



Pre-breeding Diets in the Seahorse *Hippocampus reidi*: How Do They Affect Fatty Acid Profiles, Energetic Status and Histological Features in Newborn?

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Seahorses (*Hippocampus* spp.) are exceptional marine species considering their reproductive patterns and other features. Due to the iconic characteristics of these fishes, aquarium trade, and research efforts have increased in the last years. Consequently, novel rearing techniques have been developed; however, there is a need for improvements on a series of issues, namely reproduction success enhancement. The tropical species *Hippocampus reidi* is the most traded seahorse but many aspects of breeding and its impact on the quality of neonates are still poorly understood. In the present study, we assessed the effects of two pre-breeding diets on newborn quality and viability considering biochemical characteristics, energetic status, and ultrastructural aspects of muscular tissue. During the whole pre-breeding season (5 months), the breeders were fed on one of the following diets: M0 (adult non-enriched *Artemia*) and M5 (adult non-enriched *Artemia* + mysidaceans). From the onset of the reproduction period, all breeders were fed for 6 months on diet M5. Breeding success and energetic status (ATP, total adenylic nucleotides, AEC, and NAD) of newborns resulted considerably enhanced in treatment M5. However, initial differences in neonates quality did not affect further newborn performance (survival and growth until day 7 after male's pouch release) while gaining access to high-quality preys (copepods). Besides, morphological alterations in muscle tissue were not observed. The reproduction in the species followed a capital-income continuum pattern characterized by an initial mixed capital-income period (until 70–100 days since the onset of the breeding season) followed by an income breeding period with progressive exhaustion of body reserves, especially in M0-newborns. Interestingly, the effects of pre-breeding diets were also noticed in the second half of the breeding period. Our results seemed to indicate that the requirements in essential fatty acids in *H. reidi* are lower than in other seahorse

species (e.g., *Hippocampus guttulatus*). Globally, the results achieved revealed that high-quality pre-breeding diets enhanced reproduction success and would likely result advantageous to improve newborn endurance in conditions of moderate starvation or sub-optimal feeding.

Keywords: Syngnathidae, seahorses, *Hippocampus reidi*, breeder's diet, newborn, fatty acids, energy charge, histology

INTRODUCTION

Seahorses (*Hippocampus* spp.) are flagship fishes submitted to over-exploitation in nature due to their use in several human activities, habitat loss, and the aquarium and curio trade, compromising their conservation (Vincent, 1996; Foster and Vincent, 2004; Martin-Smith et al., 2004; Vincent et al., 2011). In recent years, concern over how to reduce the exploitation of wild populations has been increasing in parallel to rising efforts to provide reliable tools for their rearing in *ex situ* facilities (Koldewey and Martin-Smith, 2010; Olivotto et al., 2011b; Cohen et al., 2016). Breeding in captivity can partially replace the capture of wild seahorses and reduce the pressure over natural populations.

There are more than thirty seahorse species but only a few are currently reared with certain success in captivity. The tropical fish *Hippocampus reidi* Ginsburg, 1933 is one of the seahorse species most in demand for the marine aquarium trade, and one of the most promising for industrial cultivation. The geographic distribution of the species ranges from Florida to Rio de Janeiro (Brazil) and Gulf of Mexico (Kuiter, 2013). Adults measure up to 26 cm in TL and inhabit shallow inshore to moderately deep water (15–55 m) (Rosa et al., 2002).

A singular characteristic of seahorses is reproduction, in which females provide most nutrients to eggs whereas males take care and also supply nutrients to embryos developing from eggs transferred by females to their brood pouches (Carcupino et al., 2002; Skalkos et al., 2020; Wittington and Friesen, 2020). Mating and parturition are highly synchronized with egg maturation so that the release of eggs and newborn occur almost simultaneously and at similar time intervals, which vary depending on species, temperature, and breeder's diet (Lin et al., 2006, 2007; Planas et al., 2010; Otero-Ferrer et al., 2020). In seahorses, the assessment of nutritional and energetic requirements in seahorse breeders is lacking. Only a few studies have focused on the impact of breeder's diet on newborn quality and further viability. Most of them were carried out in *H. guttulatus*, *H. hippocampus*, and *H. reidi* by comparing diets differing in biochemical profiles [namely fatty acids (FAs)] and nutritional quality (e.g., *Artemia*, mysidaceans, and amphipods as prey) (Woods and Valentino, 2003; Murugan et al., 2009; Otero-Ferrer et al., 2012, 2016, 2020; Palma et al., 2012; Saavedra et al., 2014; Saavedra et al., 2015; Planas et al., 2020b).

The effect of breeder's diet in fish have been usually focused on the impact of FAs and the resulting quality of fry (Bromage, 1995; Brooks et al., 1997; Izquierdo et al., 2001; Fernández-Palacios et al., 2011; Migaud et al., 2013). In seahorses, most studies have focused on the effects of diets provided during

the breeding season (Woods and Valentino, 2003; Binh and Serrano, 2012; Otero-Ferrer et al., 2012, 2020; Palma et al., 2012, 2017; Planas et al., 2020b) or shortly before reproduction (Otero-Ferrer et al., 2016). In a previous study on *H. guttulatus*, we assessed isotopically the assimilation by breeders of three diets furnished during the whole pre-breeding period and their incorporation into newborns (Planas, 2021). We demonstrated that the species follows a capital-income breeding strategy ((Williams et al., 2017). The optimization of seahorse breeding also should consider how newborn development is affected by pre-breeding dietary regimes. For that, newborn characteristics and initial viability must be properly addressed. Condition indices, energy allocation, and metabolic scope in early ontogeny aim to evaluate the physiological status of fish (Cunha et al., 2003, 2007; Di Pane et al., 2019). Adenylic energy charge (AEC) was proposed as an indicator of the energy potentially available for cellular metabolism (Atkinson, 1977; Dickson and Franz, 1980; Harms, 1992). To our knowledge, the unique study available focused on adenylic nucleotides and energetic status in fish larvae was carried out in clownfish (Olivotto et al., 2011a).

In the present study, we assessed the effects of two dietary treatments on the reproduction of the seahorse *H. reidi* by analyzing (1) breeding success and general newborn characteristics (FA profiles, endogenous nucleosides, and AEC), and (2) determining the breeding pattern of the species. To our knowledge, this is the first multiapproach study on the effect of long-term pre-breeding diets on reproductive performance and newborn energetic status in seahorses.

MATERIALS AND METHODS

Bioethics

Animal handling and sampling were conducted in compliance with all bioethics standards on animal experimentation of the Spanish Government (Real Decreto 1201/2005, 10th October) and the Regional Government Xunta de Galicia (REGA ES360570202001/15/FUN/BIOL.AN/MPO01).

Seahorse Maintenance

Adult seahorses *H. reidi* Ginsburg, 1933 were kept in captivity at the facilities of Instituto de Investigaciones Marinas (IIM-CSIC) (Vigo, Spain) for at least two generations, or donated by l'Oceanogràfic aquarium (Valencia, Spain). The fish were maintained in 320 L aquaria (Planas et al., 2008) in a semi-closed system with a 10–14% daily renewal of seawater. Seawater temperature was maintained constant at $26 \pm 0.5^\circ\text{C}$, and a natural-like photoperiod regime was applied (16L:8D). Water

quality was checked periodically for NO_2 , NO_3 , and NH_4/NH_3 content (0 mg L^{-1}). Salinity and pH levels were 37 ± 2 and 8.0 ± 0.2 , respectively. Wastes and uneaten food were removed daily by siphoning the bottom of aquaria.

The fish were routinely fed twice daily on diets comprising enriched adult *Artemia* (Iberfrost, Spain; 40–70 *Artemia* seahorse⁻¹ dose⁻¹) and frozen mysidaceans *Neomysis* sp. (Ocean Nutrition, Spain). Adult *Artemia* was produced in 100 L units at 26–28°C with gentle aeration and constant light on a mixture consisting of live microalgae *Phaeodactylum tricornutum* and *Isochrysis galbana*, dried *Spirulina*, and subsequently long-time enrichment for at least 3 days (from day 16 onward) on the commercial product Red Pepper (Bernaqua, Belgium) (Planas et al., 2017).

Experimental Design

Pre-breeding (Period 1)

Two groups of seahorses (groups M0 and M5; eight females and six males per group) were randomly selected from the seahorse broodstock and kept separate for 5 months (from November 1, 2017 to March 31, 2018) in two 320 L and maintained under the same chemical-physical conditions reported above. For each group, males and females were kept physically separate during the whole period.

The breeders were fed (*ad libitum*) twice daily according to the following feeding scheme (Figure 1):

1. Group M0: adult non-enriched *Artemia* (Iberfrost, Spain).
2. Group M5: adult non-enriched *Artemia* and frozen mysidaceans (*Neomysis* sp.; Ocean Nutrition, United States) (1:1).

Prey lengths were 5–7 mm for *Artemia* and about 1–1.5 cm for mysidaceans.

Adult non-enriched *Artemia* was produced as reported above but not enriched with Red Pepper.

The mixed diet used in group M5 was selected considering that it provides the best trophic enrichment factors in syngnathids (Planas et al., 2020a). The lipid content and FAs profile of prey are provided in Table 1.

