

# Sexual Dimorphism and Reproductive Status of the Red Swamp Crayfish *Procambarus clarkii*

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

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

## BACKGROUND

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 in several states in the continental USA and 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, 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 have a high potential for rapid increases in population size in new available habitats; indeed, *P. clarkii* 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* exerts 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 loss of biodiversity (Souty-Grosset et al. 2016). Additionally, native crayfish populations have been seriously damaged due to the crayfish plague caused by the parasitic oomycete 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 introduced regions (Scalici and Gherardi 2007; Alcorlo et al. 2008; Anastácio et al. 2009; Chucholl 2011).

After reaching sexual maturity, crayfish males that belong to the family Cambaridae, which originated in North America, exhibit a cyclical dimorphism between the reproductive phenotype (form I) and the non-reproductive phenotype (form II); after breeding season, form I males moult to form II and then, upon the return of the breeding season, form II males moult and return to form I (Scudamore 1948; Stein 1976; Taketomi et al. 1990; Payne 1996; McLay and van den Brink 2016; Kawai 2017). Form I males have longer and wider chelae, hooks on the ischia of the third and fourth pereopods for holding females during copulation, and more calcified copulatory pleopods (gonopods); form II males lack these morphological characters (Suko 1953; Stein et al. 1977; Taketomi et al. 1990 1996; Huner and Barr 1991; McLay and van den Brink 2016; Kawai 2017). In contrast to males, the form alternation

of cambarid females has only been reported for some species in the genus *Orconectes* Cope 1872 (Wetzel 2002; Wetzel et al. 2005; Buřič et al. 2010a) and for the species *Cambarus elkensis* Jezerinac and Stocker 1993 (Jones and Eversole 2011). The classification of these *Orconectes* species has been updated (Crandall and De Grave 2017), and here we use the reclassified genus name *Faxonius* Ortmann 1905. In these *Faxonius* and *Cambarus* species, form I females exhibit larger chelae and wider pleons than the form II females. In *P. clarkii*, form alternation is evident in males (Taketomi et al. 1990), but it has been assumed that females do not exhibit form alternation (Oluoch 1990; Loureiro et al. 2015). Consequently, the reproductive phase of males has been assigned by identifying either sexual form I or II, whereas that of females is assigned by examining gonad maturation; active glair glands of the ventral pleon, uropods and telson; and the eggs, juveniles and egg-remains on the pleopods (Scalici and Gherardi 2007; Alcorlo et al. 2008; Anastácio et al. 2009; Chucholl 2011). Suko (1953), however, documented the presence of different sexual morphotypes with longer (form I) or shorter (form II) chelae and the occurrence of form alternation in *P. clarkii* females, although he did not show the actual measurements of the body parts. Thus, previous reports of intra-sexual dimorphism in *P. clarkii* females have been contradictory. If *P. clarkii* females exhibit different sexual forms, their reproductive status would be more easily assessed based on the chela morphology.

The present study therefore aimed to elucidate the existence of different sexual forms in *P. clarkii* females by allometric growth analyses of some body parts with reproductive information, such as the gonad maturity condition and the presence of eggs and juveniles on the pleopods, and to highlight the intra- and inter-sexual dimorphisms in *P. clarkii*. The reproductive status of a *P. clarkii* population was then evaluated by the occurrence of sexually active form I females and males.

## MATERIALS AND METHODS

### Crayfish samples

In Japan, *Procambarus clarkii* was imported from the USA as bait for culturing bullfrogs in 1927, and approximately 20 individuals were stocked into a pond in Kamakura, Kanagawa Prefecture (Sako 1987); since then, this species has expanded its range to cover the entire country (Kawai and Kobayashi 2005; Kawai 2017). *Procambarus clarkii* is listed in the “100 of Japan’s Worst Invasive Alien Species” (Ecological Society of Japan 2002), and removal campaigns using

fishing gear such as traps and nets have been practised in an effort to eradicate the populations (Nakata 2018). In the present study, we used *P. clarkii* samples that were collected during the period from April to December 2011 through removal campaigns at the public natural park in Yokohama, Kanagawa Prefecture, Japan (35°32'22–23"N, 139°34'44–46"E). The crayfish were eliminated from a pond (~1900 m<sup>2</sup>, maximum depth ~1.5 m) with fishing gears such as bait traps (mesh size, 9–13 mm) and scoop nets (mesh size, 4 mm). The number of days that were taken to collect the crayfish and the number of crayfish collected are summarized for each month in table 1. The total numbers of female and male specimens were 566 and 476, respectively.

### Crayfish measurements

The crayfish samples were brought to the laboratory and stocked in the refrigerator at 4°C. The samples were sexed based on the morphology of the pleopods (Suko 1953) (see Fig. S1A, B). Several body parts of the intact specimens were then measured to the nearest 0.01 mm as follows (see Fig. S1C–E): for both sexes, the postorbital carapace length (POCL, from the edge of the eye socket to the posterior margin of the carapace) (Fig. S1C), and the propodus width (the widest part of the propodus) and length (from the tip of the propodus to the carpal joint) of the right and/or left chelipeds were measured (Fig. S1D); for males, the first gonopod length (from the base to the tip of the right gonopod) was also measured (Fig. S1E), while for the females, the pleon width (the widest part of the second pleonite) was assessed (Fig. S1C). Males were assigned as either form I or form II morphotypes based on the presence of hooks on the ischia of the third and fourth pereopods and calcified whitish gonopods (see Fig. S2) with developed and cornified apical lobe (Taketomi et

