Chemically mediated rheotaxis of endangered tri-spine horseshoe crab: potential dispersing mechanism to vegetated nursery habitats along the coast

Kit Yue Kwan [Equal first author, 1] , Xin Yang [Equal first author, 1] , Chun-Chieh Wang [Corresp., 2] , Yang Kuang [1] , Yulong Wen [1] , Kian Ann Tan [1] , Peng Xu [1] , Wenquan Zhen [1] , Xueping Wang [1] , Junhua Zhu [1] , Xing Huang [1]

1 College of Marine Sciences, Beibu Gulf Ocean Development Research Centre, Guangxi Key Laboratory of Beibu Gulf Biodiversity Conservation, Beibu Gulf University, Qinzhou, Guangxi, China

2 Guangxi Key Laboratory of Marine Environmental Science, Guangxi Beibu Gulf Marine Research Center, Guangxi Academy of Sciences, Nanning, China

Corresponding Author: Chun-Chieh Wang
Email address: chunchiehwang@gxas.cn

Background: An enhanced understanding of larval ecology is fundamental to improve the management of locally depleted horseshoe crab populations in Asia. Recent studies in the northern Beibu Gulf, China demonstrated that nesting sites of Asian horseshoe crabs are typically close to their nursery beaches with high-density juveniles distributed around mangrove, seagrass and other structured habitats.

Methods: A laboratory Y-maze chamber was used to test whether the dispersal of early-stage juvenile tri-spine horseshoe crab *Tachypleus tridentatus* is facilitated by chemical cues to approach suitable nursery habitats. The juvenile orientation to either side of the chamber containing controlled seawater or another with various vegetation cues, as well as their movement time, the largest distance and displacement were recorded.

Results: The juveniles preferred to orient toward seagrass *Halophila beccarii* cues when the concentration reached 0.5 g l^{-1}, but ceased at 2 g l^{-1}. The results can be interpreted as a shelter-seeking process to get closer to the preferred settlement habitats. However, the juveniles exhibited avoidance behaviors in the presence of mangrove *Avicennia marina* and invasive saltmarsh cordgrass *Spartina alterniflora* at 2 g l^{-1}. The juveniles also spent less time moving in the presence of the *A. marina* cue, as well as reduced displacement in water containing the *S. alterniflora* cue at 1 and 2 g l^{-1}. These results may explain the absence of juvenile *T. tridentatus* within densely vegetated areas, which have generally higher organic matter and hydrogen sulfide. **Conclusion:** Early-stage juvenile *T. tridentatus* are capable of detecting and responding to habitat chemical cues, which can help guide them to high-quality settlement habitats. Preserving and restoring seagrass beds in the intertidal areas should be prioritized when formulating habitat conservation and management initiatives for the declining horseshoe crab populations.

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[1] College of Marine Sciences, Beibu Gulf Ocean Development Research Centre, Guangxi Key Laboratory of Beibu Gulf Biodiversity Conservation, Beibu Gulf University, Qinzhou, Guangxi, China

[2] Guangxi Key Laboratory of Marine Environmental Science, Guangxi Beibu Gulf Marine Research Center, Guangxi Academy of Sciences, Nanning, Guangxi, China

* Corresponding Author:
Chun-Chieh Wang
98 Daling Road, Nanning, Guangxi, 530007, China
Email address: chunchiehwang@gxas.cn

Co-first Author

Abstract

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39 the presence of the *A. marina* cue, as well as reduced displacement in water containing the *S.*
40 *alterniflora* cue at 1 and 2 g l^{-1}. These results may explain the absence of juvenile *T. tridentatus*
41 within densely vegetated areas, which have generally higher organic matter and hydrogen
42 sulfide.
43 **Conclusion:** Early-stage juvenile *T. tridentatus* are capable of detecting and responding to
44 habitat chemical cues, which can help guide them to high-quality settlement habitats. Preserving
45 and restoring seagrass beds in the intertidal areas should be prioritized when formulating habitat
46 conservation and management initiatives for the declining horseshoe crab populations.
47

Introduction

49 Horseshoe crabs are an ancient group of invertebrates that are broadly distributed along the west
50 coast of the North Atlantic and Pacific Oceans. They are inshore species which are important in
51 the food web of coastal and estuarine ecosystems (*Botton, 2009*). Their eggs serve as protein and
52 lipid sources for fishes and migratory shorebirds (*Mizrahi & Peters, 2009*), whereas the juveniles
53 and adults are key predators of the benthic community in intertidal flats (*Gaines et al., 2002;*
54 *John et al., 2012; Kwan et al., 2021*). However, horseshoe crabs are heavily harvested for their
55 blood for the manufacture of *Tachypleus* and *Limulus* amebocyte lysates, the worldwide
56 standardized tests for bacterial endotoxin detection in pharmaceutical products (*Gauvry, 2015;*
57 *Tinker-Kulberg et al., 2020*). In addition to resource exploitation, habitat loss and degradation
58 from coastal development (*Tsuchiya, 2009; Nelson et al., 2015; Wang et al., 2020*), as well as
59 bycatch by artisanal fishing and discarded fishing gear, are also widely observed to cause
60 considerable threats to horseshoe crab populations (*Zauki et al., 2019; Wang et al., 2022*). The
61 Atlantic horseshoe crab *Limulus polyphemus* and tri-spine horseshoe crab *Tachypleus tridentatus*
62 are listed as "Vulnerable" (*Smith et al., 2016*) and "Endangered" (*Laurie et al., 2019*),
63 respectively, in the IUCN Red List of Threatened Species, while the status of other two Asian
64 species, the coastal horseshoe crab *T. gigas* and mangrove horseshoe crab *Carcinoscorpius*
65 *rotundicauda* are under reassessment owing to the recent reports describing substantial
66 population declines (*John et al., 2018; Wang et al., 2020*). To reverse the declining trend,
67 national and regional conservation measures have been imposed in Bangladesh, India, China,
68 Singapore, Indonesia, and in specific regions in Japan. The effectiveness of these measures in
69 protecting the remaining horseshoe crab populations may be limited (*Wang et al., 2020*),
70 possibly due to insufficient scientific knowledge, financial resources and enforcement capacity
71 (*Xie et al., 2020*).
72   The larval dispersal and settlement of marine species are critical for the persistence of
73 local populations; therefore, an enhanced understanding of their ecology and behavior,
74 particularly for endangered or locally depleted species, is useful for management and
75 conservation (*Botton & Loveland, 2003; Green et al., 2015; Whomersley et al., 2018*).
76 Horseshoe crabs have unique reproductive strategies to maximize egg hatching success and
77 subsequent larval development (*Penn & Brockmann, 1994; Vasquez et al., 2015*). The spawning
78 pairs in amplexus migrate from shallow waters to sandy estuarine beaches, and lay clusters of