Mean weight (DW) and standard length (SL) of breeders at the onset of Period 1 did not differ significantly across groups (K-W test, $n = 32$, $p = 0.951$ for DW and 0.878 for SL). Mean DW and SL were $6.17 \pm 2.07 \text{ g}$ and $14.15 \pm 1.53 \text{ cm}$ in M0, and $6.76 \pm 3.09 \text{ g}$ and $14.43 \pm 2.22 \text{ cm}$ in M5, respectively. At the end of the pre-breeding period, mean DW ($9.18 \pm 2.69 \text{ g}$ in M0; $10.99 \pm 2.96 \text{ g}$ in M5), SL ($16.03 \pm 1.42 \text{ cm}$ in M0; $17.01 \pm 1.60 \text{ cm}$ in M5) and G (daily weight-specific growth rates) ($0.355 \pm 0.196\% \text{ day}^{-1}$ in M0; $0.434 \pm 0.221\% \text{ day}^{-1}$ in M0) were similar across groups (K-W test, $n = 32$; $p = 0.242$ for DW, 0.140 for SL, and 0.124 for G).

Breeding (Period 2)

Males and females from the same experimental treatment were regrouped on April 1, 2018 and maintained until September 30, 2018 under the same conditions reported above for Period 1, except for the diet. During this period, both experimental groups were fed twice daily on a mixture of non-enriched adult *Artemia* and frozen mysidaceans *Neomysis* sp. (1:1).

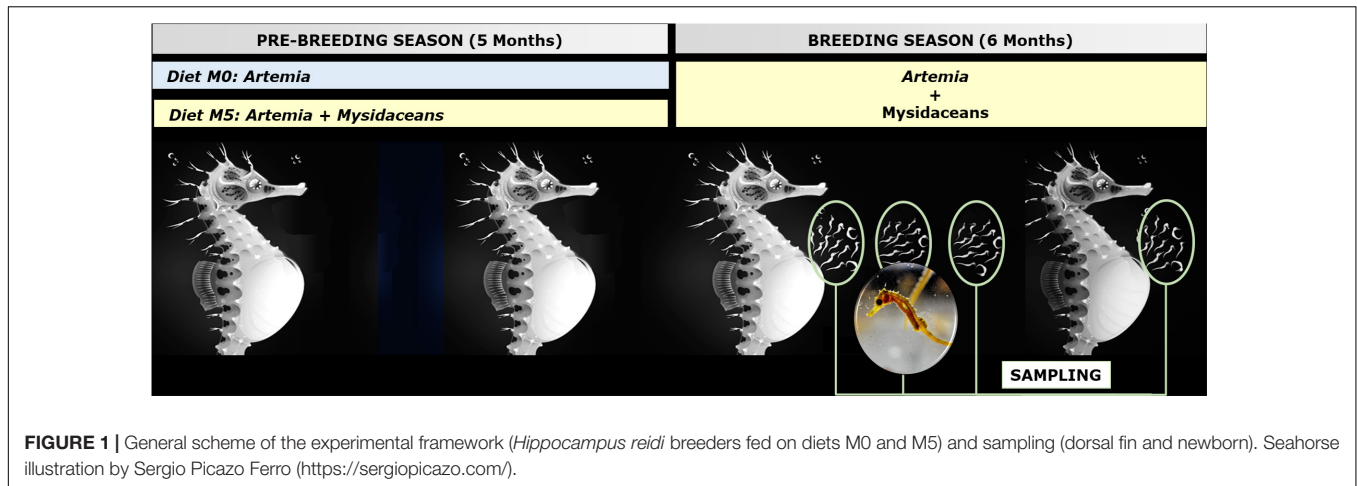
Seahorses were monitored several times a day to check for newborn releases from male's pouch. Samples of freshly released newborn were immediately collected for further analyses (electronic microscopy, total lipids, FAs, endogenous nucleotides, and energy charge calculation) and weight/size determination. Sampled juveniles were euthanized with Tricaine MS-222 (0.1 mg L^{-1} , Sigma-Aldrich). For length and weight measurements, the juveniles were transferred to Petri dishes, photographed, and weighed individually on a Sartorius microbalance MC210P ($\pm 0.01 \text{ mg}$). Curved SL were measured (SL = head + trunk + curved tail) from digital photographs using image processing software (NIS, Nikon).

After sampling, the remaining released juveniles were carefully collected, counted, and transferred (2–5 juveniles L^{-1}) to 30 L pseudo-Kreisel aquaria connected to a semi-opened

TABLE 1 | Total lipids (% dry weight) and fatty acids (relative percentage) in prey ($n = 2$) for seahorse breeders.

Fatty acids (%)	<i>Artemia</i>	Mysidacea
14:0	1.43 ± 0.09	3.41 ± 0.02
15:0	0.46 ± 0.05	0.84 ± 0.03
16:0	17.92 ± 0.92	21.63 ± 0.15
16:1n-7	8.31 ± 0.17	9.06 ± 0.04
17:0	3.22 ± 0.10	1.23 ± 0.03
17:1	0.06 ± 0.01	0.12 ± 0.09
18:0	11.44 ± 0.56	2.65 ± 0.04
18:1n-7	10.39 ± 3.98	4.11 ± 0.00
18:1n-9t	6.97 ± 0.50	5.99 ± 0.05
18:2n-6c	12.55 ± 0.68	1.17 ± 0.01
18:3n-3	0.86 ± 0.04	1.31 ± 0.01
18:3n-6c	6.36 ± 0.36	0.22 ± 0.00
18:4n-3	0.15 ± 0.03	0.18 ± 0.02
20:0	0.64 ± 0.01	0.20 ± 0.03
20:1n-9	0.30 ± 0.01	0.84 ± 0.01
20:2n-6	0.31 ± 0.00	0.52 ± 0.02
20:3n-6	0.66 ± 0.06	0.27 ± 0.00
20:4n-3	0.24 ± 0.00	0.35 ± 0.02
20:4n-6 (AA)	1.45 ± 0.05	2.33 ± 0.01
20:5n-3 (EPA)	15.43 ± 0.76	22.19 ± 0.04
22:4n-6	0.12 ± 0.01	0.28 ± 0.01
22:5n-3	0.00 ± 0.00	0.43 ± 0.08
22:5n-6	0.00 ± 0.00	0.83 ± 0.05
22:6n-3 (DHA)	0.51 ± 0.01	19.69 ± 0.03
24:0	0.20 ± 0.02	0.15 ± 0.01
Saturated	35.3	30.1
Monounsaturated	26.0	20.1
Polyunsaturated	38.7	49.8
n-3	17.2	44.2
n-6	21.5	5.6
n-3 HUFA	16.2	42.6
DHA/EPA	0.03	0.89
DHA/ARA	0.35	8.45
n-3/n-6	0.80	7.87

Sample size: 10–20 mg dry weight.



recirculation system (Blanco et al., 2014). The newborns were reared until day 7 (7 DAR) after male's pouch release (DAR) at 26°C under a constant 14L:10D photoperiod. Further details on the rearing system were reported by Planas et al. (2012).

From 0 to 5 DAR (days after male's pouch release), newborn seahorses were fed on two daily doses of *Acartia tonsa* (0.67 copepods mL⁻¹ dose⁻¹), whereas one daily dose of *A. tonsa* (0.67 copepods mL⁻¹ dose⁻¹) and one daily dose of *Artemia* nauplii (1 *Artemia* mL⁻¹ dose⁻¹) were supplied on 6–7 DAR.

Dead seahorses were daily removed (8:00 a.m. and 3:00 p.m.) and counted. Final survivals of juveniles were recorded at 7 DAR, corresponding to complete mortality in unfed seahorses (personal observation).

Microalgae and live prey (copepods and *Artemia* nauplii) were cultured as reported by Planas et al. (2020b).

Sample Analysis

Total Lipids and Fatty Acids

Total lipids from live prey and 0 DAR juveniles (10–20 mg dry weight per sample; $n = 30\text{--}60$) were extracted according to Bligh and Dyer (1959). Aliquots of total lipid extracts with known lipid content were centrifuged, resuspended with 0.5 M ammonium formate solution, freeze-dried and stored at -80°C . Total lipid content was quantified gravimetrically (Herbes and Allen, 1983). FA composition in total lipids was analyzed by gas-chromatography (GC) according to Christie (1982). Lipids were transmethylated (Lepage and Roy, 1986) and FA analyzed by GC (Perkin Elmer, GC-FID Clarus 500 gas chromatograph) (Planas et al., 2010).

Total C and N

Elemental composition (total C and N) were analyzed in dried newborn seahorses ($n > 5$; pooled individuals). The samples were measured by continuous flow isotope ratio mass spectrometry using a FlashEA1112 elemental analyzer (Thermo Finnigan, Italy) coupled to a Delta Plus mass spectrometer (FinniganMat, Bremen, Germany) through a ConFlo II interface. Further details are provided by Planas (2021).

NAD, ATP, ADP, and AMP Assay and Energy Charge Calculation

Whole seahorses, in five replicates each, were anesthetized and rapidly collected, frozen, and grounded in liquid nitrogen, weighed in aliquots, and stored at -80°C and processed according to Olivotto et al. (2011a).

All data in this work are referred to as grams of N₂-powder (five fresh weight tissues). Tissue N₂-powders (20–200 mg aliquots) were added with 6 mL g⁻¹ of 0.4 M HClO₄ containing 50 mM cAMP (300 nmol g⁻¹) as internal standard and thawed by vortexing. The perchloric extracts were sonicated (30 s at 50 watts with 0.5-s pulses) and centrifuged (16,000g, 10 min at 4°C). The supernatants were neutralized by adding 0.29 vol of 1 M K₂CO₃ and centrifuged again to remove the precipitated KClO₄. The clear supernatant, referred as to neutralized extract, was used for subsequent analyses of metabolites.

