

## Effects of body size on mating behaviour and spawning of the red swamp crayfish *Procambarus clarkii*

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### Abstract

The red swamp crayfish *Procambarus clarkii* is an invasive alien species worldwide. The sterile-male-release technique (SMRT), which uses sterile but sexually active males, may help control its population. Body sizes of males and females affect mating and spawning success through mate choice. However, mating behaviour and spawning between different-sized males and females of *P. clarkii* remain unclear. The present study elucidated the effects of body size on mating behaviour and spawning of *P. clarkii* to develop effective SMRT for this invasive species. We prepared nine test groups as a combination of males and females in three size classes, and the mating behaviour of the test pairs was videorecorded for 48 h; then, spawning and egg development rate were examined. The body size difference negatively affected the copulation frequency and duration in the pairs, and the incidence of copulation was low in the pairs between the largest males and smallest females. When mating with smaller males, females delayed spawning and the egg development rate decreased, likely because the females may be waiting an opportunity to mate with larger males with higher reproductive potential. Our results might be consequences of mutual mate choice (i.e., preference for larger mates). Our findings indicate that in SMRT, sterile medium- and large-sized males with high reproductive potential are recommended to satisfy the mutual mate choice in *P. clarkii* populations of various-sized females.

**Key words:** mate choice; copulation; invasive species; Cambaridae

### Introduction

The red swamp crayfish *Procambarus clarkii* (Girard 1852) (Decapoda; Cambaridae) is native to northeastern Mexico and south-central USA (Hobbs 1972); it is commonly used for fish bait and commercially harvested from swamps, marshes, and cultivated ponds in North America (Huner and Bar 1991). This crayfish species has been successfully introduced into all continents except Australia and Antarctica for human consumption by aquaculture and/or ornamental animal trade (Hobbs et al. 1989; Huner and Bar 1991; Chucholl 2013; Loureiro et al. 2015; Souty-Grosset et al. 2016; Oficialdegui et al. 2020).

*Procambarus clarkii* adapts to various

freshwater environments, including swamps and marshes that are periodically flooded and drained (Huner and Barr 1991). It exhibits rapid growth rates, early maturation, longer reproductive season, 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; Aquilon and Gherardi 2008a), while showing the ability to exit the water and move overland (Ramalho and Anastácio 2015). These physiological and behavioural characteristics have enabled *P. clarkii* to successfully establish self-sustaining populations after escaping or releasing into freshwater bodies from aquaculture ponds or

aquariums in introduced regions (Loureiro et al. 2015; Souty-Grosset et al. 2016).

*Procambarus clarkii* is considered one of the worst invasive species worldwide because it exerts negative impacts on the ecosystems and native species in newly colonised regions. This crayfish consumes aquatic plants and algae and preys on several aquatic species, including amphibians, molluscs, and macroinvertebrates, leading to loss of biodiversity, and it is a vector of the parasitic pathogen oomycete causing the crayfish plague, which is a highly infectious disease of indigenous crayfishes (Loureiro et al. 2015; Souty-Grosset et al. 2016). In regions invaded by *P. clarkii*, control measures such as trapping, physical methods (pond drawdown and liming), biocontrol with indigenous fish predators, and sterile-male-release technique (SMRT), which uses sterile but sexually active males, have been applied (Aquiloni et al. 2009a, 2010; Gherardi et al. 2011; Loureiro et al. 2015; Souty-Grosset et al. 2016; Manfrin et al. 2021). Spermatophores are deposited in a seminal receptacle (annulus ventralis) of females (Huner and Barr 1991) via gonopods of sterile males during copulation. SMRT has the advantage that sterile males may seek and mate with the remaining few females even at low crayfish density (Aquiloni et al. 2009a); under such a circumstance, the efficacy of mechanical removal using traps may be substantially decreased.

Body sizes of males and females affect mating and spawning success through mate choice in decapod crustaceans (Fukui 1995; Gherardi et al. 2006; Reading and Backwell 2007; Wada et al. 2011; Robertson and Butler 2013). Therefore, information of body size effects on mating and spawning is vital for developing effective SMRT.

