frontiers in Marine Science

REVIEW
published: 09 February 2021
doi: 10.3389/fmars.2021.608155

Tri-Spine Horseshoe Crab Aquaculture, Ranching and Stock Enhancement: Perspectives and Challenges

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Specialty section:
This article was submitted to Marine Conservation and Sustainability, a section of the journal Frontiers in Marine Science

Received: *19 September 2020*
Accepted: *13 January 2021*
Published: *09 February 2021*

Citation:
Xu P, Bai H, Xie X, Wang C-C, Huang X, Wang X, Zhang M, Ye Z, Zhu J, Zhen W, Cheung SG, Shin PKS and Kwan KY (2021) Tri-Spine Horseshoe Crab Aquaculture, Ranching and Stock Enhancement: Perspectives and Challenges. Front. Mar. Sci. 8:608155. doi: 10.3389/fmars.2021.608155

As a well-known example of "living fossil," horseshoe crabs are ecologically significant macroinvertebrates in coastal and estuarine ecosystems. The tri-spine horseshoe crab, *Tachypleus tridentatus*, has been widely utilized for *Tachypleus* amebocyte lysate production and food consumption since the 1980s, which led to considerable population declines along the west coast of the Pacific Ocean. The declining horseshoe crab population is expected to have ecological and social impacts. Stock enhancement through captive rearing of juveniles is cited as an important alternative to repopulate the native *T. tridentatus*, which in turn supports sustainable resource utilization and research activities. The hatchery production techniques for this species have gradually developed following the mass culture efforts in Japan since the late 1980s. However, the previous studies have primarily concerned the feed types and husbandry conditions to maximize the growth and survival of the juveniles. Little is known about the practicability and effectiveness of releasing large numbers of hatchery-bred individuals through releasing programs. In this review, we (1) summarize the available captive breeding and rearing techniques, (2) discuss the release strategies that could potentially improve the survival of released juveniles, and (3) identify the future opportunities and challenges in establishing technical frameworks to support responsible stock enhancement programs for *T. tridentatus*. The information should benefit future horseshoe crab fisheries management efforts in the attempt to restore the severely depleted populations.

Keywords: captive rearing, release strategy, optimal diet, size at release, tagging

INTRODUCTION

Current global fisheries production is severely threatened by overfishing, climate change, and their interactions (Brander, 2007). However, with the advance in aquaculture techniques, this opens up the possibility to release hatchery-reared juveniles to help restore depleted global fishery stocks (Bartley and Bell, 2008; Le Vay et al., 2008; Lorenzen et al., 2010; Han et al., 2016; Taylor et al., 2017). Uses of hatchery releases with different management objectives are referred to as restocking ("to restore depleted spawning biomass to a level where it can once again provide regular, substantial yield"), stock enhancement ("to augment a natural supply of juveniles and optimize harvest by overcoming recruitment limitation"), and sea ranching ("to harvest at a larger size in put-grow-take operations") (Bell et al., 2008). Yet, few releasing programs have claimed their management interventions successful to produce a measurable impact on fisheries stock abundance and yield. Challenges such as producing cost-effective juveniles, developing optimal release strategies and searching for appropriate monitoring and evaluation methods, have been identified (Molony et al., 2005; Wada et al., 2010). A set of elements aimed at developing a responsible approach for releasing programs has been proposed, which emphasizes the equal weight to the dynamics of biological and human components (Blankenship and Leber, 1995; Lorenzen et al., 2010). Lorenzen et al. (2010) also identified common weaknesses in most stock enhancement efforts in coastal systems, including lack of fishery stock assessments and modeling, ignorance in governance framework establishment, rare involvement of stakeholders in program development, and poor integration of adaptive management into stock enhancement plans. Compared to finfish fisheries, some coastal invertebrates may have a higher potential for effective sea ranching and stock enhancement due to their sessile or sedentary characteristic and are more likely to create self-replenishing populations within a relatively localized region (Bell et al., 2006; Gomez and Mingoa-Licuanan, 2006).

A case in point is the potential application of stock enhancement through captive rearing of juveniles as a proactive management tool to restore the dwindling population of the tri-spine horseshoe crab, *Tachypleus tridentatus*, worldwide. Horseshoe crabs are an ancient group of macroinvertebrates that use multiple inshore and estuarine habitats throughout their life cycles: adults spawn on sheltered intertidal flats near the high tide mark, the newly hatched larvae emerge from sediment and enter the sea as plankton, the larvae/juveniles settle and grow on the same or adjacent beaches, and gradually migrate into subtidal areas to reach adulthood (**Figure 1**; Chen et al., 2015; Laurie et al., 2019). Therefore, horseshoe crabs are referred to as the potential sentinel species to indicate the general health status of coastal and estuarine ecosystems (Kwan et al., 2018b).

Horseshoe crabs have a lengthy maturity period. For example, *T. tridentatus* become mature at age 13–14 (instar stage: 17–18) after undergoing 16–17 molts (**Figure 1**; Chen et al., 2010; Hu et al., 2015, for detailed information on instar, age and size). Apart from that, horseshoe crabs also exhibit low dispersal rates, long life span, small home range, and delayed reproductive development (Kwan et al., 2015b, 2020). These life-history features render them vulnerable to overharvesting and habitat loss (John et al., 2018; Laurie et al., 2019). *T. tridentatus* has also been heavily harvested since the 1980s for the production of *Tachypleus* amebocyte lysate (TAL), a substance that quantitatively detects the presence of bacterial endotoxins or fungal contamination in drugs and biomedical devices (Liao and Li, 2001; Liao et al., 2019a). *T. tridentatus* is recently listed as "Endangered (EN)" on the IUCN Red List (Laurie et al., 2019). Considering the increasing demand for pharmaceuticals and biomedical applications in emerging markets, including China, India, and Brazil, mass harvest for wild *T. tridentatus* resources will not cease.

The global decline in *T. tridentatus* populations has attracted increasing interest in searching for appropriate management tools to revert the situation. Despite the introduction of marine protected areas and the establishment of new environmental legislation can be effective, stock enhancement initiative in some cases is considered as a more proactive approach to populate the exploited stocks (Carmichael and Brush, 2012; Wang et al., 2020; Zhu et al., 2020). Releasing programs using hatchery-bred *T. tridentatus* juveniles, mostly the newly hatched first-instars or second-instars, and occasionally adults have been attempted or are underway along the Chinese coast of Fujian, Guangdong, and Guangxi provinces in the Mainland and Taiwan (Hong, 2011; Zhu et al., 2020). In total, more than 7,450,000 juveniles and 2,500 adults of *T. tridentatus* have been used for sea ranching and stock enhancement during 2010–2020, in which 42% of these programs released more than 100,000 individuals each time (**Supplementary Table 1**). Apart from that, every year by joining a conservation education program in Hong Kong, students from 30 selected secondary schools also release the laboratory-reared juveniles under their care at Ha Pak Nai, the most important nursery habitat for the local *T. tridentatus* population (Kwan et al., 2017b). These programs, however, are unlikely to have the desired result given a lack of proper formulation of release strategies, dependable evaluation, and understanding of the ecosystem.

