interesting model system.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary Files**.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because all data was compiled from the literature. No animals were collected or used in experiments.

AUTHOR CONTRIBUTIONS

All authors contributed intellectually to the work, provided data and words to the manuscript versions, and edited the manuscript and approved it for publication.

FUNDING

JM acknowledges the funding provided by FAPESP (São Paulo Research Foundation – proc. 2013/02653-1, 2014/11008-5, 2015/15447-6, 2017/16182-1, and 2018/19180-2), CNPq (National Council for Scientific and Technological Development – proc. 477233/2013–9), and CAPES (Coordination for the Improvement of Higher Education Personnel – Finance Code 001).

ACKNOWLEDGMENTS

This article emanated from discussions at the Cephalopod International Advisory Council Conference (CIAC) in 2018 at St. Petersburg, FL, United States and we thank the organizers for an invigorating conference. We also thank the guest editor EV and both the reviewers for constructive comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.01281/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling Editor declared a shared committee membership, though no other collaboration, with one of the authors, JM, in the Cephalopod International Advisory Council.

frontiers in Physiology

ORIGINAL RESEARCH
published: 31 December 2019
doi: 10.3389/fphys.2019.01558

The Tentacular Strike Behavior in Squid: Functional Interdependency of Morphology and Predatory Behaviors During Ontogeny

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This study examines the relationship between morphology and predatory behaviors to evaluate the ontogeny of the specialized tentacular strike (TS) in *Doryteuthis opalescens* squid reared under laboratory conditions [hatching to 80 day-old; 2–16 mm mantle length (ML)]. Ontogenetic morphological changes in the arm-crown and the role played by the arms and tentacles during predatory behavior was correlated with prey types captured and revealed interconnected morphological and behavior traits that enabled paralarvae to perform the TS. Hatchlings have a poorly developed arm-crown and tentacles that resemble and function as arms, in which tentacular clubs (suckerfull non-contractile portion) and stalks (suckerless contractile portion) have not yet formed. Only a basic attack (BA) behavior was observed, involving arms and tentacles, which were not ejected during prey capture. A more elaborated behavior, the arm-net (AN) was first employed by 30 day-old (>4.7 mm ML) paralarvae, in which the tentacles were eject down, but not toward the prey. The TS was first observed in 40–50 day-old (6.7–7.8 mm ML) squid, which stay stationary by sustainable swimming prior to ejecting the tentacles toward the prey. Thus, the ability to perform sustainable swimming and acquisition of swimming coordination (schooling behavior) are prerequisites for the expression of the TS. The arms played the same roles after prey was captured: hold, subdue and manipulate the prey, while the actions performed by the tentacles truly defined each behavior. Prey size captured increased with increasing squid size. Morphometric data showed that hatchlings have little ability of elongating their tentacles, but this ability increases significantly with size. Squid older than 40 days could elongate their tentacles up to 61% of their ML, whereas early paralarvae 13% on average. Paralarvae were frequently observed elongating and contracting their tentacles, while not attempting to capture prey, which could perhaps serve to adjust muscle activity and development, while specializations for the strike – stalks, clubs, muscle fibers, arm-crown and swimming coordination – are still being developed. The expression of the TS is constrained by development in early paralarvae as it involves interdependency of morphology and behavior and as such, represents a major developmental milestone in the early life history of squid.

Keywords: arm crown, cephalopod, *Doryteuthis opalescens*, feeding behavior, paralarvae, prey types, tentacles

OPEN ACCESS

Edited by:
Shigehiro Namiki,
The University of Tokyo, Japan

Reviewed by:
Letizia Zullo,
Italian Institute of Technology, Italy
Julieta Sztarker,
University of Buenos Aires, Argentina

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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *23 July 2019*
Accepted: *11 December 2019*
Published: *31 December 2019*

Citation:
Vidal EAG and Salvador B (2019) The Tentacular Strike Behavior in Squid: Functional Interdependency of Morphology and Predatory Behaviors During Ontogeny. Front. Physiol. 10:1558. doi: 10.3389/fphys.2019.01558

INTRODUCTION

The arm crown of Decapodiform cephalopods consists of ten appendages enclosing the mouth. Eight of these appendages – the arms – possess suckers along their entire length, while the other two appendages – the tentacles – possess suckers only at the distal portion. The arms and tentacles of adult squid differ fundamentally in their function, ultrastructure, and behavior (Kier, 1991; Boletzky, 1993). The primary function of the arms is prey capture and manipulation, but they are also involved in behavioral displays, locomotion stabilization, and reproduction. The tentacles are specialized for prey capture and possess a unique capacity for fast elongation (Kier, 1996, 2016).

During the tentacular strike (TS) predatory behavior, the stalks (tentacles suckerless proximal portion) are elongated so the clubs (tentacles suckerfull distal portion) make the first contact with prey and attach to it. Then the stalks are immediately contracted to bring the prey within reach of the arms, which subdue and manipulate the prey during ingestion. The tentacular stalk muscles are capable of an extremely rapid extension that reaches the prey in a straight line in a remarkable 20–40 ms, with maximum stalk extension velocities reaching over 2 ms^{-1} and peak accelerations of nearly 250 ms^{-2} (Kier, 1982; Kier and van Leeuwen, 1997).

The ultrastructure of the transverse muscle cells of the tentaclular stalk is different from all other cephalopod musculature. It shows cross-striation, short-sarcomere, and thick filaments, which are specializations that enable the fast elongation of the tentacular stalks to reach the prey during the strike (Kier, 1992, 1996). The transverse muscle fibers of the arms instead are obliquely striated and responsible for slower movements of bending and twisting to subdue and manipulate the prey (Kier and Schachat, 1992).

The musculoskeletal system of arms and tentacles in squid function as a muscular-hydrostatic mechanism in which the musculature itself serves as the hydrostatic fluid. As such, they are incompressible at physiological pressures and constant in volume. Thus, a decrease in one dimension will result in an increase in another (Kier and Smith, 1985). During the strike, elongation of the stalk is caused by the contraction of the muscle cells of the transverse muscle mass, which decreases in diameter. After prey is attached to the club's suckers, the stalk is shortened by contraction of the longitudinal muscle bundles, causing the elongation of the transverse muscle cells (Kier, 1991).

The specialization of tentacles transverse muscle fibers for fast contraction allude to the evolutionary history of coleoid cephalopods (Donovan, 1977; Boletzky, 1993; Kier and van Leeuwen, 1997). From the original five pairs of arms in the line that gave rise to the Decapodiform cephalopods, the tentacles have evolved through modifications of the fourth pair of arms in the ancestral coleoid. In this regard, it was suggested that the transverse muscle of arms and tentacles are homologous, and the cross-striated muscle fiber have evolved through a reorganization of the obliquely striated muscle cells (Kier, 1985, 1991).

In the present context, it is of significance that the tentacles of loliginid squid hatchlings do not possess the adult cross-striated ultrastructure responsible for fast contraction to perform the strike (Kier, 1996; Kier and van Leeuwen, 1997). This was demonstrated in a comprehensive study correlating predatory behaviors with tentacles muscle fiber differentiation in *Sepioteuthis lessoniana* (Kier, 1996). Thus, the predatory behavior of paralarvae and adults must differ fundamentally and paralarvae foraging techniques must evolve until they can perform the TS. These differences must arise out of constraints on development, which seems to be the most important factor influencing paralarvae foraging behavior and ecological niche.

Evaluation of the sequential underlying factors responsible for the expression of the TS can offer important insights into the way this specialized behavior is ultimately constraint in paralarvae. Indeed, the means by which specializations arise in ontogeny may provide clues on how developmental constraints impose behavioral adaptations (Boletzky, 1997). This in turn, is paramount for a better understanding of paralarvae adaptive foraging strategies and its evolutionary and ecological consequences.

Laboratory studies have been the basis for much of what is known about predatory behavior in squid paralarvae. The ontogeny of copepod predation in laboratory reared *Doryteuthis opalescens* have shown that paralarvae capture their prey using the arms and the TS was only observed in four weeks old squid (Chen et al., 1996).

Upon hatching, squid paralarvae have bell shape, limited swimming and behavioral abilities (Chen et al., 1996; Vidal et al., 2018) and an underdeveloped arm-crown and beak (Franco-Santos and Vidal, 2014). In contrast, during their first month of life, squid undergo foremost morphological, behavioral, and ecological changes, leading to enhanced swimming performance and cognitive abilities, which make them competent to swim in schools and control their distribution (Vidal et al., 2018). This coincides with the end of the paralarval dispersive phase and the transition from plankton to nekton, representing a shift in paralarvae physiological ecology (Vidal et al., 2018). In concert with these major developmental landmarks that squid goes through during early ontogeny, their predatory behavior and functional morphology changes.

The present study integrates behavioral observations with morphological and morphometric data to obtain a detailed understanding of tentacular kinematics and morphology, while also evaluating the role played by the arms and tentacles during predatory behavior in *D. opalescens* reared under laboratory conditions from hatching to 80 days of age. Importantly, comprehensive available information on feeding behavior and prey types, growth, body and beak morphology, and swimming abilities for *D. opalescens* (Chen et al., 1996; Vidal et al., 2002, 2018; Franco-Santos and Vidal, 2014; Vidal and Boletzky, 2014) provides the unique opportunity to encompass several levels of knowledge in examining how predatory behavior relates to key events during ontogeny. Thus, the aims of this study are to evaluate the ontogeny of predatory behavior, particularly

Abbreviations: AI, arm pair I; AII, arm pair II; AIII, arm pair III; AIV, arm pair IV; AN, arm-net; BA, basic attack; ML, mantle length; TL, tentacles length; TS, tentacular strike.

the TS behavior, and its relationship with morphological development. In addition, we correlated arm-crown morphology and predatory behaviors with the prey types captured by paralarvae during ontogeny.

MATERIALS AND METHODS

Rearing of Paralarvae and Video Recordings of Predatory Behavior

Doryteuthis opalescens eggs were collected by SCUBA divers in Monterey Bay (36°60′N, 121°80′W) and Southern California (34°7′N, 119°05′W), United States. Eggs were transferred to the National Resource Center for Cephalopods, University of Texas Medical Branch, Galveston, TX, and upon hatching paralarvae were raised up to 80 days after hatching on a closed recirculating system. Detailed information on rearing of eggs and paralarvae are found in Vidal et al. (2002).

Paralarvae ranging in size from 2 to 13 mm mantle length (ML) were filmed at 0, 1, 2, 6, 10, 11, 13, 14, 17, 20, 30, 40, 50, 55, and 60 days of age. Mean ML for paralarvae of these ages (0 = 2.65 mm ML, 6 = 2.7 mm ML, 14 = 3.8 mm ML, 20 = 4.1 mm ML, 30 = 4.7 mm ML, 40 = 6.7 mm ML, 55 = 7.8 mm ML, 60 = 9.8 mm ML) were obtained from Vidal et al. (2018) as filming used in the present study were conducted simultaneously with the experiments reported in that particular study. A round aquarium (7 cm H, 12 cm diameter and holding a center core of 9 cm) was constructed as a miniature of the large rearing tanks, in which filming was performed from above (dorsal perspective). A rectangular aquarium (14 cm L, 15 cm H, 3 cm W) was used to film early paralarvae (0–30 day-old), and a larger rectangular aquarium (30 cm L, 20cm H, 6.5 cm W) was used to film larger paralarvae (40–60 day-old). In the rectangular aquaria, filming was performed from the side (lateral perspective), that in combination with the dorsal view (round aquarium), resulted in a three-dimensional understanding of predatory behaviors. Conditions of the large holding tanks were reproduced in the small aquaria (i.e., temperature was kept at 16°C, a small current was generated to homogeneously distribute the paralarvae and prey, and the walls were covered on the outside with a flat-black plastic to enhance prey contrast and to reduce reflectance).

From 8 to 20 paralarvae of each age were collected at random and transferred from the large rearing tanks (200 L) to the small aquaria from 4 to 5 h prior to filming. They were fed *ad libitum* on *Artemia* spp. nauplii enriched with SUPER SELCO (INVE), juvenile and adults mysid shrimp (*Americamysis almyra*) and wild zooplankton, composed mainly by copepods (adults and copepodits), but containing also a wide variety of other zooplankton organism such as crustacean zoeae and myses, Cladocera, Cirripedia nauplii, etc. Feeding a variety of prey types and sizes is important to maintain high survival rates, which during the first 60 days of rearing ranged from 42 to 60% (Vidal et al., 2002). More information on live prey composition, size and developmental stages offered to paralarvae and juveniles during rearing can be found in Vidal et al. (2002) and Vidal and Boletzky (2014).

A Sony CCD-TR930 Digital Hi8 camcorder fitted with #1.5 close–up lens and operating at 30 frames s^{-1} was used to film paralarvae and record their predatory behavior. Frame-by-frame analyses were performed with a Sony CVD-1000 Hi8 editing deck. The camera was mounted next to the aquaria at a 90° angle; the frame of view for filming was 3.6 × 3.6 cm. A thin ruler was positioned inside the aquaria to set the scale for each image and distance calibration was performed prior to each filming session. The camera was set to operate in manual mode and the focus was adjusted to the ruler with a focal distance of 1–3 cm in toward the center of the aquaria. The autofocus and zoom functions of the camera were turned off and the lens aperture was locked to maintain a constant depth of field. Paralarvae were videotaped when they were in focus for the small depth of field. The erros resuting from the positioning of the paralarvae along the optical axis were estimated to be below 15% for hatchlings and decreased as paralarvae increased in size.

To evaluate the role played by the arms and tentacles during prey capture paralarvae were filmed during attack attempts and prey capture sequences. Approximately 36 hrs of filming observations were analyzed frame-by-frame (from 2 to 2.5 h for each age filmed). There were over 500 predator-prey interactions, proportionally distributed among the ages evaluated, where the behavior of paralarvae toward a prey could be analyzed and the role played by each arm and the tentacles observed. During filming behavior, each prey captured by paralarvae was recorded and correlated with the predatory behavior employed. Behavior monitoring was performed through a TV set so that the paralarvae were not disturbed during filming. To obtain prey sizes, a sample (10–15 individuals) of the main prey types offered to paralarvae was taken from the zooplankton maintenance tanks and fixed in 4% formaldehyde–seawater for subsequent identification and size measurement under a dissecting stereomicroscope equipped with an ocular micrometer.

Arm-Crown Morphological Development and Measurements of Tentacular Elongations

Morphological development of arms and tentacles of paralarvae and juveniles were observed in 30 specimens from 1 to 80 days of age (2–16 mm ML) at every 10 d interval through a dissecting stereomicroscope, when mean tentacles length (TL) was measured.

The measurements of tentacular elongations and ML from the same paralarva were performed on the public domain software NIH Image (version 1.61) using the images recorded. The ruler placed inside the aquaria provided a reference scale and length-values were stored in the Result Window of the NIH Image software. To ensure the accuracy and precision of measurements, TL was only measured when the squid were within a predefined distance and orientation to the camera, when their eyes were exactly parallel to the video camera and in focus. To obtain the length of the tentacles, it was necessary to use an external landmark, as the base of the tentacles is enclosed within the arm crown of paralarvae. Thus, the anterior margin of the lens of the

eyes was used as this landmark following the methodology of Kier and van Leeuwen (1997). Measurements of fully elongated and contracted tentacles from the same individual were obtained from 8 to 20 squid of each age during the first 60 days of age. These measurements were obtained from filmed performed on the rectangular aquaria, when paralarvae were not attempting to capture prey. The contracted TL was measured as the distance between the anterior margin of the eye lens and the extremity of fully contracted tentacles and, the elongated TL as the distance between the anterior margin of the eye lens and the extremity of fully elongated tentacles.

The ability of paralarvae to elongate its tentacles was calculated as the length difference between fully elongated and contracted tentacles (tentacular elongation). The results were normalized by the ML and converted in percentage. The tentacular strain (ε) was calculated according to Kier and van Leeuwen (1997) as:

$$\varepsilon = (l - l_0)/l_0,$$

where l = final TL and l_0 = initial TL.

Data Analysis

To compare the relationship between contracted and elongated TL and ML, TL was log-transformed and the differences between regression slopes and intercepts of growth curves were tested by analysis of covariance (ANCOVA; Sokal and Rohlf, 1981). The validity of growth curves was accepted only when the slopes and intercepts between the regression lines of contracted and elongated tentacles and ML showed significant differences and when slopes were significantly different from zero (Sokal and Rohlf, 1981).

Differences in the percentage of tentacular elongation and in the tentacular strain between paralarvae of different ages were tested by analysis of variance (ANOVA). The age groups analyzed were 1–10, 14, 20, 30, 40, 55, and 60 day-old paralarvae. The first category (1–10) included length measurements taken from 1, 6, and 10 day-old paralarvae. Overall statistically significant differences between groups were reported by ANOVA and *post hoc* pairwise comparisons among all groups' means were conducted with Tukey HSD test to specify which age groups were significantly different from each other. The data was fourth root (tentacular elongation) and square root (tentacular strain) transformed prior to analysis to satisfy normality assumptions.

Ethics Statement

This study was conducted in compliance with the Guidelines for the Care and Welfare of Cephalopods (Fiorito et al., 2014, 2015) and the principles of the European Directive (2010/63/EU), which regulate animal research, including cephalopods, in the European Union (E 121 U; Smith et al., 2013) and, with recommendations of the ARRIVE Guideline (Kilkenny et al., 2010) for reporting *in vivo* experiments with research animals. The Institutional Animal Care and Use Committee (IACUC) of the University of Texas where this study was conducted did not require researchers to submit protocols for the ethical treatment of invertebrate larvae when this research was performed.

RESULTS

Arm-Crown Complex Morphological Development During Ontogeny

At Hatching (2.0–2.7 mm ML; Mean TL = 1.58 ± 0.04 mm)

Hatchlings have a rudimentary arm crown, with arm pair I (AI) arrested at the bud stage, arm pair II (AII) has only 1 sucker, arm pair III (AIII) has about 5 suckers and the fourth pair (AIV) has 2 suckers. The arm formula is III:IV:II:I (**Figure 1A**). The tentacles are easily discernible from the other arms, being larger and thicker than AIII. The tentacles possess about 18–20 suckers that are distributed along their entire length. As such, the tentacles resemble an arm as neither the tentacular clubs nor the stalks are differentiated (**Figure 1A**). No suckers are present at the tips of the arms or tentacles.

30 Day-Old (3.5–5.0 mm ML; Mean TL = 2.36 ± 0.3 mm)

The length of arms and tentacles and the number of suckers on them increased when compared to hatchlings (**Figure 1B**). AI has 1 sucker, AII 6, AIII 17, and AIV 7. On the arms, the suckers are distributed in two alternate rows as in the adults. The tentacles have about 38–40 suckers in four rows, covering about 80% of the tentacle's length, the remaining 20% represents the stalks.

60 Day-Old (9.5–13.0 mm ML; Mean TL = 5.8 ± 0.83 mm)

Major morphological changes occurred in the tentacles and arm-crown between 30 and 60 days of age. The tentacles have about 24 distal sucker rows which occupy approximately 50% of the tentacular length, forming a long club, clearly separated from the stalk, which represents nearly 50% of the tentacular length (**Figure 1C**). On the clubs, the 4 sucker rows that characterize the Genus are already present on the manus, but the carpus and dactylus are not yet differentiated. The arms show considerable increase in both length and number of suckers, particularly AI and AIV. AI has about 14 suckers, AII 24, AIII 28, and AIV 30. Swimming keels (lateral expansions) are present on the aboral surface of AIV (**Figure 1C**).

80 Day-Old (12.0–16.0 mm ML; Mean TL = 8.7 ± 1.5 mm)

The length of the arms and their suckers increased considerably. AI has about 35 suckers, AII 42, AIII 46 and AIV 52. The tentacular clubs are well defined occupying approximately 35–40% of the tentacular length (**Figure 1D**). On the clubs, there are 10 rows of suckers in the manus and the dactylus is well differentiated, having about 30 rows of suckers (**Figure 1D**). Some modification in the relative length of the manus and dactylus will still occur before the clubs attained their final adult shape (i.e., in the adults the manus is thicker with larger suckers and the dactylus is longer and thinner (see Jereb and Roper, 2005, pp. 62).

Tentacles Morphometry and Behavior

Paralarvae were often videotaped elongating and contracting their tentacles while not attempting to capture a prey. Sequential

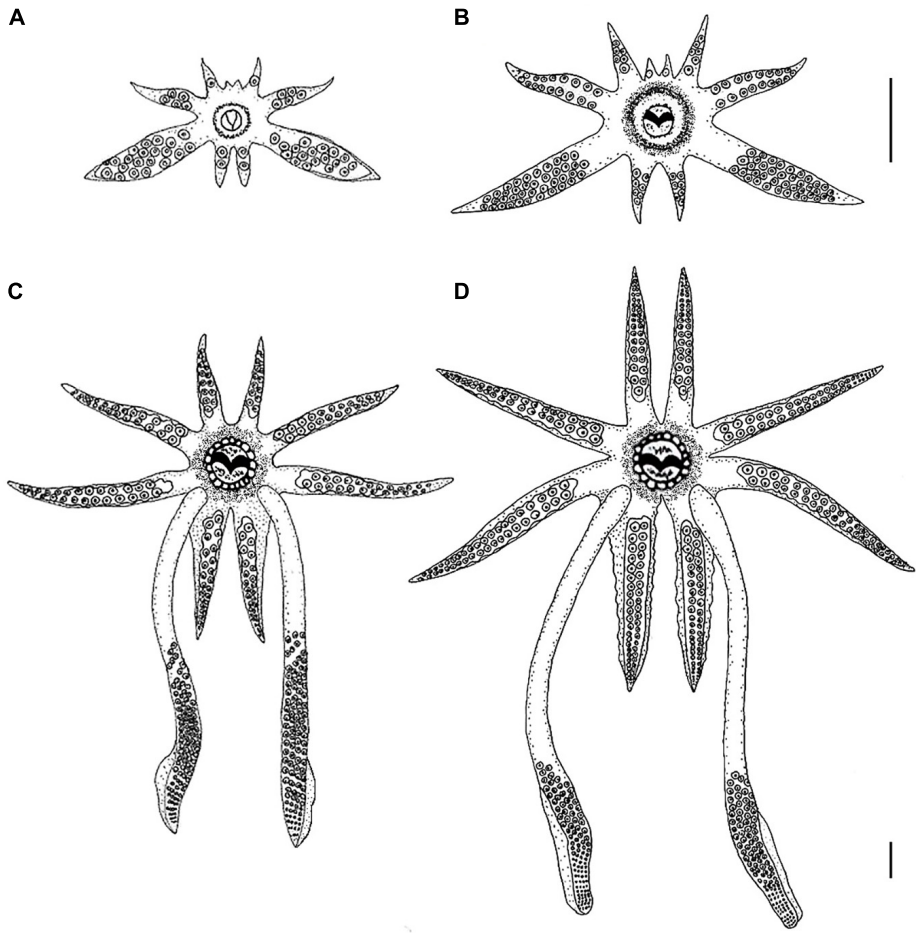

FIGURE 1 | Morphological development of the arm-crown complex of *Doryteuthis opalescens* paralarvae and juveniles. **(A)** 2.7 mm ML (1 day-old); **(B)** 4.7 mm ML (30 day-old); **(C)** 10.0 mm ML (60 day-old); **(D)** 13 mm ML (80 day-old). The upper scale bar applies to panels **(A)**, **(B)**, and **(C)**; the bottom scale bar refers to panel **(D)**. Scale bars = 1 mm. Panel **(A)** is reprinted by permission from Springer: Hydrobiologia, **725**, 85–103. *Beak development of early squid paralarvae (Cephalopoda: Teuthoidea) may reflect an adaptation to a specialized feeding mode*, by R. M. Franco-Santos and E. A. G. Vidal, Copyright 2013 Springer Science + Business Media Dordrecht.

video frames of paralarvae practicing tentacular elongations and contractions showed a progression of this movement according to size and age (**Figure 2**).

Early paralarvae (<20 day-old and <4 mm ML) showed low variability between contracted and elongated TL. In contrast, in older paralarvae there was large variability and differences between contracted and elongated TL (**Figures 2**, **3**), which resulted in significantly different regression slopes ($p < 0.05$, ANCOVA) in the relationship between contracted and elongated TL and ML (**Figure 3**). A wide range of elongation values were recorded for the same ML in larger squid, illustrating their enhanced ability to elongate their tentacles when compared to smaller paralarvae (**Figure 3**).

Paralarvae younger than 30 days were only able to perform elongations from 3 to 30% of ML (mean 13%), while older paralarvae became capable of elongating their tentacles up to 61% of ML (**Figure 4A**). The results from the ANOVA showed overall significant differences in proportions of tentacular elongation between paralarvae age groups [$F(6, 75) = 19.41$, $p < 0.0001$; **Figure 4A**]. Pairwise comparisons between groups revealed that the elongation means of 40, 55, and 60 day-old paralarvae were significantly longer than 1–10, 20, and 30 day-old paralarvae (Tukey HSD, $p < 0.01$). Significant differences were also observed between 14–55 and 14–60 age groups ($p < 0.01$).

The enhanced ability of elongating the tentacles in older and larger squid was also evidenced by the velocities for a full elongation and contraction cycle. While in early paralarvae elongation lengths (difference between fully elongated and contracted tentacles) were very small, resulting in velocities <0.2 mm s^{-1}, squid older than 40 days (>6.7 mm ML) became capable of longer elongation lengths (up to 6 mm), reaching velocities of up to and higher than 1.0 mm s^{-1} for a full elongating and contracting movement (**Figure 4B**). Additionally, paralarvae older than 40 days were observed bending the distal tip of their tentacles outward for the first time, exposing the clubs

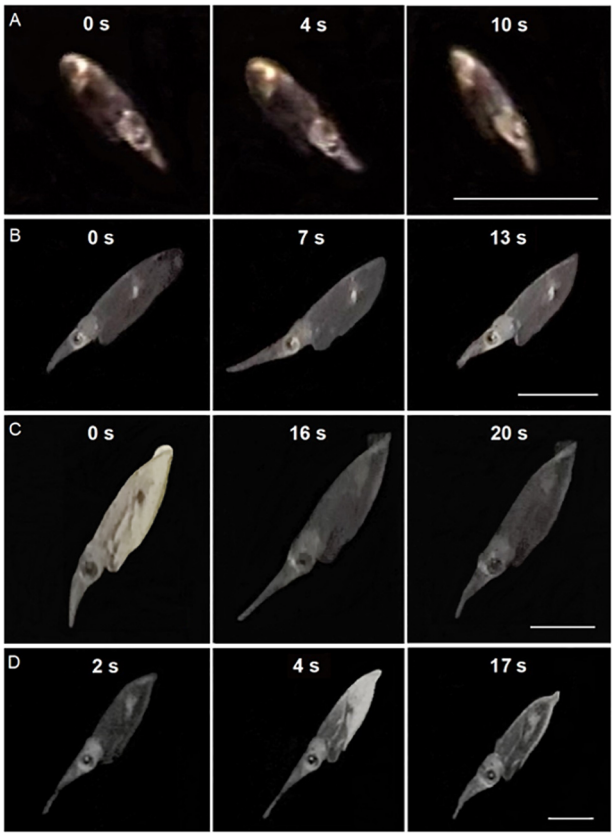

FIGURE 2 | Sequential video frames of tentacular elongation and contraction cycles in *Doryteuthis opalescens*. **(A)** 14 day-old; **(B)** 40 day-old; **(C)** 50 day-old; and **(D)** 60 day-old. In sequence **(D)**, tentacles are already being elongated in the first image (2 s). Time (s) are indicated between frames. Scale bar = 5 mm.

just after elongation to their maximum length and before fast contraction (**Figure 5**).

Significant differences between paralarvae age groups were also observed in relation to tentacular strain [$F(6, 70) = 8.64$, $p < 0.0001$]. Larger differences occurred between 60 day-old compared to 1–10 and 30 age groups (Tukey HSD, $p < 0.001$). Differences were also significant between 1–10 and 55, 20–40, 20–60, 30–40, and 30–55 age groups ($p < 0.05$). The mean (±standard error) strains measured in 1–10, 14, 20, 30, 40, 55, and 60 day-old paralarvae were 0.39 ± 0.05, 0.67 ± 0.11, 0.46 ± 0.05, 0.37 ± 0.03, 0.76 ± 0.10, 0.77 ± 0.05, and 0.87 ± 0.13, respectively.

Predatory Behaviors

Three predatory behaviors were observed during the first 60 days after hatching. In general aspects, these behaviors (see below) were similar to those described by Chen et al. (1996). Here, we expanded this initial study providing other details and documenting the role played by the arms and tentacles during predatory behavior. Each predatory behavior was divided into four sequential stages (shown in rows; **Figures 6–8**) and diagrammed from three perspectives; lateral, anterior and dorsal (shown in columns; **Figures 6–8**). Each row represents one moment in time seen from three different angles (lateral, anterior and dorsal). The dark oval shape represents the prey item and it was omitted from the anterior perspective to show unobstructed views of arms and tentacles.

Basic Attack (BA)

This behavior is observed in hatchlings and up to 40 day-old paralarvae (2.0–6.7 mm ML; **Table 1**). After reacting to the prey, the paralarvae orient and move toward it until reaching an attack distance. The arms and tentacles are held together in a tight cone (**Figure 6**). The paralarvae begin jetting forward along a curve, with the eyes directly toward the prey and positioning its arms underneath the prey as the arms spread out to expose the suckers. The tentacles are held straight and slightly spread apart, and their distal tips are bent outward, exposing the suckers (**Figure 6**). Usually, the first contact with the prey is made with AII and AIII that are then brought down onto the "platform" created by the tentacles. Arm pair IV are held straight. The tentacles have suckers along their entire length, which increase the area for prey attaching (**Figure 1A**). Tentacles are used in the same way as the arms to hold the prey. After the arms securely grasped the prey, the tentacles hang limp and are not involved in prey manipulation. The arms play a primary role in prey holding and handling. After prey capture, the arms quickly manipulate crustacean prey (copepods, decapod zoeae, mysid shrimp) so their dorsal exoskeleton is placed in contact with the buccal mass and prey is held in this position during ingestion. The BA was not observed in paralarvae older than 40 days as it was replaced by the arm-net (AN) and the Tentacle strike.

Arm-Net (AN)

The AN was first observed in 30 day-old paralarvae (>4.7 mm ML) and was still present through day 60. After the paralarvae orient toward the prey, so that the prey is positioned directly in front of the cone formed by the arms and tentacles, the AI, AII and AIII are peeled back slightly. The tentacles are ejected down and out but not directly at the prey and at the same instant the paralarvae begin a forward jet in a straight trajectory (**Figure 7**). The dorsal arms fully open and spread apart as the paralarvae jet toward the prey, while AIV is held straight. The ejection of the tentacles downward almost in parallel orientation with arm pair AIV aids to prevent the prey from escaping underneath the dorsal arms, improving prey interception and capture. As the prey contacts the dorsal arms, they are closed and hold the prey. The AN behavior was sometimes employed by older paralarvae/early juveniles to capture motionless prey on the bottom or walls of the aquaria. The tentacles play no role in holding and handling the prey (**Figure 7**). Prey handling and ingestion behavior is identical to that of the BA.

Tentacular Strike (TS)

This behavior was first observed in 40–50 day-old squid (>6.7 mm ML). The prey is approached in a similar way

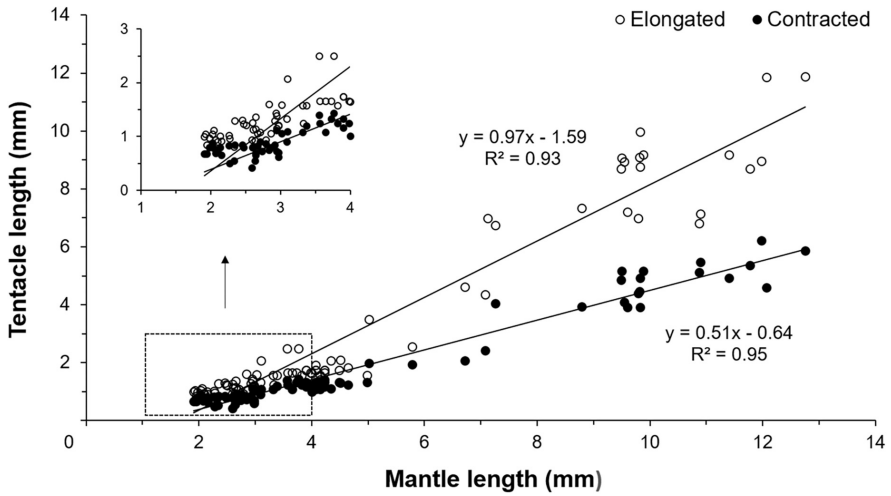

FIGURE 3 | Relationship between contracted and elongated tentacles length and mantle length (ML) of *Doryteuthis opalescens* reared in the laboratory from hatching to 60 days of age (n = 79 contracted, n = 86 elongated).

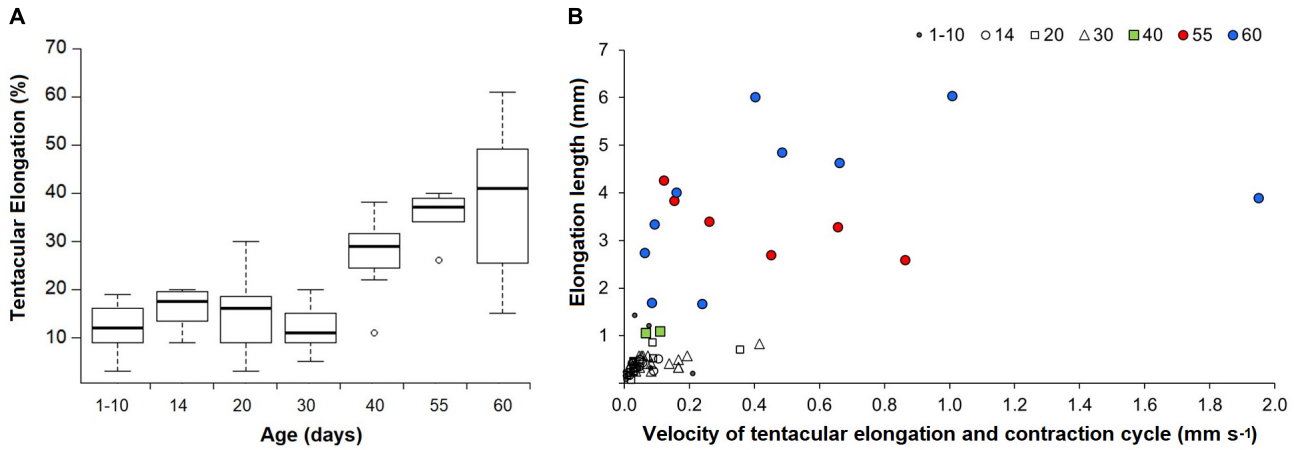

FIGURE 4 | *Doryteuthis opalescens*. **(A)** Tentacular elongation percentage between contracted and elongated tentacle modes in relation to age classes. Tentacular elongation percentage was calculated by the difference between contracted and elongated tentacles; the results were normalized by the squid mantle length and converted into percentages; n = 6–21 for each age class. **(B)** Tentacular elongation lengths in relation to velocities for a full elongation and contraction cycle. Tentacular elongation lengths were calculated by the difference between fully contracted and elongated tentacles; n = 2–19 for each age class.

as in the AN, however, the AI, AII, and AIII began to peel back from the tentacles prior to the strike, while AIV is straight, allowing the tentacles to be ejected forward in a straight trajectory directly at the prey (**Figure 8**). The first contact with the prey is made by the suckered surface of the tentacular clubs. By the time the tentacles reach and attach to the prey, the AI, AII, and AIII are fully opened and spread apart, while AIV is straight. During the TS, paralarvae are stationary and their position is maintained by strong fin beats. The tentacles then contract and pull the prey into the open arms that hold and manipulate the prey. As in the other two behaviors, the tentacles play no role in holding and handling the prey; the arms usually quickly flip the prey into the dorsal side prior to immobilization followed by ingestion. This was particularly noticeable with large prey, such as mysid shrimp. The prey is ingested in the same manner as in the two previous behaviors (**Figure 8**).

Comparison of Predatory Behaviors

In the BA, the tentacles are used like another pair of arms during prey capture. This is the first and only feeding behavior exhibited early after hatching; it begins to wane in 30–40 day-old squid (4.7–6.7 mm ML; **Figure 9** and **Table 1**). The BA is not observed in squid older than 40 days, when the AN is firmly developed showing the highest frequency of occurrence (**Figure 9**). The TS is first observed in 40 day-old squid, but with a lower frequency of occurrence (9%); however, the occurrence of this behavior increases rapidly

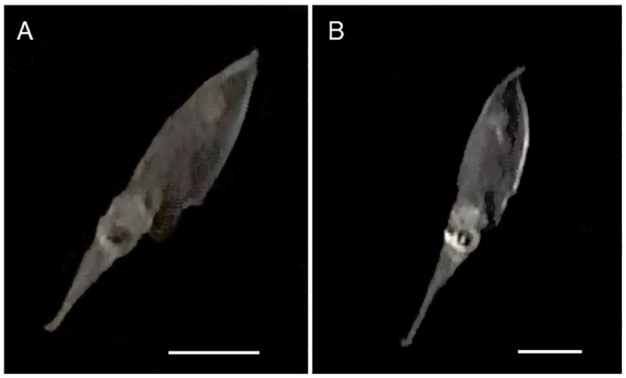

FIGURE 5 | Captured images of 50 day-old *Doryteuthis opalescens* specimens bending the tips of their tentacles immediately after elongation to the maximum length and before fast contraction. **(A)** 9 mm ML; **(B)** 8.7 mm ML. Scale bars = 5 mm.

and it is more frequent than the AN in 55–60 day-old squid (**Figure 9**).

The tentacles are ejected in both the AN and the TS. But in the AN the first contact with the prey is made by the dorsal arms and the tentacles are ejected downward, not directly at the prey (**Table 1**).

Main Prey Types Captured by Paralarvae

The main prey types captured by paralarvae during filming were enriched *Artemia* spp. nauplii, copepods and mysid shrimp. *Artemia* spp. nauplii ranged in size from 0.3 to 0.6 mm. Copepods were composed mainly by copepodits and adults of several species. Their sizes ranged from small (0.5–1.1 mm, *Corycaeus* spp., *Euterpina acutifrons*, *Paracalanus* spp.), to medium (0.8–1.6 mm, *Acartia tonsa*, *Acartia lilljeborgi*, *Calanopia* sp., *Centropages velificatus*, *Temora turbinata*, *Temora stylifera*) and large composed by Pontellid copepods (1.5–4.0 mm, *Anomalocera ornata*, *Labidocera aestiva*, *Pontella* spp.). Mysid shrimp, *A. almyra* juveniles and adults, ranged in total size from 2 to 11 mm. Paralarvae also captured other types of prey, such as decapod crustacean zoeae, Cladocera, Cirripedia larvae, etc., which were included in the category "others" for simplification (**Figure 10**).