*Procambarus clarkii* males can identify the sex of a conspecific during mating interactions through olfaction alone and females through a combination

of olfaction and vision (Aquiloni et al. 2009b). Aquiloni and Gherardi (2008b) examined the effects of body size, chela size, and chela symmetry, social status (dominant or subdominant), and mating status (mated or virgin) of a partner on mutual mate choice in *P. clarkii* males and females by conducting a binary choice experiment, in which two same-sex crayfish (targets) with opposing characteristics were simultaneously presented to an opposite-sex crayfish (chooser). They demonstrated that both sexes were significantly attracted by large-sized targets and were not affected by other characteristics except for selecting virgin females by males. Aquiloni and Gherardi (2008c) observed the mating behaviour of virgin females paired with either small or large males and examined the number, size, and weight of offspring (i.e., eggs and juveniles). They reported that females approached large males more often than small males, and they spawned a similar number of eggs regardless of the mate size but produced larger offspring when mated with larger males.

Thus, body size may affect reproductive success in *P. clarkii*, leading to male-size dependent SMRT success. However, mating behaviour and spawning have only been evaluated between females with a designated body size and males from two size classes (small or large). In the present study, to improve our knowledge of reproductive biology and ecology of *P. clarkii* as a basis for developing effective SMRT for controlling invasive populations, we aimed at elucidating the effects of male and female body size on mating behaviour and spawning.

## Materials and Methods

### Test animals

In the Kanto region (35°31–48'N, 139°34–49'E)

of Japan, the *P. clarkii* population exhibit continuous copulation and spawning incidences throughout the year, with high reproduction noted in two seasons: September to October (autumn, most prolific) and May to June (spring) (Suko 1956 1958; Hamasaki et al. 2020). In the present study, we used *P. clarkii* collected in ponds at two public natural parks in the Kanto region during 1 June to 5 August 2016 before beginning the main reproductive season of this species.

Approximately 100 crayfish were transferred into a laboratory at the Tokyo University of Marine Science and Technology, Tokyo, Japan. They were sexed and measured for the postorbital carapace length (POCL, from the edge of the eye socket to the posterior margin of the carapace) to the nearest 0.1 mm using a digital caliper. They were then cultured individually in plastic cups with lids (13 cm diameter and 10 cm height), which were set in a 400-L rectangular stock tank by sex under natural photoperiod conditions between 13L: 11D and 14L: 10D at room temperatures (20–26°C). Culture cups had many small holes that allowed tap water to enter the cup from the stock tank. Aeration was provided to the stock tanks, and the water in the tanks was renewed five times per week using dechlorinated tap water stocked in another containers. Crayfish were fed with commercial formula feeds for crayfish (Kyorin, Himeji, Hyogo, Japan) ad libitum every 2 days.

In mid-August, we selected crayfish with all intact appendages (33 females and 33 males) considering the minimum size at sexual maturity of this species (21–22 mm POCL) (Suko 1953; Hamasaki et al. 2020). The selection of males also relied on the reproductive phenotype, exhibiting hooks on the ischia of the third and fourth pereopods and calcified copulatory pleopods (Taketomi 1990, 1996). They were measured for POCL, and the individuals of each sex were then

divided into three size groups (mean POCL  $\pm$  standard deviation, range): large [male (LM), 40.9  $\pm$  1.7 mm, 38.2–44.2 mm, n = 10; female (LF), 40.1  $\pm$  1.9 mm, 38.1–44.8 mm, n = 10], medium [male (MM), 34.4  $\pm$  1.1 mm, 33.1–36.2 mm, n = 10; female (MF), 34.9  $\pm$  1.1 mm, 33.3–36.1 mm, n = 10], and small [male (SM), 27.8  $\pm$  1.3 mm, 25.2–29.5 mm, n = 13; female (SF), 28.6  $\pm$  1.0 mm, 26.9–30.2 mm, n = 13] crayfish.

### **Mating experiments and spawning**

We conducted mating experiments from 24 August to 29 September 2016 by pairing different sizes of males and females: LM-LF (n = 3 each), LM-MF (n = 3 each), LM-SF (n = 4 each), MM-LF (n = 3 each), MM-MF (n = 3 each), MM-SF (n = 4 each), SM-LF (n = 4 each), SM-MF (n = 4 each), and SM-SF (n = 5 each).