In this review, we summarize the available aquaculture technologies for hatchery production of juvenile *T. tridentatus*. Based on our best knowledge regarding biological and ecological characteristics of the species, release strategies that could potentially improve the survival of released *T. tridentatus* were discussed. Future opportunities and challenges in establishing technical frameworks to support responsible stock enhancement for *T. tridentatus* were also identified. Relevant articles and references were searched and identified mostly through databases and search engines, including Web of Science, Scopus, China National Knowledge Infrastructure, Scholarly and Academic Information Navigator, Japan, and Google Scholar. To include as many studies as possible, reference searching using local names of *T. tridentatus* in varying languages was also conducted. The ultimate goal is to establish effective and economically viable methods for the mass release of environmentally fit juveniles in the attempt to restore the severely depleted *T. tridentatus* populations.

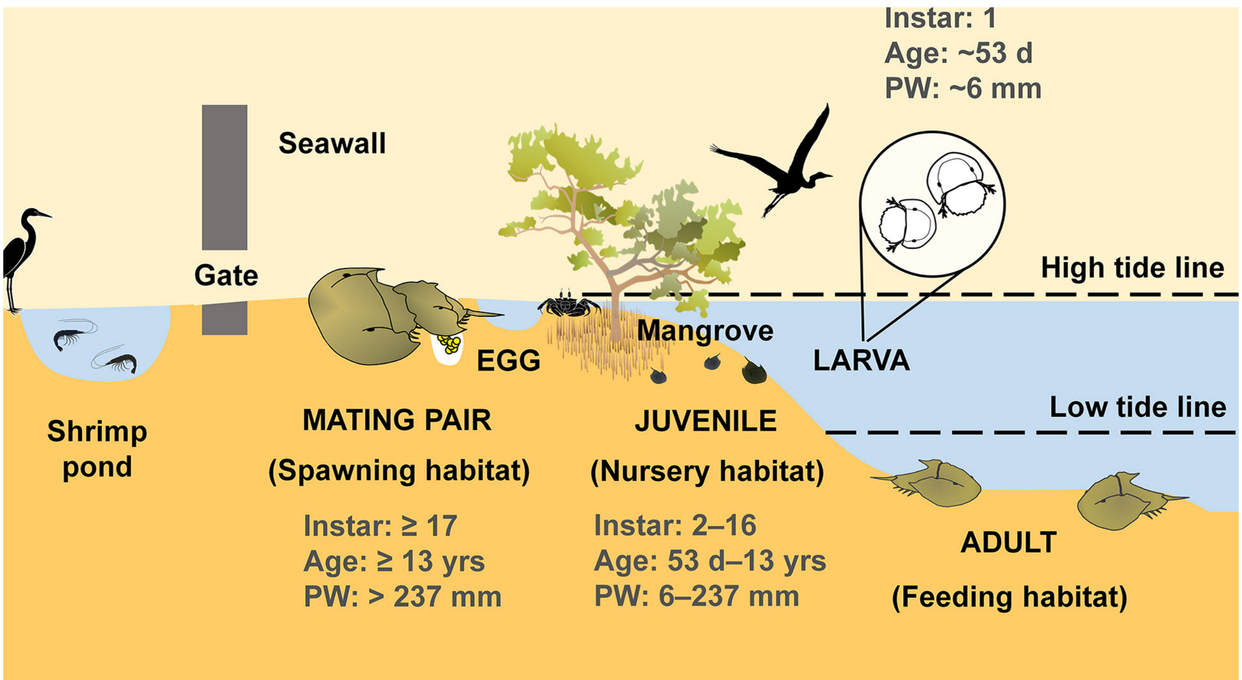

FIGURE 1 | Life cycle and the associated habitats for *T. tridentatus*. PW, prosomal width (size); d, day; yrs, years.

DEVELOPMENT OF HATCHERY PRODUCTION

There is considerable demand in the development of hatchery production technology for rearing horseshoe crab larvae and juveniles due to the decline in horseshoe crab populations (Carmichael and Brush, 2012). However, the capacity of hatcheries to producing a sufficient number of high-quality juveniles in a cost-effective manner is the prerequisite of any releasing activity. Culturing *T. tridentatus* in the laboratory was initiated by the Japanese scientist, Koichi Sekiguchi in 1972 from artificially fertilized eggs with an aim to study the growth and development of juveniles (Sekiguchi et al., 1988). A broader scale of *T. tridentatus* culture efforts has later been attempted in China (e.g., Liang, 1987) and Japan (e.g., Tsuchiya, 2009) since the 1980s. While most previous studies concerned how diet compositions and water quality variables affect the survival and growth of horseshoe crab embryos and juveniles (Carmichael and Brush, 2012; Hu et al., 2013a, 2014, 2018; Liao et al., 2019b), the mass production of cost-effective, high-quality seedstocks has presented a bottleneck in the releasing programs for *T. tridentatus* (Kwan et al., 2014; Zhu et al., 2020). We compiled and discussed the available rearing techniques in maintaining *T. tridentatus* embryos and juveniles in hatcheries with the ultimate goal in defining effective culture conditions (**Figure 2**).

Spawning and Fertilization

The development of *T. tridentatus* aquaculture has been enabled by breakthroughs in artificial breeding. Earlier in the 1940s, Oka (1943) reported his trial on artificial insemination following Patten (1894) to obtain *T. tridentatus* embryos for developmental mechanism study. Sekiguchi et al. (1988) further improved the fertilization rate by spreading the diluted sperm evenly over the eggs after a shorter preparation procedure. The sperm and eggs were collected by cutting the soft tissue on the ventral sides of the prosoma of male and female *T. tridentatus* from the appendage base toward the lateral margin (Chen et al., 2010). Nonetheless, this invasive method would further deplete the remaining population of *T. tridentatus*. Non-invasive electrical stimulation for egg and sperm collection was applied in the Atlantic counterpart, *Limulus polyphemus*. By adopting a similar method, however, a very limited amount of eggs/sperm could be collected from *T. tridentatus* (Sekiguchi et al., 1988; Li, 2008). Alternatively, the natural spawning method has been adopted by keeping amplexed mating pairs of horseshoe crabs in tanks with approximately 10-cm sediment layer underneath (Liang, 1987; Chen and Xia, 2002). The authors also claimed that the released eggs obtained from natural spawning were mostly mature, which can considerably enhance the fertilization rate (Liang, 1987), compared to that from electrical stimulation or artificial insemination.