79 eggs beneath the sediment in the intertidal zones (*Smith et al., 2017*). The eggs hatch into
80 planktonic trilobite larvae and settle in the vicinity of the shoreline (*Botton & Loveland, 2003;*
81 *Botton, Tankersley & Loveland, 2010*). Most hatched larvae emerge from the sediment at high
82 spring tides when the water reaches the height of the nests (*Botton & Loveland, 2003; Ehlinger*
83 *et al., 2003*), facilitating larval dispersal from the nesting locations.
84   While the spawning biology of horseshoe crabs may share common characteristics, the
85 existing information for Asian species is limited and mostly descriptive. Similar to their Atlantic
86 counterpart, the distribution of newly settled and early-stage juvenile *T. tridentatus* and *C.*
87 *rotundicauda* populations is non-random and has a high tendency to stay close to mangrove,
88 seagrass and other structured habitats (*Kwan et al., 2016; Kaiser & Schoppe, 2018; Xie et al.,*
89 *2020; Meilana, Hakim & Fang, 2021*). Recent spawning habitat surveys in the northern Beibu
90 Gulf, following the last report in 1984 in China (*Cai, Lin & Huang, 1984*), demonstrated that the
91 identified nesting beaches were adjacent to nursery habitats for juveniles (*Kwan et al., 2022*).
92 Little is known regarding the movement behavior of the larvae and early-stage juveniles under
93 field conditions. Previous laboratory studies on *L. polyphemus* suggest that their directed
94 movements to water flow (i.e., rheotaxis) change upon exposure to habitat chemical cues
95 (*Medina & Tankersley, 2010; Butler & Tankersley, 2020*). A rheotaxis can either be positive by
96 turning face into the current to hold their position rather than being swept downstream, or
97 negative to avoid oncoming currents (*Kobayashi et al., 2014*). In the experiment of *Butler &*
98 *Tankersley (2020)*, *L. polyphemus* larvae exhibited a positive rheotaxis in the presence of
99 chemical cues from seagrass associated with their settlement sites, which may imply that the
100 strong tendency of early juveniles to remain close to the beach is a consequence of upstream
101 movement behavior mediated by habitat chemical cues. However, the mechanism of post-larval
102 orientation and settlement is likely species- and/or site-specific, depending on the perceived
103 coastal environmental conditions (*Rossi et al., 2019*).
104   In this study, we examined whether the early-stage juvenile *T. tridentatus* are able to
105 detect and respond to chemical cues associated with varied coastal vegetations available in their
106 nursery habitats. We predict that the habitat chemical cues can influence the orientation and
107 movement behaviors of juvenile, providing guidance to preferred settlement habitats, which
108 shapes the distribution patterns of early juveniles in the immediate vicinity of the shoreline. The
109 Beibu Gulf, a semi-closed gulf located off the coast of southern China and northern Vietnam, is
110 broadly considered to be one of the most important habitats for the remaining high-density
111 population of endangered *T. tridentatus* (*Brockmann & Smith, 2009; Sekiguchi & Shuster, 2009;*
112 *Liao et al., 2019*). The spawning and nursery habitats of Asian horseshoe crabs in the gulf are
113 typically characterized by extensive mangrove fringes along the coastline with patches of
114 seagrass *Halophila* spp. and invasive saltmarsh cordgrass *Spartina alterniflora* scattered on the
115 intertidal flats (*Xie et al., 2020; Kwan et al., 2022*). These characteristics of the spawning and
116 nursery habitats serve as a good opportunity to test our prediction of the orientation and
117 movement behaviors of the endangered *T. tridentatus* juveniles for exploring the ecological
118 importance to settlement in suitable habitats.

119

Materials & Methods

Larval and juvenile horseshoe crab rearing

Tachypleus tridentatus larvae were obtained from the Guangxi Institute of Oceanology, China.
The use of hatchery-bred animals was approved by the Department of Agriculture and Rural
Affairs of Guangxi Region, China (approval number 2022-0131). Mating pairs of *T. tridentatus*
were kept in indoor tanks with an approximately 10-cm sediment layer underneath. The released
eggs were incubated in hanging baskets from the surface of culture water with continuous,
vigorous airflow pumping below the baskets (*Xu et al., 2021*). Most eggs developed and hatched
into trilobite larvae after one-month rearing under the following environmental conditions:
temperature 26–30°C, salinity 32–33 ppt, pH 7.6–7.9, dissolved oxygen 6–7 mg l⁻¹.