al. 1990 1996; Kawai 2017). Females were checked for eggs or juveniles on their pleopods. All male and female specimens were dissected, and the gonads were removed and weighed to the nearest 1 mg. Some minute gonads could not be removed from the specimens (36 females and 6 males). The gonadosomatic index (GSI), an effective tool for representing gonad maturity condition in crayfish species, was used and calculated as [gonad weight / body weight] × 100 (Alcorlo et al. 2008; Chucholl 2011; McLay and van den Brink 2016; Jin et al. 2019). In the present study, there were specimens that lost both chelipeds (13 females and 7 males) or one cheliped (54 females and 34 males), and form I crayfish with larger chelipeds may be heavier than same-sized form II, leading to bias in the calculations of the weight-based GSI values. Therefore, we calculated the GSI as [gonad weight / POCL<sup>3</sup>] × 1000. Alcorlo et al. (2008) reported that oocyte diameter increased with increasing GSI values in *P. clarkii* females. To examine the oocyte development using GSI values, the gonads of 14 females, with GSI values from 0.9 to 13.6, were preserved in 10% formalin solution. The maximum diameters of oocytes were then measured to the nearest 1 μm using a Nikon stereomicroscope (MZ-800; Nikon Corp., Tokyo, Japan) equipped with a digital camera and an image analysing system (Nikon Digital Sight and NIS-Elements software). The number of oocytes measured for each gonad specimen ranged from 153 to 291, and frequency distributions of the diameters of oocytes were illustrated for respective gonads.

The carapace length (CL, from the tip of the rostrum to the posterior margin of the carapace) or the total length (TL, from the tip of the rostrum to the posterior margin of the telson) are often measured as body size of *P. clarkii* (Fig. S1C). To compare results of the present study, which employed POCL, to other studies that employed the CL or TL measurements,

**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.

the POCL, CL and TL were measured for additional specimens (50 males and 50 females) collected in October and November 2019 from the same pond as the 2011 collections. The linear regression equations used to convert POCL to CL and TL values were as follows (Fig. S3): CL =  $-0.5871 + 1.321 \text{ POCL}$  ( $R^2 = 0.9832$ ,  $F_{1,98} = 5726$ ,  $P < 0.0001$ ); and TL =  $3.6814 + 2.4523 \text{ POCL}$  ( $R^2 = 0.9757$ ,  $F_{1,98} = 3935$ ,  $P < 0.0001$ ). In the present study, the statistical analyses were performed with R statistical software (R3.5.2; R Core Team 2018) at a 5% significance level.

### Intra- and inter-sexual dimorphisms

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

Suko (1953) stated that in *P. clarkii* females, the reproductive form I has longer chelae than those of form II of the same body size. In the present study, two different morphotypes with larger or smaller chelae were distinguished by the allometric growth equations with the aid of the information about reproductive status, such as the gonad maturity condition represented by the GSI values and the presence of eggs and juveniles on the pleopods. The GSI values of the *P. clarkii* females increased with oocyte development, and the oocyte diameter particularly increased after the gonad reached the GSI value of 3, as shown in figure S5. Therefore, we considered females with GSI values  $> 3$  to be candidates for the reproductive morphotype (form I). Additionally, one ovigerous female and five females carrying juveniles were found in the collected specimens. The chela propodus widths and lengths were plotted against the POCL in these potentially or actively reproductive

females, with the exception of one ovigerous female that had lost both chelipeds, and are shown in figure S6. The allometric growth equations between the POCL and chela dimensions were estimated as follows: chela propodus width,  $y = 0.0725x^{1.4043}$  (statistics between  $\ln y$  and  $\ln x$ :  $n = 41$ ,  $R^2 = 0.8756$ ,  $F_{1,39} = 274.5$ ,  $P < 0.0001$ ); and chela propodus length,  $y = 0.2408x^{1.3752}$  (statistics between  $\ln y$  and  $\ln x$ :  $n = 41$ ,  $R^2 = 0.9373$ ,  $F_{1,39} = 582.7$ ,  $P < 0.0001$ ). Our female samples appeared to have two morphological groups with relatively large or small chelae, and the chela dimensions of the potentially or actively reproductive females were scattered within the larger chela group (see the RESULTS section). Here, the above mentioned allometric growth curves of these potentially or actively reproductive females decreased in parallel with decreasing intercept values, so that the lowest data plots for these females just matched the adjusted allometric growth curves (see Fig. S6) (chela propodus width,  $y = 0.0633x^{1.4043}$ ; chela propodus length,  $y = 0.2184x^{1.3752}$ ); these adjusted allometric growth curves successfully discriminated between the larger and smaller chelae groups (see the RESULTS section). We assigned a female as form I when either the chela propodus width or chela propodus length were beyond the discriminant value calculated by substituting its POCL into the adjusted allometric growth equation or form II when both the chela propodus width and chela propodus length were below the discriminant values.