However, there is relatively little information available regarding the effects of diet and culture conditions on adult *T. tridentatus* survival, breeding, and cellular health. Chen and Xia (2002) reported that natural spawning occurred in breeding tanks during May–July when the mating pairs were fed with shrimp and fish meat, and maintained at water temperature 23–26°C, salinity 25–30, and pH 7.8–8.2. Chow (2019) observed stimulation of *T. tridentatus* spawning when the water salinity

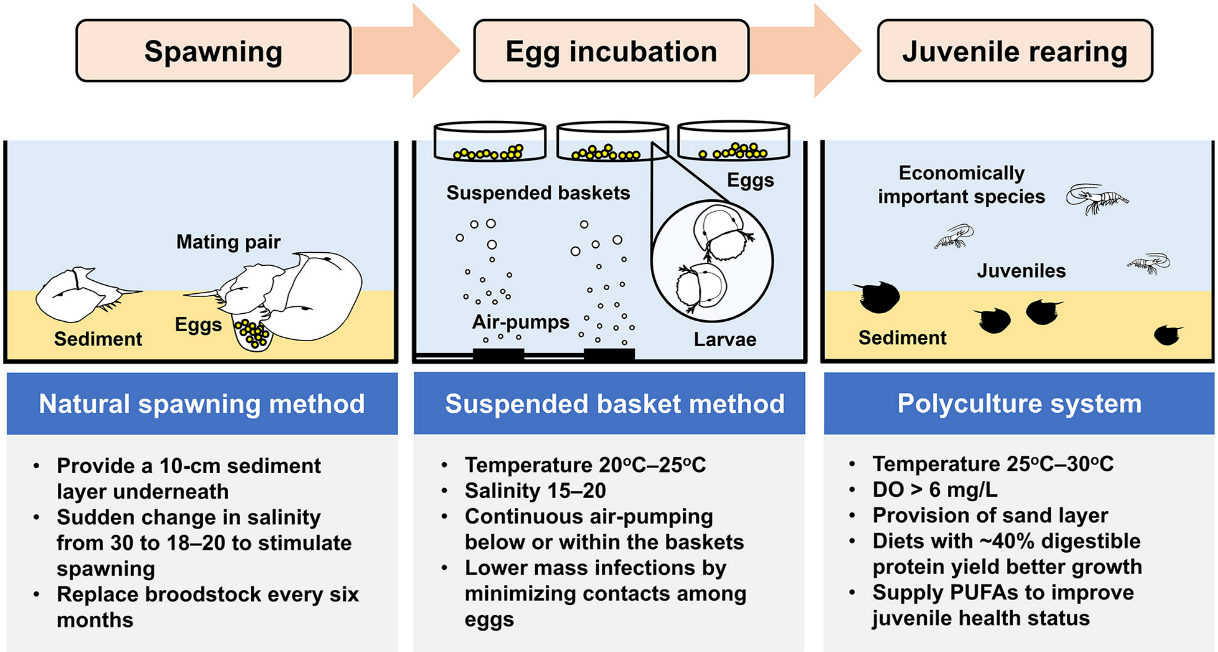

FIGURE 2 | Important techniques and operating procedures on artificial breeding as well as larval and juvenile culture for *T. tridentatus*.

was gradually lowered from 30 to 18–20. A recent study demonstrated that the addition of copper supplements into the feed for adult *T. tridentatus* could improve their immune enzyme activity and hemolymph quality (Xu et al., 2020). Future efforts should be concentrated on developing prototypical nutritional and aquaculture standards for adult horseshoe crab husbandry to support long-term aquaculture management (Tinker-Kulberg et al., 2020a,b,c). Otherwise, the broodstock should be replaced every 6 months. Sustained aquaculture in the facilities has been linked to low survival rates, possibly due to poor nutritional conditions and water quality which would increase their susceptibility to bacterial and parasitic infections (Nolan and Smith, 2009).

Egg Incubation

To maintain relatively stable environmental conditions, the culture of *T. tridentatus* embryos was mostly confined in indoor husbandries or laboratories. In most cases, the fertilized eggs were extracted from the sediment several days after the spawning events, and transferred to an individual culture system for egg incubations. Many authors reported generally higher survival rates (>60%) of fertilized eggs under the following incubation conditions: water temperature 20–35°C, salinity 15–35, pH 8.1–8.3 under continuous aeration with water renewal once or twice a day (Sekiguchi et al., 1988; Li et al., 1999; Li, 2008; Hong et al., 2009, 2011; Chen et al., 2010; Wu et al., 2014; Carmichael and Brush, 2012; Miao et al., 2020).

Despite the fact that most previous studies overlooked the effect of sole environmental factor on fertilization, survival, and hatching of *T. tridentatus* eggs (**Table 1**), Li et al. (1999) found surprisingly high fertilization (>95%) and survival (>98%) rates when the eggs were cultured under salinity ranged 16–33. Li (2008), however, observed interactive effects between temperature and salinity, in which the egg survivorship was deleteriously affected (<30%) at salinity 25 and 30 when the water temperature was kept at 32°C. No hatching was noted during the 90-day experiment in all treatment groups with salinity at 15 (Li, 2008).

Bacterial/fungal infections and dissolved oxygen deficiencies seem to be the most prominent issue in the rearing of horseshoe crab eggs. Faizul et al. (2015) identified four species of bacteria (*Shewanella putrefaciens*, *Bacillus cereus*, *Corynebacterium* sp., and *Enterococcus faecalis*) and fungi (*Aspergillus niger*, *Aspergillus* sp., *Penicillium* sp., and *Gliocladium* sp.), respectively, in infected horseshoe crab eggs. The authors suggested that *S. putrefaciens* and *A. niger* possess primary threats to egg survival and development. While possible treatments to the infections are largely lacking, a 3- to 12-min freshwater bath, and/or addition of chlorine or other disinfectant agents, may be useful in removing the pathogens in hatcheries (Li, 2008; Nolan and Smith, 2009; Shinn et al., 2015). To lower the spread of diseases, it is also important to maintain an appropriately low density of eggs or embryos within each incubator, even though the optimal density information is currently unknown.

Dissolved oxygen (DO) plays a critical role in promoting horseshoe crab embryonic developments (Penn and Brockmann, 1994; Vasquez et al., 2015). Consequently, many attempts were made to maximize the DO concentrations in the culture system for *T. tridentatus* eggs and embryos. Suspended culture, which involves hanging baskets from the surface of culture water, was the most widely reported method to be efficient in egg hatching (Liao, 2011, 2015; Liao et al., 2015). Continuous air-pumping

TABLE 1 | Effects of various environmental factors on fertilization and survival of *T. tridentatus* embryos in hatcheries (–, no data).

Factors	Source	Experimental groups	Day of experiment	Fertilization rate	Survival rate
Salinity	Li et al. (1999)	10 groups ranged 3–70	44	Greater than 95% at 16, 26, and 33	Greater than 98% at 16, 26, and 33
	Li (2008)	15, 20, 25, 30	90	–	No significant difference among salinity groups at 20°C and 25°C, but significantly higher survival rates in salinity 15 and 20, compared to those in salinity 25 and 30 at 32°C
Temperature	Li (2008)	20, 25, 32°C	90	–	20°C and 25°C groups have significantly higher survival rates than 32°C, whereas 20°C and 25°C groups are statistically similar

below or within the baskets where the eggs housed provides more DO. The vigorous airflow can also agitate the eggs constantly to avoid mass infections due to the prolonged contact among eggs. Keeping the eggs/embryos within a moist and sterile sediment layer in husbandries can also be another alternative to be attempted.

Culture of Juveniles

The success of sea ranching and stock enhancement programs is predominantly determined by rearing technologies in the mass production of seedstocks and juveniles for release. However, since most *T. tridentatus* individuals in the releasing programs were the newly hatched first-instar larvae (Zhu et al., 2020; **Supplementary Table 1**), the development of improved culture techniques to produce environmentally fit, older juveniles has been greatly impeded. To date, most available rearing results were derived from laboratory experiments (**Table 2**), in which their practicability in large-scale seedstock production is virtually unknown.

