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journal or publication title	Zoological Studies
volume	59
page range	7
year	2020-02
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URL	http://id.nii.ac.jp/1342/00001943/

doi: 10.6620/ZS.2020.59-07.

1 Sexual Dimorphism and Reproductive Status of the Red Swamp Crayfish

2 *Procambarus clarkii*

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10 The red swamp crayfish *Procambarus clarkii* is native to northeastern Mexico and the south-
11 central USA, and it has expanded its distribution worldwide and negatively impacted the
12 ecosystems in the invaded regions. The dynamics of the *P. clarkii* populations have been
13 studied as the basis for the development of effective control measures against this invasive
14 alien species. Adult males of *P. clarkii* exhibit a cyclical dimorphism between two sexual
15 morphotypes; reproductive form I has large chelae and non-reproductive form II has small
16 chelae. However, whether *P. clarkii* females have two sexual morphotypes and exhibit form
17 alternation has not been resolved, and little is known about the degree of intra-sexual
18 dimorphism of the chelae even among males. We employed allometric growth analysis for the
19 chelae dimensions of *P. clarkii* females and males that were collected from a small pond in
20 Yokohama, Japan. Our analysis demonstrated the existence of form I, which has larger chelae,
21 and form II, which has smaller chelae, in *P. clarkii* females and highlighted the intra- and
22 inter-sexual dimorphisms in the chelae of this species. The reproductive cycle of the
23 population was successfully traced by the reproductive status of *P. clarkii* based on the
24 occurrence patterns of each sexual morphotype; the form I crayfish occurred throughout the
25 sampling period from April to December, while the occurrences of form I females and males
26 were highly correlated, peaking in October. Our results suggested that alternation of sexual
27 forms occurs in *P. clarkii* females. The ability to discriminate between the sexual
28 morphotypes based on chelae allometric growth would allow us to evaluate the female
29 reproductive status more easily and precisely in invasive *P. clarkii* populations.

30
31 **Key words:** Form alternation, Reproductive ecology, Allometric growth, Sexual maturity,
32 Invasive alien species.

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BACKGROUND

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Red swamp crayfish *Procambarus clarkii* (Girard 1852) (Decapoda; Cambaridae) is native to northeastern Mexico and south-central USA (Hobbs 1972); it inhabits various freshwater environments, including swamps and marshes that are periodically flooded and drained (Huner and Barr 1991). This species has been introduced into several states in the continental USA and into many other countries in Asia, Africa and Europe for aquaculture purposes (Hobbs et al. 1989; Loureiro et al. 2015), and now its aquaculture industry is growing in the USA, China and Spain (Souty-Grosset et al. 2016). *Procambarus clarkii* has also been introduced outside its native range as prey for aquaculture organisms such as bullfrogs (Sako 1987; Kawai and Kobayashi 2005).

Procambarus clarkii exhibits rapid growth rates, early maturation at a small body size, year-round egg production, and extended maternal care in which hatchlings and juveniles are attached to the mother's pleon (Suko 1953, 1956, 1961; Huner and Barr 1991; Paglianti and Gherardi 2004; Scalici and Gherardi 2007). Thus, the life history characteristics of *P. clarkii* may promise a high potential for rapid increases in population size in new available habitats; indeed, *P. clarkii* has successfully established self-sustaining populations after escaping into freshwater bodies from aquaculture ponds in much of the introduced range (Loureiro et al. 2015; Souty-Grosset et al. 2016). This crayfish species has also become a popular ornamental animal and has expanded its populations through releases of pets from aquariums in some regions (Chucholl 2011, 2013; Souty-Grosset et al. 2016).

Procambarus clarkii has exerted negative impacts on the ecosystems of newly colonized environments through the consumption of aquatic plants and algae and predation on several aquatic species, including amphibians, molluscs, and macroinvertebrates, leading to biodiversity loss (Souty-Grosset et al. 2016). Additionally, native crayfish populations have been seriously damaged due to the crayfish plague caused by the parasitic oomycete that is derived from vector *P. clarkii* (Souty-Grosset et al. 2016; Martín-Torrijos et al. 2018). In regions invaded by *P. clarkii*, control measures such as trapping; biocontrol with indigenous fish predators; sterile male release techniques, which use sterile but sexually active males; and the use of pheromones as bait for traps have been applied to manage populations of this invasive alien species (Aquiloni et al. 2009, 2010; Aquiloni and Gherardi 2010; Gherardi et al. 2011; Loureiro et al. 2015). To manage the populations of biological resources, information on population structure and dynamics is crucial; therefore, population ecology, such as growth and reproduction, has been extensively studied for *P. clarkii* populations in

68 introduced regions (Scallici and Gherardi 2007; Alcorlo et al. 2008; Anastácio et al. 2009;
69 Chucholl 2011).

70 After reaching sexual maturity, crayfish males that belong to the family Cambaridae,
71 which originates in North America, exhibit a cyclical dimorphism between the reproductive
72 phenotype (form I) and the non-reproductive phenotype (form II); after breeding season, form
73 I males moult to form II and then, upon return of the breeding season, form II males moult
74 and return to form I (Scudamore 1948; Stein 1976; Taketomi et al. 1990; Payne 1996; McLay
75 and van den Brink 2016; Kawai 2017). Form I males have longer and wider chelae, hooks on
76 the ischia of the third and fourth pereopods for holding females during copulation, and more
77 calcified copulatory pleopods (gonopods); form II males lack these morphological characters
78 (Suko 1953; Stein et al. 1977; Taketomi et al. 1990, 1996; Huner and Barr 1991; McLay and
79 van den Brink 2016; Kawai 2017). In contrast to males, the form alternation of cambarid
80 females has only been reported for some species of the genus *Orconectes* Cope 1872 (Wetzel
81 2002; Wetzel et al. 2005; Buřič et al. 2010a) and for the species *Cambarus elkensis* Jezerinac
82 and Stocker 1993 (Jones and Eversole 2011). The classification of these *Orconectes* species
83 has been updated (Crandall and De Grave 2017), and here we use the reclassified genus name,
84 *Faxonius* Ortmann 1905. In these *Faxonius* and *Cambarus* species, form I females exhibit
85 larger chelae and wider pleons than the form II females. In *P. clarkii*, form alternation is
86 evident in males (Taketomi et al. 1990), but it has been assumed that females do not exhibit
87 form alternation (Oluoch 1990; Loureiro et al. 2015). Consequently, the reproductive phase of
88 males has been assigned by identifying either sexual form I or II, whereas that of females is
89 assigned by examining gonad maturation; active glair glands of the ventral pleon, uropods and
90 telson; and the eggs, juveniles and egg-remains on the pleopods (Scallici and Gherardi 2007;
91 Alcorlo et al. 2008; Anastácio et al. 2009; Chucholl 2011). Suko (1953), however,
92 documented the presence of different sexual morphotypes with longer (form I) or shorter
93 (form II) chelae and the occurrence of form alternation in *P. clarkii* females, although he did
94 not show the actual measurements of the body parts. Thus, previous reports of intra-sexual
95 dimorphism in *P. clarkii* females have been contradictory. If *P. clarkii* females exhibit
96 different sexual forms, their reproductive status would be more easily assessed based on the
97 chela morphology.

98 The present study therefore aimed to elucidate the existence of different sexual forms in
99 *P. clarkii* females by allometric growth analyses of some body parts with reproductive
100 information, such as the gonad maturity condition and the presence of eggs and juveniles on
101 the pleopods, and to highlight the intra- and inter-sexual dimorphisms in *P. clarkii*. The

102 reproductive status of a *P. clarkii* population was then evaluated by the occurrence of sexually
103 active form I females and males.

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105

MATERIALS AND METHODS

106

107 **Crayfish samples**

108

109 In Japan, *P. clarkii* was imported from the USA as bait for culturing bullfrogs in 1927,
110 and approximately 20 individuals were stocked into a pond in Kamakura, Kanagawa
111 Prefecture (Sako 1987); since then, this species has expanded its range in the whole country
112 (Kawai and Kobayashi 2005; Kawai 2017). *Procambarus clarkii* is listed in the “100 of
113 Japan's Worst Invasive Alien Species” (Ecological Society of Japan 2002), and removal
114 campaigns using fishing gear such as traps and nets have been practised in an effort to
115 eradicate the populations (Nakata 2018). In the present study, we used *P. clarkii* samples that
116 were collected during the period from April to December 2011 through removal campaigns at
117 the public natural park in Yokohama, Kanagawa Prefecture, Japan (35°32'22–23"N,
118 139°34'44–46"E). The crayfish were eliminated from a pond (~1900 m², maximum depth ~
119 1.5 m) with fishing gears such as bait traps (mesh size, 9–13 mm) and scoop nets (mesh size,
120 4 mm). The number of days that were taken to collect the crayfish and the number of crayfish
121 collected are summarized for each month in Table 1. The total numbers of female and male
122 specimens were 566 and 476, respectively.