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

### Reproductive status

To statistically evaluate the seasonal changes in the reproductive status of the *P. clarkii* population, a generalized linear model (GLM) (*glm* function with a Gaussian error distribution) was performed to evaluate

the differences in GSI values (response variable) among the months (April–December) and the sexual morphotypes (form I and II) (categorical explanatory variables) of each sex. Next, the numbers of form I and II crayfish after reaching the size of onset of sexual maturity (females, 22 mm POCL; males, 21 m POCL) (see the RESULTS section) were summarized for the different body size groups (22–25.99 mm (female), 21–25.99 mm (male), 26–29.99 mm, 30–33.99 mm, 34–37.99 mm, 38–41.99 mm (both sexes) POCL) and months (April–December) (see Table S1 for the number of crayfish and figures S7 and S8 for the size frequency distributions of crayfish). Then, differences in the proportions form I (response variable) among the different body size groups and months (categorical explanatory variables) were evaluated for each sex using the GLM analysis (*glm* function with a quasibinomial family (logit link), taking into account the overdispersion of the data). All females with  $\geq 22$  mm POCL were form I in October, and all females and males were form I in the body size class with  $\geq 38$  mm POCL; these data were excluded from the analyses because reliable coefficients could not be estimated by the GLM analysis. The statistical significance of the explanatory variables in the GLM analysis was evaluated with an *F*-test using the *Anova* function (Type II) (Fox and Weisberg 2011). Pearson's product moment correlation coefficient (*r*) was used with a *t*-test to evaluate the relationship between the overall monthly proportions of form I females and males. The sex ratio was calculated as the [number of males / number of total crayfish], and a binomial test was used to test the null hypothesis for the sex ratio in each month ( $H_0$ : sex ratio = 0.5).

## RESULTS

### Intra- and inter-sexual dimorphisms

The females could be divided into two morphotypes with relatively large or small chelae by the discriminant allometric growth equations between the POCL and chela propodus width or length that were derived from the potentially reproductive and sexually active females (Fig. 1A, B). These females with larger or smaller chela should correspond to form I or II because potentially or actively reproductive females were included in the group with larger chela. Form I occurred in females that had grown to a POCL of 22 mm or greater. Among the three log-transformed allometric growth equation models applied for the chela dimensions, model 1 and model 2 were selected as the best (Table 2), indicating the existence of intra-

sexual dimorphism in the chelae of *P. clarkii* females. Model 1 was also the best for pleon width (Table 2) as the differences in the intercept and slope were small between the morphotypes (Table 2, Fig. 1C). Males were assigned as either form I or II based on their external reproductive characteristics, and form I occurred in males that had grown to POCL of 21 mm or larger (Fig. 2A, B). The form I and II males had relatively large and small chelae, respectively. As seen in the females, the allometric growth analyses detected intra-sexual dimorphism among the males, and there was distinct dimorphism in the chelae (Table 2, Fig. 2A, B) but small dimorphism in the first gonopod (Table 2, Fig. 2C). The chelae showed positive allometric growth ( $b = \sim 1.2\text{--}1.5$ ) regardless of the sexual morphotype in both sexes, and the pleon widths and gonopod lengths showed approximately isometric growth ( $b = \sim 0.9\text{--}1.1$ ) (Table 2).

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

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

### Reproductive status

The GSI values significantly varied among the months and sexual morphotypes for both females (month,  $F = 9.5279$ , *d.f.* = 8,  $P < 0.0001$ ; morphotype,  $F = 157.77$ , *d.f.* = 1,  $P < 0.0001$ ) and males (month,  $F = 26.697$ , *d.f.* = 8,  $P < 0.0001$ ; morphotype,  $F = 28.316$ , *d.f.* = 1,  $P < 0.0001$ ). The form I females showed higher GSI values than the form II females, and the form I females with higher GSI values were found in April and September (Fig. 6A). One ovigerous female (23.2 mm POCL) and five females carrying juveniles

(26.8–37.7 mm POCL) were found in the specimens collected in April 24, 2011. The differences in the GSI values between form I and II males were small, and the GSI values tended to slightly decrease or vary less from April to July, then increase until September and decrease again until December (Fig. 6B).

The proportions of form I crayfish significantly varied among the months and body size groups in the females (month,  $F = 3.1070$ ,  $d.f. = 7$ ,  $P = 0.0248$ ; body size,  $F = 23.653$ ,  $d.f. = 3$ ,  $P < 0.0001$ ) and males (month,  $F = 6.6543$ ,  $d.f. = 8$ ,  $P = 0.0003$ ; body size,  $F = 12.0611$ ,  $d.f. = 3$ ,  $P < 0.0001$ ). The proportions of form I crayfish tended to increase with increasing body size, and the largest group ( $\geq 38$  mm POCL) comprised all form I

females and males (Fig. 7). The monthly proportions of form I females and males were significantly correlated ( $r = 0.7743$ ,  $t = 3.2369$ ,  $d.f. = 7$ ,  $P = 0.0143$ ) (Fig. 8). The overall proportions of form I females and males fluctuated around 50% from April to September, increasing to 83–100% in October. The proportion of form I was higher in males (76%) than in females (50%) in November and decreased to the same level in both sexes in December (Fig. 8). The sex ratio fluctuated around 0.5, but significant unbalanced sex ratios that favoured the females and males were detected in April ( $P = 0.0161$ ) and November ( $P = 0.0275$ ), respectively (Fig. 8). The sex ratio appeared to be male biased in December, but this was not statistically significant due

**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 representing 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	-	<b>-982.7</b>	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	<b>-1476.8</b>	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	<b>-1988.9</b>	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	<b>1230.3</b>	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	<b>1230.3</b>	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	<b>-618.8</b>	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
		2	-1.8203	1.4755	0.3760	-	<b>-922.2</b>	0.9525	4674	2	466	< 0.0001
		3	-2.9988	1.8806	-	-	-231.0	0.7918	1776	1	467	< 0.0001
	First gonopod length	1	-1.3167	1.0637	0.6268	-0.1782	<b>-1341.8</b>	0.9263	1977	3	472	< 0.0001
		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
	Gonad weight	1	-5.4075	2.3708	2.7552	-0.6876	<b>540.5</b>	0.6405	276.8	3	466	< 0.0001
		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
	GSI	1	1.5002	-0.6292	2.7552	-0.6876	<b>540.5</b>	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.