In the culture environment, the first-instar larvae were not fed until after the first post-hatch molt (Carmichael et al., 2009; Schreibman and Zarnoch, 2009; Chen et al., 2010) since the previous studies suggested that the larval digestive system was rather incomplete and appeared to subsist on the yolk of the embryos for nutrition (Botton et al., 1992; Carmichael et al., 2009). For juvenile culture, many studies concerned their growth and survival under varying dietary conditions, except a small amount of research investigated their responses toward different environmental variables such as temperature, DO, light-dark cycle, and substrate type (**Table 2**). Among frozen food sources commonly available for aquarium fish, hatchery-bred first-year juveniles (instars 1–6) fed with brine shrimp, *Artemia* sp. had higher growth and survival rates due to the increased consumption rate (Hu et al., 2013a). Biswal et al. (2016) provided small, chopped earthworms to the second-instar juvenile coastal horseshoe crab, *Tachypleus gigas*, and observed most molted into the fourth-instar within 90 days from the hatching. For older juveniles, clam meat, which has higher crude protein, can significantly promote their growth, compared to sandworms, *Marphysa sanguinea* and brine shrimp that contain indigestible chaete and exoskeleton, respectively (Kwan et al., 2014). Feed size is another vital factor determining juvenile feeding rates and prey sources (Gao et al., 2003). Gaines et al. (2002) found that the prey selection of *L. polyphemus* in varying growth stages depends largely on their mouth size, in which the younger juveniles (instars 2–7) mainly forage on benthic and suspended particulate organic matters (POM), whereas instars 8 or older juveniles shifted their diets to a combination of POM and small benthic invertebrates such as crustaceans and polychaetes. Nonetheless, feeding cultured juveniles with natural diets is costly, and is difficult to be tailored to fulfill the different nutritional requirements in accordance with the ontogenetic development of *T. tridentatus*.

In the search of the optimal diets for hatchery-reared juvenile *T. tridentatus*, Hu et al. (2014) formulated nine artificial diet treatments in different levels of digestible protein and energy. While the fifth-instar juveniles fed with brine shrimp had the highest feeding rate, the formulated diet containing 40% digestible protein yielded the best growth and feed utilization. The authors suggested that excessively high digestible protein diets such as brine shrimp, fish and clam meat, could reduce juvenile feed consumption and pollute the culture environment. Providing the juveniles with artificial feeds using poultry by-products (PB) as well as meat and bone meals (MB) to replace the traditional fish meal were also attempted to lower the production cost (Hu et al., 2018). The experimental results are promising that a mixture of MB and PB in 2:1 resulted in similar growth performance to that fed with fish meals. However, a longer acclimation period may be required with these dry, hard artificial feeds as horseshoe crabs prefer softer food sources in both natural habitats and captive environments (Zhou and Morton, 2004; Kwan et al., 2015a; Razali et al., 2020).

Since the production of high-quality juveniles is essential in any releasing activity, technological development of seed production has gradually shifted its focus from quantity to quality (e.g., Fushimi, 2001). Concerning the health status of cultured juvenile *T. tridentatus*, Kwan et al. (2014) observed a general decline in juvenile hemolymph quality during a 12-week feeding experiment, even though high-protein diets were provided. To further verify the presence of captivity-related stress, Kwan (2016) kept wild juvenile *T. tridentatus* in the laboratory for 3 months under the best husbandry practices. Significant declines in health indicators, including amebocyte density, amebocyte morphological states, and plasma protein level, were consistently found. Such a decrease in juvenile health status may indicate deficiencies of essential diet compositions, movement constraints and/or absence of tidal rhythms in the culture systems (Kwan et al., 2014). To address the problems,

TABLE 2 | Effects of various environmental factors and diet conditions on optimal growth, survival and health status of juvenile *T. tridentatus* in culture environments.

Factors	Source	Instar	Experimental groups	Day of experiment	Growth rate	Survival rate	Feeding rate	Health condition
Temperature	Lee and Morton (2005)	5–9	18–22°C and 28–32°C	60	Considerably higher molting occurrence (50%) in 28–32°C than those (10%) in 18–22°C	Marginally higher (30%) in 18–22°C compared to those (25%) in 28–32°C	–	–
	Liao et al. (2019b)	16	9 groups ranged 0–40°C	7	–	Significantly lower at 40°C	25°C and 30°C are significantly higher	–
Dissolved oxygen	Shin et al. (2014)	4	2, 4, 6 mg L^{-1}	6	Scope for growth: Significantly higher in DO 6 group on 0 d	100% in all treatments	–	–
Substrate	Hong et al. (2009)	1–2	No substrate, sand, sediment	152	Max. 35% PW increase in sand treatment group	>90% in sand and sediment treatment group	–	–
Day/night time	Gao et al. (2003)	13–16	Day time, night time	2	–	–	Optimal 1.4–2.5% d^{-1} at night time	–
Feed type	Hu et al. (2013a)	3	Brine shrimp, opossum shrimp, chironomus larva	100	BW: Significantly higher in brine shrimp group	Significantly higher in brine shrimp group	Significantly higher in brine shrimp group	–
	Hu et al. (2014)	4	Brine shrimp, nine diets in different levels of digestible protein (DP, 36, 40, 43%) and energy (DE, 14, 16, 18 kJ g^{-1})	84	Max. BW in diets with 40% DP, 14 kJ g^{-1} DE	100% in most treatment groups except for diets with 43% DP	Max. in brine shrimp treatment group	–
	Hu et al. (2018)	5	Fish meal with blood meal (FB), meat and bone meal (MB), poultry by-product meal (PB)	84	BW: MB + PB in 2:1 group is significantly higher than 1:1	100% in feeds with FBM, 25% and 50% MB + PB in 2:1, 25% MB + PB in 1:1	MB + PB in 2:1 group is significantly higher than 1:1	–
	Kwan et al. (2014)	7	Brine shrimp, clam, sandworm, clam plus sandworm	84	Significantly higher in clam group	79–96%	Significantly higher in clam group	Unaffected
	Gao et al. (2003)	13–16	Fish, peanut worm, mussel, squid, shrimp	7	–	–	Max. 2% d^{-1} in squid treatment group	–
Feed size	Gao et al. (2003)	13–16	0.3–0.5, 0.5–1.0, 1.5, 2.0 cm	8	–	–	≥1 in 0.3–0.5 cm	–
Diet supplement	Kwan et al. (2017a)	8	Unicellular green algae *Dunaliella tertiolecta* (DT), golden-brown flagellated microalgae *Isochrysis galbana* (IG)	84	Unaffected	93–100%	–	Higher amebocyte viability and granular-spherical amebocyte state with fed with IG
	Kwan et al. (2017a)	8	Microalgae supplement level: 5%, 10% dw	84	Unaffected	93–100%	–	Unaffected

PW, prosomal width; BW, body weight; ind., individual; d, day; dw, dry weight; max., maximum; –, no data. Instar stage of juveniles is referred to Chen et al. (2010).