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124 **Crayfish measurements**

125

126 The crayfish samples were brought to the laboratory and stocked in the refrigerator at
127 4 °C. The samples were sexed based on the morphology of the pleopods (Suko 1953) (see Fig.
128 S1A, B). Several body parts of the intact specimens were then measured to the nearest 0.01
129 mm as follows (see Fig. S1C–E): for both sexes, the postorbital carapace length (POCL, from
130 the edge of the eye socket to the posterior margin of the carapace) (Fig. S1C), and the
131 propodus width (the widest part of the propodus) and length (from the tip of the propodus to
132 the carpal joint) of the right and/or left chelipeds were measured (Fig. S1D); for males, the
133 first gonopod length (from the base to the tip of the right gonopod) was also measured (Fig.
134 S1E), while for the females, the pleon width (the widest part of the second pleonite) was
135 assessed (Fig. S1C). Males were assigned as either form I or form II morphotypes based on

136 the presence of hooks on the ischia of the third and fourth pereopods and calcified whitish
137 gonopods (see Fig. S2) with developed and cornified apical lobe (Taketomi et al. 1990, 1996;
138 Kawai 2017). Females were checked for eggs or juveniles on their pleopods. All male and
139 female specimens were dissected, and the gonads were removed and weighed to the nearest 1
140 mg. Some minute gonads could not be removed from the specimens (36 females and 6 males).
141 To represent the gonad maturity condition in crayfish species including *P. clarkii*, the
142 gonadosomatic index (GSI) was used and calculated as [gonad weight / body weight] \times 100
143 (Alcorlo et al. 2008; Chucholl 2011; McLay and van den Brink 2016; Jin et al. 2019). In the
144 present study, there were specimens that lost both chelipeds (13 females and 7 males) or one
145 cheliped (54 females and 34 males), and form I crayfish with larger chelipeds may be heavier
146 than same-sized form II, leading to the bias in the calculations of the weight-based GSI
147 values. Therefore, we calculated the GSI as [gonad weight / POCL³] \times 1000. Alcorlo et al.
148 (2008) reported that oocyte diameter increased with increasing GSI values in *P. clarkii*
149 females. To examine the oocyte development in accordance with GSI values, the gonads of 14
150 females, which had GSI values ranged from 0.9 to 13.6, were preserved in 10% formalin
151 solution. The maximum diameters of oocytes were then measured to the nearest 1 μ m using a
152 Nikon stereomicroscope (MZ-800; Nikon Corp., Tokyo, Japan) equipped with a digital
153 camera and an image analysing system (Nikon Digital Sight and NIS-Elements software).
154 Number of oocytes measured for each gonad specimen ranged between 153 and 291, and
155 frequency distributions of the diameters of oocytes were illustrated for respective gonads.

156 The carapace length (CL, from the tip of the rostrum to the posterior margin of the
157 carapace) or the total length (TL, from the tip of the rostrum to the posterior margin of the
158 telson) are often measured as body size of *P. clarkii* (Fig. S1C). To allow the comparison of
159 the present study, which employed the POCL, and other studies that employed the CL or TL
160 measurements, the POCL, CL and TL were measured for additional specimens (50 males and
161 50 females) collected in October and November 2019 from the same pond as the 2011
162 collections. The liner regression equations used to convert from the POCL to CL and TL
163 values were as follows (Fig. S3): CL = $-0.5871 + 1.321$ POCL ($R^2 = 0.9832$, $F_{1, 98} = 5726$, P
164 < 0.0001); and TL = $3.6814 + 2.4523$ POCL ($R^2 = 0.9757$, $F_{1, 98} = 3935$, $P < 0.0001$). In the
165 present study, the statistical analyses were performed with R statistical software (R3.5.2; R
166 Core Team 2018) at a 5% significance level.

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168 **Intra- and inter-sexual dimorphisms**

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170 The intra- and inter-sexual dimorphisms were evaluated based on an allometric growth
171 analysis. The relative growth of the body dimensions to a reference dimension (POCL) was
172 examined using the following allometric growth equation (Huxley 1932): $y = ax^b$, where x is
173 POCL, y is the measurement for another body-part (chela propodus width and length, pleon
174 width, and gonopod length), b is the allometric growth coefficient, and a is the initial growth
175 constant. The relative growth patterns were defined as follows: $b > 1$ indicates positive
176 allometric growth, or faster growth of y than x ; $b = 1$ indicates isometric growth, or the same
177 growth rate for y and x ; and $b < 1$ indicates negative allometric growth, or slower growth of y
178 than x . The parameters were estimated by applying a general linear model (*lm* function) to the
179 log-transformed equation $\ln y = \ln a + b \ln x$. The right and left chelae were basically
180 symmetrical, and some specimens appeared to have regenerating smaller right or left
181 chelipeds, as shown in figure S4. Therefore, we selected the larger measurement between the
182 right and left chelae as chela dimensions.

183 Suko (1953) stated that in *P. clarkii* females, the reproductive form I has longer chelae
184 than those of form II of the same body size. In the present study, two different morphotypes
185 with larger or smaller chelae were distinguished by the allometric growth equations with the
186 aid of the information about reproductive status, such as the gonad maturity condition
187 represented by the GSI values and the presence of eggs and juveniles on the pleopods. The
188 GSI values of the *P. clarkii* females increased in accordance with the oocyte development, and
189 the oocyte diameter particularly increased after the gonad reached the GSI value of 3, as
190 shown in figure S5. Therefore, we considered females with GSI values > 3 to be candidates
191 for the reproductive morphotype (form I). Additionally, one ovigerous female and five
192 females carrying juveniles were found in the collected specimens. The chela propodus widths
193 and lengths were plotted against the POCL in these potentially or actively reproductive
194 females, with the exception of one ovigerous female that had lost both chelipeds, and are
195 shown in figure S6. The allometric growth equations between the POCL and chelae
196 dimensions were estimated as follows: chela propodus width, $y = 0.0725x^{1.4043}$ (statistics
197 between $\ln y$ and $\ln x$: $n = 41$, $R^2 = 0.8756$, $F_{1, 39} = 274.5$, $P < 0.0001$); and chela propodus
198 length, $y = 0.2408x^{1.3752}$ (statistics between $\ln y$ and $\ln x$: $n = 41$, $R^2 = 0.9373$, $F_{1, 39} = 582.7$, P
199 < 0.0001). Our female samples appeared to have two morphological groups with relatively
200 large or small chelae, and the chela dimensions of the potentially or actively reproductive
201 females were scattered within the larger chela group (see the RESULTS section). Here, the
202 above mentioned allometric growth curves of these potentially or actively reproductive
203 females were lowered in parallel by reducing the intercept values so that the lowest data plots

204 for these females just matched the adjusted allometric growth curves (see figure S6) (chela
 205 propodus width, $y = 0.0633x^{1.4043}$; chela propodus length, $y = 0.2184x^{1.3752}$); these adjusted
 206 allometric growth curves successfully discriminated between the larger and smaller chelae
 207 groups (see the RESULTS section). We assigned a female as form I when either the chela
 208 propodus width or chela propodus length were beyond the discriminant value calculated by
 209 substituting its POCL into the adjusted allometric growth equation or form II when both the
 210 chela propodus width and chela propodus length were below the discriminant values.

211 To statistically infer the intra- and inter-sexual dimorphisms in females and males, we
 212 applied three models that included a continuous explanatory variable (POCL) and a
 213 categorical explanatory variable (sexual morphotype (MT), form I or II; Sex, female or male)
 214 with the *lm* function as follows: model 1, $\ln y \sim \ln \text{POCL} + \text{MT or Sex} + \ln \text{POCL} \times \text{MT or Sex}$;
 215 model 2, $\ln y \sim \ln \text{POCL} + \text{MT or Sex}$; and model 3, $\ln y \sim \ln \text{POCL}$, where y is the
 216 measurement for another body-part; then, we selected the best model with the lowest AIC
 217 value (Akaike 1973; Burnham and Anderson 2002). The models showed whether the
 218 allometric growth equation had different intercepts and slopes (model 1), different intercepts
 219 and the same slope (model 2), or same intercept and slope (model 3) between the sexual
 220 morphotypes or sexes. When model 1 or 2 was selected, intra- and inter-sexual dimorphisms
 221 are detected. The allometric growth analysis was also applied to the gonad weight and GSI
 222 values in each sex.

223

224 **Reproductive status**

225

226 To statistically evaluate the seasonal changes in the reproductive status of the *P. clarkii*
 227 population, a generalized linear model (GLM) (*glm* function with a Gaussian error
 228 distribution) was performed to evaluate the differences in GSI values (response variable)
 229 among the months (April–December) and the sexual morphotypes (form I and II) (categorical
 230 explanatory variables) of each sex. Next, the numbers of form I and II crayfish after reaching
 231 the size of onset of sexually maturity (females, 22 mm POCL; males, 21 m POCL) (see the
 232 RESULTS section) were summarized for the different body size groups (22–25.99 mm
 233 (female), 21–25.99 mm (male), 26–29.99 mm, 30–33.99 mm, 34–37.99 mm, 38–41.99 mm
 234 (both sexes) POCL) and months (April–December) (see Table S1 for the number of crayfish
 235 and figures S7 and S8 for the size frequency distributions of crayfish). Then, differences in
 236 the proportions form I (response variable) among the different body size groups and months
 237 (categorical explanatory variables) were evaluated for each sex using the GLM analysis (*glm*

238 function with a quasibinomial family (logit link), taking into account the overdispersion of the
239 data). All females with ≥ 22 mm POCL were form I in October, and all females and males
240 were form I in the body size class with ≥ 38 mm POCL; these data were excluded from the
241 analyses because reliable coefficients could not be estimated by the GLM analysis. The
242 statistical significance of the explanatory variables in the GLM analysis was evaluated with
243 an *F*-test using the *Anova* function (Type II) (Fox and Weisberg 2011). Pearson's product
244 moment correlation coefficient (*r*) was used with a *t*-test to evaluate the relationship between
245 the overall monthly proportions of form I females and males. The sex ratio was calculated as
246 the [number of males / number of total crayfish], and a binomial test was used to test the null
247 hypothesis for the sex ratio in each month (H_0 ; sex ratio = 0.5).