The main prey types and sizes captured by paralarvae changed during ontogeny, prey size and diversity increased with increasing squid size. The predominant prey of early paralarvae (<20 day-old, <4.0 mm ML) were *Artemia* nauplii, representing 62% of capture frequency, followed

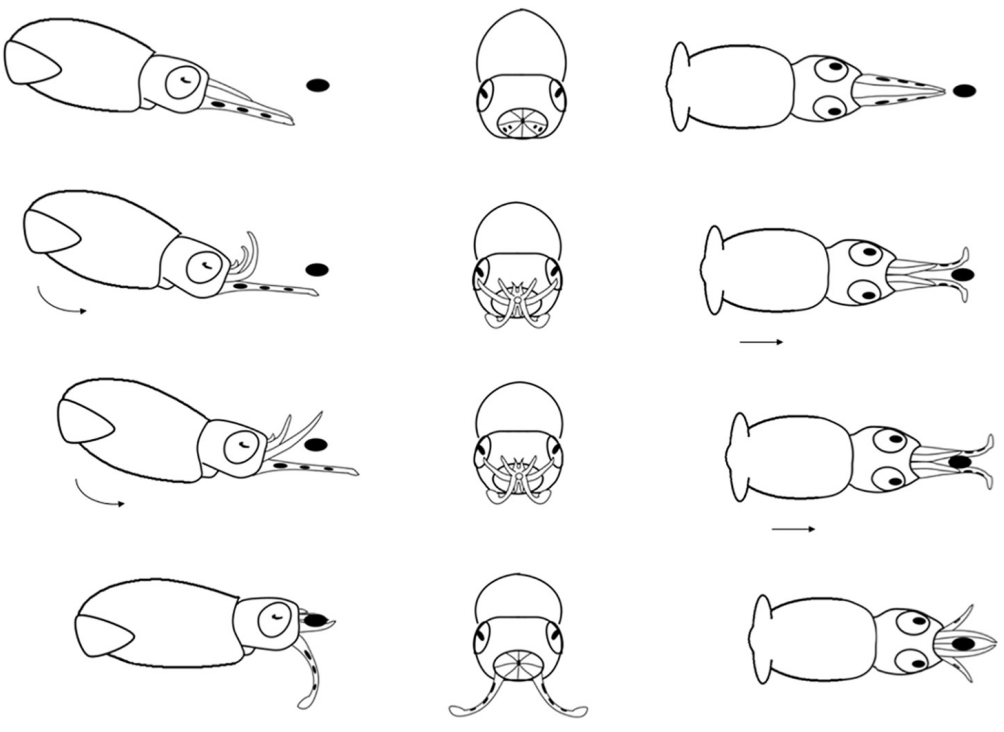

FIGURE 6 | Diagrammatic sequence of the role played by the arms and tentacles of *Doryteuthis opalescens* paralarvae during the Basic Attack behavior (BA). The behavior was divided into 4 sequential stages (shown in rows) and diagrammed from three perspectives; lateral, anterior and dorsal (showed in columns). Each row represents one moment in time seen from three different angles. The dark oval represents the prey item and it was omitted from the anterior perspective because it would obfuscate the diagram. Paralarvae orient toward the prey. Their arms and tentacles are held together in a tight cone as they jet forward along a curve, positioning the arms underneath the prey. The arms spread out and the tentacles are held straight. The first contact with the prey is made with arm pairs II and III that are brought down onto the "platform" created by the tentacles. Prey is hold by the arms and the tentacles are not involved in prey holding.

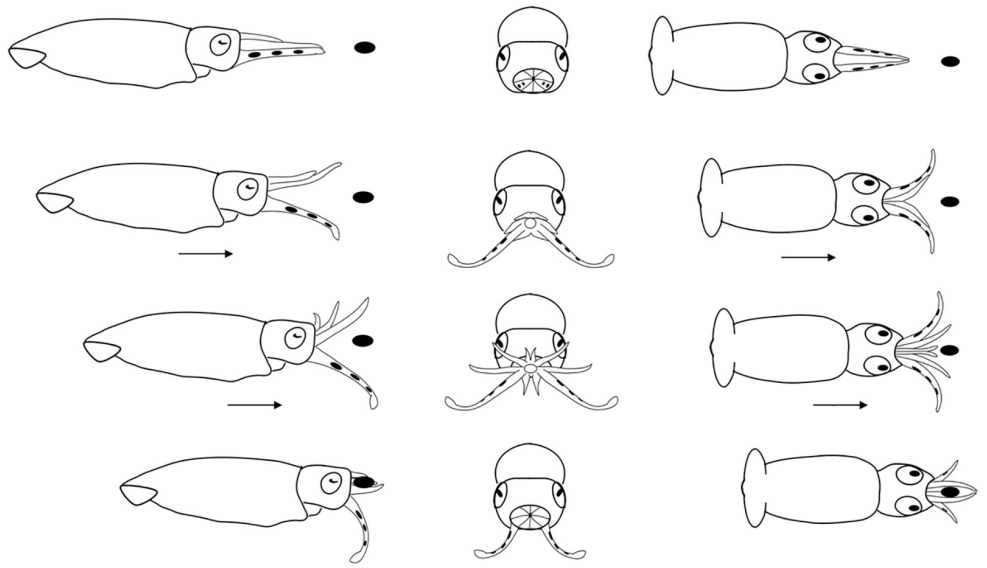

FIGURE 7 | Diagrammatic sequence of the role played by the arms and tentacles of *Doryteuthis opalescens* paralarvae during the Arm-net behavior (AN). The behavior was divided into 4 sequential stages (shown in rows) and diagrammed from three perspectives; lateral, anterior and dorsal (showed in columns). Each row represents one moment in time seen from three different angles. The dark oval represents the prey item and it was omitted from the anterior perspective because it would obfuscate the diagram. Paralarvae orient toward the prey. The arms are peeled back slightly. The tentacles are ejected down and out but not directly at the prey and at the same instant the paralarvae begin a forward jet in a straight trajectory. The arms fully open as the paralarvae jet toward the prey. As the prey come into contact with the arms, they are closed and hold the prey. The tentacles play no role in prey holding.

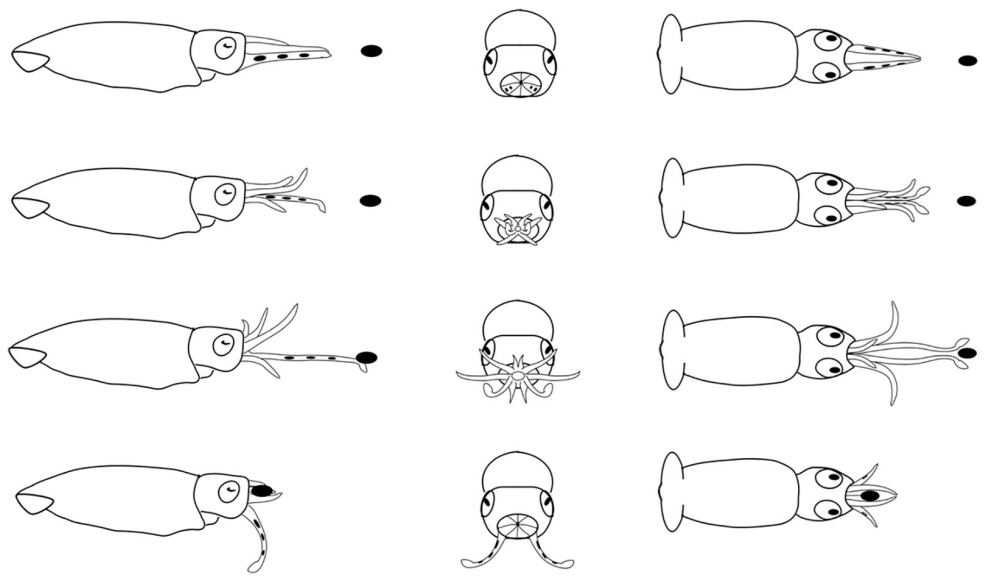

FIGURE 8 | Diagrammatic sequence of the role played by the arms and tentacles of *Doryteuthis opalescens* squid during the tentaclular strike behavior (TS). The behavior was divided into 4 sequential stages (shown in rows) and diagrammed from three perspectives; lateral, anterior and dorsal (showed in columns). Each row represents one moment in time seen from three different angles. The dark oval represents the prey item and it was omitted from the anterior perspective because it would obfuscate the diagram. Squid orient toward the prey and when the prey is positioned in front of arms and tentacles, the arms begin to peel back from the tentacles prior to the strike. The tentacles are ejected forward in a straight trajectory directly at the prey. The first contact with the prey is made by the tentacular clubs. The tentacles contracted and pulled the prey into the open arms that hold the prey. The tentacles play no role in prey holding.

by copepods with 21% in total (**Figure 10A**). As paralarvae reach 20–30 day-old, capture of juvenile mysids increased to 22%, while *Artemia* nauplii became less frequent (44%) and copepods represented 21% in total (**Figure 10B**). For older paralarvae (>40 day-old, >6.7 mm ML), juvenile and adult mysids were the predominant prey, representing 50%

TABLE 1 | Comparison of the role played by the arms and tentacles during predatory behaviors in *Doryteuthis opalescens* reared in the laboratory from hatching to 60 days of age.

	Basic attack	Arm-net	Tentacular strike
Age (days)	0 – 40	>30	>40
Size (mm ML)	2.0 – 6.7	>4.7	>6.7
Tentacles	Not ejected	Ejected down	Ejected at the prey
Arm pairs (I, II, and III)	Fully opened	Fully opened	Fully opened
Body movement	Forward jet	Forward jet	Stationary
First contact with prey	Arms and tentacles	Arms	Tentacular clubs
Prey holding and manipulation	Arms	Arms	Arms

ML, mantle length.

of capture frequency, followed by copepods with 22% in total (**Figure 10C**).

DISCUSSION

Tentacles Morphological Development

Herein we have shown that the tentacles of *D. opalescens* early paralarvae resemble and function as arms during predatory behavior as they do not possess clubs and stalks yet formed. Both stalks and clubs play a crucial role during the strike and their arrested development imposes a morphological constraint for the performance of the strike behavior. The stalks and clubs develop progressively as squid grow. In hatchlings, the tentacles possess suckers on their entire length and the area with suckers is non-extensible (Kier and van Leeuwen, 1997). The stalk must growth until it represents at least 50% of the tentacles' length, as it contains the specialized transverse cross-striated muscles fibers responsible for fast elongation (Kier, 1996, 2016), and clubs need to be formed to effectively attach to prey during the strike. In fact, our results have demonstrated that the tentacles of paralarvae younger than 40 days (>6.7 mm ML) do not possess the morphological and anatomical differentiation to perform the TS, confirming and expanding previous studies (Chen et al., 1996; Kier, 1996; Kier and van Leeuwen, 1997). It turns out that while muscle fibers, stalks and clubs develop concurrently, tentacles' muscle elongations are repeated over and over again, perhaps to adjust muscle activity pattern and its developmental process through use. It seems that flexible muscle activation patterns would be important for paralarvae to cope with the ontogenetic changes observed in the morphology and structure of the tentacles, as well as to perfect the TS performance. Our results have shown ample variability in the kinematic pattern of tentacular elongations that along with its constant repetition by paralarvae suggests that elongations could be modulated during the strike according to several cues from the prey, such as type, size, and escape response, among others. This possibility shall be a very interesting topic for future studies. For example, octopus can extend its arm to seize a prey by a wave-like propagation of a bend that travels from the base of the arm toward the extremity (Kier and Smith, 1985; Gutfreund et al., 1996). To achieve point-to-point movement, they use a quasi-articulated structure that has two bends that divide the arm into three segments, which are dynamically adjusted. The segment lengths appear the multijointed, articulated limbs of animals with rigid skeletons (Sumbre et al., 2005).

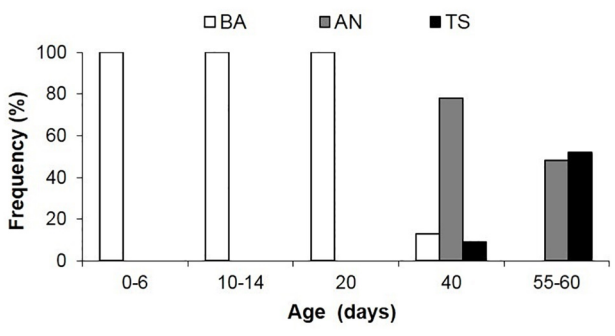

FIGURE 9 | Frequency of occurrence of predatory behavior in *Doryteuthis opalescens* squid reared in the laboratory from hatching to 60 days of age. BA = Basic Attack; AN = Arm-net; TS = Tentacular strike. Age classes 0–6 (n = 19), 10–14 (n = 12), 20 (n = 23), 40 (n = 27), 55–60 (n = 30).

In paralarvae, results from the morphometric analysis showed intrinsically correlated changes with behavior, illustrating that early paralarvae have little or no capacity to elongate their tentacles. This ability increased with age and size and those older than 40 days (>6.7 mm ML) could elongate the tentacles up to 61% of their ML (**Figure 4A**). Considering that in adult loliginid squids the strike involves an elongation of 40–80% of the tentacles resting length (Kier and van Leeuwen, 1997; van Leeuwen and Kier, 1997), the elongation percentage values obtained in the present study for squid older than 40 days were quite similar. It is also interesting to notice that the mean tentacular strain values (0.46–0.87) recorded in the present study for *D. opalescens* paralarvae older than 20 days were surprisingly similar to peak strains registered for adults *Loligo pealei* (0.43–0.80; Kier and van Leeuwen, 1997). Such comparison must consider that these authors calculated strain values based on the estimated tentacular stalks length (not including the clubs), while our measurements considered the total length of the tentacles. Thus, as the clubs become smaller and the stalk portion increases relative to the size of the tentacles in older paralarvae/early juveniles, strains values would become even more comparable to those recorded from adults. On the other hand, although older paralarvae were able to perform a complete tentacular elongation and contraction cycle much faster ($\geq$1 mm s^{-1}) than younger paralarvae (**Figure 4B**), this is indeed a slow movement – performed while paralarvae were not attempting to capture prey – and should not be compared with the elongation velocities of adults reported by Kier and van Leeuwen (1997), which considered only the fast elongation phase until the prey was reached.

An optimal matching of tentacles structure and functional behavioral expression occurs in squid of 6.7–7.8 mm ML (40–50 day-old), when the TS behavior was observed for the first time. Simultaneously, observations of tentacular elongations within this ML window also revealed, for the first time, squid bending up

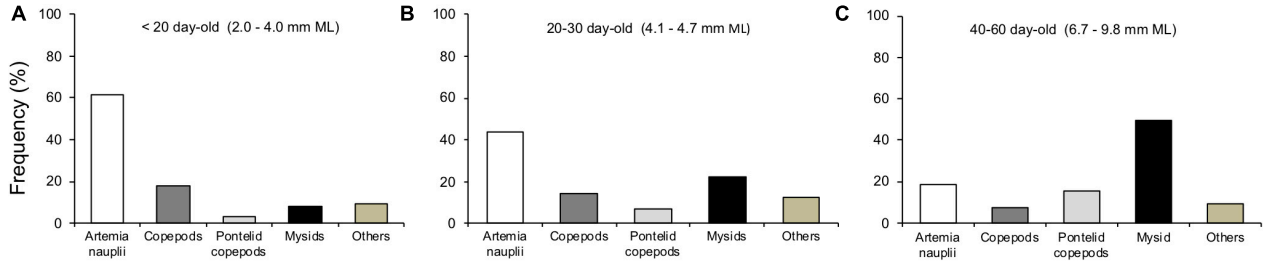

FIGURE 10 | Feeding frequency of *Doryteuthis opalescens* on different prey types according to three size classes. **(A)** $n = 42$, **(B)** $n = 32$, and **(C)** $n = 55$.

the tips of their tentacles and exposing the clubs immediately after tentacles were elongated to their maximum length (**Figure 5**), indicating that the TS was functional.

Correlated Arm-Crown Morphology, Predatory Behavior, and Prey Types During Early Ontogeny

Arm-crown morphology and predatory behaviors revealed close adjustments with progressive complexity and efficiency as paralarvae go through major developmental milestones. Hatchlings have a rudimentary arm-crown and fins, bell-shaped body, limited swimming abilities (Vidal et al., 2018) and display only a basic predatory behavior (BA). In this behavior, the arms and tentacles frame prey from underneath (**Figure 6**). These paralarvae fed mainly on small prey such as *Artemia* nauplii and copepods (nauplii, copepodits and adults), but also on other small planktonic prey, such as Crustacea brachyuran zoeae (**Figure 10**). Observations of prey handling by hatchlings revealed that their short arms were particularly advantageous to manipulate these preys, which have many appendages and spines (Vidal, EAG unpublished data). The underdeveloped arm-crown of hatchlings seems to make them specialized in foraging smaller prey (**Figures 1A**, **10**). Nevertheless, paralarvae broaden their foraging repertoire by performing kleptoparasitism – feeding on prey already subdue by another squid – an imitative foraging behavior that allows several paralarvae to feed on a larger prey, such as mysid shrimp (Vidal et al., 2018).

Additionally, the ontogeny of predatory behavior of *D. opalescens* paralarvae on copepods revealed a refinement of the BA that consisted in circling copepods to find the best attack position (Chen et al., 1996). These authors have shown that through trial and error paralarvae learned to refine the BA position from posterior to anterior and approach copepods head-on, which increased the capture success.

A more elaborated behavior, the AN was first observed in 30 day-old (>4.7 mm ML) paralarvae and was depicted by the ejection of the tentacles downward (not toward the prey) with a forward jet in straight line to intercept the prey. When the arm-crown of 30 day-old paralarvae is compared with that of hatchlings, the fast development of all arms, but AI is quite conspicuous (**Figures 1A,B**). Although these paralarvae can eject their tentacles, this ability is still limited, as the stalk portion represents only 20% of the tentacles' length. During the AN behavior, AIV is positioned almost in parallel orientation to the stalks (**Figure 1B**), which increases the area with suckers for prey interception, compensating for the lack of suckers on the stalk. This in turn should reduce the chances of prey escaping below the dorsal arms, likely improving capture success on larger prey, such as Pontellid copepods and adult mysids (**Figure 10**).

The AN is a transition for the TS behavior. Nevertheless, it was sometimes employed by older paralarvae/early juveniles to capture sluggishly moving or motionless prey. It is quite interesting that this predatory behavior was observed in adults *Doryteuthis pealeii* also toward slowly moving or moribund shrimp prey (Kier and van Leeuwen, 1997), in adult ommastrephid squid (Flores, 1983), and in cuttlefish (Duval et al., 1984). Certainly, there will be variations of the AN between adults and paralarvae, but once it is established at the end of the first month of life it seems to persist until adulthood. On the other hand, after the AN is expressed, the BA frequency of occurrence was reduced until it vanished completely.

The TS was first observed in 40 day-old paralarvae (>6.7 mm ML) in combination with the BA and AN, with the latter being largely predominant (**Figure 9**). However, in 60 day-old squid the specialized TS behavior was already more frequent than the AN. In these squid, the stalks represented at least 50% of the tentacle's length, and the clubs were clearly defined (**Figure 1C**). The clubs and AIV show well-developed keels, which are expanded muscular membranes to provide better hydrodynamics and as such should play a role in the strike. Actually, it was suggested that AIV provides stability and alignment to the tentacular stalks during the entire elongation phase of the strike (Kier and van Leeuwen, 1997) and its fast development (compared to the other arms) might explain its prominent role in the strike. As paralarvae grow, the size (and weight) of the head and arm-crown complex relative to the ML increases, which might be necessary to stabilize the body and counterbalance the development of fins (Vidal et al., 2018), providing locomotion stabilization.

When the three foraging behaviors observed in the present study are compared, they revealed that the arms played the main role of prey capture from hatching to up to 40 days of age. This indicates an adaptive foraging strategy in paralarvae, as the TS is not employed in prey capture. Nonetheless, after prey was captured, the roles played by the arms were stereotyped, as they did almost the same tasks in all behaviors: hold, subdue and manipulate the prey during ingestion. Most importantly, in all three behaviors, after prey was brought to the arms, the

tentacles were not involved further in prey manipulation and ingestion as happens in adults (Kier and van Leeuwen, 1997). The existence of a forward jet during the BA and AN, and the actions performed by the tentacles (not ejected, ejected downward or ejected toward the prey) (**Table 1**), are what truly defined each predatory behavior during early ontogeny of *D. opalescens*. The expression of these behaviors and its connection to prey-capture learning might have profound effects on the ontogeny of brain development and cognition in squid.

When the TS of a 60 day-old juvenile described in the present study is compared with that of the adults (Kier and van Leeuwen, 1997), it is clear that some modifications will still occur before the adult behavior is fully established. In juveniles, the relative length of the clubs is longer and the stalks shorter. This might influence the performance of the strike as well as the type and size of the prey that can be captured by juveniles, perhaps defining their niche occupation.

A study on the development of the beak, another essential feeding structure of cephalopods, have shown that in *D. opalescens* paralarvae the rostrum protrudes and becomes pigmented in both jaws between 30 and 50 days of age (>4.5 mm ML) (Franco-Santos and Vidal, 2014). The intensity and extent of beak pigmentation (darkening) and rostrum protrusion were suggested as having an important influence on prey selection and feeding behavior in *Octopus vulgaris* paralarvae (Hernández-García et al., 2000; Franco-Santos et al., 2014). The darkening of the chitin serves as an indication of beak hardness that together with the arm-crown morphology hint to the level of development required to feeding on prey that is hard to capture, hold on to, and dilacerate for ingestion. Therefore, it is worthy of mention that the acquisition of a robust beak in *D. opalescens* occurs coincidently just prior to the display of the TS for the first time. Squid older than 40 days (>6.7 mm ML) were able to capture larger prey such as adult mysids using the TS behavior (**Figures 9**, **10**). Furthermore, it has been proposed that foremost ontogenetic morphological changes, such as those observed in *D. opalescens* at about 40 days of age (namely rostrum protrusion, TS behavior, school formation, see below), could be also indicative of changes in vertical distribution related to prey distribution at different depths (Karpov and Cailliet, 1979; Shea and Vecchione, 2010; Vidal et al., 2018).

Although the prey types and sizes offered to paralarvae during the present study were largely influenced by the suite of prey available during zooplankton collections and the fact that *Artemia* nauplii was offered daily due to its easy accessibility, it was possible to observe a change in the main prey type, an increase in prey size and diversity with increasing squid size (**Figure 10**).

Developmental Constraints and the Performance of the Specialized TS

Our behavioral observations revealed that immediately before shooting the tentacles at the prey, squid stayed stationary holding their position against the current by means of fin movements. It is noteworthy that no forward jet was involved, contrary to observations of the TS in adults *D. pealeii*, which swim forward at velocities of 0.7–1.2 ms^{-1} as the stalks elongate (Kier and van Leeuwen, 1997). Therefore, to be able to perform the TS for the first time, squid need to be competent at holding their position against a current (sustainable swimming).

A major ecological and behavioral transition occurs in the early life history of *D. opalescens* at about 40 days of age, when they can perform sustained swimming and hover against a current (Vidal et al., 2018). The development of fins during early ontogeny has a crucial role for sustained swimming as fins act as stabilizers of the body during locomotion (Stewart et al., 2010; Vidal et al., 2018). This enhanced swimming control is a precursory stage for the formation of schools.

At this point, it should be emphasized that the TS was first observed in *D. opalescens* squid of 6.7–7.8 mm ML, this size ranges overlaps to the sizes that they start to swim in schools (see data reported in Vidal et al., 2018). This is no coincidence, as early juveniles perform sustainable swimming immediately prior to ejecting their tentacles at prey (**Figure 8**). This indicates that the sequential underlying factors responsible for the TS expression are complex and involves different levels of development. Besides the needed morphological development of the tentacles – involving muscle fibers, stalks and clubs – squid must have acquired swimming control. The ability to perform parallel synchronized and fine-motor control swimming with nearest neighbor squid during schooling requires advanced swimming coordination and plasticity of the neurolocomotor system, which are absent in early paralarvae (Gilly et al., 1991). In addition, binocular vision, and thus precise convergent eye movements to determine the proper distance to a prey (Budelmann and Young, 1993), is another prerequisite for the TS. It seems that several features might have evolved together in squid to design the TS. Not surprisingly, the TS expression represents a major developmental milestone and demands interaction of structures on several levels of organization.

Interestingly, in octopus paralarvae the differentiation of tissue layers, including the ganglionic structure and the majority of the musculature of the arms occurs during the planktonic paralarval phase and is maintained until the end of the animal life (Nödl et al., 2015). This seems consistent with the developmental pattern in squid paralarvae, in which ultrastructural and morphological development of the tentacles is delayed to several weeks after hatching, constraining the expression of the TS during prey capture.

Hydrodynamic Environment and Morphological Changes of Squid During Ontogeny

Over the growth of paralarvae its hydrodynamic environment is transformed radically due to changes in morphology and size and this may impose adaptive constraints on feeding mechanisms. Hydrodynamic theory provides a basis for interpreting how growth would affect important biological processes, which in turn are dependent on the interactions of an organism with its

environment (Koehl, 2000). The Reynolds number (Re) expresses the relative contribution of inertial and viscous forces to the total force acting on an animal's body. Newly hatched squid live in an environment within intermediate Re numbers ($10 < Re < 200$), in which both viscous and inertial flow forces play important roles (Bartol et al., 2009; Vidal et al., 2018). However, as they grow, paralarvae need to adapt to the differing functional demands of viscous and inertial regimes.

Due to the viscous drag production it was suggested that it would be easier for a hatchling to move its entire bell-shaped body (predicted to generate relatively low drag) to seize a prey instead of shooting the tentacles (Vecchione, 1987). Indeed, this is exactly what we have shown during the BA and AN behaviors (**Figures 6**, **7**). Even if the tentacles of hatchlings would be morphologically functional to be eject during prey capture, hatchlings would not be able to hold their position against a current prior to shooting their tentacles at prey, because they are passive drifters. The precision of the TS involves swimming control. This might partially explain why there is a forward jet to intercept the prey in the BA and AN behaviors. As emphasize previously, the TS is only employed after *D. opalescens* can perform sustainable swimming and also attain a size (>6 mm ML) that afford them to transition occasionally to the inertia dominated realm (reaching high Re values), making the transition from plankton to nekton just after its first month of life (Vidal et al., 2018). Interestingly, the hatchlings of other squid families, such as Onychoteuthidae and Cranchiidae also hatch out with short tentacles, without differentiated stalks and clubs (Sweeney et al., 1992), which resemble arms as in loliginid squid. Based on the results of the present study, one can predict that these hatchlings will most likely capture their prey with a predatory behavior similar to the BA described here, involving a forward jet and an arm-strike in which the tentacles function as arms.

Overall, prey size in loliginid squid hatchlings seems to be limited by their rudimentary arm-crown and poor morphological development. It was suggested that the tentacles have evolved from modification of the AIV (Boletzky, 1993; Kier and van Leeuwen, 1997), but in hatchlings they still function as arms because of morphological constraints that impose displacement of the TS to later ontogenetic stages. This in turn called for a change in feeding strategy toward smaller prey and an adaptative predatory behavior (BA) allowing hatchlings to explore an ecological niche different from the adults. Ultimately, there is a trade-off between the poor morphological development of loliginid squid hatchlings and their exponential growth rates. The latter appears to be a selection on paralarvae to reach the adult morphology as rapidly as possible.

CONCLUSION

This study documented the ontogeny of the predatory behavior in *D. opalescens* paralarvae as they undergo critical developmental milestone. Our results were combined with available literature to demonstrate that arm-crown morphology, swimming abilities and predatory behaviors of paralarvae show interdependency and progressive complexity during ontogeny. Hatchlings have overall poor morphological development, limited swimming abilities and little or no capacity to elongate their tentacles. The tentacles of hatchlings resemble and function as arms during early predatory behavior (BA), as the stalks and clubs are not yet formed. Over the first month of life, the ability to eject the tentacles develops progressively as paralarvae experience major changes in body form, swimming performance, and arm-crown morphology and structure. Paralarvae were often observed elongating and contracting their tentacles, while not attempting to capture prey, suggesting that this behavior could perhaps serve to adjust muscle activity and its developmental process through use, while specializations for the strike (stalks, clubs, muscle fibers, arm-crown complex) are not yet fully formed.

When paralarvae reach 30 days of age, the AN behavior was firmly in place and prevailing over the others. This predatory behavior represents a transition from the BA to the TS and continues to be employed by adults. Squid older than 40 days (>6.7 mm ML) became capable of performing tentacular elongations significantly larger (up to 61% of the ML) and a complete tentacular elongation and contraction cycle much faster ($\geq$1 mm s^{-1}) than the younger ones. An optimal coordination between tentacles structure and functional behavior happened in squid of 40–50 days of age (6.7–7.8 mm ML), when clubs and stalks were formed, and squid were observed bending up the tips of the tentacles to expose the clubs immediately after fully elongations. This event also coincided with the expression of the TS behavior for the first time.

The TS was first observed in *D. opalescens* simultaneously with their ability to swim in schools (see Vidal et al., 2018), as swimming control is a prerequisite for the performance of the strike in early juveniles. This emphasizes that the sequential underlying factors responsible for the TS expression are complex and involves different levels of development: muscle fibers, stalks and clubs differentiation, arm-crown (particularly arm IV), swimming coordination (schooling) and binocular fixation of the prey.

The arms played the main role of prey capture in squid younger than 40 day-old (<6.7 mm ML) as the TS was not functional. After prey was captured, the roles played by the arms were stereotyped, as they did almost the same tasks in all behaviors: hold, subdue and manipulate the prey during ingestion. On the contrary, the actions played by the tentacles were what really defined each predatory behavior during squid early ontogeny. However, after prey was brought to the arms, the tentacles were not involved further in prey manipulation and ingestion as happens in adults. The predatory behaviors of paralarvae/early juveniles are adapted to the functional demands imposed by the developing morphology, structure and mechanics of the tentacles, as well as swimming coordination (Vidal et al., 2018).

By correlating behavioral observations with morphological and morphometric data, this study documented interconnected morphological and behavior traits that enabled squid to perform the TS, offering new insights into the interdependency of morphology and predatory behaviors during squid ontogeny.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because The Institutional Animal Care and Use Committee (IACUC) of the University where this study was conducted did not require researchers to submit protocols for the ethical treatment of invertebrate larvae when this research was performed.

AUTHOR CONTRIBUTIONS

EV conceived and designed the study, conducted the experiments, collected and analyzed the data, and drafted the manuscript. BS helped with the data analysis, visualization, preparations, and made improvements to the manuscript. Both authors worked together to interpret the findings and approved the final version.

FUNDING

EV was supported by the Brazilian National Research Council (CNPq; Grant # 312332/2018-1). This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

ACKNOWLEDGMENTS

We would like to thank Ben L. Stafford and J. Guilherme F. Bersano for their invaluable help with film analysis and copepods maintenance and identification, respectively. We thank the reviewers for their constructive comments. EV also thank the Brazilian National Research Council – CNPq.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 04 February 2020
doi: 10.3389/fphys.2020.00022

Changes in Biochemical Composition and Energy Reserves Associated With Sexual Maturation of *Octopus maya*

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Climate conditions are related to changes in the biochemical composition of several tissues and associated to the processes of growth and sexual development in cephalopods. The biochemical composition (protein, glucose, cholesterol, acylglycerides) and hemocytes of the hemolymph, the hepatosomatic and gonadosomatic indices, and the reserves of the gonad, hepatopancreas and muscle (lipids, glycogen, and caloric value of muscle) of *Octopus maya* were determined and related to sex and season. A total of 154 wild animals were used (≈50 caught per season) and the multivariate analysis of the biochemical indicators of the tissues allowed following the variations during winter, dry and rainy season. The permutational MANOVA showed that both sex and season contributed significantly to variations in metabolites and energy reserves. However, the non-significant interaction term indicated that the biochemical composition changed with the seasons in a similar way and regardless of sex. The pattern observed in metabolites and reserves indicates a variation associated with growth and the reproductive peak, but may also reflect a physiological response to seawater temperature. The present study provides reference values for several physiological indicators in *O. maya* that may be useful for programs monitoring wild populations, as well as to design diets and management protocols to produce octopus under controlled conditions.

Keywords: body size, season, energy reserves, hemolymph, *Octopus maya*

OPEN ACCESS

Edited by:
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University of Lisbon, Portugal

Reviewed by:
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Portuguese Institute for the Ocean and Atmosphere (IPMA), Portugal
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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *31 May 2019*
Accepted: *13 January 2020*
Published: *04 February 2020*

Citation:
Pascual C, Cruz-Lopez H, Mascaró M, Gallardo P, Sánchez A, Domingues P and Rosas C (2020) Changes in Biochemical Composition and Energy Reserves Associated With Sexual Maturation of Octopus maya. Front. Physiol. 11:22. doi: 10.3389/fphys.2020.00022

INTRODUCTION

Cephalopods are thought to be the most evolved group among marine molluscs (O'Dor and Webber, 1986). Along with fish, cephalopods comprise a significant part of the ocean biomass (Grisley and Boyle, 1985, 1988), and their position in the food chain grants them a relevant place in energy transfer among different trophic levels (Jackson et al., 1998). Important commercial species such as fish and lobsters among their prey, whereas their predators are marine mammals, fish, and birds (Guerra, 1992; Hernández-García, 1995; Rocha and Vega, 2003). The red octopus (*Octopus maya*) is endemic to southeast Mexico and is one of its most important fishery resources (Voss and Solís-Ramírez, 1966; Solís-Ramírez and Chávez, 1986; Solís-Ramírez, 1988). Red octopus fishery

begun its development in 1949 (Solís-Ramírez et al., 1997) and its commercial value has driven its growth; during the period 1998–2016 national catches varied between 9,000 and 35,000 t/year (Markaida et al., 2017).

Octopus maya has a semelparous reproductive strategy, with females reproducing only once and dying shortly after the eggs hatch, as is common in many cephalopod species (Cortéz et al., 1995; Zúñiga-Romero et al., 1995; Rocha et al., 2001). In captivity, *O. maya* life span is between 8 and 12 months at 25–30°C (Van Heukelem, 1983; Hanlon and Forsythe, 1985). The study of the population structure and maturation indicates that *O. maya* grows and matures during the fishing season (August to December). Adults show functional maturity during the winter storm season (November to February), when seawater temperature oscillates around 25°C (Angeles-González et al., 2017). Spent females predominate in January and February, revealing a year-long life cycle (Markaida et al., 2017). Embryonic development lasts 40 to 50 days, depending on temperature stability under cultured conditions (Rosas et al., 2014), and 50 to 65 days in natural environments (Solís-Ramírez, 1967). Voss and Solís-Ramírez (1966), reported that *O. maya* produces the largest eggs among octopodids, measuring up to 17 mm. Direct development and high adaptability to captivity make *O. maya* one of the most promising cephalopod species for commercial aquaculture (Boletzky and Hanlon, 1983; Tercero et al., 2015).

Octopus maya inhabits shallow waters adjacent to the continental shelf of the Yucatan Peninsula (states of Campeche, Yucatan, and Quintana Roo). This species is often found in areas covered by sea grass (*Thallassia testudinum*), gastropod empty shells and coral fragments (Voss and Solís-Ramírez, 1966). At the Yucatan Peninsula, a transition site between the Caribbean Sea and the Gulf of Mexico, three climatic seasons can be identified: (1) the winter season ("nortes;" November to February), with low atmospheric temperatures (mean 23°C) and moderate precipitation (40–150 mm/month); (2) the dry season (March to May), with atmospheric temperatures around 36–38°C and low precipitation (0–30 mm/month); and (3) the rainy season (June to October), with atmospheric temperatures higher than 38°C and precipitation above 220 mm/month, even higher than 350 mm/month if hurricanes occur (Herrera-Silveira et al., 1998).

Cephalopods are highly sensitive to environmental variations, which directly impact their abundance and lifecycle (Pecl et al., 2004; Leporati et al., 2008; Pierce et al., 2008), both in natural populations (Cortéz et al., 1999), and under cultured conditions (Hanlon and Forsythe, 1985; DeRusha et al., 1987). Clarke et al. (1994) reported *Illex argentinus* biochemical composition associated to energy demands during growth and sexual maturation. Otero et al. (2007) observed that the weight of the digestive gland in *Octopus vulgaris* females increased proportionally to the maturity index. Likewise, De Moreno et al. (1998) found greater biochemical variations in female *Illex argentinus* probably associated with oogenesis.

Lipid energy reserves have an important role in the physiology of marine animals. They provide food supply for oocytes, ensuring the viability of the larvae (Giese, 1966; Giese et al., 1967). Several studies on this topic have been conducted in cephalopods. Rosa et al. (2002, 2004, 2005) studied changes in the biochemical composition of different tissues in several cephalopod species with diverse life and sexual maturation strategies. They found that the biochemical composition of the digestive gland and muscle is related to feeding, food availability, spawning and incubation. Nutritional status influences the time and intensity of the reproductive events (Sibly and Calow, 1986) and breeding frequently depletes animals energy reserves. Pollero and Iribarne (1988) studied the biochemical changes in different tissues during the reproductive cycle of *Octopus tehuelchus*. They reported that females undergo greater biochemical changes: lipid contents change primarily in gonadal tissue, increasing during sexual maturation and decreasing during the incubation period.

Reserves have a strong influence on sexual maturation, leading to nutrient mobilization and accumulation. A better understanding of the tissues biochemical composition is crucial to assess population health status and energy flow in ecosystems. Climatic seasons have been found to have a marked effect on tissues biochemical composition in other molluscan species and are associated with sexual development (Gámez-Meza et al., 1999; Berthelin et al., 2000; Orban et al., 2002). In this context, the present study aimed to characterize the biochemical composition of hemolymph, gonad, digestive gland, and muscle of wild *O. maya*, in order to assess seasonal variations and their relation to sexual maturation.

MATERIALS AND METHODS

Octopus Sampling

Between May 2007 and February 2008, wild adult organisms were captured in coastal areas of Sisal, Yucatan, Mexico (21°09′N; 90°01′W). A total of 52 wild adult organisms were analyzed in three different climatic seasons and were assigned based on local seasonality: (1) the winter season (February); (2) the dry season (May) and (3) the rainy season (September). Six sampling days were performed per season, and three captures were conducted at 2 days intervals. Organisms were caught using the "gareteo" technique that allows catching the organisms without harming them (Solís-Ramírez, 1988). The physicochemical parameters of water (pH, temperature, salinity, and dissolved oxygen) were recorded at every capture using a Van Dorn bottle and a multi-sensor (Hatch), at an average depth of 8 meters. On board, organisms were placed inside a black tank with 1000 L of sea water in constant replacement by using a submersible pump. Transportation from the capture areas to the laboratory facilities took less than 2 h. Organisms were settled in individual green tanks (45 cm in diameter, 60 cm in depth, with 80 L of seawater) in a closed area with controlled photoperiod (12:12 light-dark), with constant aeration system and daily seawater flow exchange equivalent to 300% (28 ± 1°C, 37 ± 1 PSU, pH 8.1 ± 0.2). Samples were obtained the next day and organisms were not fed to avoid influencing the indicators of nutritional and physiological status.

Tissues Samples and Plasma Analyses

Octopus were individually immersed in cold water (17°C below the maintenance system temperature) for 2 to 4 min

(Cruz-López, 2010; Roumbedakis et al., 2017). When the respiratory rate and locomotor activity decreased, the animals were removed, and hemolymph was drawn from the aorta with a sterile catheter-cold connected to a 5 mL Falcon tube (Cruz-López, 2010). Approximately 0.5 mL of the hemolymph were transferred into tubes for hemocyanin and hemocytes analysis, and then stored (2–8°C) for 4 h. The samples were centrifuged at 800 × g for 5 min at 4°C to separate plasma, which was then used to assess the metabolites concentration in the plasma. Glucose, cholesterol, and acylglycerides concentrations were determined using enzymatic/colorimetric methods with commercial kits: Glucose Bayer (Sera Pak Plus B014509-01); acylglycerides (Sera Pak Plus, B01455101) and cholesterol (Bayer B01 4507-01). A total of 10 μL of plasma were added to 200 μL of the appropriate enzyme reagent for each sample. Protein concentrations were determined by the method of Bradford (1976), plasma was previously diluted with sterile water (400×) and 10 μL were mixed with 200 μL of Bradford reagent (Bio-Rad laboratories Cat. 500-0006). Absorbance values of all metabolites were recorded with a microplate reader (Benchmark Plus Bio-Rad). All samples were analyzed in triplicate and concentrations of metabolites (mg mL^{-1}) were calculated using the standard curves.

The hemolymph was diluted into 1:100 using distilled water and measured at 335 nm (Thermo-Genesys 10 uv) and hemocyanin concentration (mM L^{-1}) was determined using an extinction coefficient of 17.26 (Chen and Cheng, 1993a,b). Total hemocytes were counted in a Neubauer chamber from the hemolymph aliquot fixed with 4% formaldehyde in Alsever solution (115 mM $C_6H_{12}O_6$, 30 mM $Na_3C_6H_5O_7$, 338 mM NaCl, 10 mM EDTA.Na_2, pH 7.0) with a 1:3 dilution (Roumbedakis et al., 2017). Samples were kept at 2–8°C, for a maximum period of 10 days before analysis. All samples were tested in duplicate and expressed as cells/mm^3.