Circular plastic containers (61 cm diameter and 20 cm height) with pebble substrate and 7-cm-deep water were prepared for observing the mating behaviour of crayfish in a temperature-controlled room (~25°C) under natural photoperiod conditions between 11L: 13D and 13L: 11D. A brick (20 cm  $\times$  9.5 cm  $\times$  5.5 cm) was laid at the centre of the bottom of each container as a resting place for the crayfish. Two to four containers were used on the same experiment day, and they were rinsed thoroughly with freshwater after the end of each experiment. Two culture cups, each containing a male or female, were placed on either side of the brick of the test container bottom at approximately 18:00 of the day before the beginning of the experiment. At 12:00 on the experiment day, male and female individuals were released gently into the container. The behaviour of the paired crayfish was then recorded for 48 h using a video recording system with infrared cameras and infrared light sources (850 nm) installed over the observation containers.

After finishing the video recording, crayfish were removed from the containers, and females were individually stocked in plastic cups and cultured in another stock tank for spawning until 30 November 2016. Female egg-laying occurred between 20 September and 16 November. Eggs were removed from the pleopods 11 days after egg-laying. We then counted the number of eggs and determined if each egg was developed by observing the appearance of embryo at the nauplius stage (Huner and Bar 1991; Shui et al. 2021) under a stereomicroscope. Egg development rate was calculated as (number of developed eggs/number of eggs attached to the female pleopods).

### Video analysis

We observed the mating behaviour of *P. clarkii* on the time-stamped video recordings as previously reported (Ameyaw-Akumfi 1981; Peddio et al. 2019). We divided mating behavioural process into the following three phases: phase 1, the male grasps the female by her chelipeds, antennae, antennules or rostrum with his claws while attempting to climb on her back; phase 2, the male then begins to turn the female over, the female then is turned either side while stretching her chelipeds ahead and her pleon on the straight, and the male attempt to adjust her ventral parts as he positions himself while holding the partner; phase 3, the pair completely falls to one side while in close contact of their ventral surfaces (i.e., male gonopods and a female annulus ventralis) and maintain its position for the entire period of copulation. Some test pairs of crayfish did not exhibit phase 1 and others ended in phase 1 or phase 2 or proceeded to phase 3 in mating behaviour.

To analyse the mating behaviour quantitatively in the paired crayfish, we counted the number of grasping events by males to females (phase 1), number of holding events by males to females for

proceeding copulation (phase 2), number of copulation events in the pairs (phase 3), and total duration (in seconds) of the copulation position (phase 3).

### Statistical analysis

Statistical analyses were performed using R statistical software (R4.1.1; R Core Team 2021) at a 5% significance level. We used a generalised linear model (GLM) to evaluate the effects of body size (explanatory variable) on mating behaviour and spawning (response variables) in the test pairs. In the GLM analyses, the following response variables were considered: incidence of grasping behaviour, incidence of holding and copulation behaviour [presence (1) or absence (0)], number of grasping events, number of holding events and number of copulation events, copulation success rate (number of copulation events/number of holding events), total copulation duration, mean duration per copulation, and number of days from copulation to spawning. We used body size difference (BSD) between male and female (male POCL – female POCL) for a body size parameter in a test pair. A positive BSD value indicated that males were larger than females, and *vice versa*. The test time (TT) [i.e., number of elapsed days from the initial test date (24 August)] was also included in the explanatory variables because mating behaviour and spawning may be stimulated during the main reproductive season of this species (September and October). We prepared the different size combinations of test pairs to be presented evenly during the test period, so that BSD of the test pairs did not significantly differ among the test times (one-way analysis of variance:  $F_{1,31} = 0.9966$ ,  $p = 0.3259$ ).

In our mating experiments, three females (each from the MM-MF, SM-LF, and SM-MF pairs) spawned eggs without having copulated with their

experimental partner. This suggests that females copulated prior to collections. This may underestimate the influence of BSD on mating behavioural intensity. Therefore, we also conducted the GLM analysis after removing the behavioural data of these three test pairs.

The relationship between incidence of copulation [presence (1) or absence (0)] (explanatory variable) and incidence of female spawning [presence (1) or absence (0)] (response variable) in the test pairs was evaluated with the GLM analysis. The number of eggs attached to the pleopods primarily depends on female body size in *P. clarkii* (Suko 1958; Oluoch 1990; Jin et al. 2019). We therefore analysed the effects of female POCL and BSD in the test pairs (explanatory variables) on the number of eggs attached to the female pleopods (response variable). We also assessed the egg development rate (number of developed eggs/number of eggs attached to the female pleopods) (response variable) in relation to BSD or mating behaviour (number of copulation events or total copulation duration) (explanatory variables).

