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Authors

Tsuboko-Ishii, Satomi
Burton, Ronald S

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7 **Authors**

8 Satomi Tsuboko-Ishii¹, Ronald S. Burton¹

9

10 **Affiliations**

11 ¹Marine Biology Research Division, Scripps Institution of Oceanography, University of
12 California, San Diego, La Jolla, California, United States of America

13

14 **Corresponding author**

15 Satomi Tsuboko-Ishii

16 E-mail: tsuboko.medaka@gmail.com; stsuboko@alumni.u-tokyo.ac.jp

17 Tel: +1 (858)-534-7827; +81 (50)-5539-7627

18

19 **Author contributions**

20 STI: conceptualization, methodology, experimental design and execution of the behavioral
21 observation, data curation, data analysis, data interpretation, visualization, and writing.

22 RSB: conceptualization, methodology, experimental design and execution of the spermatophore
23 examination, data curation, data interpretation, and writing.

24

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32

33 **Data Accessibility Statement:** The original data is available at Dryad:

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35 **Conflict of Interest Statement:** There is no conflict of interest to declare.

36 **Title**

37 Prezygotic reproductive barriers in precopulatory behavior of tidepool copepod species

38 **Abstract**

39 Complexity in prezygotic mating behavior can contribute to the emergence of sexual
40 incompatibility and reproductive isolation. In this study, we performed behavioral tests with two
41 tidepool copepod species of the genus *Tigriopus* to explore the possibility of precopulatory
42 behavioral isolation. We found that interspecific mating attempts failed prior to genital contact,
43 and that this failure occurred at different behavioral steps between reciprocal pairings. Our
44 results suggest that prezygotic barriers may exist at multiple points of the behavioral process on
45 both male and female sides, possibly due to interspecific differences in mate-recognition cues
46 used at those “checkpoints”. While many copepod species are known to show unique
47 precopulatory mate-guarding behavior, the potential contribution of prezygotic behavioral factors
48 to their isolation is not widely recognized. The pattern of sequential mate-guarding behaviors
49 may have allowed diversification of precopulatory communication and contributed to the
50 evolutionary diversity of the *Tigriopus* copepods.

51 **Keywords**

52 Prezygotic isolation, precopulatory behavior, speciation, mate-guarding behavior, Copepoda,
53 *Tigriopus*

54 **Main text**

55 **Introduction**

56 Speciation is an evolutionary process resulting in reproductive isolation between populations.
57 Mechanisms underlying reproductive isolation are often composite, consisting of both prezygotic
58 and postzygotic factors. While postzygotic isolation has been a major concern in genetic and
59 molecular studies (Orr 2005), prezygotic isolation, including that by geographical, mechanical,
60 and behavioral barriers, is also considered to play a significant role in speciation, as it often
61 arises on a similar or even shorter time scale compared to postzygotic isolation (Mayr 1966;
62 Coyne and Orr 1997; Orr 2005; Lowry et al. 2008; McNabney 2012). Complexity in
63 precopulatory process can increase the probability of sexual incompatibility emergence, thereby
64 facilitating reproductive isolation between populations (Edward et al. 2015; Langerhans et al.
65 2016). Studies with a wide range of animals, including mollusks, insects and teleosts, have
66 demonstrated cases where variations in courtship patterns are possibly involved in reproductive
67 isolation between species as well as conspecific populations (Ehrman 1964; Kupfermagel and
68 Baur 2011; Dean et al. 2021; Sumarto et al. 2021; Tanaka et al. 2022).

69
70 Copepods (class: Maxillopoda; *subclass: Copepoda*) are a group of small aquatic crustaceans
71 with a high abundance and evolutionary diversity (Razouls et al. 2005; Bron et al. 2011; Walter
72 2019). Among them, copepods of the genus *Tigriopus*, often abundant in splash pools, exhibit a
73 wide geographic distribution across continents and have evolved adaptations to local
74 environments (Pereira et al. 2016; Pereira et al. 2017; Barreto et al. 2018). Inhabiting intertidal or
75 supratidal rock pools, local populations of *Tigriopus* often show high levels of genetic

76 divergence even when separated by geographical barriers of only a few hundreds of meters
77 (Burton 1997). Mating experiments in laboratory environments have demonstrated sterility or
78 low viability of hybrids between some populations from the Pacific coast of the North America
79 in *Tigriopus californicus* (Ganz and Burton 1995; Peterson et al. 2013a), and complete
80 reproductive isolation has been observed between two closely related species, *T. californicus* and
81 *T. japonicus* (Ito 1988), inhabiting the east and west coast of the Pacific Ocean, respectively.
82 Recent studies suggested involvement of incompatibilities between mitochondrial and nuclear
83 factors in inviability of some hybrids between conspecific populations (Foley et al. 2013;
84 Peterson et al. 2013; Barreto et al. 2018; Lima et al. 2019). However, there is no direct evidence
85 that such postzygotic barriers play a significant role in explaining the origin or maintenance of
86 reproductive isolation observed between *Tigriopus* species (Burton 2022).

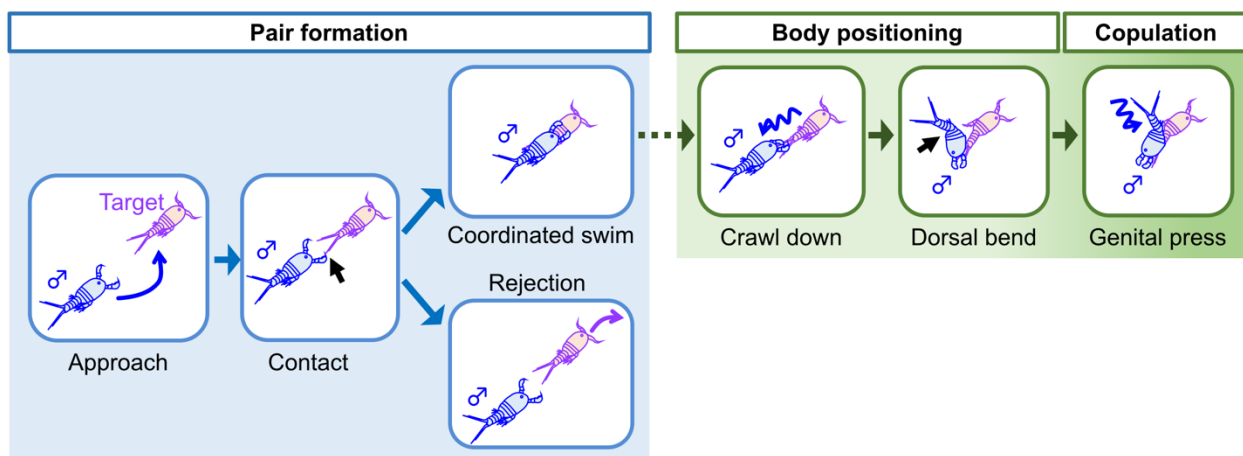
87
88 To determine the potential role of prezygotic mechanisms in the reproductive isolation of
89 *Tigriopus* species, we focused on their mating behavior. In many copepod species, males exhibit
90 precopulatory mate-guarding behavior, in which they secure a potential mate prior to copulation
91 by utilizing elongated and/or geniculate antennules, antennae, or maxillipeds (Boxshall 1990;
92 Bagøien and Kiørboe 2005; Goetze and Kiørboe 2008; Wasserman and Vink 2014). In
93 *Tigriopus*, males clasp a guarding target and swim as a pair prior to copulation (Figure 1) (Fraser
94 1936; Ito 1970; Burton 1985; Lazzaretto et al. 1994; Snell 2010; Alexander et al. 2014; Tsuboko-
95 Ishii and Burton 2017, 2018). In the formation of a mate-guarding pair, an adult male approaches
96 a guarding target (juvenile or adult). The male contacts the target with the geniculate first
97 antennae and then clasps the target on its prosome (beneath the caudal rim of cephalosome) to
98 initiate coordinated swimming. Males continue to clasp the target's prosome until the target

99 reaches the adult stage. Males then crawl down the mate's body toward its caudal end and
100 undertake a dorsal body bend to take a copulatory position. The whole process proceeds in a
101 sequential matter (Kelly et al. 1998; Tsuboko-Ishii and Burton 2017, 2018).