Kwan et al. (2018a) invented a double-layered water-circulating enclosure, allowing the simulation of the daily ebb and flow tidal conditions. A high survival rate was obtained, as the circulating system can keep good water quality for at least 3 months and prevent disturbance to the juveniles since frequent water renewal is not required. The system also enabled the juvenile horseshoe crabs to accommodate natural feeding activities under low tides. Supplementing diets with microalgal powder with high polyunsaturated fatty acids, notably eicosapentaenoic acids, also improved the hemolymph quality of juvenile *T. tridentatus* (Kwan et al., 2017a).

In terms of environmental variables (**Table 2**), juvenile *T. tridentatus* can survive in a broad range of rearing temperatures (15–30°C), except for the significantly higher mortality that occurred at 40°C (Liao et al., 2019b). It seems that *T. tridentatus* population in the higher latitudes such as that in Japan, is better adapted to the lower temperatures (Shuster and Sekiguchi, 2009; Liao et al., 2019b). The optimal water temperature for growth and survival was observed within the range of 25–30°C. Deficiencies in DO (2 or 4 mg L^{-1}) lowered the scope of growth in juvenile *T. tridentatus*, but their physiological performance can be recovered to the normal level once the short-term hypoxic conditions were ceased on day 4 (Shin et al., 2014). Gao et al. (2003) showed higher juvenile feeding activities during the night, which is consistent with previous studies, suggesting that horseshoe crabs are nocturnally active (Rudloe, 1979; Wada et al., 2016). Provision of a sand layer in the culture environment that simulates the nursery habitat conditions of juveniles can also effectively enhance their growth and survival by 10% and 42%, respectively (Hong et al., 2009).

Production Modes

The current market demand for mass production of hatchery-bred juvenile *T. tridentatus* is mainly for releasing programs organized by local governments and research institutes (**Supplementary Table 1**; Zhu et al., 2020). In hatcheries, large numbers of fertilized eggs are incubated using suspended baskets or other similar methods within indoor cement ponds (**Figure 2**; Liao, 2011, 2015; Liao et al., 2015). For the third-instar or older juveniles, a very limited amount has been produced from laboratories to serve research (Chen et al., 2010; Kwan et al., 2019) and conservation educational purposes (Kwan et al., 2017b). The production of adults for TAL biomedical industry through captive rearing is potentially technically feasible (Tinker-Kulberg et al., 2020a,b,c). Such an approach could not only reduce the need to harvest wild horseshoe crabs for such bleeding practices, but would also provide a cost-effective indirect approach for the production of seedstock, including the polyculture of juveniles for a limited amount of time for subsequent release for stock enhancement purposes. Such captivity studies have been recently reported with replacement, reduction and refinement as important drivers of change for horseshoe crab conservation (Gorman, 2020). To the best of our knowledge, however, no study has successfully reared the early instar *T. tridentatus* juveniles till adulthood. Sekiguchi et al. (1988) obtained the 10th-instar (prosomal width, PW: 48.4 mm) juveniles after 7 years of laboratory culture, whereas Huang and Tsai (2011) spent only 4 years to successfully rear three 10th-instar juveniles from a total of 12,000 first-instar larvae in research facilities.

To produce more cost-effective juvenile *T. tridentatus* for releasing programs, Chen et al. (2016) proposed to adopt a polyculture system to rear the juveniles with other economically important aquaculture species in husbandries. In this case, the juveniles can be a "by-product" from other rearing activities without the additional investments in husbandry management and maintenance. To examine the practicability of such production mode, a trial on mixed cultivation of juvenile *T. tridentatus* (instars 1–2; 203,000 individuals) with a scavenging species, juvenile spotted Babylon snail, *Babylonia areolata* (338,000 individuals) was conducted in outdoor cement ponds for 150 days (Chen et al., 2016). Despite the fact that the temperature maintenance system was unavailable during the winter and spring, about half of the juvenile *T. tridentatus* molted into the second- or third-instar with an acceptably high survival rate of 33%. Meanwhile, 87% of juvenile *B. areolata* survived through the entire experiment. Chen et al. (2016) explained that the outdoor culture system had provided larger movement space and a relatively stable rearing environment to the juveniles, compared to the typically small experimental tanks within the research facilities. The juveniles can feed on overgrown benthic algae in the outdoor ponds, which are important food sources for these young individuals (Gaines et al., 2002; Carmichael et al., 2009). The polyculture with juvenile *B. areolata* also can enhance the growth performance of juvenile *T. tridentatus* by providing better water quality and culture environment due to their complementing feeding niches. Rearing juvenile *T. tridentatus* with shrimp and other mollusks is also worth to be attempted to identify more practical, cost-effective methods for seedstock production.

The important techniques and operating procedures on artificial breeding, as well as larval and juvenile culture for *T. tridentatus* are summarized in **Figure 2**. All in all, the experimental findings suggest that the optimal rearing techniques of juvenile *T. tridentatus* should be based on their biology and ecology. Therefore, an improved understanding of the released species and ecosystems can compensate for the limitations in providing older captive-bred juveniles and increase the potential success of sea ranching and stock enhancement. While captivity-related and other health issues are largely unsolved, mass production of the juveniles using more natural, ecologically relevant methods, e.g., outdoor ponds with sediment substrates, may be more promising.

RELEASE STRATEGIES

The success of stock enhancement programs depends largely on the survival of the hatchery-bred juveniles to natural habitat conditions. The mortality of released juveniles, particularly those of invertebrates, is typically higher than 60% within the first month (Oliver et al., 2005; Dixon et al., 2006; Purcell and Simutoga, 2008). Thus, potential reasons that can cause high mortality of released juveniles, including predation, habitat conditions, environmental stress, and extreme weather, should be addressed (Lorenzen, 1996; Lorenzen, 2000; Hines et al., 2008; Ceccarelli et al., 2018; Poh et al., 2018). Of these, Bartley and Bell (2008) perceived predation as the key obstacle to the survival of released juveniles. While relevant experimental results in developing optimal release strategy for juvenile *T. tridentatus* are largely lacking, we discuss these based on our understanding of the biology and ecology of juvenile *T. tridentatus* in the estuarine ecosystem.

Selection of Release Sites, Season and Timing

Horseshoe crabs have specific spawning and nursery requirements related to a combination of beach topography and physico-chemical parameters such as dissolved oxygen, chlorophyll a content, total sulfide content, and sediment grain size (Hsieh and Chen, 2009; Vasquez et al., 2015; Cheng et al., 2016; Xie et al., 2020). Therefore, the identification of optimal nursery habitats for releases is the first step. Among the 14 nursery sites distributed along the northern Beibu Gulf, China, a high density of wild juveniles was found in the intertidal areas along the outer fringe of mangrove forest, especially near the outflows of tidal creeks (Xie et al., 2020). In addition, at the nursery site where seagrass patches are available, areas with higher seagrass coverage and coarser sediment had more abundant juvenile *T. tridentatus* individuals (Xie et al., 2020). Most wild juveniles were found forage actively in areas with surface water with 1–10 mm depth during ebb tides to keep the juvenile book gills wet and enable respiration while feeding under high surface temperatures (Lee and Morton, 2009; Kwan et al., 2020). Based on the above findings, releasing hatchery-produced juveniles at the existing nursery sites on the intertidal areas along the outer fringe of mangroves or near the seagrass patches seems to enhance their survival rate.