Tissues Biochemical Composition

Immediately after obtaining hemolymph, the octopus were euthanized by cerebral incision (Boyle, 1976; Fiorito et al., 2015). The animals were then weighted and dissected; the hepatopancreas and gonad were removed and weighted separately to estimate their proportion in relation to total weight (TW): hepatosomatic (HSI) and gonadosomatic (GSI) indices were calculated as follows: HSI = (DGW/TW) × 100; GSI = (GW/TW) × 100. Subsequently, those tissues and muscle were cut for lipids and glycogen analyses. Tissue sections were frozen in liquid nitrogen and stored at −40°C until analysis. Subsample of muscle were used for caloric analysis; the muscle was dried in the oven at 60°C until constant weight, and then placed in a calorimetric pump (Parr instruments).

Total lipids were assessed according to Folch et al. (1957); digestive gland, gonad and muscle tissues (0.5 g) were homogenized with a Teflon tip in 20 ml of chloroform/methanol solution (2:1) at 5000 × *g* for 2 min. The mixture was agitated for 15 min in an orbital shaker at ambient temperature. The homogenized was filtered (Whatman paper No 42). Samples were washed with water-methanol solution and placed in a separation funnel until two layers were formed. The chloroform-lipid fold was recovered and placed in a desiccation hood at 60°C with an air flow to complete evaporation. Glycogen in the tissues (digestive gland, gonad, and muscle) was extracted in trichloroacetic acid and determined by a reaction with sulfuric acid and phenol (Dubois et al., 1965). Approximately 0.4 g of the tissue was macerated with 200 μL of trichloroacetic acid (5%) and centrifuged for 6 min at 5000 × *g* (Eppendorf microcentrifuge 5415). Supernatant (100 μL) was pipetted into a tube and mixed with five volumes of 95% ethanol. Tubes were placed in an oven at 37°C for 3 h. After precipitation, the tubes were centrifuged again, the supernatant was discarded, and the tubes were drained. Finally, 200 μL of 5% phenol and 1 mL of sulfuric acid was added and samples were shaken with a vortex; subsequently 200 μL were placed in a microplate and measured (490 nm) using a microplate reader (Benchmark Plus Bio-Rad). The total amount of lipids and glycogen were analyzed in triplicate and expressed as mg g^{-1} of tissue.

Statistical Analyses

A set of 14 descriptors measured in sexually mature *O. maya* were used to characterize changes in the biochemical composition and energy reserves of male and female octopus sampled in three different seasons (winter, dry, and rainy season). Descriptors included: hemocyanin concentration (Hemocyan, mM L^{-1}), total hemocyte count (Hemocytes, cells mm^{-3}), plasmatic proteins (Protein, mg mL^{-1}), glucose (Glucose, mg mL^{-1}), cholesterol (Cholest, mg mL^{-1}), acylglycerides (Acylglycer, mg mL^{-1}), lipids in gonads (LipidGonad, mg mL^{-1}), hepatopancreas (LipidHepat, mg mL^{-1}) and muscle (LipidMuscle, mg mL^{-1}), glycogen in hepatopancreas (GlucoHepat, mg mL^{-1}) and muscle (GlucoMuscle, mg mL^{-1}), and caloric content of muscle (CaloricCont, cal g^{-1}). The total wet weight, hepatopancreas and gonad weight (g) were obtained to calculate the hepatosomatic index (HSI,%) and the gonadosomatic index (GSI,%).

Non-metric multidimensional scaling was used to obtain configuration maps with reduced dimensions that would order and separate samples considering differences in all 14 descriptors simultaneously. This was achieved by calculating Gower's dissimilarity index between all pairs of samples (n = 154; Legendre and Legendre, 1998). A permutational MANOVA (Anderson, 2001) was then used to examine variations in these descriptors amongst octopus combining sex and season of collection. The underlying experimental design was a two-way model with sex (2 levels) and season (3 levels). Sampling stations (3 levels) were nested within each season. Octopus total weight was included in the model as a covariable, thus forcing the use of a Type I sum of squares for the partitioning of total variation. Octopus total weight was included in the model as a covariable, thus forcing the use of a Type I sum of squares for the partitioning of total variation. A maximum of 9,999 unrestricted permutations of raw data were used to obtain the empirical distribution of *pseudo-F* values (Anderson, 2001; McArdle and Anderson, 2001). Multivariate paired comparisons between centroids were applied following a similar procedure to calculate empirical *pseudo-t*

values. Statistical analyses were performed using PRIMER v6 plus PERMANOVA (Anderson et al., 2008).

RESULTS

The physicochemical characteristics of seawater at the bottom of capture sites were stable in salinity (35.4 to 36.4 PSU), pH (8.2 to 8.3), and dissolved oxygen (7.1 to 7.3 mg/L). The largest variation was recorded in seawater temperature from 25°C in the winter to 27°C in the dry season and 28°C in the rainy season (**Table 1**).

The ratio of female and male specimens caught was consistent with the reproductive dynamics of *O. maya*: during the rainy season the ratio was 1:1.1 and changed to 1:1.3 and 1:1.4 during the dry and winter storm seasons, reflecting a lower proportion of females due to parental care and/or death after hatching.

The 154 *O. maya* comprised 87 males and 67 females. Wet weight of specimens varied strongly among seasons (from 131 to 1023 g). Octopus with the lowest total weights were recorded in the rainy season (392 ± 20 g), followed by those collected in the winter (447 ± 24 g). The largest specimens were caught in the dry season (535 ± 23 g).

The 2-dimensional NMDS showed an effective separation of samples according to the season in which octopus were collected (**Figure 1**). The horizontal axis ordered samples with the highest values of hemocyanin and acylglycerids in right hand side corresponding to individuals collected during the rainy season (light and dark blue). These samples however, were low in hemocytes, cholesterol and GSI. By contrast, samples from individuals collected during the winter season were located in the lower left of the configuration map (light and dark green) and had high hemocyte count, glucose and glucose in hepatopancreas, but relatively low values of hemocyanin and acylglicerids. Octopus collected during the dry season (light and dark orange) were located in the upper half of the configuration map, corresponding to samples with high lipid content in muscle, but low glucose, glucose in hepatopancreas and HSI. Samples from female octopus were consistently more disperse than those from males (**Figure 1**). A stress value of 0.25 was obtained with the 2-D plot configuration, indicating a sufficiently adequate configuration that requires caution in graphical interpretation.

The permutational MANOVA (**Table 2**) showed that differences between the season in which octopus were collected contributed significantly to explain the variation in biochemical composition of the tissues sampled (*pseudo-F* = 7.3; $p < 0.001$). It also showed significant differences in biochemical descriptors between male and female octopus (*pseudo-F* = 6.1; $p < 0.05$). However, the non-significant interaction term indicated that the biochemical composition changed with the seasons in a similar way regardless of sex (*pseudo-F* = 1.3; $p = 0.279$). Further multivariate pairwise comparisons revealed significant differences between all three seasons (**Table 3**), thereby statistically confirming the graphical separation of samples previously described. The analysis also showed a significant contribution of total weight as a covariate in the model (*pseudo-F* = 8.6; $p < 0.001$). This result indicates there was considerable variation in the biochemical descriptors that can be attributed to octopus biomass, and suggests that total weight should be included in the analyses in order to unmistakably distinguish changes amongst seasons. Differences between sampling stations nested within each season were also significant (*pseudo-F* = 5.4; $p < 0.001$), but had no influence on the main trends detected. **Table 4** shows the hemocytes concentration and biochemical descriptors of tissues (hemolymph, hepatopancreas, gonads, and muscle) of *O. maya* caught in three seasons (winter, dry, and rainy season).

TABLE 1 | Temperature, salinity, dissolved oxygen and pH of seawater samples taken at the collecting sites (8 m depth) of *Octopus maya* in each season.

Month	Winter, February	Dry, May	Rainy, September
Temperature, °C	25 ± 0.5	27 ± 0.6	28 ± 0.7
Salinity, PSU	36.4 ± 0.1	35.3 ± 0.1	36.3 ± 0.1
Dissolved oxygen, mg mL^{-1}	7.08 ± 0.1	7.21 ± 0.1	7.30 ± 0.1
pH	8.2 ± 0.1	8.26 ± 0.1	8.24 ± 0.1

Values are mean ± SE.

DISCUSSION

The effects of climatic season and sex on the biochemical composition of *O. maya* were assessed for the first time. The gonadosomatic index (GSI) has been widely used to estimate the stage of sexual maturity and to determine the reproductive dynamics in cephalopods belonging to the family Octopodidae (Mangold, 1983). The highest GSI values were recorded in organisms caught in the dry and winter seasons, which is consistent with the reproductive period. According to the gonad maturation stages and to variations in progesterone and testosterone levels in the gonad of *O. maya,* the reproductive season occurred from February to June (Avila-Poveda et al., 2015). GSI in *O. maya* increased progressively concurring with the reproductive period. The same pattern was reported by Zamora and Olivares (2004) in female *Octopus mimus,* De Moreno et al. (1998) in *I. argentinus*, and Otero et al. (2007) in *O. vulgaris*. GSI in *O. maya* males varied less between seasons (0.75, 0.72, and 0.56% in winter, dry and rainy season respectively), indicating reproductive readiness from 160 to 1000 g. Considering that its lifecycle is short and dies after breeding, male maturity throughout the year represents an ecological advantage allowing more opportunities for reproduction. This behavior has also been reported in *O. mimus* males (Ishiyama et al., 1999) and in *O. vulgaris* in the Mediterranean Sea and in the eastern Atlantic Ocean (Mangold, 1983).

Environmental factors have a strong impact on animal reproduction; among these, light has been signaled out as the main factor involved in the reproductive activities of different species (Zúñiga-Romero et al., 1995). *O. vulgaris* size and the age at sexual maturity seems to depend primarily on light, temperature, and feeding (Mangold, 1983; Forsythe, 1993). Reserves have a strong influence on sexual maturation, leading to nutrient mobilization and accumulation. The present results showed a distinct seasonal variation in the biochemical

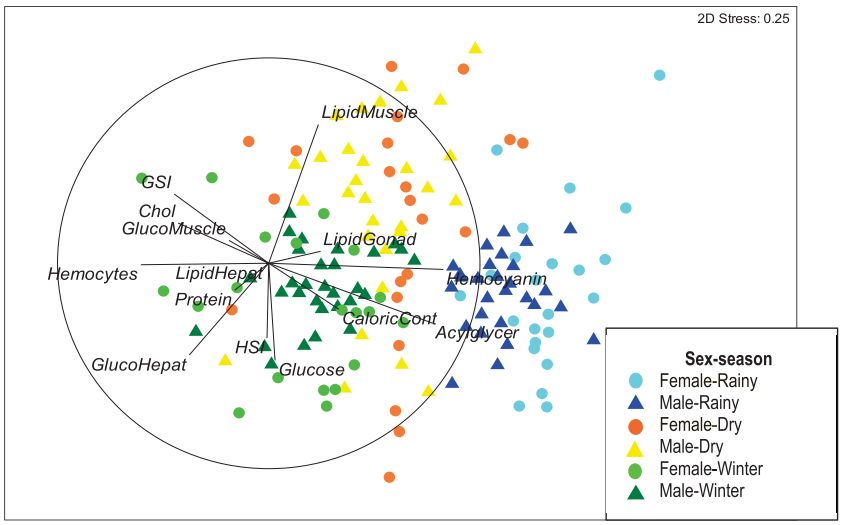

FIGURE 1 | Non-metric Multidimensional Scaling (NMDS) of 14 descriptors biochemical composition: hemolymph (hemocyanin, hemocyte concentration, total protein, glucose, cholesterol and acylglycerides), gonads, hepatopancreas and muscle (glycogen concentration and total lipids), caloric value of muscle, hepatosomatic index (HSI) and gonadosomatic index (GSI) in *Octopus maya* caught in three seasons (winter, dry, and rainy season).

composition of *O. maya* tissues. The digestive gland stored more lipid and glycogen than the gonad and muscle. This has also been reported in other cephalopods (Boucaud-Camou and Yim, 1980; Rosa et al., 2004, 2005; Sieiro et al., 2006). Lipid concentration in the digestive gland could be required for production and maturation of gametes, as well as during the egg-caring period, when females stop feeding. A recent research in post-spawning *O. maya* females reported the loss of 40% in wet weight during the incubation period, with higher weight loss in the gonad and hepatopancreas (Roumbedakis et al., 2017).

During the winter storm and the dry seasons, *O. maya* gonadal glycogen increased, possibly associated with the organisms' response to the high demand of polysaccharides for the metabolic processes at the final phase of oogenesis and during the eggs caring period, which would be consistent with the species functional maturity. Carbohydrates are intermediary metabolic precursors for energy and non-essential amino acid production, and are components of ovary pigments (Harrison, 1990). These findings were also reported in *O. vulgaris* gonads (Rosa et al., 2004). The percentage of reproductively mature females and males is higher in the winter storm season than in the dry and rainy seasons, when the sea-surface temperature oscillates around 25°C (Angeles-González et al., 2017).

Higher values of lipid in muscle were recorded in the organisms caught in the dry season, which were the largest organisms captured, and therefore size could be associated with an increase in food intake to comply with the metabolic demands of growth. Similar results were reported in male and female *O. tehuelchus* muscle at intermediate maturation developmental stage (Pollero and Iribarne, 1988). These results are consistent with those reported by Rosa et al. (2005), for the muscles of the squids *Illex coindetii* and *Todaropsis eblanae* during the reproductive period.

TABLE 2 | Results of the permutational MANOVA applied to hemocyte concentration and the biochemical composition of tissues (hemolymph, hepatopancreas, gonads, and muscle) of *Octopus maya* caught in three seasons (winter, dry and rainy season).

Source	df	SS	MS	*Pseudo -F*	*P* (perm)	Unique permutations
Total weight	1	2375	2375	8.638	0.0001	9932
Season	2	10122	5061	7.265	0.0001	9788
Sex	1	1298	1298	6.086	0.0106	9948
Sampling (Season)	6	4259	709	5.374	0.0001	9850
Season x Sex	2	458	229	1.339	0.2797	9941
Sex x Sampling (Season)	6	1009	168	1.273	0.1167	9877
Residuals	133	17568	132			
Total	150	37091				

Degrees of freedom (df); sum of squares (SS); Mean square (MS); pseudo-F value obtained with a permutational MANOVA; P (perm) proportion of F values smaller than the pseudo-F value obtained with permutations; number of unique permutations used to calculate previous values.

TABLE 3 | Multivariate pairwise comparisons amongst centroids calculated based on hemocyte concentration and biochemical descriptors measured in the tissues (hemolymph, hepatopancreas, gonads, and muscle) of *Octopus maya* collected in three seasons (winter, dry, and rainy season).

Pairwise comparisons	*pseudo-F*	*P* (perm)	Unique permutations
Dry *vs.* Rainy	2.8	<0.001	720
Dry *vs.* Winter	1.8	<0.01	719
Rainy *vs.* Winter	3.5	<0.001	718

pseudo-F value obtained with a permutational MANOVA; P (perm) proportion of F values smaller than the pseudo-F value obtained with permutations; number of unique permutations used to calculate previous values.

TABLE 4 | Hemocytes concentration and biochemical composition (mean ± standard error) of tissues (hemolymph, hepatopancreas, gonads, and muscle) of *Octopus maya* caught in three seasons (winter, dry, and rainy season).

	Winter		Dry		Rainy	
	Females	**Males**	**Females**	**Males**	**Females**	**Males**
Number	21	30	22	29	24	28
Total wet weight, g	438 ± 43	473 ± 34	511 ± 29	557 ± 34	360 ± 27	407 ± 30
Hepatosomatic index (HSI,%)	3.71 ± 0.21	2.59 ± 0.12	2.58 ± 1.14	2.12 ± 0.09	2.97 ± 0.09	2.91 ± 0.08
Gonadosomatic index (GSI,%)	1.70 ± 0.57	0.75 ± 0.05	1.39 ± 0.43	0.72 ± 0.21	0.35 ± 0.17	0.56 ± 0.05
Hemocyanin, mM L^{-1}	1.69 ± 0.06	1.80 ± 0.06	2.03 ± 0.05	2.06 ± 0.05	2.73 ± 0.05	2.69 ± 0.05
Hemocytes, cells mm^{-3}	10567 ± 2072	18329 ± 2203	12501 ± 2088	18289 ± 2165	7030 ± 1201	8404 ± 1615
Proteins, mg mL^{-1}	141 ± 11	152 ± 7	111 ± 14	141 ± 10	119 ± 9	101 ± 15
Cholesterol, mg mL^{-1}	0.025 ± 0.001	0.026 ± 0.001	0.050 ± 0.005	0.048 ± 0.005	0.014 ± 0.001	0.016 ± 0.003
Glucose, mg mL^{-1}	0.122 ± 0.01	0.125 ± 0.01	0.119 ± 0.01	0.106 ± 0.01	0.115 ± 0.01	0.114 ± 0.01
Acylglycerides, mg mL^{-1}	0.035 ± 0.001	0.037 ± 0.001	0.036 ± 0.005	0.033 ± 0.003	0.080 ± 0.003	0.082 ± 0.001
Lipids in hepatopancreas, mg mL^{-1}	42.99 ± 2.77	42.61 ± 1.76	37.84 ± 1.37	42.37 ± 2.08	40.97 ± 2.45	43.17 ± 1.94
Lipids in gonads, mg mL^{-1}	24.03 ± 1.81	20.87 ± 1.16	32.60 ± 2.70	29.48 ± 2.11	32.21 ± 1.71	22.86 ± 0.95
Lipids in muscle, mg mL^{-1}	5.66 ± 0.41	5.33 ± 0.35	9.56 ± 0.73	9.71 ± 0.66	8.50 ± 0.73	7.00 ± 0.37
Glycogen in hepatopancreas, mg mL^{-1}	4.30 ± 0.40	4.04 ± 0.25	4.07 ± 0.43	3.44 ± 0.34	3.49 ± 0.23	3.22 ± 0.12
Glycogen in gonads, mg mL^{-1}	3.42 ± 0.50	0.77 ± 0.08	2.98 ± 0.66	1.87 ± 0.62	1.42 ± 0.48	0.58 ± 0.17
Glycogen in muscle, mg mL^{-1}	0.41 ± 0.08	0.25 ± 0.02	0.28 ± 0.02	0.25 ± 0.01	0.27 ± 0.03	0.25 ± 0.02
Caloric content muscle, g^{-1}cal	4373 ± 124	4421 ± 73	4653 ± 88	4355 ± 51	4566 ± 51	4601 ± 48

The caloric value of muscle in both sexes increased during the rainy season, which could reflect a higher intake and/or provision of food prior to the reproductive period. In the winter season the caloric value decreased, possibly associated to the reproductive effort. Sexual maturation and breeding are the most energy-intensive investment periods of cephalopods lifecycle (Rosa et al., 2004). During the periods of catabolism and high energy demand, tissue proteins are mobilized to provide energy (Castro et al., 1993; Jackson and Mladenov, 1994).

Although the biochemical composition of the digestive gland, gonad, and muscle has been well studied in cephalopods, analysis of hemolymph components is still poorly known. This work provides a more comprehensive approach on the seasonal dynamic of cephalopods reserves mobilization and use. *O. maya* hemocyanin concentration has distinct seasonal variations, decreasing during the winter and dry seasons when reproduction takes place. Cholesterol is an important precursor of vitamin D and steroid hormones, a structural component of cellular membranes and precursor of sexual hormones involved in reproductive control (Kanazawa, 2001). The present study found that cholesterol concentration in hemolymph was higher in the largest organisms caught in the winter and dry seasons, coincident with the main reproductive period (Avila-Poveda et al., 2015; Markaida et al., 2017). Heras and Pollero (1989) also reported cholesterol variations in plasma associated with *O. tehuelchus* sexual maturation. Cephalopods are known to have a low capacity to synthetize sterols (Voogt, 1973; Goad, 1978), therefore cholesterol comes mainly from the diet (Rosa et al., 2004).

In crustaceans, hemocyanin is used as energy substrate and amino acid source in situations of high energy demand and starvation periods (Rosas et al., 2002; Pascual et al., 2003). In cephalopods, hemocyanin is probably involved as a source of metabolic energy. The present results indicate that hemocyanin could be associated with protein transfer for sexual maturation, which concurs with the highest values of GSI. Protein concentration in plasma did not change with seasons, but displayed an opposite pattern to hemocyanin concentration, which could reflect the unfolding of the hemocyanin molecule and release of proteins. These proteins could be used as energy substrate and as source of amino acids for gametes production. They are also structural components of tissues and can be used as reserves at the final development stages.

The decrease in *O. maya* plasma acylglycerides concentration at the pre-reproductive period may reflect lipid mobilization, transported by hemolymph primarily to gonadal tissue. Hemocyanin, a lipoprotein, has an important role in lipid transport (Heras and Pollero, 1990). Heras and Pollero (1989) found plasma acylglycerides variations associated with sexual maturation of *O. tehuelchus*. They also determined the lipid and fatty acid composition of hemocytes and plasma at different sexual development stages. These authors reported that the highest concentrations of lipids linked to eggs development were constituted by esters, triacylglycerols and diacylglycerols. Heras and Pollero (1990, 1992), detected the presence of three lipoproteins that transport mainly cholesterol and phospholipids.

The pattern observed in metabolites and reserves indicates a seasonal variation associated with the growth of the organisms and the reproductive peak, but it may also reflect the physiological response to seawater temperature. Studies on reproductive conditions of *O. maya* suggest that variations in population parameters could be linked to the geographic distribution of thermal zones (Angeles-González et al., 2017). Low values of hemocyanin, acylglycerides, lipids, and glycogen concentrations could possibly reflect the metabolic cost

of being exposed to high temperatures. High temperatures (28 to 31°C) have been demonstrated to affect *O. maya* reproductive capability by inhibiting spawning (Juárez et al., 2015), compromising male maturation (López-Galindo et al., 2018), health status (Pascual et al., 2019) and affecting the viability of eggs and progeny growth (Sánchez-García et al., 2017). Further studies on the physiological condition of wild organisms are required to better understand the relation between nutritional status and environmental variations. Improving our knowledge on cephalopods response to temperature is highly relevant in the wake of a global warming scenario. Monitoring the environmental conditions in ecosystems with important commercial species to anticipate relevant changes has been widely recommended. This is particularly important in thermosensitive species narrowly distributed, such as *O. maya*.

The present study complements the baseline information on this species. It provides reference values of several physiological indicators for *O. maya* sub-adult and adult organisms that may be useful for monitoring programs of wild populations, as well as to design diets and management protocols to produce octopus under controlled conditions. Nowadays there is a huge interest in establishing cephalopods aquaculture and a better understanding of nutrient use and mobilization is relevant to advance knowledge on energy demand during sexual maturation and reproductive activity.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The Mexican oficial norm (NOM-062-ZOO-1999) on the technical specifications for the production, use and care of laboratory animals does not include marine invertebrates, and regulations on the matter are scarce. We followed the Guide for the Care and Use of Experimental Animals in Research and Teaching of the National Aotonomous University of Mexico.

AUTHOR CONTRIBUTIONS

CP, CR, MM, and PG conceived and designed the study. CP, HC-L, and AS conducted the experimental procedures. MM and CP analyzed and interpreted the data. MM, CP, HC-L, PG, and PD wrote the original draft.

FUNDING

We appreciate the financial support granted by the Dirección General de Asuntos del Personal Académico of UNAM through projects IN229819, IN204019, and IT20111.

ACKNOWLEDGMENTS

We specially thank Vianey Sosa, Richard Mena, Claudia Caamal, and Karla Escalante for their valuable technical assistance and our colleagues from the Universidad Nacional Autónoma de México (UNAM) for their collaboration in many aspects of this work.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 20 May 2020
doi: 10.3389/fphys.2020.00501

Antagonistic Interactions and Clutch-Dependent Sensitivity Induce Variable Responses to Ocean Acidification and Warming in Squid (*Doryteuthis pealeii*) Embryos and Paralarvae

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OPEN ACCESS

Edited by:
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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *27 June 2019*
Accepted: *23 April 2020*
Published: *20 May 2020*

Citation:
Zakroff CJ and Mooney TA (2020) Antagonistic Interactions and Clutch-Dependent Sensitivity Induce Variable Responses to Ocean Acidification and Warming in Squid (Doryteuthis pealeii) Embryos and Paralarvae. Front. Physiol. 11:501. doi: 10.3389/fphys.2020.00501

Ocean acidification (OA) and warming seas are significant concerns for coastal systems and species. The Atlantic longfin squid, *Doryteuthis pealeii*, a core component of the Northwest Atlantic trophic web, has demonstrated impacts, such as reduced growth and delayed development, under high chronic exposure to acidification (2200 ppm), but the combined effects of OA and warming have not been explored in this species. In this study, *D. pealeii* egg capsules were reared under a combination of several acidification levels (400, 2200, and 3500 ppm) and temperatures (20 and 27°C). Hatchlings were measured for a range of metrics [dorsal mantle length (DML), yolk sac volume (YV), malformation, and hatching success] in three trials over the 2016 breeding season (May – October). Although notable resistance to stressors was seen, highlighting variability within and between clutches, reduced DML and malformation of the embryos occurred at the highest OA exposure. Surprisingly, increased temperatures did not appear to exacerbate OA impacts, although responses were variable. Time to hatching, which increased with acidification, decreased much more drastically under warming and, further, decreased or removed delays caused by acidification. Hatching success, while variable by clutch, showed consistent patterns of greater late stage loss of embryos under acidification and greater early stage loss under warming, highlighting the potential difference in timing between these stressors for this system, i.e., that acidification stress builds up and causes impacts over time within the egg capsule as the embryos grow and respire. High OA-exposed hatchlings from the warmer conditions often showed reduced impacts compared to those reared in ambient temperatures. This may be due to the increased developmental rate and subsequently reduced OA exposure time of embryos in the higher temperature treatment. These results indicate a substantive potential plasticity to multiple stressors during the embryonic development of this species of squid, but do not predict how this species would fare under these future ocean scenarios.

Keywords: cephalopod, hypercapnia, Myopsida, temperature, stress, multifactor, malformation

INTRODUCTION

Coastal ecosystems are seeing levels of acidification and warming today that are not predicted for the open ocean for hundreds of years (Pachauri and Meyer, 2014). OA can be enhanced in coastal regions due to increased pH variability from freshwater influx, urbanization, and pollution (Gledhill et al., 2015). Rapid coastal warming, particularly in ocean warming hotspots like the northwest Atlantic and the Gulf of Maine region, is already causing substantive impacts to vital marine services and valuable fisheries (Hobday and Pecl, 2014; Pershing et al., 2015; Saba et al., 2016). The complexity of this scenario is further compounded by the potential for interactive effects, whether additive, synergistic, or antagonistic, between these stressors for a range of potentially sensitive organisms and processes (Crain et al., 2008; Kroeker et al., 2013; Breitburg et al., 2015). Further, it is becoming clear that while data in global change is limited and generalizations are to some degree necessary, many organismal responses to multistressor scenarios are context specific to population and/or region (Kroeker et al., 2017). Life stage specific responses, for example, particularly for early development and dispersal where sensitivities are often thought to be highest, are foundational to understanding how populations may be impacted under multistressor scenarios (Byrne, 2011; Haigh et al., 2015).

Coastal squids, the Myopsid squids, are a fundamental component of shelf and nearshore food webs, and the longfin inshore squid, *Doryteuthis pealeii*, serves this role along the northwest Atlantic shelf system (Jacobson, 2005). These squid also support a valuable fishery in New England, with landings of 11,000 mt in 2018 (NOAA, 2019). These benthopelagic squid overwinter in the deeper, warmer waters of the shelf, but from May to October they aggregate in the shallow nearshore along the northwest Atlantic coastline to breed (Jacobson, 2005). They lay their eggs in mucous-bound capsules (tens to hundreds of eggs per capsule), which are tied to benthic structures or the substrate, often in large masses consisting of many capsules (Shashar and Hanlon, 2013). These eggs are stationary and must develop under whatever environmental conditions and variability they are exposed. Observers have recorded eggs laid at depths of up to 50 m, in temperatures of 10–23°C, and salinities of 30–32, which contextualizes a presumed preferred laying habitat for this species (McMahon and Summers, 1971; Jacobson, 2005). Less is known about the preferences for pH of egg laying habitat for this species, but pH_t calculated from shelf carbonate system profiles throughout their habitat range indicate a typical exposure range of 7.88–8.2 (600–250 ppm pCO_2) during the breeding season (Wang et al., 2013; Zakroff et al., 2019). Navarro et al. (2018) reported preferred egg laying habitat for the California market squid, *Doryteuthis opalescens*, as requiring pH_t greater than 7.8 and O_2 concentrations greater than 160 μmol with no apparent temperature limitation within the region. These static ranges do not account for the possible variability of temperature and carbonate system measures across temporal scales, as have been observed in this nearshore system, but at least provide a framework for ideal tolerance windows (Hong et al., 2009; Connolly and Lentz, 2014).

Developing embryos are often thought to be particularly sensitive to added stressors because they have limited available energy stores and are actively building the machinery needed to maintain homeostasis (Steer et al., 2004; Hu et al., 2010). This paradigm is challenged, however, by the inherent need for coastal embryos to cope with a highly variable environment and the potential plasticity of an embryo given active and adaptive developmental pathways (Hamdoun and Epel, 2007). The question, then, becomes one of the limits of resilience and plasticity during embryonic development, and when human-driven global ocean change will push systems past those limits.

A growing body of research has examined the impacts of various stressors on the early life stages of Myopsid squid. Egg capsules of *D. opalescens* reared under acidification (pH 7.57, $pCO_2 \sim 1440$ ppm) and hypoxia (80 μM O_2) showed delays to development, potential reductions in YV, and decreases in statolith size relative to the embryo (Navarro et al., 2016). Pimentel et al. (2012) exposed egg capsules of the European market squid, *Loligo vulgaris*, to warming (+2°C above regional seasonal averages) and described a 28-fold increase in oxygen consumption during embryogenesis, resulting in rapid depletion of available oxygen and causing metabolic suppression in late stage embryos. Further work with individualized *L. vulgaris* embryos (embryos removed from the protections of the egg capsule) under +2°C warming resulted in metabolic suppression that encouraged premature hatching, with an increase in malformations observed among the hatched paralarvae (Rosa et al., 2012). This study also noted that warming-exposed paralarvae, as opposed to encapsulated embryos, activated an integrated stress response of heat shock proteins and antioxidant enzymes, suggesting the transition to planktonic life could come with the addition of a more robust stress response toolbox (Rosa et al., 2012; Robin et al., 2014). Few studies of warming and acidification have been performed with Myopsid squid embryos. Rosa et al. (2014) exposed seasonal clutches of individualized *L. vulgaris* embryos to the combined effects of warming and acidification (+2°C and $pCO_2 \sim 1650$ ppm) and observed decreased hatching success (47%) and increased premature hatching and abnormalities, most strongly within the summer clutch. They also observed delays in development time and decreases in oxygen consumption under acidification that were antagonistic to the increases in these rates caused by warming. The effects of individualization, removing squid eggs from the environmental protections imbued by the egg capsule during embryonic development, have not been determined.

Despite their ecological and economic importance, we know little regarding the interactive effects of OA and temperature on *D. pealeii*. Studies with this species have only focused on acidification. Kaplan et al. (2013); experiments ran in 2011 reared *D. pealeii* egg capsules under a low, facility ambient (550 ppm), and high acidification (2200 ppm), observing development delays of about 1 day, a decrease in DML of the hatchling paralarvae, and decreased size and quality of the statoliths. Subsequently, Zakroff et al. (2019; experiments ran in 2013) expanded upon

Abbreviations: DML, dorsal mantle length; ESL, Environmental Systems Laboratory; KW, Kruskal–Wallis test; LR, linear regression; MBL, Marine Biological Laboratories; OA, ocean acidification; YV, yolk sac volume.

this preliminary work, exposing *D. pealeii* egg capsules to a range of pCO_2 levels (from 400 to 2200 ppm), noting delays in development time, decreases in DML, and smaller, rougher statoliths that indicated a potential dose response threshold of 1300 ppm. In addition, this study highlighted notable variability in response intensity across the breeding season, demonstrating potentially different physiological strategies or responses to acidification stress within the egg capsules that expressed as different levels of sensitivity or resistance across the season (Zakroff et al., 2019). Behavioral experiments run in parallel to and following Zakroff et al. (2019) showed full years where paralarvae showed little to no response to acidification even up to the original 2200 ppm level, suggesting greater levels of resilience than had been expected (Zakroff et al., 2018; experiments ran from 2013 to 2015).

In this study, we examined the extent of stress resistance and response variability in developing *D. pealeii* embryos by both increasing the acidification exposure (2200 ppm was considered a variable response, while 3500 ppm was used as a positive control) and by adding warming (+2°C above peak breeding season temperature for Vineyard Sound, MA, United States) as a potentially compounding stressor. Metrics analyzed are the same as those in Zakroff et al. (2019) except that proportions of malformation in the paralarvae were added based on their prevalence in work by Rosa et al. (2012, 2014) and statolith data are not included to maintain concision in this manuscript. Further, unlike most of the previously cited work, which used wild collected or lab produced egg capsules most likely sourced from multiple parents, egg capsule maternity was monitored, allowing for an examination of both between and within clutch variability of stress responses.

MATERIALS AND METHODS

Experiments were performed between May and October of 2016 at the Environmental Systems Laboratory (ESL) of the Woods Hole Oceanographic Institution, Woods Hole, MA, United States during peak breeding season of *Doryteuthis pealeii* for this region (Arnold et al., 1974; Jacobson, 2005). Methods presented throughout are similar to those reported in detail in Zakroff et al. (2019) so will be reiterated in brief, but with differences noted.

Squid Capture and Care

Squid were acquired from the Marine Biological Laboratory (MBL) Marine Resources Center from trawls performed at 10–30 m depth in Vineyard Sound at the Menemsha Bight of Martha's Vineyard. Squid were either selected on ship or following offloading from the ship, but prior to deposition into holding aquaria at the MBL. Females of 15–25 cm DML that exhibited the least signs of stress (calmly resting at bottom or gently hovering with no damage or lesions to fins or skin) and had bright orange accessory nidamental glands were selected. Per trip, three adult females were hand-selected from the catch and each carefully placed into their own seawater filled cooler. The squid were then driven quickly and gently to the ESL. Each female squid was gently transferred from the coolers into one of three flow-through round tanks (120 cm in diameter, 70 cm depth). Overall, time from capture to introduction to the tanks at the ESL was less than 6 h from capture to tank.

Each female had her own tank with no males or other squid present. These tanks were fed by Vineyard Sound seawater that had been sand-filtered and cooled to 15°C (Salinity = 32, pH_{nbs} = 7.96). As noted in Zakroff et al. (2019) this temperature occurs during the breeding season (9.60–25.40°C from May to October 2016, Station BZBM3, US DOC/NOAA/NWS/NDBC > National Data Buoy Center), but is typically lower than the ambient mean (19.57°C from May to October 2016, Station BZBM3, US DOC/NOAA/NWS/NDBC > National Data Buoy Center), and was used to avoid increased damage and stress due to increased metabolism and activity under higher temperatures. Each aquarium had a ca. 2 cm thick layer of sand at the bottom, was continuously bubbled with air, and was covered throughout the day to avoid startling. Each tank included false egg capsules, comprised of the inflated fingers of seawater soaked and cleaned nitrile gloves zip-tied to a weight, to encourage egg-laying on a clean surface away from the substrate or air hose. Squid were fed once per day with local killifish, *Fundulus heteroclitus*. All female squid were fed and maintained in the ESL until they died following egg laying.

New female squid were brought in for each trial. Female squid typically laid egg capsules after 2 days of capture, producing small mucous-bound masses of around 2–30 egg capsules, with each capsule containing 80–300 eggs. These female squid fertilized eggs with stored sperm from breeding that occurred prior to capture, so paternity was unknown (and potentially complex; Buresch et al., 2001), but maternity of all eggs was known. Tanks were checked for eggs each morning. If eggs were present in any single tank, they were immediately hand-transferred into a clean 5-gallon bucket of 15°C, filtered seawater and taken to the room containing the egg culturing system. Only egg capsules of high quality (orange-tinted, thin, and oblong fingers with no notable air pockets or other damages) were selected and randomly hand sorted into the cups of the experimental system to initiate a trial (described below). Only one trial was run at a time, so the eggs used were always from a single female/tank, and had been laid the night prior to discovery and introduction to the system. No additional eggs, laid by the same squid or other females of the same catch, were collected for any trial of the experiments described here.

Squid Egg Culture System: Acidification and Warming

Details of the culture and acidification system are the same as those reported in Zakroff et al. (2019). In brief, 15°C, 10 μm filtered, and UV-treated Vineyard Sound water was fed into an air-bubbled header tank, which gravity-fed three H-shaped PVC equilibration chambers that each contained four airstones (two per leg) bubbling with gas mixtures for each acidification treatment line (400, 2200, and 3500 ppm CO_2). Treatment gases were not tested with a CO_2 analyzer as they were in Zakroff et al. (2019) because treatments exceeded the range of the meter. Water in the ESL increases in pCO_2 compared to

environmental ambient, from 400 ppm to about 550 ppm, so the water in the 400 ppm control was first degassed with N_2 in one H-chamber and then re-equilibrated with ambient air in two additional, subsequent chambers. Given the several stages of storage and filtration input water goes through as it enters both the ESL and the acidification system, it is presumed that it is not subject to small-scale environmental variability due to mixing and integration of water over time. No such variability was noted in temperature, salinity, or pH, but moderate variability in alkalinity was observed within and between trials (**Table 1**).

Acidified water left the equilibration chambers and entered PVC manifolds and was carried by drip lines to the individual experimental culture cups (pre-soaked, 1-L PET containers, Solo Foodservice, Lake Forest, IL, United States; 5 cups per treatment * 3 treatments per water bath = 15 cups per water bath). Each cup was sealed with a lid through which a drip line was fed to the bottom of the cup. A bubbling line of the appropriate gas concentration was also fed through the lid to about half way up the cup and aligned underneath the outflow window, so as to not disturb the egg capsule during development, but push hatched paralarvae away from the outflow screen. Water outflowed through a 5 μm mesh window in the treatment cups into the surrounding water bath, which then outflowed to the drain.

Each water bath was maintained at 15°C by an aquarium chiller (Oceanic Aquarium Chiller 1/10hp, Oceanic Systems, Walnut Creek, CA, United States) and heaters (JÄGER 3603, EHEIM GmbH and Co., Deizisau, DE) to match the maternal holding tanks until introduction of eggs for a trial. Although in Zakroff et al. (2019) temperature acclimation between holding tanks and experimental tanks was not performed, as transfer from 15 to 20°C showed no impact to the eggs, the shock from 15 to 27°C was highly impactful to egg capsule survival in preliminary experiments and so methods were changed to acclimate eggs slowly to temperature. Upon introduction of eggs, water baths were increased in temperature 1 degree every 2 h until desired treatments temperatures were reached: 20°C, the average seasonal temperature, and 27°C, two degrees above peak temperature (25°C) in Vineyard Sound from May to October (2011–2016, Station BZBM3, US DOC/NOAA/NWS/NDBC > National Data Buoy Center). Water bath temperatures were swapped, the 20°C bath changed to 27°C and vice versa, between trials in order to reduce any potential impact of water bath or position in the room on the temperature treatments. Each water bath was monitored with a HOBO data logger (HOBO pendant model UA-004-64, Onset Data Loggers, Bourne, MA, United States), which recorded temperature and ambient light every 15 min. The culture room used ceiling mounted fluorescent lighting, which was set to a 14:10 light:dark photoperiod (broadly that of the natural system during this time).

An Experimental Trial: Egg Rearing and Monitoring

Trials were initiated by the presence of eggs in one of the maternal holding tanks and were demarcated by lay date (June 19, July 28, and September 14). Egg capsules were randomly sorted by hand: one capsule each into four out of the five cups in each treatment (with the last cup acting as an abiotic control for monitoring of seawater chemistry). A "full" trial would therefore be comprised of 12 egg capsules per water bath (four capsules, one per cup, in each of the three acidification treatments), requiring 24 capsules for the two temperature treatments/water baths. This "full" number of egg capsules was not always reached in each trial, so egg capsules were sorted to prioritize each treatment having as many treatment replicates as possible (see number of egg capsules in **Table 1**).