102

103 Based on nuclear and mitochondrial DNA sequences, *T. californicus* and *T. japonicus* are more
104 closely related to each other compared to other *Tigriopus* species (Ki et al. 2008; Park et al.
105 2014; Barreto et al. 2018). While interspecific pair formation was observed between these two
106 species in previous studies, the further progress of the process (which only resulted in infertile
107 eggs) was not investigated (Ito 1988; Kelly et al. 1998). In this study, we performed quantitative
108 evaluation of precopulatory behavior to examine its involvement in the infertility between the
109 two closely related species and possible mechanisms underlying the reproductive isolation in the
110 genus *Tigriopus*.

111



112

113 **Figure 1 Mating process of *Tigriopus* copepods.** Illustrations of behavioral steps in mate-
114 guarding pair formation and copulation of *Tigriopus*.

115 [Materials and Methods](#)

116 [Animals](#)

117 **Populations and collection.** We used stocks available from permitted collection sites as well as
118 from our collaborator. Original culture stocks were collected from high intertidal rock pools in
119 San Diego, California, USA (SD: 32° 45' N, 117° 15'W), La Bufadora, Mexico (BUF: 31° 43' N,
120 116° 43'W), and San Roque, Mexico (SRQ: 27° 11' N, 114° 23'W) for *T. californicus*, and in
121 Stanley, Hong Kong (HK: 22° 13' N, 114° 13' E) for *T. japonicus*. The *T. japonicus* stocks were
122 kindly gifted to us by Dr. Suzanne Edmands (University of Southern California, Los Angeles,
123 CA). Unless specified otherwise, individuals of the SD population were used as *T. californicus*
124 samples. *T. californicus* is genetically diverse along the Pacific coast of North America,
125 especially in southern areas from Baja California, Mexico to California, USA (Edmands 2001).
126 We chose SRQ and BUF populations as geographically close and genetically distant groups to
127 SD population to explore if there is prezygotic isolation between local populations of the same
128 species. For *T. japonicus*, populations around South China Sea are reported to be genetically
129 most distant from *T. californicus*. We chose a population from this area (HK) expecting to
130 observe a distinct behavioral incompatibility with *T. californicus*.

131 **Rearing.** Animals were reared in artificial seawater (salinity: 35 ppt) and fed with finely ground
132 fish food by following a previously established method (Tsuboko-Ishii and Burton 2018).
133 Culture beakers and multi-well plates were maintained in an incubator at 20 °C with a 12-hour
134 light-dark cycle. The animals were obtained from gravid adult females randomly selected from
135 the culture stocks and kept in the mass culture of siblings during nauplius stages. They were
136 collected as soon as they developed to copepodid stage I, approximately 6 days after hatching.
137 Each collected juvenile was individually maintained in a well on a 24-well cell culture plate until

138 it developed to an adult and kept virgin until the tests. Animals were subjected to the tests about
139 one week after the final molt. Staging and sex identification of animals were executed under a
140 stereoscopic microscope (Stemi SV 6, Carl Zeiss Microscopy, Switzerland) following previously
141 established methods (Tsuboko-Ishii and Burton 2018).

142

143 Behavioral tests

144 **Test condition.** All the behavioral tests were performed during the light period of the light-dark
145 cycle following previously established methods (Tsuboko-Ishii and Burton 2018). Animals were
146 fed with their normal diet at least one hour before a behavioral test and allowed to eat it for 30
147 minutes. Each animal was then collected from a rearing well and rinsed by gently pipetting with
148 a glass Pasteur pipet in a succession of four wells of a 24-well cell culture plate filled with clean
149 artificial seawater (approximately 2 mL in each well) to prevent carry-over of debris and exuviae
150 from culture wells. This rinsing process was previously shown to increase frequency of
151 approaches by males with no significant effects on velocity of clasped targets and on duration of
152 pairing (Tsuboko-Ishii and Burton 2018). After that, each animal was transferred to a well on a
153 48-well cell culture plate containing 400 μ L of artificial seawater and kept in the well for 30
154 minutes for adjustment. Each target was transferred to a well of a capturer (adult) male and
155 exposed to the capturer for 15 minutes (Figure 2A).

156 **Video recording.** A digital camera (EOS REBEL T6i, Canon, Japan) mounted above a test plate
157 was used for recording and observation of behavior. The video recording was started after the
158 30-minute adjustment period and performed at 30 frames per second. The recording was stopped
159 after the 15-minute observation period.

160 **Examination of spermatophores.** To investigate whether copulation was executed in
161 interspecific pairs, transfer of spermatophores was examined after males and females were
162 allowed to stay in a pair for an extended period. Each pair was kept in a well on a 24-well cell
163 culture plate for 2.5 hours to enable observation of transferred spermatophores before their
164 detachment from female genitalia. After the 2.5-hour period, each female was placed on a slide
165 glass with the artificial seawater and covered with a cover glass. The females were examined for
166 the presence of spermatophores on their genitalia at a lateral view (Figure 2C) under a
167 stereoscopic microscope at a 100x magnification, which was achieved with an additional screw-
168 on 2x objective lens, or at 200x under a compound microscope.

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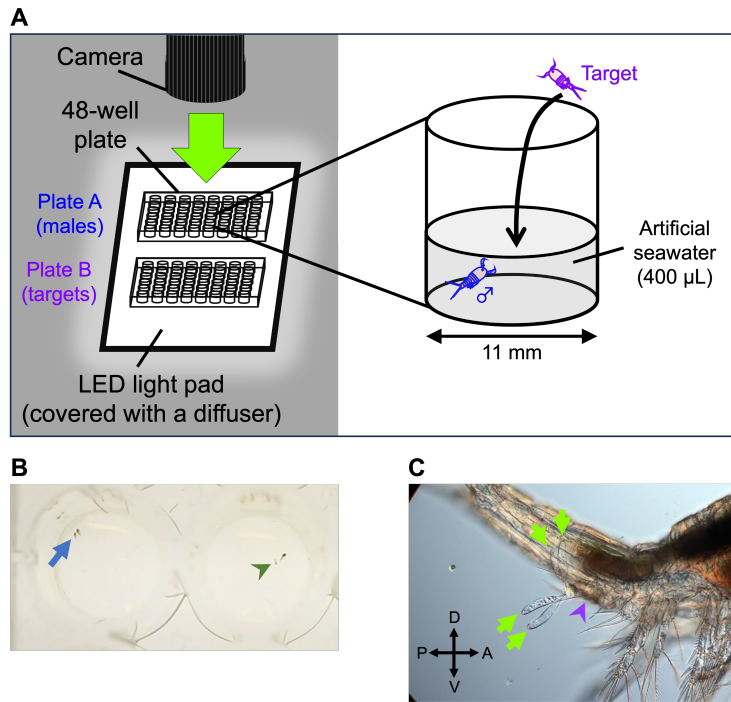
170 Behavioral analysis

171 **Characterization of behavioral steps.** Initiation and termination timing of the following events
172 were examined by manual observation of recorded videos according to descriptions and visual
173 materials provided in previous studies (Snell 2010; Tsuboko-Ishii and Burton 2018). (1) Contact
174 (initiation of pairing): An antenna of a male contacts any body part of a target following a swift
175 (<0.5 s) chase or pounce, which is shown as “Approach” in Figure 1. (2) Crawl down: A male
176 crawls down with its antennae to the caudal end of a target’s body. (3) Dorsal bend: A male
177 dorsally bends its body while holding the body of a target. (4) Genital press: A male repetitively
178 presses its urosome against that of a target. Frequency of the press is several times per second.
179 (5) Termination of pairing: Both antennae of the male detach from a body of a target.