The hatched larvae were transported to the laboratory and cultured in aquarium tanks
(dimension: $120 \times 40 \times 25$ cm) equipped with a water filtration system, thermostatic heaters and
ultraviolet sterilizers. A 4-cm sediment layer was provided underneath. Seawater was maintained
at the rearing conditions similar to egg incubation. The water quality was monitored weekly, and
half of the volume of water was changed every month or whenever water ammonia concentration
was above 0.1 mg l⁻¹. Frozen brine shrimp larvae were provided thrice per week when the larvae
had developed into second-instar juveniles.

Experimental setup and conditioned water preparation

The experimental setup consisted of a laboratory Y-maze acrylic chamber and two reservoirs
containing control and conditioned waters, separately (Fig. 1a). A water pump was placed within
each reservoir to pump the test waters into the inflow end at each side of the Y-maze chamber.
The chamber was filled with seawater to 6 cm depth with a 1-cm sand layer underneath, so as to
keep all experimental juveniles completely submerged under the water. Prior to the experiment,
two acrylic movable plates were inserted near the outflow end of the chamber (Fig. 1a) to
maintain the water level and avoid the immediate mixing between control and conditioned
waters. The experiment began after the experimental waters had been flowing in the chamber for
at least 10 mins. The flow rate was calculated by measuring the volume of outflowing seawater
per unit time. A standard flow rate (200 mL/min) was maintained throughout the experiments by
adjusting the control valve on each water tube connected to the water pumps until reaching stable
equilibrium from each side with the aid of different dyed waters (water-soluble ink). Two video
cameras were installed on each side to record juvenile directional movements relative to the flow
of water.

Conditioned waters were prepared using three dominant vegetation sources, including
mangrove *Avicennia marina*, seagrass *Halophila beccarii* and saltmarsh cordgrass *Spartina
alterniflora*, which can be found in *T. tridentatus* nursery habitats along the coast of the northern
Beibu Gulf, China (*Xie et al., 2020*). Fresh fallen leaves of mangrove, seagrass and saltmarsh
cordgrass were collected at the identified nursery sites (*Kwan et al., 2021*) during low tides in the
summer (May–September) of 2020. The collected samples were rinsed repeatedly, freeze-dried

159   for at least one week, and ground into the powder with a mortar and pestle. The dried samples
160   were weighed, dissolved into artificial seawater at salinity 30 ppt, homogenized and filtered after
161   12 h, to prepare the conditioned waters at concentrations of 0.25, 0.50, 1.00 and 2.00 g l^{-1}. The
162   levels were selected based on the concentration range (0.3–30 g l^{-1}) described in *Butler &*
163   *Tankersley (2020)*. However, the preparation method of conditioned seawater in the present
164   study (dissolution of ground vegetation powder) was slightly different from those in the previous
165   study (24-h incubation of fresh vegetation), which should cause different actual levels of
166   chemical cues available in the conditioned waters. The trials with concentrations higher than 2 g
167   l^{-1} were not conducted because the conditioned water would become too turbid and the juvenile
168   behavioral parameters could not be quantified. Artificial seawater at salinity 30 ppt was used as
169   the control. All experimental waters were subjected to experiments within 12 h of preparation.
170
171   **Orientation and movement behaviors toward chemical cues**
172   To quantify the movement responses during the settlement process, the orientation and various
173   behavioral data from 60 juveniles were collected per treatment. During each treatment, a second-
174   instar juvenile *T. tridentatus* (prosomal width: 7.5–8.8 mm, wet weight: 35.9–55.3 mg) was
175   randomly chosen and introduced into the intersection area of the chamber (Fig. 1a). The juvenile
176   was given 30 mins to respond to the flow by moving upstream to either side of the chamber
177   containing control or conditioned water, or downstream toward the outflow end. The orientation,
178   movement time, the largest movement distance and displacement of the juvenile were quantified
179   based on the video recordings. After the completion of data collection from 10 juveniles, the
180   inner surface and sand layer of the chamber was rinsed completely. Another group of 10
181   juveniles was used for the same treatment by alternating the inflow of conditioned water from
182   the left to the right arm of the chamber, to test if the choice of seawater source by the juveniles
183   was non-random. The set of experiment was repeated three times, and all juveniles were only
184   used once per observation (each treatment: 10 juveniles × 2 positions × 3 replicates).
185   Because none of the experimental juveniles traveled downstream throughout the
186   experiment, the orientation parameter was used to quantify the percentage of individuals moving
187   upstream to choose control/conditioned water. The orientation toward conditioned or control
188   water of each juvenile was recorded by a single video, and the percentage of individuals moving
189   up to either side of the Y-maze chamber was calculated based on the video recordings from 10
190   different juveniles. A juvenile that failed to travel in either direction during the first 10 mins was
191   considered "unresponsive" and would be replaced by another juvenile. The proportion of
192   "unresponsive" juveniles was very low, which ranged from 0–1 individuals in each experimental
193   replicate. Movement time was the total time the juveniles spent crawling or swimming in the
194   chamber. The largest movement distance was defined as the longest length traveled in a single
195   upstream direction, whereas displacement was the length between the initial and final points of
196   movement within the allowed experimental time, i.e., 30 mins (Fig. 1b). Artificial lighting was
197   used to ensure that all animals were exposed to the same conditions. None of the juveniles was