248

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RESULTS

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Intra- and inter-sexual dimorphisms

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253 The females could be divided into two morphotypes with relatively large or small chelae
254 by the discriminant allometric growth equations between the POCL and chela propodus width
255 or length that were derived from the potentially reproductive and sexually active females (Fig.
256 1A, B). These females with larger or smaller chela should correspond to form I or II because
257 potentially or actively reproductive females were included in the group with larger chela.
258 Form I occurred in females that had grown to POCL of 22 mm or greater. Among the three
259 log-transformed allometric growth equation models applied for the chela dimensions, model 1
260 and model 2 were selected as the best models (Table 2), indicating the existence of intra-
261 sexual dimorphism in the chelae of *P. clarkii* females. Although model 1 was also the best for
262 pleon width (Table 2), the differences in the intercept and slope were small between the
263 morphotypes (Table 2, Fig. 1C). Males were assigned as either form I or II based on their
264 external reproductive characteristics, and form I occurred in males that had grown to POCL of
265 21 mm or larger (Fig. 2A, B). The form I and II males had relatively large and small chelae,
266 respectively. As seen in the females, the allometric growth analyses detected intra-sexual
267 dimorphism among the males, and there was distinct dimorphism in the chelae (Table 2, Fig.
268 2A, B) but small dimorphism in the first gonopod (Table 2, Fig. 2C). The chelae showed
269 positive allometric growth ($b = \sim 1.2-1.5$) regardless of the sexual morphotype in both sexes,
270 and the pleon widths and gonopod lengths showed approximately isometric growth ($b = \sim 0.9-$
271 1.1) (Table 2).

272 When the allometric growth models were tested for the chela dimensions between the
 273 sexes in each sexual morphotype, model 1 and model 2 were selected as the best models
 274 (Table 3), indicating the existence of inter-sexual dimorphism in the chelae of *P. clarkii*. In the
 275 comparison of the chela dimensions among crayfish with the same POCL, the form I males
 276 were found to have wider and longer chelae than the form I females, the form I females had
 277 wider chelae than the form II males, and the form II males showed wider and longer chelae
 278 compared with the form II females after reaching the size at onset of sexual maturity (~21–22
 279 mm POCL) (Fig. 3).

280 The allometric growth of the gonad weight and GSI were best described by model 1 for
 281 both sexes (Table 2); however, these values varied widely in form I females (Fig. 4). In males,
 282 the gonad weight tended to increase with increasing body size, whereas the GSI values tended
 283 to decrease in larger males (Fig. 5). The gonad weight and GSI values of the males tended to
 284 be larger in form I than those in form II (Table 2), but the scatter plots of these values against
 285 the POCL largely overlapping between the form I and II males.

286

287 **Reproductive status**

288

289 The GSI values significantly varied among the months and sexual morphotypes for both
 290 females (month, $F = 9.5279$, $df = 8$, $P < 0.0001$; morphotype, $F = 157.77$, $df = 1$, $P < 0.0001$)
 291 and males (month, $F = 26.697$, $df = 8$, $P < 0.0001$; morphotype, $F = 28.316$, $df = 1$, $P <$
 292 0.0001). The form I females showed higher GSI values than the form II females, and the form
 293 I females with higher GSI values were found in April and September (Fig. 6A). One
 294 ovigerous female (23.2 mm POCL) and five females carrying juveniles (26.8–37.7 mm
 295 POCL) were found in the specimens collected in April 24, 2011. The differences in the GSI
 296 values between form I and II males were small, and the GSI values tended to slightly decrease
 297 or vary less from April to July then increase until September and decrease again until
 298 December (Fig. 6B).

299 The proportions of form I crayfish significantly varied among the months and body size
 300 groups in the females (month, $F = 3.1070$, $df = 7$, $P = 0.0248$; body size, $F = 23.653$, $df = 3$, P
 301 < 0.0001) and males (month, $F = 6.6543$, $df = 8$, $P = 0.0003$; body size, $F = 12.0611$, $df = 3$, P
 302 < 0.0001). The proportions of form I crayfish tended to increase with increasing body size,
 303 and the largest group (≥ 38 mm POCL) comprised all form I females and males (Fig. 7). The
 304 monthly proportions of form I females and males were significantly correlated ($r = 0.7743$, $t =$
 305 3.2369 , $df = 7$, $P = 0.0143$) (Fig. 8). The overall proportions of form I females and males

306 fluctuated around 50% from April to September, and then the proportions increased to 83–
307 100% in October. The proportion of form I was higher in males (76%) than in females (50%)
308 in November and decreased to the same level in both sexes in December (Fig. 8). The sex
309 ratio fluctuated around 0.5, but significant unbalanced sex ratios that favoured the females
310 and males were detected in April ($P = 0.0161$) and November ($P = 0.0275$), respectively (Fig.
311 8). The sex ratio appeared to be male biased in December but was not statistically significant
312 due to the small sample size ($n = 8$, $P = 0.7266$).

313

314

DISCUSSION

315

Intra- and inter-sexual dimorphisms

317

318 Males of the American cambarid crayfish could be assigned as either sexually active
319 form I or sexually inactive form II morphotypes based on the presence of hooks on the ischia
320 of the third and fourth pereopods and more calcified rigid gonopods (Suko 1953; Stein et al.
321 1977; Taketomi et al. 1990, 1996; Huner and Barr 1991; McLay and van den Brink 2016;
322 Kawai 2017), and form I males have longer and wider chelae than form II males (Suko 1953;
323 Stein et al. 1977; Huner and Barr 1991; McLay and van den Brink 2016; Buřič et al. 2010b).
324 Sexually active females (form I) of the American cambarids have been identified based on the
325 presence of developed glair glands, oocytes in the gonopores, and/or eggs and juveniles on the
326 pleopods (Wetzel 2002; Buřič et al. 2010a; Jones and Eversole 2011), the architecture of
327 annulus ventralis (Wetzel et al. 2005; Jones and Eversole 2011) and the occurrence of mating
328 with males (Wetzel 2002; Buřič et al. 2010a); then, the body dimensions such as chela size
329 and pleon width were compared between form I and form II females. Wetzel et al. (2005)
330 documented that form I and form II females of *Faxonius pardalotus* (Wetzel, Poly and Fetzner
331 2005) exhibited different morphologies of annulus ventralis: form I females have a corneous
332 sternum and strongly convoluted structures, whereas those of form II females are less
333 cornified and convoluted. Kawai (2017) found these two characters in the annulus ventralis of
334 *P. clarkii* and suggested the existence of form I and form II morphotypes in *P. clarkii* females.

335 In the present study, based on the documentation by Suko (1953) that different sexual
336 morphotypes with longer (form I) or shorter (form II) chelae were found in *P. clarkii* females,
337 we attempted to elucidate the existence of different sexual forms in *P. clarkii* females by
338 allometric growth analyses of some body parts with reproductive information, such as the
339 gonad maturity condition and the presence of eggs and juveniles on the pleopods. Females

340 could be assigned into two morphotypes with relatively large or small chelae, corresponding
341 to form I or II because potentially or actively reproductive females were included in the
342 morphotype with larger chela. We also identified the sexual morphotypes of males based on
343 the external morphological characteristics and conducted the allometric growth analyses of
344 some body parts. Our analyses demonstrated the existence of different sexual morphotypes in
345 *P. clarkii* females and highlighted the intra- and inter-sexual dimorphisms in the chelae of this
346 species (Figs. 1–3); the chelae were largest in the form I males, followed by the form I
347 females and form II males, and were the smallest in the form II females (Fig. 3). To further
348 elucidate the reproductive characteristics of form I and form II females of *P. clarkii*,
349 architecture of annulus ventralis should be examined in relation to the sexual morphotypes
350 revealed by the present study and confirm the occurrence of mating of these females with
351 form I males.

352 Suko (1953) illustrated the relative growth patterns between the TL and chela propodus
353 length of *P. clarkii* females and males collected from Urawa (35°52'N, 139°35'E), Saitama
354 Prefecture, Japan as similar to those listed in figure 3 of the present study, while the actual
355 measurements and allometric growth coefficients are not shown. Suko (1953) stated that the
356 form I females and males occurred from 56 mm TL (21.3 mm POCL) and 54 mm TL (20.5
357 mm POCL), respectively, which corresponded to the size at onset of sexual maturity of the
358 males (21 mm POCL) and females (22 mm POCL) in the present study. To evaluate the
359 reproductive potential of the decapod crustacean populations, the size at which 50% of the
360 animals reach maturation has been evaluated (Pescinelli et al. 2016; Waller et al. 2019). In *P.*
361 *clarkii*, form alternation is evident in males (Taketomi et al. 1990) and it might also occur in
362 females as discussed below, so that in our crayfish specimens, the form II crayfish might
363 include the subadults that have never moulted to the sexually active form I and adults that
364 have moulted and returned to the sexually inactive form II. To determine the size at which
365 50% of the crayfish reach their first maturation in *P. clarkii* populations, the characteristics of
366 the subadults and form II adults need to be clarified.

367 Stein (1976) suggested that chelae of crayfish may be used for (1) prey capture and
368 manipulation, (2) defence against predators, (3) inter- and intraspecific interactions, and (4)
369 reproductive activities. *Faxonius propinquus* (Girard 1852), which have large chelae, are less
370 vulnerable to predation by fish and superior competitors in intraspecific interactions (Stein
371 1976). Ueno and Nagayama (2015) reported that large *P. clarkii* with smaller chelae were
372 beaten by small ones with larger chelae. Thus, selection might favour large chelae in *P.*
373 *clarkii*, resulting in the positive allometric growth of the chelae regardless of the sex or the

374 sexual morphotype, as demonstrated by our allometric growth analyses. The shift of the
375 chelae to large sizes in form I cambarid males suggests that the chelae of sexually active
376 males might be under stronger directional selection. Large chelae should provide an
377 advantage for males in competition to acquire receptive females. In *F. propinquus*, males with
378 larger chelae more successfully mate with females than similar-sized males with smaller
379 chelae (Stein 1976). The *P. clarkii* form I females also had larger chelae than form II
380 conspecifics (Figs. 1, 3), which is known to occur in *Faxonius limosus* (Rafinesque 1817)
381 (Buřič et al. 2010a) and *C. elkensis* (Jones and Eversole 2011). Reproductive *P. clarkii*
382 females remain in the burrows to lay and brood eggs (Huner and Barr 1991). Laboratory
383 experiments demonstrated that in *P. clarkii*, maternal (carrying eggs or juveniles) female
384 residents won a significantly higher proportion of their contests for shelters than did non-
385 maternal residents, regardless of whether the intruders were males or non-maternal females
386 (Figler et al. 1995; Peeke et al. 1995). Large chelae might also be advantageous for defence of
387 offspring by maternal *P. clarkii* females. The males of *P. clarkii* use their chelae to grasp and
388 hold female chelae during copulation (Ameyaw-Akumfi 1981). Selection might favour larger
389 chelae of sexually active females to match the chelae size of males for successful courtship.