Following the introduction of eggs and temperature acclimation, water samples were taken from every cup for carbonate system measurements. These methods mirror exactly those described in Zakroff et al. (2019) except that salinity was no longer taken with bottle samples and was instead measured using a salinity probe (Orion Star^{TM} A329, Thermo Fisher Scientific Inc., Waltham, MA, United States). Data from spectrophotometric pH_t, alkalinity, salinity, and temperature were input into CO2SYS (Pierrot et al., 2006), calculated with dissociation constants from Mehrbach et al. (1973) and sulfate constants from Dickson (1990) to produce pCO_2 values for the seawater treatments (**Table 1**). After a trial's initiation, these measurements were performed weekly on the abiotic control cup (twice more, usually). The pH_{nbs} of all cups was measured every 3 days using a three-point standard calibrated pH probe (Orion Star^{TM} A329, Thermo Fisher Scientific Inc., Waltham, MA, United States). These pH measurements were used primarily to monitor the stability of the pH in the system and ensure pH of the biotic cups did not vary notably from the abiotic controls.

Egg capsules were left to develop undisturbed, with particular care taken during chemical monitoring, within the treatment system. Cups were checked daily to observe development and check for hatchlings. Under ambient pCO_2, hatching typically initiated after 13–15 days in the 20°C temperature control, and 8–10 days in the 27°C warming treatment. Each hatching day, all paralarvae were removed, counted, and subsampled for the various measurements described below. All the paralarvae that were not subsampled for analysis were anesthetized with 7.5% w/v $MgCl_2$ mixed with equal part seawater and preserved in 70% ethanol in microcentrifuge tubes (0.65 mL and 1.7 mL Costar microcentrifuge tubes, Corning, Inc., Corning, NY, United States). Handling and preservation of subsampled paralarvae is described below. No hatched squid remained in the cups across days, so all paralarvae included in the data are from their day of hatching (less than 1 day old).

Water Quality

Water chemistry, particularly temperature, salinity, and pH_t, were quite stable within and between experiments (**Table 1**). Seawater alkalinity varied the most in this system, which may have contributed to the variability of the pCO_2 equilibrations. Within a treatment, pH_t and calculated pCO_2 were consistent across cups (KW, $p > 0.05$ for all treatments in all trials). Input gas mixtures were the same across trials, but resultant pCO_2 equilibrations were variable between temperature treatments and across trials (**Table 1**). This variability was most likely due to the flow-through nature of the system and fluctuations in seawater input flow rates, although it may also represent

TABLE 1 | Seawater chemistry, maternal wet weight, number of egg capsules, and number of paralarvae subsampled for each treatment of each trial.

Laying date	Mother wet weight (g)	Temp (°C)	Treatment pCO_2 (ppm)	# Egg capsules	pH_{total}	Salinity	A_T (mmol $kgSW^{-1}$)	Ω_{Arag}	$pC0_2$ (ppm)	n	
										DML	YV
June 19	44.5	20.86	400	3	7.98 (0.02)	32.82 (0.16)	2222.6 (45.6)	2.44 (0.12)	474.96 (17.7)	94	96
			2200	3	7.41 (0.02)	32.84 (0.19)	2236.6 (37.1)	0.76 (0.04)	2005.07 (53.2)	59	80
			3500	2	7.25 (0.02)	32.87 (0.17)	2201.2 (60.1)	0.53 (0.04)	2922.38 (133.7)	89	30
		27.04	400	3	7.93 (0.04)	32.94 (0.14)	2172.1 (16.3)	2.72 (0.19)	523.64 (55.4)	105	107
			2200	3	7.41 (0.03)	33.01 (0.12)	2241.6 (36.5)	0.96 (0.07)	2092.08 (137.0)	93	58
			3500	2	7.26 (0.03)	32.99 (0.17)	2185.0 (15.0)	0.68 (0.04)	2917.18 (207.0)	59	38
July 28	56.5	19.86	400	4	8.00 (0.04)	33.19 (0.17)	2143.5 (7.77)	2.40 (0.21)	426.05 (46.3)	115	111
			2200	4	7.43 (0.03)	33.23 (0.32)	2154.4 (22.2)	0.73 (0.07)	1856.25 (138.9)	93	104
			3500	3	7.26 (0.04)	33.11 (0.18)	2146.0 (13.9)	0.52 (0.06)	2729.6 (290.0)	91	48
		27.26	400	3	7.93 (0.06)	33.53 (0.16)	2147.8 (25.2)	2.76 (0.33)	512.67 (85.9)	45	28
			2200	3	7.41 (0.04)	33.45 (0.20)	2146.6 (14.5)	0.95 (0.09)	1985.6 (187.7)	101	54
			3500	3	7.25 (0.04)	33.42 (0.14)	2140.0 (13.5)	0.67 (0.06)	2889.3 (289.2)	58	28
September 14	62.5	19.46	400	3	8.03 (0.03)	33.33 (0.18)	2099.7 (19.4)	2.44 (0.11)	387.25 (28.3)	94	90
			2200	3	7.41 (0.02)	33.60 (0.16)	2126.0 (16.2)	0.69 (0.02)	1908.70 (88.8)	59	57
			3500	3	7.26 (0.01)	33.48 (0.11)	2116.1 (11.9)	0.50 (0.02)	2687.75 (89.1)	89	67
		27.47	400	3	7.97 (0.03)	33.83 (0.15)	2093.65 (15.0)	2.87 (0.19)	452.46 (41.6)	105	100
			2200	3	7.42 (0.02)	33.78 (0.14)	2119.13 (9.8)	0.96 (0.05)	1907.1 (108.2)	93	85
			3500	3	7.28 (0.02)	33.97 (0.43)	2154.9 (76.9)	0.73 (0.06)	2691.1 (86.9)	59	51

some uncontrolled for seawater variability due to the natural sourcing of water from Vineyard Sound. Equilibrations at higher CO_2 concentrations were much more challenging to maintain, resulting in seawater pCO_2 values somewhat lower than the input gas concentrations (e.g., 2729.6 ppm for the 20°C × 3500 ppm treatment in the July 28 trial; **Table 1**). Despite these variations, data are reported across trials by the input gas concentrations for concision and clarity. However, it should be understood that these three concentrations, 400, 2200, and 3500 ppm, are acting more as a negative control, variable response level, and positive control, respectively, across these experiments rather than a precise representation of response at that equilibrated seawater CO_2 concentration.

Metrics

Methods for measurements of DML and YV were the same as those reported in Zakroff et al. (2019). In brief, paralarvae anesthetized in 7.5% w/v $MgCl_2$ in equal parts seawater (around 10 per treatment for the first 4 days of hatching) were photographed under dissecting scope (SteREO Discovery.V8, Carl Zeiss AG, Oberkochen, DE) and measured for DML using ImageJ (National Institutes of Health, Rockville, MD, United States; **Figure 1**). Paralarvae measured for DML were preserved in ethanol with the rest of the day's hatch. YV was measured by anesthetizing, fixing, and staining paralarvae with oil red O following Gallager et al. (1986) and processing in ImageJ (**Figure 1**) following the methods of Vidal et al. (2002a). No premature paralarvae, those with external yolk remaining, were included in either the DML or YV datasets.

Hatching time and success likewise were as described in Zakroff et al. (2019). The process of counting and preserving hatchling paralarvae continued each day until 2 days with no paralarvae found in the cup was reached. The egg capsule would then be removed, photographed, and dissected under dissecting scope. The remaining unhatched embryos were counted and categorized by simple visual discrimination of their stage of development (early: stages 1–16, middle: stages 17–26, and late: stages 27–30) adapted from Arnold et al. (1974).

Malformation

On the day with the greatest proportion of hatching for each cup, a random subsample of around 50 paralarvae were taken and categorized for malformations (sample sizes varied depending on hatching dynamics, but only samples of 20 paralarvae or more were used in the analysis). The subsampled paralarvae were categorized as either *Normal*: showing no external yolk or malformations, *Premature*: showing external yolk remaining post-hatch, but no other notable malformations, *Eye Bulge*: showing an inflation of the membrane around the eyes, or *Malformed Head*, showing a misshapen, often pointed or oblong head, occasionally also with odd growths or a malformed mantle.

Statistics

Statistical analyses were run in a Jupyter Notebook (Project Jupyter) using Python (version 3.5.5, Python Software Foundation). Data were first tested for normality with Shapiro–Wilks tests ($p > 0.05$) and through assessment of the linearity of quantile plots and the shapes of histograms. DML data are reported as means ± one standard deviation, primarily for easier relation to their visualizations. Log-transformed YV data are reported as the back transformed mean and values ± one standard deviation. Parametric data are often presented with a LR trend line, but these are not presented for statistical power; they serve as visual aids of trends.

Normally distributed data (DML and log-transformed YV) were then processed for group differences of means with multi-factor Type II ANOVAs. For the ANOVA model, pCO_2, temperature, and trial were all treated as independent factors. Trials were intended to serve as experimental replicates, however, variability in response, likely caused by parentage and seasonality, interferes with this assumption. Replicate trials independent of external influences were not achievable for us for this organism and experimental system. Experimental cups serve as treatment replicates within a trial. Since a single egg capsule was used per cup, the effects of cup versus capsule (represented by number of eggs) variability cannot be disentangled statistically and are not included in the ANOVA's discussed here (data was therefore compiled across cups; an egg number ANOVA is discussed below). ANOVA data are presented with calculated effect sizes (ω^2). A Tukey's HSD *post hoc* test was used to determine which groups showed statistically significant ($p < 0.05$) differences.

Non-parametric water quality data were analyzed with Kruskal–Wallis (KW) tests for difference between treatments. Non-parametric distributional data (hatching time curves, hatching success, and malformation) were analyzed using *G*-tests and are described for trends with LR, though the statistical power

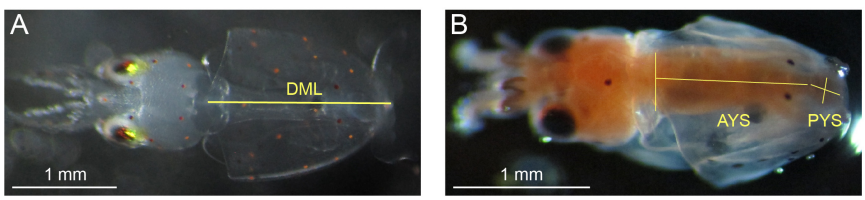

FIGURE 1 | Morphometrics measured on *Doryteuthis pealeii* paralarvae through microscope imagery. **(A)** Dorsal mantle length (DML; the superimposed yellow line) of an anesthetized paralarvae. **(B)** Squid paralarvae were fixed and stained with oil red O for measurement of YV. The length and width of both the anterior yolk sac (AYS), which was modeled as either a cone or cylinder (cone, in this case), and the posterior yolk sac (PYS), which was modeled as an ellipsoid were measured (superimposed yellow lines). Each image has a unique 1 mm white scale bar at bottom left.

of these regressions is low due to low sample size (data for these metrics is per cup/egg capsule; maximum $n = 4$; **Table 1**).

Assumptions

Although each trial in this experiment contains eggs from a single, separate mother, since replicates of different mothers were not run at or very near the same time point, the effects of maternity and seasonality could not be disentangled in our data. Maternal wet weight is noted in **Table 1**, but is not included in statistical models, as both the low sample size of mothers and the experimental design did not allow for it to be statistically distinguishable from trial effects.

Number of embryos within an egg capsule was noted in Zakroff et al. (2019) as a potentially impactful continuous variable on the state of hatched paralarvae, particularly for DML. As each cup contained only a single egg capsule in these experiments, any effects particular to the culture cup could not be disentangled from effects of egg capsule (or number of eggs per capsule). Type II ANOVA's were run with number of eggs per capsule as a continuous covariate to examine the potential impact of cup/number of eggs per capsule on response to acidification and warming.

On top of trial and cup effects, Zakroff et al. (2019) highlighted different responses to stressors across the days of the squid eggs hatching. While responses likely change across hatching days here as well, digging into them is beyond the scope of this manuscript. Further, samples were taken over fewer days of hatching in this dataset (4 days compared to six) making the dataset less robust for that type of analysis. Statistical models are presented with the assumption that effects of hatching date are occurring, but can be ignored in order to investigate the overall impacts of the stressors.

RESULTS

Dorsal Mantle Length

Dorsal mantle length of the paralarvae was impacted by both acidification and warming, but responses varied substantially between trials (**Figure 2**). Control treatment (20°C × 400 ppm) DML shifted across trials similar to the pattern reported in Zakroff et al. (2019). Notably, this pattern of seasonal DML shift appears unrelated to maternal weight: DML started around the typical paralarval size of 1.8 mm (June 19, 44.5 g mother: 1.80 ± 0.11 mm), reached its minimum at the peak of summer (July 28, 56.5 g mother: 1.64 ± 0.13 mm), and then increased again (September 14, 62.5 g mother: 1.74 ± 0.07 mm). Compiled across trials, the data indicate that while interactions between all factors are significant, the individual factors of trial ($\omega^2 = 0.257$), pCO_2 ($\omega^2 = 0.119$), and temperature ($\omega^2 = 0.054$) had the greatest effects on DML (**Supplementary Table S1**). Significant, relatively strong effects of acidification in ANOVA's and LR's across all trials are driven primarily by the consistently strong response at the 3500 ppm positive control (**Figure 2**). Assessments of responses to acidification were therefore focused on results from the 2200 ppm treatment.

Paralarvae from the June 19 trial were resistant to both stressors in terms of DML, only showing a notable decrease in size at the 3500 ppm (20°C: 1.63 ± 0.13 mm; 27°C: 1.64 ± 0.13 mm) positive control acidification level (**Figure 2** and **Supplementary Table S1**). The 2200 ppm exposed paralarvae from the 27°C treatment (1.74 ± 0.11 mm) showed a slight decrease relative to their acidification control (1.81 ± 0.14 mm), but were not different from the 2200 ppm from the 20°C water bath (1.78 ± 0.12 mm; **Figure 2**). Interactions between pCO_2 and temperature were not significant in this trial (**Table 1**).

The July 28 trial showed a substantial response to temperature in the DML data (decreasing to 1.49 ± 0.10 mm at the 27°C × 400 ppm treatment), but no effect of acidification at the 2200 ppm level (**Supplementary Table S1** and **Figure 2**). The 3500 ppm positive control resulted in pCO_2 having the greatest effect size in this trial ($\omega^2 = 0.132$), but temperature was nearly as impactful ($\omega^2 = 0.077$), and these stressors appeared to interact slightly ($\omega^2 = 0.029$, **Supplementary Table S1**).

Decreases in DML were seen in the September 14 trial with both acidification at the 2200 ppm treatment (20°C: 1.63 ± 0.07) and warming (27°C × 400 ppm: 1.53 ± 0.10; 27°C × 2200 ppm: 1.49 ± 0.09; **Figure 2**). Both acidification ($\omega^2 = 0.165$) and warming ($\omega^2 = 0.252$) had significant impacts on DML, as did their interaction ($\omega^2 = 0.090$), which was the largest of all the trials (**Supplementary Table S1**).

Notably, warming did not simply transpose the acidification impact downward or exacerbate the slope/severity of acidification effects (**Figure 2**). Rather, in trials where warming had a significant effect (July 28 and September 14), acidification impacts in the warming treatment were decreased (e.g., order of magnitude decrease in slope in September 14; 20°C LR: $-6.97^{*}10^{-5}$, 27°C LR: $-8.10^{*}10^{-6}$). In the compiled data, this results in a shift from a significant decrease with increasing acidification (20°C, LR, slope = $-5.75^{*}10^{-5}$, $R^2 = 0.824$, $p < 0.001$) to a slight decrease with increase acidification under warming (27°C, LR, slope = $-2.27^{*}10^{-5}$, $R^2 = 0.406$, $p = 0.065$; **Figure 2**).

Variance of DML Data

Variance in DML showed broadly similar patterns between the June 19 and September 14 trials, with variance increasing with acidification at 20°C and decreasing with acidification at 27°C, while the July 28 trial showed the opposite trends (**Figure 2**). As a result, the compiled data show a weak increasing trend with acidification at 20°C (LR, slope = $1.19^{*}10^{-6}$, $R^2 = 0.445$, $p = 0.057$) that diminishes to roughly flat line at 27°C (LR, slope = $4.19^{*}10^{-7}$, $R^2 = 0.025$, $p = 0.682$; **Figure 2**). Individual paired t-tests of variance between treatments broadly showed no significant changes in DML variance [two-sample $t(2)$, $p > 0.05$ for most treatment pairings within in each trial], except in the September 14, 20°C × 400 ppm vs. 20°C × 3500 ppm test [two-sample $t(2) = -2.96$, $p = 0.042$], although these results are likely impacted by low sample sizes (**Table 1**: number of egg capsules per treatment).

Distributions of DML for each capsule within a treatment were relatively similar in shape, indicating consistency in responses among the egg capsules of a mother's clutch (September 14: **Figure 3**; June 19: **Supplementary Figure S1**; July 28: **Supplementary Figure S2**). In the September 14 trial, where

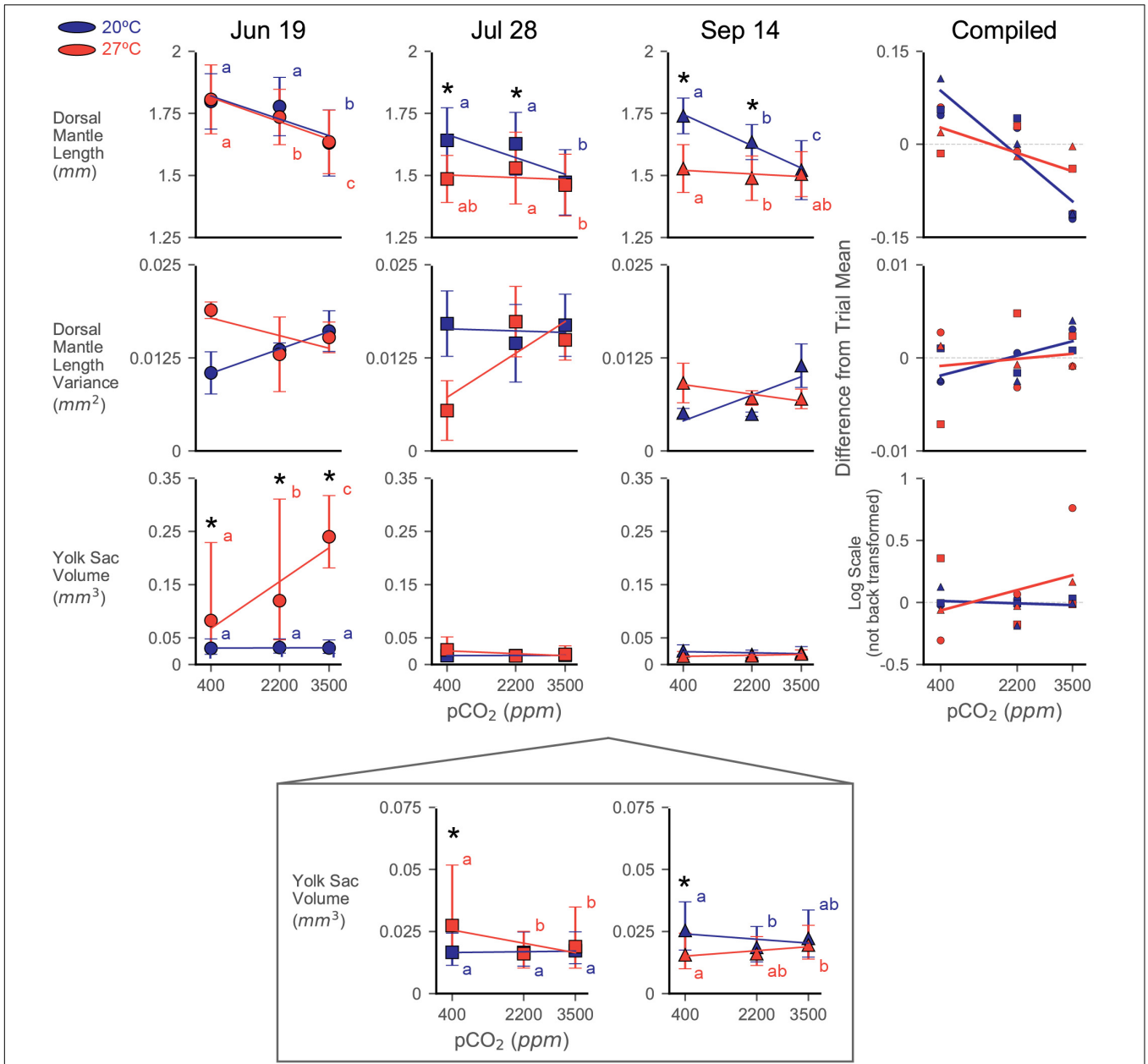

FIGURE 2 | Dorsal mantle length, its variance, and YV data from three experiments rearing squid eggs under acidification (in parts per million CO_2; *x*-axis) and warming (color; blue/dark = 20°C, red/light = 27°C). Yolk data were transformed to logarithmic scale for statistical analyses and has been back transformed for the depiction of trial data. A zoom box is provided beneath the July 28 and September 14 YV plot in order to see statistical difference and trends. Symbols represent trial, demarcated by dates eggs were laid (titles). Symbols depict means with error bars of one standard deviation. Letters indicate statistical groups across acidification levels within a temperature treatment from a Tukey's HSD. Asterisks indicate statistically significant differences between temperature treatments at the same acidification level. Regression lines are presented primarily as an aid to visualizing trends in the data and are not intended to indicate statistical power. The compiled plots show data from all trials normalized by subtracting the average value for a trial from its data; relative differences in yolk data have not been back transformed from the logarithmic scale. Error bars have been removed and symbols shrunk in order to emphasize trendlines.

DML was sensitive to both stressors, egg capsule distribution demonstrated wider spread with decreased peaks under warming (**Figure 3**). At 20°C, acidification caused September 14 egg capsules to translated to decreased sizes at 2200 ppm, but distributions retained the same shape, before flattening, spreading, and become more varied at 3500 ppm (**Figure 3**).

Yolk Sac Volume

Yolk sac volume responses appear to have been consistently affected (compiled data Type II ANOVA, $p < 0.001$ for all factors; **Supplementary Table S1**) by both temperature ($\omega^2 = 0.045$) and pCO_2 ($\omega^2 = 0.008$), but the direction and intensity of those responses shift strongly between trials ($\omega^2 = 0.383$, **Figure 2**),

particularly due to the interaction between trial and warming response ($\omega^2 = 0.153$). Control treatment hatchling YV followed a similar pattern as DML across trials, decreasing to its minimum in the July 28 trial (20°C × 400 ppm: June 19, 0.030 mm^3 [0.019–0.048 mm^3]; July 28, 0.017 mm^3 [0.011–0.024 mm^3]; September 14, 0.025 mm^3 [0.018–0.037 mm^3]).

In the June 19 trial, warming appeared to have the most substantial effect ($\omega^2 = 0.410$; **Supplementary Table S1**) on remaining paralarval yolk reserves, which increased under warming and increased further under combined warming and acidification (**Figure 2**). Paralarvae reared at 3500 ppm in the 20°C water bath hatched with internal YV of 0.031 mm^3 (0.021–0.046 mm^3), similar to the control, while YV of those in the 27°C water bath were 0.240 mm^3 (0.181–0.317 mm^3).

For the July 28 trial, while all factors were significant (**Supplementary Table S1**), the interaction between warming and acidification had the greatest impact ($\omega^2 = 0.045$) on paralarval YV. Similar to the June 19 trial, acidification had no notable effect on YV in the 20°C water bath and warming increased (though by much less than in June 19) remaining YV at the 400 ppm treatment (0.027 mm^3 [0.014–0.052 mm^3]). In contrast to June 19, however, YV in the 27°C water bath decreased with increasing acidification in this trial (3500 ppm, 0.019 mm^3 [0.010–0.035 mm^3]; **Figure 2**).

Paralarvae in the September 14 clutch showed weak, but significant, overall responses in YV, with temperature having the greatest effect ($\omega^2 = 0.109$; **Supplementary Table S1**). Unlike the other trials, the September 14 paralarvae showed a slight decrease in hatching YV with increasing acidification in the 20°C water bath (**Figure 2**). Also unique to the September 14 trial, warming to 27°C resulted in paralarvae hatched with less YV at the 400 ppm treatment (0.016 mm^3 [0.010 mm^3–0.024 mm^3]). Increasing acidification resulted in slightly increased remnant YV in the 27°C water bath, similar to, but much weaker than, the June 19 data (**Figure 2**).

Patterns of YV variance were inconsistent across trials and broadly showed no notable trends across acidification [two-sample $t(2)$, $p > 0.05$ for most treatment pairings within in each trial]. A significant decrease in YV variance was seen in the 27°C water bath of the June 19 paralarvae between both the 400 ppm [1.044 ± 0.079 mm^6; two-sample $t(2) = 13.30$, $p < 0.001$] and 2200 ppm [0.935 ± 0.061 mm^6; two-sample $t(2) = 13.92$, $p = 0.005$] treatments and the 3500 ppm sample (0.083 ± 0.007 mm^6).

Comparing DML and YV

In order to investigate clutch-specific patterns of physiological response to both acidification and warming stress, YV was plotted against DML for each egg capsule of each treatment (**Figure 4**). The 27°C treatment of the June 19 showed the strongest trend (LR, slope = −8.60, $R^2 = 0.865$, $p = 0.002$; **Figure 4**), with warming and acidification having resulted in smaller paralarvae

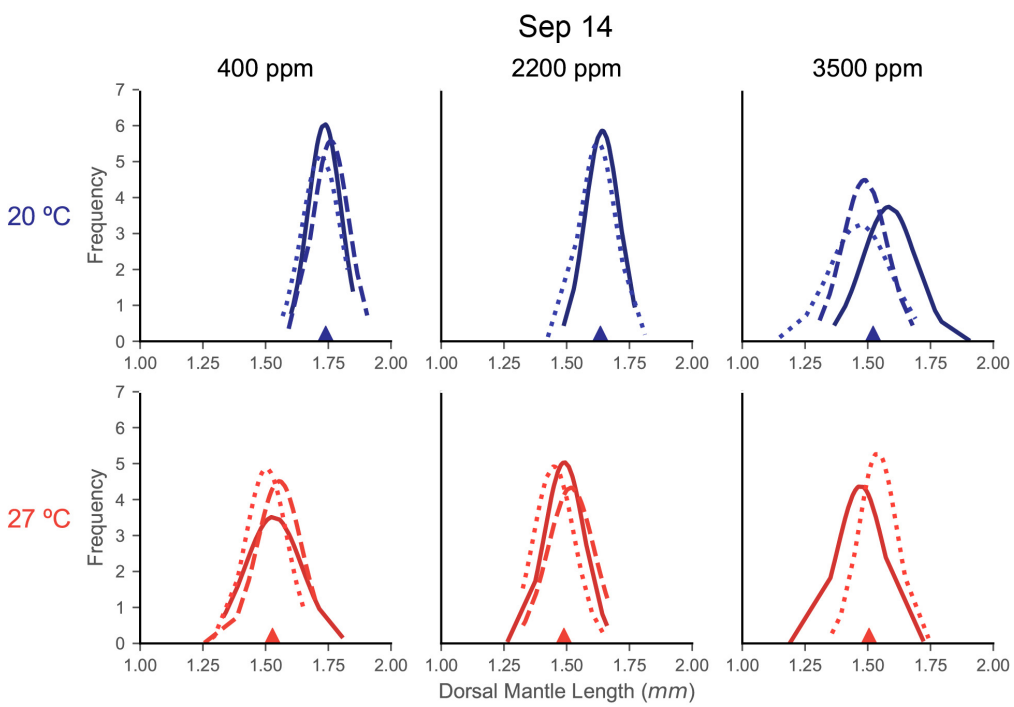

FIGURE 3 | Fitted normal distributions of DML histogram density from individual egg capsules in each treatment of the September 14 trial. Histograms are not shown for clarity of curves, but DML data were segmented in 0.05 mm bins. Each plot is a treatment combination, with column determining acidification (titles) and row determining temperature treatment (also differentiated by color: top/blue = 20°C, bottom/red = 27°C). Each line represents the curve from the sampling of an individual egg capsule for DML (*x*-axis). Lines are shaded and patterned to help differentiate individual egg capsules within a plot, but this carries no relationship or meaning across the plots. The filled triangle on the *x*-axis marks the mean value for the compiled sample of that treatment.

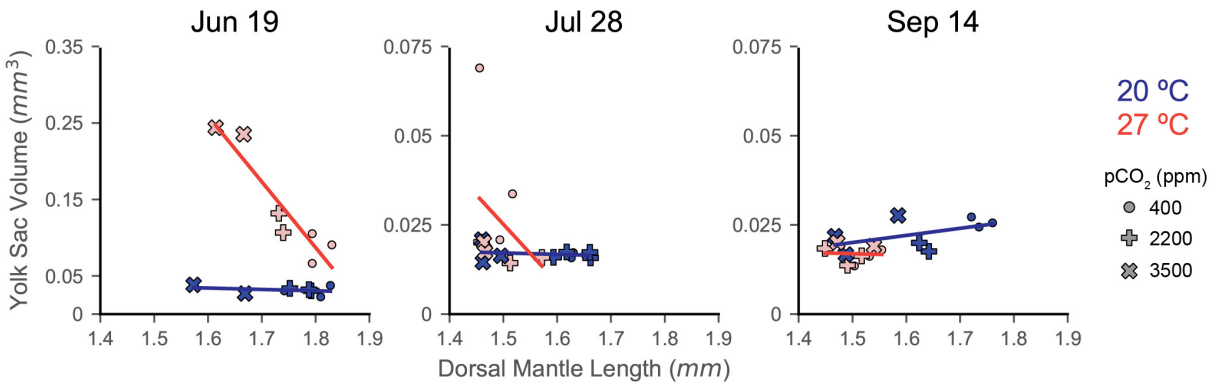

FIGURE 4 | Response of individual egg capsules within a clutch to acidification and warming as indicated by comparing YV (*y*-axis; note shift in scale from June 19 to July 28) with DML (*x*-axis). Trials are indicated by title. Symbol shape indicates acidification treatment (circle = 400 ppm, plus sign = 2200 ppm, *X* = 3500 ppm), while color denotes temperature (blue/dark = 20°C; red/light = 27°C). Symbols represent the means for a single egg capsule (one cup within the experimental system). No error bars are depicted for visual clarity. Trend lines are regressions of each temperature treatment, but are only presented as visual aides.

with less consumed yolk before hatching. The July 28 eggs, conversely, showed a weak trend of smaller paralarvae hatching with more yolk consumed under the same conditions. The September 14 clutch demonstrates a much weaker trend under both acidification and warming, but of a similar response type to the June 19 clutch. This trial also differs by having the only positive slope of the 20°C exposures (LR, slope = 0.020), with paralarvae having hatched smaller and with less yolk under increased acidification (**Figure 4**).

Egg Number

Type II ANOVA's were run with number of eggs per capsule as an independent continuous covariate, but did not have a significant effect on DML or YV in these experiments (multi-factor Type II ANOVA, $p > 0.05$ for all trials and in combined data). For this reason, this factor was not included in the model and statistics and these data not presented.

Hatching Time

Increased temperature increased the rate of embryonic development, resulting in 27°C egg capsules consistently hatching sooner (around 9 days) than their 20°C counterparts (around 14–15 days) in all trials (**Figure 5A**). While time to hatching increased for the 20°C treatments across the breeding season, as was seen in Zakroff et al. (2019); this seasonal increase to hatching time disappears in the 27°C treatments (see y-intercepts in **Figure 5B**). Increasing acidification broadly delayed hatching by around 1.5 days, but these impacts were somewhat dampened by warming, although responses to combined stressors varied across trials (see slopes in **Figure 5B**).

In the June 19 eggs, time to 50% hatching was delayed in the 20°C treatment from 14.09 ± 0.40 days at 400 ppm to 14.79 ± 0.10 days at 2200 ppm and 15.13 ± 0.32 days at 3500 ppm (**Figure 5B**). Hatching distributions were significantly different between the 400 ppm and both increased acidification treatments [$G(7), p << 0.001$ for both pairs] at this temperature, but the 2200 and 3500 ppm curves were not statistically distinct [$G(6) = 4.699$, $p = 0.583$]. In the 27°C treatment, time to 50% hatching was delayed from 9.67 ± 0.08 days at 400 ppm and 9.83 ± 0.05 days at 2200 ppm to 11.01 ± 0.32 days at 3500 ppm CO_2. At this temperature, all hatching curves were different from each other [$G(7), p << 0.001$ for all pCO_2 treatment pairs], but the differences between 400 and 2200 ppm were two orders of magnitude lower (G statistic of around 50 compared to around 1000) than pairings with the 3500 ppm treatment.

Delays in hatching occurred more consistently and progressively with increasing acidification in the July 28 trial (**Figure 5**). Within each temperature treatment, each hatching distribution at each pCO_2 treatment was significantly different from each other [20°C, $G(6), p << 0.001$ and 27°C, $G(11), p << 0.001$ for all pCO_2 treatment pairs]. Time to 50% hatching increased at 20°C from 15.04 ± 0.49 days at 400 ppm to 15.92 ± 0.27 days at 2200 ppm and 16.54 ± 0.36 days at 3500 ppm. At 27°C, 50% hatching was delayed from 9.81 ± 0.59 days, to 10.52 ± 0.70 days, then to 11.22 ± 1.62 days with increasing acidification (**Figure 5B**).

While hatching was clearly delayed in the 20°C water bath of the September 14 trial, acidification responses were strongly dampened at 27°C (**Figure 5**). Distributions of cumulative percent hatching were statistically distinct in both the 20°C [$G(6), p << 0.001$ for all pCO_2 treatment pairs] and 27°C [$G(11), p << 0.001$ for all pCO_2 treatment pairs] water baths, but the differences (as assessed by G statistics and p values) are an order of magnitude higher in the 20°C samples. Time to 50% hatching had the greatest delay in the 20°C samples of the September 14 trial, increasing from 15.83 ± 0.06 days at 400 ppm to 17.87 ± 0.452 at 3500 ppm (**Figure 5B**). Contrastingly, this trial also had the smallest delay in its 27°C samples, increasing from 9.54 ± 0.18 days to 10.04 ± 0.14 days.

Hatching Success

Hatching success decreased both with acidification and warming, with increased acidification typically resulting more late stage losses, while warming resulted in more

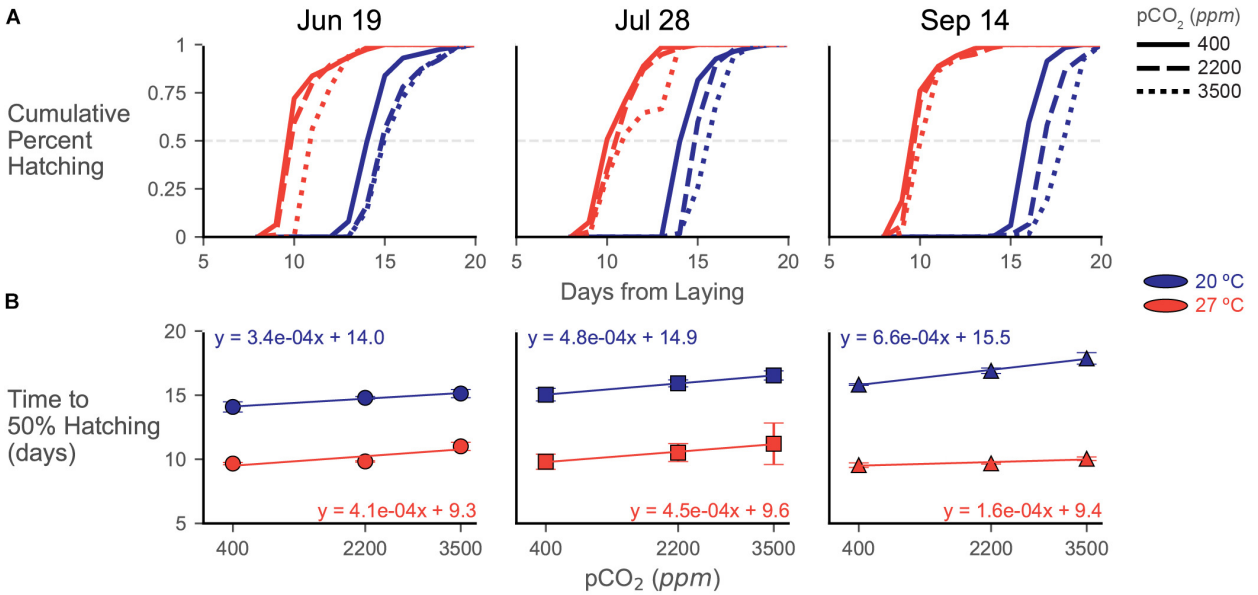

FIGURE 5 | Hatching data from experiments depicted as **(A)** cumulative hatching curves and **(B)** plots of time to 50% hatch. **(A)** Cumulative hatching curves are shown for each trial (lay date in title). Patterning of lines represents acidification treatments (smaller hash = more acidic), while color represents temperature treatment (blue/dark = 20°C, red/light = 27°C). Error bars/shading omitted for visual clarity of the curves. The gray dashed line represents the 50% hatching mark. **(B)** Time to 50% hatching data across acidification exposures, calculated from the curves in **(A)**, plotted for each trial. Symbols depict means (and represent trial) with errorbars of one standard deviation. Color represents temperature treatment as in **(A)**. Regression lines with related equations are presented to visualize trends and assess changes in slope and y-intercept across treatments and trials.

early to middle stage losses (**Figure 6**). Response patterns in hatching success were unique in each trial, as with previous metrics.

In the July 19 trial, hatching was quite high in the 400 (98.9 ± 1.0%) and 2200 ppm (92.7 ± 3.9%) treatments of the 20°C and the 400 ppm at 27°C (96.1 ± 2.9%). Distributions of staged failed embryos and hatched paralarvae were significantly different for all pCO_2 treatment combinations within each temperature [$G(3)$, $p < 0.001$ for all pCO_2 treatment pairs] and for all pCO_2 comparisons across temperatures [$G(3)$, $p < 0.001$ for comparison of 2200 and 3500 ppm across temperatures] except at 400 ppm [$G(3) = 5.313$, $p = 0.150$; **Supplementary Table S2**]. The 2200 ppm at 27°C sampled had a single egg capsule completely fail at the middle stages, which drove down over hatching success for the treatment (59.2 ± 42.2%). The 3500 ppm treatments at both temperatures (20°C: 73.2 ± 10.3%; 27°C: 84.1 ± 6.6%) had decreased hatching due to losses at late stages.

Egg capsules of the July 28 trial showed high hatching success across acidification treatments at 20°C (all above 90%; **Supplementary Table S2**), but had substantive decreases in the 400 (34.0 ± 31.9%) and 3500 ppm (54.7 ± 36.3%) treatments at 27°C. Embryos halted development in multiple egg capsules of the 400 ppm treatment at all stages, but mostly early, while late stage losses drove the decrease in hatching success at 3500 ppm (**Supplementary Table S2**). Hatching success distributions were all distinct for all treatments within this trial [$G(3)$, $p < 0.001$ for almost all pCO_2 and temperature treatment pairs; **Supplementary Table S2**], though the 400 and 3500 ppm treatments at 20°C were nearly the same due to the presence of slight late stage losses [$G(3) = 10.77$, $p = 0.013$].

Hatching success was highest in the 400 (94.0 ± 5.0%) and 2200 ppm (93.8 ± 2.8%) treatments at 27°C in the September 14 data (**Supplementary Table S2**), which showed very similar distributions with only slight early stage losses [$G(3) = 3.524$, $p = 0.318$]. All other treatment combinations showed significant differences in hatching success distribution within this trial [$G(3)$, $p < 0.001$; **Supplementary Table S2**]. The 3500 ppm treatment at 27°C had low hatching success (56.8 ± 40.7%) driven by near complete early stage loss in a single capsule, as well as slight early stage losses in the other capsules. The 400 ppm treatment at 20°C had relatively high hatching success (85.1 ± 8.3%), although lower compared to the 27°C due to high early stage losses. Increased acidification at 20°C had much higher decreases in hatching success, however, due to large late stage losses in both the 2200 (57.8 ± 41.0%) and 3500 ppm (74.2 ± 15.0%) treatments.