In our analyses, quasi-Poisson GLM (log link), binomial GLM (logit link), quasi-binomial GLM (logit link), and Gamma GLM (log link) were applied for count data (number of events, days, and eggs), binary data (incidence of behaviour), proportion data (copulation success rate), and time data (copulation duration), respectively. The quasi-Poisson and quasi-binomial GLM analyses were employed considering the overdispersion of the count and proportion data (Zuur et al. 2009). The GLM analyses were conducted using the *glm* function.

## Results

### Mating behaviour

A total of 995 grasping, 393 holding, and 221 copulation events occurred in 33 test pairs. Copulation events were observed throughout the experimental period for 48 h, but the majority of copulation events occurred by the morning (6 a.m.) of the second test day (73% cases, 161/221) (Fig. 1).

Grasping behaviour was observed in all test

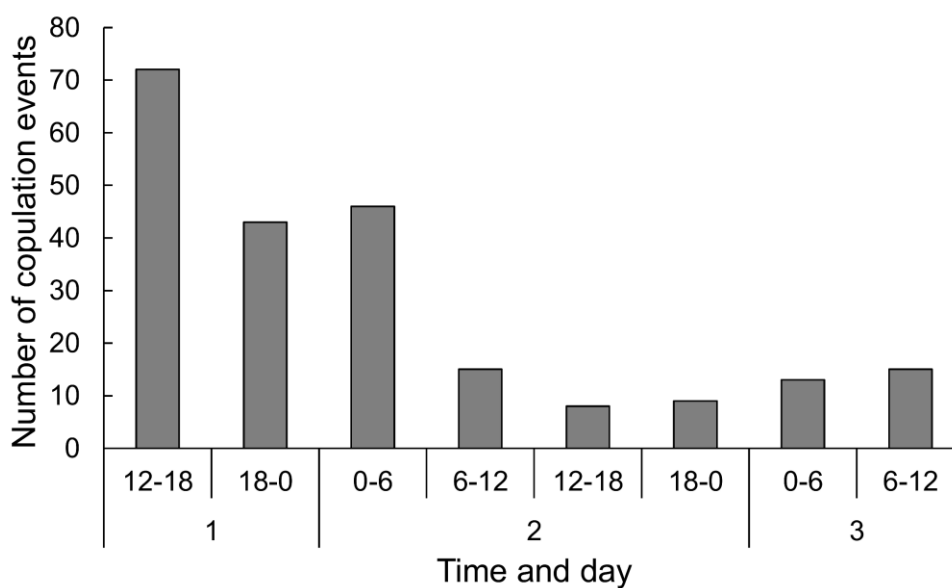


Fig. 1. Frequency distribution of 221 copulation events that occurred in 33 mating pairs of the red swamp crayfish *Procambarus clarkii* in relation to the time and day during the 48-h observation periods.

pairs, except in two from the LM-SF group. Holding behaviour and copulation did not occur in eight pairs: three from the LM-SF group, two from the SM-SF group, and one each from the MM-MF, SM-LF, and SM-MF groups. The BSD did not significantly affect the incidence of grasping behaviour ( $p = 0.1530$ ) or the incidence of holding and copulation behaviour ( $p = 0.3976$ ) (Table 1; Fig. S1A, B). After excluding three pairs that spawned without copulation, the negative influence of BSD on the incidence of holding and

copulation behaviour was nearly significant ( $p = 0.0508$ ) (Table S1; Fig. S2B).

The number of grasping and copulation events significantly decreased with increasing BSD values ( $p = 0.0416$  and  $0.0128$ , respectively), and a similar trend was found in the number of holding events ( $p = 0.0584$ ) (Table 1; Fig. 2A–C). Copulation success rate was not significantly influenced by BSD ( $p = 0.7940$ ) (Table 1; Fig. S1C). BSD exerted a significant negative effect on total copulation duration ( $p = 0.0010$ ) (Table 1; Fig.