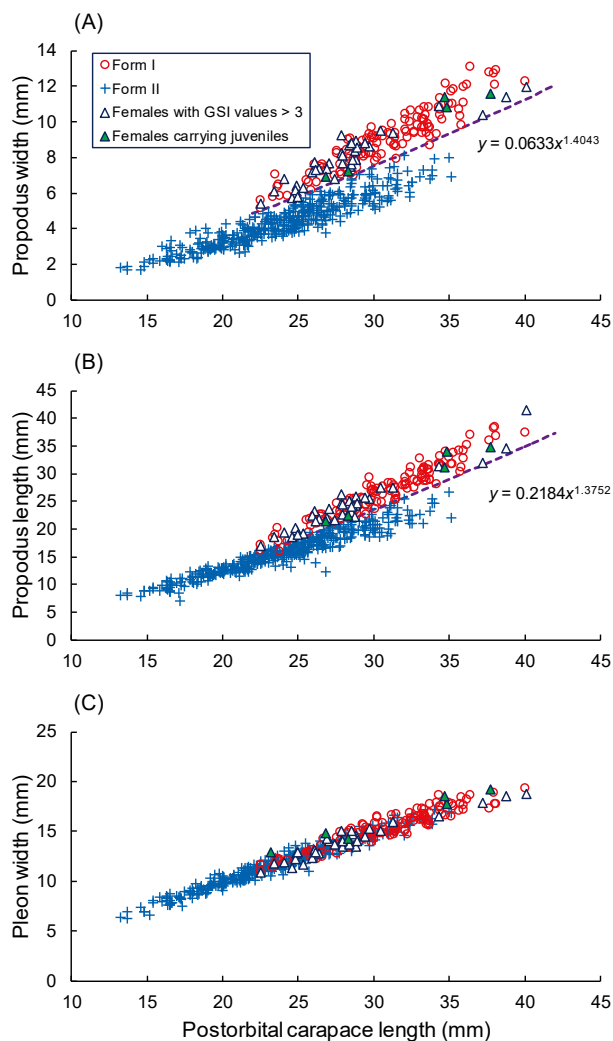
to the small sample size ( $n = 8, P = 0.7266$ ).

## DISCUSSION

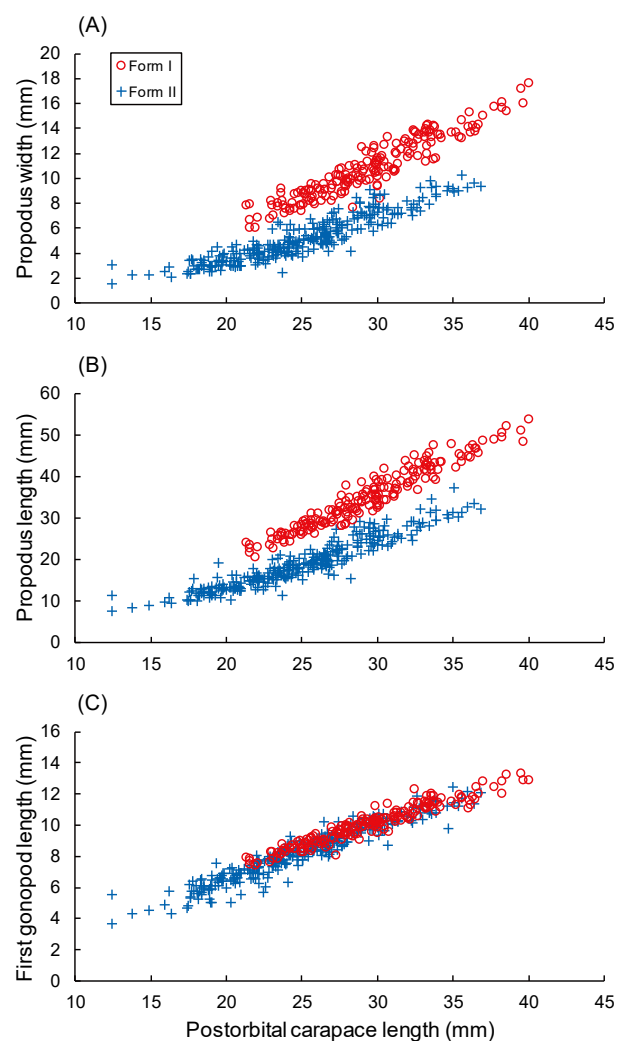
### Intra- and inter-sexual dimorphisms

Males of the American cambarid crayfish occur as either sexually active form I or sexually inactive form II morphotypes based on the presence of hooks on the ischia of the third and fourth pereopods and more calcified rigid gonopods (Suko 1953; Stein et al.

