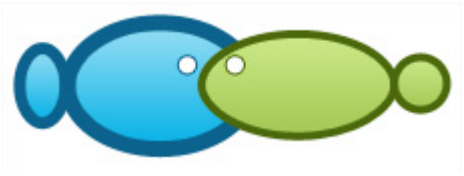

Intermediate culture of juvenile horseshoe crab (*Tachypleus tridentatus*) mixed with juvenile spotted babylon (*Babylonia areolata*) for restocking horseshoe crab populations

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Abstract. The populations of the 'living fossil' horseshoe crab *Tachypleus tridentatus* have decreased dramatically. Releasing juveniles in large quantities, preferably at up to the third instar stage and with better survival in the wild, would be an effective method for replenishing the populations. However, the mortality between second instar and third instar stages is high according to indoor, monospecies culture results. We conducted an outdoor culture of juvenile crabs mixed with juvenile spotted babylon (*Babylonia areolata*) and found that this technique could enhance the horseshoe crabs' survival, accelerate their molting and produce large numbers of juveniles. At the final harvest time, after running the mixing culture for 150 days, horseshoe crab juveniles exhibited survival of 32.5% and production of 66,000 individuals with ages beyond the second instar. Spotted babylon had a survival rate of 87.3%. The resultant survival and abundance levels were greater than those previously reported. We propose that these profound results can be attributed to 1) abundant benthic algae grow in outdoor ponds under sunlight, 2) the commensal lifestyle of juvenile horseshoe crab and spotted babylon, which likely prefer different food particle sizes, 3) the behaviors of spotted babylon, such as burrowing and moving in the sediment substrata, probably create an environment that favors horseshoe crab growth, 4) large amounts of space in the outdoor ponds, which improves growth for unknown reasons, and 5) suitable weather conditions from November to April in Guangxi.
Key Words: mixing culture, intermediate culture, juvenile horseshoe crab, gastropod spotted babylon, horseshoe crab conservation.

Introduction. Horseshoe crabs are known as 'living fossils' because they have been on Earth for over 400 million years (Rudkin & Young 2009). *Tachypleus tridentatus* lives in sandy coastal zones in Japan, China and the South China Sea (Sekiguchi 1988; Hsieh & Chen 2015). This species is extremely valuable in medicine because its amebocytes are used to produce *Tachypleus* Amoebocyte Lysate (TAL) for detecting the presence of human-pathogenic endotoxins in injectable drugs and implanted medical devices (Levin & Bang 1968; Swan 2001). Because of habitat degradation and loss, great demand for bleeding for TAL reagent production, seafood consumption and fishery bycatch, the populations of *T. tridentatus* have decreased dramatically over its historical distribution ranges (Botton 2001; Chen et al 2004; Hsieh & Chen 2009; IUCN 2012; Chen et al 2015). To replenish the populations, releasing large amounts of juveniles into the wild has been considered to be an important step. In fact, this approach had been practiced by releasing first instars in Taiwan in 2002 and releasing first and the second instars in China in 2005 (Chen et al 2014). However, the consequences of these release efforts were unsatisfactory due to difficulties in rearing large quantities of high-quality horseshoe

crab juveniles in indoor culture facilities (Chen et al 2004; Hong et al 2009; Carmichael & Brush 2012) and due to a lack of restocking techniques (Chen et al 2014).