198 sacrificed during the experiment, and the study protocol was approved by the Committee for
199 Animal Welfare of the Beibu Gulf University.
200
201 **Statistical analysis**
202 Data were first examined for normality and homogeneity of variance by Shapiro-Wilk and
203 Levene's tests, respectively. Student's *t* and Mann-Whitney U tests were conducted to check
204 whether the choice of the left/right arm of the chamber by the juveniles was non-random. The
205 data from two groups of 10 juveniles were pooled for subsequent behavioral parameter analyses
206 after the differences were found to be statistically similar (Table S1). Since the orientation data
207 were non-normal, non-parametric binomial tests were performed to examine the possible
208 differences in juvenile orientation between control and conditioned waters at individual
209 concentration. The test proportion of the binomial model was set at 0.50. Student's *t* tests were
210 used for other behavioral parameters analyses. To understand the overall effects of various
211 vegetation sources at different concentrations on juvenile behaviors, the data were analyzed
212 using two-way analysis of variance (ANOVA: source [fixed] × concentration [fixed]). Multiple
213 pair-wise comparisons among sources/concentrations were applied using post hoc Tukey's tests
214 with Bonferroni adjustments when a significant difference was identified. All the above analyses
215 were implemented using IBM SPSS Statistics Software (version 26, New York, USA).
216
217 **Results**
218 Between 17%–82% of juvenile *T. tridentatus* traveled to the side containing habitat cues from
219 different vegetation sources at various concentrations (Fig. 2a-c). Binomial tests between control
220 and treatment groups revealed that statistically higher proportions of juveniles responded to *H.*
221 *beccarii* chemical cues at 0.50 and 1.00 g l^{-1}, while significantly lower percentages of juveniles
222 moved upstream approaching *A. marina* chemical cues at 2.00 g l^{-1} and *S. alterniflora* chemical
223 cues at 2.00 g l^{-1} (Fig. 2a-c). For other behavioral parameters, a significant reduction in juvenile
224 movement time for >57% was detected in water containing *A. marina* cue at 2.00 g l^{-1}, while the
225 juvenile largest movement distance and displacement performed in seawater sources with
226 chemical cues were similar to those recorded in the control (Table 1, Fig. 2d).
227   When the overall effects of various vegetation sources at different concentrations were
228 simultaneously considered, both source and concentration of chemical cues were found to
229 significantly alter the displacement of juveniles, but only source and concentration were noted to
230 affect juvenile movement time and the largest distance, respectively (Table 2, Fig. 3). A
231 significant decrease in juvenile displacement at 1.00 and 2.00 g l^{-1} was also observed in water
232 containing *S. alterniflora* cue (Fig. 3a). In terms of movement time, the juveniles were more
233 active in seawater containing *H. beccarii* cue than those in *A. marina* (Fig. 3b). A significant
234 reduction in the largest movement distance of juveniles was also recorded at 2 g l^{-1}, compared to
235 those at 0.25 g l^{-1}, regardless of the vegetation sources (Fig. 3c).
236

Discussion

237
238 There is increasing interest in studying horseshoe crab populations due to their biomedical
239 importance and use in various fisheries, and understanding the factors that may contribute to
240 larval recruitment is a worthwhile investigation. Recent studies provided useful information on
241 the nesting/nursery habitat distributions and larval hatching processes of Asian horseshoe crabs
242 (*Itaya et al., 2022; Kuang et al., 2022; Kwan et al., 2022*). However, little is known regarding
243 the role of chemoreceptive and olfactory capabilities in larval transport and settlement, despite
244 the fact that high densities of juvenile Asian horseshoe crabs are known to occur in the upper
245 intertidal beaches adjacent to mangrove, seagrass and other structured habitats (*Xie et al., 2020*).
246 In this study, the use of chemical cues in seeking preferred settlement habitat by *T. tridentatus*
247 was tested using a laboratory Y-maze chamber. Our results provided evidence that early-stage
248 juvenile *T. tridentatus* are capable of detecting and responding to chemical cues associated with
249 the typical vegetations available in nursery habitats. Overall, the juveniles were attracted to the
250 seagrass *H. beccarii* cue when the concentration reached 0.5 or 1 g l^{-1}. On the other hand, the
251 juveniles tended to avoid chemical cues from mangrove *A. marina* and saltmarsh cordgrass *S.*
252 *alterniflora* at relatively high concentrations (i.e., 2 g l^{-1} in this study).
253   The use of chemical cues was documented in examples of marine decapod crustaceans
254 and fish (*Havel & Fuiman, 2015; Foretich et al., 2017; Hinojosa et al.; 2018; Arvedlund &*
255 *Kavanagh, 2020*). Horseshoe crabs are known to possess a variety of chemoreceptors on the
256 gills, flabellum, chilaria and walking legs, which would respond to oxygen in seawater and
257 varying chemical cues associated with food (*Quinn, Paradise & Atema, 1998; Mittmann &*
258 *Scholtz, 2001; Saunders et al., 2010*). In Cape Cod, U.S.A., *L. polyphemus* were observed to
259 locate their preferred food, *Mya arenaria*, which were completely buried within the sediment
260 (*Smith, 1953*). There is also evidence of chemical cue use by male *L. polyphemus* in locating
261 spawning females. *Hassler and Brockmann* (*2001*) found that a cement model with conditioned
262 seawater collected from spawning females was more attractive to males. Previous studies also
263 demonstrated that *L. polyphemus* would use other sensory cues in addition to chemical cues to
264 adapt to the overall complexity of signals in coastal and estuarine environments. The use of
265 visual cues enables male *L. polyphemus* to see and respond to females at night (*Barlow, Ireland*
266 *& Kass, 1982; Herzog, Powers & Barlow, 1996*), and pair up with larger females (*Hassler &*
267 *Brockmann, 2001; Barlow & Powers, 2003*).
268   Relatively little is known about the use of multisensory cues by larvae and juvenile
269 horseshoe crabs to identify preferred habitats. *Limulus polyphemus* larvae were noted to be more
270 active at nighttime and positively phototactic to dim light sources such as moonlight (*Rudloe,*
271 *1979*; *Botton & Loveland, 2003*). The major releases of hatched larvae from the nesting sites are
272 shown to be associated with high water conditions such as hydration, hypoosmotic shock and
273 agitation (*Ehlinger & Tankersley, 2003; Botton, Tankersley & Loveland, 2010; Kuang et al.,*
274 *2022*). These exogenous cues are possibly detected by mechanoreceptors available on the entire
275 surface of prosoma, spines and walking legs (*Wyse 1971*), to facilitate the dispersal of larvae
276 away from the spawning locations. Our results, together with previous studies on *L. polyphemus*