390 Form I females have wider pleons than form II females in some *Faxonius* species
391 (Wetzel 2002; Buřič et al. 2010a) and *C. elkensis* (Jones and Eversole 2011), and wider pleons
392 in females is believed to provide more space for egg incubation (Buřič et al. 2010a; Jones and
393 Eversole 2011). In *P. clarkii*, however, form I and II females with identical POCLs appeared
394 to have a similar-sized pleons with near isometric growth (Fig. 1). Pleopods of crayfish
395 females are used to carry eggs and juveniles, and the pleopod length of *P. clarkii* females
396 showed positive allometric growth (Kato and Miyashita 2003), suggesting that pleopods
397 rather than pleons might be under directional selection for the ability to carry more offspring.
398 Buřič et al. (2010b) reported that form I males of *F. limosus* possess longer gonopods than
399 form II males. However, the length of the first gonopod was similar between the sexual
400 morphotypes of the *P. clarkii* males with the same POCL, and the gonopod showed
401 approximately isometric growth (Fig. 2). Kato and Miyashita (2003) reported similar
402 allometric growth coefficients for the gonopods of *P. clarkii* form I males as were shown in
403 the present study, and they suggested that gonopods have been under stabilizing selection to
404 allow copulation with females of various sizes.

405 The gonad weight and GSI were correlated with the male body size (Fig. 5). The gonads
406 were slightly heavier in the form I males than in the form II males with the same POCL and
407 largely overlapped between the sexual morphotypes (Figs. 4, 5). Taketomi et al. (1996)

408 classified *P. clarkii* males into five developmental stages, A–E, based on the gonopod
409 morphology and the histologically examined testis development. They revealed that the
410 development of gonopods was complete and hooks (reversed spines) were present on the
411 ischia of the third and fourth pereopods in stage E, which is identical to form I, and that testes
412 became sexually mature in stage D before reaching the morphologically mature stage E.
413 Because of this, some form II males in the present study may have shown similar gonad
414 weights to the form I males with the same POCL. The GSI values tended to increase in
415 smaller males (Fig. 5), suggesting that smaller males might invest in gonads to fertilize more
416 eggs when they mate with larger females.

417

418 **Reproductive status**

419

420 The sexually active form I females and males occurred throughout a sampling period
421 from April to November, while the occurrences of form I females and males were highly
422 correlated, peaking in October in our *P. clarkii* population in Yokohama, Japan (Figs. 7, 8).
423 Although there were few females with high GSI values, probably because the females with
424 mature gonads may be less active in their burrows during egg laying, one female and five
425 females with GSI values > 10 were detected in April and September, respectively (Fig. 6), and
426 one ovigerous female and five females carrying juveniles were found in April. The GSI values
427 of the form I males appeared to fluctuate in conjunction with those of the form I females (Fig.
428 6). The reproductive cycle in the Yokohama *P. clarkii* population generally agreed with that of
429 a previously investigated population in Urawa, Saitama Prefecture, Japan, which showed
430 continuous copulation and spawning incidences throughout the year with two seasons of high
431 reproduction; the most prolific season was from September to October (autumn) and the other
432 was from May to June (spring) (Suko 1956, 1958). The synchronization of reproductive
433 cycles between females and males has also been shown in European *P. clarkii* populations
434 (Alcorlo et al. 2008; Anastácio et al. 2009).

435 Suko (1958) summarized the main reproductive cycles of the Urawa *P. clarkii* population
436 but did not show population dynamics data, such as growth or proportions of sexual
437 morphotypes; the crayfish hatched in the late autumn of the main reproductive season rapidly
438 grow after the warm spring, moult to the sexually active form I in early autumn, copulate and
439 spawn, and then moult and return to the sexually inactive form II during the long period from
440 November to June; crayfish born in spring do not mature within the year and overwinter as
441 juvenile stage and moult to form I in the next spring, and after breeding, they moult and return

442 to form II in autumn. In the present study, the occurrence of form I crayfish varies among
443 different body size groups, and the proportions of form I individuals in the smaller groups
444 showed higher values in spring (April) and autumn (October) in both sexes (Fig. 7). Changes
445 in the proportions of the sexual morphotypes in smaller crayfish of the Yokohama population
446 appeared to support the form alternation events of the autumn- and spring-born crayfish of the
447 Urawa population. In the Yokohama population, the overall proportions of form I crayfish
448 peaked and reached >80% in October, and the proportion of form I females decreased in
449 November, whereas that of the form I males was still high at 76% in November (Fig. 8). The
450 form I males might be actively seeking receptive females and might be more likely to be
451 collected by bait traps than females; therefore, the sex ratio might be biased in favour of the
452 males in November (Fig. 8). However, the reason for the female-biased sex ratio in April is
453 not known. Many females exhibited a form I phenotype in October, but their GSI values were
454 not as high in the Yokohama population (Figs. 6–8). In the autumn season, female *P. clarkii*
455 require approximately 1.5 months to incubate the eggs and hatchlings to rear stage 3 juveniles
456 that depart from mother's pleopods (Suko 1956, 1961). Almost all form I females of *P. clarkii*
457 laid eggs once during the intermoult period and they require several months for gonad
458 development before oviposition, and the females that copulated with males in early winter
459 spawned in the next spring in the Urawa population (Suko 1958). In the Yokohama population,
460 the form I females that were collected after late October might include females that had
461 already bred and females that would oviposit in the next spring as females carrying eggs and
462 juveniles were found in April.

463 Buřič et al. (2010a, b) examined the moulting events in relation to form alternation in *F.*
464 *limosus* for 8–10 months in captivity and reported that the majority of females (58%) and
465 males (84%) moulted twice and showed form alternation (form I → form II → form I); the
466 remainder of the females (42%) and males (9%) moulted once without form alternation (form
467 I → form I), and some males (7%) did not moult. They also documented that the initial body
468 size was smallest in the twice moulted crayfish, followed by the once moulted or not moulted
469 crayfish. Taketomi et al. (1990) documented that the proportions of form I males tended to
470 increase with increasing body size in *P. clarkii* collected from Kumamoto (32°46'N,
471 130°45'E), Japan. They also stated that many of the form I males with 25–30 mm POCL
472 changed to form II at their autumn/winter moult and returned to form I at the next
473 spring/summer moult, whereas form I males greater than 30 mm POCL did not undergo an
474 autumn/winter moult, but they did not refer to the next spring/summer moult of these crayfish.
475 In general, the intermoult periods increased with increasing body size, and thus, the moulting

476 events during the year decrease with growth in decapod crustacean species (Kurata 1962). In
477 the Yokohama *P. clarkii* population, the body size groups became larger and higher and less
478 variable proportions of form I occurred (Fig. 7), suggesting less form alternation occurs in
479 larger crayfish. Cambarid crayfish may exhibit size-dependent moulting and form
480 alternations. This hypothesis should be tested by laboratory culture experiments for *P. clarkii*
481 populations.

482 483 CONCLUSIONS

484
485 Our allometric growth analyses demonstrated the existence of two sexual morphotypes
486 in the females of the red swamp crayfish *Procambarus clarkii* and highlighted the inter-sexual
487 dimorphism of the chela dimensions in this species. In *P. clarkii* populations, the reproductive
488 status of males is evaluated by determining the sexual morphotypes, whereas that of females
489 has historically been conducted by examining gonad maturation; active glair glands; and the
490 eggs, juveniles and egg-remains on the pleopods (Scallici and Gherardi 2007; Alcorlo et al.
491 2008; Anastácio et al. 2009; Chucholl 2011). If we relied on only the traditional criteria using
492 GSI values for evaluating the female reproductive status, we would not have been able to
493 follow the reproductive cycle among the different body size groups in the Yokohama *P. clarkii*
494 population. The discrimination of the sexual morphotypes based on chela allometric growth
495 would allow us to evaluate the female reproductive status more easily and precisely in
496 conjunction with the traditional reproductive criteria, and this might help us to understand the
497 *P. clarkii* population dynamics as a basis for developing control measures for this invasive
498 alien species.

499
500 **Acknowledgements:** We would like to acknowledge the people who participated in
501 elimination campaigns of red swap crayfish in the public natural park, Yokohama, Japan. We
502 thank Keisuke Morimoto and Naoki Ishiyama for helping with the field and laboratory works.
503 We are grateful to two anonymous reviewers for their valuable comments and suggestions,
504 which have improved the manuscript substantially.

505
506 **Authors' contributions:** KH designed the study. NS and NI performed the sample collections
507 and measurements. KH, SD, and SK analysed the data and prepared the manuscript. All
508 authors approved the final manuscript.

509

510 **Competing interests:** The authors declare that they have no conflicts of interest.

511

512 **Availability of data and materials:** All data are provided within the manuscript and
513 supplementary materials.

514

515 **Consent for publication:** Not applicable.

516

517 **Ethics approval consent to participate:** The present study complies with current Japanese
518 laws.