The endogenous nucleotides and the internal standard cAMP were quantified by UV C18-HPLC analysis, both under reverse-phase and ion-pairing conditions. For details see Mori et al. (2014). The column temperature was 8°C and the eluate absorbance was monitored by a diode-array detector (Shimadzu SPD-M10A). For quantitation, the integrated peak areas, expressed by the Shimadzu LC solution v1.24 software used as mAU s⁻¹, were first converted into mAU × ml by appropriate factoring, then into nanomoles dividing by the appropriate extinction coefficient at 260 nm (18.6 mM⁻¹ cm⁻¹ for NAD; 15.4 mM⁻¹ cm⁻¹ for ATP, AMP, and cAMP, respectively). The extraction yield calculated from cAMP recovery was 95% in all samples.

For the AEC evaluation, the following equation was used (Atkinson and Walton, 1967):

$$AEC = [ATP + \frac{1}{2}ADP] / [ATP + ADP + AMP]$$

Transmission Electron Microscopy

Abdominal portions of newborns (three newborns per dietary treatment) were processed for Durcupan ACM (Fluka) resin embedding as follows: pieces fixed in 2.5% glutaraldehyde in 0.2 M phosphate-buffered saline (pH 7.4), for 2 h at 4°C, were washed repeatedly in the same buffer and postfixed in

1% osmium tetroxide in 0.2 M phosphate-buffered saline for 1 h at 4°C. Then, the tissues were dehydrated with increasing alcohol concentrations. The dehydrated pieces were embedded in Durcupan ACM (Squadrito et al., 2017). Finally, the sections were obtained with a Reichert Jung Ultracut E. Semi-thin sections (1 μm) were stained with toluidine blue and examined with a light microscope. Ultrathin sections (740 Å) were obtained from selected areas of the semi-thin sections, stained with uranyl acetate and lead citrate, and examined and photographed with a transmission electron microscope (JEOL JEM 1400 Flash) (Viña et al., 2014).

Data Analysis

Statistical analyses were conducted in R v.3.6.3 (R Core Team, 2020a). Variability across treatments was examined by non-parametric Kruskal-Wallis test for independent samples (Kassambara, 2020a). When significant, the means were compared by Wilcoxon range test (Pgirmess v1.6.9 package of R) (Giraudoux et al., 2018) or Dunnett's test (Kassambara, 2020a). Correlations (Pearson's correlation coefficients) and hierarchical clustering linkage (complete method) of variables and samples for newborn batches were performed with factoextra v1.0.7 (Kassambara, 2020b) for computational analysis and igraph v1.2.6 (Csardi and Nepusz, 2006) for plotting. Additionally, principal component analyses (PCA) were performed to summarize and visualize the information newborn datasets. For that, we used factoMineR v2.3 (Husson et al., 2020), factoextra v1.0.7 (Kassambara, 2020b), and corrplot v0.8.4 (Wei et al., 2017) packages in R. The data values were standardized (mean = 0; SD = 1) for clustering and PCA. Data normality was checked with the Shapiro-Wilk normality test (stats v3.6.2 R package) (R Core Team, 2020b). Significance levels were set at $p < 0.05$ and results are presented as mean \pm standard deviation. Graphics were constructed using ggplot2 v3.3.0 (Wickham et al., 2020) and lattice v0.20-41 (Sarkar et al., 2020) packages in R.

The present study was carried out on an endangered/threatened species. Bioethics referred to these organisms imply the use of a number of specimens as much reduced as possible (strictly the necessary). Besides this, the availability of endangered species (particularly breeders) and large aquaria is certainly limited. For that reason, only one aquarium was used for each dietary treatment. Consequently, the breeders were considered pseudoreplicates and the aquaria were the basic experimental units. There is literature available that justify the use of pseudoreplicates in some circumstances and recommends that pseudoreplication not be invoked when reviewing research manuscripts and research proposals (see Planas, 2021).

RESULTS

Breeding Success

The total number of newborn batches produced in treatments M0 and M5 were 11 and 22, respectively, whereas total newborn

production was 4,397 in M0 and 7,298 in M5 (Figure 2). The average batch size in treatments M0 (400 ± 182 newborns) and M5 (332 ± 155 newborns) were similar (K-W test, $n = 36$, $p = 0.321$). Survivals of juveniles at 7 DAR did not differ similarly across dietary treatments (K-W test, $n = 33$, $p < 0.219$), ranging from $86 \pm 7\%$ in group M5 to $88 \pm 10\%$ in group M0 (Figure 2).

Breeders from group M5 produced larger newborn both in SL (K-W test, $n = 36$, $p = 0.002$) and DW (K-W test, $n = 36$, $p < 0.001$) (Figure 2). Mean weights of newborns were 0.22 ± 0.03 mg in M0 and 0.28 ± 0.04 mg in M5, whereas mean SL were 8.0 ± 0.3 mm in M0 and 8.6 ± 0.6 mm in M5.

Total Lipids and Fatty Acids

Overall, total lipids content in newborns ranged from 14.6 ± 1.7 to $14.4 \pm 1.6\%$ dry weight and did not differ among treatments (K-W test, $n = 33$, $p = 0.942$) but decreased continuously in group M5, especially during the first half of the breeding season (Figure 3).

Regarding FA groups across dietary treatments, the relative content (percentage of total FA) in saturates (37.4–37.7%), monoenes (20.6–23.7%) and polyenes (38.9–41.7%) was similar (K-W test, $n = 33$; $p = 0.643$, 0.417 and 0.153, respectively) (Figure 3). Total n-3 HUFA accounted for 28.4–29.6% (K-W test, $n = 33$, $p = 0.942$).

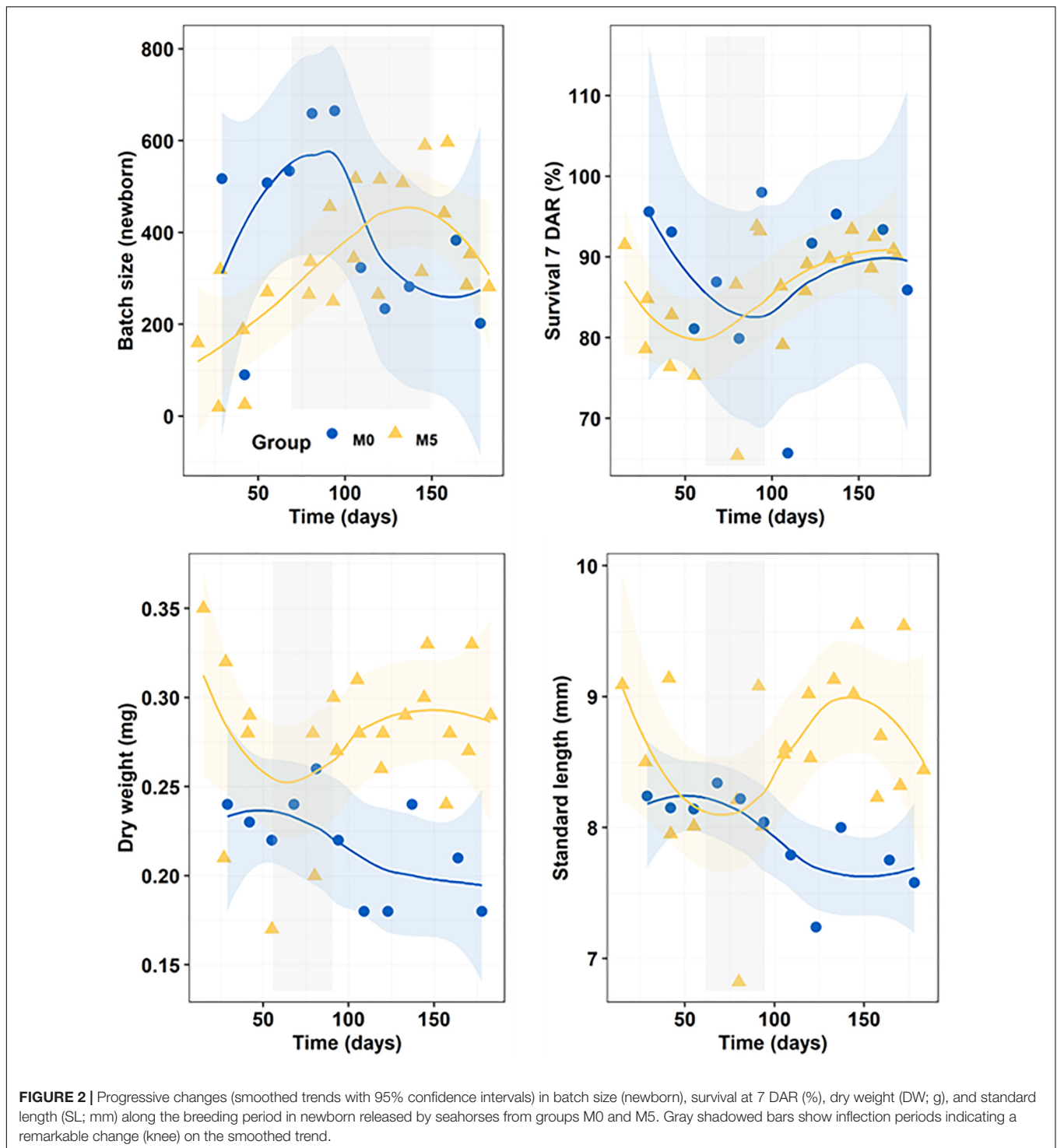
DHA/EPA (range: 3.33–3.36), DHA/AA (4.31–4.70) and DHA/DPA (4.73–4.91) values gradually increased during the breeding season but average ratios for the whole period did not differ among treatments (K-W test, $n = 33$, $p = 0.817$, 0.113 and 0.589, respectively) (Figure 3).