277   (*Medina & Tankersley, 2010; Butler & Tankersley, 2020*), suggest that chemical cues are
278   involved in the settlement and habitat selection process. Horseshoe crab larvae and juveniles
279   were more directed toward chemical cues from seagrasses (*Medina & Tankersley, 2010; Butler
280   & Tankersley, 2020*). The responses were perceived as a shelter-seeking behavior, since high-
281   density juvenile *T. tridentatus* populations in the northern Beibu Gulf, China were found in areas
282   of seagrass patches, mainly *Halophila* species (*Xie et al., 2020*). Apart from providing refuge
283   from predation, other studies also revealed that the juveniles predominantly assimilated energy
284   from seagrass as basal production sources in the food web (*Kwan et al., 2015; Fan et al., 2017;
285   Kwan et al. 2021*). However, it is rare to find juvenile Asian horseshoe crabs near *S. alterniflora*
286   in the field, even though the invasive plant has expanded rapidly throughout the Chinese
287   coastline (*Meng et al., 2020*), and highly overlapped with horseshoe crab habitats (*Kwan et al.,
288   2015; Xie et al., 2020; Kwan et al., 2021*). Our data also showed that juvenile *T. tridentatus*
289   showed stronger preferences for the native *H. beccarii* habitat over the one with invasive *S.
290   alterniflora*.
291   Although not addressed in our study, biofilm available on the plants and other substrata
292   can also act as settlement cues for a broad variety of marine invertebrate larvae, including
293   mollusks (*Liang et al., 2020*), crustaceans (*Siddik & Satheesh, 2019*), polychaetes (*Freckelton et
294   al., 2022*), gastropods (*La Marca et al., 2021*), cnidarians (*Petersen et al., 2021*) and
295   echinoderms (*Huggett et al., 2006*). Marine biofilms are complex, heterogenic microbial
296   communities, mainly bacteria and diatoms, surrounded by a matrix of extracellular polymeric
297   substances (*Antunes, Leão & Vasconcelos, 2019*). Larval settlement responses to different
298   bacteria can be species-specific. Similarly, the bacterial community on the surfaces and/or roots
299   of habitat plants can also be important as settlement and behavioral cues for early-stage *T.
300   tridentatus*. While the role of bacteria in larval settlement of horseshoe crabs is currently unclear,
301   their larvae and early-stage juveniles are known to feed primarily on sedimentary organic matter
302   (*Gaines et al., 2002; Kwan et al., 2021*), which is dominated by benthic diatoms (e.g.,
303   Naviculaceae and Cymbellaceae in Beibu Gulf region, Table S2). Alternatively, chemical
304   compounds released during the decay processes of coastal plants may also attract the settlement
305   of marine invertebrate larvae, as seen in several amphipod species associated with seagrass bed
306   (*Edgar 1992*) and the mangrove jellyfish *Cassiopea xamachana* (*Hofmann et al., 1996; Fleck &
307   Fitt, 1999*). However, the preparation of conditioned seawater using dried plant materials in the
308   current study may lower the effects of live bacteria and decayed plant compounds on settlement
309   behaviors of the early-stage juvenile *T. tridentatus*.
310   In this study, the chemically-mediated orientation and movement behaviors of the
311   juveniles were generally concentration-dependent. As noted in Figure 2(b), the juveniles
312   preferred the water containing seagrass chemical cues at a concentration of 0.5 and 1 g l^{-1}.
313   However, the effect on directional choice toward seagrass cue was ceased at 2 g l^{-1} and became
314   statistically similar to that observed in control water. The results can be interpreted as the
315   movement process of the juveniles seeking settlement habitats (*Medina & Tankersley, 2010*):
316   their movements become more directed when the juveniles get closer to the source, which is