To enhance the effectiveness of releases, producing large amounts of healthy juvenile horseshoe crabs in a cost-effective way is critical. Three subjects need to be studied. First, what kinds of food should be used? Brine shrimp (*Artemia* spp.) have been fed to juvenile horseshoe crabs in most of the indoor culture experiments because it is a live zooplankton diet and easy to handle (Carmichael & Brush 2012). However, brine shrimp may not be an accessible diet for juvenile horseshoe crabs because the crabs are benthic and spend most of their time in sedimentary substrata. Therefore, the crabs are much less likely to feed on the zooplankton. In addition, a study on the diet of field-collected juvenile *T. tridentatus* in Hong Kong showed that polychaetes and oligochaetes were the main foods of juveniles from the third to the ninth instars (Zhou & Morton 2004). This indicates that benthic, not pelagic, invertebrates should be used to rear juvenile horseshoe crabs. Data on the changes in $\delta^{15}N$ signatures in juveniles of the horseshoe crab *Limulus polyphemus* revealed that trophic positions of younger instars were close to herbivorous, and shifting to carnivorous at later stages (Gaines et al 2002). This suggests that, in culture systems, seagrass and/or algae should be given to the horseshoe crab instars when they are at early stages.

Second, which instar stages will yield better survival and growth when the instars are released into the wild after intermediate cultivation? In coastal areas, the first instars hatch from nests on sandy beaches and disperse into mud flats (Hsieh & Chen 2015). The first instars do not feed; the second instars commence feeding and forage on sand/mud flats (Sekiguchi 1988; Carmichael & Brush 2012). The third instars have a depleted yolk and must eat enough food to survive. Based on their natural life histories, the first and second instars can be released to the wild if the coastal area is healthy. However, in degraded environments, where most horseshoe crab restoration projects are conducted, older juveniles at the third instar or beyond would be better.

According to data from laboratory cultivation of *T. tridentatus*, there is still a bottleneck in molting enhancement due to high mortality occurring between the second and third instar stages (the survival rate was approximately 30% in the first author and corresponding authors' personal observations; 0.5% in Li 2008). Chen et al (2010) have reported higher survival rate of approximately 80% for the second and the third instar stages but a very low hatching rate of 0.79%, resulting in approximately 54% survival rate from the first instar to the third instar. In their study, only few tens of the third instars were produced. Therefore, an advanced intermediate culture technology that can produce massive amounts of third-instar or older juveniles is urgently needed for restocking the troubled species *T. tridentatus*.

Third, could juvenile horseshoe crabs be cultured in combination with other commercially important species, such as spotted Babylon, *Babylonia areolata*? Both *T. tridentatus* and *B. areolata* are benthic invertebrates, living in similar sedimentary habitats and eating similar food items (Zhou & Morton 2004; Kritsanapuntu et al 2006a; Chen et al 2011). Their relationships are size-dependent, either prey-predator or commensal. The adult *T. tridentatus* eat *B. areolata* as seen in lab culture but adult *B. areolata* do not eat *T. tridentatus* juveniles (personal observations).

The cultivation of *B. areolata* from egg mass to market size has been operated commercially (Kritsanapuntu et al 2006a; Chen et al 2011). In addition, *B. areolata* have been mixed-cultured with economically important fishes, such as the milk fish, *Chanos chanos* and the sea bass, *Lates calcarifer* (Chaitanawisuti et al 2001; Kritsanapuntu et al 2006a, 2006b, 2008). This mixed-culture success can be attributed to the differentiation of habitats in cultural ponds among these partner species. By contrast, *B. areolata* has not been mixed cultured with other macrobenthos. Therefore, intermediate culture of juvenile horseshoe crabs mixed with juvenile spotted babylon appears to be worth of study.

The purpose of this study is to examine whether mixed cultures of the juvenile *T. tridentatus* and *B. areolata* in large outdoor ponds can increase the production of advanced instars, preferably up to the third instar stage. The study also addresses concerns about the effectiveness of restocking *T. tridentatus* juveniles.

Material and Method

Management of culture ponds. Two cement ponds, Pond A and B, located in Beihai, Guangxi, China were used. One was 2,365 m^2, and the other was 2,150 m^2. Each pond was 1.5 m in height, and sea water was maintained at 60 to 100 cm in depth. Before culturing, the moss, macro-algae, and mud within the ponds were removed, and the ponds were exposed to air for ten days. Then, the bottom of the pond was covered with clean coarse sand grains ranging from 0.3 to 0.5 mm in diameter, to a thickness of 5 cm. After the ponds were filled with sea water, aqueous crude extract of tea seed (*Camellia* sp.) oil cake and bleach were added in water to kill fishes, shrimps, and crabs.