Malformation

The patterns of malformation were relatively consistent across trials, with increasing acidification producing greater proportions of premature and eye bulge paralarvae, while warming dampened the acidification impacts slightly and increased the proportion of malformed head paralarvae (**Figure 7B**). In all trials, proportionally less premature paralarvae were seen in the 3500 ppm treatment at 27°C than at 20°C (**Supplementary Table S3**). Malformation distributions compared across trials, showed significant shifts as a result of trial for the

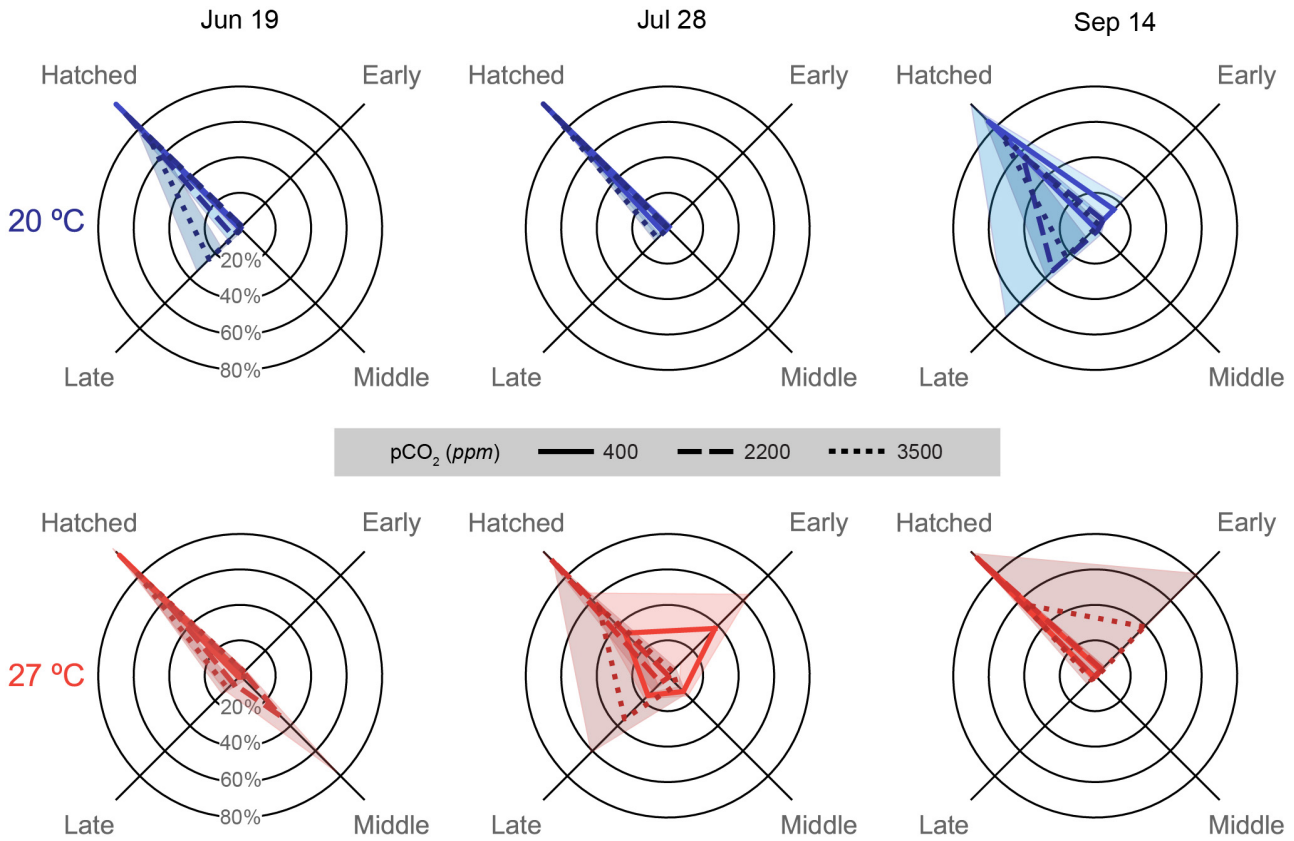

FIGURE 6 | Embryonic survival data depicted for each trial (column title) on spider plots: axes represent categorical variables, while rings represent proportions of embryos in each category (labeled in June 19 plots). Categories include, clockwise from top left, Hatched: embryos that successfully hatched from the egg capsules (includes premature and malformed hatchlings), and embryos that ceased development either Early: Arnold stages 1–16, Middle: Arnold stages 17–26, or Late: Arnold stages 27–30 (Arnold et al., 1974). Lines represent means, while shading represents one standard deviation. Line patterns and shade of color represent acidification treatments (smaller hash/darker color = more acidic). Plot position and data coloration represent temperature treatment (top/blue = 20°C, bottom/red = 27°C).

20°C × 2200 ppm treatment and all 27°C treatments [$G(6)$, $p < 0.001$ for listed treatments].

Paralarvae from the June 19 trial generally demonstrated the pattern described above, but with particularly notable increases in malformed head paralarvae in all acidification levels at 27°C (**Figure 7** and **Supplementary Table S3**). All distributions in all within temperature and within pCO_2 pairings in this trial significantly differed from each other [$G(3)$, $p < 0.01$; **Supplementary Table S3**].

In the July 28 trial, paralarvae showed broadly similar patterns of malformation across temperatures, with acidification impacts being slightly more prominent in the 20°C and temperature impacts being minimal (in part possibly driven by sample size shifts between temperatures; **Supplementary Table S3**). Although all within-temperature comparisons of malformation distributions between pCO_2 treatments were significant, those at 27°C were less different than those at 20°C [$G(3)$, $p \leq 0.001$; see exponents in **Supplementary Table S3**]. Notably, distributions of malformations between pCO_2 treatments across temperatures were either comparatively weakly, in the case of 400 ppm [$G(3) = 11.57$, $p = 0.009$], or not significantly different, for 2200 [$G(3) = 4.485$, $p = 0.214$] and 3500 ppm [$G(3) = 0.338$, $p = 0.953$], in this trial.

The September 14 paralarvae also demonstrated more prominent acidification impacts at 20°C. These impacts were slightly dampened with warming, under which there only showed a slight, but weak, increase in malformed head proportions (**Figure 7**). Distributions of malformations were significantly different between all pCO_2 treatment pairs within temperatures [$G(3)$, $p < 0.001$; **Supplementary Table S3**] owing to increasing premature and eye bulge proportions with increasing acidification. Differences across temperatures, driven by the dampening and shifts described with warming above, were significant in the 400 ppm [$G(3) = 16.32$, $p < 0.001$] treatment and neared significance in the 2200 [$G(3) = 6.622$, $p = 0.085$] and 3500 ppm [$G(3) = 6.829$, $p = 0.078$] treatments.

DISCUSSION

These experiments demonstrated clutch-dependent sensitivity to the combination of high levels of acidification and warming stress

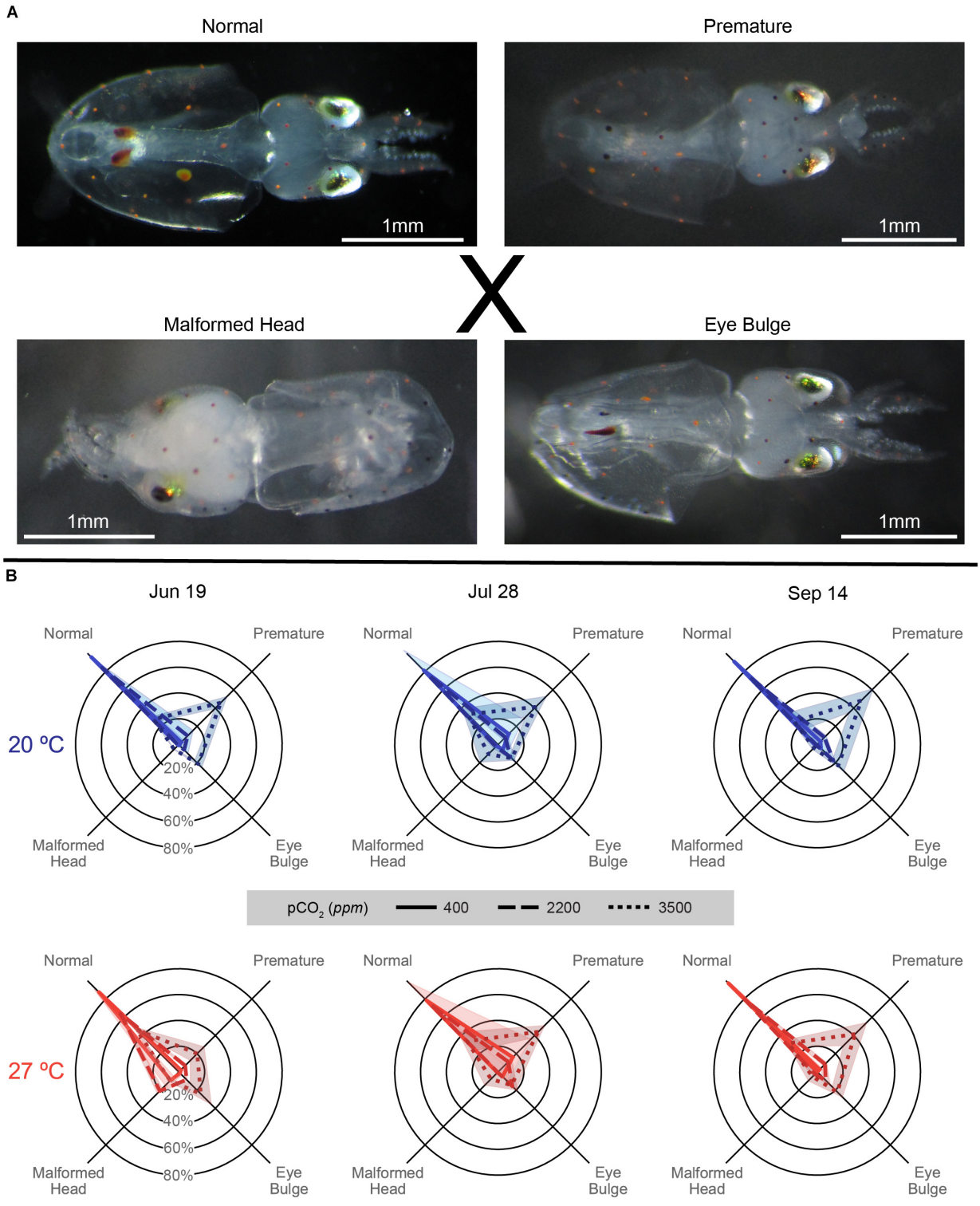

FIGURE 7 | (A) Images of types of hatched *Doryteuthis pealeii* paralarvae corresponding to the axes categories in the data figure **(B)**, as referenced by the 'X.' From top left: Normal: a typical hatchling paralarvae, Premature: a paralarvae showing remaining external yolk, Eye Bulge: a paralarvae with inflation of the membrane around the eye, and Malformed Head: a paralarvae with misshapen head, can also present with strange growths or misshapen mantle. All images have a unique 1 mm white scale line in the bottom left. **(B)** Malformation data for each trial (column title) on spider plots: axes represent categorical variables, while rings represent proportions of embryos in each category (labeled in June 19 plots). Categories include, clockwise from top left, Normal, Premature, Eye Bulge, and Malformed Head as depicted in **(A)**. Lines represent means, while shading represents one standard deviation. Line patterns and shade of color represent acidification treatments (smaller hash/darker color = more acidic). Plot position and data coloration represent temperature treatment (top/blue = 20°C, bottom/red = 27°C).

in the egg capsules of *D. pealeii* across the 2016 breeding season. Embryos appear to be capable of developing normally, at least in terms of size, yolk consumption, and survival up to at least 2200 ppm CO_2, a value which is not predicted in 'no reduction of emissions' scenarios in the open ocean until at least the year 2300 (Caldeira and Wickett, 2003). The consistent hatching delay, as well as the increased proportion of late stage loss and premature paralarvae observed, suggest that acidification may cause metabolic suppression, particularly late in development, as was described for *L. vulgaris* embryos under warming (Pimentel et al., 2012; Rosa et al., 2012). Metabolic suppression was long suggested as an expected impact of OA on squid because squid hemocyanins are very sensitive to pH, squid operate at the very peak of blood oxygen utilization, and the resultant Bohr shift would starve the animal of oxygen (Pörtner, 1990, 1994; Fabry et al., 2008; Seibel, 2016). While metabolic suppression was observed in jumbo squid, *Dosidicus gigas*, exposed to 1000 ppm CO_2, more recent studies have demonstrated no metabolic impacts to adults of bigfin reef squid, *Sepioteuthis lessoniana*, and pygmy reef squid, *Idiosepius pygmaeus*, as well as adults and juvenile *D. gigas*, and *D. pealeii* at equal or greater levels of acidification (Rosa and Seibel, 2008; Hu et al., 2014; Birk et al., 2018; Spady et al., 2019). It is possible that metabolic sensitivity to acidification is life stage dependent in cephalopods with juveniles and adults having the robust physiological machinery needed to manage under OA stress, while the gears of development may be slowed in embryos. Of particular interest, then, is the metabolic scope of the paralarvae under acidification, which are thought to be quite sensitive based on aquaculture studies (pH range of 8.1–8.4 for loliginid paralarvae), and the transition from paralarvae to juvenile in squid (Vidal et al., 2002b).

Observed impacts of acidification to the squid suggest systems of pH and ionic/osmotic balance may be strained, particularly under the severe dosage of 3500 ppm. Zakroff et al. (2019) discussed potential mechanisms of acidification impact to DML and YV, in the scope of a limited energy store and energy budget, suggesting that reductions in growth and YV under hatching are a potential product of upregulation and increased activity of energetically costly proton secreting transporters in ion-transport epithelia (Hu et al., 2010, 2013). The increased proportion of paralarvae showing inflation of the membrane around the eyes under increased OA may further suggest a breakdown in osmoregulatory controls, particularly given the prevalence of ionocytes in the epidermis of cephalopod embryos (Hu et al., 2011). Though it is also plausible that this inflation is related to poorly known osmotic mechanisms that cause the swelling of the egg capsule during development (Hu and Tseng, 2017).

In our sensitive clutches (July 28 and September 14), warming strongly impacted development time, DML and YV, hatching success, and malformations, likely through an increase in metabolic and developmental rates. Warming of +2°C is a standard experimental choice given predicted scenarios under no emission reductions (Pachauri and Meyer, 2014). The warming temperature used, 27°C (+2°C above peak for Vineyard Sound), is within the habitat window reported for *D. pealeii* juveniles and adults, but well above the 23°C maximum reported for egg laying habitats (Jacobson, 2005). Cephalopod eggs typically demonstrate hatching curves wherein success is quite high (>80%) within the preferred thermal window and then drops off rapidly and precipitously above and below certain temperature thresholds (Cinti et al., 2004; Sen, 2005; Staaf et al., 2011; Zeidberg et al., 2011). This threshold was reported to be between 22 and 25°C in *D. opalescens*, but appears to be higher for *D. pealeii* (at least 27°C; Zeidberg et al., 2011). Loss of embryos under warming was primarily in early- and mid-stages of development, suggesting, despite acclimation, a clear and immediate impact to physiology that could easily push embryos past their limits. Disruption of the developmental machinery by warming likely also explains the increased proportion of paralarvae with malformed heads and odd growths, as has been described in *L. vulgaris* (Rosa et al., 2012).

Contrary to some of the observations of temperature and acidification compounding stress effects in *L. vulgaris* embryos and paralarvae, in this work these stressors appear to act antagonistically for most of the factors we measured in the early life stages of *D. pealeii* (Rosa et al., 2014). In part, this antagonism may be due to the much stronger effect size of warming in stress sensitive clutches. In the DML data, for example, warming may have driven embryos to their size floor, the minimum size viable for a paralarvae to hatch, and therefore no further decreases due to acidification could be observed. Previous studies have shown that these two stressors counteract most clearly in hatching time, where warming increases oxygen consumption and developmental rate, while acidification causes developmental delay and potentially metabolic suppression (Pimentel et al., 2012; Rosa et al., 2014; Navarro et al., 2016; Zakroff et al., 2019). In hatching success and malformation, the dampening of acidification impacts is likely driven by a reduction in acidification exposure time as a result of the drastic decrease in time to hatching. The data compiled suggests that warming impacts *D. pealeii* eggs early in development with disruptions, like malformed bodies, potentially propagating to hatching if development doesn't cease altogether. Acidification, conversely, appears to be a slow burn across development, compounding the buildup of CO_2 and acidification that would naturally occur due to respiration and thereby causing greater impacts to late stage embryos (Gutowska and Melzner, 2009; Long et al., 2016).

Each clutch of eggs (each trial) demonstrated a different set of responses to acidification and warming across most metrics, particularly DML and YV. As in Zakroff et al. (2019), the comparison of these metrics provides potential insight into the range of physiological coping responses available to *D. pealeii* embryos under multiple stressors (**Figure 4**). In the June 19 trial, paralarvae from eggs under warming and acidification were slightly smaller in size with substantially less consumed yolk, suggesting a possible overall metabolic suppression that resulted in a relatively resistant clutch. However, the substantive increase in yolk under warming (**Figure 2**) coincided with an increased loss of embryos in the middle stages of development (**Figure 6**) and increased premature paralarvae in the hatch (**Figure 7**). Taken together, these data suggest that the combined stressors, but especially warming, impacted this clutch most during mid-stage development resulting in a notable proportion of unviable hatchlings with too much internal and external yolk

left unconsumed, which would be unlikely to survive (Vidal et al., 2002b; Martins et al., 2010; Vidal and Von Boletzky, 2014). July 28 trial showed the reverse, with more yolk consumed in the smaller paralarvae of the combined acidification and warming treatment, suggesting warming outpaced acidification, and the response of the taxed embryos in this case was to consume more yolk in order to cope. The September 14 clutch showed larger paralarvae with slightly more yolk in the control condition, indicating both acidification and warming taxed the energy budgets of these developing embryos. Unfortunately, while it is possible to culture Myopsid paralarvae in aquaria, it is a very challenging proposition for *D. pealeii* that we tried, but could not accomplish (Vidal et al., 2002b). Thus, a question that remains is: given these differential responses to the same stressors between clutches, which strategy would produce the most viable paralarvae in a stressful ocean?

There were two aspects of clutch variability highlighted in these experiments, the first of which is variability between clutches/mothers (which cannot be disentangled from seasonality in our data). Parental conditioning has been shown to impact sensitivities conferred to offspring in fishes and corals (Miller et al., 2012; Putnam and Gates, 2015; Schunter et al., 2016, 2018) Murray et al. (2014) described seasonal pH conditioning of parents in a coastal fish, *Menidia menidia*, which resulted in differential pH sensitivity in offspring. In cephalopods, embryos from winter and summer cohorts of *L. vulgaris* were shown to respond very differently to acidification and warming stress, with summer cohorts being more sensitive (Rosa et al., 2014). Scientists, staff, and fisherman that work with *D. pealeii* at the various scientific institutions in Woods Hole, MA, United States anecdotally acknowledge the presence of cohorts within the breeding season, or at least a succession of size classes, but this shift has only roughly been described in the literature as a transition between an early 2-year-old cohort and the new 1-year-old cohort (Arnold et al., 1974; Mesnil, 1977). In Zakroff et al. (2019) sensitivity to acidification started strong and decreased as the season went on, while here, the earliest trial was the most resistant and the latest the most sensitive. This appears to indicate some form of change in parental conferred sensitivity across the 2013 and 2016 seasons from the early summer squid to the early autumn squid, although in opposite directions between these years, which may support the idea of shifting cohorts within the breeding season and suggests that it is parentage rather than seasonality that is driving offspring sensitivity.

The second form of egg clutch variability we examined is variability between the egg capsules of a single mother's clutch. Even among egg capsules of a single female, squid parentage is a complex proposition since mating can occur with, and sperm can be stored from, multiple males (Buresch et al., 2006). Stress responses and statolith elemental composition have been observed to vary between egg capsules in *D. opalescens* (Navarro et al., 2014, 2016). Ikeda et al. (1999) noted variability within the paralarvae from a single *S. lessoniana* mother, indicating a range in DML correlated with statolith size and hatching time. Our results suggest, at least for DML, that not only does the variation of paralarval sizes within each egg capsule produce an approximately normally distributed curve, but also that egg capsules from the same mother produce very similar distributions (**Figure 3**). Variability in the sample of hatchling DML has been noted previously as a possible consequence of egg position within the egg capsule and, in a natural setting, of egg capsule within the egg mass, both of which can impact oxygen availability and thus developmental rate and ultimately paralarval size and yolk content (Steer and Moltschaniwskyj, 2007; Vidal and Von Boletzky, 2014). Under additional stressors, these distributions appear to shift. Relatively light stress appears to simply shift the distributions, while heavier stressors cause flattening and spreading of these curves (**Figure 3**). It has been theorized that size variation among offspring acts as a kind of adaptation to unpredictable environments, with selection pressures (in this case increased environmental stress) acting upon both the mean and variance of an offspring distribution (Marshall et al., 2008).

In cephalopods, paralarval size is, on a taxonomic level, known to correlate to egg size (Laptikhovsky et al., 2013). Adult squid do not retain lipid reserves, so maternal investment in reproduction is primarily driven by the allocation of resources between somatic vs. reproductive growth (Pecl and Moltschaniwskyj, 2006). Energy for egg production is captured through recent feeding, so while successive clutches of eggs may degrade in quality as the state of the mother degrades, maternal input within a clutch may, as appears to be supported by our DML evidence, be relatively consistent across egg capsules (Steer et al., 2004). It is plausible then that the breakdown of similarities between egg capsules under stress could owe to differences in genetic background due to paternity, though this is purely speculative without much more robust experimentation.

This study demonstrated that *D. pealeii* embryos and paralarvae reared under severe, chronic acidification and warming could show a range of responses from sensitive to resistant. These responses are driven by between clutch differences, which are likely representations of parentage, but may also be influenced by seasonality. Responses are also variable given the complexity of interacting and antagonistic physiological processes influenced by warming and acidification in this system. These experiments were limited in a number of key ways. As an in lab experiment, factors of flow, egg capsule density, and variability that occur in the natural system are not represented here. Variability of pH in natural systems is thought to decrease impacts in some organisms by reducing exposure time, which appears to be an important factor in acidification's impact on *D. pealeii* eggs (Shaw et al., 2013). While a growing body of literature is beginning to suggest squid, at least embryos and adults (there is still a great deal left to understand with respect to paralarvae), may be fairly robust in the face of OA, these responses may be taxon, population, or region specific making it difficult to generalize (Kroeker et al., 2017; Birk et al., 2018; Spady et al., 2019). Warming, however, clearly has its limits in *D. pealeii* embryonic development, but squid have the advantage of mobility in coping with that (Doubleday et al., 2016). A fecund, plastic, year class species, such as *Doryteuthis pealeii*, appears well suited to rapid adaptability under rapid global ocean change. It is important, therefore, to continue to describe the signs and understand the mechanisms of that adaptability, and to investigate its limits, in order to inform how

we design experiments to diagnose sensitivity and adaptability in other marine taxa.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

Ethical guidelines for animal research are institutionally specific in the United States and research involving cephalopods does not require ethical review or approval from the Woods Hole Oceanographic Institution's IACUC. Transport, care, and treatment of the cephalopods used here was done following international guidelines in the literature, particularly those which have since been summarized in Fiorito et al. (2015).

AUTHOR CONTRIBUTIONS

CZ and TM designed the experiments. CZ performed the experiments and collected the data. The data analysis and statistics was performed by CZ with assistance from TM. CZ wrote the first draft of the manuscript. CZ and TM revised the manuscript. Both authors read and approved the submitted version of the manuscript.

FUNDING

This research was supported by the National Science Foundation Grant No. 1220034 to TM and the National Science Foundation Graduate Research Fellowship under Grant No. 1122374 to CZ.

ACKNOWLEDGMENTS

We would like to express our gratitude to the Cephalopod International Advisory Council for the opportunity to present this work at their triennial conference and contribute to this special issue. We also thank D. Remsen, the MBL Marine Resources Center staff, and MBL *Gemma* crew for their help acquiring squid. R. Galat and WHOI facilities staff aided with set up, maintenance, and system support at the ESL. D. McCorkle provided guidance and insight on the acidification system and water quality monitoring, as well as the spectrophotometric pH system, with methods from M. White. Assistance and advice from A. Schlunk and L. Fitzgerald during these experiments was incredibly beneficial.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00501/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

ORIGINAL RESEARCH
published: 30 June 2020
doi: 10.3389/fphys.2020.00697

Short and Long-Term Effects of Anesthesia in *Octopus maya* (Cephalopoda, Octopodidae) Juveniles

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OPEN ACCESS

Edited by:
Erica A. G. Vidal, Federal University of Paraná, Brazil

Reviewed by:
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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *28 August 2019*
Accepted: *28 May 2020*
Published: *30 June 2020*

Citation:
Roumbedakis K, Alexandre MN, Puch JA, Martins ML, Pascual C and Rosas C (2020) Short and Long-Term Effects of Anesthesia in Octopus maya (Cephalopoda, Octopodidae) Juveniles. Front. Physiol. 11:697. doi: 10.3389/fphys.2020.00697

This study aimed to explore different substances (or cold sea water) as potential anesthetic agents to facilitate short-term handling in *Octopus maya* juveniles. We investigated oxygen consumption before (baseline), during (first 600 s of exposure) and after anesthesia (recovery) of octopuses (n = 98; 1.67 ± 0.5 g) exposed to cold sea water (SW; 11 and 13°C), ethanol (EtOH; 0.5; 1.5 and 3.0%), magnesium chloride ($MgCl_2$; 0.75; 1.5 and 3.75%), ethanol combined with magnesium chloride (Mix; 1.5:0.75%; 0.75:1.13%; and 2.25:0.37%) and clove oil (0.15 mL L^{-1}). After exposure, the animals were handled for 180 s (exposed to air) and weighted. Two experimental groups not exposed to anesthetics (with or without handling) were also evaluated. The criteria for general anesthesia were analysed. Times of induction and recovery, incidence of attack response after recovery and possible longer-term effects of repeated general anesthesia on growth and mortality of the octopuses were evaluated. During anesthesia, *O. maya* juveniles exposed to SW (11 and 13°C), EtOH (0.5; 1.5 and 3.0%), Mix (0.75:1.13%), and clove oil, presented a significant decrease on oxygen consumption. In animals exposed to different concentrations of EtOH and Mix 0.75:1.13%, this decrease was registered after an increase on oxygen consumption. Animals exposed to $MgCl_2$ did not show significant changes on oxygen consumption, except for animals exposed $MgCl_2$ 3.75%, which showed a significant increase on oxygen consumption. At the end of recovery, except for octopuses exposed to clove oil and $MgCl_2$ 0.75%, the values of oxygen consumption observed were comparable to the ones registered during baseline. Animals exposed to SW 11°C, EtOH 3.0%, Mix 1.5:0.75% and $MgCl_2$ 3.75% fulfilled the criteria defined for general anesthesia. Exposure to $MgCl_2$ (all concentrations), SW 13°C and clove oil reduced or inhibited the incidence of attack response after recovery. Except for animals exposed to clove oil, growth of the juveniles was not affected by the exposure to the different substances. Short-term handling (180 s) of *O. maya* juveniles can eventually be carried out without anesthesia. However, to facilitated handling, we suggest the use of EtOH 3.0% or cold sea water 11°C.

Keywords: anesthetic, cephalopods, oxygen consumption, criteria, growth, mortality, animal welfare

INTRODUCTION

General anesthesia may be defined as a controllable and reversible state, which includes loss of consciousness, analgesia, suppression of reflex activity, and muscle relaxation (Flecknell, 2015). In aquatic animals, general anesthesia is commonly used for husbandry (e.g., handling, transport, and artificial reproduction), veterinary examination (e.g., blood sampling and pathological analysis), and for research purposes (e.g., to perform experimental procedures and surgery, facilitate manipulation, prevent injuries, reduce stress, and promote animal welfare). Regarding cephalopods, the topic has gained special attention in recent years, since their inclusion in the European legislation Directive 2010/63/EU on the protection of animals used for scientific purposes (European Parliament Council of the European Union, 2010).

Surprisingly, despite the recent increased interest, current information is available only for a few species using a limited variety of substances potentially acting as anesthetic agents: only about 20 substances have been investigated to anesthetize cephalopods (reviewed by Sykes et al., 2012; Gleadall, 2013b; Fiorito et al., 2015). Moreover, with few exceptions (Andrews and Tansey, 1981; Pugliese et al., 2016; Butler-Struben et al., 2018) the majority of anesthetic studies in cephalopods do not explore in-depth the efficacy of the commonly used agents and the physiological effects of anesthesia on the animals. Another limitation is the lack of standardized anesthesia protocols for cephalopods, since distinct methods are described in the literature.

Cephalopods are highly specialized invertebrates with particular characteristics, such as sophisticated brains and nervous systems (Shigeno et al., 2018) visual, mechanoreceptive, olfactory, and chemosensory abilities (Cooke et al., 2019b) complex behavioral and learning capabilities (Hanlon and Messenger, 2018) among others. These characteristics are important to improve the current understanding of cephalopods welfare requirements. In this sense, Ponte et al. (2019) provided a list of biological features that should be considered while assessing cephalopod welfare. From this list, three aspects are, in our view, particularly relevant in the context of cephalopod anesthesia: (i) respiration, including possible limitations that may affect the animals; (ii) inter-individual differences in behavioral repertoire and responses; and (iii) responses to stressors and negative experiences.

Physiological (e.g., respiration and heart rate) and physical aspects (e.g., skin color and body morphology) as well as behavioral responses (e.g., swimming activity, coordination, and inking) are utilized to evaluate and monitor anesthesia in cephalopods (Andrews and Tansey, 1981; Gonçalves et al., 2012; Gleadall, 2013b; Butler-Struben et al., 2018). Regarding physiological aspects, monitoring the respiration is a common practice during cephalopod anesthesia: reduction and even cessation of respiration have been utilized as criteria for anesthesia (Gleadall, 2013b; Fiorito et al., 2015) although increased respiration have been eventually observed during anesthesia induction (Andrews and Tansey, 1981; Messenger et al., 1985; Gonçalves et al., 2012). Nevertheless, potential physiological effects caused by oxygen consumption changes in cephalopods exposed to anesthetics have never been explored.

The effectiveness of anesthetics in relation to individual characteristics, including biological aspects (e.g., age, life stage, and sex), physiological condition and health status, is a gap in knowledge of cephalopod anesthesia (O'Brien et al., 2018). Inter-individual behavioral responses of cephalopods to anesthetics may differ depending on the species/taxon and the substance used (Butler-Struben et al., 2018). As observed in fishes (Readman et al., 2017) the responses to different anesthetic agents seem to be specie-specifics also in cephalopods (e.g., Andrews and Tansey, 1981; Gleadall, 2013b; Butler-Struben et al., 2018). Possible intra-specific differences may also occur depending on the life cycle stage and size/age of the animals or due to environmental variables (Sykes et al., 2012). Therefore, investigate different anesthetic agents and concentrations in the most commonly used cephalopod species for research and aquaculture purposes is fundamental to allow adequate handling and to ensure animal welfare to the highest standards.

The exposure to a stressor might cause short and long-term consequences in aquatic animals, affecting physiological and behavioral aspects, for example, directly impairing food intake, growth, reproduction, or other aspects of the normal performance (Ross and Ross, 2008). The purpose of use of anesthesia in animal experimentation is to contribute to improve animal welfare, however, some aspects should be considered. Before, during and after anesthesia, cephalopods can be exposed to potentially stressful situations, such as non-desirable side-effects due to exposure to substances as putative anesthetics and inadequate handling of the animals (Fiorito et al., 2015). Physiological consequences and eventual long-term effects due to the exposure of the animals to a stressor might occur, especially if the exposure is repeated. Food intake as well as body weight have been recommended as physiological indicators of cephalopod welfare, and, therefore, altered feeding behavior and/or reduction of body weight are considered objective measures of health and welfare status (for details, see Table 5C in Fiorito et al., 2015).

Within the scope of the Directive 2010/63/EU, animal research workers should reduce discomfort, pain, and suffering of animals used for research to an absolute minimum. In addition, it is legally required to assign the prospective severity classification of scientific procedures on live animals, as well as reporting the actual severity experienced by each animal used during the course of such procedures. To assist and guide cephalopod scientists in assessing severity classification of procedures using live cephalopods for research purposes, Cooke et al. (2019a) evaluated 50 scenarios covering different types of procedures. From these scenarios, 12 included the use (or lack) of anesthesia for different purposes, such as handling for growth rate comparisons, hemolymph sampling, surgery, among others.

During aquaculture and research practices, handling is commonly used for many purposes (e.g., selection, weighting, transport, management of broodstock, etc.). While handing cephalopods, a few aspects should be considered. For instance, their delicate skin can be eventually harmed (particularly relevant for squids and cuttlefishes) when removed from the water (Fiorito et al., 2014). A part from physical damage, stress

caused by inadequate handling can promote a suppression of the immune response; in both cases, opportunistic secondary infections can eventually occur afterward (Sykes et al., 2019). Furthermore, some cephalopod species may be difficult to manipulate without sedation or anesthesia (Oestmann et al., 1997). This is especially important when handling octopuses, since the strength, flexibility, and dexterity of their arms can easily interfere or difficult handling and/or procedures (Gleadall, 2013b).

Octopus maya (Voss and Solís Ramírez, 1966) is an endemic warm-water species of the Yucatan Peninsula, which inhabits shallow waters of the continental shelf at depths ranging from 0 to 50 m (Jereb et al., 2014). This species has a great social and economic importance for fisheries and is considered one of the most promising candidates for aquaculture diversification (for review, see Rosas et al., 2014). Studies in many areas, such as reproduction, embryonic development, nutrition, physiology, immunology, and climate change, have been carried out for *O. maya* (Domingues et al., 2007; Rosas et al., 2008; Avila-Poveda et al., 2009, 2016; Juárez et al., 2015, 2016; Linares et al., 2015; Caamal-Monsreal et al., 2016; Roumbedakis et al., 2018; López-Galindo et al., 2019; Pascual et al., 2019). However, to date, no information regarding anesthesia is available for this species.

This study explored, for the first time, the use of different substances (or cold sea water) to be used as potential anesthetic agents in *O. maya* juveniles. Specifically, we aimed to: (1) investigate the efficacy of a range of presumed anesthetic agents to induce a general anesthetic state in *O. maya* juveniles; (2) explore the metabolic consequences of acute exposure to the presumed general anesthetic agents; (3) analyze possible longer-term effects of repeated general anesthesia in the juvenile cephalopod *O. maya*; (4) define appropriate agent(s) for short-term handling and non-invasive procedures in *O. maya* juveniles.

MATERIALS AND METHODS

Ethics Statement

In Mexico, cephalopods used for scientific purposes are not included in any animal welfare regulation. However, the experiments described here were previously submitted and approved at institutional level by the Animal Ethics Committee of the Faculty of Chemistry at Universidad Nacional Autónoma de México (UNAM, Sisal) (Permit Number: Oficio/FQ/CICUAL/099/15). The studies were conducted in accordance with the published Guidelines for the Care and Welfare of Cephalopods (Fiorito et al., 2015), which align with the principles of the European Directive (2010/63/EU) regulating animal research, including cephalopods, in the European Union (EU) (Smith et al., 2013). The recommendations of the ARRIVE Guidelines[1] (Kilkenny et al., 2010) for reporting *in vivo* animal research were followed as closely as possible.

[1]https://www.nc3rs.org.uk/arrive-guidelines

Experimental Animals: Origin, Husbandry, and Housing

Juvenile *O. maya* were obtained from eggs of wild-caught adults captured by the traditional fishing method "gareteo" (small drifting boats with bamboo sticks placed at the bow and stern in which artisanal fishing lines containing crabs as baits are attached; Pech-Puch et al., 2016) off the coast of Sisal, Yucatan, Mexico. After capture, the adults were transported in tanks containing aerated sea water and kept at the laboratory of the UNAM, Sisal, according to best practices for this species as described by Rosas et al. (2014). Fertilized females were housed individually in 320-L rectangular dark tanks until spawning. The animals were fed twice a day with frozen crabs (*Callinectes* spp. weighing about 130 g; half crab in the morning and half in the afternoon). The tanks were cleaned daily to remove food leftovers and feces. Water quality parameters were monitored daily and maintained within the range recommended for *O. maya* (Rosas et al., 2014). Food was withdrawn if either the female octopus stopped feeding spontaneously or when spawning was observed.

After spawning, the eggs were artificially incubated for approximately 45 days, following the protocol established for this species (see details in Rosas et al., 2014). Hatchlings were cultured for around 2 months in 7.5 m^2 (5.0 × 1.5 × 0.4 m) rectangular dark tanks at a density of 50–60 individuals m^{-2}. Each tank contained at least one or two gastropod shells (*Melongena corona bispinosa* or *Strombus pugilis*, depending on the juvenile's size) per animal to provide refuge. Animals were fed twice a day *ad libitum*, with a semi-dried crab-squid paste bound with gelatine (Rosas et al., 2008; Vidal et al., 2014). Every 20 days, the animals were weighed and separated according to size (±1 g of wet weight) to reduce competition and avoid cannibalism. During embryonic development and hatchlings rearing, temperature, salinity, pH, and dissolved oxygen (DO) concentration were maintained within the adequate ranges for this species, as previously described (Rosas et al., 2014). For the experiments, 2-month-old juveniles were housed individually as described below. The animals were fed, twice a day *ad libitum*, using the same diet as the hatchlings. During the experimental period, the plastic containers housing the juvenile octopuses (see details below) were cleaned daily to remove food leftovers and feces. Sea water quality parameters were recorded in a daily basis and maintained within the adequate ranges for this species, as follows: 24 ± 1°C; 35–38 PSU, pH > 8, DO > 5 mg L^{-1} and a 10:14 light/dark photoperiod regime with an intensity of 30 lux cm^2.

Investigation of Potential Anesthetic Agents

Study Design

A total of 98 juveniles (n = 7 animals/treatment), aged approximately 2 months, were weighed (wet weight mean ± SD: 1.67 ± 0.5 g) on a digital semi-analytical balance (OHAUS Scout SC2020) and randomly assigned to the experimental groups (see below; **Table 1**). The sex of the juveniles cannot be determined at this age. The animals were housed individually in square plastic containers (500 mL) inside a tank with recirculating sea water to acclimatize for 10-days. Each container had two apertures

TABLE 1 | Summary of the experimental groups: *Octopus maya* juveniles (n = 98; wet weight 1.67 ± 0.5 g) exposed or not to the substances (or cold sea water) to be used as potential anesthetic agents.

Experimental group	Concentration	Temperature
SW – H	–	25°C
SW + H	–	25°C
SW 11°C	–	11°C
SW 13°C	–	13°C
EtOH 0.5%	0.5%	25°C
EtOH 1.5%	1.5%	25°C
EtOH 3.0%	3.0%	25°C
$MgCl_2$ 0.75%	0.75%	25°C
$MgCl_2$ 1.5%	1.5%	25°C
$MgCl_2$ 3.75	3.75%	25°C
Mix 1.5:0.75%	1.5% EtOH:0.75% $MgCl_2$	25°C
Mix 0.75:1.13%	0.75% EtOH:1.13% $MgCl_2$	25°C
Mix 2.25:0.37%	2.25% EtOH:0.37% $MgCl_2$	25°C
Clove oil	0.15 mL L^{-1}	25°C

SW: sea water (25°C, unless if specified); SW – H: sea water, without handling; SW + H: sea water, with handling (see text for details). EtOH, ethanol; $MgCl_2$, magnesium chloride; Mix, ethanol in combination with magnesium chloride.

(∼5 × 7 cm) covered with plastic mesh (5 mm) on each side to allow the flow of water (**Supplementary Figure S1**); the meshes were cleaned daily. A gastropod shell (*Strombus pugilis*) was provided as den.

After the acclimatization period, the oxygen consumption in sea water before, during and after exposure to the potential anesthetic agents was measured in a continuous flow respirometer (see details below). Subsequently, the octopuses were reared for another 14 days, and were then once again exposed to the potential anesthetic agents and the criteria for general anesthesia was analyzed. In the second exposure, each animal was exposed to the same substance and concentration as used during the first exposure (for oxygen consumption measurements purposes). Following, octopuses were reared for another 14 days. Surviving animals were used in subsequent studies in the laboratory following recovery. The experimental timeline is summarized in **Figure 1**, which also shows the key measurements made at each time point.