Chiu and Morton (2004) demonstrated that juvenile *T. tridentatus* buries in the sediment and remains inactive when the water temperature drops below 20°C. In such cases, decisions related to factors linked to the ecological requirements of the juveniles, including microhabitat, season and timing play critical roles for the release success. To improve the survival of released juveniles, they should be placed on the intertidal areas during low tides in summer when the surface water temperature exceeds 20°C. This can also mimic the spawning period of *T. tridentatus* typically recorded in late spring (Cai et al., 1984) and would coincide with the summer peak in juvenile production.

If there are problems in locating any actively utilized nursery habitats for *T. tridentatus* for releases, the programs can be conducted at any suitable intertidal habitats where the juveniles were known to occur in the past but are now extirpated. However, it is important to ensure the current state of the intertidal areas fits the macrohabitat requirements of the juveniles, e.g., mangroves, seagrass beds, and tidal creeks. For microhabitat conditions, juvenile *T. tridentatus* tends to concentrate in regions where sediment comprised medium-sized (median particle: 0.1–0.5 mm) sand with relatively higher chlorophyll a content (1–4 μg/g) and/or total organic content (0.1–0.7%) (Hsieh and Chen, 2009; Xie et al., 2020), which presumably indicate a higher abundance of food from the marine algae-derived particulate organic matter sources (Gaines et al., 2002). For optimal surface water quality, salinity should range between 12 and 34, dissolved oxygen content 7–9 mg/L, and pH 7.3–7.9 (Hsieh and Chen, 2009; Xie et al., 2020), although Xie et al. (2020) found that sediment physico-chemical conditions, rather than surface water parameters, can better explain the distribution pattern of wild juvenile *T. tridentatus* population.

To date, however, no study has looked specifically at the survival, growth, and behavioral responses of hatchery-reared juvenile *T. tridentatus* at the release sites. During a release trial with 60,000 individuals of cultured first-instar larvae (∼53-day old) in mangrove and seagrass areas at Yuzhouping, Guangxi region, China (Zhu et al., 2020), an actively utilized nursery habitat for *T. tridentatus*, none of the released larvae had been recovered during monthly belt-transect population survey in the following 4 months, which was possibly due to the high predation rates. Size at release is, therefore, another critical component to be considered prior to the releases.

Size at Release

Size at release is one of the key elements in a successful stock enhancement. Natural mortality rates within natural fish (possibly also invertebrate) populations are approximately inversely proportional to their lengths (Lorenzen, 1996). Meanwhile, the predation risk at the released site is inversely related to their age and size. Carmichael et al. (2003) found that only very few *L. polyphemus* in Cape Cod, Massachusetts, United States survived their first year (instars 1–6), in which the cumulative mortality rate can be as high as 99%. Conversely, most juveniles survived to reach adulthood given that they survived through the first year (Carmichael et al., 2003). While there is very limited information regarding key predators of juvenile horseshoe crabs (Botton, 2009), releasing using hatchery-bred juveniles at their sixth instar or older seems to be an optimal decision to minimize potential predation by fish and hermit crabs, and to increase the success of releases.

Releasing undersized juveniles, e.g., the first-instar larvae, is unlikely to be effective, but the effectiveness of releases at small sizes can be increased significantly through the selection of optimal habitats and times as well as acclimation and soft release strategy (refer to sections "Selection of Release Sites, Season and Timing" and "Stress Management"). Apart from that, the fitness of juveniles under prolonged captivity is another area of concern. Despite the development of rearing technologies for Asian horseshoe crabs has been initiated in the 1980s, Kwan et al. (2014) suggested that the present culture models may not guarantee the continuing fitness of juveniles. To maximize the survival rate of released juveniles, it is important to identify the captivity-related issues such as deficiencies in essential dietary components (Kwan et al., 2017a) that are widely present in the current hatchery systems. Nevertheless, it is also important to account for ecological differences between wild and hatchery-produced juveniles (Lorenzen, 2005). Natural mortality rates of hatchery-reared juveniles tend to be substantially higher than those of wild conspecifics of similar size (Lorenzen, 2000; Fleming and Petersson, 2001). Reproductive success of captive-bred salmonids, for example, is generally higher than that of their wild conspecifics (Fleming and Petersson, 2001). However, no strong evidence on their differences in growth was reported (Svåsand et al., 2000; Fleming and Petersson, 2001).

Tagging Methods

The development of cost-effective tagging methods is necessary to identify the released individuals from the wild stock to evaluate the effectiveness of stock enhancement programs (Bartley and Bell, 2008). In fact, little research has focused on selecting appropriate tags and markers for juvenile horseshoe crabs. No tagging experiment was currently conducted on the most commonly released size, the first-instar larvae. For other released size, only physical tags have been attempted. Hu et al. (2013b) marked 300 individuals of the second-instar *T. tridentatus* (∼134-day old; prosomal width, PW ∼8 mm) using visible implant elastomer (VIE, i.e., colored tag; Northwest Marine Technology Inc., WA, United States) and found the 22-d survival rate was 96%. Nonetheless, no information on the juvenile survival rate at the released site was provided. Tag retention is another important consideration when injecting the implants of fluorescent colors under the juvenile carapace. Although the tag retention time in juvenile horseshoe crabs is virtually unknown, Brennan et al. (2005) observed such tag loss in 2% of tagged snook *Centropomus undecimalis* after 6 months. Kwan et al. (2015b) examined the recovery and movement of a total of 150 individuals of the 7th- to 10th-instar *T. tridentatus* (1–2-year old; PW 31–59 mm) with colored plastic tape, passive integrated transponder tag (PIT; 134.2 kHz, Biomark, WA, United States) and their combinations. The mortalities induced by the three kinds of tags were minimal, and the cumulative recovery rates of the released juveniles within 2 months in summer were ranged from 70 to 82% (Kwan et al., 2015b). The PIT tag seems not to affect the juvenile foraging activity as their utilization distribution area using these three tagging methods was statistically similar.

More tagging studies were conducted in adult horseshoe crabs to investigate their migratory, spawning, and post-bleeding behavior (James-Pirri et al., 2005; Anderson et al., 2013; Bopp et al., 2019). Disk tags were developed by U.S. Fish and Wildlife Service, in which the tagging involves drilling a small hole through the lower back corner of horseshoe crab prosoma. Mattei et al. (2011) found no mortality in tagged adult Atlantic horseshoe crab, *L. polyphemus*, and reported an 11–20% recapture rate within the 44-day field observation. James-Pirri et al. (2005) determined movement patterns of spawning *L. polyphemus* during 2000–2002 with a double T-bar anchor tag (model SHD, Floy Tag Inc., WA, United States). However, the recovery of these conventional passive tags can be labor-intensive and time-consuming, particularly when only a small number of tagged individuals were released to an open area of the appropriate habitat. Ultrasonic transmitters, which allow wider coverage of detection in submerged habitat, were useful in tracking the distribution and movement of adult horseshoe crabs at varying temporal and spatial scales (Schaller et al., 2010; Wada et al., 2016; Owings et al., 2019). Since the transmitter size is much larger and heavier compared to that of conventional physical tags, telemetry technologies are only suitable for adult horseshoe crabs. However, restocking with the adults, should not be encouraged, except for those bled or confiscated from illegal wildlife trade. In fact, all captive adult horseshoe crabs are harvested from the wild stocks because of their lengthy life histories, and thereby impractical to rear them till adulthood.