180 **Tracking and velocity.** Two-dimensional tracking of newly formed pairs was performed on
181 recorded videos with ImageJ (Version 1.52a) (Schneider et al. 2012) and its motion-tracking
182 plugin MTrackJ (Meijering et al. 2012) by following a previously established method (Tsuboko-

183 Ishii and Burton 2018). Trajectories in the first 3 s of a guarding attempt were tracked to measure
184 mean velocity.

185 **Analysis.** All statistical analyses were performed with Prism 9 for Mac OS X (Version 9.5.1)
186 except for F tests that were performed with Microsoft® Excel for Mac (Version 16.43). Data
187 were tested for normal distribution with a D'Agostino-Pearson omnibus normality test and for
188 equal variances with F test. When no normal distribution could be assumed, Mann-Whitney U
189 test was used to compare two groups. When both normal distribution and equal variance were
190 assumed (*i.e.*, data showed in Figures 5A and 6A), Student's t-test was also used to compare the
191 groups and to confirm consistency of the results with those obtained by Mann-Whitney U test.
192 Pairs that showed no guarding attempt were excluded from the analyses of pairing duration.
193 Attempts made after copulation were excluded from the analyses of velocity and pairing duration
194 to avoid influence from postcopulatory behavioral change of females (Tsuboko-Ishii and Burton
195 2017).



196

197 **Figure 2 Behavioral procedure.** (A) Setup for behavioral tests and video recording of
198 prezygotic behavioral process. This figure has been modified from a previous study (Tsuboko-
199 Ishii and Burton 2018). (B) Sample image from a recorded video. The pair in the left well is
200 exhibiting coordinated swim (marked with a blue arrow; also see Figure 1). The male in the right
201 well is transitioning from crawl down to dorsal bend for precopulatory body positioning (marked
202 with a dark green arrowhead; also see Figure 1). (C) Spermatophores (marked with green
203 arrows) attached to a female genital (marked with a purple arrowhead). D: dorsal side. V: ventral
204 side. A: anterior side. P: posterior side.

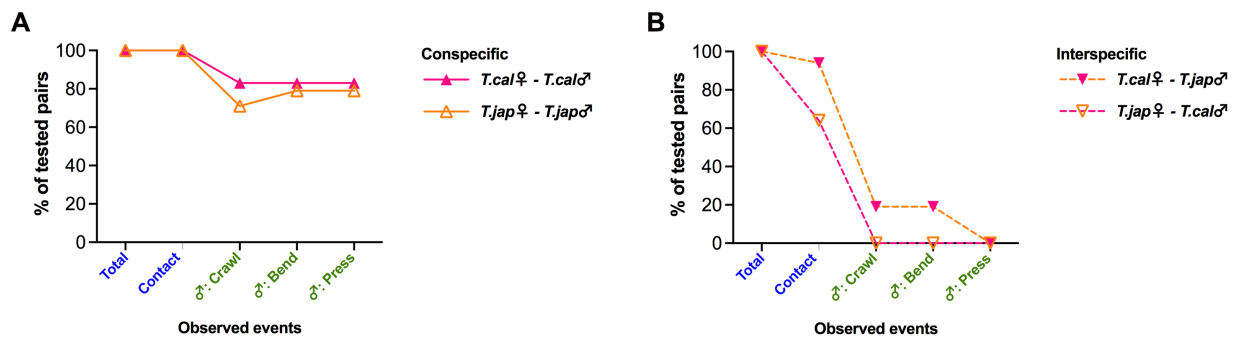
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206 Results

207 Interspecific pairs showed a lower copulatory rate compared to conspecific pairs

208 To explore if there are prezygotic reproductive barriers, we tracked progress of the precopulatory
209 behavioral process and assessed copulatory rates of conspecific and interspecific pairs with *T.*
210 *californicus* and *T. japonicus* individuals. While the majority of the conspecific pairs
211 successfully completed the whole precopulatory process and experienced the normal genital
212 press by a male (Figure 3A; 83% in *T. californicus* and 79% in *T. japonicus*), none of the
213 interspecific pairs reached that step within the 15-minute testing window (Figure 3B).
214 Consistently, in examinations with an extended 2.5-hour observation window, females in the
215 approximately 50% of conspecific pairs were confirmed to have received spermatophores from
216 males (Table S1; 47% of *T. californicus* pairs and 50% of *T. japonicus* pairs), while less than 5%
217 of females in interspecific pairs had spermatophores attached on their genitalia (Table S1). These
218 results suggest the existence of some barriers that hinder the prezygotic behavioral process
219 across these species.

220



221

222 **Figure 3 Progress of prezygotic behavioral process in conspecific and interspecific pairs.**

223 Percentage of tested pairs that initiated each step in the precopulatory behavioral process. (A)

224 Conspecific pairs. *T. californicus* female and *T. californicus* male (n = 12); *T. japonicus* female
225 and *T. japonicus* male (n = 14). (B) Interspecific pairs. *T. californicus* female and *T. japonicus*
226 male (n = 16); *T. japonicus* female and *T. californicus* male (n = 14).

227

Pairing type	Female	Male	Number of females with spermatophores attached to genitalia	Number of tested pairs
Conspecific	<i>T. californicus</i> SD	<i>T. californicus</i> SD	9	19
Conspecific	<i>T. japonicus</i> HK	<i>T. japonicus</i> HK	12	24
Interspecific	<i>T. californicus</i> SD	<i>T. japonicus</i> HK	1	23
Interspecific	<i>T. japonicus</i> HK	<i>T. californicus</i> SD	1	28
Conspecific	<i>T. californicus</i> SRQ	<i>T. californicus</i> SRQ	4	10
Conspecific	<i>T. californicus</i> SRQ	<i>T. californicus</i> SD	0	20
Conspecific	<i>T. californicus</i> SD	<i>T. californicus</i> SRQ	2	14

228 **Table S1 Transfer of spermatophores in conspecific and interspecific pairs.**