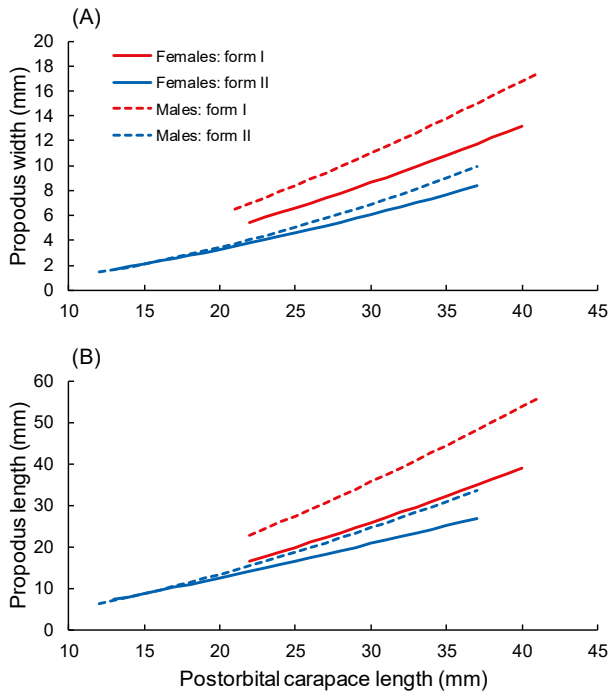
1977; Taketomi et al. 1990 1996; Huner and Barr 1991; McLay and van den Brink 2016; Kawai 2017); form I males have longer and wider chelae than form II males (Suko 1953; Stein et al. 1977; Huner and Barr 1991; McLay and van den Brink 2016; Buřič et al. 2010b). Sexually active females (form I) of the American cambarids have been identified based on the presence of developed glair glands, oocytes in the gonopores, and/or eggs and juveniles on the pleopods (Wetzel 2002; Buřič et al. 2010a; Jones and Eversole 2011), the architecture of annulus ventralis (Wetzel et al. 2005; Jones and Eversole 2011) and the occurrence of mating with



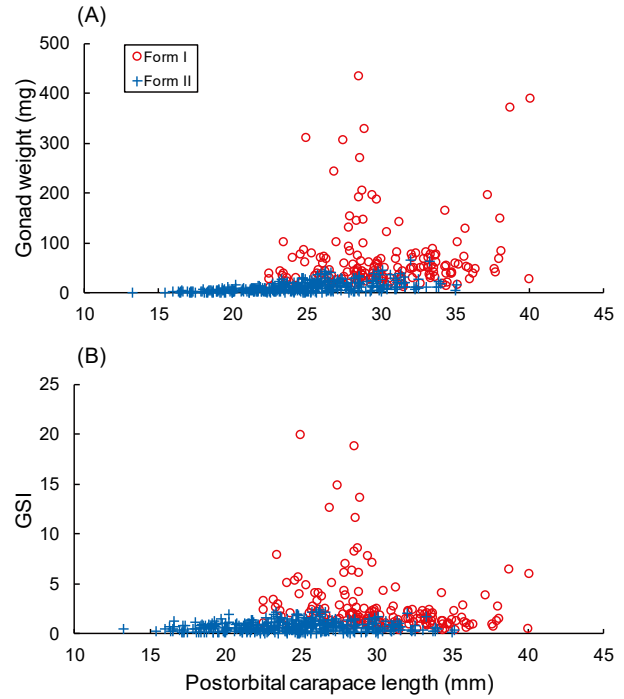
**Fig. 1.** Growth of the chela propodus width (A), chela propodus length (B) and pleon width (C) relative to the postorbital carapace length in female red swamp crayfish *Procambarus clarkii*. Data are shown for the different sexual morphotypes, reproductive form I and non-reproductive form II, the potentially reproductive females with GSI values > 3 and the reproductive females carrying juveniles. Allometric growth curves discriminating the different sexual morphotypes are shown for chela propodus width and length. See figure S6 for discriminant functions.



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



**Fig. 3.** Inter-sexual dimorphism of the chela propodus width (A) and chela propodus length (B) in red swamp crayfish *Procambarus clarkii*. Allometric growth curves were calculated based on the equations estimated for females and males in the respective sexual morphotypes shown in table 3.



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

**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 representing the change in the response variable relative to the baseline category (female)

		Coefficient estimates					AIC	$R^2$	F	df1	df2	P
Form	Response variable	Model	Intercept	lnPOCL	Sex-Male	lnPOCL × 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	-	<b>-815.8</b>	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	-	<b>-1051.0</b>	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	<b>-871.4</b>	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	<b>-1381.3</b>	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.