317   indicated by the increased concentration of seagrass chemical cues. When the concentration is
318   too high (e.g., 2 g l^{-1} in this study), the juveniles may perceive the signal as the arrival to the
319   preferred settlement habitats, and therefore their behavioral responses would become weaker. In
320   contrast, the juveniles exhibited avoidance behaviors when getting too close to mangrove and
321   saltmarsh cordgrass, as indicated by selecting the side with control seawater when the source
322   concentrations had reached 2 g l^{-1}. Other movement behaviors, including reduced time spent on
323   movement and/or shorter displacement, also recorded a similar trend (Figs. 2d and 3a). A
324   possible explanation for such avoidance behaviors is that the densely vegetated saltmarsh
325   cordgrass and mangrove areas have slower water movement and accumulation of the fine-
326   grained, poorly drained substratum, which would result in higher concentrations of organic
327   matter and hydrogen sulfide in the areas (*Wang et al., 2015; Rossi et al., 2019; Su et al., 2020; Li*
328   *et al. 2021*). A recent study in the Beibu Gulf region also demonstrated that the *Spartina*
329   occupation reduced the diversity of macroinvertebrate assemblages on intertidal flats (*Su et al.,*
330   *2020*), and therefore may affect the availability of food sources for the juveniles (*Kwan et al.,*
331   *2021*). The presence of high tannin, phenolics and other plant defensive compounds in *A. marina*
332   and *S. alterniflora* extracts (*Zhou et al., 2010; Zhang et al., 2021*) were found to negatively
333   affect benthic invertebrates (*Alongi, 1987; Lee, 1999*), probably also reducing the rheotaxis of
334   juvenile *T. tridentatus* toward these vegetations.
335   While the induction by a single source of vegetation cues never exists in the marine
336   environment, and the actual contribution of these factors in the field is poorly understood, similar
337   laboratory experiments are common and useful to investigate the mechanism of larval settlement
338   and habitat selection in marine invertebrates (*Suárez-Rodríguez, Kruesi & Alcaraz, 2019;*
339   *Gravinese et al., 2020; Brooker et al., 2022*). For example, Jensen & Morse (1990) identified an
340   inductive organic molecule that induced larval settlement in marine polychaete *Phragmatopoma*
341   *californica* in the laboratory and also triggered the same processes in the ocean. Previous
342   research on horseshoe crabs, to the best of our knowledge, has not compared the potential
343   behavioral difference between hatchery-bred individuals and those in the field. However, given
344   that horseshoe crab populations are threatened and even endangered across the distribution
345   range, previous studies suggest the use of artificially cultured horseshoe crabs is useful to explain
346   the habitat selection mechanisms and distribution patterns of the wild populations (e.g., *Medina*
347   *& Tankersley, 2010; Hieb et al., 2015; Kwan et al., 2020; Chan et al., 2022*). Apart from this,
348   horseshoe crabs are also likely to use multiple sensory cues, particularly visual cues, in
349   settlement habitat selection. As the entire exclusion of the multisensory factors is challenging, in
350   this study, we can observe some discrepancies in the juvenile orientation results. In Figure 2, the
351   juveniles showed (1) avoidance behavior toward mangrove *A. marine* cues at 2.00 g l^{-1} but not at
352   0.25, 0.50 and 1.00 g l^{-1}; and (2) there is neither preference nor avoidance of cordgrass *S.*
353   *alterniflora* except at the highest concentration of 2.00 g l^{-1}. Therefore, consideration of the
354   simultaneous use of multiple sensory cues is needed in horseshoe crabs to make further
355   conclusions on the process and mechanism of juvenile habitat selections. Another possibility of
356   the discrepancies is due to the lower resolution of orientation data compared to the other

357 behavioral parameters: multiple video recordings from a group of juveniles were required to
358 obtain each percentage orientation sample value, but only one video recording per juvenile
359 would be needed to collect each of the other behavioral parameter values.
360   Collectively, our results demonstrated the differential orientation behaviors of juveniles
361 between seagrass and mangrove/cordgrass chemical cues, which may provide useful navigation
362 to juvenile *T. tridentatus* to identify and settle on the upper intertidal flats adjacent to seagrass
363 habitats, and avoid getting too close to densely vegetated areas of mangroves and saltmarsh
364 cordgrass. The results of nursery habitat selection can maximize the chance to obtain high-
365 quality food and avert adverse environmental conditions, and thereby increasing the survival rate
366 of the juveniles. Additional emphasis on mating, food searching and predation avoidance should
367 also be addressed in *T. tridentatus* and other Asian species to make further conclusions on the
368 role of chemical cues in horseshoe crabs. From a management perspective, preserving coastal
369 and estuarine habitats, particularly those with seagrass beds, should be prioritized in management
370 measures for conservation of the declining Asian horseshoe crab populations. Active seagrass
371 restoration in the upper and middle portion of intertidal areas can also benefit Asian horseshoe
372 crab conservation by providing more suitable nursery habitats for shelter and basal production
373 sources in the juvenile food web.
374

Conclusions

376 Our findings demonstrated that early-stage juvenile *T. tridentatus* are capable of detecting and
377 responding to varying sources of habitat vegetation. Positive rheotaxis was exhibited in the
378 presence of seagrass *H. beccarii* cue at 0.5 and 1 g l^{-1}, but juveniles avoided mangrove *A. marina*
379 and invasive saltmarsh cordgrass *S. alterniflora* cues when the concentrations were too high at 2
380 g l^{-1}. Juvenile displacement was also significantly reduced in water containing *S. alterniflora* cue
381 at 1 and 2 g l^{-1}. These behaviors may help guide juveniles to high-quality settlement habitats, as
382 seagrass is known to serve as basal production sources in the *T. tridentatus* food web, as well as
383 prevent juveniles from getting too close to the mangrove and saltmarsh cordgrass, which are
384 generally higher in organic matter and hydrogen sulfide. The present study provided valuable
385 evidence on the scope of larval dispersal and habitat selection mediated by habitat chemical cues,
386 which is useful to improve the management efforts for the declining Asian horseshoe crab
387 populations.
388

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394

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650

Statistical results of Student's *t* tests (d.f. = 4) showing the effects of each vegetation source at varying concentrations compared to the control on various movement behaviors of juvenile *T. tridentatus*.

Significant *p* values (<0.05) are highlighted in bold. Conc. = concentration.

1 **Table 1.** Statistical results of Student's *t* tests (d.f. = 4) showing the effects of each vegetation
2 source at varying concentrations compared to the control on various movement behaviors of
3 juvenile *T. tridentatus*.

Habitat cue	Conc. (g l⁻¹)	Movement time		Largest distance		Displacement	
		t	*p*	*t*	*p*	*t*	*p*
A. marina	0.25	-0.825	0.413	0.196	0.846	0.248	0.805
	0.50	-0.169	0.866	-1.725	0.090	-0.935	0.354
	1.00	1.446	0.153	0.672	0.504	1.668	0.101
	2.00	-3.081	**0.003**	-1.033	0.306	-1.314	0.194
H. beccarii	0.25	0.088	0.930	0.987	0.328	0.474	0.637
	0.50	-0.857	0.395	-0.283	0.778	-0.568	0.572
	1.00	1.175	0.245	0.450	0.655	-0.015	0.988
	2.00	1.018	0.313	0.335	0.739	0.383	0.703
S. alterniflora	0.25	-0.712	0.479	1.507	0.137	0.595	0.554
	0.50	0.117	0.907	0.118	0.907	0.313	0.755
	1.00	-1.71	0.093	-0.571	0.570	-0.227	0.821
	2.00	-0.279	0.781	-1.254	0.215	-1.225	0.226

6 Significant *p* values (<0.05) are highlighted in bold. Conc. = Concentration.

Statistical results of two-way ANOVA showing the effects of different source and concentration of chemical cues (g l^{-1}) on movement behaviors of juvenile *T. tridentatus*.