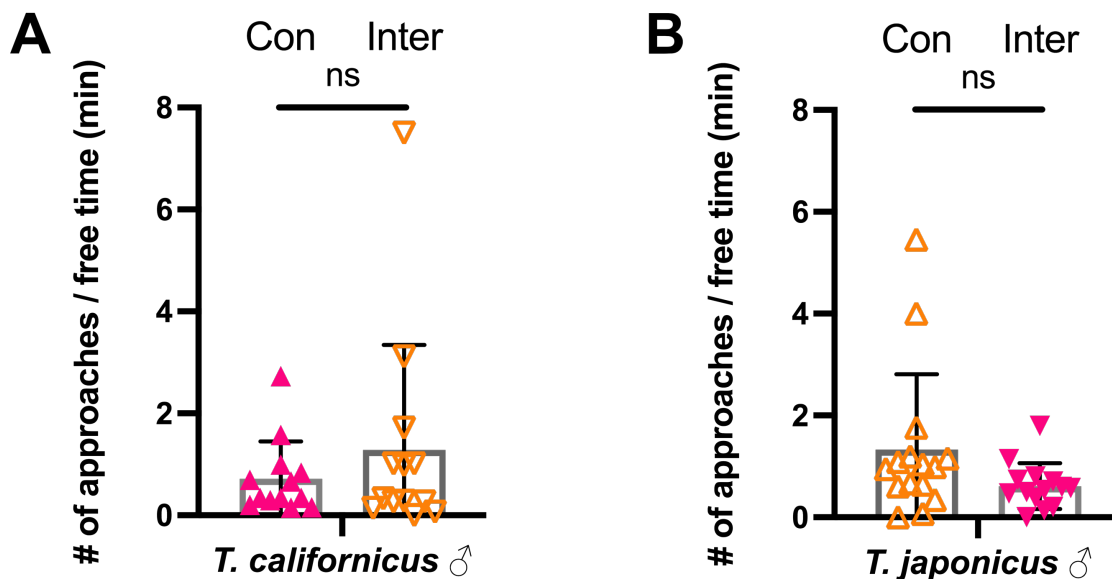
229 Transfer of spermatophores was examined after males and females were allowed to stay in a pair
230 in wells on a 24-well culture plate for an extended 2.5-hour observation window.

231

232 Males of both species did not avoid interspecific pairing

233 To investigate if a possible barrier exists in the initiation of the mate-guarding behavior by
234 males, we presented either a conspecific or heterospecific adult female to individual males and
235 calculated frequency of approaches by males to examine whether males actively avoid
236 interspecific pairing. In both *T. japonicus* and *T. californicus* males, no significant difference
237 was detected in the frequency of approaches depending on species of a target (Figure 4; $p = 0.96$
238 for *T. californicus* males, $p = 0.071$ for *T. japonicus* males, Mann-Whitney U test). These results
239 suggest that males are attracted to both conspecific and heterospecific females at the initiation
240 step of a mate-guarding attempt.

241



242

243 **Figure 4** Difference in frequency of approaches by males between conspecific and
244 **interspecific pairs.** Each triangle symbol represents data from one tested pair. One approach

245 (shown as “Approach” in Figure 1) is defined as a swift (<0.5 s) chase or pounce by a male.
246 Filled symbols represent frequencies against *T. californicus* females and open symbols represent
247 frequencies against *T. japonicus* females. Bars and whiskers represent medians and interquartile
248 range (IQR) respectively. “Con”: conspecific pairs. “Inter”: interspecific pairs. (A) *T.*
249 *californicus* SD male to *T. californicus* SD female ($n = 13$); *T. californicus* SD male to *T.*
250 *japonicus* HK female ($n = 14$). No significant difference was detected by Mann-Whitney U test
251 ($p = 0.96$). Consistently, no significant difference was detected by Mann-Whitney U test with an
252 outlier datapoint omitted from the conspecific pairs group ($p = 0.69$). (B) *T. japonicus* HK male
253 to *T. japonicus* HK female ($n = 15$); *T. japonicus* HK male to *T. californicus* SD female ($n =$
254 14). No significant difference was detected by Mann-Whitney U test ($p = 0.071$).

255

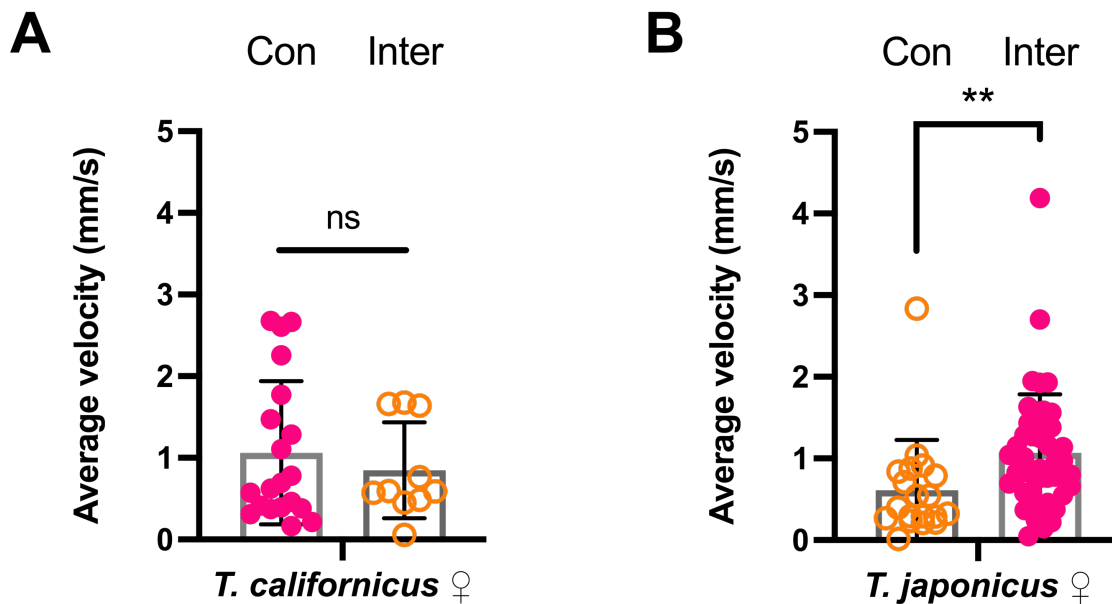
256 *Females of T. japonicus showed rejective response toward a T. californicus male*

257 A qualitative study on reproductive behavior of *T. californicus* and *T. japonicus* reported that
258 interspecific pairing between the two species last for shorter times than conspecific pairing, often
259 letting females escape from a capturer male (Ito 1988). Recent studies with conspecific
260 individuals suggested that reproductively unreceptive *Tigriopus* individuals (*e.g.*, already mated
261 females) exhibit rejective response toward a capturer male, represented by vigorous movement
262 with increased swimming velocity, leading to avoidance of reproductively unbeneficial pairing
263 between conspecifics (Tsuboko-Ishii and Burton 2017, 2018).

264 To examine if females exhibit such rejective responses against interspecific guarding attempts,
265 we presented either a conspecific or heterospecific adult male to individual females (adult

266 virgins) and calculated their average velocity in the first three seconds of capture. *T. japonicus*
267 females captured by a *T. californicus* male showed significantly higher velocity compared to
268 those captured by a conspecific male (Figure 5B; $p = 0.006$, Mann-Whitney U test) and were
269 released in a shorter time from an interspecific pair (Figure 6B; $p = 0.0004$, Mann-Whitney U
270 test). *T. japonicus* female escaped from interspecific pairing with a *T. californicus* male in less
271 than one minute on average (mean = 43.6, median = 10; $n = 14$), while those in a conspecific pair
272 spent more than seven minutes on average in a pair (mean = 472.1, median = 374.8; $n = 14$). On
273 the other hand, *T. californicus* females showed no significant difference in velocity (Figure 5A; p
274 = 0.95, Mann-Whitney U test; $p = 0.50$, unpaired t-test) and the average duration of pairing
275 (Figure 6A; $p = 0.47$, Mann-Whitney U test; $p = 0.42$, unpaired t-test) depending on the species
276 of a capturer male. *T. californicus* females stayed for more than three minutes on average both in
277 pairs with a conspecific male (median = 98; $n = 13$) and those with a *T. japonicus* male (median
278 = 222.7; $n = 13$). These results suggest that although active rejection by *T. japonicus* females is
279 involved in the interruption of interspecific pairing with *T. californicus* males, such rejective
280 behavior was not evident in *T. californicus* females captured by *T. japonicus* males.