Table 1. Coefficient estimates with standard errors (SE) for the generalised linear model (GLM) to evaluate the effects of body size difference (BSD) between male and female and test time (TT) (explanatory variables) on mating behaviour and spawning (response variables) in the test pairs of the red swamp crayfish *Procambarus clarkii*.

| Response variable  | n  | Coefficient | Estimate | SE     | t or z values | p        |
|--|----|-------------|----------|--------|---------------|----------|
| Incidence of grasping behaviour [presence (1) or not (0)]                      | 33 | Intercept   | 11.1652  | 7.7217 | 1.446         | 0.1480   |
|  |    | BSD         | -0.6742  | 0.4716 | -1.430        | 0.1530   |
|  |    | TT          | -0.1129  | 0.1426 | -0.792        | 0.4290   |
| Incidence of holding and copulation behaviour [presence (1) or not (0)]        | 33 | Intercept   | 1.2336   | 0.7379 | 1.672         | 0.0945   |
|  |    | BSD         | -0.0486  | 0.0574 | -0.846        | 0.3976   |
|  |    | TT          | -0.0037  | 0.0323 | -0.115        | 0.9081   |
| Number of grasping events  | 33 | Intercept   | 2.9247   | 0.3147 | 9.295         | < 0.0001 |
|  |    | BSD         | -0.0460  | 0.0216 | -2.129        | 0.0416   |
|  |    | TT          | 0.0212   | 0.0123 | 1.723         | 0.0951   |
| Number of holding events   | 33 | Intercept   | 1.7130   | 0.4450 | 3.849         | 0.0006   |
|  |    | BSD         | -0.0552  | 0.0281 | -1.968        | 0.0584   |
|  |    | TT          | 0.0328   | 0.0165 | 1.987         | 0.0561   |
| Number of copulation events  | 33 | Intercept   | 1.4116   | 0.3120 | 4.524         | 0.0001   |
|  |    | BSD         | -0.0581  | 0.0220 | -2.647        | 0.0128   |
|  |    | TT          | 0.0201   | 0.0121 | 1.652         | 0.1090   |
| Copulation success rate (number of copulation events/number of holding events) | 25 | Intercept   | 0.8852   | 0.6106 | 1.450         | 0.1610   |
|  |    | BSD         | -0.0110  | 0.0415 | -0.264        | 0.7940   |
|  |    | TT          | -0.0282  | 0.0220 | -1.283        | 0.2130   |
| Total copulation duration (in seconds)   | 25 | Intercept   | 8.2969   | 0.2534 | 32.743        | < 0.0001 |
|  |    | BSD         | -0.0822  | 0.0216 | -3.796        | 0.0010   |
|  |    | TT          | 0.0237   | 0.0112 | 2.124         | 0.0452   |
| Mean copulation duration (in seconds)  | 25 | Intercept   | 6.5816   | 0.1696 | 38.803        | < 0.0001 |
|  |    | BSD         | -0.0038  | 0.0145 | -0.263        | 0.7950   |
|  |    | TT          | 0.0091   | 0.0075 | 1.211         | 0.2390   |
| Number of days from copulation to spawning                                     | 24 | Intercept   | 3.9015   | 0.1304 | 29.930        | < 0.0001 |
|  |    | BSD         | -0.0369  | 0.0127 | -2.911        | 0.0084   |
|  |    | TT          | -0.0288  | 0.0064 | -4.510        | 0.0002   |

2D), but the mean copulation duration did not vary depending on the BSD values ( $p = 0.7950$ ) (Table 1; Fig. 2E). TT affected some mating behavioural traits: when the TT was progressing [i.e., the main reproductive season (September and October) approached], the number of holding events tended

to increase ( $p = 0.0561$ ) (Table 1) or total copulation duration significantly increased ( $p = 0.0452$ ) (Table 1; Fig. 2D). After removing from the analyses the three females that spawned without copulating during the mating trials, BSD and TT significantly affected the numbers of