After the ponds were treated with the aforementioned cleansing procedures, juveniles of both species, *T. tridentatus* and *B. areolata*, were introduced to the ponds. Seawater temperature, salinity, and pH were measured every day during culture. A proportion of 40% to 50% of seawater in each pond was changed by using high tide flows twice every month; a total of eight changes were performed throughout the culture period of 150 days. When the water pH was lower than 8.0, calcium oxide was added to maintain the pH. During the mixed culture, water temperature ranged from a low of 14^oC to a high of 23.5 ^oC. The water salinity was 32 to 33 psu.

Mixing culture of juvenile T. tridentatus with juvenile B. areolata. Several batches of sperm and ova of *T. tridentatus* were collected by drawing from the gonopores. Artificial fertilization occurred from July to September 2013. Fertilized eggs were maintained in indoor ponds for collection of hatchlings. The first instars hatched in September, and the second instars appeared in October. Approximately 203,000 juvenile *T. tridentatus*, consisting of 67% first instars and 33% second instars, were cultured in mixing with juvenile *B. areolata* (8 to 15 mm in shell length) in the two pretreated outdoor ponds through a duration of 150 days from November 20, 2013 to April 20, 2014 when *B. areolata* were harvested. Initial stocking densities of juvenile *B. areolata* and juvenile *T. tridentatus* were 75 individuals/m^2 and 45 individuals/m^2, respectively, with a mixing ratio of 5:3 in each pond. Estimating and combining the juvenile abundance in the two ponds, a total of 203,000 *T. tridentatus* juveniles and 338,000 *B. areolata* juveniles were cultivated initially.

For food, the fresh meat of white-leg shrimp (*Litopenaeus vannamei*), flower crabs (*Portunus pelagicus*), Japanese jack mackerel (*Trachurus japonicus*), and clam (*Meretrix meretrix*) were chopped into small pieces and given to the juvenile *B. areolata* once daily. None of the additional food was given to the juvenile *T. tridentatus*. The amount of food provided was 3% to 5% of the weight of *B. areolata*. Occasionally multivitamin powder was added to the food.

A timeline of the study, including indoor and outdoor culture, subsequent sampling, and developmental stages of the juveniles, is shown in Figure 1.

Estimation of molting rate of T. tridentatus and survival rates of both species. To estimate the molting and survival rates of juvenile *T. tridentatus* throughout the mixed culture, individuals were collected at a fixed sampling effort: a period of 30 mins every 15 days on average from December 5, 2013 until March 30, 2014, for a total of 115 days (Figure 1). The number of individuals in each sampling effort was counted, and their body length was measured. The frequency distribution of body length was constructed to estimate the juvenile's instar stage based on leap growth occurring between the two consecutive molts. Changes in the compositions of instar stages, expressed as percentages, were analyzed over time. Molting rate was the ratio that denoted increasing numbers of individuals from one instar stage to the next instar stage and was calculated as follows:

Molting rate = number of individuals observed at the (n + 1)-th instar stage / number of individuals observed at the n-th instar stage

Linear regression was used to analyze the changes in the numbers of *T. tridentatus* instars over the course of 115 days. Survival rates of the instars were estimated by calculating the ratio of the number of instars observed on day 115 to those on day 1 using this linear regression equation. The survival rate calculated here was for an instar group consisting of different ages, which had blended instars from the first to the fifth stage. An overall survival rate was also estimated for each species for the whole study period of 150 days. At this time, the shell lengths of *B. areolata* ranged from 20 to 50 mm.

Estimation of the shortest developing time from the first instar to subsequent instar stages. The development time for a given instar stage was counted as days from the earliest hatchling of the first instar to an individual, which was the first one to molt at the given stage. The time taken by the first instar in indoor culture was included in the count (first instar first appeared in September 2013, Figure 1).