Statistical mean comparisons for individual FAs (area percentage) for the whole Period 1 only revealed significant differences in 16:0 (K-W test, $n = 33$, $p = 0.021$), 18:0 ($p = 0.025$), 18:3n-3 ($p = 0.045$), 20:2n-6 ($p = 0.049$), and 22:4n-6 ($p < 0.001$) (Figure 4).

Nucleotides and Energy Charge

Figure 5 reports the progress of endogenous nucleotide (AMP, ADP, ATP, and NAD) content and AEC in newborn seahorses along the breeding season. With regards to both AMP and ADP content, no significant differences were detected between M0 (5.49 ± 3.42 and 8.33 ± 3.52 pmol/μg of proteins for AMP and ADP, respectively) and M5 (5.60 ± 3.08 and 8.63 ± 2.75 pmol/μg of proteins for AMP and ADP, respectively) groups. Differently, M0-group showed significantly ($n = 19$; $p < 0.05$) lower values of ATP (13.26 ± 5.51 pmol/μg of proteins) and NAD (2.33 ± 0.81 pmol/μg of proteins) content as compared to M5-group (30.16 ± 10.41 and 3.35 ± 0.82 pmol/μg of proteins for ATP and NAD, respectively). Finally, M0-group (0.62 ± 0.14) evidenced a significantly ($p = 0.001$) lower AEC compared to M5-group (0.78 ± 0.05).

ATP/ADP ratios in M5 and M0 treatments (3.69 ± 1.20 and 1.82 ± 0.83 , respectively) were significantly different ($n = 17$; $p = 0.005$).



Pooled adenylic nucleotides were significantly correlated with total C ($df = 15$, $r = 0.587$, $p = 0.01$), ADP ($r = 0.641$, $p = 0.006$), and especially ATP ($r = 0.925$, $p < 0.0001$) and NAD ($r = 0.630$, $p < 0.007$). Furthermore, NAD was also positively correlated with total C ($r = 0.544$, $p = 0.024$), AMP ($r = 0.541$, $p = 0.025$), ADP ($r = 0.693$, $p < 0.002$) and ATP ($r = 0.630$, $p = 0.007$). Also, AEC was positively correlated with ATP ($r = 0.640$, $p = 0.006$) and

ATP/ADP ratio ($r = 0.722$, $p = 0.001$), and negatively correlated with AMP ($r = -0.556$, $p = 0.020$).

Changes With Breeding Season Progress

Average values for the whole breeding period in the variables assessed in newborns did not differ significantly in most cases. However, the smoothed trends showed in **Figures 2–6** revealed

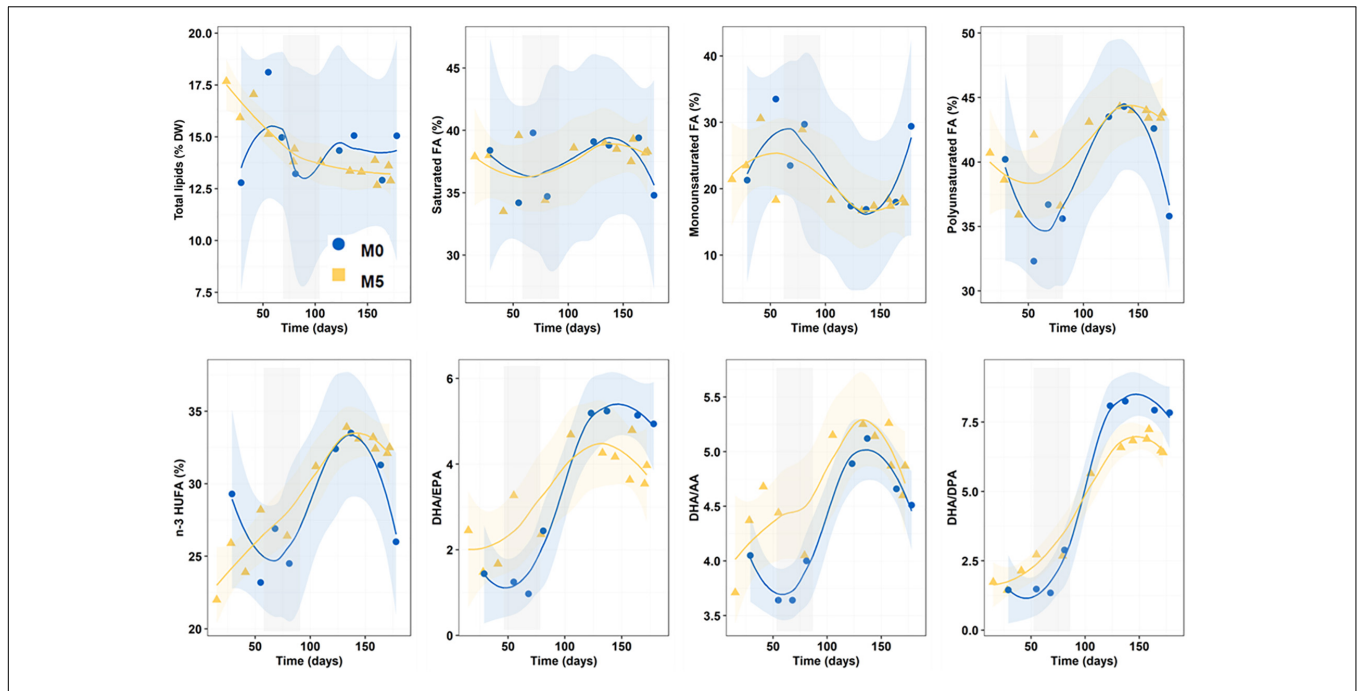


FIGURE 3 | Progressive changes (smoothed trends with 95% confidence intervals) in total lipids (dry weight percentage), fatty acid series (percentage of total FA) and fatty acid ratios along the breeding period in newborn released by seahorses from groups M0 and M5. Gray shadowed bars show infection periods indicating a remarkable change (knee) on the smoothed trend.