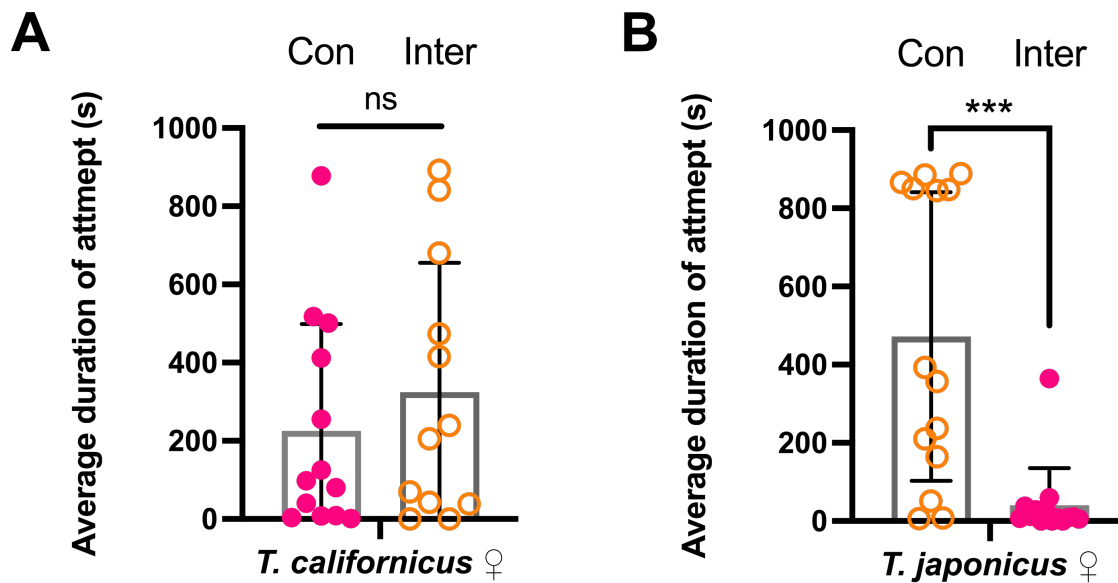
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282

283 **Figure 5** Difference in mean velocity of captured females between conspecific and
284 **interspecific pairs.** Each circle symbol represents data from one observed attempt. Filled
285 symbols represent velocities against *T. californicus* males and open symbols represent velocities
286 against *T. japonicus* males. Bars and whiskers represent medians and IQR respectively. “Con”:
287 conspecific pairs. “Inter”: interspecific pairs. (A) *T. californicus* SD female against *T.*
288 *californicus* SD male (n = 20); *T. californicus* SD female against *T. japonicus* HK male (n = 10).
289 No significant difference was detected by Mann-Whitney U test (p = 0.95) and unpaired t-test (p
290 = 0.50). (B) *T. japonicus* HK female to *T. japonicus* HK male (n = 19); *T. japonicus* HK female
291 against *T. californicus* SD male (n = 47). **p<0.01 by Mann-Whitney U test.

292



293

294 **Figure 6** Difference in pairing duration between conspecific and interspecific pairs. Each
295 circle symbol represents data from one tested pair. Filled symbols represent duration of pairs
296 with *T. californicus* males and open symbols represent duration of pairs with *T. japonicus* males.
297 Bars and whiskers represent medians and interquartile range (IQR) respectively. “Con”:
298 conspecific pairs. “Inter”: interspecific pairs. (A) *T. californicus* SD female against *T.*
299 *californicus* SD male (n = 13); *T. californicus* SD female against *T. japonicus* HK male (n = 13).
300 No significant difference was detected by Mann-Whitney U test (p = 0.47) and unpaired t-test (p
301 = 0.42). (B) *T. japonicus* HK female against *T. californicus* SD male (n = 14); *T. japonicus* HK
302 female to *T. japonicus* HK male (n = 14). ***p<0.001 by Mann-Whitney U test.

303

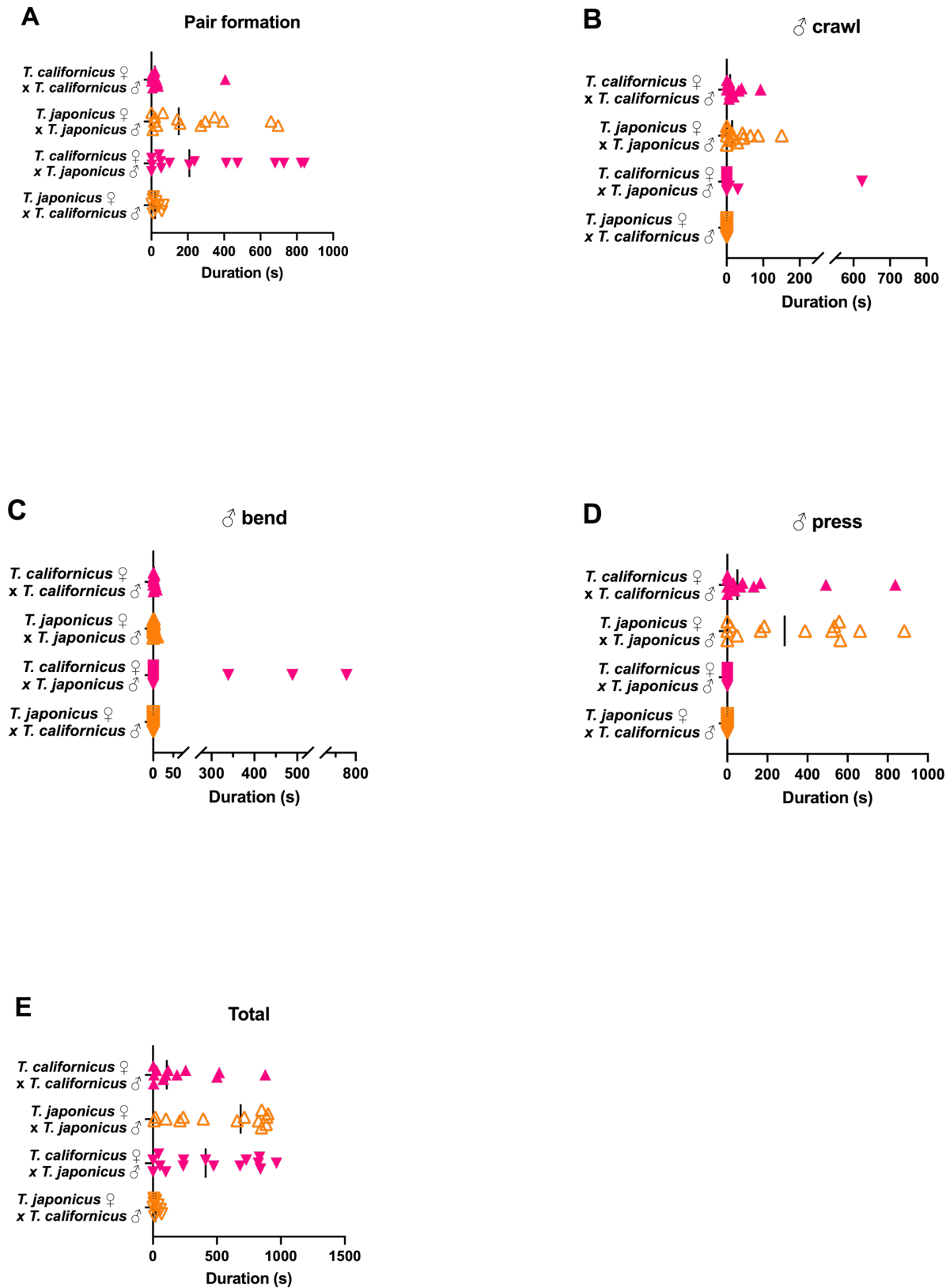
304 Prezygotic prevention of insemination was observed at different behavioral steps between
305 reciprocal pairings