519

520

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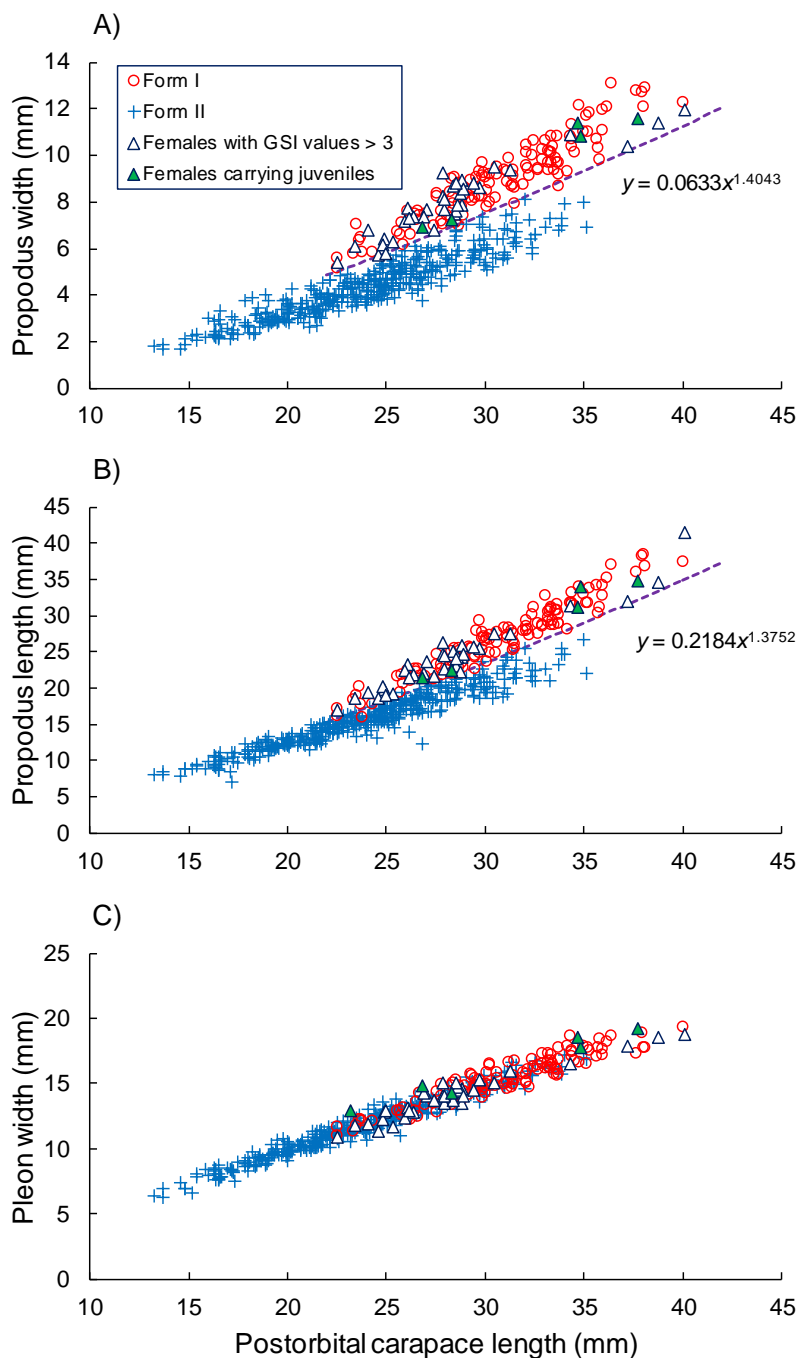
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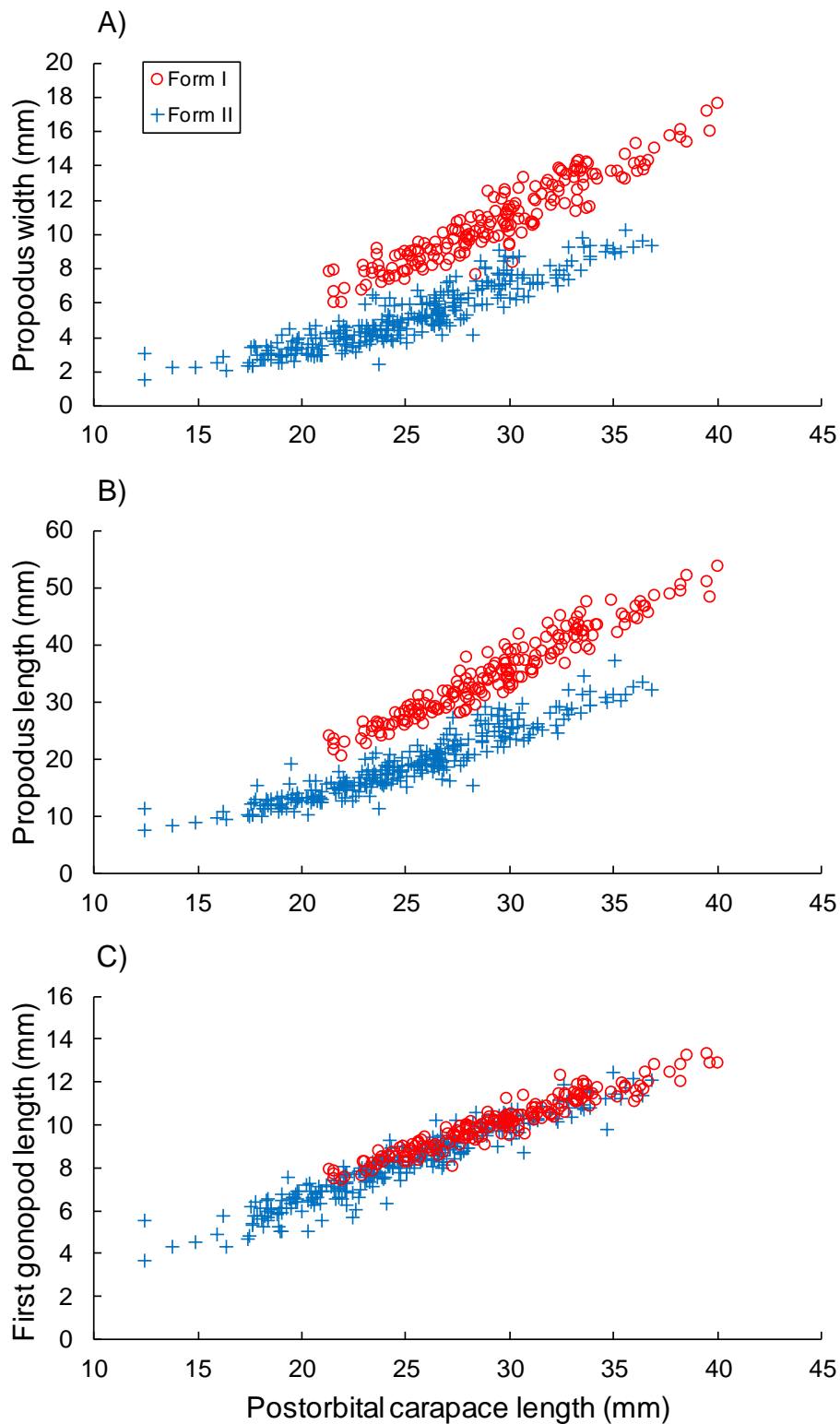
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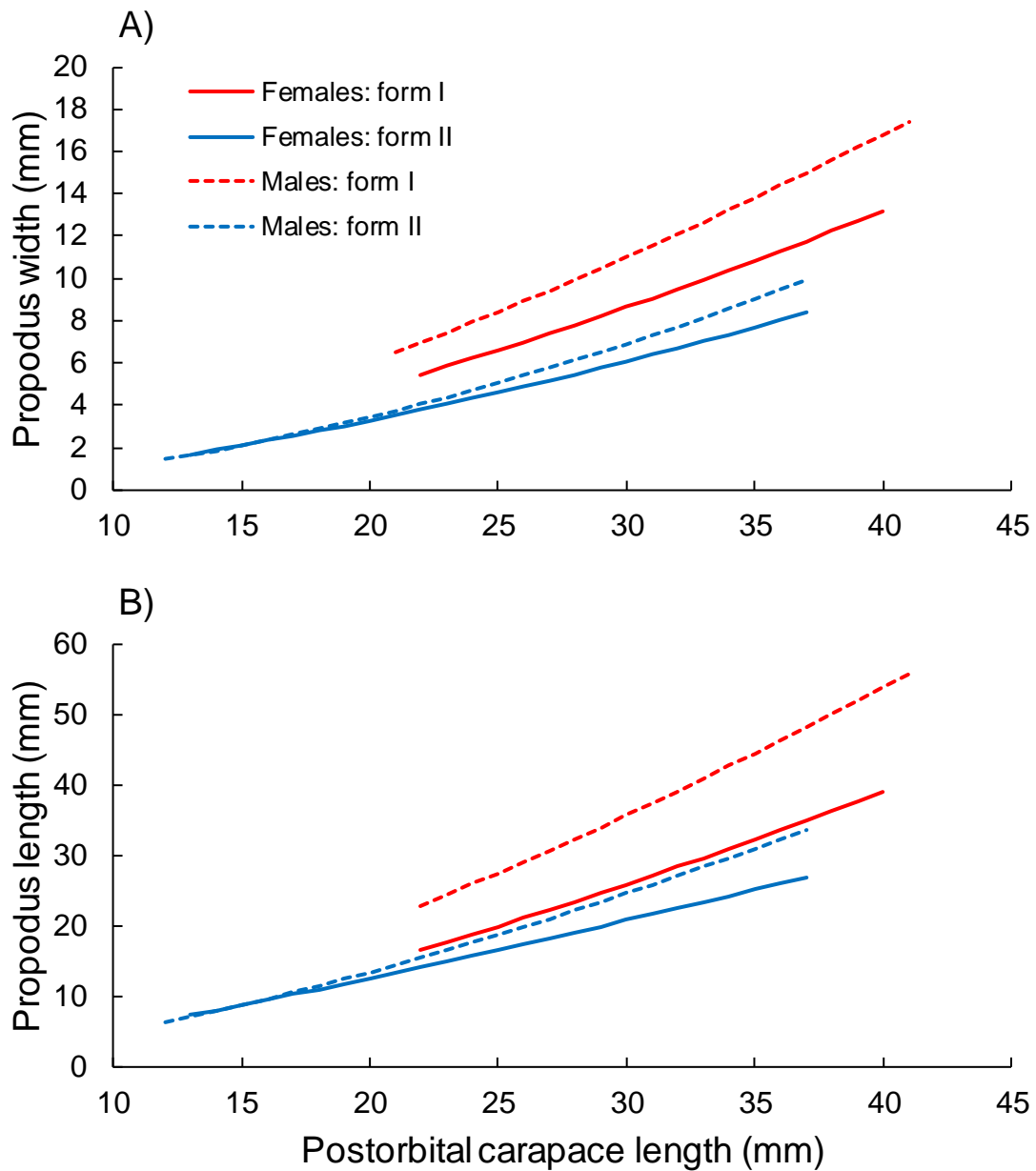
668 **Fig. 1.** Growth of the chela propodus width (A), chela propodus length (B) and pleon width
669 (C) relative to the postorbital carapace length in female red swamp crayfish *Procambarus*
670 *clarkii*. Data are shown for the different sexual morphotypes, reproductive form I and non-
671 reproductive form II, the potentially reproductive females with GSI values > 3 and the
672 reproductive females carrying juveniles. Allometric growth curves discriminating the different
673 sexual morphotypes are shown for chela propodus width and length. See figure S4 for
674 discriminant functions.