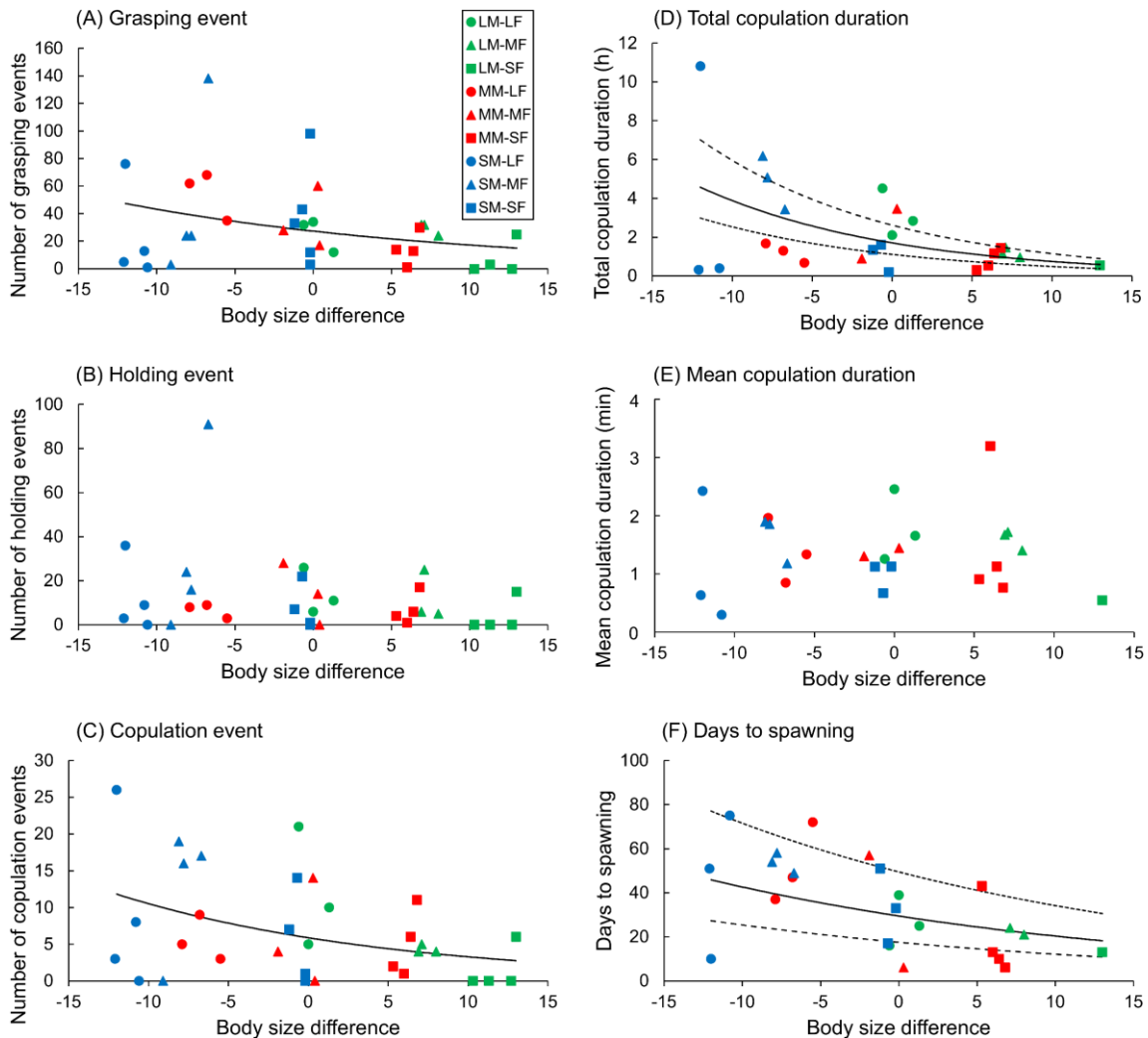


Fig. 2. Relationships between body size difference (male postorbital carapace length – female postorbital carapace length) and numbers of grasping (A), holding (B) and copulation events (C), and total copulation duration (D), mean copulation duration (E), and number of days from copulation to spawning (F) in the test pairs of the red swamp crayfish *Procambarus clarkii*. Nine combination groups were evaluated using three size classes (L, large; M, medium; and S, small) of males (M) and females (F). Effects of body size difference (BSD) and the test time (TT) [number of elapsed days from the initial test date (24 August)] (explanatory variables) on mating behaviour and spawning (response variables) were evaluated with a generalised linear model (GLM). When the GLM analysis generated significant coefficient estimates for BSD and TT, theoretical values of response variables were drawn for BSD at the minimum (dotted line), median (solid line), and maximum (broken line) TT values. When the GLM analysis generated a significant coefficient estimate only for BSD, theoretical values of response variables were drawn for BSD at the median TT value. See Table 1 for the GLM analyses.