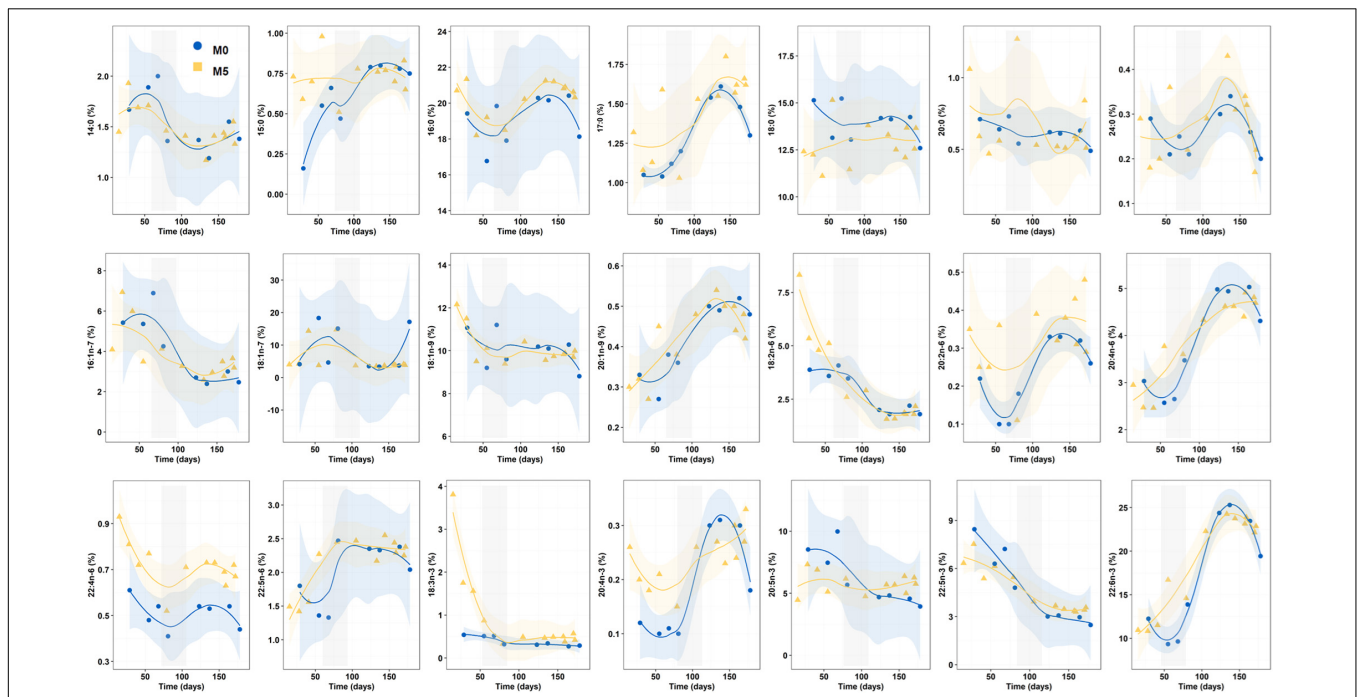
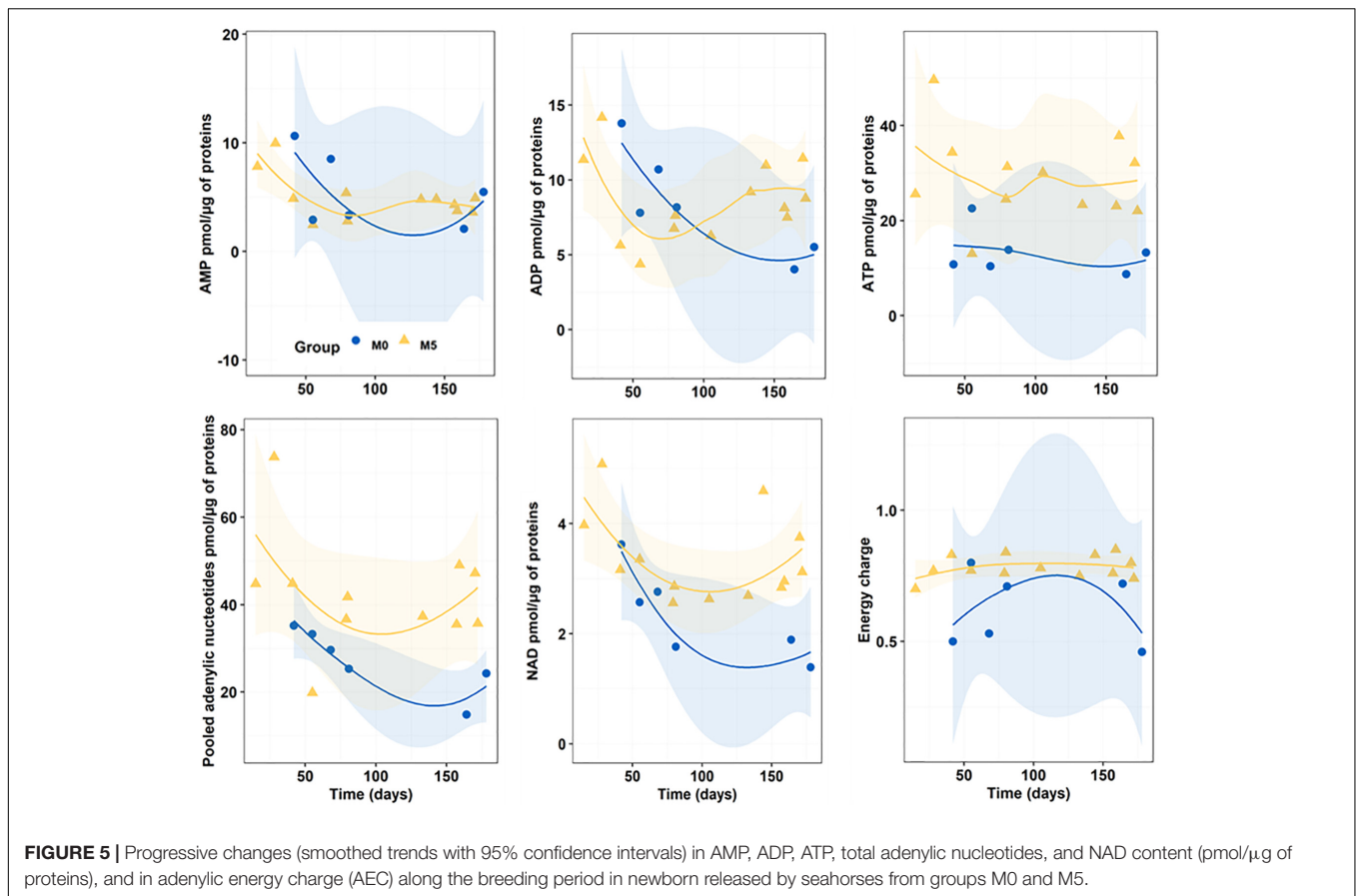


FIGURE 4 | Progressive changes (smoothed trends with 95% confidence intervals) for fatty acids (percentage of total FA) along the breeding period in newborn released by seahorses from groups M0 and M5. Gray shadowed bars show infection periods indicating a remarkable change on the smoothed trend.

that the diverse characteristics of the batches released differed along the breeding season. Accordingly, the characteristics of batches released in the first part of the season (Period 1) differed

in most cases from those released later (Period 2). Hence, a comparison between both periods was more informative than the analyses performed over the entire breeding period.



An intermediate phase, tentatively ranging from day 70 to day 100 (see gray bars in **Figures 2–4**), would indicate a transitional change between stages 1 and 3. The comparative analyses revealed that DW and SL in newborn released on stage 3 were lower in M0 than in M5 batches (K-W test, $n = 33$, $p < 0.01$). However, survivals at 7 DAR did not differ with treatments nor stages (K-W test, $n = 33$, $p = 0.370$). Concerning total lipids and FAs, the most significant differences occurred between stages 1–2 and 3, whereas M0 and M5 batches released in stage 1 showed similar profiles and wide ranges for most FA. Interestingly, DHA (22:6n-3), AA (20:4n-6), and 11-eicosenoic acid (20:1n-9) contents in M0 and M5 batches from stage 1 were significantly lower than those in stage 3 ($n = 20$, $p = 0.002$).

About AMP, ADP, ATP, NAD, and AEC across groups M0 and M5, no statistically significant differences were revealed by the comparisons carried out on newborn batches produced on the first and second periods of the breeding season.

Additional data and results on statistical tests performed on newborn characteristics, lipid and FAs content, endogenous nucleotides, AEC and NAD are provided in **Supplementary Figures 1–4**. Data on the content in total C, total N and protein are shown in **Supplementary Figure 5**.

Transmission Electron Microscopy

Ultrathin sections of abdominal portions of newborn seahorses were studied by transmission electron microscopy (TEM)

to analyze the ultrastructural aspects of muscular tissue. Longitudinal sections from groups M0 and M5 showed the sarcoplasm filled with myofibrils oriented parallel to the long axis of the cell. The sarcomeres displayed the characteristic Z lines, light isotrope bands I, dark anisotrope bands A, and central H-bands bisected by M lines. That sarcoplasmic reticulum appeared regularly spaced. The sarcotubules formed a continuous system around and between individual myofibrils. Sarcoplasmic reticulum cisternae in contact with invaginations of the sarcolemma, T tubules, where they formed the sarcoplasmic triad have, also, been observed. These were located at the Z line level of sarcomeres (**Figures 6a,b**, Upper).

The oblique (**Figures 6a,b**, Middle) and transverse sections (**Figures 6a,b**, Lower) from groups M0 and M5 of a striated muscle through the A and I bands showed that the myofibrils sectioned at various levels through the sarcomeres revealed the regular different arrays of filaments. The myofibrils were represented by closely packed irregular polygonal profiles and this hexagonal organization determined by the arrangement of the thick and thin of the myofilaments was clearly visible. These are separated by a small amount of sarcoplasm containing rows of mitochondria regularly organized among the sarcomeres. The nuclei of fiber muscles, myonucleus, were located in the peripheral layer of the fiber beneath the sarcolemma. Numerous satellite cells, identified by their location inside the basal lamina and outside the sarcolemma, have been seen. They were