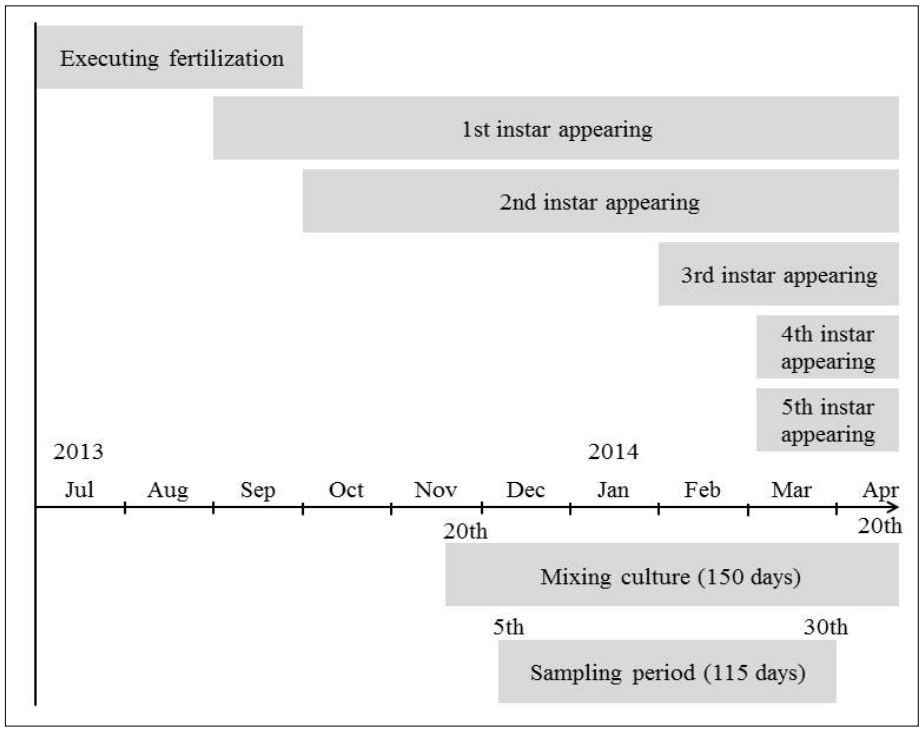

Figure 1. Timeline of the study of a mixed culture of juvenile *T. tridentatus* and juvenile *B. areolata*. Time starts from fertilization and extends to sampling juveniles for growth-related measurements and the end of the mixed culture, when *B. areolata* were harvested.

Results

Instar stage estimated from body length of juvenile T. tridentatus. The body-length distribution curve of all juvenile *T. tridentatus* sampled during the mixing culture clearly displayed five disconnected groups, indicating that each group is a separate instar stage (Figure 2). Therefore, instar stage, instead of body length, is used hereafter. The body lengths of instars at the first, second, third and fourth instar stages averaged 0.7 cm, 1.17±0.17 cm, 1.95±0.16 cm and 2.8±0.10 cm, respectively. Only one individual with a body length of 4 cm was found at the fifth instar stage.