Table 2. Coefficient estimates with standard errors (SE) for the generalised linear model (GLM) to evaluate the relationship between incidence of copulation (IC) [presence (1) or absence (0)] (explanatory variable) and incidence of spawning [presence (1) or absence (0)] (response variable), the effects of female postorbital carapace length (FPOCL) and body size difference (BSD) between male and female (explanatory variables) on the number of eggs attached to the female pleopods (response variable), and the effects of BSD, number of copulation events (NC) or total copulation duration (TCD) (explanatory variables) on egg development rate (number of eggs developed/number of eggs attached to the female pleopods) (response variable) in the test pairs of the red swamp crayfish *Procambarus clarkii*.

| Response variable     | n       | Coefficient | Estimate | SE     | t or z values | p      |
|-----------------------|---------|-------------|----------|--------|---------------|--------|
| Incidence of spawning | 33      | Intercept   | -0.5108  | 0.7303 | -0.699        | 0.4843 |
|                       |         | IC          | 3.6889   | 1.2550 | 2.939         | 0.0033 |
| Number of eggs        | 24      | Intercept   | 3.4608   | 0.9682 | 3.574         | 0.0018 |
|                       |         | FPOCL       | 0.0619   | 0.0272 | 2.276         | 0.0334 |
|                       |         | BSD         | 0.0379   | 0.0210 | 1.807         | 0.0852 |
| Egg development rate  | 24      | Intercept   | 3.4297   | 0.7162 | 4.789         | 0.0001 |
|                       |         | BSD         | 0.2093   | 0.0798 | 2.623         | 0.0155 |
|                       |         | Intercept   | 4.7225   | 1.4733 | 3.205         | 0.0041 |
|                       |         | NC          | -0.1849  | 0.0892 | -2.073        | 0.0501 |
|                       |         | Intercept   | 4.4770   | 1.1240 | 3.981         | 0.0006 |
| TCD                   | -0.0002 | 0.0001      | -2.484   | 0.0211 |               |        |

BSD: male postorbital carapace length – female postorbital carapace length.

## Discussion

The present study detected a possible effect of body size on the mating behaviour of *P. clarkii*. The numbers of grasping, holding, and copulation events and total copulation duration tended to decrease with increasing BSD values (i.e., increasing male size and decreasing female size) (Table 1; Fig. 2A–D). In particular, copulation and spawning did not occur in 75% of the trials (3/4), and mating activities were rare in the LM-SF pairs with the highest BSD values (10–13 mm) (Fig. 2A–C). This may be a result of male mate preference for large females, as demonstrated by Aquiloni and Gherardi (2008b) during the binary choice experiments in which large or small females (two targets) were simultaneously presented to a male (chooser); specifically, males visited large females more frequently and spent more time in their proximity.

In our experiments, however, there may be a possibility that females and males were not

sexually responsive in five test pairs without having copulated and spawned. Moreover, three females spawned fertilised eggs without having copulated with their experimental partner. In future experiments, we should confirm the sexual responsiveness of test crayfish by pairing them with different partners. Additionally, females that had moulted in captivity should be prepared because the spermatophore in the annulus ventralis are lost when females moult (McLay and van den Brink 2016; Johović et al. 2020).

*Procambarus clarkii* males did not avoid the opportunities to copulate with smaller females in the LM-MF and MM-SF pairs with the second largest BSD values (5–8 mm) (Fig. 2C). *Procambarus clarkii* reproduce all year round, with seasonal peaks (Suko 1956, 1958; Huner and Barr 1991; Hamasaki et al. 2020). The overall sex ratio of *P. clarkii* populations is nearly 1:1, but male predominance is observed in certain periods during the peak reproductive seasons (Anastácio et