Oxygen Consumption Measurements

Oxygen consumption (VO_2) was measured using a continuous flow respirometer comprised of a closed chamber connected to a recirculating sea water system (Rosas et al., 2008). Prior to the measurements, the juvenile octopuses were fasted for 24 h. Then, the animals were placed in 90 mL respirometric chambers with an approximate sea water flow rate of 100 mL min^{-1}, in which they remained for 12 h before the experimental measurements. A *St. pugilis* shell was placed inside each chamber as a den for the octopuses. A chamber without any octopus but containing a shell was used as a control. Measurements of DO were recorded every 15 s for each chamber (input and output) with oxygen sensors attached to flow-cells connected by optical fiber to an Oxy 10 mini-amplifier (PreSens, Germany). The sensors were calibrated with seawater gassed with air to produce sea water with 100% DO and with a 5% sodium sulfate solution (0% DO). Sea water temperature in the chambers was maintained at 25°C, except during exposure to cold sea water (see **Table 1** for details).

Data of oxygen consumption obtained 45 min before anesthesia were considered as a baseline of the treatments. Subsequently, the potential anesthetic agent or cold sea water was introduced into the chambers via the flow-through seawater system that filled the chambers. This procedure allowed a gradual exchange (around 1 min) of the water in the chambers for the potential anesthetic solution or cold sea water, without provoking an additional stress to animals. When the octopuses met the criteria for general anesthesia (see below) they were immediately removed from the respirometer, transferred to a container (exposed to air) for a standardized brief period of handling and weighing (180 s; protocol comparable to Gonçalves et al., 2012). While the animals were being handled, the respirometric chambers were emptied and refilled with fresh aerated sea water at 25°C and 35–38 PSU. After handling, the animals were returned to the respirometric chambers for recovery and the oxygen consumption measured for another 45 min. At the end of this period, food was offered to the octopuses and the presence of an attack response within 300 s was used as an indicator of recovery as described by Gonçalves et al. (2012) for *Sepia officinalis*. The animals were then returned to their individual home plastic containers.

Criteria for General Anesthesia

Criteria for general anesthesia in cephalopods has been the subject of several original publications (Andrews and Tansey, 1981; Messenger et al., 1985; Gonçalves et al., 2012; Polese et al., 2014; Butler-Struben et al., 2018) and reviews (Andrews et al., 2013; Gleadall, 2013b; Fiorito et al., 2015). In this article, investigating potential agents for the first time in *O. maya* we used the following criteria: (i) a decrease in chromatophore tone (paling) of the skin of the mantle, head and arms; (ii) a loss of muscle tone manifest as flaccidity of the arms and mantle; (iii) reduced or absent sucker adhesiveness indicated by inability to adhere to the walls of the chamber or by touching; (iv) loss of normal posture and immobility; (v) cessation of breathing indicated by absence of obvious contraction of the mantle; (vi) absence of a response to a noxious mechanical stimulus (pinch of the skin of the arms or the orbit). To assess general anesthesia, the octopuses were transferred from their home plastic containers to a circular, white plastic container (250 mL). The time to induce general anesthesia is defined as the time at which all the above criteria were met after introduction of the animal in the potential anesthetic solution. However, as one of the aims of this study was to identify agents which could be used to induce anesthesia relatively quickly for routine handling and non- or minimally invasive procedures and from which the animals would recover quickly we set a maximum time of 600 s for an agent to fulfill the criteria (Gonçalves et al., 2012). Time to recovery is the time after transfer to aerated sea water to when the animals were breathing, had regained a normal posture and colouration as assessed by visual inspection. A 600 s cut off was used for measurement of recovery time. In the first part of the experiment, during which animals were exposed to the potential anesthetic solutions

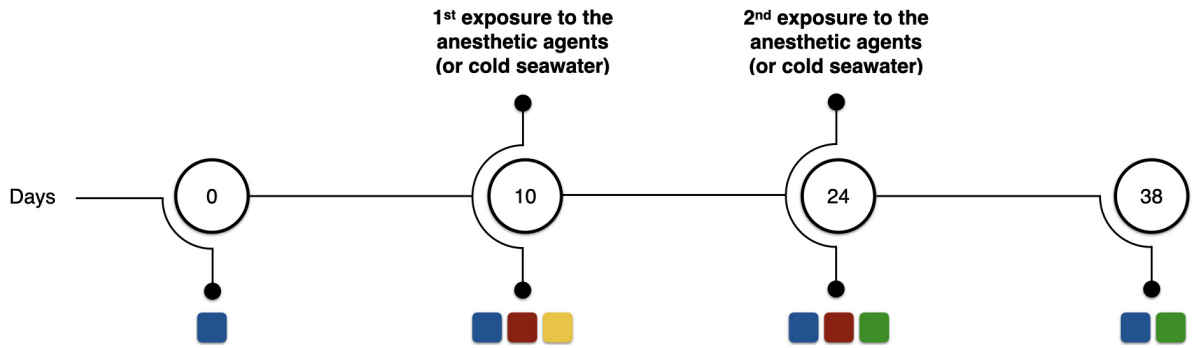

FIGURE 1 | Schematic representation of the experimental timeline. Blue squares represent wet weight measurements; red squares assessment of criteria to anesthesia; yellow square represents oxygen consumption measurements before, during, and after exposure to the presumed anesthetic agents; and green squares represent assessment of mortality rates in relation to the precedent anesthesia. Numbers inside the circles represent the day of the experimental period in which the analyses were carried out.

in the respirometer chambers, it was only possible to visually assess criteria (i) to (v) as described above. In the subsequent exposure, carried out in an open chamber, all six criteria were assessed. In both studies, the same handling and attack response protocols were used, however, after the first exposure, the attack response was assessed after the full period of oxygen consumption measurements during recovery (45 min) while in the second exposure, the attack response was assessed immediately after recovery. The attack response was recorded only as positive (+) or negative (−). During all the experiments, data were assessed always by the same two people and in such way that one of them made the observations and the other made the records.

Growth and Mortality

Possible longer-term effects of repeated exposure to the anesthetic agents were determined by measuring growth and mortality. The weight of the animals was determined at the beginning and at the end of the experimental protocol, as well as at each anesthesia event (during handling) (**Figure 1**). Daily growth coefficient (DGC) expressed as %BW d^{-1}, where BW is body weight (wet weight) in g. DGC was calculated for each octopus taking into account the experimental periods in which the animals were anesthetized and/or manipulated (day 0 to 10, day 10 to 24, and day 24 to 38); total growth rate was calculated considering all the experimental period (from day 0 to 38). DGC was calculated using the equation: DGC (%BW d^{-1}) = [(Ln BW_2 − Ln BW_1)/t] × 100, where BW_2 and BW_1 are the final and initial wet weights of the juveniles, respectively, Ln the natural logarithm and t the number of days of the experimental period. Mortality was calculated as the number of deaths in the 14 days following each anesthetic exposure.

Experimental Groups

Exposure to cold sea water (SW + temperature), ethanol (EtOH), magnesium chloride ($MgCl_2$), combinations of ethanol with magnesium chloride (Mix), and clove oil were selected to be tested for their potential as general anesthetic agents (for details, see **Table 1**) for *O. maya* juveniles. Anesthetic agent concentrations were selected based on the literature (Andrews and Tansey, 1981; Messenger et al., 1985; Mooney et al., 2010; Gonçalves et al., 2012; Gleadall, 2013b; Fiorito et al., 2015). The temperatures of cold sea water were based on the Critical Thermal Minima (CTMin) established for *O. maya* juveniles by Noyola et al. (2013a) at which the metabolic rate is nearest to zero (i.e., 11.6°C). Exposure to sea water at 11 and 13°C was achieved by decreasing the temperature with bottles of frozen sea water. The solutions containing the anesthetic agents were prepared just before use. Ethanol (Merck, Mexico) concentrations (0.5–3.0%) were obtained by direct dilution in sea water. Solutions containing magnesium chloride (Hexahydrate; Sigma Aldrich, Mexico) (0.75–3.75%) were obtained adjusting distilled and sea water volumes, in order to maintain the salinity similar to the sea water of the acclimation tank. For clove oil (Sigma Aldrich, Mexico), a stock solution was dissolved in 96% ethanol at a ratio of 1:10 and then diluted in sea water to obtain the final concentration (0.15 mL L^{-1}). Two experimental groups not exposed to any anesthetic agent, without (SW − H) or with handling (SW + H), were also evaluated (**Table 1**).

Statistical Analysis

All data were tested for normality distribution. Chi-squared test was used to compare if anesthetic agents induce complete anesthesia and affect the incidence of attack response after recovery. Data on wet weight were transformed on Ln and analyzed by linear regression. ANCOVA analysis was applied to known differences between changes in time of wet weight of animals manipulated along the experimental period. The result of this analysis showed that the changes in wet weight of octopus anesthetized with clove oil were statistically different from octopus from other treatments. For this reason, a new ANCOVA analysis was developed comparing data of wet weight changes of animals exposed to clove oil and the remaining treatments, including the treatments were animals were not exposed to any anesthetic agent. Regression analysis and ANCOVA tests were done using Prism software (6.0). To evaluate if the anesthetic agent and handling affected the growth rate of the animals (%BW d^{-1}), a one-way ANOVA was applied to the data at the end of the experiment. Additionally, one-way ANOVA followed

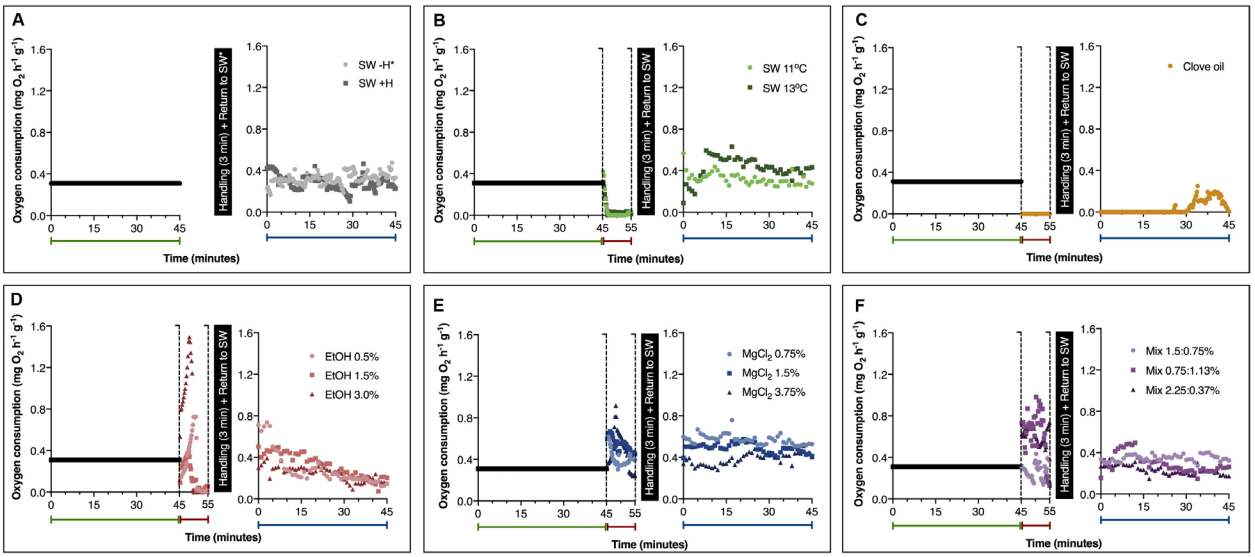

FIGURE 2 | Oxygen consumption of *Octopus maya* juveniles in sea water and before, during and after the exposure to different substances (or cold sea water). Green, red and blue labels represent oxygen consumption before (baseline), during and after anesthesia and/or handling (recovery), respectively. **(A)** Animals not exposed to any anesthetic agent; Animals exposed to **(B)** cold sea water; **(C)** clove oil; **(D)** ethanol; **(E)** magnesium chloride; and **(F)** ethanol in combination with magnesium chloride. SW: sea water (25°C, unless if specified); SW – H: sea water, without handling; SW + H: sea water, with handling (see text for details). EtOH, ethanol; $MgCl_2$, magnesium chloride; Mix, ethanol in combination with magnesium chloride (concentrations are provided in this order). *SW – H measurements were performed without any interruption. Data obtained from the first exposure to anesthesia. Note: As the baseline oxygen consumption was similar in the experimental groups ($p > 0.05$), we used the calculated mean value as a visual reference of the baseline oxygen consumption of animals (see section "Results" for details).

by Newman–Keuls was used to test the effect of the anesthetic agents on induction and recovery times of animals anesthetized and manipulated. Oxygen consumption measurements were analyzed considering three different stages: baseline (before anesthesia) (all 14 treatments: 12 exposed to the anesthetic agents or cold sea water and two not exposed); anesthesia (11 treatments, excluding the treatments in which animals were not exposed to any anesthetic agent and the treatment in which the animals were exposed to clove oil, since this treatment produced non-detectable values of oxygen consumption, considered as zero) and recovery (after anesthesia and/or handling) (all 14 treatments). The results for the baseline were analyzed using a one-way ANOVA. The effects of each treatment and the interaction with the time of exposure were considered while analyzing the data obtained from the animals during and after anesthesia. Each group of data, anesthesia, and recovery were analyzed separately with a two-way ANOVA, followed by the Tukey Honesty test to determine significant differences among the experimental groups. In ANOVA tests, homogeneity of variances was evaluated using the Cochran test. A histogram was used to test the normality of the data. Differences were considered statistically significant at the $p < 0.05$. For all ANOVA tests we used STATISTICA software.

TABLE 2 | Results of two-way ANOVA on oxygen consumption of *Octopus maya* juveniles exposed to the different anesthetic agents.

	SS	DF	MS	*F*	*p*
Anesthetic agent	131.6	9	14.62	320.6	0.00*
Time	31.5	39	0.81	17.7	0.00*
Anesthetic agent vs time	103.1	351	0.29	6.4	0.00*

*This analysis was made considering the data obtained from the 11 experimental groups, excluding the groups in which the animals were not exposed to any anesthetic and the data obtained from animals anesthetized with clove oil, since this treatment produced only non-detectable values of oxygen consumption (considered as zero). *Statistical differences at p < 0.0001 level.*

RESULTS

Oxygen Consumption Measurements

Mean values on oxygen consumption obtained from animals exposed to the different substances (or cold sea water) before, during (first 600 s) and after anesthesia and/or handling are presented in **Figure 2**. Baseline oxygen consumption (i.e., before exposure to the presumed anesthetic agents) was similar in the experimental groups ($p = 0.052$; $F = 93.28$). For this reason, a mean value was calculated (0.31 ± 0.09 mg O_2 h^{-1} g^{-1}). During exposure to the presumed anesthetic agents, the results on oxygen consumption showed that the metabolic rate was affected by treatment and time of exposure (**Table 2**). In octopuses exposed to both temperatures of cold sea water (11 and 13°C), a significant decrease on oxygen consumption was recorded after 120 s of exposure, producing non detectable values (considered here as zero) of metabolic rate ($p < 0.00001$) (**Figure 2B**). Juvenile octopuses exposed to different concentrations of EtOH also showed a significant reduction on oxygen consumption to non-detectable values (**Figure 2D**). These values were reached

earlier on animals exposed to EtOH 3.0% (after 285 s of exposure) than in animals exposed to EtOH 0.5 and 1.5% (after about 360 s of exposure) ($p < 0.014$). However, it should be noted that animals exposed to EtOH 3.0% showed an increase on oxygen consumption after 180–240 s of exposure, reaching the highest values registered among all treatments (**Figure 2D**). The mean value calculated for this period of exposition to EtOH 3.0% was 1.46 ± 0.4 mg O_2 h^{-1} g^{-1}, 4.7 times higher than the mean value calculated for the baseline. A part from hyperventilation, represented by an initial increase in oxygen consumption of the animals exposed to EtOH concentrations during the first 300 s of exposure, no apparent stressful reactions were observed in these animals. In general, oxygen consumption of the animals exposed to all concentrations of $MgCl_2$ remained practically constant during the 600 s of exposure. A significant increase on oxygen consumption of octopuses exposed to $MgCl_2$ 3.75% after 180–210 s of exposure was observed, with values reaching 0.84 ± 0.3 mg O_2 h^{-1} g^{-1} ($p < 0.01$). No significant differences were observed in the other values registered ($p > 0.05$; **Figure 2E**). In octopuses exposed to Mix 1.5:0.75%, oxygen consumption in the first 260 s of exposure was lower (0.23 ± 0.05 mg O_2 h^{-1} g^{-1}) than in the animals exposed to Mix 0.75:1.13% and 2.25–0.37% (mean values of 0.66 ± 0.16 mg O_2 h^{-1} g^{-1}) ($p < 0.0012$; **Figure 2F**). Likewise, an increase on the oxygen consumption of the animals exposed to Mix 1.5:0.75% was registered between 360 and 450 s of exposure, with mean values of 0.82 ± 0.17 mg O_2 h^{-1} g^{-1} ($p < 0.023$), followed by a quick reduction reaching 0.15 ± 0.02 mg O_2 h^{-1} g^{-1} after 540–600 s of exposure ($p < 0.0134$; **Figure 2F**). Exposure to clove oil cause a reduction in the oxygen consumption of the animals during anesthesia, however, a full recovery with values of oxygen consumption comparable to the baseline was missing at the end of 45 min after exposure (**Figure 2C**).

After manipulation and during recovery, in animals not exposed to any anesthetic agent, oxygen consumption was similar to the values registered during baseline (**Figure 2A**). In *O. maya* juveniles exposed to SW 11°C, the oxygen consumption during recovery was comparable to the baseline, with a mean value of 0.32 ± 0.10 mg O_2 h^{-1} g^{-1} (**Figure 2B**). Similarly, oxygen consumption of the animals exposed to SW 13°C was also comparable to baseline oxygen consumption in the first 10 min of recovery. Although, a small increase of oxygen consumption was detected after this period in these animals when compared to octopuses exposed to SW 11°C, at the end of the recovery period, both groups presented values comparable to baseline oxygen consumption (**Figure 2B**). Values of oxygen consumption comparable to the baseline were also observed in juvenile octopuses exposed to EtOH, $MgCl_2$, and Mix (**Figures 2D–F**), except by animals exposed to $MgCl_2$ 0.75%, which showed, at the end of the recovery period, oxygen consumption significantly higher than the ones registered during the baseline (**Figure 2E**; $p < 0.01$).

Criteria for General Anesthesia

Except for clove oil, all other anesthetic agents induced (in a given concentration) general anesthesia in the juvenile *O. maya*. All animals exposed to sea water at 11°C, EtOH 3.0%, $MgCl_2$ 3.75% and Mix 1.5:0.75% fulfilled all the criteria for general anesthesia in the pre-established time (600 s). Ethanol 1.5% and Mix 2.25:0.37% induced general anesthesia in 86% of the animals, whereas only 26% exposed to Mix 0.75:1.13% fulfilled all the criteria. In contrast, all animals exposed to sea water at 13°C, EtOH 0.5%, $MgCl_2$ 0.75 and 1.5% and clove oil did not fulfill the criteria for induction to anesthesia (**Figure 3A**). Although the juveniles exposed to clove oil met some of the criteria (except for criteria ii and iii), typical adverse response pattern (TARP) as described by Gleadall (2013a) were observed in all animals. Reactions included fast disoriented swimming behavior and erratic swimming movements, attempts to escape and jetting against the container's wall, violent contraction of the mantle, sometimes causing inability to breath (closing of the funnel), rapid color changes, exaggerated raising papillae, abnormal lageniform appearance of the mantle (see Gleadall, 2013b for details) and inking. One animal was not able to completely release the ink due to mantle rigidity and closure.

Induction and recovery times (see section "Materials and Methods" for definitions and protocol) in the animals that fulfilled the criteria for general anesthesia are presented in **Figure 3B**. Induction times were similar between the treatments with exception of animals exposed to EtOH 3.0% which had lower induction times ($p < 0.05$) when compared to animals exposed to Mix 2.25:0.37%. On the other hand, animals exposed to sea water at 11°C, EtOH 1.5% and Mix 2.25:0.37% recovery was slower ($p < 0.05$) than animals exposed to Mix 0.75:1.13%.

The use of anesthetic agents and/or handling affected the attack response of *O. maya* juveniles ($p < 0.001$). Animals that were not exposed to anesthesia or handling (SW − H) promptly responded to the presence of food, whereas in animals submitted only to handling (SW + H), a reduced incidence of attack response was observed. After exposure to EtOH 1.5 and 3.0%, more than 80% of the animals showed predatory behavior. In animals exposed to SW at 11°C, EtOH 0.5% and all concentrations of the mixed solutions, incidence of attack responses varied between 57 and 71%. On the other hand, incidence of attack response was reduced or inhibited in animals exposed to all concentrations of $MgCl_2$, SW 13°C and clove oil (**Figure 4**).

Growth and Mortality

Except for the animals exposed to clove oil, the growth of *O. maya* juveniles was similar in all other groups [wet weight (g) = $1.98e^{0.021(T,days)}$; **Figure 5**]. The animals exposed to clove oil had a reduced growth rate compared to all other experimental groups after the first and second exposure [$p < 0.0006$; wet weight (g) = $1.66e^{0.009(T,days)}$; **Figure 5**, **Supplementary Tables S1**, **S2**, and **Supplementary Figure S2**]. Mortality of the juveniles after the first and the second exposure to the anesthetic agents are presented in **Figures 6A,B**, respectively. After the first exposure, mortality was observed only in animals exposed to $MgCl_2$ 3.75%, Mix 0.75:1.5%, and clove oil. Nevertheless, after the second exposure, natural mortality was observed in nine of the 14 experimental groups, including in the groups where animals were not exposed to anesthesia (i.e., SW − H and SW + H).

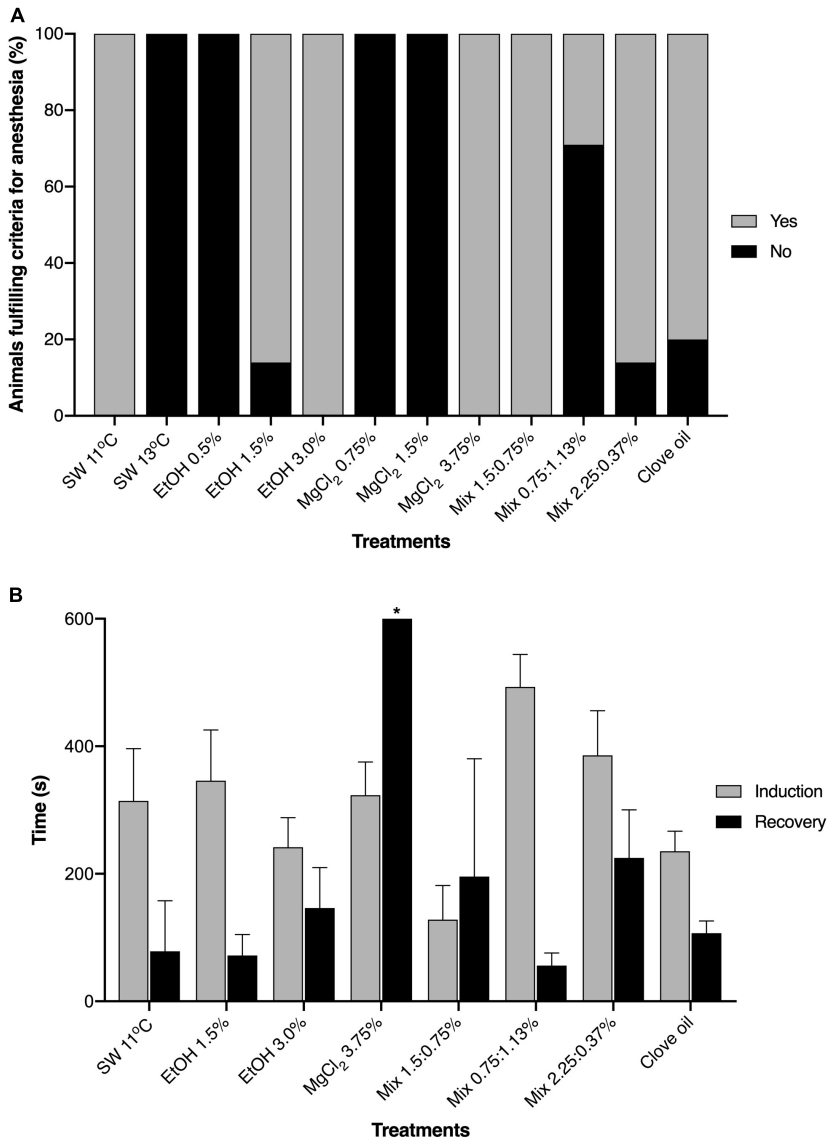

FIGURE 3 | (A) Percentage of *Octopus maya* juveniles that fulfilled the criteria (see section "Materials and Methods") for complete induction to general anesthesia when exposed to different substances (or cold sea water). **(B)** Times of induction and recovery from anesthesia. SW, sea water; EtOH, ethanol; $MgCl_2$, magnesium chloride; Mix, ethanol in combination with magnesium chloride (concentrations are provided in this order). Data obtained from the second exposure to anesthesia. *Animals exposed to $MgCl_2$ 3.75% did not fulfill the criteria for recovery from anesthesia in the pre-established time of 600 s (which does not mean that they did not recovery from anesthesia).

DISCUSSION

Here, we provide, for the first time, original data regarding anesthesia in the warm-water species *O. maya*, including the physiological effects of exposure to anesthetics on octopuses' oxygen consumption. We also include additional information on possible longer-term effects of repeated anesthesia on the growth and mortality of juvenile octopuses. As one of our goals was to define an agent which induces anesthesia in a short period (less than 600 s) to perform quick handling (>180 s), we focused the discussion mainly in the substances considered, according to our requisites, adequate for these purposes. For this reason, we will not discuss in-depth the data obtained from animals exposed to clove oil, since it is clearly not suitable to anesthetize this species. Although our experiments were carried out with *O. maya* juveniles, TARP was also observed in adult *O. maya* (unpublished data), thus, we strongly believe that clove oil does not produce effective anesthesia in this species.

Considerations Regarding Anesthesia and Anesthetic Agents in Cephalopods

Immersion baths in the anesthetic solution are the most commonly method utilized to anesthetize cephalopods.

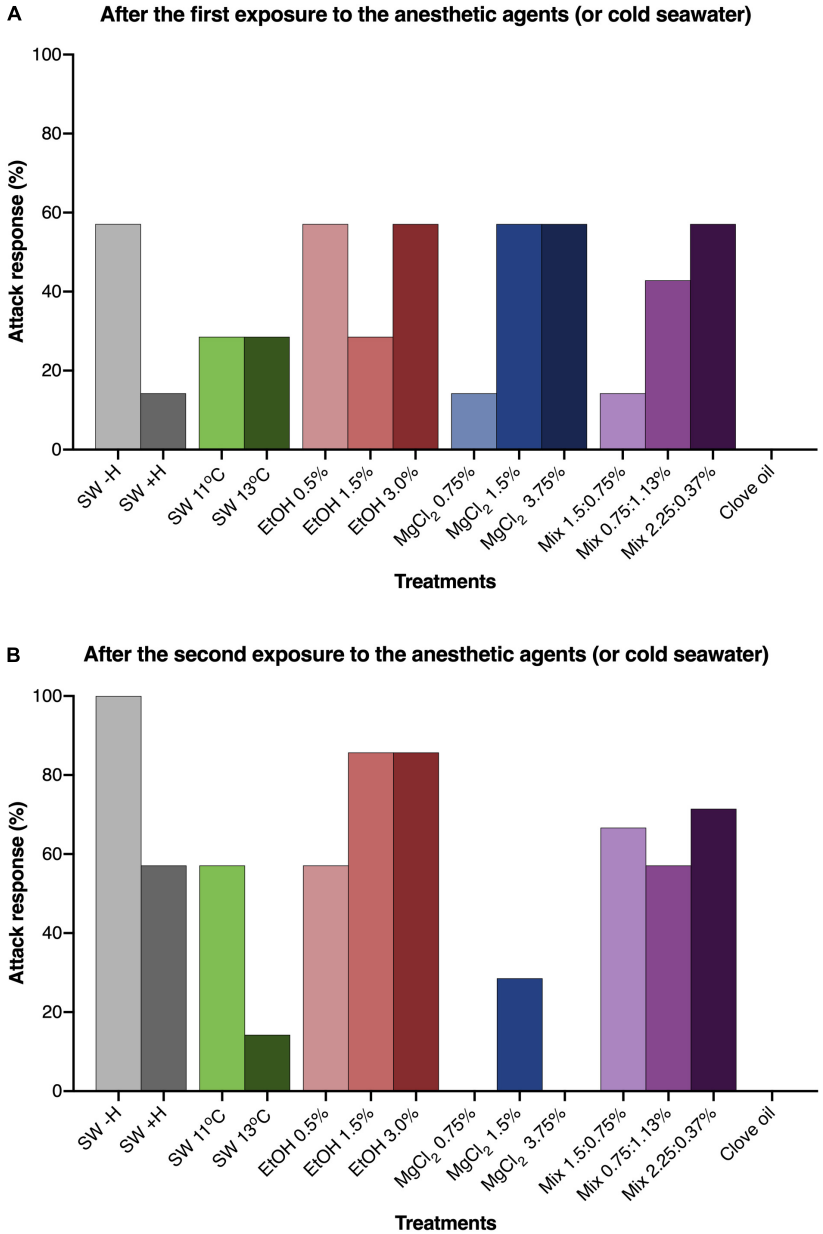

FIGURE 4 | Percentage of *Octopus maya* juveniles that presented attack response when food was offered after the first **(A)** and the second **(B)** exposure to the presumed anesthetic agents (or cold sea water) and in the experimental groups that were not exposed to any anesthetic agent (SW – H; SW + H). SW: sea water (25°C, unless if specified); SW – H: sea water, without handling; SW + H: sea water, with handling (see text for details). EtOH, ethanol; $MgCl_2$, magnesium chloride; Mix, ethanol in combination with magnesium chloride (concentrations are provided in this order). Chi^2 analysis were made to test if the attack response were affected by the type of presumed anesthetic after the first **(A)** and the second **(B)** exposure. For **(A)**: Calculated $Chi^2 = 38.1$; Table $Chi^2 = 31.3$; GF = 14; $p < 0.005$, in this analysis two experimental groups in which the animals were not exposed to the anesthetic agents were added. For **(B)**: Calculated $Chi^2 = 63.2$; Table $Chi^2 = 19.67$; GF = 11; $p < 0.001$. Both testes indicate that the type of presumed anesthetic affected significantly the attack response of *O. maya* juveniles in two testes **(A,B)**.

A range of substances, such as urethane, ethanol, tricaine methanesulfonate (MS-222), benzocaine, clove oil, menthol, magnesium chloride, and sulfate, as well as cold sea water, have been tested as anesthetic agents for cephalopods (see review in Gleadall, 2013b; Fiorito et al., 2015). However, the use of some of these substances were discontinued due to negative and stressful reactions caused to the animals during exposure, low effectiveness of anesthesia, or toxicity to humans. Despite the potential of use of volatile (e.g., isoflourane and sevoflourane) and injectable (e.g., ketamine and propofol) anesthetic agents, commonly utilized in vertebrates, studies with these agents in cephalopods are rare or inexistent (Polese et al., 2014).

The most commonly used substances and considered, to date, the most effective in anesthetizing cephalopods

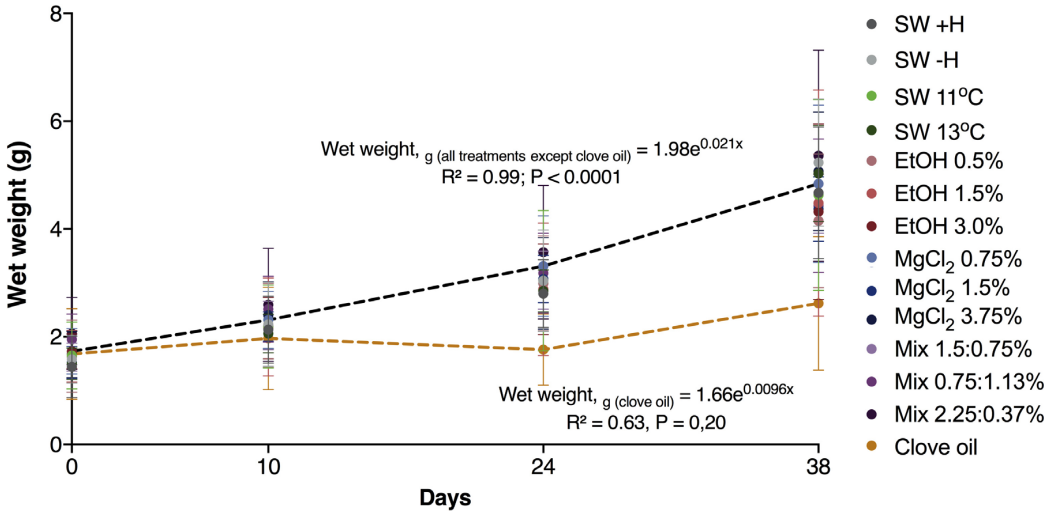

FIGURE 5 | Mean wet weight (±SD) of *Octopus maya* juveniles exposed or not to the anesthetic agents. All treatments except clove oil (continuous line): Wet weight (g) = $1.60e^{0.28(T,days)}$; clove oil (dashed line): wet weight (g) = $1.53e^{0.0089(T,days)}$. ANCOVA analysis showed differences in slopes at $p < 0.0006$ levels between clove oil and the rest of the treatments (see **Supplementary Table S2**).

are the solutions containing ethanol and magnesium chloride, used separately or in combination (Gleadall, 2013b; Fiorito et al., 2015; Butler-Struben et al., 2018). Both substances are of relatively low cost and ease access, manipulation and application. Although ethanol and magnesium chloride are generally not accepted to anesthetize animals from other taxa, satisfactory results have been obtained in cephalopods.

Ethanol in concentrations between 1.0 and 3.0% have been successfully used to anesthetize different species of cuttlefishes, squids and octopuses (for review, see Gleadall, 2013b; Fiorito et al., 2015). However, some authors (Froesch and Marthy, 1975; Andrews and Tansey, 1981) described adverse reactions of the animals after immersion in the anesthetic solution, such as escape attempts and inking. In addition, incomplete induction may occur at low temperatures (Gleadall, 2013b), probably due to a reduction of the narcotizing effect of ethanol (Moore et al., 1964). For this reason, solutions containing ethanol might be ineffective to anesthetise cold-water species.

Magnesium chloride is probably the oldest substance used for anesthetizing invertebrates (Ross and Ross, 2008). Successful induction to anesthesia using different formulations of magnesium chloride (i.e., diluted directly in sea water or in a combination of sea water and distilled water) have been achieved for cephalopod species of different sexes, ages, and sizes (Messenger et al., 1985; Gore et al., 2005; Gonçalves et al., 2012; Gleadall, 2013b; Pugliese et al., 2016; Butler-Struben et al., 2018). Likewise, magnesium chloride had been effectively used for long-duration anesthesia (Mooney et al., 2010). Nonetheless, similarly to ethanol, stressful reactions and inking were eventually noted (Garcia-Franco, 1992). Controversial opinions whether magnesium chloride produces adequate general anesthesia or simply act as neuromuscular blocking agent are discussed in the literature (Andrews et al., 2013; Polese et al., 2014; Fiorito et al., 2015; Butler-Struben et al., 2018; Winlow et al., 2018), however, no consensus is completely achieved to date.

Cold sea water (commonly also referred as hypothermia) as anesthetic agent has the advantage of avoiding the use of chemicals (and potential associated negative effects) (Gleadall, 2013b) and, despite the popularity of use in the past, its anesthetic properties are questionable (Andrews and Tansey, 1981; Gleadall, 2013b; Fiorito et al., 2015; Winlow et al., 2018). Muscle relaxation is usually reported to lack in animals exposed to cold sea water (e.g., Andrews and Tansey, 1981). Immersion in cold sea water may be effective for immobilization rather than inducing general anesthesia (Gleadall, 2013b) and thus, its use in potentially invasive procedures should not be considered.

Clove oil has been successfully used to anesthetize many species of fish (Taylor and Roberts, 1999; Javahery et al., 2012; Priborsky and Velisek, 2018) and aquatic invertebrates (Araujo et al., 1995; Bilbao et al., 2010). Although the potential use of clove oil was speculated for humanely killing cephalopods (Andrews et al., 2013; Fiorito et al., 2015) effectiveness of anesthesia using this agent in some species is uncertain. Satisfactory results were reported only for *O. minor* (Seol et al., 2007). For other species (e.g., *O. vulgaris* – including different life stages, *Dorytheutis pealeii*, *Se. officinalis*), similarly to our results for *O. maya*, no anesthetic effect and/or highly stressful reactions were observed (Mooney et al., 2010; Estefanell et al., 2011; Gonçalves et al., 2012; Escánez et al., 2018).

Effects of the Anesthetics on Animals' Oxygen Consumption

As active animals, oxygen consumption rates of cephalopods are directly related to their activity (Segawa and Hanlon, 1988). For instance, the oxygen consumption measured during active swimming is higher than in resting cephalopods: these values can increase around 2.4 times in *O. vulgaris* (Wells et al., 1983b) up

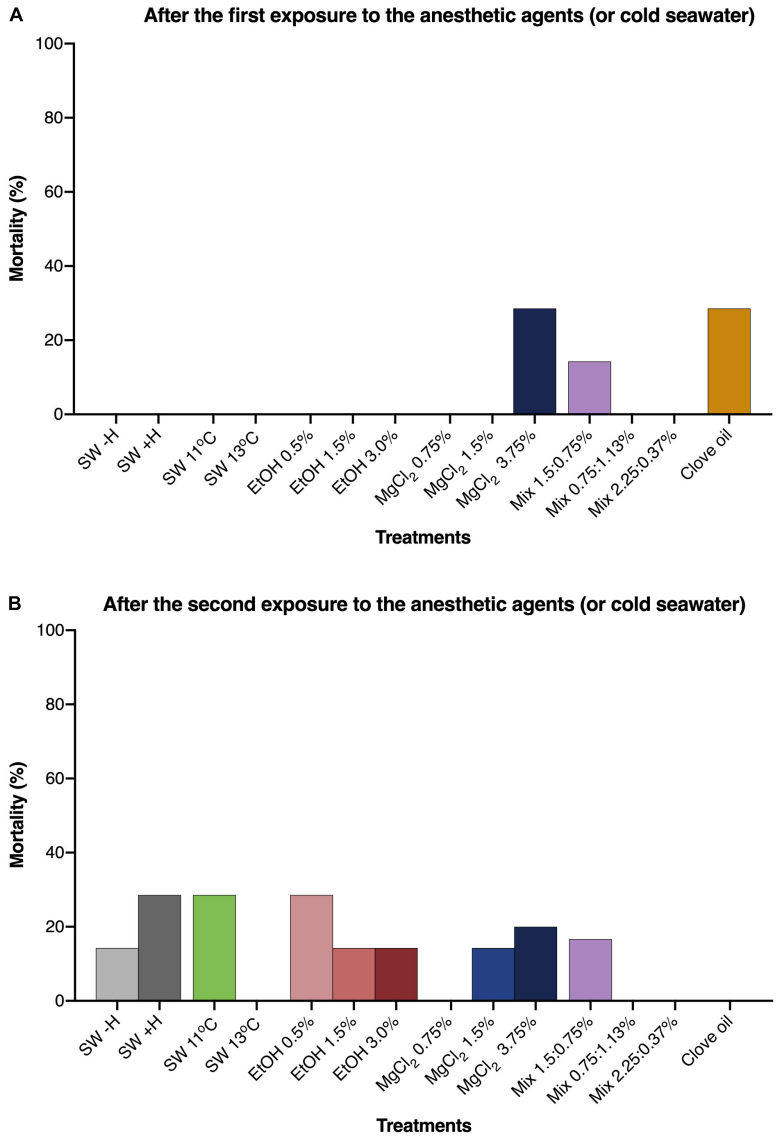

FIGURE 6 | Mortality rates of *Octopus maya* juveniles after the first **(A)** and the second **(B)** exposure to the anesthetic agents (or cold sea water) and in the experimental groups that were not exposed to any anesthetic agent (SW – H; SW + H). SW: sea water (25°C, unless if specified); SW – H: sea water, without handling; SW + H: sea water, with handling (see text for details). EtOH, ethanol; $MgCl_2$, magnesium chloride; Mix, ethanol in combination with magnesium chloride (concentrations are provided in this order).

to 2.6–3.4 times in *Loligo opalescens* (O'Dor, 1982; Webber and O'Dor, 1985) and five times in *Illex illecebrosus* (Hoeger et al., 1987). Similarly, oxygen consumption during and after feeding also increase when compared to fasted cephalopods (Hirtle et al., 1981; Wells et al., 1983a; Rosas et al., 2008).