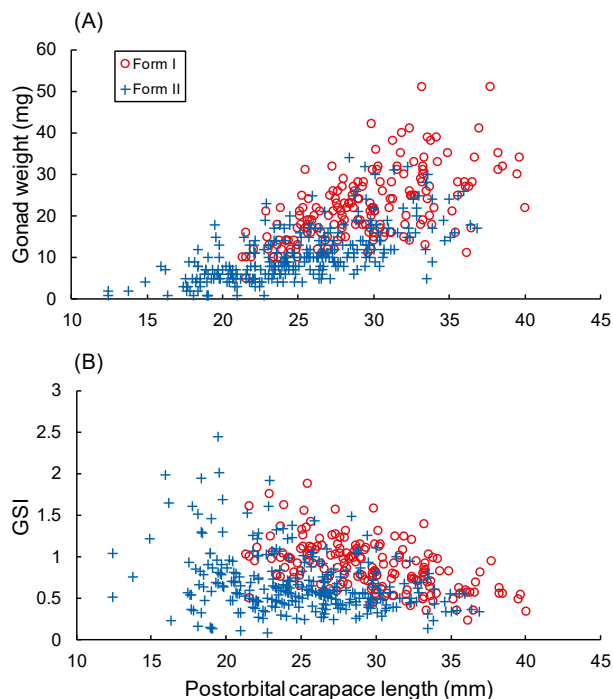
males (Wetzel 2002; Buřič et al. 2010a); then, the body dimensions such as chela size and pleon width were compared between form I and form II females. Wetzel et al. (2005) documented that form I and form II females of *Faxonius pardalotus* (Wetzel, Poly and Fetzner 2005) exhibited different morphologies of annulus ventralis: form I females have a corneous sternum and strongly convoluted structures, whereas those of form II females are less cornified and convoluted. Kawai (2017) found these two characters in the annulus ventralis of *P. clarkii* and suggested the existence of form I and form II morphotypes in *P. clarkii* females.

In the present study, based on the documentation by Suko (1953) that different sexual morphotypes with longer (form I) or shorter (form II) chelae were found in *P. clarkii* females, we attempted to elucidate the existence of different sexual forms in *P. clarkii* females by allometric growth analyses of some body parts with reproductive information, such as the gonad maturity condition and the presence of eggs and juveniles on the pleopods. Females could be assigned into two morphotypes with relatively large or small chelae, corresponding to form I or II because potentially or actively reproductive females were included in the morphotype with larger chela. We also identified the sexual morphotypes of males based on the external

morphological characteristics, and conducted allometric growth analyses of some body parts. Our results confirm the existence of different sexual morphotypes in *P. clarkii* females and highlight the intra- and inter-sexual dimorphisms in the chelae of this species (Figs. 1–3); the chelae were largest in the form I males, followed by the form I females and form II males, and were the smallest in the form II females (Fig. 3). To further elucidate the reproductive characteristics of form I and form II females of *P. clarkii*, the architecture of annulus ventralis should be examined in relation to the sexual morphotypes revealed by the present study and confirm the occurrence of mating of these females with form I males.

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

Stein (1976) suggested that chelae of crayfish may be used for (1) prey capture and manipulation, (2) defence against predators, (3) inter- and intraspecific interactions, and (4) reproductive activities. *Faxonius propinquus* (Girard 1852), which have large chelae, are less vulnerable to predation by fish and superior competitors in intraspecific interactions (Stein 1976). Ueno and Nagayama (2015) reported that large *P. clarkii* with smaller chelae were beaten by small ones with larger chelae. Thus, selection might favour large chelae in *P. clarkii*, resulting in the positive allometric growth of the chelae regardless of sex or the sexual morphotype, as demonstrated by our allometric growth analyses. The observation that form I cambarid males have large chelae suggests that the chelae of sexually



**Fig. 5.** Relationships between the postorbital carapace length and gonad weight (A) and the gonadosomatic index (GSI) (B) in male red swamp crayfish *Procambarus clarkii*. Data are shown for the different sexual morphotypes, reproductive form I and non-reproductive form II.

active males might be under stronger directional selection. Large chelae should provide an advantage for males competing to acquire receptive females. In *F. propinquus*, males with larger chelae more successfully mate with females than similar-sized males with smaller chelae (Stein 1976). The *P. clarkii* form I females also had larger chelae than form II conspecifics (Figs. 1, 3), which is known to occur in *Faxonius limosus* (Rafinesque 1817) (Buřič et al. 2010a) and *C. elkensis* (Jones and Eversole 2011). Reproductive *P. clarkii* females remain in the burrows to lay and brood eggs (Huner and Barr 1991). Laboratory experiments demonstrated that in *P. clarkii*, maternal (carrying eggs or juveniles) female residents won a significantly higher proportion of their contests for shelters than did non-maternal residents, regardless of whether the intruders were males or non-maternal females (Figler et al. 1995; Peeke et al. 1995). Large chelae might also be advantageous for maternal *P. clarkii* females defending offspring. The males of *P. clarkii* use their chelae to grasp and hold female chelae during copulation (Ameyaw-Akumfi 1981). Selection might favour larger chelae in sexually active females to match the chelae size of males for successful courtship.

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

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

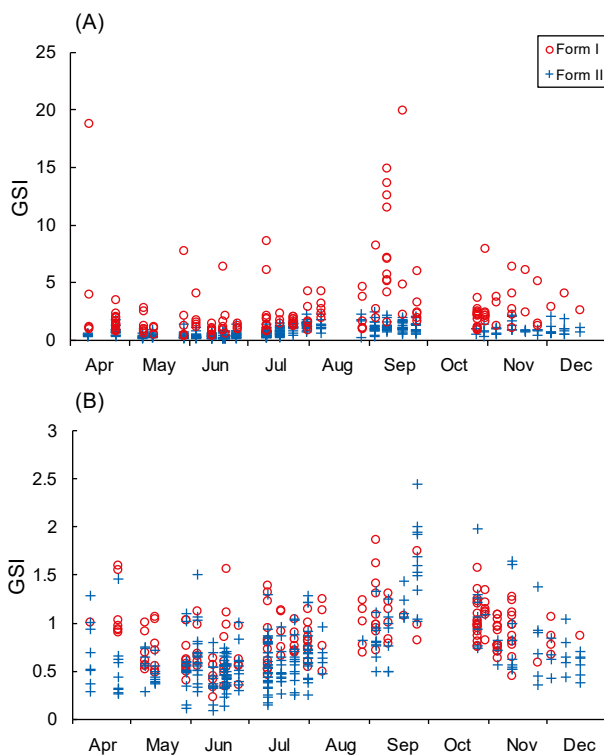
developmental stages, A–E, based on the gonopod morphology and the histologically examined testis development. They revealed that the development of gonopods was complete and hooks (reversed spines) were present on the ischia of the third and fourth pereopods in stage E, which is identical to form I,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