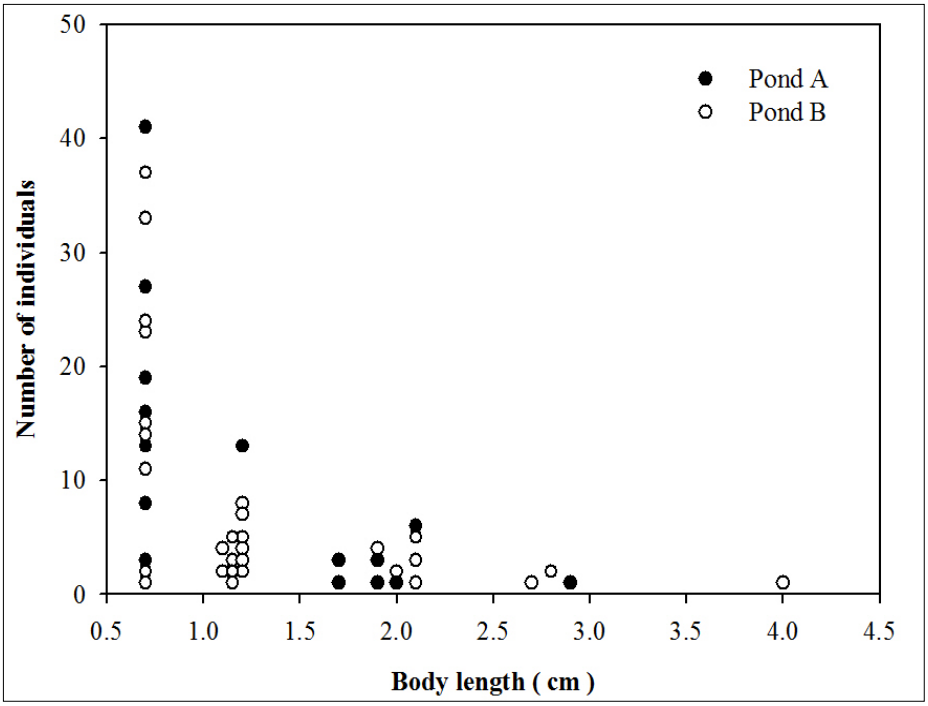

Figure 2. The body-length distributions of juvenile *T. tridentatus*. Measurements were taken in each pond during the mixed culture. Each sampling for measurements was conducted for 30 min in each pond at an interval of 15 days during a culture period of 115 days.

Changes in instar compositions and molting rate in juvenile T. tridentatus. During approximately 4 months of mixed culture, the compositions of *T. tridentatus* instars through 5 instar stages were similar between the two ponds (Table 1). At the end of mixed culture, no individual remained in the first instar in Pond A, and only two individuals were still at the first instar in Pond B.

Numbers of individuals steadily decreased with time at a rate of -0.42 during 115-day observation (Figure 3). The molting rate for the first instars to molt to second instars was 46.2% (145 second instars/314 first instars, Table 1). The same estimation method revealed a molting rate of 32.4% for second instars growing to third instars (47 third instars/145 second instars) and 8.5% for a third instar molting to a fourth instar during the 115-day survey.

The first third instar appeared on February 3, 2014 after 60 days of cultivation in outdoor mixed culture ponds, and most of the second and third instars appeared on the 90th day. At this time, the second and the third instars consisted of 39.7% (23/58) and 27.6% (16/58) of the total survivors (Table 1). The fourth and fifth instars occurred on March 16, 2014, which was 105 days after the start of the outdoor mixed culture (Table 1, Figure 4).

Table 1

Numbers of horseshoe crab instars (*T. tridentatus*) estimated for each instar stage during a culture period of 115 days.
Instar stages were determined using body-length distributions based on an abrupt increment in body length. Determination of instar stage is described in the Results

Sampling date	*Instar*															*Total*
	1			*2*			*3*			*4*			*5*			
	Pond		*Sub-total*	*Pond*		*Sub-total*	*Pond*		*Sub-total*	*Pond*		*Sub-total*	*Pond*		*Sub-total*	
	A	*B*		*A*	*B*		*A*	*B*		*A*	*B*		*A*	*B*		
2013																
December 5	27	37	64	12	9	21			0			0			0	85
December 20	41	33	74	10	2	12			0			0			0	86
2014																
January 3	19	23	42	11	9	20			0			0			0	62
January 18	27	24	51	7	7	14			0			0			0	65
February 3	16	15	31	4	8	12	1		1			0			0	44
February 20	13	14	27	5	5	10	1	1	2			0			0	39
March 4	8	11	19	13	10	23	9	7	16			0			0	58
March 16	3	1	4	8	7	15	6	3	9		1	1		1	1	30
March 30	0	2	2	9	9	18	12	7	19	1	2	3			0	42
Total			314			145			47			4			1	