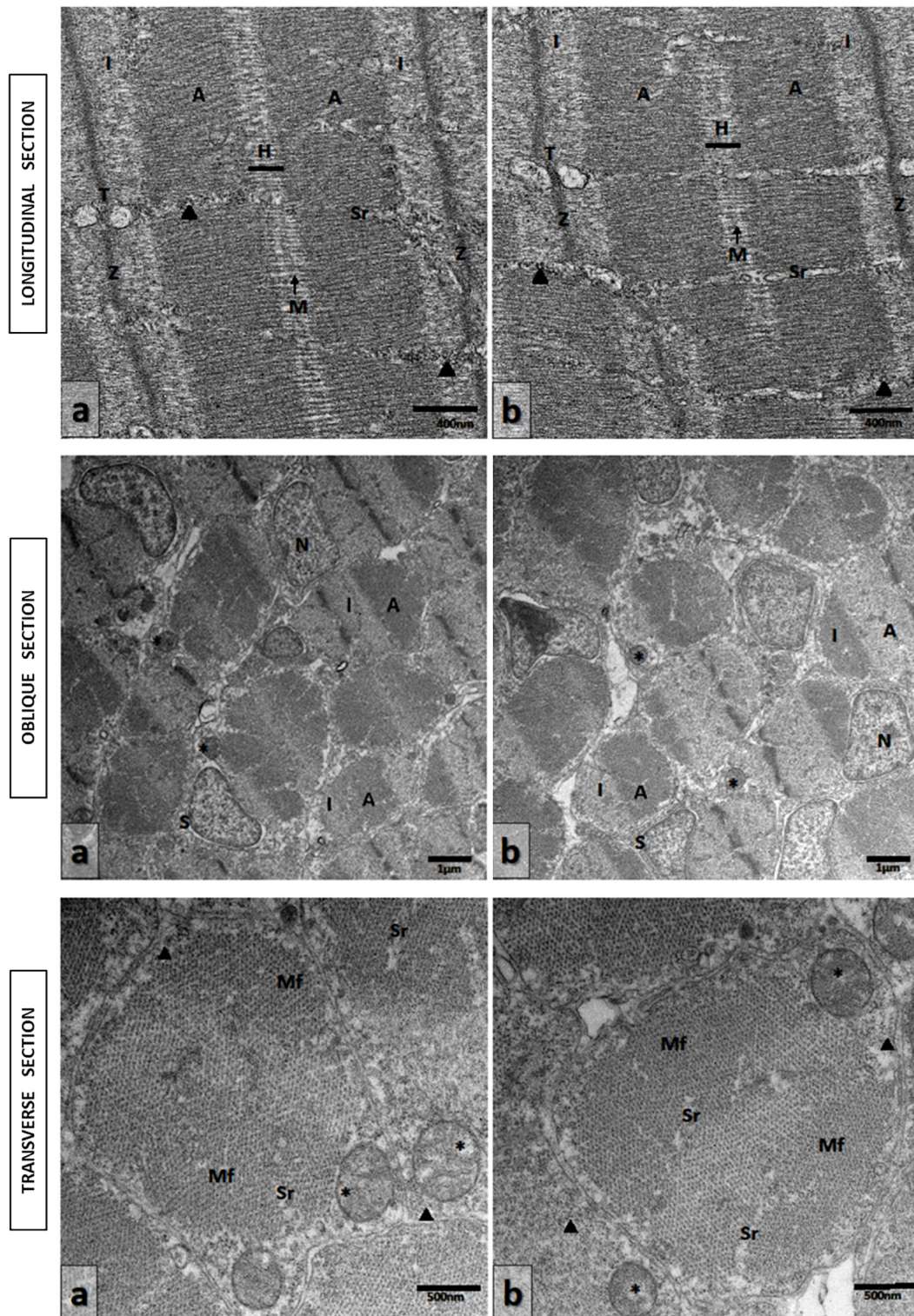


FIGURE 6 | Transmission electron micrographs of skeletal muscle in newborn seahorses from groups (a) M5 and (b) M0. *Upper:* Longitudinal sections. Sarcomeres with characteristic Z lines, I bands, A bands, and central H-bands bisected by M lines. Triad (T) located at the Z line level. Sarcoplasmic reticulum (Sr). Glycogen granules (arrowheads). Scale bars: 400 nm. *Middle:* Oblique sections at various levels through the sarcomeres. Light isotrope band (I); dark anisotrope band (A); myonucleus (N); mitochondria (asterisks); muscle satellite cell (S). Scale bars: 1 μm. *Lower:* Transverse sections. Myofibrils (Mf); mitochondria (asterisks). Sarcoplasmic reticulum (Sr). Glycogen granules (arrowheads). Scale bars: 500 nm.

characterized by a very little cytoplasm around the nucleus, devoid of myofilaments and few small mitochondria. Glycogen granules were also observed (Figures 6a,b, Upper and Lower).

Global Assessment

The PCA performed on datasets (see variables included in Figure 7) revealed that factors 1 and 2 explained 61.4% of total data variability. Newborn batches released on the first half of the breeding season were displayed on the left side of the plot, whereas samples released on the second half were closer to each other and located on the right side. Factor 1 was positively associated with time (days elapsed since the onset of the breeding period), 17:0, 20:1n-9, 20:4 n-6, 22:6n-3, DHA/EPA, and DHA/DPA, and negatively related with 16:1n-7 and 22:5n-3. The plot also showed a higher similarity between samples collected on the second half of the breeding period (batches M0-2 and M5-2) compared to those on the first half of that period (M0-1 and M5-1). Factors 3 and 4 accounted for 18.2% of total variability, which was mostly explained by the content in 18:0 (M0 < M5).

%

With regards to PCA results on endogenous nucleotides and energy charge in newborn seahorses (Figure 7), Factor 1 (36.8%) was positively associated with NAD, ATP, and newborn weight, whereas factor 2 (21.7%) was positively related to time and survival at 7 DAR, and negatively associated with lipid content. The plot showed clear discrimination among treatments, with lower endogenous nucleotide levels in treatment M0, particularly in M0-2. The main finding in factors 3 (12.9%) and 4 (9%) biplot was the higher energy charge in newborn from treatment M5 during the whole breeding season.

DISCUSSION

Diets and Breeding Success

The results achieved on the assessment of effects of dietary treatments on the pre-breeding season in the tropical seahorse *H. reidi* denoted significant improvements in size, weight, endogenous nucleotide content and AEC in newborn, as well as several changes in the biochemical composition (FA profiles). The use of *Artemia* as the unique prey (treatment M0) resulted in lower initial fitness in newborns as compared to those fed on the mixed diet including mysidaceans (treatment M5). Since some aspects of the influence of the diet on reproductive potential and success in *H. reidi* have been assessed previously (Planas, 2021), this discussion will be mostly focused on biochemical characteristics, energetic status and tissue ultrastructure in newborns.

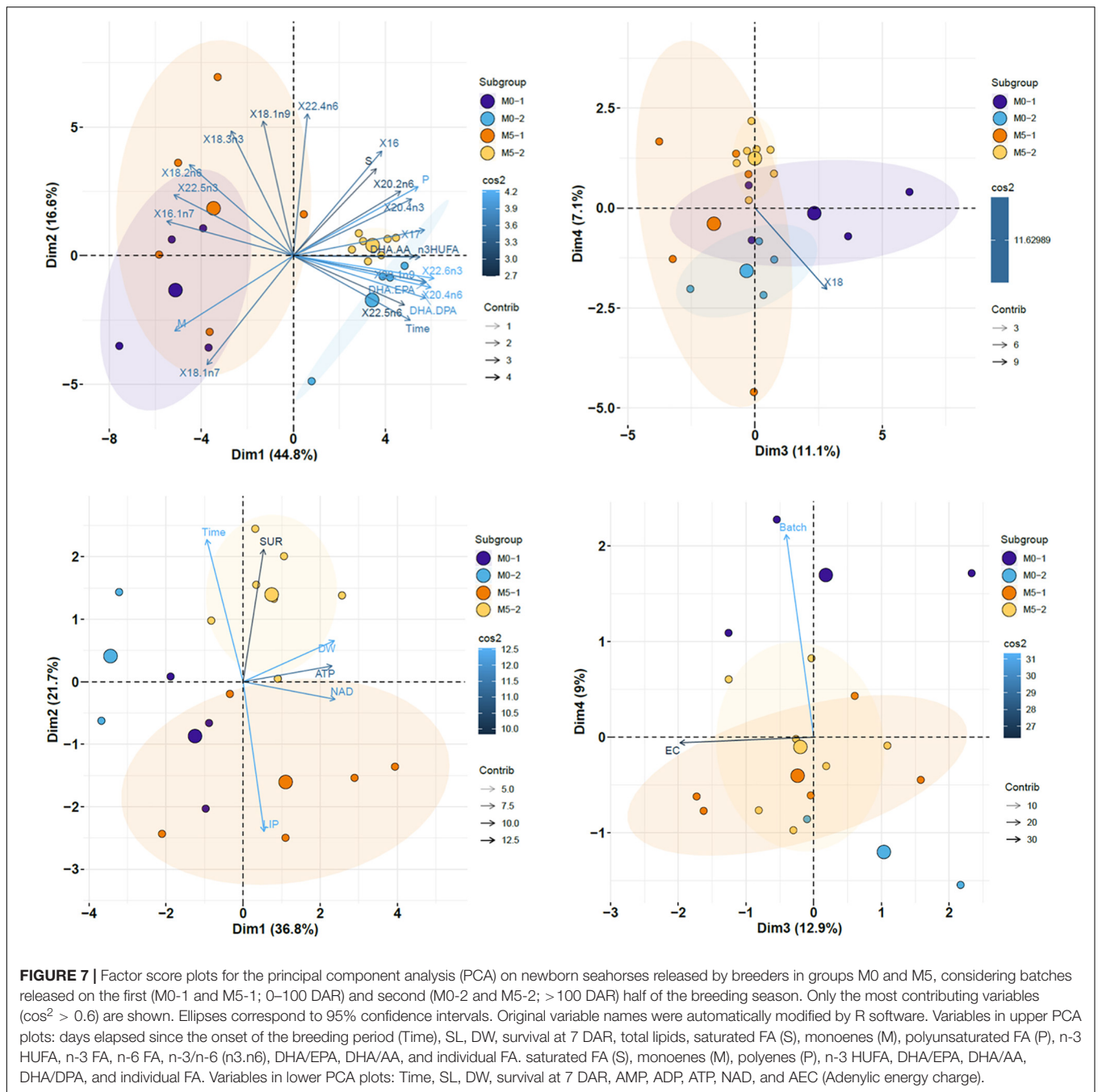
The experimental diets were efficiently incorporated into the breeder's tissues as assessed by stable isotopes (Planas, 2021). Hence, the results achieved would rely mainly on the biochemical characteristics of the diets provided during the pre-breeding season. The negative effect of low-quality diets (i.e., non-enriched adult *Artemia*) on fish breeding has been demonstrated in many fish species (Izquierdo et al., 2000, 2001; Piccinetti et al., 2012). Providing a mixed diet including a potential high-quality prey (i.e., mysidaceans, amphipods, or carideans) or

supplementary enrichment (Murugan et al., 2009; Otero-Ferrer et al., 2012; Saavedra et al., 2014) would certainly enhance breeding success as reported in *H. guttulatus* (Palma et al., 2012, 2017; Planas et al., 2020b). Neither average lipid content nor initial survivals for the whole reproduction season resulted significantly affected by the type of pre-breeding diet offered to breeders. However, overall breeding performance (spawning events and total newborn produced), and neonates size and weight were markedly enhanced in breeders fed on the mixed diet (M5-group).