### Reproductive status

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

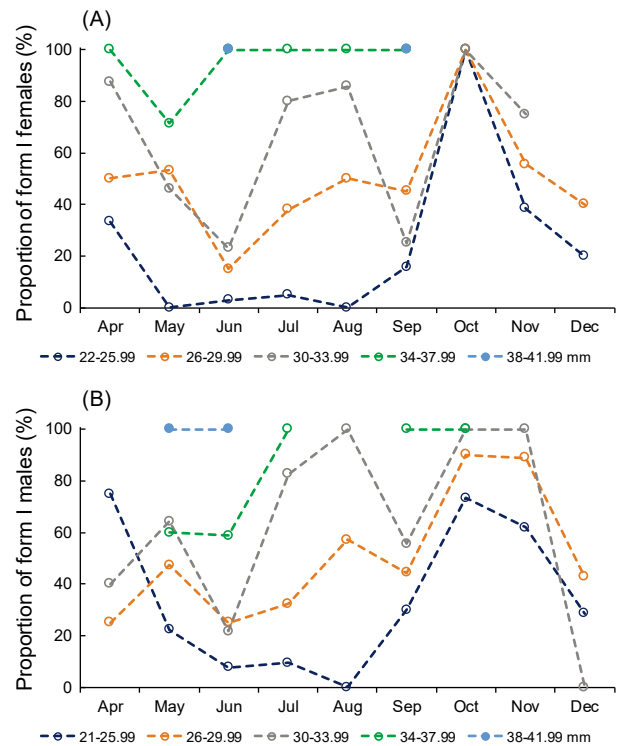
Suko (1958) summarized the main reproductive cycles of the Urawa *P. clarkii* population, but did not show population dynamics data, such as growth or proportions of sexual morphotypes; the crayfish hatched in the late autumn of the main reproductive season rapidly grow after the warm spring, moult to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

moult and return to form II in autumn. In the present study, the occurrence of form I crayfish varies among different body size groups, and the proportions of form I individuals in the smaller groups showed higher values in spring (April) and autumn (October) in both sexes (Fig. 7). Changes in the proportions of the sexual morphotypes in smaller crayfish of the Yokohama population appeared to support the observation that autumn- and spring-born crayfish of the Urawa have alternative forms. In the Yokohama population, the overall proportions of form I crayfish peaked and reached > 80% in October, and the proportion of form I females decreased in November, whereas that of the form I males was still high at 76% in November (Fig. 8). The form I males might be actively seeking receptive females and might be more likely to be collected by bait traps than females; therefore, the sex ratio might be biased towards males in November (Fig. 8). However, the reason for the female-biased sex ratio in April is not known. Many females exhibited a form I phenotype in October, but their GSI values were not as high in the Yokohama population (Figs. 6–8). In the autumn season, female *P. clarkii* require approximately 1.5 months for their eggs to hatchlings to rear stage 3 juveniles that depart from mother’s pleopods (Suko 1956 1961).

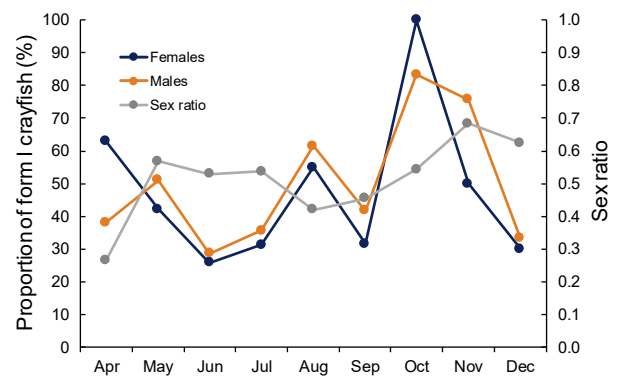


**Fig. 6.** Changes in the gonadosomatic index (GSI) in red swamp crayfish *Procamburus clarkii* females (A) and males (B) from April to December 2011. Data are shown for the different sexual morphotypes, reproductive form I and non-reproductive form II.

Almost all form I females of *P. clarkii* laid eggs once during the intermoult period, and they require several months for gonad development before oviposition, and the females that copulated with males in early winter spawned the next spring in the Urawa population (Suko 1958). In the Yokohama population, the form I females that were collected after late October might include females that had already bred and females that would



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



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

oviposit in the next spring, as females carrying eggs and juveniles were found in April.