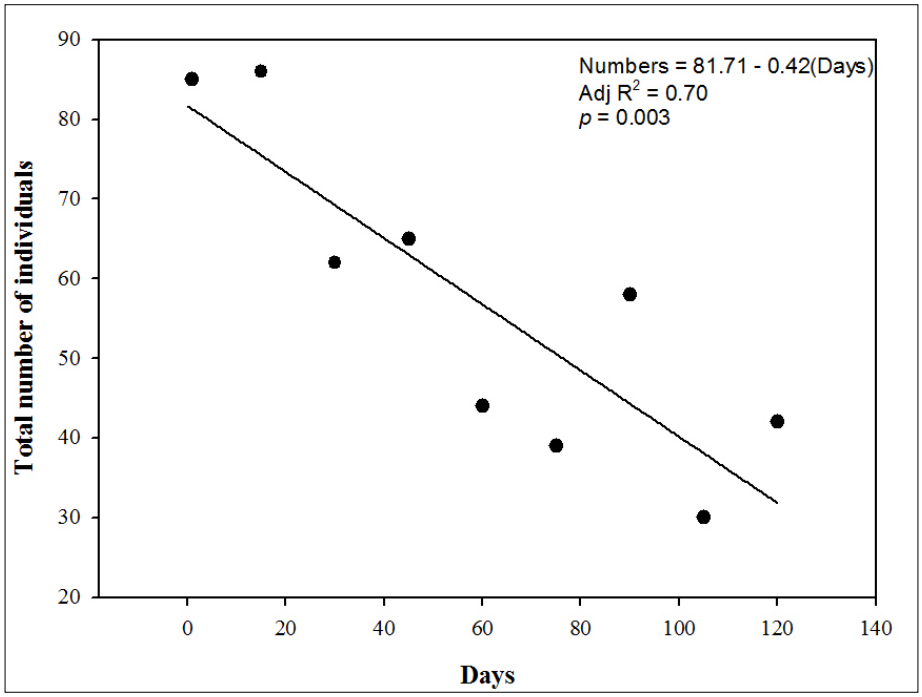

Figure 3. Changes in the numbers of juvenile *T. tridentatus* collected while they were grown in mixed culture with *B. areolata* for 115 days.

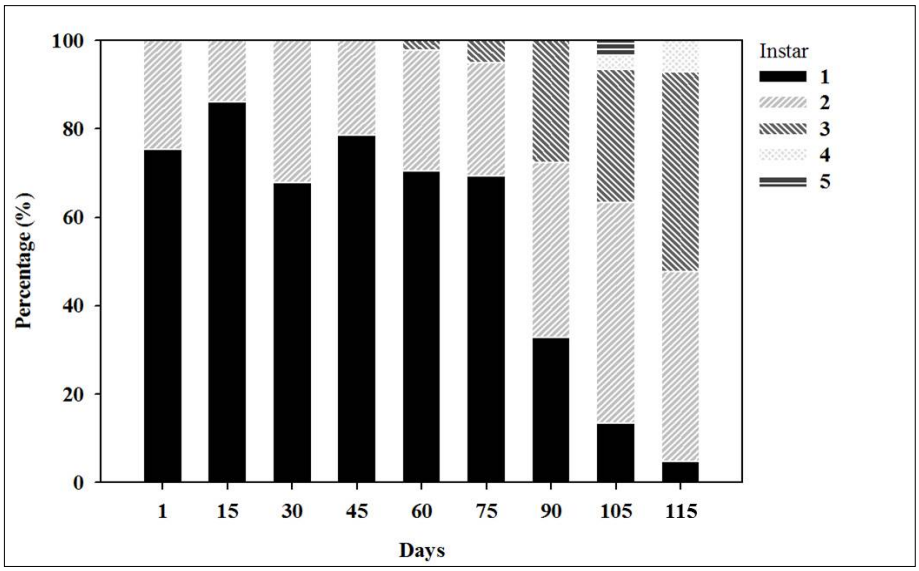

Figure 4. Composition changes of horseshoe crab instars (*T. tridentatus*) during the 115-day mixed culture. Data were combined from the two ponds. Labels of instar stages 1 to 5 are shown in the right panel.