306 Interspecific pairing between *T. californicus* females and *T. japonicus* males has been reported to
307 be infertile (Ito 1988). Nevertheless, *T. californicus* females did not exhibit active escape from *T.*
308 *japonicus* males (Figure 5A) and stayed in interspecific pairs for a comparable length of time to
309 conspecific pairs (Figure 6A). To investigate if there are any prezygotic barriers related to the
310 infertility, we tracked duration of each step in their precopulatory behavioral process and
311 compared it with that of conspecific pairs. In interspecific pairs between *T. californicus* females
312 and *T. japonicus* males, 81% of the males did not start to crawl down a female's body to take a
313 copulatory position (Figure 3B). Although the rest of the *T. japonicus* males did start crawling
314 down and take a copulatory position by bending their body, they did not proceed to genital press
315 to execute interspecific copulation (Figure 3B). Even without actual copulation, their pairing was
316 maintained for a comparable length of time to the conspecific pairs (Figure 7E), consistently
317 with the previous result (Figure 6A).

318 In contrast, interspecific pairing between a *T. japonicus* female and a *T. californicus* male was
319 interrupted in less than 70 seconds in most of the tested pairs (Figures 6B and 7E) and no *T.*
320 *californicus* male crawled down a body of a *T. japonicus* female before the termination of the
321 interspecific pairing (Figure 3B).

322 These results suggest that extension and/or prevention of behavioral steps in the precopulatory
323 process contribute to prezygotic isolation between the two species.

324



326 **Figure 7 Duration of each step of prezygotic behavioral process in conspecific and**
327 **interspecific pairs.** Symbols represent average duration of each behavioral step per tested pair.
328 Bars represent medians. Pair formation: time spent from body contact to either initiation of crawl
329 down or termination of pairing, whichever the earlier. *T. californicus* SD female and *T.*
330 *californicus* SD male (n = 12); *T. japonicus* HK female and *T. japonicus* HK male (n = 14); *T.*
331 *californicus* SD female and *T. japonicus* HK male (n = 16); *T. japonicus* HK female and *T.*
332 *californicus* SD male (n = 14).

333

334 Discussion

335 Prezygotic isolation between *T. californicus* and *T. japonicus*

336 The present results suggest that reproductive barriers exist in the precopulatory behavior between
337 *T. californicus* and *T. japonicus*. A previous study reported that pair formation between these
338 two sister species does occur, but whether or not copulation actually occurred was not
339 determined (Ito 1988). With video recording and quantitative analysis of behavior, this study
340 illustrates the sequential behavioral process executed in their mating attempts (from approach to
341 copulation) and demonstrates the steps at which interspecific mating attempts are interrupted.

342 In previous studies, postzygotic systems were suggested as major mechanisms behind
343 reproductive isolation within and between species in *Tigriopus*. Differences in chromosome
344 structures have been discussed since the early 1960's (Ar-rushdi 1962), and more recently,
345 incompatibilities between mitochondrial and nuclear genes have been suggested to decrease
346 hybrid viability and sterility, and thus to contribute to speciation (Barreto et al. 2018). This study

347 suggests that reproductive isolation results from more direct interspecific barriers involving
348 behavioral mechanisms that operate at early points in the mating process.

349

350 Possible “checkpoints” in the precopulatory process

351 While reproductive attempts by males were unsuccessful in both reciprocal pairings, the steps at
352 which interruption occurred were different between the pairings. Prezygotic barriers may exist at
353 multiple points in the precopulatory process, probably due to differences in mate-recognition
354 cues used at those “checkpoints” by the tested species (Figure 8).

355 Previous studies suggest that not only behavioral responses by a captured target (Tsuboko-Ishii
356 and Burton 2017, 2018) but also distant cues (*e.g.*, chemical substances and water distortions)
357 from potential partners (Lazzaretto 1990; Kelly et al. 1998; Tsuboko-Ishii and Burton 2017) play
358 roles in reproductively successful pair formation for males in *Tigriopus* (Figure 8 “Approach”).
359 The present and some previous studies have demonstrated low species specificity of such distant
360 cues used by males; *Tigriopus* males could be attracted to and would make capturing attempts
361 toward heterospecific females within and even across genera (Figure 4) (Goetze and Kiørboe
362 2008; Tsuboko-Ishii and Burton 2017). The low species specificity is reasonable given the
363 scarcity of other zooplankton species in their habitats. Meanwhile, some studies reported that *T.*
364 *californicus*, *T. japonicus*, or *T. brevicornis* males were more attracted to individuals or
365 diffusible cues of the same species over those of another species when given a choice, although
366 the preference was not exclusive (Lazzaretto 1990; Lazzaretto et al. 1994; Kelly and Snell 1998;

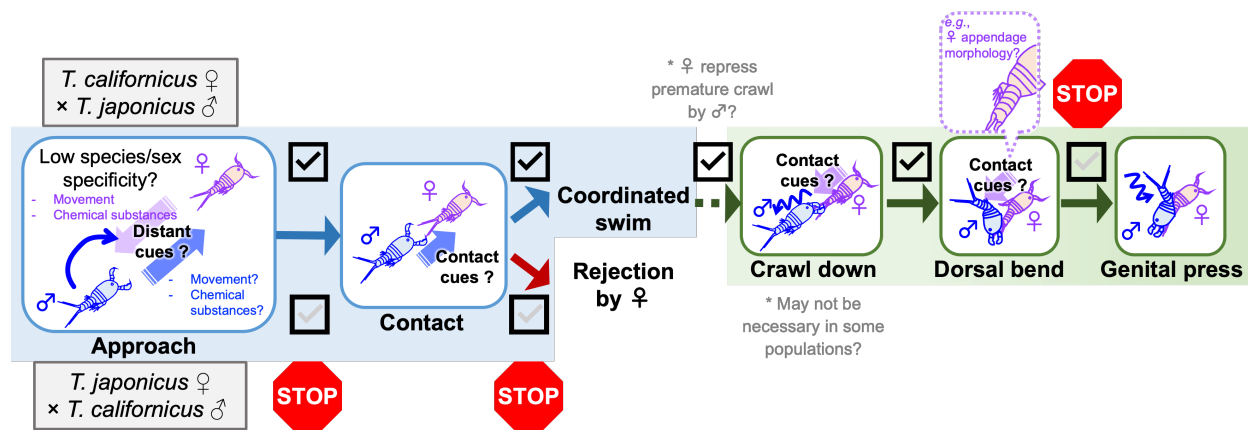
367 Kelly et al. 1998). Diversification of chemical cues could possibly reinforce the prezygotic
368 isolation between *Tigriopus* species.

369 Regarding mate recognition by females, the present results suggest that mate recognition and
370 rejective response by *T. japonicus* females contributes to prevention of interspecific mating with
371 *T. californicus* males (Figures 5 and 6). Considering their mating system in which females mate
372 only once in their lifetime and produce up to several hundred offspring (Burton 1985), an ability
373 of females to recognize and evaluate a potential partner may have evolved to increase their
374 fitness. Pairs between *T. japonicus* females and *T. californicus* males showed a lower contact
375 rate (Figure 3B), possibly because the females escaped from approaching heterospecific males
376 by recognizing distant cues (Figure 8 “Approach”). In addition, *T. japonicus* females showed
377 higher velocity when captured (Figure 5B), suggesting they also use contact cues (Figure 8
378 “Contact”). In later steps of pairing, contact cues are possibly used in mate recognition and
379 involved in the prevention of interspecific copulation between *T. californicus* females and *T.*
380 *japonicus* males (Figure 3B), either on female’s or male’s side.