Significant *p* values (<0.05) are highlighted in bold. d.f. = Degree of freedom.

2 **Table 2.** Statistical results of two-way ANOVA showing the effects of different source and

3 concentration of chemical cues (g l^{-1}) on movement behaviors of juvenile *T. tridentatus*.

Response	d.f.	*F*	*p*
Movement time (min)			
Source	2	4.344	**0.025**
Conc.	3	0.751	0.533
Source × Conc.	6	8.316	**< 0.001**
Error	24		
Largest distance (cm)			
Source	2	2.488	0.104
Conc.	3	3.090	**0.046**
Source × Conc.	6	1.756	0.151
Error	24		
Displacement (cm)			
Source	2	4.728	**0.019**
Conc.	3	4.884	**0.009**
Source × Conc.	6	2.398	0.060
Error	24		

6 Significant *p* values (<0.05) are highlighted in bold. d.f. = Degree of freedom.

Figure 1

(a) The experimental setup comprises a Y-maze chamber used to measure the rheotaxis and movement behavior of juvenile *T. tridentatus* in response to control (blue) versus conditioned seawater with chemical cues (red).

(b) A typical example of juvenile movement in the chamber. The movement pathway is indicated by a dashed line. P_i = initial position, P_f = final position when the allowed experimental time ended, i.e., 30 mins.

a)

Juvenile
placement area

Drain

Movable
plate

40 cm

Arm for observation

Control

Conditioned seawater

80 cm

8 cm

12 cm

Connected to
reservoir

Connected to
reservoir

b)

Largest distance

Displacement

P_i

Swim

Crawl

P_f

Juvenile

Sand layer
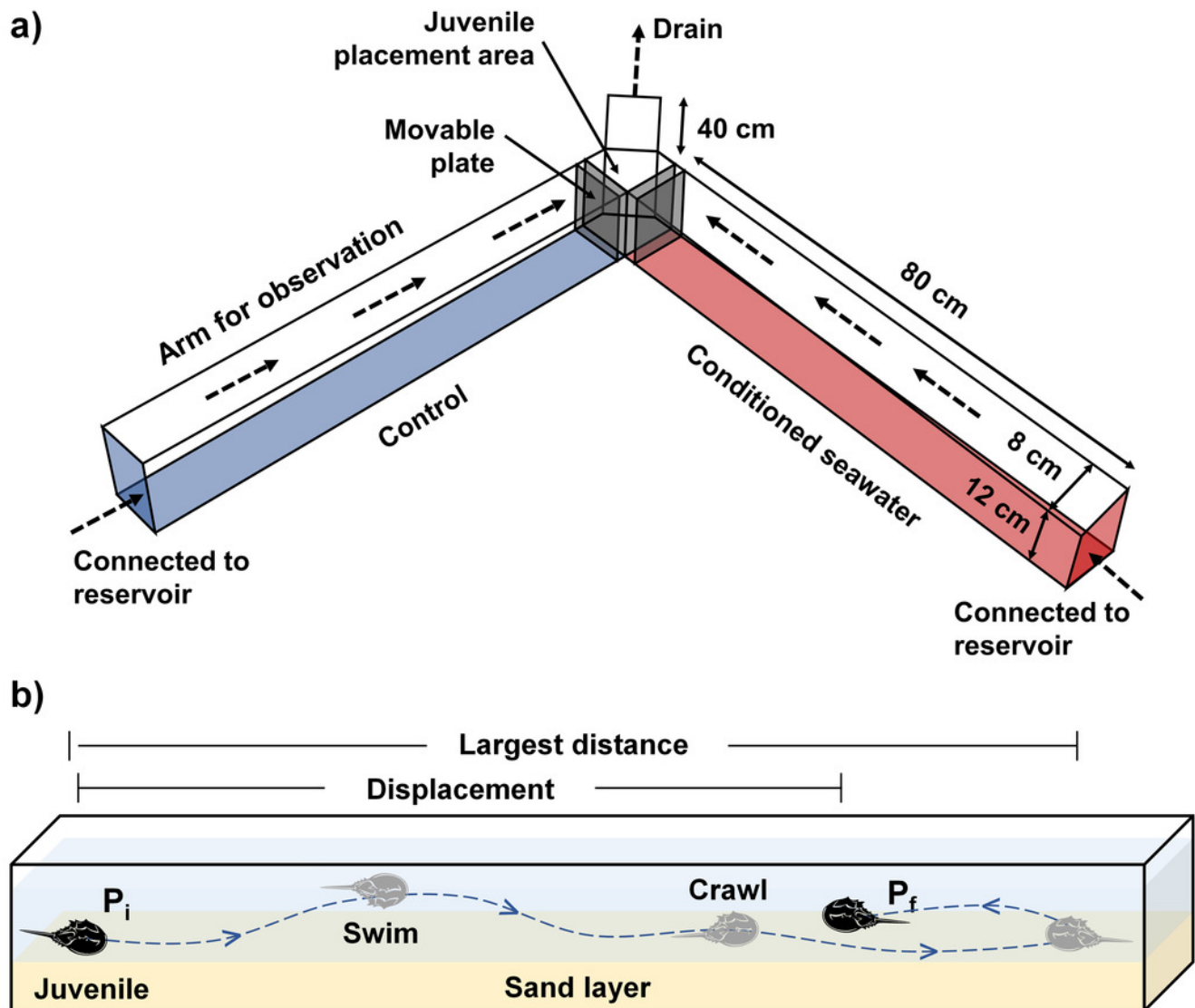
Figure 2

Effects of each vegetation source at varying concentrations compared to the control on rheotaxis (a-c), and movement time (d) of juvenile *T. tridentatus*.