675

676

677 **Fig. 2.** Growth in the chela propodus width (A), chela propodus length (B) and first gonopod
 678 length (C) relative to the postorbital carapace length in male red swamp crayfish
 679 *Procambarus clarkii*. Data are shown for the different sexual morphotypes, reproductive form
 680 I and non-reproductive form II.



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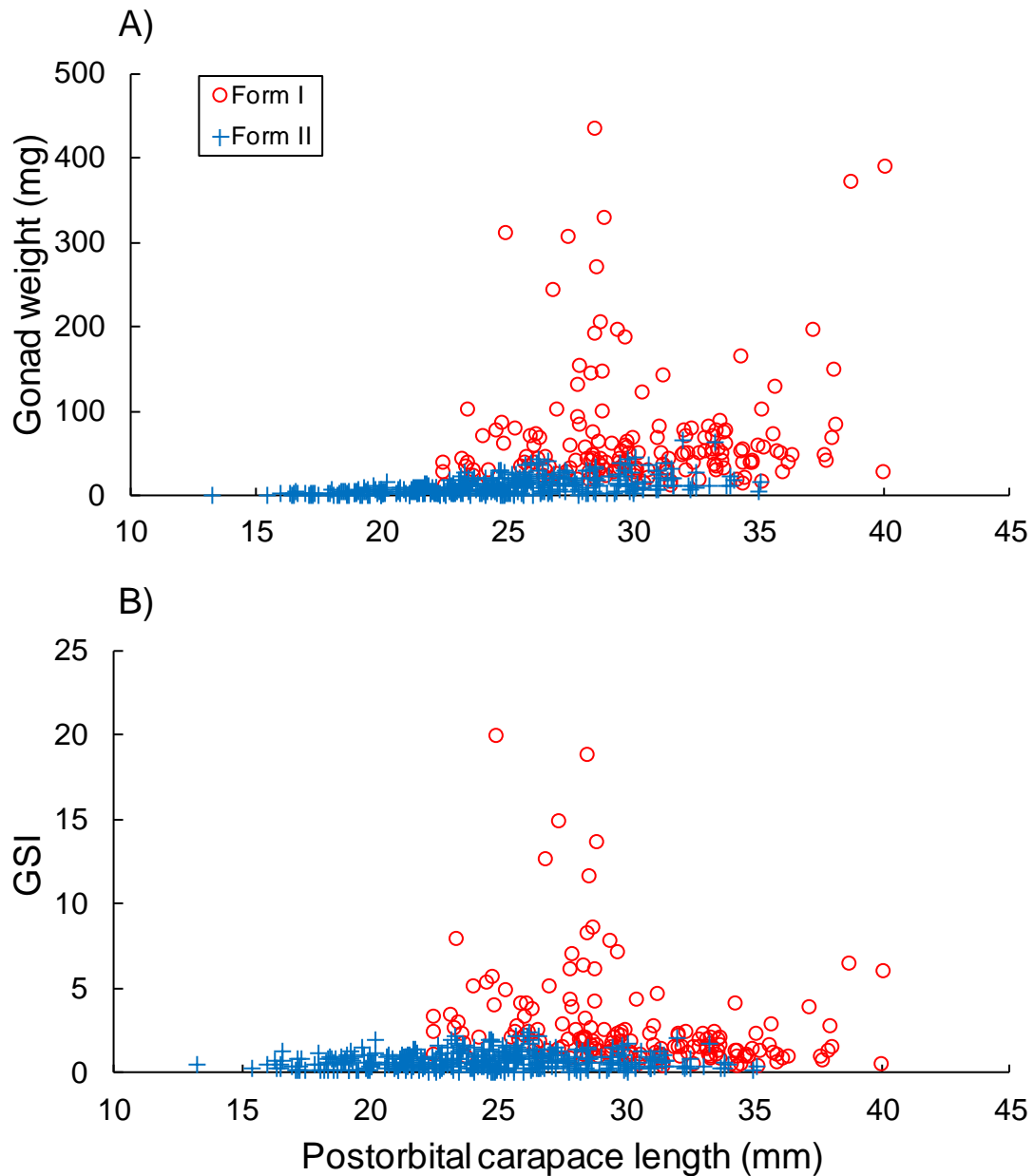
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683 **Fig. 3.** Inter-sexual dimorphism of the chela propodus width (A) and chela propodus length684 (B) in red swamp crayfish *Procambarus clarkii*. Allometric growth curves were calculated

685 based on the equations estimated for females and males in the respective sexual morphotypes

686 shown in Table 3.

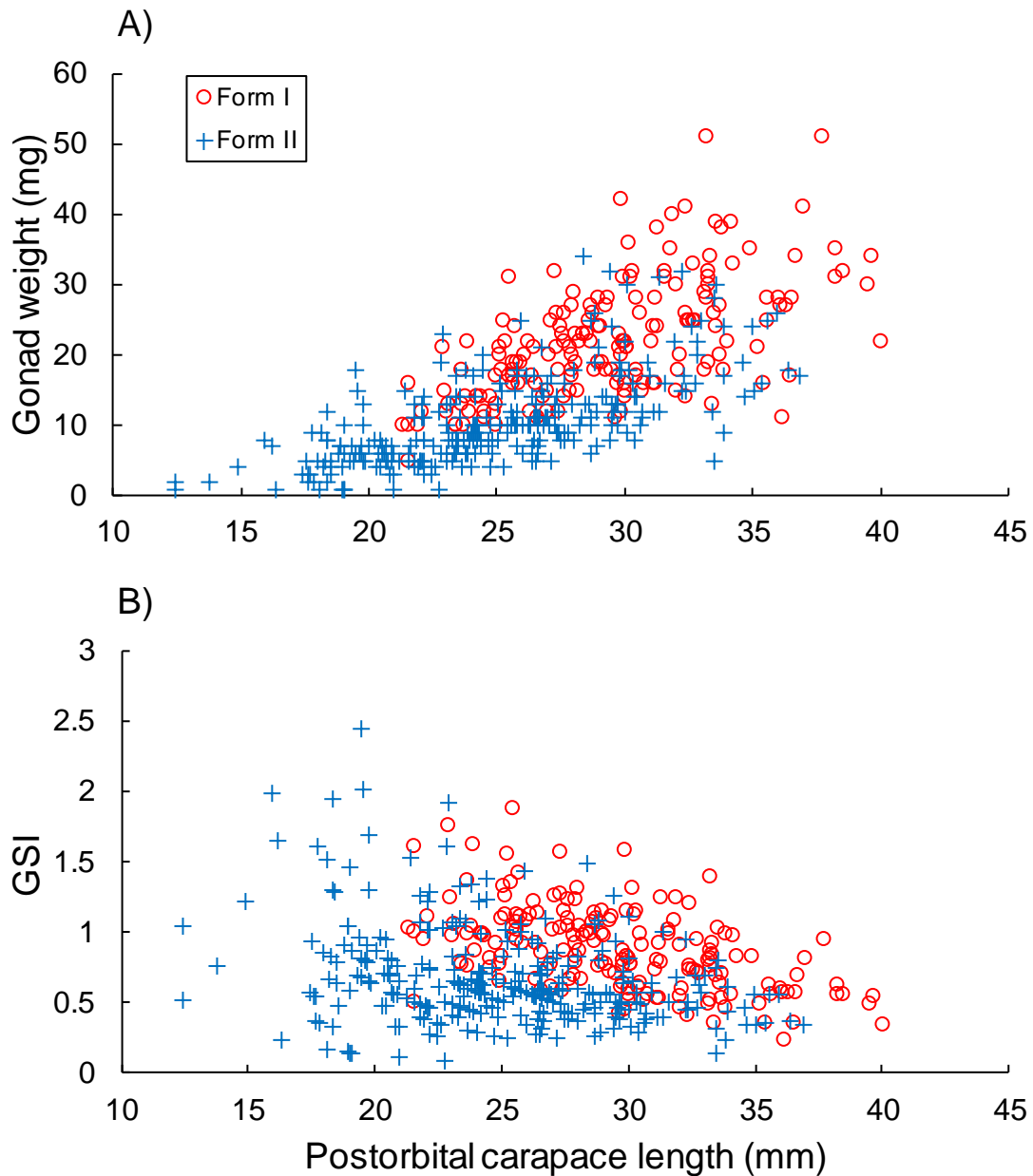
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689

690 **Fig. 4.** Relationships between the postorbital carapace length and gonad weight (A) and the
 691 gonadosomatic index (GSI) (B) in female red swamp crayfish *Procambarus clarkii*. Data are
 692 shown for the different sexual morphotypes, reproductive form I and non-reproductive form
 693 II.