In the present study, the mean value calculated for baseline oxygen consumption obtained from *O. maya* juveniles (i.e., 0.31 ± 0.09 mg O_2 h^{-1} g^{-1}) was similar to the values previously registered in fasted juveniles of the same species (0.31 ± 0.06 mg O2 h−1 g^{-1}, Rosas et al., 2008; 0.31 ± 0.04 mg O_2 h^{-1} g^{-1} at 22°C, Noyola et al., 2013b). After immersion in the anesthetic solution, hyperventilation is frequently reported in cephalopods (e.g., Andrews and Tansey, 1981; Messenger et al., 1985; Gonçalves et al., 2012) and, consequently, an increase in oxygen consumption is expected. Increased ventilation frequency can be considered an indicator of physiological stress (Fiorito et al., 2015). Thus, this should be reduced to a minimum (Gleadall, 2013a) and be followed by a significant reduction in ventilation frequency (and, consequently, in oxygen consumption), expected to occur as a result of an adequate anesthesia induction and lower activity levels of the animal.

In fact, our results indicate that animals exposed to different concentrations of EtOH, Mix 0.75:1.13%, and $MgCl_2$ 3.75% showed an increase in oxygen consumption during the beginning of the exposure. Nonetheless, in general, the values registered during anesthetic exposure are comparable to the ones obtained

for *O. maya* juveniles during feeding (up to 3.4 times higher than in fasted animals, depending on the diet) (Rosas et al., 2008). Although oxygen consumption obtained from the juveniles exposed to EtOH 3.0% was higher (∼4.7 times than baseline values), this lasted less than 300 s and was followed by a significant decrease in these values. Interestingly, in animals exposed to the mixed solutions (EtOH + $MgCl_2$) this effect was much lower or absent when compared to the animals exposed to EtOH alone. This is probably due to the combined effects of both substances. The combination of substances to obtain general anesthesia, each contributing to the overall effect, is a common practice in laboratory animals (Flecknell, 2015) but still need to be better explored in cephalopods.

Exposure to cold sea water caused a direct reduction in oxygen consumption of the juvenile octopuses, as expected, since lower temperatures decreases animal's metabolism. When considering the use of cold sea water to "tranquilize" or immobilize cephalopods, CTMin and time of exposure should be considered to avoid excessive stress. The exposure of animals to temperatures below the CTMin may induce disorganized locomotory responses, signs of stress and atypical behaviors, ultimately leading to death (Noyola et al., 2013a). Nonetheless, CTMin is not yet known for the great majority of the commonly studied species. In addition, to avoid a possible cold-shock stress, gradual cooling of sea water might be more appropriate.

The relationship between oxygen consumption and the exact moment at which the animal fulfills the criteria for anesthesia, which includes immobility and marked reduction or absence of respiration, should be further investigated. During recovery, almost all experimental groups presented values of oxygen consumption comparable to baseline values. There was no evidence for an overshoot in oxygen consumption after a period with reduced or no ventilation.

Criteria for General Anesthesia and Requisites for Selecting Anesthetic Agents

Anesthetic agents and concentrations are frequently used indiscriminately of the species and following protocols already developed for the most commonly studied or cultured species (Zahl et al., 2012). However, the anesthetic agent and the protocol used for one species may not be appropriate for others or even for a diverse life stage of the same species. This is similarly important when defining criteria for anesthesia. For instance, Escánez et al. (2018) considered *O. vulgaris* paralarvae anesthetized when they "lost the ability to swim and remained perfectly still on the bottom," which differs from the criteria commonly used for adults of the same species (Andrews and Tansey, 1981; Estefanell et al., 2011; Butler-Struben et al., 2018).

In the present study, we tried to be the most precise as possible when defining the criteria for assessing general anesthesia in order to facilitate the standardization of a protocol to be used for *O. maya* juveniles. The criteria defined here represent behavioral indicators that accomplish, in general lines, the definition of anesthesia. Although we do not accessed consciousness, a recent study has demonstrated that magnesium chloride and ethanol are considered "genuine anesthetics" for cephalopods (Butler-Struben et al., 2018).

One of the criteria considered to evaluate general anesthesia in cephalopods is the decrease and, eventually, cessation of ventilation (Gleadall, 2013b; Fiorito et al., 2015). Cessation of ventilation would be an unacceptable criterion for mammals. Therefore, this might be questionable for cephalopods as well. Although cutaneous oxygen uptake has been demonstrated in cephalopods (Wells and Wells, 1983; Madan and Wells, 1996) its contribution for oxygen acquisition only recently has been systematically explored (Birk et al., 2018). In addition, the effects on brain function and/or possible tissue damages after hypoxia are not well understood.

For our purposes, we define that a given anesthetic agent and concentration should produce quick induction and recovery (600 s), with side-effects and stressful reactions reduced to a minimum, and allow animals' handling during 180 s. Following these definitions, except for clove oil, all the anesthetic agents tested induced (in a given concentration) general anesthesia in *O. maya* juveniles. All octopuses exposed to SW 11°C, EtOH 3.0%, and Mix 1.5:0.75% fulfilled the criteria defined for general anesthesia. Animals exposed to $MgCl_2$ 3.75%, despite fulfilling the criteria for general anesthesia, presented recovery times exceeding 600 s.

Another relevant factor to be considered for anesthetic selection, are the induction and recovery times. Anesthesia should have rapid action, with stress reactions and hyperactivity, reduced to a minimum; similarly, recovery should not last long and none or minimum side-effects are desirable (Winlow et al., 2018). For fishes, recommended times of induction and recovery should be preferably not longer than 3 and 5 min, respectively (Ross and Ross, 2008). In the present study, times of induction were, in general, similar among the substances that induce complete anesthesia according to our criteria and varied from around 180 (EtOH 3.0%) to 480 s (Mix 0.75:1.13%). Recovery times were shorter, varying from around 60 s (SW 11°C) and 240 s (Mix 2.25:0.37%). Animals exposed to $MgCl_2$ 3.75% did not recovery in 600 s, which does not mean that they did not recovery from anesthesia, but simply that they did not meet the requisites established for this study.

Short- and Long-Term Effects of Anesthesia

The exposure to an anesthetic can result in important direct consequences in the physiology and behavior of the animals and eventually impair food intake, growth and other important aspects in the long-term (Ross and Ross, 2008). Our results show significant changes in attack response to food in animals exposed to the different anesthetic agents. The attack response in animals exposed to all concentrations of $MgCl_2$, SW 13°C and clove oil, differently from the other experimental groups, was inhibited after the second exposure. This inhibition could be an adverse effect of repeated exposure to these agents. After the second anesthesia, animals exposed to EtOH 1.5 and 3.0% showed higher attack response when compared to animals exposed to other anesthetic agents and animals that were just

handled (SW + H). Although different species may have distinct responses, similar results were observed by Gonçalves et al. (2012) in cuttlefishes exposed to EtOH and clove oil using similar concentrations. On the other hand, different responses in cuttlefishes exposed to $MgCl_2$ were observed. It should be noted that these results might be influenced by species, size of animals and the applied methodology (including differences in concentration of some anesthetics).

To the best of our knowledge, long-term effects of anesthesia have never been explored in cephalopods. In the present study, clove oil was the only substance that affect *O. maya* juveniles' growth, which reinforces that this agent is not suitable to anesthetise this species. A high number of deaths in the end of the experiment (four out of seven) was observed in animals exposed to $MgCl_2$ 3.75%. Pugliese et al. (2016) observed that the worst cardiac performance of isolated hearts of *O. vulgaris* previously exposed to anesthetics was obtained with a concentration similar to the one used in the present study (i.e., $MgCl_2$ 3.5% dissolved in sea- and distilled-water; 1:1). These findings could possibly explain the high mortality rate in animals exposed to $MgCl_2$ 3.75% observed in our study. Nonetheless, we are aware that the data obtained for mortality should be analyzed with caution, since natural mortality was also observed in other experimental groups, including the ones where animals were not exposed to anesthesia.

CONCLUSION

In conclusion, our study provides insights into the metabolic and behavioral responses of juvenile octopuses to anesthesia. Our results showed that during short-term handling (not longer than 180 s) of *O. maya* juveniles that do not cause pain, distress, suffering or lasting harm to the animals, the use of anesthetic agents can be eventually suppressed. If the handling will be carried out easily in an anesthetized/tranquilized animal, we suggest the use of EtOH 3.0% or cold sea water 11°C. These agents presented the most appropriate results according to our criteria: satisfactory metabolic responses; absence or reduced stressful reactions during induction and recovery from anesthesia; induction and recovery times within the maximum pre-specified time range (600 s); adequate growth and low mortality rates. The data presented here might serve as a reference for other cephalopod species, specially, juvenile warm-water octopuses. Ultimately, the protocol described in this study might contribute to improve *O. maya* welfare during laboratory practices, as well as the quality of research data.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Ethics Committee of the Faculty of Chemistry at Universidad Nacional Autónoma de México (UNAM, Sisal). Permit Number: Oficio/FQ/CICUAL/099/15.

AUTHOR CONTRIBUTIONS

KR and CR conceived and designed the study, and drafted the manuscript. KR, MA, and JP conducted the experiments. CR and CP performed the data analysis. All authors interpreted the findings together and approved the final version of the manuscript.

FUNDING

KR was supported by the Coordination for the Improvement of Higher Education Personnel (CAPES) for Ph.D. (Brazil) and Ph.D. Sandwich (6419/2014-03; Mexico) scholarships. This research was partially financed by the project CAPES Ciências do Mar 43/2013 and PAPIIT – UNAM program IN204019 to CR.

ACKNOWLEDGMENTS

We would like to thank our colleagues from the UNAM (Mexico), specially Claudia Camaal, Elisa Chan, and Ariadna Sánchez for their technical collaboration. We are grateful for the substantial contributionsmade by Prof. Paul Andrews (Associate Fellow, SZN) during various steps of this manuscript. KR is grateful to the Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil), the Ministry of Foreign Affairs and International Cooperation (MAECI, Italy) and the Association for Cephalopod Research CephRes–a non-profit organization (Italy). UNAM and CephRes supported the costs of this publication. This work is considered a contribution to the activities of the COST Action FA1301 Cephs*In*Action A network for improvement of cephalopod welfare and husbandry in research, aquaculture and fisheries http://www.cephsinaction.org/.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2020.00697/full#supplementary-material

FIGURE S1 | Daily Growth Coefficient (DGC, %BW d^{-1}) of *Octopus maya* juveniles exposed or not to the anesthetic agents. (SW − H; SW + H). SW: sea water (25°C, unless if specified); SW − H: sea water, without handling; SW + H: sea water, with handling (see text for details). EtOH, ethanol; $MgCl_2$, magnesium chloride; Mix, ethanol in combination with magnesium chloride (concentrations are provided in this order).

FIGURE S2 | Plastic containers for housing *Octopus maya* juveniles (not to scale).

TABLE S1 | Changes on wet weight (BW, ww, g) and daily growth coefficient (DGC; %BW d^{-1}) of *Octopus maya* juveniles exposed or not to the different

substances (or cold sea water) and handling. Values as mean ± SD (g). Different letters mean statistical differences between row data at $p < 0.05$ level. SW: sea water (25°C, unless if specified); SW − H: sea water, without handling; SW + H: sea water, with handling (see text for details). EtOH, ethanol; $MgCl_2$, magnesium chloride; Mix, ethanol in combination with magnesium chloride.

TABLE S2 | ANCOVA analysis of the relationship between wet weight and experimental time of *Octopus maya* juveniles exposed or not to the different substances or cold sea water and handling. Note that two groups were formed: one of the animals anesthetized with clove oil and manipulated and the other with the remaining treatments.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Physiology

CORRECTION
published: 07 October 2020
doi: 10.3389/fphys.2020.599873

Corrigendum: Short and Long-Term Effects of Anesthesia in *Octopus maya* (Cephalopoda, Octopodidae) Juveniles

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Keywords: anesthetic, cephalopods, oxygen consumption, criteria, growth, mortality, animal welfare

OPEN ACCESS

Approved by:
Frontiers Editorial Office, Frontiers Media SA, Switzerland

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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *28 August 2020*
Accepted: *01 September 2020*
Published: *07 October 2020*

Citation:
Roumbedakis K, Alexandre MN, Puch JA, Martins ML, Pascual C and Rosas C (2020) Corrigendum: Short and Long-Term Effects of Anesthesia in Octopus maya (Cephalopoda, Octopodidae) Juveniles. Front. Physiol. 11:599873. doi: 10.3389/fphys.2020.599873

A Corrigendum on

Short and Long-Term Effects of Anesthesia in *Octopus maya* (Cephalopoda, Octopodidae) Juveniles

by Roumbedakis, K., Alexandre, M. N., Puch, J. A., Martins, M. L., Pascual, C., and Rosas, C. (2020). Front. Physiol. 11:697. doi: 10.3389/fphys.2020.00697

In the original article, there was an error in the Acknowledgments statement.

A correction has been made to ***Acknowledgments Statement***. It should read as follow:

"We would like to thank our colleagues from the UNAM (Mexico), specially Claudia Camaal, Elisa Chan, and Ariadna Sánchez for their technical collaboration. We are grateful for the substantial contributions made by Prof. Paul Andrews (Associate Fellow, SZN) during various steps of this manuscript. KR is grateful to the Coordination for the Improvement of Higher Education Personnel (CAPES, Brazil), the Ministry of Foreign Affairs and International Cooperation (MAECI, Italy) and the Association for Cephalopod Research CephRes—a non-profit organization (Italy). UNAM and CephRes supported the costs of this publication. This work is considered a contribution to the activities of the COST Action FA1301 Cephs*In*Action A network for improvement of cephalopod welfare and husbandry in research, aquaculture and fisheries http://www.cephsinaction.org/."

The authors apologize for this error and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

frontiers in Physiology

HYPOTHESIS AND THEORY
published: 23 July 2020
doi: 10.3389/fphys.2020.00765

Can Cephalopods Vomit? Hypothesis Based on a Review of Circumstantial Evidence and Preliminary Experimental Observations

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In representative species of all vertebrate classes, the oral ejection of upper digestive tract contents by vomiting or regurgitation is used to void food contaminated with toxins or containing indigestible material not voidable in the feces. Vomiting or regurgitation has been reported in a number of invertebrate marine species (*Exaiptasia diaphana*, *Cancer productus*, and *Pleurobranchaea californica*), prompting consideration of whether cephalopods have this capability. This "hypothesis and theory" paper reviews four lines of supporting evidence: (1) the mollusk *P. californica* sharing some digestive tract morphological and innervation similarities with *Octopus vulgaris* is able to vomit or regurgitate with the mechanisms well characterized, providing an example of motor program switching; (2) a rationale for vomiting or regurgitation in cephalopods based upon the potential requirement to void indigestible material, which may cause damage and ejection of toxin contaminated food; (3) anecdotal reports (including from the literature) of vomiting- or regurgitation-like behavior in several species of cephalopod (*Sepia officinalis*, *Sepioteuthis sepioidea*, *O. vulgaris*, and *Enteroctopus dofleini*); and (4) anatomical and physiological studies indicating that ejection of gastric/crop contents *via* the buccal cavity is a theoretical possibility by retroperistalsis in the upper digestive tract (esophagus, crop, and stomach). We have not identified any publications refuting our hypothesis, so a balanced review is not possible. Overall, the evidence presented is circumstantial, so experiments adapting current methodology (e.g., research community survey, *in vitro* studies of motility, and analysis of indigestible gut contents and feces) are described to obtain additional evidence to either support or refute our hypothesis. We recognize the possibility that further research may not support the hypothesis; therefore, we consider how cephalopods may protect themselves against ingestion of toxic food by external chemodetection prior to ingestion and digestive gland detoxification post-ingestion. Reviewing the evidence for the hypothesis has identified a number of gaps in knowledge of the anatomy (e.g., the presence of sphincters) and physiology (e.g., the fate of indigestible food residues, pH of digestive secretions, sensory innervation, and digestive gland detoxification mechanisms) of the digestive tract as well as a paucity of recent studies on the role of epithelial chemoreceptors in prey identification and food intake.

Keywords: digestive tract, motility, nutrition, *Octopus vulgaris*, regurgitation, *Sepia officinalis*, vomiting, welfare

OPEN ACCESS

Edited by:
Rui Rosa,
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Reviewed by:
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Specialty section:
This article was submitted to Invertebrate Physiology, a section of the journal Frontiers in Physiology

Received: *05 July 2019*
Accepted: *11 June 2020*
Published: *23 July 2020*

Citation:
Sykes AV, Almansa E, Ponte G, Cooke GM and Andrews PLR (2020) Can Cephalopods Vomit? Hypothesis Based on a Review of Circumstantial Evidence and Preliminary Experimental Observations. Front. Physiol. 11:765. doi: 10.3389/fphys.2020.00765

INTRODUCTION

In the obligative act of eating, animals expose themselves to the ingestion of food potentially contaminated with toxins, which may not have been detected by vision, olfaction, or gustation prior to swallowing (Davis et al., 1986; Glendinning, 2007); these systems are considered the first line of defense. To avoid or minimize the potential effects of toxins once ingested, they must be detected pre-absorption and/or post-absorption (at low concentrations) and trigger physiological mechanism(s) for rapid removal in bulk from the body either *via* the mouth (vomiting) or anus (diarrhea).

Vomiting in vertebrates describes the forceful ejection of upper digestive tract contents from the body *via* the mouth in a single coordinated action with the animal usually adopting a characteristic posture assumed to optimize the mechanics of ejection and reduces strain on the body musculature (Stern et al., 2011; Andrews and Rudd, 2015). The word regurgitation is sometimes used interchangeably with vomiting, as it can also result eventually in oral expulsion of upper digestive tract contents if combined with "spitting," but regurgitation should be used to describe movement of previously swallowed solids or liquids only to the buccal cavity. However, regurgitation is used to describe the process by which some mammals and birds (Lecomte et al., 2006) feed their young and by which some birds (e.g., owls) void pellets of indigestible material (Duke et al., 1976).

Vomiting is only one of the strategies by which animals defend against toxins in the food. Other strategies in vertebrates include, for example, vision, taste, smell, learned aversions, ingestion of clay to adsorb toxins, and hepatic detoxification (Davis et al., 1986; Glendinning, 2007; Stern et al., 2011). This hypothesis focuses on the possibility of vomiting or regurgitation in cephalopods, so a detailed discussion of all the potential toxin defensive mechanisms is outside the immediate scope of this paper, and functions of vomiting or regurgitation may extend beyond toxin defense (see below). However, as vomiting and regurgitation are only one of the potential defensive strategies, we consider some of the other mechanisms in the section on Testing the Hypothesis, where the implications should cephalopods eventually be proven to neither vomit nor regurgitate are discussed.

Vomiting or regurgitation has been reported in representative species of all vertebrate classes, although the mechanics differ and the functions extend beyond ejection of toxic food, but a review is outside the scope of this paper, so the reader is referred to the following papers: fish, Andrews and Young (1993); Sims et al. (2000); amphibia, Naitoh et al. (1989); Naitoh and Wassersug (1992), reptiles, Andrews et al. (2000); birds, Duke et al. (1976); and mammals, Horn et al. (2013).

In contrast to the vertebrates, there are relatively few published reports of either vomiting or regurgitation in invertebrates, although the ability of arthropods to regurgitate is well described (e.g., *Apis mellifera*: Chapman, 1969; *Locusta migratoria*: Freeman, 1968; *Schistocerca emarginata*: Sword, 2001). To provide a background, we firstly briefly review the literature on vomiting or regurgitation in marine invertebrates. We then consider why cephalopods may need to vomit, observational evidence, and anatomical and physiological data on the digestive tract to provide insights into potential mechanisms and identify any constraints, which would make either vomiting or regurgitation difficult (e.g., see Horn et al., 2013, for discussion of inability of rodents to vomit). It is important to note that there are no published studies which have directly investigated either vomiting or regurgitation in cephalopods, so we can only include evidence that is supportive of our hypothesis. However, we describe studies using currently available methodologies which could confirm or refute our hypothesis and consider the implications for how cephalopods may defend against toxic foods if our hypothesis is refuted by direct experimental studies. While the focus is on vomiting and regurgitation, collating evidence for our hypothesis has necessitated a detailed examination of a number of aspects of cephalopod digestive tract anatomy and physiology, resulting in identification of a number of knowledge gaps.

VOMITING AND REGURGITATION IN MARINE INVERTEBRATES: SOME EXAMPLES

We describe examples of vomiting/regurgitation from three classes (*Anthozoa*, *Malacostraca*, and *Gastropoda*) of marine invertebrate solely to illustrate that this ability is not confined to marine vertebrates (see above). Only publications where the authors have themselves referred to an event as either regurgitation or vomiting and have provided a clear description of the phenomenon are reviewed here. In the sections below, we adopt the terminology used in the original publication (i.e., vomiting or regurgitation).

The sea anemone *Exaiptasia diaphana* (cited as *Aiptasia pallida*) regurgitated pellets made from squid meat mixed with an extract of the tunicate *Tridemnum solidum* (Lindquist and Hay, 1995). The tunicate extract contains the secondary metabolites and didemnins, used to chemically defend the larvae from predation by fish.

The mechanics of regurgitation of the foregut contents following feeding have been described in both the red rock crab (*Cancer productus*) and the graceful crab (*Metacarcinus gracilis*, originally referred to as *Cancer gracilis*) in response to exposure to air and reduced salinity (McGaw, 2006, 2007; for review see McGaw and Curtis, 2013). The ejection is by initial relaxation of the foregut with subsequent intense contraction of the foregut muscles (pyloric and cardiac stomach) pushing the material into the esophagus, which is already opened and from where it exits *via* the mouth. In some animals, the entire foregut contents were ejected in a few minutes. As the normal motility cycle in the upper digestive tract is under the control of the stomatogastric nervous system (commissural ganglion, esophageal, and stomatogastric ganglia), it is likely that regurgitation occurs by motor program switching in these ganglia (see below).

The vomiting, regurgitation, and rejection mechanism in the gastropod mollusk *Pleurobranchaea californica* has been studied in detail (McClellan, 1982, 1983; Croll et al., 1984). *Pleurobranchaea*, which had swallowed a mixture of fresh squid homogenate mixed with rotten squid homogenate, expelled it from the body *via* the buccal cavity with the "vomiting phase" lasting 46.1 ± 6.9 s

(McClellan, 1982). The author proposed that the initial propulsive force for ejection was provided by contraction of the body wall and gut muscles. Vomiting was accompanied by tonic shortening of the esophagus, but reverse peristalsis of the esophagus was not observed and final ejection from the buccal cavity was due to cyclical movements of the radula. Vomiting was also evoked if fresh squid was mixed with a dilute solution of liquid detergent, a stimulus capable of inducing vomiting in dogs and humans (Weaver and Griffiths, 1969). It was also reported that animals would reject pieces of rubber tube mixed with palatable food, but this ejection differed from vomiting as the ejection was from the buccal cavity and not the stomach. The neural control of swallowing, vomiting, and rejection provides a good example of "motor program switching" as is also the case for vomiting in vertebrates (Stern et al., 2011).

Studies in *Aplysia californica* have shown the egestion of material (seaweed) from the buccal cavity and esophagus (Jing and Weiss, 2001) and reduced feeding behavior when food was paired with a negative reinforcer (Susswein et al., 1986). Egestion of food by modification of the feeding pattern has also been reported in the pulmonate gastropod *Lymnaea* (Elliott and Susswein, 2002).

The above brief survey of published evidence provides clear, but limited, evidence that the ability to void material from the gut *via* the mouth is not confined to vertebrates, but clearly additional studies are required to identify the circumstances under which ejection of previously ingested food or other material occurs in the wild.

WHY IS IT IMPORTANT TO KNOW IF CEPHALOPODS CAN VOMIT?

The answer to this question is approached by analogy with species in which vomiting or regurgitation is known to be present and by considering components of the cephalopod diet.

The Diet May Contain Indigestible Components Which Cannot Be Voided in the Feces

Although there are some data on fecal color (Taki, 1941), data on analysis of feces in either captive or wild cephalopods are very rare, so it is not known with certainty what, if any, indigestible material can be voided by this route; but see Bidder (1957, p. 143) and Wells and Wells (1989, p. 220) for rare examples. The presence of small amounts of indigestible material in the feces does not exclude the possibility that the same material is ejected in larger quantities *via* the mouth following digestion of food in the upper digestive tract. Passage of indigestible material beyond the stomach, should it occur, risks obstruction and damage to distal structures with the cecal lamellae being at particular risk together with the typhlosoles in the intestine (particularly prominent in *Nautilus*; Budelmann et al., 1997).

So what evidence is there for the presence of indigestible material in the cephalopod digestive tract? In the earliest published description of the digestive tract of *Nautilus*, Owen (1832, p. 24) commented "*The whole alimentary canal was filled with fragments of Crustaceans among which portions of branchiae, claws, and palpi were distinctly recognisable…[…] The crop in particular was densely filled with these fragments*." Examination of the stomach contents in wild caught *Sepia officinalis* (Guerra et al., 1988) also revealed the presence of fragments of crustacean exoskeleton, bones, and scales from fish and beaks from sepioid cephalopods. Pieces of crustacean exoskeleton, with attached soft tissue cleaned, were present in 90% of the cuttlefish examined and in animals >100 mm dorsal mantle length (DML) pieces of skeletal material ~5–17 mm long and ~2.5–5 mm wide were found; the esophagus diameter was 2–3 mm in animals in this size range. A further example is provided by the deep-sea octopus *Graneledone* c.f., *boreopacifica*, in which the gut was found to contain gastropod shells, shell fragments, polychaete bristles, and jaws (Voight, 2000). Polychaetes (*Hermione hystrix*) with indigestible cetae are part of the diet in octopods (including *Octopus vulgaris*; Nixon and Budelmann, 1984).

In *O. vulgaris* fed on crabs or dead fish, gill leaflets were found in the crop 2 h post prandial (Altman and Nixon, 1970), and in *O. vulgaris* fed on 10 g of sardine, fish bones were visible in the stomach 3 h after feeding when the stomach contents had a clay-like appearance (Andrews and Tansey, 1983). Some sardine bones were also observed in the intestine of animals killed >1 h post feeding. The cephalopod stomach does not secrete hydrochloric acid, which contrasts with the stomach in most vertebrates in which the gastric pH is between 1 and 2 (Babkin, 1950). It is the acid which is responsible for digestion of bone in the vertebrate diet. The pH of stomach contents of *Nautilus pompilius* and *O. vulgaris* is only mildly acidic with a pH of ~5.1–5.8 (Mangold and Bidder, 1989, p. 351, Table XVI), consistent with the acidic pH optima of many digestive enzymes in cephalopods (Linares et al., 2015; Gallardo et al., 2017).

Overall, while there is a body of evidence showing that the crop and stomach of cephalopods contains indigestible residues after the soft tissue has been removed, the fate of this material remains unknown. Quantifying the ability of cephalopod digestive tract secretions with a pH 5–6 to degrade small fish bones, scales, and fragments of crustacean exoskeletons at body temperature will provide part of the answer to their fate in the digestive tract. However, detailed studies of the composition of feces (as proposed by Ponte et al., 2017) are also required to enable a more informed conclusion to be reached about voiding by defecation vs. the possibility that indigestible residues are voided by vomiting or regurgitation.

Cephalopods May Ingest Food Contaminated With Toxins Known to Induce Vomiting in Vertebrates

The most likely source of an emetic agent (i.e., a vomit-inducing chemical) is the food cephalopods eat (mollusks, crustacea and fish; see Villanueva et al., 2017, for review), which may contain harmful algal blooms producing a range of toxins as secondary metabolites. It is estimated that at least 100 species of microalgae produce structurally diverse toxins and many of these cause nausea and vomiting in humans (Sobel and Painter, 2005; Berdalet et al., 2015).

Of particular relevance to cephalopods is domoic acid, shown to accumulate in multiple tissues in cephalopods (Lopes et al., 2013, 2018), and which can induce vomiting in humans (see Pulido, 2008, for review) and Cynomolgus monkeys (Tryphonas et al., 1990). Although there is no evidence that consumption of mussels contaminated with domoic acid alters food intake in *O. vulgaris* (Lopes et al., 2013), there are also no studies examining a wide dose-range of domoic acid on the functioning of the digestive tract either *in vivo* or *in vitro*. Electrophysiological studies of the effect of domoic acid on neurotransmission in slices of *O. vulgaris* vertical lobe demonstrated potent effects on the AMPA-kainate type glutamate receptor with a calculated EC_{50} of 0.28 ± 0.05 μM, making it the most potent of the agonists used (domoic acid > SYM2208 > > CNQX >> L-glutamate > > kynurenic acid; Langella, 2005). Domoic acid exposure resulted in irreversible neurotoxicity in the vertical lobe slices (Langella, 2005). There is evidence for glutamate receptors (both AMPA-kainate and NMDA type) in cephalopod central and peripheral neural tissue (for references see Lima et al., 2003; Di Cosmo et al., 2006; Lee et al., 2013). Transcriptomic evidence supports the presence of glutamate receptors in the gastric ganglion of octopus (Zarrella, et al., 2019; Ponte, personal communication) but they have not been demonstrated in the enteric nervous system although this seems very likely, making the digestive tract neural control mechanisms a potential target for toxic effects of domoic acid. As domoic acid is widely distributed in visceral tissues (e.g., digestive gland, posterior salivary glands, kidney, gills, and systemic heart; Costa et al., 2005) and the brain (Lopes et al., 2018), it is highly likely that the neurones in the wall of the gut and the gastric and buccal ganglia will also be exposed. The gastric ganglion has a variety of putative neurotransmitters and receptors (Andrews and Tansey, 1983; Baldascino et al., 2017), so even if glutamate receptors are present and the associated neurones are damaged, a total loss of functionality is unlikely but the ability of the gastric ganglion to coordinate digestive tract motility may be disrupted.

Saxitoxin accumulates in cephalopod tissues (Lopes et al., 2014), acts on voltage-gated sodium channels (Wiese et al., 2010), and is emetic in humans (James et al., 2010), but there is no evidence that it has deleterious effects on cephalopods (Lopes et al., 2014).

Although the above section has focused on algal toxins there are at least two other potential sources of toxins which could act as an emetic stimulus.

i. *Plastics*. Ingestion of plastics has been demonstrated in a diverse range of marine species (e.g., Law, 2017), particularly predators (e.g., Nelms et al., 2018) including one species of cephalopod, the jumbo squid *Dosidicus gigas* (Braid, et al., 2012; Rosas-Luis, 2016). Examination of gastric contents in *D. gigas* revealed the presence of fishing line, plastic pellets, and pieces of polyvinyl chloride (PVC; ~1 cm) fishing floats as well as rocks, sand, and plant matter (Braid, et al., 2012; Rosas-Luis, 2016). Larger pieces of plastics could evoke vomiting or regurgitation by triggering mechanisms detecting indigestible food residues (see below) and smaller pieces could accrete, leading to digestive tract obstruction (e.g., bezoars in humans and cats). A further possibility is that the chemicals released from the plastics could themselves act as emetic stimuli. For example, phthalates used as plasticizers in PVC production can cause nausea and digestive tract discomfort in humans (Diaz, 2015).

ii. *Heavy metals*. Many heavy metals in trace quantities are essential for normal operation of a number of metabolic processes (e.g., enzymes), but heavy metals can also be toxic to the organism. The digestive gland in cephalopods accumulates a range of heavy metals and is implicated in their detoxification (e.g., Penicaud et al., 2017; Rodrigo and Costa, 2017). However, a number of heavy metals cause acute nausea and vomiting in humans when ingested in higher concentrations including mercury, copper, and zinc (Cushny, 1918). Copper sulfate has been extensively as an emetic in experimental studies of emesis in vertebrates to induce emesis by gastric administration including in fish (see Table 1 in Tiersch and Griffiths, 1988).

Vomiting Can Be a Symptom of Disease

Vomiting, particularly if chronic, is recognized as a symptom of disease in both human and veterinary medicine. In the context of animals in laboratory-based research (e.g., under Directive 2010/63/EU; Fiorito et al., 2015), vomiting or regurgitation would be an important indicator of poor welfare or an adverse reaction to a procedure, particularly one involving pharmacologically active agents as occurs frequently in mammals (e.g., Percie du Sert et al., 2012). Regurgitation was included in the list of possible indicators of ill health and poor welfare in cephalopods (see Table 5 in Fiorito et al., 2015). The possibility that cephalopods experience nausea (or a functionally equivalent negative hedonic sensation; Stern et al., 2011) should not be excluded particularly in view of the recent discussions of the capacity of cephalopods to experience pain (e.g., Sneddon et al., 2014; Sneddon, 2015; Key and Brown, 2018).

In attempting to answer the question "*Why it is important to know if cephalopods can vomit?*" we have drawn on knowledge of the functions of vomiting and regurgitation in other species and considered how they could apply to cephalopods. Assuming that cephalopods are able to vomit or regurgitate, the most likely functions are periodic ejection of indigestible material (e.g., crustacean exoskeleton pieces, fish bones and scales, and plastic fragments) and acute ejection of toxic food before the toxin can be absorbed in sufficient quantity to have systemic toxic effects. Obviously, these functions will depend on the diet of the species, so it is conceivable that not all species may have the capacity to either vomit or regurgitate, so formal investigation may require studies in representatives of at least each sub-class.

BEHAVIORAL OBSERVATIONS SUPPORTING THE PRESENCE OF VOMITING IN CEPHALOPODS

The data summarized below are primarily anecdotal reports from the literature but with limited support from incidental observations by the laboratory of two of the present authors.

All the reports below are from cephalopods in captivity. In the wild, vomiting/regurgitation may be very difficult to observe (see the section on Testing the Hypothesis-iv below), but we are unaware of any published reports claiming to have observed this phenomenon in the wild and we have not identified any studies systematically investigating the ability, or not, of cephalopods to either vomit or regurgitate in the laboratory.

Immersion of the Caribbean reef squid, *Sepioteuthis sepioidea*, in the anesthetic agent magnesium sulfate (3 and 4% in filtered sea water) evoked "regurgitation of stomach contents" in some animals (García-Franco 1992, p. 121) and most animals defecated. The same reaction was not observed when the animals were immersed in a solution of magnesium chloride in sea water (2 and 3%). Vomiting or regurgitation and defecation in response to exposure to putative general anesthetic agents has been observed in juvenile/adult *S. officinalis* if they were not fasted for 24 h prior to exposure to the anesthetic agent (Sykes, unpublished observations).

There is an anecdotal report of the occurrence of vomiting/regurgitation in *Enteroctopus dofleini* during net-feeding in captivity (Gleadall, personal communication cited in Andrews et al., 2013).

Vomiting- or regurgitation-like events have been observed in free swimming juvenile/adult *S. officinalis* during which the animals appear to adopt a characteristic posture (Sykes, unpublished observations). Although data on the incidence have not been gathered, the impression of those who inspect the animals daily is that it is not a rare event. The accompanying video clips (taken using Sony Action Cam HDR-AS10) from intermittently monitored tanks of breeding cuttlefish fortuitously captured the behavior accompanying a "spontaneous" suspected vomiting-like event in cuttlefish[1]. Similar events were observed directly by technical staff in fed animals.

Over a 2-year period, four adult (weight 1–2.5 kg) *O. vulgaris* have been observed to have a vomiting- or regurgitation-like event following feeding a crab, but the time after feeding is not known as the suspected vomit was found when the tank was inspected the next day and in one case the animal ejected material when it was moved between tanks (Almansa, unpublished observation). The latter suggests that vomiting could be induced by handling in fed octopus, in a similar way to *S. officinalis* (see above). Suspected vomit had the appearance (mucoid, viscid, and particulate) and color (brown) of partially digested gastric contents and could be readily distinguished from feces, which were in ropes and were a lighter orange, white, or brown color (according to the diet) compared to gastric contents.

The above observations reporting vomiting- or regurgitation-like events in cephalopods from the literature and unpublished observations should all be treated with caution until confirmed by more systematic studies designed specifically to investigate this phenomenon (see the section on Testing the Hypothesis).

Additional indirect support for our hypothesis comes from a paper, published while this paper was initially under review, analyzing fossilized regurgitalites (orally ejected stomach contents) containing aptychi (calcitic lower jaws of ammonites) from late Jurassic Solnhofen deposits (Hoffmann et al., 2019). From a detailed study of the regurgitalites, data on fossilized cephalopod digestive tract contents and other fossils found in the same or related deposits, the authors built a case (using some of the same literature reported here) that coleoid cephalopods and most likely vampyropods were the predators responsible for the origin of the regurgitated ammonite remains.

[1]https://youtu.be/0tFz2ppx_DY; https://youtu.be/c02EZcjWNSE

THE POSSIBLE MECHANICS OF VOMITING OR REGURGITATION IN CEPHALOPODS

Here, we consider if it is theoretically possible for cephalopods to vomit or regurgitate by focusing on *S. officinalis* and *O. vulgaris* as relevant anatomical and physiological data are available in the literature, which we supplement by some additional data.

The key issues are: (a) Where could the force required to expel the contents from the crop/stomach into the esophagus originate? (b) What are the resistances to the movement of material?

Anatomical Considerations

Buccal Mass

The buccal mass is the final structure through which material if ejected from the crop/stomach *via* the esophagus would need to transit, prior to ejection from the body. The buccal mass is formed from the chitinous beak, the mandibular muscles (superior, inferior, and lateral; Boyle et al., 1979), the lateral buccal palps, and the radula and associated bolster muscles (see Messsenger and Young, 1999, p. 163, Figure 1). To minimize resistance to material emerging from the esophagus, into the pharynx and subsequently the mouth, the lateral palps would need to be retracted and the beak opened as occurs at the start of the "bite cycle" (described in *O. vulgaris* by Boyle et al., 1979, p. 59, Figure 6). *In vitro* studies of the buccal mass in *O. vulgaris* showed that the opening phase of the bite cycle lasted 5.6 ± 2.3 s during a spontaneous bite; this would provide sufficient time for material to be ejected if opening was coordinated with the arrival of crop/stomach contents into the pharynx. Cyclical activity of the radula plays a role in egestion in *P. californica*, but we are unable to comment whether the radula working in the opposite way to the "backward-forward," chain saw-like, motion (Messenger and Young, 1999) utilized in swallowing food could be involved in the ultimate ejection of material. The radula is not critical in swallowing (Boyle et al., 1979), so even if it is involved in ejection its role may not be essential.

The movements of the buccal mass are regulated by a "programme of actions" (Boyle et al., 1979) generated in the inferior buccal ganglion, which provides the innervation to the muscles (Young, 1971). The inferior buccal ganglion also innervates the esophagus and crop, and the gastric ganglion is also innervated by the visceral nerves (Young, 1967). Thus, all the key structures, which may be involved in vomiting or

regurgitation (buccal mass, esophagus, crop, and stomach) are innervated either directly or indirectly (*via* the gastric ganglion) from the inferior buccal ganglion. This makes the buccal ganglion a likely site for the genesis of a vomiting or regurgitation "motor program" initiated in response to inputs from the superior buccal lobe of the brain, which itself may be innervated from the subvertical lobe (Young, 1971). Potential pathways are reviewed in more detail in the "Discussion" section.

The Length and Diameter of the Esophagus

To reach the buccal cavity, gastric or crop contents must transit the full length of the esophagus. Measurements taken immediately *post mortem* of the length of the esophagus *in situ* in fresh specimens show that the length is 68.9 ± 8.5% of the DML in *S. officinalis* (mean ± SD: body weight 367.5 ± 82 g; DML 140 ± 7 mm, $N = 10$) and 35.5 ± 2.1% in *O. vulgaris* (mean ± SD: body weight 890 ± 57 g; 130.7 ± 4.3 mm, $N = 7$). Based on a dissection photograph (see Guerra, 2019, p. 24, Figure 3.11) of *Loligo vulgaris*, we estimate that the esophagus to stomach distance is at least 50% of the DML and the same appears true for *Loligo pealei* (see Berk et al., 2009, p. 2, Figure 1f). The relatively narrow diameter of the esophagus at the junction of the crop/stomach is a resistance to retrograde flow of contents. If the esophagus in either octopus or cuttlefish acts as a purely passive conduit for the passage of material ejected by crop/gastric contractions, then digesta will not be visible in the beak until the volume ejected from the crop/stomach exceeds the volume of the esophagus; for *O. vulgaris* and *S. officinalis* (in the above weight range and using the mean length), this is calculated to be 0.5 and 1.2 ml, respectively.