Movement time of the juveniles in seagrass *H. beccarii* and saltmarsh cordgrass *S. alterniflora* cues was statistically similar to those in the control, and was not shown in the figure. The data are expressed in mean ± standard deviation. Different lowercase letters represent statistical differences (p < 0.05) between control (white bars) and conditioned (grey bars) waters at the corresponding concentration.
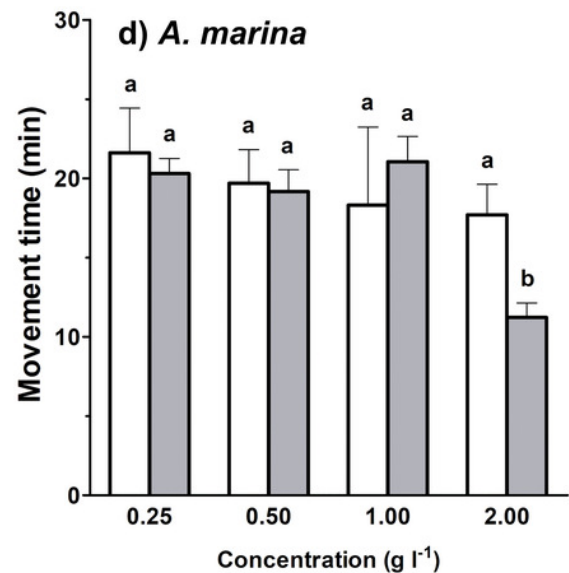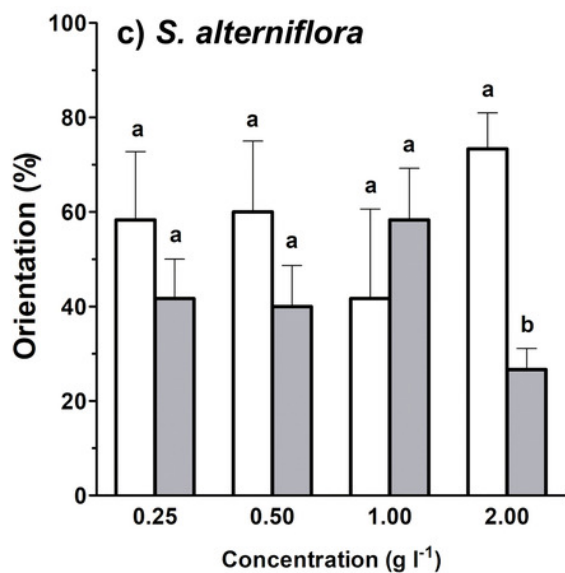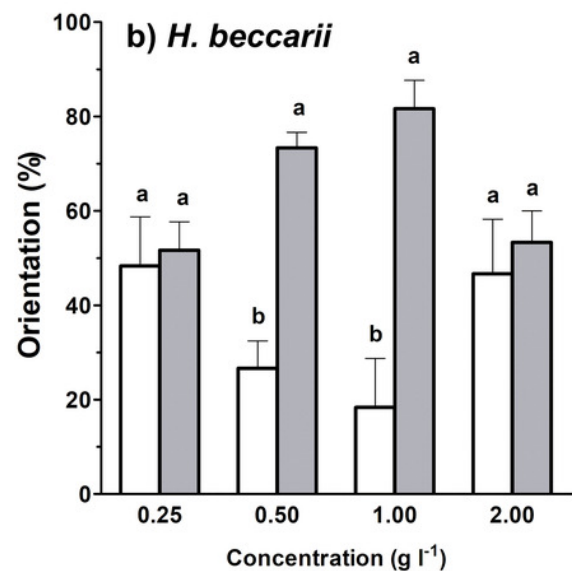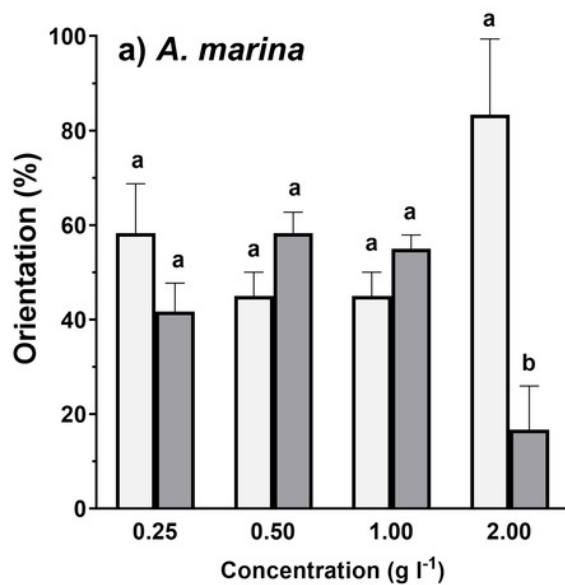
Figure 3

Movement behaviors (mean ± standard deviation) of juvenile *T. tridentatus* exposed to varying habitat chemical cues.

The data were tested by two-way ANOVA, followed by multiple post hoc Tukey tests with Bonferroni adjustments. Different lowercase letters represent statistical differences ($p < 0.05$) among vegetation sources at the same concentration, whereas different capital letters indicate significant differences among concentrations of individual source (a). Since only source and concentration were noted to affect juvenile movement time (b) and the largest distance (c), respectively, the statistical differences among treatment groups are indicated by different lowercase letters.