The search for optimal tagging methods for the first-year juveniles (instar 1–6) is urgently needed, despite the fact that their natural mortality rates are too high (Carmichael et al., 2003) and should not be used for releasing programs. As mentioned earlier, since the selection of release size is a balance between survival rates and production costs, we predict that the use of the first-instar larvae would still be predominant in the releasing programs, unless the hatchery techniques have been largely improved and the production costs can be lowered to an acceptable level. The application of physical tags to these juveniles would be challenging due to their minute body size (PW $<$ 30 mm, wet weight $<$ 2 g), frequent molting (50–290 days for each instar stage), and burying behavior during high tides. The development of biological tags such as genetic markers and some visible morphological characters for the early instar juveniles is worth attempting. Non-invasive genetic markers are developed based on the selection of a rare DNA variant that distinguishes the released batch from the native stock (Romana-Eguia, 2006). Maternally inherited mitochondrial DNA markers and biparentally inherited nuclear DNA markers (such as microsatellite DNA and allozyme electrophoresis) are commonly used and effective for assessing the success of releasing activities and possible loss of genetic variability in both released and wild stocks. Nevertheless, the use of genetic markers can be ineffective when the genetic variation within and between stocks is negligible.

Stress Management

Proper management of stress derived from handling and transport can also improve the survival of released juveniles. Purcell et al. (2006) found that prolonged transport duration (i.e., 12 and 24 h) suppressed the normal sand burrowing behavior of juvenile sea cucumber, *Holothuria scabra*. In addition to transport duration, inappropriate storage temperature and medium, as well as juvenile density during transport can elevate both lethal and non-lethal stress of released invertebrates, which in turn, increase their predation risk upon release (Heasman et al., 2004; Purcell et al., 2006). Unfortunately, to the best of our knowledge, no study has focused on the effects of handling and transport stress on juvenile *T. tridentatus* before release. However, previous studies suggested that health conditions of horseshoe crabs would be affected by hypoxia (Shin et al., 2014), increased temperature (Coates et al., 2012), and aerial exposure originated from transport and holding procedure of biomedical bleeding (Hurton et al., 2009). The changes in water chemistry in transport containers can also be relevant to the survival of released juveniles. Heasman et al. (2004) found increasing temperature, in the combination with elevated ammonia and lowered dissolved oxygen in transport containers, has resulted in substantial mortality of juvenile blacklip abalone, *Haliotis rubra*, during the transport to the release site. Keeping juvenile *T. tridentatus* at cooler and constant temperatures during the transport can be attempted to reduce their metabolic activities, which was proven to be effective in the transport of hatchery-reared grouper, *Epinephelus* larvae (Estudillo and Duray, 2003). Further examinations of an optimal temperature and juvenile density during transport are also necessary for *T. tridentatus*.

Burying behavior is an important measure for juvenile horseshoe crabs to avoid predation. During high tides when large numbers of diurnal predators can access the upper intertidal zones, juvenile horseshoe crabs tend to bury themselves into sediments. Lee and Morton (2009) observed that less than 5% of juvenile *T. tridentatus* emerged from sediments during simulated high tides in a laboratory experiment. In such a case, transport with a thin sediment layer prior to release would be an important approach to lower the transport stress. A similar approach has been undertaken by Juinio-Meñez et al. (2012) showing that sand-conditioned juvenile *H. scabra* took a shorter time burying themselves into the sediment at the released site, which possibly improves their survival in the wild. Alternatively, the juvenile horseshoe crabs can be placed in acclimation cages at the release site for 48 h before the release, in which the technique has been demonstrated successful in improving post-release survival, growth and site fidelity in many fish species such as juvenile winter flounder, *Pseudopleuronectes americanus* (total length:

130–175 mm, Fairchild et al., 2009) and juvenile common snook, *Centropomus undecimalis* (fork length: 76–251 mm, Brennan et al., 2006). Gil et al. (2014) demonstrated that hatchery-produced juvenile meagres, *Argyrosomus regius* had adverse body conditions due to starvation during the first few days after the release, which could cause a high mortality rate.

Genetic Considerations

Genetic factors deserve serious considerations in releasing programs. A study of natural population genetic structure should be conducted once stock enhancement programs have been considered. A systematic review by Kitada (2018) found clear evidence of substantial gene flow from hatcheries, in which the magnitude was attributed to the numbers of broodstock and stocking intensity. Half of 38 empirical studies on marine stock enhancement initiatives demonstrated a reduction in genetic diversity and changes in the population structure of wild populations (Kitada, 2018).

However, no study has investigated the impact of releasing initiatives on the genetic variability of wild horseshoe crab stocks. Sugawara et al. (1988) found no genetic differences among *T. tridentatus* populations from Japanese and Chinese waters. The recent application of amplified fragment length polymorphism on *T. tridentatus* populations from the three coastal provinces in southern China also drew a similar conclusion (Xu et al., 2011). In contrast, at a finer scale, several previous studies also suggested the existence of genetically distinct subdivisions between that on Kinmen Island and Magong Island in Taiwan Strait (Yang et al., 2007), between Ninghai and Danzhou along the Chinese coast (Weng et al., 2013), as well as between eastern and western shores around Kyushu, Japan (Nishida and Koike, 2009).

For *L. polyphemus*, a clear isolation-by-distance model was evident, in which six major zones of genetic discontinuity were identified, including the Gulf of Maine, Mid-Atlantic, Southeast, Florida Atlantic, Northeast Gulf, and Yucatán Peninsula of Mexico (Smith et al., 2017). Within each zone, considerable gene flow was found to occur between geographically proximate localities. The genetic variation patterns may be due to the limited potential of female migration between embayments (King et al., 2005; Swan, 2005) and larval dispersal (Botton and Loveland, 2003).

While the population genetic structure of *T. tridentatus* is insufficiently clear at present, the wild populations are likely to have very low genetic diversity due to their considerably low population sizes and densities. Mass release of seedstocks from a small number of breeders in the hatchery may have substantial effects on the genetic diversity of wild horseshoe crab populations. A large number of parents from geographically proximate estuaries as "genetic repositories" is generally required for the larger stocking rates to maintain genetic diversity in populations (Kitada et al., 2009). Releasing multiple cohorts of juveniles from mating pairs or replacing the breeders regularly can be the alternative way to avoid genetic degradation caused by inbreeding from mass release. Interactions between wild and released stocks in the environment are worth to be investigated in the future.