381 Although no noticeable difference is known in morphology of male first antennae (Ito 1988),
382 physical differences, especially in females, may mechanically contribute to the early termination
383 of interspecific pairing (Figure 6B; between *T. japonicus* females and *T. californicus* males)
384 and/or the suspension of copulatory body positioning (Figure 3B; between *T. californicus*
385 females and *T. japonicus* males). For example, morphologies of fifth thoracic appendages are
386 reported to be different between *T. japonicus* and *T. californicus* females, with those of *T.*
387 *californicus* females shorter and possessing more distinct spines on a bristle (Ito 1988). Since
388 these legs are positioned proximately to the urosome, their morphological difference may hinder

389 *T. japonicus* males from suitable body positioning for genital contact with heterospecific females
 390 (Figure 8 “Dorsal bend”).

391



392

393 **Figure 8 Possible mate recognition cues and precopulatory “checkpoints” between**

394 ***Tigriopus* species.** A schematic based on the present and previous studies (Tsuboko-Ishii and
 395 Burton 2017; Tsuboko-Ishii and Burton 2018; Lazzaretto 1990; Kelly et al., 1998; Goetze and
 396 Kiørboe 2008; Ito 1988).

397

398 Possible barriers between local populations within a species

399 Reproductive incompatibilities are reported even between conspecific local populations of *T.*
 400 *californicus* (Foley et al. 2013; Peterson et al. 2013; Barreto et al. 2018; Lima et al. 2019).

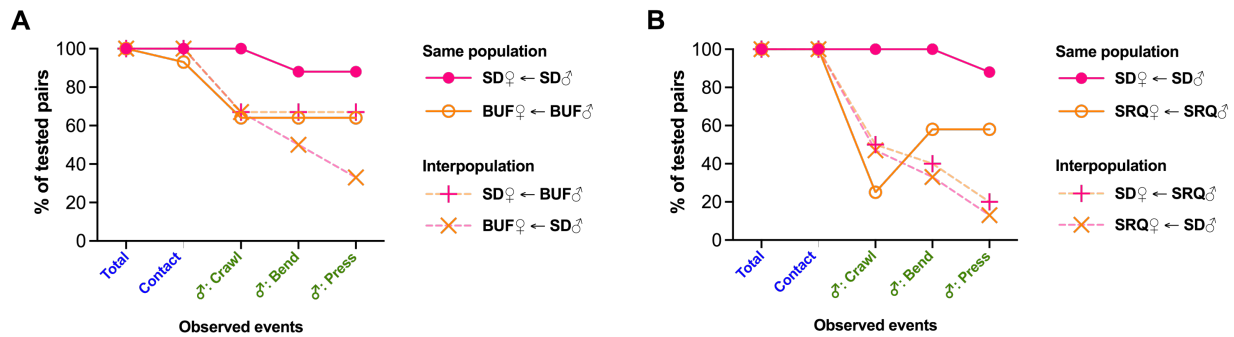
401 Behavioral tests with local populations showed relatively lower copulatory rates with some
 402 interpopulational pairs compared to same-population pairs (Figure S1). Among the

403 interpopulational pairs, those with the larger geographic and genetic distances of source

404 populations (between SD and SRQ) showed relatively lower copulatory rates compared to those
405 with the smaller geographic and genetic distances (between SD and BUF). This possibly reflect
406 development of intraspecific behavioral incompatibility with genetic drift along time. In
407 examinations of spermatophore transfer between SD and SRQ, SD females paired with SRQ
408 males received spermatophores in 2 of 14 pairings, while no SRQ females received
409 spermatophores in 20 pairings with SD males. In contrast, females in nearly half of
410 intrapopulation pairs (47% of SD pairs and 40% of SRQ pairs) were confirmed to have
411 received spermatophores (Table S1). These results suggest possible asymmetric precopulatory
412 barriers exist even between conspecific populations, though this was not apparent in the shorter-
413 time video observations.

414 In addition to that, BUF pairs and SRQ pairs respectively showed lower copulatory rates
415 compared to SD pairs, despite being paired with a partner of the same population (Figure S1).
416 These results may reflect interpopulational variations in properties of the precopulatory
417 behavioral process, which could lead to miscommunication between individuals of different
418 populations. In SRQ pairs, some capturer males clasped a target female on its caudal part and
419 directly proceeded to dorsal bend and genital press without crawling down the body of the target
420 (Figure S1B). This suggests that the clasp on a female's prosome is not a necessary condition for
421 the proceeding of the precopulatory behavioral process at least in SRQ pairs (Figure 8 "Crawl
422 down"). Again, this may reflect diversification of precopulatory "checkpoints", which possibly
423 lead to future reproductive isolation.

424



425

426 **Figure S1 Progress of prezygotic behavioral process in intrapopulation and**

427 **interpopulational pairs within a species.** Percentage of tested pairs that initiated each step in

428 the precopulatory behavioral process. Circle symbols represent percentage of intrapopulation

429 (*i.e.*, same population) pairs and cross symbols represent percentage of interpopulational pairs.

430 (A) *T. californicus* SD female and *T. californicus* SD male (n = 8); *T. californicus* BUF female

431 and *T. californicus* BUF male (n = 14); *T. californicus* SD female and *T. californicus* BUF male

432 (n = 6); *T. californicus* SRQ female and *T. californicus* SD male (n = 12). (B) *T. californicus* SD

433 female and *T. californicus* SD male (n = 13); *T. californicus* SRQ female and *T. californicus*

434 SRQ male (n = 12); *T. californicus* SD female and *T. californicus* SRQ male (n = 10); *T.*

435 *californicus* SRQ female and *T. californicus* SD male (n = 15).

436

437 **Emergence of female rejective response in development**

438 Adult males often clasp immature individuals (*i.e.*, juveniles of copepodid stages from CII to

439 CV) as pairing targets in both *T. californicus* and *T. japonicus*. A previous study on the

440 relationship between the two species reported that *T. californicus* CV copepodids (one stage

441 prior to the adult stage) were larger than *T. japonicus* adult males and capable of swinging off the
442 heterospecific males when captured (Ito 1988). In our present study, however, *T. californicus*
443 adult females did not exhibit higher velocity against *T. japonicus* males (Figure 5A) and stayed
444 in interspecific pairs for comparable times to conspecific pairs (Figure 6A). Considering these
445 results together, another prezygotic barrier between *T. californicus* females and *T. japonicus*
446 males may reside in the rejective response by premature females, in addition to the observed
447 suspension of the precopulatory body positioning by males on adult females (Figure 3B, Figure 8
448 top right).

449 The same previous study also reported that pairing between *T. japonicus* CV copepodids and *T.*
450 *californicus* adult males was maintained while resulting no offspring (Ito 1988). This suggests
451 that the rejective response by *T. japonicus* females (Figure 5B, Figure 8 bottom) expresses, or
452 becomes intense enough to resolve interspecific pairing, after sexual maturation.