We did not observe premature egg clutch dropping nor premature newborn release in breeders fed exclusively on *Artemia*, which is in agreement with previous findings in *H. guttulatus* (Planas et al., 2020b) but not with those in *H. hippocampus* (Otero-Ferrer et al., 2012; Saavedra et al., 2015) or *Hippocampus kuda* (Saavedra et al., 2014). Therefore, the quality of breeder's diet during the pre-reproduction period should not affect postnatal viability if offspring are subsequently fed on high-quality prey such as copepods. However, it is known that the quality of male's diet offered from 1 month before conception may result in large neonates with low viability (Otero-Ferrer et al., 2016). An effect of diet quality in males has also been reported on other syngnathids (Sagebakken et al., 2017). Our findings might not be extrapolated to other syngnathid species as the early performance of newborns would likely depend upon specific characteristics such as the balance between brood size, newborn size, and survival (Planas et al., 2020b), and on parental size and age (Dzyuba et al., 2006; Faleiro et al., 2016). In that respect, our breeder's groups were similar in average weight but there were within-group differences in ranges for both size and age. Since those characteristics seem to affect further juvenile performances, it would have been interesting to analyze both growth and survival until more advanced developmental stages and to identify parents involved in each batch produced as suggested by the results obtained by Dzyuba et al. (2006). Unfortunately, parental identities for each batch produced could not be identified in the present study.

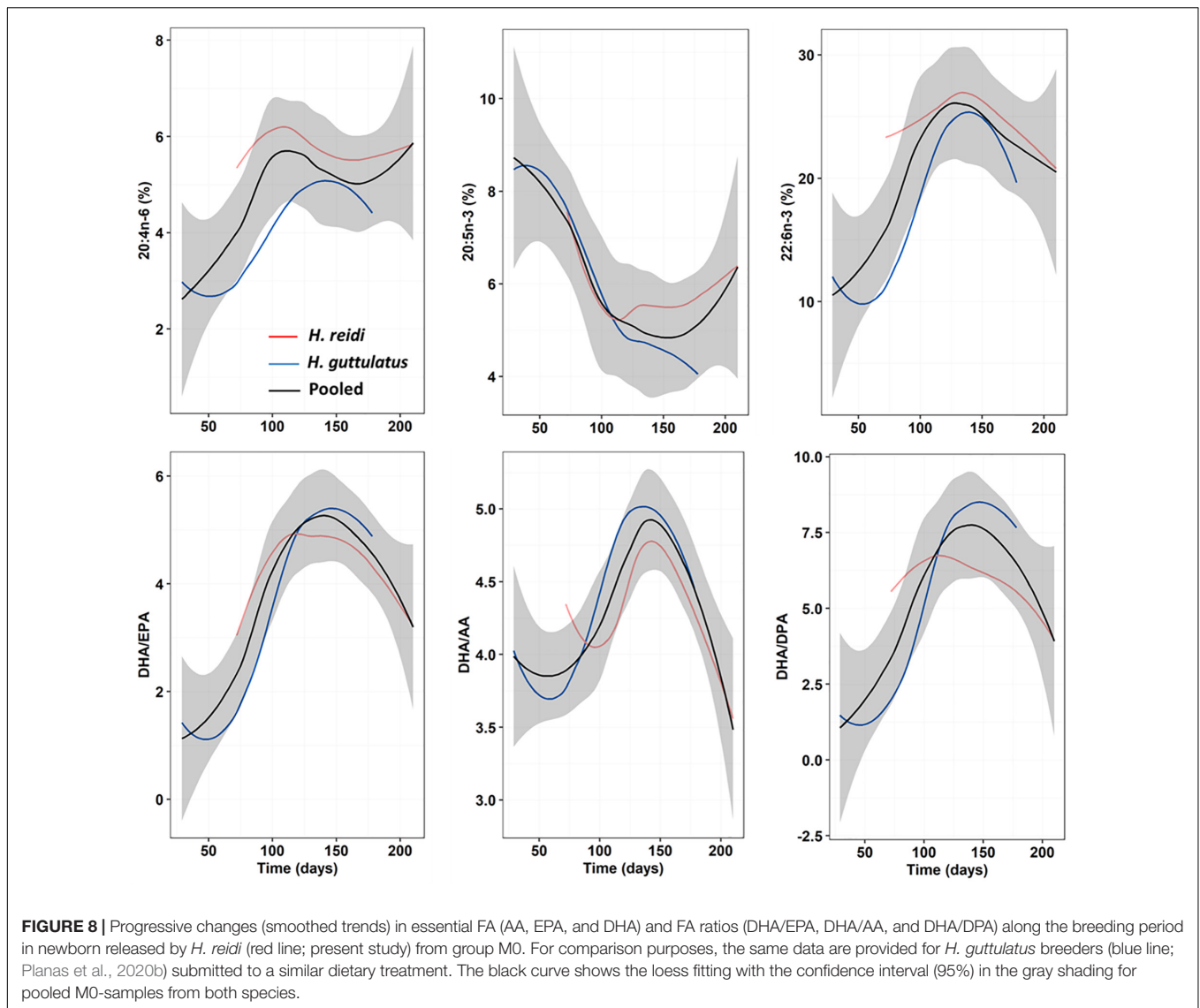
Fatty Acids Profiles

The importance of essential FAs, namely n-3 HUFA, to marine and freshwater fish larvae has been studied extensively (Sargent et al., 1999; Izquierdo et al., 2000; Kanazawa, 2003; Olivotto et al., 2010; Tocher, 2010; Zarantoniello et al., 2018), but only a few studies have been published in eggs or newborn seahorses. FA profiles in seahorse eggs, neonates and also adults largely differ depending on species, temperature and breeder's diet (Chang and Southgate, 2001; Lin et al., 2008; Faleiro and Narciso, 2010; Otero-Ferrer et al., 2010, 2012; Planas et al., 2010, 2020b; Saavedra et al., 2014, 2015; Buen-Ursue et al., 2015), hence, comparative analyses are difficult to assess. Despite the large differences in the proportion of dietary FA groups, average relative proportions in saturates, monoenes, polyenes, and n-3 HUFA in newborn seahorses were rather similar across our dietary treatments. That finding indicates that the production of eggs/neonates in *H. reidi* is accomplished following a conservative plan for global FA groups, essential FAs (arachidonic and docosahexaenoic acids) and FA ratios.



Interestingly, the shapes of the patterns followed by those FAs and ratios in *H. reidi* resembled those in *H. guttulatus* if time lags and differences in values are not considered (Figure 8 and Supplementary Figure 6). Conversely to AA and DHA, EPA values in both species along the second phase of the breeding season followed different patterns. These three FA, especially DHA and AA, are essential biochemical compounds in marine fish larvae, being highly dominant within the total pool of FA (Rainuzzo, 1993; Rainuzzo et al., 1997; Sargent et al., 1999). DHA is the main FA in both vitellogenin and developing embryos of marine fishes (Sargent et al., 1999; Tocher, 2003). However, their

stores in young seahorses may be partially used as fuel, especially EPA, which is consumed at a higher rate (Faleiro and Narciso, 2010). Differences in EPA catabolism compared to AA and DHA were likely reflected in differences in the patterns of change discussed above. When comparing *H. reidi* and *H. guttulatus*, time lags observed for certain FA and ratios (e.g., AA, DHA/EPA, and DHA/DPA) might be due to species-specific differences on inter-batch intervals and temperature thresholds. The ratio DHA/EPA and the content in AA seem to be higher in species from warmer water temperatures (Dey et al., 1993; Ogata et al., 2004; Ibáñez, 2018) but the validity of those assumptions would



likely depend on a series of factors other than temperature (e.g., breeder's diet).

The interest of analyzing by TEM the ultrastructure of muscular tissue in newborns was due to a previous study in which newborn of *H. guttulatus* released by breeders fed on *Artemia* during the reproduction season showed the presence of ultrastructural muscle alterations (Planas et al., 2020b), likely due to deficiencies in DHA (Cong-cong et al., 2019). The observations by TEM have not detected morphological alterations nor differences as for both analyzed groups M0 and M5, suggesting that both administered diets ensured the regular development of muscular tissue.