694

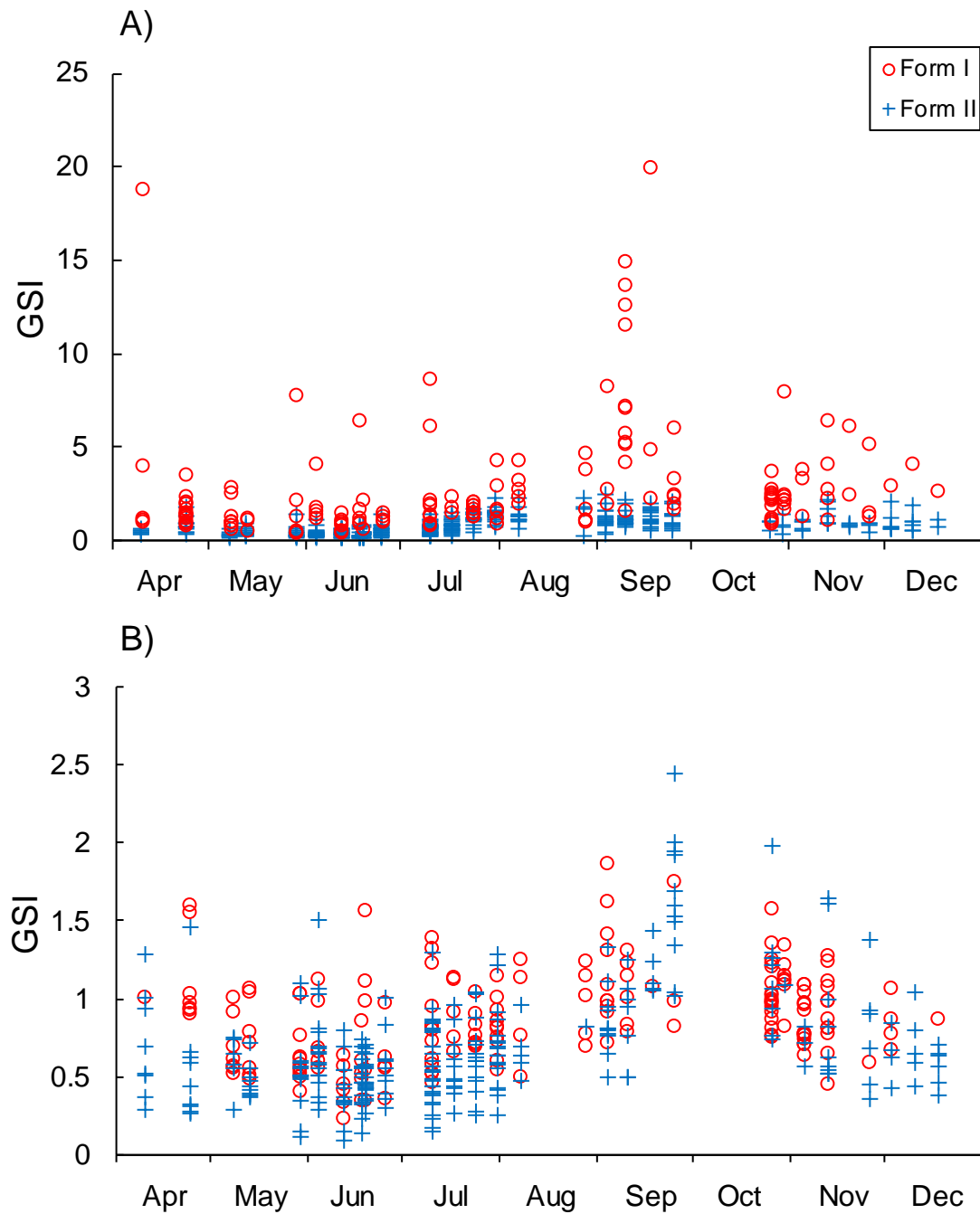
695

696 **Fig. 5.** Relationships between the postorbital carapace length and gonad weight (A) and the697 gonadosomatic index (GSI) (B) in male red swamp crayfish *Procambarus clarkii*. Data are

698 shown for the different sexual morphotypes, reproductive form I and non-reproductive form

699 II.

700



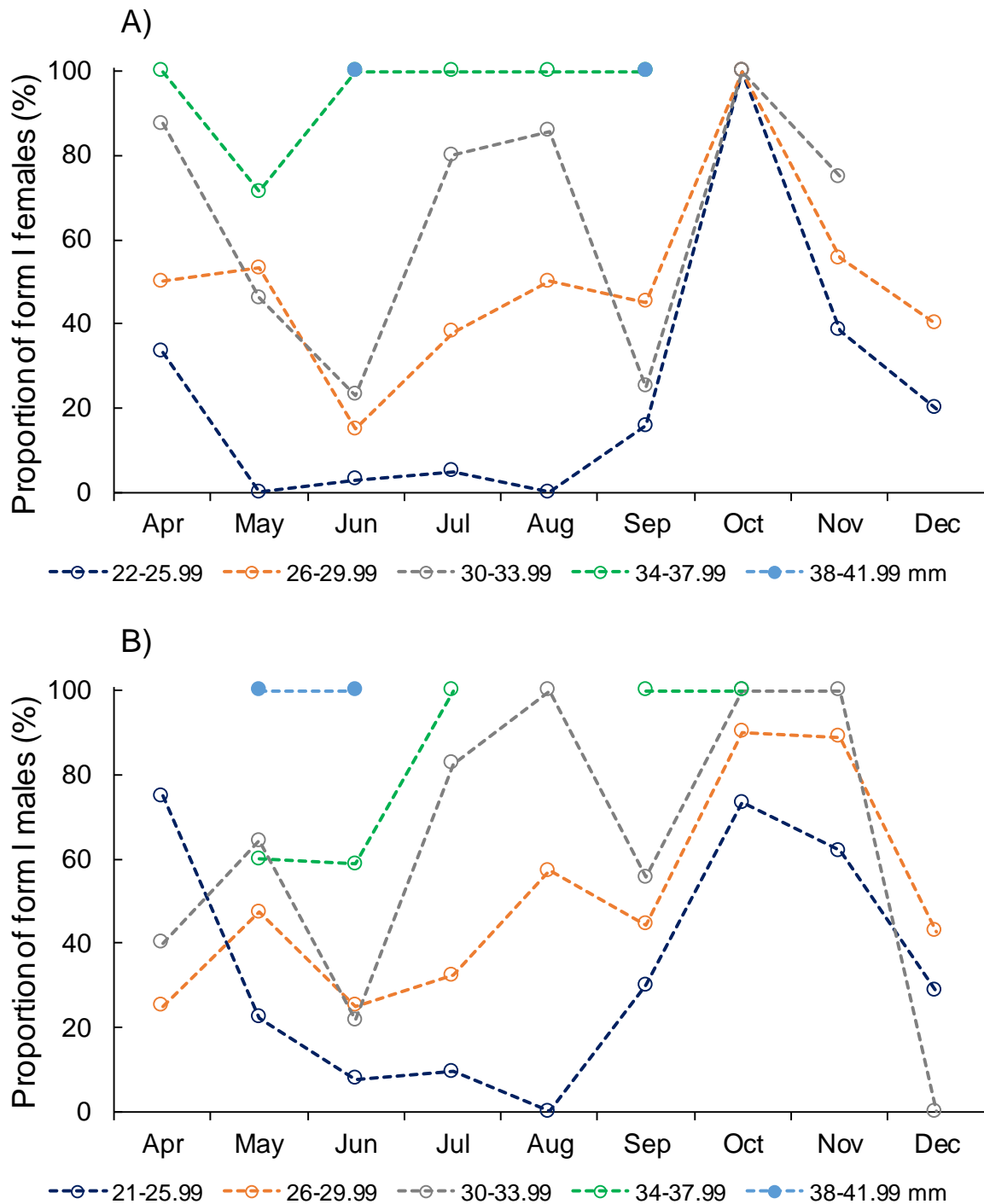
701

702

703 **Fig. 6.** Changes in the gonadosomatic index (GSI) in red swamp crayfish *Procamburus clarkii*

704 females (A) and males (B) from April to December 2011. Data are shown for the different