Buřič et al. (2010a b) examined the moulting events in relation to form alternation in *F. limosus* for 8–10 months in captivity and reported that the majority of females (58%) and males (84%) moulted twice and showed form alternation (form I → form II → form I); the remainder of the females (42%) and males (9%) moulted once without form alternation (form I → form I), and some males (7%) did not moult at all. They also documented that the initial body size was smallest in the twice moulted crayfish, followed by the once moulted or not moulted crayfish. Taketomi et al. (1990) documented that the proportions of form I males tended to increase with increasing body size in *P. clarkii* collected from Kumamoto (32°46'N, 130°45'E), Japan. They also stated that many of the form I males with 25–30 mm POCL changed to form II during their autumn/winter moult and returned to form I during the next spring/summer moult, whereas form I males greater than 30 mm POCL did not undergo an autumn/winter moult, but they did not refer to the next spring/summer moult of these crayfish. In general, the intermoult periods increased with increasing body size; and thus, the moulting events during the year decrease with growth in decapod crustacean species (Kurata 1962). In the Yokohama *P. clarkii* population, higher and less variable proportions of form I occurred in larger body size groups (Fig. 7), suggesting that less form alternation occurs in larger crayfish. Cambarid crayfish may exhibit size-dependent moulting and form alternations. This hypothesis should be tested by laboratory culture experiments for *P. clarkii* populations.

## CONCLUSIONS

Our allometric growth analyses demonstrated the existence of two sexual morphotypes in the females of the red swamp crayfish *Procambarus clarkii* and highlighted the inter-sexual dimorphism of the chela dimensions in this species. In *P. clarkii* populations, the reproductive status of males is evaluated by determining the sexual morphotypes, whereas that of females has historically been conducted by examining gonad maturation; active glair glands; and the eggs, juveniles and egg-remains on the pleopods (Scallici and Gherardi 2007; Alcorlo et al. 2008; Anastácio et al. 2009; Chucholl 2011). If we had relied on only the traditional criteria using GSI values for evaluating the female reproductive status, we would not have been able to follow the reproductive cycle among the different body size groups in the Yokohama *P. clarkii* population. The discrimination of the sexual morphotypes based on

chela allometric growth would allow us to evaluate the female reproductive status more easily and precisely in conjunction with the traditional reproductive criteria, and this might help us understand the *P. clarkii* population dynamics as a basis for developing control measures for this invasive alien species.

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

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

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

**Consent for publication:** Not applicable.

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

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## Supplementary Materials

**Fig. S1.** Ventral view of female (A) and male (B) showing female pleopods and male gonopods (dotted lines), dorsal views of female (C) and its right chela (D) and ventral view of male (E) showing body parts for size measurements of red swamp crayfish *Procambarus clarkii*. Second gonopods were removed from the male specimen shown in the panel E. Abbreviations: CL, carapace length; GpL, first gonopod length; POCL, postorbital carapace length; PrL, propodus length of the cheliped; PrW, propodus width of the cheliped; PW, pleon width; TL, total length. The POCL of the specimens: female, 34.7 mm (A, C, D); male, 33.1 mm (B, E). (download)

**Fig. S2.** Dorsal (A) and ventral views (B, C) of form I and form II males of red swamp crayfish *Procambarus clarkii* with an identical postorbital carapace length (31.1 mm). Arrows in the panel B indicate the hooks on the ischia of the third and fourth pereopods of a form I male. Dotted lines in panels B and C indicate the first and second gonopods. (download)

**Fig. S3.** Relationships between the postorbital carapace length and carapace length (A) and the total length (B) of red swamp crayfish *Procambarus clarkii*. The measured parts are as follows: postorbital carapace

length, which covers the edge of the eye socket to the posterior margin of the carapace; carapace length, which covers tip of the rostrum to the posterior margin of the carapace; and total length, which covers the tip of the rostrum to the posterior margin of the telson. (download)

**Fig. S4.** Scatter plots between the dimensions of right and left chelae in females (A, B) and those of the males (C, D) of red swamp crayfish *Procambarus clarkii*. The line indicates the same size between the right and left chelae. (download)

**Fig. S5.** Frequency distributions of the diameters of oocytes in ovaries with various gonadosomatic index (A–N) in red swamp crayfish *Procambarus clarkii*. The gonadosomatic index is shown in parentheses in each panel. (download)

**Fig. S6.** Growth in the chela propodus width (A) and chela propodus length (B) relative to the postorbital carapace length in female red swamp crayfish *Procambarus clarkii*. The red dotted allometric growth curves were estimated for females with gonadosomatic index (GSI) values exceeding 3 and females carrying juveniles. The red dotted curves decreased in parallel with the decreasing intercept values so that the lowest data plots just matched the adjusted allometric growth curves (broken dark blue curves). (download)

**Fig. S7.** Size frequency distributions of the reproductive form I and non-reproductive form II females of red swamp crayfish *Procambarus clarkii* collected from a small pond in Yokohama, Japan, from April through December 2011. The sexual morphotypes of some females could not be determined because of the loss of their both chelipeds. (download)

**Fig. S8.** Size frequency distributions of the reproductive form I and non-reproductive form II males of red swamp crayfish *Procambarus clarkii* collected from a small pond in Yokohama, Japan, from April through December 2011. (download)

**Table S1.** Number of individuals assigned to either sexual morphotype, reproductive form I or non-reproductive form II, among the crayfish that had reached the size of sexual maturity (females, 22 mm in postorbital carapace length (POCL); males, 21 mm POCL) in the respective size classes of the red swamp crayfish *Procambarus clarkii* collected from a small pond in Yokohama, Japan in 2011. (download)