Adenylic Energy Charge

The energy status of an organism is fully charged when it consists of ATP alone (adenylate charge = 1.0). A charge value of 0.5 indicates that only ADP is present, whereas the energy charge (AEC) is zero when all adenine nucleotide pool is in

form of AMP (system fully discharged) (De la Fuente et al., 2014). The AEC might be a relatively conservative property and changes in AEC are always accompanied if not preceded by changes in the quantity of adenylate nucleotides (Atkinson, 1968). Commonly, AEC values in unstressed organisms are above 0.80 (Ivanovici, 1980; Vetter and Hodson, 1982), so that AEC was proposed as an indicator of the energy potentially available for cellular metabolism (Atkinson, 1977; Dickson and Franz, 1980). Interestingly, ATP concentration, total adenylate concentration, and AEC might be useful indices of environmental impact (i.e., stress) (Vetter and Hodson, 1982; Dehn and Schirf, 1986; Methling et al., 2017) and condition factor in fishes (Giesy, 1988). However, AEC level did not always reflect the growth of organisms, as reported by Harms (1992) in sub-optimally fed larvae of the crab *Hyas araneus*, in which pooled adenylic nucleotides were found to be correlated with the content in total C and protein, reflecting differences in the experimental diets. In our study, pooled adenylic nucleotides were correlated with

turbot (*Psetta maxima*) larvae. Those authors reported a daily weight-specific growth rate of $29.6\% \text{ day}^{-1}$, and suggested a trade-off between fast growth and viability in the larvae. With growth rates of up to about $25\% \text{ day}^{-1}$, the energy demands for growth represent a large fraction of the energy available (Pedersen, 1997). The ATP/ADP ratio is a robust readout of the cellular energy status and its value should remain high in order to maximize the energy status and cell metabolism. Conversely, energy depletion with an accumulation of AMP would deplete the cell's stores of ATP and ADP leading to cell death. Considering the higher AEC values in M5-newborn compared to M0-newborn (3.69 ± 1.20 and 1.82 ± 0.83 , respectively), a greater amount of energy would be available in the former for growth and cell metabolism. The values of ATP/ADP ratio in newborn seahorses support that statement, showing that suboptimal feeding conditions in breeders were reflected in the nucleotide pool and energy charge of newborn. In this context, higher initial growth rates would be expected in M5-newborns. However, neither survivals nor specific growth rates until day 7 differed significantly (SGR: 17.7 ± 7.4 and $20.8 \pm 4.5\% \text{ day}^{-1}$ in M5- and M0-juveniles, respectively) (data not provided). Those growth rates were high but markedly lower than in larvae of other fish species, likely due to low digestion and assimilation efficiencies in early developing seahorses (Novelli et al., 2015; Ofelio et al., 2018; Valladares and Planas, 2021). Seahorse newborn from treatment M0 enhanced their AEC values along the first stage of the breeding season, suggesting a progressive energetic improvement. Increasing AEC values in newborn might facilitate a rapid resumption of synthetic activity when accessing high-quality food (Harms, 1992) such as copepods (Olivotto et al., 2008; Palma et al., 2017; Randazzo et al., 2018).

NAD plays an important role as energy and signal transducer in cellular metabolism and can be synthesized from NMN (nicotinamide mononucleotide) and ATP. Overall, ATP, total adenylic nucleotides and endogenous NAD in seahorse newborns were highly correlated, and their levels in group M5 were about 2.3-, 1.7-, and 1.4-fold higher than in newborns from group M0. Studies on this subject in marine fish larvae are extremely scarce but our findings agree with those reported in developing clownfish (*Amphiprion ocellaris*) larvae by Olivotto et al. (2011a). In that study, the authors suggested that the enhancement in HUFA content in preys may improve larval development through the presence of better-structured mitochondria, and higher energy status and ATP synthesis compared to the control. Consequently, growth performance and survivals enhanced in clownfish larvae supplemented with HUFA and the larval phase was shortened, resulting in better larval condition to undertake metamorphosis.

In seahorse neonates, yolk reserves are lacking or almost exhausted by the time of male's pouch release (Novelli et al., 2015; Ofelio et al., 2018). Hence, the quantity of lipid of energy reserves would be particularly important for the newborns to withstand starvation or low-quality prey supply, especially in species lacking yolk sac at hatching (e.g., seahorses). However, the presence of ontogenetic shifting in the

energy allocation strategy (growth vs storage) demonstrated in other fish larvae (Cunha et al., 2007; Olivotto et al., 2011a; Di Pane et al., 2019) is unknown in seahorses, deserving further investigation.

Breeding Strategy

The pattern of change throughout the breeding season was investigated to identify and assess the effects with time of pre-breeding feeding on the variables analyzed (see **Figures 2–5** and **Supplementary Figure 6**) and the timing of energy allocation to reproduction. The detection of knee-phases and their onset on general patterns for each variable were used to determine the breeding strategy followed by *H. reidi*. In a previous study, we applied a similar assessment to the temperate seahorse *H. guttulatus*, whose breeders fed on different diets (but similar to those used in the present study) during the breeding season (Planas et al., 2020b). Our results in *H. reidi* were highly similar to those achieved in *H. guttulatus*. Both species displayed a mixed capital-income strategy, which implies a progressive decrease in newborn quality along the breeding season (McBride et al., 2015; Griffen, 2018). Due to species-specific characteristics concerning temperature and inter-brood intervals, the knee-phase in *H. reidi* occurred about 40–50 days earlier than in the temperate species. Inter-brood intervals are about 14–16 days at 25–27°C in *H. reidi* (Otero-Ferrer et al., 2016) and 27–35 days at 18–20°C in *H. guttulatus* (Planas et al., 2010).

In most cases, knee-periods identified in general trends for *H. reidi* were located within the period comprising days 70 and 100 since the onset of the breeding season, depending on the variable and treatment considered. Hence, samples of newborn released before day 100 were notably different to those subsequently released, as confirmed by the PCS analysis and the hierarchical cluster analysis provided in **Figure 9**. The similarity dendrograms also showed high differences among groups from both dietary treatments, particularly for those released before day 100. Accordingly, three stages can be recognized along the whole breeding period:

1. Period 1 (mixed capital-income sources): this stage would extend until days 70–100 since the onset of the breeding season. Most energy and nutritional resources allocated to eggs/newborn originated from the diet provided before the onset of the breeding period (capital breeding). However, the importance of income breeding (sources gained to the experimental diet) increases gradually.
2. Inflection period (income sources): this is a transitory period shown by a knee on smoothed curves. Considering the whole datasets, the duration of this stage would be about 1 month, indicating the end of the capital-income breeding period and the use of nutritional resources from the breeder's diet.
3. Period 2 (income sources and exhaustion of body reserves): globally, the similarity between dietary treatments increased compared to samples released in Period 1. This statement is especially true for FAs profiles. Conversely, differences for total adenylic nucleotides and NAD were maximal by the end of this period. The drop observed

in those products and in survivals from group M0 was noticeable. These findings together with the values for DHA and DHA/EPA in late batches suggest different levels of body resources exhaustion, particularly notorious in M0-newborn as the result of limitation in the availability of nutrients for egg/newborn formation. In this period, brood size and newborn size, and weight were considerably lower in treatment M0.

Breeding patterns are a conditional reproductive strategy since patterns along the capital–income continuum may shift with ontogeny or environmental conditions (McBride et al., 2015; Williams et al., 2017). Shiftings on the acquisition of nutritional and energy supplies for newborns along the breeding season were reported in *H. guttulatus* (Planas et al., 2020b) and validated for *H. reidi* in the present study. Shifting patterns, timing and effective duration of feeding treatments are crucial factors to understand breeding success in synchronous species such as seahorses. The duration of the improvement in eggs or fry quality is a pivotal aspect that must be precisely determined. However, the data available are rather contradictory. Some characteristics in newborn resulted notably enhanced in most M5-batches along the whole reproductive period compared to those from treatment M0. This finding agrees with our previous study on *H. guttulatus*. However, the effect of enhanced diets provided to *H. kuda* females on egg size was limited to the first spawning (Saavedra et al., 2014). From a nutritional and energetical point of view, the roles played by seahorse males and females in the production of eggs and neonates remain unclear, even though the contribution of males (and the effect of their diets) seems to be important in nutritional supply (Skalkos et al., 2020) and a key determinant in newborn quality (Otero-Ferrer et al., 2020).

CONCLUSION

The results achieved in this study in *H. reidi* demonstrate the importance of feeding breeders on a high-quality diet (M5-treatment) during the pre-breeding period. The improved diet, including *Artemia* and mysidaceans during the whole breeding season, resulted in more reproductive events, and larger size and higher energetic status (ATP, total adenylc nucleotides, AEC, and NAD) in neonates. Initially, the effect of dietary treatments on n-3 and n-6 HUFA, especially on DHA, were lower than expected but noticed in FA ratios. This finding suggests (but not demonstrates) lower requirements in n-3 HUFA than other seahorse species. The species displayed a mixed capital-income breeding continuum, as previously demonstrated in *H. guttulatus*, in which the effects of the pre-reproductive diet were mainly noticed on the first 2–3 months of the breeding season. Even though the further newborn performance was not jeopardized when the juveniles gained access to high-quality prey, the effect of parental diets should deserve further investigation under low quality or availability of food to seahorse juveniles.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories (<http://dx.doi.org/10.17632/n6zt5zw22w.1>).

ETHICS STATEMENT

The animal study was reviewed and approved by the Xunta de Galicia (REGA ES360570202001/15/FUN/BIOL.AN/MPO01).

AUTHOR CONTRIBUTIONS

MP: funding acquisition, project administration, conceptualization, methodology, fatty acids and other analyses, data curation, formal analysis, visualization, writing—original draft preparation, and writing—review and editing. IO: formal analysis, data curation, visualization, and writing—review and editing. MG: methodology, fatty acids analysis, and data curation. RL: methodology, TEM analysis, formal analysis, visualization, and writing—review and editing. CA and AA: methodology, nucleotides analysis, data curation, and writing—review and editing. MZ: methodology, nucleotides analysis, data curation, formal analysis, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2021.688058/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.

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