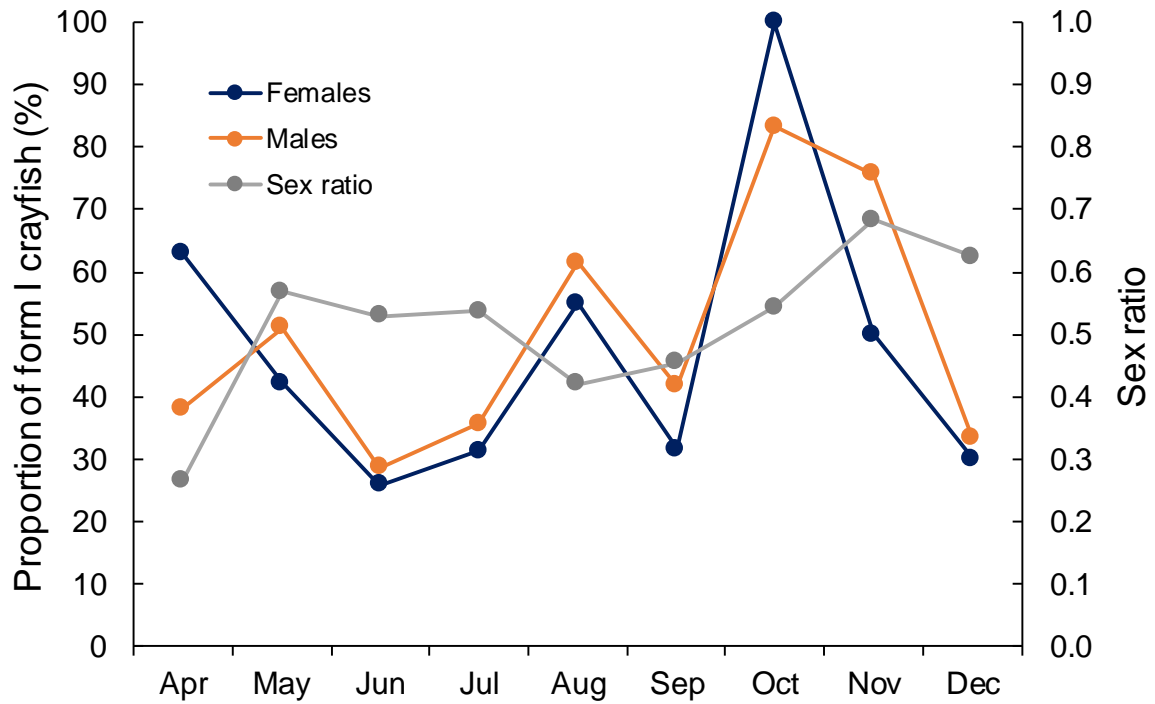
705 sexual morphotypes, reproductive form I and non-reproductive form II.



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708 **Fig. 7.** Changes in the proportions of reproductive form I females (A) and males (B) in
 709 different body size groups (postorbital carapace length, POCL) after sexual maturity (females,
 710 > 22 mm POCL; males, > 21 mm POCL) in red swamp crayfish *Procambarus clarkii*.



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712

713 **Fig. 8.** Changes in the proportions of reproductive form I females and males after sexual
 714 maturity (females, > 22 mm POCL; males, > 21 mm POCL) and the sex ratio [number of
 715 males / number of total crayfish] in red swamp crayfish *Procambarus clarkii*.

716

Table 1.

Collection records of red swamp crayfish *Procambarus clarkii* from a small pond in Yokohama, Japan, in 2011.

Month	Number of days collecting crayfish	Number of crayfish		
		Female	Female (ND)	Male
April	2	43	2	26
May	3	56	3	54
June	5	117	2	105
July	4	130	2	122
August	2	30	0	15
September	4	97	1	54
October	2	28	1	34
November	4	34	0	46
December	3	18	2	20

ND: The sexual maturity of some females could not be determined because of the loss of both chelipeds.

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Table 2.

Intra-sexual dimorphism evaluated with a general linear model for some body parts of red swamp crayfish *Procambarus clarkii*. Three models were applied: model 1, $\ln y \sim \ln \text{POCL} + \text{MT} + \ln \text{POCL} \times \text{MT}$; model 2, $\ln y \sim \ln \text{POCL} + \text{MT}$; and model 3, $\ln y \sim \ln \text{POCL}$, where POCL is the postorbital carapace length and MT is the sexual morphotype (form I, reproductive type; form II, non-reproductive type). The coefficient estimates of the categorical explanatory variable including the MT were outputted for form I and it represents the change in the response variable relative to the baseline category (form II).

Sex	Response variable	Model	Coefficient estimates				AIC	R^2	F	df_1	df_2	P
			Intercept	$\ln \text{POCL}$	MT-Form I	$\ln \text{POCL} \times \text{MT-Form I}$						
Female	Chela propodus width	1	-3.3995	1.5293	0.6040	-0.0737	-981.9	0.9486	3374	3	548	<0.0001
		2	-3.3622	1.5175	0.3561	-	-982.7	0.9485	5059	2	549	<0.0001
		3	-4.6273	1.9418	-	-	-384.9	0.8474	3055	1	550	<0.0001
	Chela propodus length	1	-1.2164	1.2489	-0.3876	0.1787	-1476.8	0.9642	4921	3	548	<0.0001
		2	-1.3066	1.2775	0.2135	-	-1461.0	0.9630	7151	2	549	<0.0001
		3	-2.0651	1.5319	-	-	-919.0	0.9010	5004	1	550	<0.0001
	Pleon width	1	-0.7177	1.0039	0.2013	-0.0573	-1988.9	0.9645	4976	3	549	<0.0001
		2	-0.6882	0.9945	0.0087	-	-1986.2	0.9642	7411	2	550	<0.0001
		3	-0.7190	1.0049	-	-	-1984.2	0.9640	14740	1	551	<0.0001
	Gonad weight	1	-8.8117	3.4061	10.6352	-2.7842	1230.3	0.6235	283.7	3	514	<0.0001
		2	-6.9706	2.8283	1.2888	-	1254.8	0.6037	392.3	2	515	<0.0001
		3	-12.1032	4.5324	-	-	1426.9	0.4454	414.4	1	516	<0.0001
	GSI	1	-1.9039	0.4061	10.6352	-2.7842	1230.3	0.3782	104.2	3	514	<0.0001
		2	-0.0628	-0.1717	1.2888	-	1254.8	0.3455	135.9	2	515	<0.0001
		3	-5.1955	1.5324	-	-	1426.9	0.0841	47.37	1	516	<0.0001
Male	Chela propodus width	1	-3.9124	1.7184	1.2432	-0.2287	-618.8	0.9364	2283	3	465	<0.0001
		2	-3.7314	1.6619	0.4815	-	-611.7	0.9352	3361	2	466	<0.0001
		3	-5.2405	2.1807	-	-	20.0	0.7496	1398	1	467	<0.0001
	Chela propodus length	1	-1.8619	1.4885	0.5511	-0.0526	-921.2	0.9526	3116	3	465	<0.0001

First gonopod length	2	-1.8203	1.4755	0.3760	-	-922.2	0.9525	4674	2	466	<0.0001
	3	-2.9988	1.8806	-	-	-231.0	0.7918	1776	1	467	<0.0001
	1	-1.3167	1.0637	0.6268	-0.1782	-1341.8	0.9263	1977	3	472	<0.0001
Gonad weight	2	-1.1779	1.0203	0.0334	-	-1319.2	0.9224	2811	2	473	<0.0001
	3	-1.2825	1.0563	-	-	-1293.7	0.9178	5291	1	474	<0.0001
	1	-5.4075	2.3708	2.7552	-0.6876	540.5	0.6405	276.8	3	466	<0.0001
GSI	2	-4.8662	2.2017	0.4652	-	545.5	0.6351	406.4	2	467	<0.0001
	3	-6.3169	2.7008	-	-	639.9	0.5520	576.6	1	468	<0.0001
	1	1.5002	-0.6292	2.7552	-0.6876	540.5	0.2095	41.18	3	466	<0.0001
	2	2.0415	-0.7983	0.4652	-	545.5	0.1977	57.52	2	467	<0.0001
	3	0.5908	-0.2992	-	-	639.9	0.0149	7.076	1	468	0.0081

The bold AIC value is the lowest among the three models for each body part.

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Table 3.

Inter-sexual dimorphism evaluated with a general linear model for the chelae of red swamp crayfish *Procambarus clarkii*. Three models were applied: model 1, $\ln y \sim \ln \text{POCL} + \text{Sex} + \ln \text{POCL} \times \text{Sex}$; model 2, $\ln y \sim \ln \text{POCL} + \text{Sex}$; and model 3, $\ln y \sim \ln \text{POCL}$, where POCL is the postorbital carapace length and Sex is female or male. The coefficient estimates of the categorical explanatory variable including the Sex is outputted for male and it represents the change in the response variable relative to the baseline category (female).

Form	Response variable	Model	Coefficient estimates				AIC	R^2	F	$df1$	$df2$	P
			Intercept	$\ln \text{POCL}$	Sex-Male	$\ln \text{POCL} \times \text{Sex-Male}$						
I	Chela propodus width	1	-2.7954	1.4556	0.1263	0.0342	-814.2	0.8994	1025	3	344	<0.0001
		2	-2.8620	1.4751	0.2421	-	-815.8	0.8993	1540	2	345	<0.0001
		3	-2.4061	1.3777	-	-	-368.9	0.6341	599.6	1	346	<0.0001
	Chela propodus length	1	-1.6040	1.4276	0.2932	0.0083	-1049.0	0.9528	2316	3	344	<0.0001
		2	-1.6202	1.4324	0.3214	-	-1051.0	0.9528	3483	2	345	<0.0001
		3	-1.0149	1.3030	-	-	-247.4	0.5223	378.3	1	346	<0.0001
II	Chela propodus width	1	-3.3995	1.5293	-0.5129	0.1892	-871.4	0.8662	1444	3	669	<0.0001
		2	-3.6608	1.6119	0.0894	-	-859.6	0.8635	2119	2	670	<0.0001
		3	-3.6928	1.6340	-	-	-785.2	0.8471	3716	1	671	<0.0001
	Chela propodus length	1	-1.2164	1.2489	-0.6455	0.2396	-1381.3	0.9107	2273	3	669	<0.0001
		2	-1.5473	1.3536	0.1173	-	-1337.3	0.9043	3167	2	670	<0.0001
		3	-1.5893	1.3826	-	-	-1103.1	0.8641	4266	1	671	<0.0001

The bold AIC value is the lowest among the three models for each body part.