453

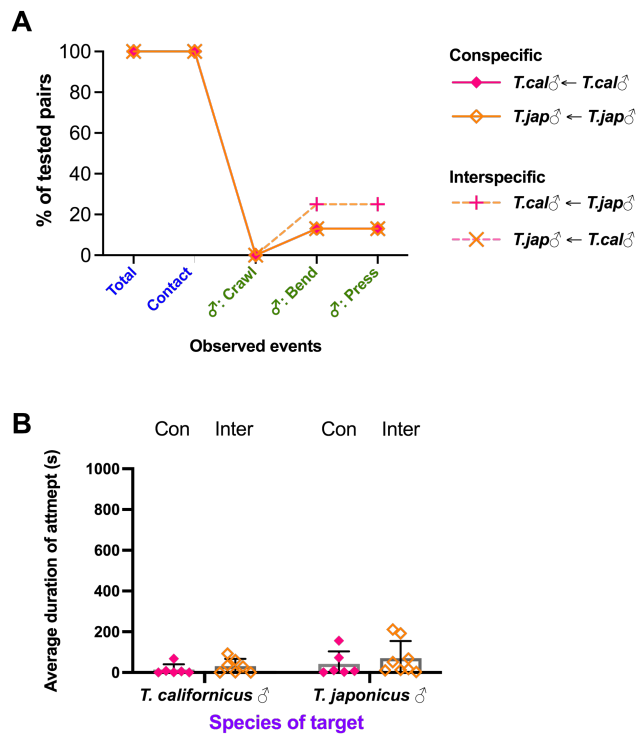
454 [Mate-guarding attempts between heterospecific males](#)

455 *Tigriopus* copepods are known to show high accuracy of male-female pairing in both natural and
456 laboratory environments (Lazzaretto 1990; Peterson et al. 2013; Tsuboko-Ishii and Burton 2017).
457 Elements in a system that contributes to the successful male-female pairing may partly overlap
458 with those involved in the interspecific prezygotic barriers. A previous study demonstrated that
459 *T. californicus* males increase velocity when captured by a conspecific male and actively reject
460 male-male pairing (Tsuboko-Ishii and Burton 2018), as *T. japonicus* females did against
461 heterospecific capturers in the present study (Figure 5B). Consistently, when we tested with *T.*
462 *californicus* and *T. japonicus* males, male targets escaped from both conspecific and interspecific

463 male-male pairing in a relatively shorter time (Figure S2B) compared to female targets in
464 conspecific male-female pairing (Figure 6). This early termination of pairing may help males
465 avoid completion of a fruitless copulating attempt.

466 However, the interspecific/interpopulational precopulatory checkpoints that we proposed above
467 do not fully function to prevent such male-male copulatory attempts. In the tests with males of *T.*
468 *californicus* and *T. japonicus*, we found some capturer males pressed their genitalia against a
469 prosome, not urosome, of a target male; in both conspecific and interspecific male-male pairs,
470 some capturer males exhibited dorsal bend and genital press without crawling down to the caudal
471 end of the target (Figure S2A). This latter observation suggests that the dorsal bend is not
472 autonomously triggered by the precedent crawl down behavior observed in male-female pairing,
473 raising a possibility that captured females send a signal to a capturer male to repress premature
474 crawl (Figure 8; between “Coordinated swim” and “Crawl down”).

475



476

477 **Figure S2 Progress of prezygotic behavioral process in male-male pairs.** *T. californicus* SD
 478 male to *T. californicus* SD male (n = 6); *T. japonicus* HK male to *T. japonicus* HK male (n = 6);
 479 *T. californicus* SD male to *T. japonicus* HK male (n = 8); *T. japonicus* HK male to *T.*

480 *californicus* SD male (n = 7). (A) Percentage of tested pairs that initiated each step in the
 481 precopulatory behavioral process. Filled symbols represent percentage of conspecific pairs and
 482 open symbols represent percentage of interspecific pairs. (B) Duration of pairing. Each symbol
 483 represents data from one tested pair. “Con”: conspecific pairs. “Inter”: interspecific pairs.

484

485 **Contribution of sequential precopulatory process to evolution of related species**

486 Genetic analyses have shown that local populations of *Tigriopus* can be sharply differentiated
 487 indicating substantial periods of geographic isolation (Burton 1997). While this isolation has
 488 permitted adaptation to habitats with varied environmental conditions (e.g., temperature, salinity,

489 and sympatric species) (Leong et al. 2017; Tangwancharoen et al. 2018; Harada et al. 2019), it
490 may also have resulted in the modified precopulatory communications among populations
491 (Figure S1), perhaps initiated by genetic drift. With the short generation time (about one month),
492 the generally high population density punctuated by irregular population bottlenecks, and long, if
493 not continuous, separation from the pelagic zone, populations in rock pools are presumed to be
494 prone to evolution by random events (Edmands 2001 overviews the elevated divergence rates in
495 *T. californicus* populations and the possible backgrounds). When an isolated population is
496 reconnected with nearby and/or distant populations by external factors (*e.g.*, dispersal by animal
497 or meteorological transmitters, geographic alternations, and sea level change), accumulated
498 genetic difference could cause a reproductive incompatibility. Although a sympatry of *T.*
499 *californicus* and *T. japonicus* is not directly observed, *Tigriopus* copepods are suggested to be
500 capable of and have experienced occasional long-distance transmarine transport events up to
501 several kilometers (Handschumacher et al. 2010). This poses a possibility of their interspecific
502 encounters in the past and future. Recent studies suggested genetic loci responsible for genetic
503 incompatibilities between *T. californicus* populations vary among populations (Lima et al. 2019;
504 Pereira et al. 2021). Further genetic analyses may reveal the genetic basis of the apparent
505 prezygotic behavioral barriers within and between *Tigriopus* species.

506 To this date, studies have demonstrated the absence of sex chromosomes in the two *Tigriopus*
507 species and the polygenic background of their sex determination (Takeda 1941; Ar-rushdi 1962;
508 Voordouw and Anholt 2002; Harrison and Edmands 2006; Foley et al. 2013; Alexander et al.
509 2015; Richardson et al. 2023). In addition to possible quantitative trait loci (QTL) distributed
510 among several chromosomes (Alexander et al. 2015), environmental factors including
511 temperature are also suggested to affect sex ratio of the *Tigriopus* copepods (Takeda 1941;

512 Voordouw and Anholt 2002). Such complexity in the expression of their sexual traits, together
513 with their adaptative and neutral evolution in isolated local environments, could have contributed
514 to polymorphisms in phenotypes that are used in their prezygotic communication.

515 Many planktonic and some benthic and parasitic copepod species exhibit precopulatory mate-
516 guarding behavior that consists of distinct phases and involves chemical and/or mechanical
517 communications (Uchima and Murano 1988; Boxshall 1990; Titelman et al. 2007). Distant and
518 contact cues are suggested to transmit information of species, sex, and/or reproductive maturity
519 of a potential partner among some species (Strickler and Bal 1973; Anstensrud 1992; Goetze
520 2008; Heuschele and Selander 2014). The extent to which these sequential phases of
521 precopulatory behavior provide checkpoints for the prevention of interspecific hybridization is
522 poorly understood. Here we have documented an asymmetry in the prezygotic isolating
523 mechanisms between two congeneric copepod species, *i.e.*, a difference in stages at which
524 interspecific mating is interrupted between the reciprocal crosses. This suggests that mate
525 recognition systems have differentiated between the congeners, involving random and/or
526 directional modifications of behavioral patterns, chemical signals, and morphologies.

527 Overall, the present study provides new insight into the evolution of prezygotic barriers between
528 *Tigriopus* species. Although not yet many, there are some studies that show examples of
529 asymmetric reproductive isolation in copepods and other zooplankters that exhibit composite
530 precopulatory interactions (Lee 2000; Chin et al. 2019). In the future, comprehensive studies
531 may reveal a relationship between complexity of prezygotic processes and asymmetric
532 development of reproductive isolation.

533

534 **Data Accessibility:** The original datasets for the figures and the table are available at Dryad
535 (<https://doi.org/10.5061/dryad.qz612jmm1>).

536

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