Sphincters

There is no evidence in the literature for the existence of a sphincter (i.e., a structure formed from a thickened layer of circular muscle, resulting in a region with elevated pressure compared to adjacent regions) at the junction between the pharynx and the esophagus (e.g., Boyle et al., 1979; Guerra et al., 1988; Messenger and Young, 1999). However, in the coleoid cephalopods the esophagus passes between the supra-esophageal and sub-esophageal masses of the brain (**Figure 1**) located dorsal and ventrally, respectively (see e.g., Grimaldi et al., 2007). This anatomical organization will limit the size of pieces of food, which can pass to (swallowing/ingestion) or from (regurgitation/vomiting) the crop/stomach. In *Nautilus*, the brain is less well-developed and the circumesphageal connections are less likely to constrain bolus size.

At the junction between the esophagus and its entry into the crop in *O. vulgaris*, the circular muscle has a sphincter-like appearance, fluid in the crop did not reflux readily into the esophagus *post mortem* and resistance to the passage of a cannula was reported (Andrews and Tansey, 1983). Best and Wells (1983) also reported that fluid in the crop of *O. vulgaris* did not pass readily into the esophagus. However, there is no physiological evidence demonstrating a zone of elevated pressure between the esophagus and crop in octopus or the esophagus and stomach in cuttlefish. Still, Boucaud-Camou and Boucher-Rodoni (1983, p. 164) comment that "*sphincters enable both the caecum and the stomach to be isolated from the rest of the digestive tract*." This statement was not referenced, but we have concluded that it refers to observations from *S. officinalis* published in Boucaud-Camou (1977) and/or from drawings in the anatomical monograph of *S. officinalis* by Tompsett (1939, Figure 46) showing sphincters at the esophagus-stomach, stomach-caecum, and stomach-intestine junctions. A recent guide to the functional anatomy of cephalopods does not describe sphincters between any of these structures in the section on the digestive tract (Guerra, 2019), and this is also the case for a chapter including a survey of the histology of the digestive tract (Anadon, 2019), and a research paper describing the anatomical and histochemical features of the *O. vulgaris* digestive tract (Fernandez-Gago et al., 2019). More detailed histological and functional studies are required to resolve the issue of the presence of sphincters between key regions of the cephalopod digestive tract.

Direct observations of the *O. vulgaris* digestive tract *in vitro* and *in vivo* have identified a reciprocal exchange of contents between the crop and stomach with functional evidence indicating that in octopuses (both adults and paralarvae), the crop (when present) and the stomach operate as a single functional unit (Andrews and Tansey, 1983; Nande et al., 2017). In the absence of a sphincter, the flow of contents between these regions of the digestive tract will depend upon the intra-luminal pressure differential between adjacent regions, the diameter of the lumen at the junction between the adjacent regions, the direction (aboral or oral) and amplitude of the peristaltic contractions, and the viscosity of contents (see below).

Physiological Considerations

Motility in the Esophagus

Peristaltic activity in the esophagus moving contents in an aboral direction has been reported in *O. vulgaris* (Andrews and Tansey, 1983) and *Doryteuthis pealeii* (Wood, 1969) *in vitro*. Although retrograde peristalsis has not been reported in cephalopods, there is no *a priori* reason why it could not occur as is the case in the mammalian small intestine immediately prior to vomiting (Lang, 2016) and the esophagus of birds during regurgitation (Duke et al., 1976). Retro-peristalsis would provide a mechanism for ejection of material delivered from the crop or stomach into the esophagus. For this to occur the enteric nervous system pathways for the oro-anal peristalsis need to be overridden and this is most likely to occur by extrinsic nerves from the buccal or gastric ganglia. Longitudinal shortening of the esophagus by contraction of the inner layer of longitudinal obliquely striated muscle (Budelmann et al., 1997) would also facilitate expulsion of material from the crop/stomach.

In *Nautilus*, Owen (1832) reported that the esophagus was only three-fourth of an inch (~2 cm) long before entering the relatively large crop (known to contain indigestible food residues), but the size of the animal was not reported. In diagrams of the *Nautilus* digestive tract [e.g., Figure 1 in Westermann et al. (2002, p. 1618)], the esophagus is relatively short when comparing its length in similar diagrams of the digestive tract in other cephalopods and could shorten further by longitudinal contraction facilitating ejection of crop contents if retropulsive contractions occurred in the crop.

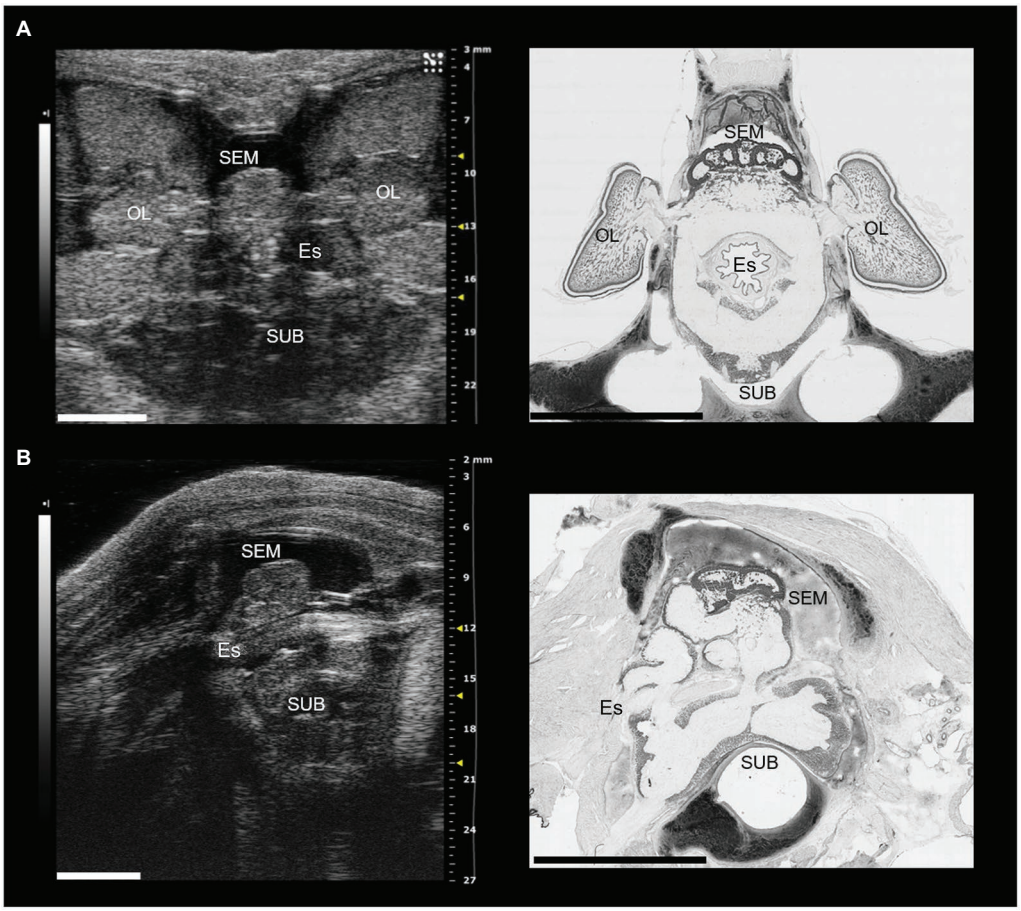

FIGURE 1 | The brain of *Octopus vulgaris* as it appears using sonographic scanning (for additional details see Grimaldi et al., 2007) and histological sections after Nissl staining (in black and white). **(A)** Ultrasound examination **(left)** of the entire cerebral mass in the coronal plane, and the corresponding histological section **(right)**. The optic lobes are visible bilaterally and appear connected through the optic tracts to the central brain (supraesophageal mass, SEM). The subesophageal mass (SUB) lies ventrolateral to the esophagus (Es). **(B)** Ultrasound examination of the central brain in the sagittal plane **(left)**, and the equivalent histological section **(right)**. The two masses (SEM and SUB: dorsally and ventrally, respectively) are clearly visible with the esophagus in the middle. OL, optic lobe; Es, esophagus; SEM, supraesophageal mass; SUB, subesophageal mass. Scale bars: 5 mm.

Motility in the Crop and Stomach

Because of the differences in digestive tract morphology between cuttlefish, squid, and octopus digestive tracts, we will discuss them separately. In cuttlefish[2] and squid tonic contraction of the stomach could propel gastric contents directly into the esophagus from where retro-peristaltic contractions could transport them to the buccal cavity. Orally directed peristaltic contractions (8–13 mm/s) have been reported in the stomach of the squid *Doryteuthis pealii* (Wood, 1969) and such contractions could push material toward the esophagus, but a regular cycle of oral/aboral contractions would be expected as a component of normal gastric motility to triturate the food and mix it with digestive secretions.

In octopus, tonic contraction of the longitudinal and circular muscle of the crop would push contents into the esophagus, if this was accompanied by contraction of the stomach and relaxation of the relevant sphincters (if present). Contraction of the stomach can propel semi-solid material into the crop but the impact of stomach contractions on crop pressure will be low because of the relatively thin walls of the crop and its ability to relax to accommodate contents. *In vitro* the digestive tract of *O. vulgaris* shows peristaltic contractions originating in the crop caudal to the entry of the esophagus, sweeping along the length of the distal crop and pushing *boli* of food into the stomach with the crop/stomach junction narrowed as the bolus enters the stomach (**Figure 2**). The passage of the peristaltic contraction over the distal crop is accompanied by longitudinal shortening of the crop (**Figure 3**). However, once the constriction of the circular muscle between the crop and stomach relaxes gastric contents reflux into the crop accompanied by a gastric contraction (**Figure 2**). This cycle is repeated as the next crop contraction passes to the stomach (**Figure 3**). This observation shows that there is no barrier to the passage of gastric contents into the crop other than the relative contractile activity of the adjacent regions. The contractions of the stomach combined with transient inactivity in the crop are clearly capable

[2]https://youtu.be/Pymql7ncWjI

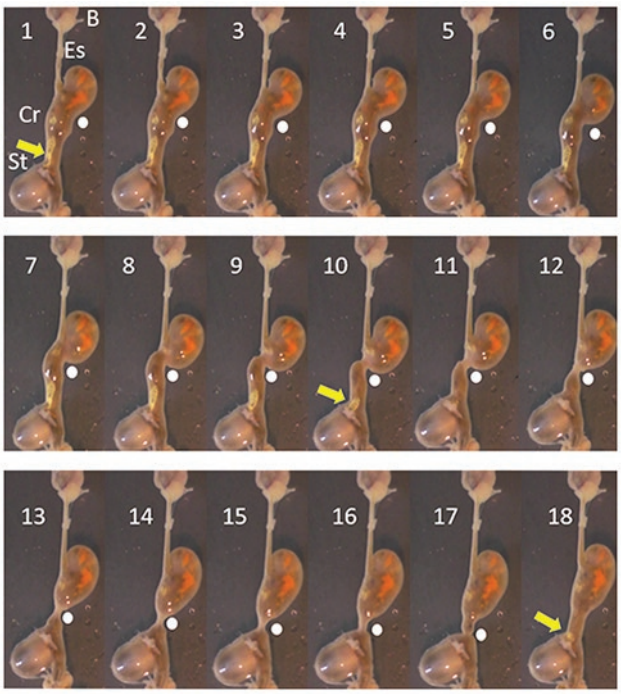

FIGURE 2 | Pictures taken each second (1–18 s) from a video recording of the digestive tract of *O. vulgaris*, *in vitro* (in sea water gassed with air). The animal had been fed a crab 1 h before killing (see Fiorito et al., 2015). The sequence shows the progression of a peristaltic wave (indicated by a white dot) from close to its origin and progressing aborally to the crop/stomach junction. The pressure created by the contraction moves material (yellow arrow) from the crop to the stomach and it is no longer visible after Frame 10. In Frame 16, the crop contraction reaches the stomach and in frames 17 and 18, the contraction subsides allowing material (yellow arrow) to reflux from the stomach toward the crop. The vertical axis of the frames is ~8 cm. B, beak; Es, esophagus; Cr, crop; and St, stomach.

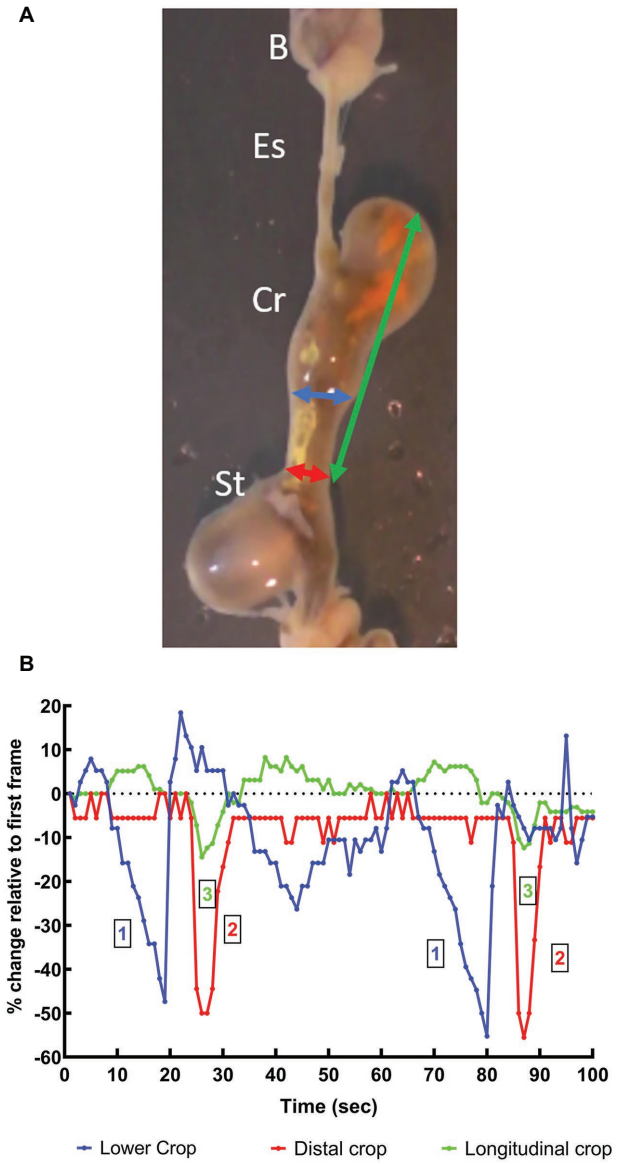

FIGURE 3 | (A) Video frame showing the beak, esophagus, crop, and stomach *in vitro* (see above text for details) from an *O. vulgaris* fed a crab 1 h before killing. The vertical axis of the frame is ~8 cm. Arrows indicate the measurements made of the apparent diameter of the lower crop (blue), distal crop (red) at the crop-stomach junction and the length of the crop (green). B, beak; Es, esophagus; Cr, crop; St, stomach. **(B)** Measurements of the changes in the dimensions of the crop made *in vitro* from an animal fed a crab 1 h before killing. The measurements defined above are made each second from 100 s of video recording (see **Figure 2**) and show two cycles of contraction. All measurements are expressed as a percentage change relative to those taken at $t = 1$ s. The two contractile cycles closely duplicate each other ~1 min apart. The graph shows the wave of contraction of the lower-crop (1) passes to the distal crop (2). The contraction of the distal crop is accompanied by longitudinal shortening of the crop (3). Note that both the lower and distal crop change apparent diameter by ~50% during the passage of the peristaltic contraction.

of pushing semi-digested food into the crop, which would be a preparatory phase for ejection *via* the esophagus. *Note*: this study used tissue removed *post mortem* from animals killed according to current guidelines (Fiorito et al., 2015) and is not covered by EU/2010/63 (Baldascino et al., 2017). The digestive tract was placed in sea water at ambient temperature (~22°C) and gassed with air; tissue remained active for at least 1 h.

We hypothesize that material could be moved into the esophagus in octopus by: (i) tonic contraction of the stomach both pushing gastric contents into the crop and preventing their return; (ii) cessation of orthograde peristalsis in the crop and its replacement by retrograde contractions originating at the crop/stomach junction, and (iii) gastric/crop contents are pushed into the relaxed posterior esophagus from where retrograde contractions of the circular muscle of the esophagus propel the *boli* to the buccal cavity for ejection possibly involving the radula.

Pressure Generation in the Mantle

Forceful, rapid contraction of the mantle muscle could cause an increase in pressure within the crop/stomach to move contents into the esophagus (and possibly beyond) if the exits from the mantle (opercula and funnel) were transiently occluded; a situation very similar to the events occurring

during jetting locomotion when the intra-mantle pressure rises rapidly and is then released by opening the funnel. Trueman and Packard (1968) recorded intra-mantle pressure pulses of duration 150 and 180 ms in *S. officinalis* (250 g body weight) and *L. vulgaris* (350 g), respectively, and of 200 ms in *Eledone moschata* (600 g) and 600 ms in *O. vulgaris* (370 g). The peak pressures recorded ranged from ~100 cm H_2O (73.5 mmHg) in *S. officinalis* to ~400 cm H_2O (294 mmHg) in *E. moschata*. These pressures are in the range of the mean and peak pressures recorded in the human stomach during vomiting (Iqbal et al., 2008), when the main expulsive force is provided by contraction of the diaphragm (excluding crura) and the anterior abdominal muscles compressing the stomach (Andrews and Rudd, 2015).

Overall, we consider it unlikely that mantle contraction would be responsible for ejection of contents as the pressure pulse is very brief and the location of the crop and stomach makes efficient compression unlikely.

The Physical Nature of the Crop/Stomach Contents

The force required to move crop or gastric contents will depend upon their physical nature. Although there have been detailed analyses of upper digestive tract contents in cephalopods, their focus has been on identifying prey types and indigestible residues (see above) rather than on establishing content characteristics such as viscosity and size and rigidity of solid matter, which would provide insights into the forces needed for ejection. Measurement of viscosity of crop/stomach contents at various stages of digestion would be useful for theoretical modeling of the forces required for vomiting/regurgitation in cephalopods. However, such modeling is likely to be challenging as full mathematical modeling of defecation in penguins (*Pygoscelis antarcticus* and *Pygoscelis adeliae*) was confounded by the inability to measure the viscosity of fecal samples due to the presence of crustacean cuticle, fish bones and scales, and other solid fragments (Meyer-Rochow and Gal, 2003).

DISCUSSION

The above review of the literature supplemented by limited observations of behavior *in vivo* and the digestive tract *in vitro* lead us to hypothesize that cephalopods are likely to have the ability to vomit or regurgitate. Below, we summarize the proposed mechanics and discuss the physiological mechanisms to show that the key components of a plausible mechanism are present before proposing how the hypothesis can be tested and the implications if the hypothesis is unsubstantiated by further studies.

A Conceptual Model of Vomiting or Regurgitation in Cephalopods

Mechanics of Ejection

We propose that the most likely way in which contents of the stomach or crop could be ejected is the following: (i) Contraction of the stomach pushes contents into the crop (when present), which is expected to have aboral peristalsis inhibited and tone reduced to accommodate material; (ii) in species lacking a crop, contraction of the stomach will push material directly into the esophagus, providing that the esophagus/stomach junction or sphincter is relaxed and sphincters (if present) between the stomach and intestine/cecum are constricted. The stomach in cuttlefish (as represented by *S. officinalis*) and squid (as represented by *L. vulgaris*) are more sacculated and have thinner muscle compared to the "gizzard-like" stomach in octopus, making it possible that these types of stomach have retrograde peristalsis as reported for *D. pealii* by Wood (1969); (iii) in species with a crop, it is proposed that retrograde contraction of the crop pushes material into the esophagus. While relatively little is known of the control of peristalsis in the cephalopod digestive tract, it is likely that it is coordinated by the myenteric plexus neurons of the enteric nervous system described by Alexandrowicz (1928) as is the case in vertebrates (Furness, 2006). The hypothesized retrograde contraction leading to retropulsion of contents would need to overcome any resistance at the esophagus/crop junction (or the esophagus/stomach junction in squid and cuttlefish) unless the muscle in the junctional zone is relaxed; and (iv) as crop/stomach contents enter the esophagus retrograde peristaltic contractions would propel it to the buccal cavity for ejection possibly involving the radula (c.f., opisthobranch mollusks discussed above) or buccal musculature with the beak open (see section Buccal Mass above).

Coordination of the Mechanical Events

The gastric ganglion in cephalopods is located at the junction of the crop/esophagus, stomach, cecum, and intestine, so it is ideally located to coordinate motility between the various regions *via* nerves projecting to each region (Young, 1967; see also Figure 1 in Baldascino et al., 2017). In *O. vulgaris*, the gastric ganglion has been shown to be involved in the control of the crop and stomach movements (Andrews and Tansey, 1983). The diversity of putative peptide and non-peptide transmitters and receptors in the gastric ganglion, its relatively large size, and complex internal organization support the proposal that it has an important function in coordinating the functions of the various regions of the digestive tract (Andrews and Tansey, 1983; Baldascino et al., 2017). It is proposed that the motility of the stomach, crop, and esophagus required for ejection of material will be coordinated by the gastric and inferior buccal ganglia, in the same way that the stomatogastric system coordinates comparable events in *Pleurobranchea* and *Aplysia* (see above).

The gastric ganglion is implicated in the control of digestive tract motility post-prandially in cephalopods but how this occurs has not been investigated (Andrews and Tansey, 1983; Baldascino et al., 2017). By analogy with the stomatogastric nervous system in crustacea and opisthobranch mollusks (see above), control involves microcircuits, but changes in activity of such microcircuits can lead to a switch from ingestive to egestive behavior (Jing et al., 2007; Daur et al., 2016). The neural activity of the stomatogastric ganglion in crab is subject to modulation by a range of amines and peptides delivered

in the circulation (Marder, 2012). In *Aplysia*, a command-like interneuron, which usually elicits food ingestion has its activity changed to evoking egestive behavior by neuropeptide Y (Jing et al., 2007). The presence of neural circuits for both ingestion and egestion in crustacea and opisthobranch mollusks indicates that both occur in the wild as well as in response to experimental stimuli. Modulation of microcircuits in the cephalopod gastric or the inferior buccal ganglion by a neuroactive agent (including toxins) arriving in the blood and leading to motor program switching provides a plausible mechanism by which vomiting or regurgitation could be initiated.

Triggering the Process

If one of the functions of the hypothesized vomiting or regurgitation is ejection of indigestible material, then this is most likely to be detected by mechanoreceptors in the muscle of the crop or stomach, activated by stretch as the material accumulates. Mechanoreceptive afferents sensitive to stretch and contraction of the mammalian stomach are well characterized (e.g., Iggo, 1957; Andrews et al., 1980; Page et al., 2002). There is no neurophysiological evidence for mechanoreceptive afferents in the cephalopod digestive tract but indirect evidence is provided by: (i) a relationship between the tendency to attack and crop distension in *O. vulgaris* (Young, 1960) and (ii) limited histological evidence for the presence of sensory cells in the upper digestive tract of octopus (Botar, 1967). In gastropod mollusks, distension sensitive receptors have been implicated in regulation of food intake with section of the stomatogastric nerves innervating the esophagus and crop, leading to hyperphagia in *Pleurobranchaea* (Croll et al., 1987). Neurophysiological studies are required in cephalopods to investigate the presence of afferent nerves in the digestive tract.

Studies in mammals have demonstrated the presence of visceral afferents in the vagus nerve with receptive fields in the digestive tract mucosa sensitive to mechanical stimulation of the mucosa by stroking (Page et al., 2002); such receptors, if present in cephalopods, would be suited to detection of abrasive indigestible material (e.g., crustacean skeletons and plastic fragments); however, they would not function in areas where there is a thick chitinous covering of the epithelium (e.g., thick stomach muscle in *O. vulgaris*). Mucosal receptors in the mammalian stomach and intestine also act as chemoreceptors detecting nutrients (e.g., glucose, lipids, and proteins; Brookes et al., 2013; Williams et al., 2016) and irritants including substances capable of induced nausea and vomiting when given luminally (e.g., copper sulfate: Endo et al., 1995; TRPA1 agonists: Bellono et al., 2017; and hypertonic NaCl: Clarke and Davidson, 1978). The anatomical correlate of the mucosal receptor is an enteroendocrine cell releasing neuroactive agent (e.g., 5-hydroxytryptamine, substance P, and cholecystokinin) at its basal surface to activate receptors located on vagal afferents terminating in close proximity (Grundy, 1988; Reybold, 2010; Powley et al., 2011; Bohorquez et al., 2015; Bellono et al., 2017); this is termed "neurocrine signaling."

The existence of enteroendocrine cells in the cephalopod digestive tract with afferents terminating in proximity has not been investigated, but neurites arising from bipolar cells in subepithelial plexus supplying the mucosa are argued to be sensory (Alexandrowicz, 1928).

The triggering of vomiting or regurgitation by activation of digestive tract afferents (projecting either to the peripheral ganglia or to the brain) is the most likely mechanism but other options include: (i) a direct effect of a systemic toxin (e.g., domoic acid) on the gastric ganglion, inferior buccal ganglion or possibly the brain [e.g., superior buccal lobe; c.f., centrally acting emetics in mammals; Stern et al. (2011)] and (ii) release of a hormone from the digestive tract mucosa in response to an ingested toxin or irritant resulting in an effect on the inferior buccal ganglion, gastric ganglion, or brain (e.g., superior buccal lobe).

TESTING THE HYPOTHESIS

Four pieces of evidence reviewed above support our hypothesis that cephalopods are likely to have the capacity to vomit/regurgitate: (1) the mollusk *P. californica* is able to vomit/regurgitate with the muscular and neural mechanisms well characterized. We acknowledge that gastropods and cephalopods are phylogenetically distant classes but there are similarities in digestive tract morphology and innervation, which support the mechanisms we propose for vomiting or regurgitation in cephalopods; (2) a rationale for the requirement to vomit or regurgitate is presented based upon voiding indigestible food residues, which may cause damage if they transit beyond the stomach and the ingestion of potential toxins in the food; (3) preliminary reports of vomiting- or regurgitation-like behavior in several species of cephalopod and indirect supporting evidence from the fossil record of regurgitolites; and (4) a conceptual model showing that all the key mechanisms exist in cephalopods (exemplified particularly by *O. vulgaris*) by which ejection of gastric/crop contents could occur.

We fully recognize that the evidence presented is largely circumstantial and is not conclusive, but we reiterate that we have been unable to identify any publications, which refute the ability of cephalopods to either vomit or regurgitate. However, we consider that on balance the limited evidence supports our hypothesis that cephalopods are likely to possess the ability to either vomit or regurgitate; so how can definitive data to confirm or refute our hypothesis be obtained using currently available methodology?

i. Vomiting and regurgitation may be rare events. Obtaining proof may be challenging as the ejection event is likely to be brief (a few seconds) and while the animal may adopt a characteristic posture or exhibit prodromal behavior as occurs in vertebrates (see chapter 8 in Stern et al., 2011), it may attempt to hide while doing this to make itself less vulnerable to predation. The arms may also obscure direct observation of the beak to observe ejection. To gather data to better assess its occurrence (or not), we request that the readers of this paper send reports of what they consider to be vomiting- or regurgitation-like behavior in cephalopods in either the wild or captivity. Additionally, Citizen Science

Programs (such as the Cephalopod Citizen Science project – https://www.researchgate.net/project/Cephalopod-Citizen-Science) can be used as a resource to search historical social media posts or to request video that may possess evidence of cephalopods vomiting from the SCUBA community.

ii. Analyze long duration video recordings of *Nautilus*, cuttlefish, and octopus post feeding in captivity to identify vomiting- or regurgitation-like events to provide more robust evidence in one or more species of cephalopod. Collection and analysis of the suspected vomit is essential to understanding the function of vomiting or regurgitation (should either occur) in cephalopods. Detailed analysis of the fecal composition of cephalopods fed on natural diets known to contain indigestible residues and diets containing indigestible artificial markers will enable firmer conclusions to be drawn about how the digestive tract in cephalopods handles indigestible food residues. Studies of *Nautilus* will be particularly important because of its ancestral role for the most recent coleoid cephalopods (e.g., Tanner et al., 2017) and the clear descriptions of relatively large pieces of indigestible material in the stomach from the original description by Owen (1832) to more recent authors (Haven, 1972).

iii. Undertake *in vitro* studies using isolated digestive tracts to investigate the presence of sphincters between the esophagus/crop/stomach, the existence of retrograde contractile activity in the esophagus, crop, and stomach, and investigate the potential role of the gastric ganglion in switching motility in the upper digestive tract from a digestive pattern to a vomiting or regurgitation pattern. The above studies would use a combination of studies on isolated strips already used in squid and octopus digestive tract (Wood, 1969; Andrews and Tansey, 1983), video recording and quantification of movements in the isolated digestive tract (as exemplified by **Figures 2, 3**) but using more sophisticated analytical techniques, such as those used in fish and mammalian intestine (e.g., Kendig et al., 2016; Brijs et al., 2017), and finally recording of intra-luminal pressure, a technique widely used in vertebrates including fish (e.g., Andrews and Young, 1993).

iv. *In vivo* studies may be necessary to provide definitive proof of the existence of vomiting or regurgitation. Among the mammals, although the ability to vomit is widespread, it is not present in rodents and lagomorphs (Sanger et al., 2011; Horn et al., 2013), so it would be unwise to extrapolate either positive or negative findings from one cephalopod group to another. Studies of multiple species of cephalopod should only be contemplated if studies outlined in i–iii above are not definitive as otherwise it will not be possible to demonstrate to an Ethical Review Committee that there is no alternative to *in vivo* studies. In the European Union, it is a requirement of the legislation (2010/63/EU) regulating animal experimentation (see Fiorito et al., 2015, for details) that all alternative methods, which could answer the scientific question posed have been explored before undertaking *in vivo* studies that meet the threshold for a regulated procedure (see Cooke et al., 2019, for details). By analogy with other experimental procedures in cephalopods, we consider that *in vivo* experiments to demonstrate vomiting or regurgitation would be prospectively classed as "moderate severity" under 2010/63/EU (see Cooke et al., 2019, for details). Studies to demonstrate vomiting or regurgitation face two practical challenges:

1. *Reliable induction of vomiting or regurgitation*. In the section on "Triggering the Process" above, we identified potential pharmacological stimuli. The study would require administration of a range of substances intravenously (e.g., Agnisola et al., 1996) or by direct administration to the crop/stomach by gavage (e.g., Berk et al., 2009; Sykes et al., 2017) or inclusion in the food. The study would need to be designed to minimize the number of animals required to identify the dose effective in all animals (ED_{100}).
2. *Ensuring the origin of ejected material*. The mouth is obscured by the arm crown making observation of the final ejection of digestive tract contents problematic, and also ensuring that the ejected material originated from the crop/stomach rather than from residues in the buccal cavity or trapped in the arm crown or arm web. Delivering food (e.g., pieces of fish, mussel, or cephalopod) directly into the stomach mixed with an indigestible radio-opaque or fluorescent marker would permit monitoring of the appearance (either by vomiting, regurgitation, or defecation) of food and the indigestible marker in the water in close proximity to the animal. Marking food ingested by the animal with a "dye," which changes color when exposed to mildly acidic pH (5–6) and proteases would avoid the need for gavage. To permit monitoring the animal would need to be adapted to a relatively small tank during study, and hence, such studies would be difficult to perform in squid.

WHAT IF CEPHALOPODS LACK THE ABILITY TO VOMIT?

Although we consider it likely that at least some species of cephalopod (e.g., *Nautilus*) can vomit or regurgitate upper digestive tract contents, the possibility remains that they do not have this ability, so here we discuss the implications if this is the case in one or more species.

If cephalopods lack the ability to vomit or regurgitate, then the digestive tract must be able to break down indigestible food residues sufficiently for them to pass through all post-gastric parts of the digestive tract without damaging the mucosa or producing an obstruction of the intestine (particularly where it narrows due to the typhlosole) with the animal finally able to pass the material *via* the anal sphincter. However, vomiting is also one of the mechanisms by which organisms eject contaminated food to reduce the systemic toxic load, so if cephalopods are unable to either vomit or regurgitate then how could they defend themselves against food containing toxins?

An insight into the above question comes from mammals. As far as is known, among the mammals, rodents and lagomorphs are unique in lacking an ability to vomit accounted for by anatomical constraints, differences in brainstem pathways

integrating and coordinating the motor outputs, and the motilin system compared to species with an emetic reflex (Sanger et al., 2011; Horn et al., 2013). Rodents do however possess well-developed conditioned aversive responses particularly involving taste (conditioned taste aversion, CTA); following ingestion of a substance presumed to induce the sensation of nausea (or a functionally equivalent sensation), they will avoid ingestion of that substance when presented on a future occasion. It is argued that the taste of the food, and probably also the smell, appearance, and place where it was eaten, is linked to the learned aversion and subsequent avoidance by the sensation of nausea (or other negative hedonic sensation) following ingestion. CTA also occurs in mammalian species with an emetic reflex, including humans (Stern et al., 2011). In fish, learned aversion provides a mechanism by which they avoid poisonous corals (Gerhart, 1984, 1991). Aversive aspects of consumer-prey interactions in marine organisms are reviewed in detail by Paul et al. (2007) and Sotka et al. (2009). Is there evidence for chemoreception and learned aversive responses in cephalopods?

There is evidence, particularly from cuttlefish and octopus, that chemoreceptors are present on the suckers, the lips, and in the olfactory organs (Hanlon and Messenger, 2018, for overview). Distance chemoreception has been implicated in food detection (e.g., Boyle, 1983, 1986; Chase and Wells, 1986; Lee, 1992) and reproductive behavior (e.g., Cummins et al., 2011; Polese et al., 2015). Reviewing behavioral studies of the ability of *O. vulgaris* to discriminate between solutions of sucrose, hydrochloric acid, quinine, and sea water with potassium chloride, Hanlon and Messenger (2018, p. 31) commented "octopuses can probably detect quite small differences in the taste of objects that they handle." The potential role of either type of chemoreceptor in detection of toxins in the food prior to ingestion (c.f., taste in mammals) remains to be investigated but two relevant studies are discussed below.

Darmaillacq et al. (2004) in adult *S. officinalis* provided preliminary evidence for taste aversion learning in cephalopods by investigating the response of cuttlefish to crabs painted with quinine, perceived by humans as bitter tasting. After eight trials, the cuttlefish learned to avoid the crabs painted with quinine, suggesting that they had developed a learned aversion. The sensation experienced by the cuttlefish is presumed to be one with negative connotations (c.f., pain). However, it must be noted that the animals did not ingest the crab, so the situation differs from the mammalian studies in which animals ingest the contaminated food and link the negative hedonic experience (nausea or equivalent) to the taste, smell, sight of food, or the place where it was ingested. Despite the significant differences in protocol, the Darmaillacq et al. (2004) study does show that cuttlefish are able to learn to avoid a specific food based upon a negative sensory experience. Similar studies in *O. vulgaris* did not provided any evidence of taste aversion (Zarrella and Ponte, personal communication).

O. vulgaris will reject pieces of sardine marinated in 3% quinine hydrochloride (c.f., above cuttlefish study) when it comes into contact with the suckers, whereas untreated sardine was accepted (Altman, 1971). The posterior buccal lobe of the inferior frontal region controls the rejection response (Altman, 1971). The chemosensory apparatus in the suckers of cephalopods is likely to act as the first line of defense against the ingestion of noxious material and *O. vulgaris* can be trained to distinguish between hydrochloric acid, sucrose, and quinine solutions applied to the suckers (Wells, 1963). However, the range of substances to which the suckers are sensitive has not been explored using the type of molecular techniques, which have provided insights into taste transduction in mammals. For example, bitter tastants, such as quinine, act *via* the T2R receptor family in mammals (Chandrashekar et al., 2000). Studies are needed to investigate the presence of this G-protein coupled receptor (GPCR) receptor family in cephalopods (e.g., see Ritschard et al., 2019).

Further studies are required to investigate whether cephalopods can learn to avoid foods, particularly those frequently containing toxins or pathogens, which produce "illness" following ingestion. In species with a relatively narrow dietary range becoming averted to one or more of the main types of prey (e.g., crabs, mussels, or fish) may be disadvantageous, so cephalopods may lack this capability and may rely instead on the ability of the digestive gland for metabolic detoxification (Bustamante et al., 2002; Penicaud et al., 2017; Rodrigo and Costa, 2017). It is also possible that toxins in the food may be degraded by salivary enzymes with which the food is mixed during external digestion and ingestion [for review of cephalopod salivary glands see Ponte and Modica (2017)].

For a detailed discussion of strategies adopted by predators in enabling them to deal with foods, which are "chemically defended," the reader is referred to the review by Glendinning (2007). A detailed review of the role of chemoreception in prey detection and food ingestion in cephalopods is required to contribute to the discussion of whether they have the capacity for learned avoidance of potentially toxic prey, which may obviate part of the need to either vomit or regurgitate. Additionally, a detailed examination of the expression of taste receptor molecules in the suckers would give insights into the spectrum of chemosensitivty.

CONCLUSION

Collation of diverse indirect evidence from the literature, a consideration of digestive tract morphology, innervation and physiology, and limited laboratory observations leads us to propose that at least some species of cephalopod are likely to be capable of either vomiting or regurgitation. Reviewing the evidence has identified a number of gaps in knowledge of the anatomy (e.g., the presence of sphincters) and physiology (e.g., the fate of indigestible food residues, pH of digestive secretions, and digestive gland detoxification mechanisms) as well as the properties and functions of epithelial chemoreceptors. The capacity of cephalopods to either vomit or regurgitate, or neither, now requires more formal investigation. Such studies should form part of a wider consideration of other adaptations, which may enable cephalopods to identify (e.g., vision and chemosensitivity) and avoid (learned aversion) potentially toxic foods, neutralize

ingested toxins (e.g., salivary and digestive glands) and deal with indigestible material (e.g., gizzard-like stomach in octopus).

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made publicly available. There are no data that could be considered a "data set" that would be of use to others. Also, the previously unpublished data included is preliminary. The videos are available *via* a link but they are not a "dataset."

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the novel behavioral data reported on "live cephalopods" are observations made during routine husbandry and care and are neither experiments or regulated procedures (as defined in 2010/63/EU). The *in vitro* observations are from tissue removed *post mortem* and are not regulated by 2010/63/EU. The manuscript includes a note to this effect where the previously unpublished findings, which support the hypothesis, are described.

AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

FUNDING

AS was supported by Fundação para a Ciência e a Tecnologia (FCT) through Programa Investigador FCT 2014 (IF/00576/2014) and also receives Portuguese national funds from Programa Operacional Mar2020 (Portugal2020/FEAMP) – Project SEPIACUL (project number 16-02-01-FMP-53), and from FCT through Plurennial funding to CCMAR (UID/Multi/04326/2019). GP has been supported by a RITMARE Flagship Project Fellowship (MIUR and SZN). EA was supported by the Spanish Government (OCTOMICs project, AGL2017-89475-C2-2-R).

ACKNOWLEDGMENTS

We acknowledge COST Action FA1301 for facilitating the interactions that have led to this paper, which was initially presented in part at a COST FA1301 meeting in Heraklion (Greece). AVS thanks Juan Carlos Capaz and EA thanks the staff of the IEO (Spain) and in particular Beatriz C. Felipe for the observations on vomiting reported during the last 2 years. PLRA wishes to acknowledge the receipt of an honorary Research Fellowship at Stazione Zoologica Anton Dohrn, Naples and would like to thank the President of the Stazione Zoologica, Professor R. Danovaro, Dr. G. Fiorito and the Head of the Department of Biology and Evolution of Marine Organisms (Dr I. Arnone). We also wish to thank the five reviewers of this manuscript who all provided interesting comments on this controversial topic and which we have attempted to reflect where possible, within the constraints of the "Hypothesis and Theory" format.

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