Survival rates of juvenile T. tridentatus and B. areolata. Using regression equation shown in Figure 3, numbers of *T. tridentatus* juveniles were estimated as 81.4 and 33.4, on the day 1 and day 115, respectively. As a result, estimated survival rate was 41.1% (33.4/81.4) in the period of 115 days. Subsequently, after 150 days the study was terminated for harvesting *B. areolata*, 66,000 *T. tridentatus* juveniles and 295,000 *B. areolata* were harvested, resulting in an overall survival rate of 32.5% (66,000/203,000) and 87.3% (295,000/338,000) for juvenile *T. tridentatus* and *B. areolata*, respectively.

Abundances of second and third instar juveniles. After growth in a mixed culture for 115 days, 42.8% of *T. tridentatus* juveniles were second instars (18/42, Table 1) and 45.2% were third instars (19/42, Table 1). Approximately 35,700 second instars survived

(203,000 × 41.1% survival rate × 42.8%) and 37,700 third instars (203,000 × 41.1% survival rate × 45.2%) were produced. These numbers suggested that a large quantity of juvenile *T. tridentatus* with development stages beyond second instars could be obtained.

Estimation of developing time from the first instar to subsequent instar stages. With a combination of indoor culture and outdoor mixed culture, it took *T. tridentatus* approximately 150 days to develop from early hatchlings (the first instar first appeared in September 2013, Figure 1) to the third instar stage (the third instar first appeared in February 2014, Figure 1, Table 1). It took them approximately 196 days to reach the earliest fourth and fifth instars (Figure 1, Table 1).

Discussion

Benefits of mixed culture for intermediate culture of juvenile T. tridentatus. Compared to monospecific indoor cultures of *T. tridentatus* (Li 2008; Liao 2011; Chen et al 2014), this novel approach of a combination of indoor and outdoor mixed culture not only promotes the survival of juveniles, it also ensures cost-effective production that can be used for restocking.

T. tridentatus juveniles developing from their first instar to the fifth instar stage in a 115-day cultivation period exhibited 41.1% survival. This was a greater survival rate than those observed in studies using indoor monospecies culture (for the duration of the first to the fourth instar stage: 27.3% over 346 days, the first and corresponding author's personal observations; 0.5% over 2 years in Li 2008; Carmichael & Brush 2012).

Another study showed that approximately 33% of the first instar could survive to the 9th instar, and approximately 54% of the first instar survived to the third instar in indoor monospecies culture (Chen et al 2010). These data reveal a survival level comparable to or slightly better than that found in the present study, but this culture approach can only produce several tens of third instars. Such small amounts of juveniles are impractical for release into the wild.

The present study revealed that the development time of *T. tridentatus* from the first instar to the third instar was approximately 150 days. This value is comparable to that found in a study in Hong Kong, in which it took approximately 155 days for the first instar to develop into the third instar (Chen et al 2010). Caution is needed when making such comparisons. In the aforementioned case, the development time was derived from following up with the same cohorts, whereas in the present study it was estimated by tracing mixed cohorts.

This outdoor mixed culture approach appears to favor restocking and release of *T. tridentatus*. When juvenile *T. tridentatus* are mixed with an economically important shellfish, *B. areolata*, there are no additional demands on management, including maintenance of the culture facilities, expenses of feeding horseshoe crabs and work for the staff. More importantly, this approach can produce a large quantity of third instars, making release programs effective. The benefits to *B. areolata* grown in the mixed culture system remain unknown.