RECOMMENDATIONS AND FUTURE PROSPECTS

The development of hatchery and stock enhancement technology for horseshoe crabs is new. Compared to other Asian species, there are relatively larger numbers of studies focused on the growth and survival of juvenile *T. tridentatus* under varying husbandry conditions as well as their distribution pattern and habitat characteristics, all of which would benefit the development of the responsible stock enhancement programs. We summarize the important findings from previous studies into a technical framework for guiding the increasing releasing initiatives for the exploited *T. tridentatus* population, especially along the Chinese coast (**Figure 3**).

Despite the recent progress in aquaculture, the mass production of environmentally fit juveniles for releases has been largely impeded by the presence of unidentifiable captivity-related issues due to the knowledge gaps in understanding the growth requirements and complex life histories of *T. tridentatus* in their nursery habitats. The deficiencies in essential fatty acids and tidal rhythms have been identified to contribute to the lowered immune competence under prolonged culture. Other rearing alternatives such as the use of natural sediment from their nursery habitats (with unknown minor essential nutrients and minerals) to replace the commonly applied aquarium sand can be helpful in maintaining the good health and growth performance of juveniles in culture facilities. Further research is also required to explore other possible culture modes, including polyculture with other economically important aquaculture species, to produce *T. tridentatus* seedstocks cost-effectively (**Figure 3**).

Overall, the success of stock enhancement programs must be guided by enhanced knowledge on horseshoe crab biology and ecology, so that the released juveniles can ultimately contribute to spawning biomass. The effectiveness of releases can be increased significantly through responsible release strategies and pre-release acclimation. Release strategies involving the selection of optimal habitats (intertidal areas near mangrove fringes) and timing (during low tides with water temperature exceeds 20°C) with thorough genetic consideration would greatly improve the survival of released juveniles, particularly those at early instar stages. The use of acclimation cages at the release site for the first 48 h or sand conditioning in hatcheries or during the transport would be important to lower natural mortality after releases. Future research should also include the identification of key predators to avoid immediate predation on hatchery-produced juveniles at release sites. Given that efficient tagging methods for the first-year juvenile horseshoe crabs are currently lacking, the development of non-invasive tagging approaches such as genetic markers should be prioritized to evaluate the effectiveness of releasing programs (**Figure 3**). Follow-up monitoring and assessment after the releases are the prerequisite to optimizing the current stock enhancement protocol.

Apart from the releasing program itself, interventions with other management measures, including nursery habitat recovery, fishing pressure reduction and habitat use restriction during peak spawning, would also allow more promising

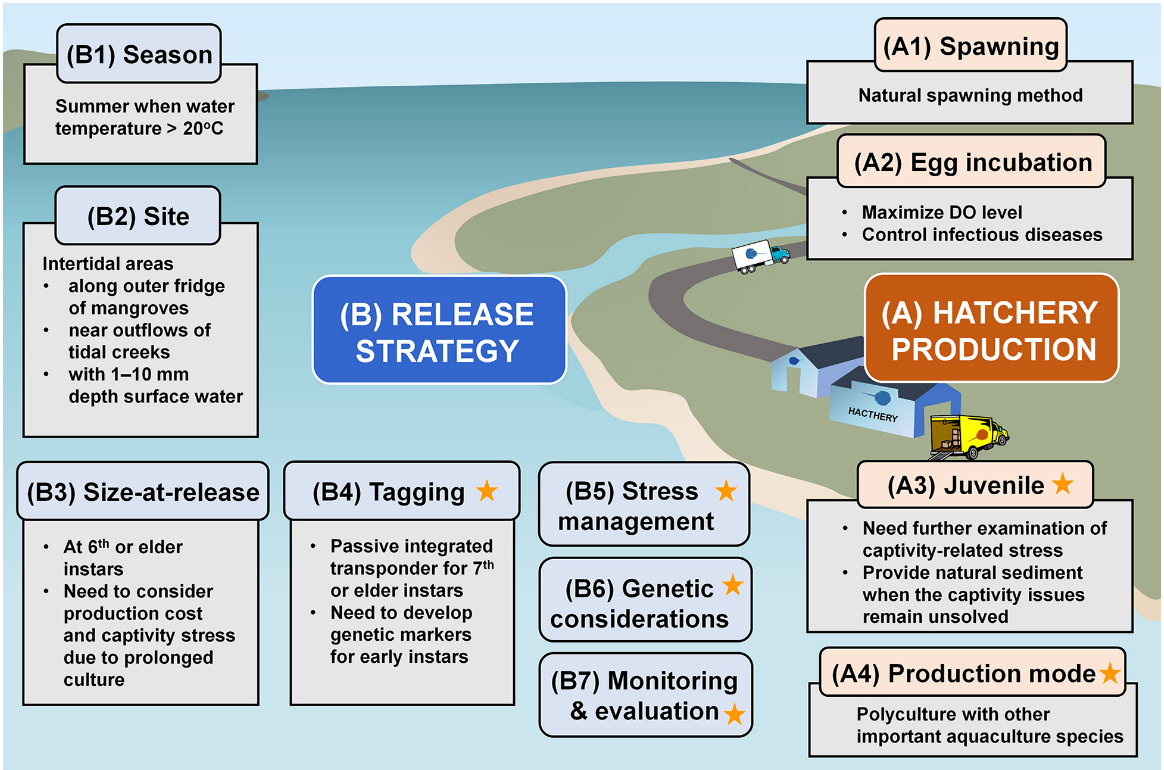

FIGURE 3 | Technical framework for developing the responsible stock enhancement programs for *T. tridentatus* conservation. The star symbols indicate the research gaps which should be focused in future studies. The sources of information are summarized in **Supplementary Table 2**.

conservation outcomes. Identification, mitigation and elimination of human-mediated disturbances at the release site must be addressed prior to the releases. Other institutional, socio-economic and legal factors should also receive equal emphasis. The involvement of local communities and other stakeholders in the releasing programs can reduce the ingrained apathy toward the environment, which often contradicts the community's established practices to consume horseshoe crabs for food and traditional medicine. While there are uncertainties in the potential role of releasing programs, we can expect to gain further insights from continuous research and trial-and-error experience in developing a responsible stock enhancement framework to meet conservation needs for *T. tridentatus*.

AUTHOR CONTRIBUTIONS

PX and HB led the writing and prepared the original draft. MZ and ZY compiled and clustered data from the published studies. XX, C-CW, XH, XW, JZ, and WZ provided input to different writing sections. SC and PS developed the overall review framework and methods. KK supervised, reviewed and edited the final draft, which was proof-read and approved by all authors. All authors contributed to the article and approved the submitted version.

FUNDING

This research was supported by the Guangxi BaGui Youth Scholars Programme, Beibu Gulf Ocean Development Research Centre under Key Research Base of Humanities and Social Sciences in Guangxi Universities, Guangxi Recruitment Program of Hundred Global Experts, the National Natural Science Foundation of China (32060129), the Guangxi Training Program of Innovation and Entrepreneurship for Undergraduates (202011607133), and Central Public-interest Scientific Institution Basal Research Fund, South China Sea Fisheries Research Institute, CAFS (2019TS21).

ACKNOWLEDGMENTS

We thank to the students from the College of Marine Sciences, Beibu Gulf for collecting relevant literature for the review. Constructive comments and suggestions from reviewers are much appreciated.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2021.608155/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.