Possible mechanisms by which the mixed culture enhances intermediate culture of T. tridentatus. We propose that the possible mechanisms explaining the performance improvements of mixed culture are as follows: 1) benthic algae can grow under sunlight, providing essential nutrients to *T. tridentatus*, 2) juvenile *T. tridentatus* and juvenile and adult *B. areolata* eat foods with different particle sizes, thus differentiating their feeding niches and preventing competition, 3) the behaviors of *B. areolata*, including feeding, burrowing and moving in the subsurface of substrata may result in positive bioturbation effects, thus favoring the growth of juvenile *T. tridentatus*, 4) outdoor ponds provide a large space for better, more stable growth than indoor and small tank laboratory culture systems, and 5) suitable weather conditions from November to April in Guangxi. Temperatures were warmer during this period compared to the extreme temperatures that induce dormancy (Sekiguchi 1988; Chiu & Morton 2004).

B. areolata, like a vacuum cleaner, uses a tube-like proboscis to sweep across the bottom for small particulates. *T. tridentatus* juveniles have chelate appendages for picking up food items and sclerotized mouthparts for cutting and grinding. The feeding apparatuses of the two species are different. In addition, diet analyses using stable carbon and nitrogen isotopes have revealed that the second and the third instars tend to assimilate particulate organic matter derived from benthic microalgae and seagrass. Thus, they are herbivores (in *L. polyphemus*, Gaines et al 2002; in *T. tridentatus*, the first and corresponding authors' manuscript in preparation). By contrast, *B. areolata* were fed with fresh meats. These data suggest that *T. tridentatus* juveniles and *B. areolata* differ in their feeding niches. As a result, they can grow together in culture.

B. areolata has been reported as a scavenger (Xue et al 2010), a species that consumes what is leftover. Scavenging is generally rapid and serves as a path for nutrient cycling (Wilson & Wolkovich 2011). Therefore, *B. areolata* is likely to maintain water quality in mixed culture ponds. *B. areolata* actively moves around when feeding in the subsurface layer of the sediment substrata; when inactive, it burrows into the subsurface. In both cases, the siphons protrude above the sediment surface (Kritsanapuntu et al 2007). These moving, feeding, or burrowing activities, so-called bioturbation, can increase sediment oxygenation, enhance nitrogenous nutrient recycling and stimulate the growth of microbes, meiofuna and some small macrofauna in the sediments, as reported from various macrobenthic assemblages (Pelegri & Blackburn 1995; Widdicombe & Austen 1999). Based on these data, therefore, we think that *B. areolata* can improve the culture of *T. tridentatus* juveniles.

T. tridentatus in the field have been known to become inactive and bury themselves in deep sediments for diapause during hot summers (air temperatures as high as 34^{o}C) or cold winters (air temperatures as low as 9 ^{o}C, Sekiguchi 1988; Chiu & Morton 2004). It has been suspected that first instar *T. tridentatus* may undergo diapause in inhospitable environments for almost six months (the authors' personal observations). In our mixed culture, the water temperature of the two outdoor ponds ranged from 14 to 23.5 ^{o}C, temperatures beyond the instar's dormant limits. This favored juvenile growth in the ponds.

To conserve *T. tridentatus* in the wild, both population restocking and habitat protection need to be implemented simultaneously (IUCN 2012; Chen et al 2015). This intermediate culture mixed with an appropriate partner species can be applied to other places, wherever local aquaculture settings are appropriate. In addition, the release sites should include protected areas or those that will potentially be protected in the future.

Conclusions. The outdoor mixed culture used in the present study was better for restocking juvenile horseshoe crabs of *T. tridentatus* than indoor monospecies cultures reported previously. The profound results include greater survival rates for first instars developing to third instars, large production of the third instar, and cost-effective operation. The economic benefits are attributed to the fact that there are no additional costs when juvenile *T. tridentatus* are grown together with a commercially important and non-predatory species, such as *B. areolata*.

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