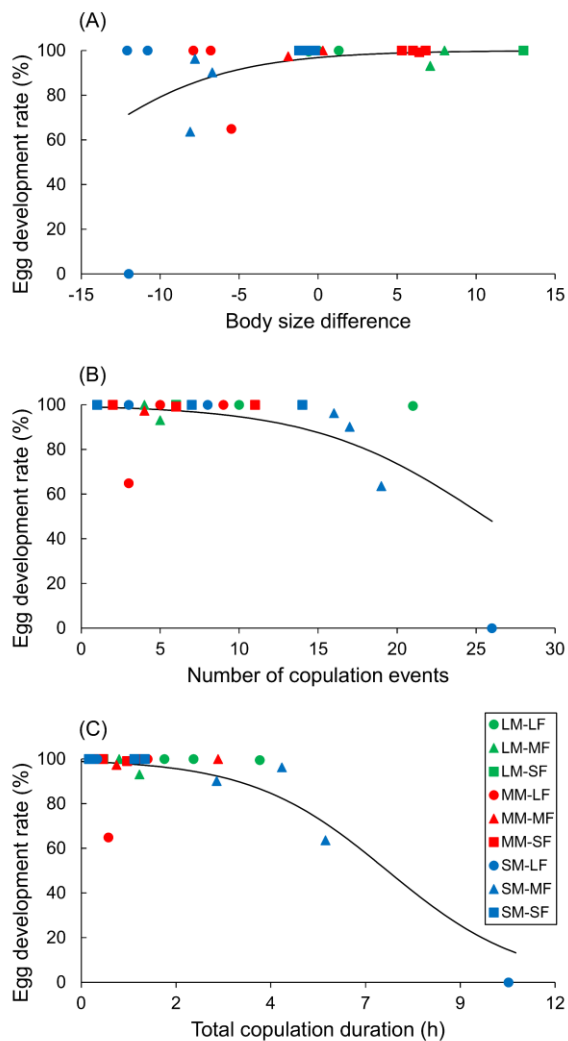


Fig. 4. Effects of body size difference (male postorbital carapace length – female postorbital carapace length) (A), number of copulation events (B), and total copulation duration (C) on egg development rate in females of the red swamp crayfish *Procambarus clarkii*. Nine combination groups were evaluated using three size classes (L, large; M, medium; and S, small) of males (M) and females (F). The theoretical values were drawn based on the GLM analyses between these variables (Table 2).

al. 2009; Peruzza et al. 2015; Jin et al. 2019; Hamasaki et al. 2020). Therefore, our results suggest that *P. clarkii* males may show mate choice while engaging in intrasexual competition for acquiring fertile females under a male-biased operational sex ratio (Emlen and Oring 1977). A similar mating strategy is documented in fiddler

crabs *Austruca mjoeberti* (Rathbun 1924) as males preferentially courted larger females with a potential high fecundity under both laboratory and natural conditions but did not avoid mating opportunities with small females, probably because the operational sex ratio was highly male biased in this species (Reading and Backwell 2007).

Besides male mate preference, low physical ability of smaller males may be another reason for increased mating behavioural intensity in the smaller BSD pairs. Relatively small males of *P. clarkii* appeared not to control the female position easily during a mating behavioural process on our video recordings. Eventually, smaller males of *P. clarkii* might not be able to transfer their spermatophores into a female annulus ventralis well, so they might persistently attempt to copulate with larger females. This was supported by decreasing egg development rate with decreasing BSD values or increasing number of copulation events and total copulation duration (Table 2; Fig. 4). A similar phenomenon has been reported for the Japanese spiny lobster *Panulirus japonicus* (von Siebold 1824) (Jinbo et al. 2017).

Our experiments also demonstrated that body size significantly affected the spawning of *P. clarkii* (Fig. 2F; Table 1). Females delayed spawning when they copulated with smaller males, as the number of days from copulation to spawning significantly increased with decreasing BSD values in the test pairs. *Procambarus clarkii* females favour larger males (Aquiloni and Gherardi 2008b, c). Female mate choice for larger males will offer the benefits to the female herself. For instance, larger males are more likely to win intrasexual fights for acquiring or defending burrows to the brooding females (Oluoch 1990; Huner and Barr 1991; Figler et al. 2005; Aquiloni and Gherardi 2008b). Additionally, larger males

will provide females enough sperms to fertilise eggs (Fig. 4). Therefore, *P. clarkii* females that hastened to copulate with smaller males may delay spawning to wait for the opportunity to re-copulate with larger males.

It has been demonstrated that females of *P. clarkii* spawned similar number of eggs regardless of the mate size (small or large) but produced large eggs and juveniles, probably with higher chances of survival, when pairing with large males (Aquiloni and Gherardi 2008c). In the present study, the number of eggs attached to the female pleopods depended primarily on female body size (Fig. 3), and BSD of the test pairs did not affect the female egg number, although the coefficient estimate of BSD was positive (Table 2). We did not examine egg loss at spawning and during the incubation period, which commonly occurs in decapod crustaceans (Kuris 1991), including *P. clarkii* (Huner and Barr 1991; Aquiloni and Gherardi 2008c). Moreover, we did not measure the offspring size. Further studies will be required to measure the more accurate fecundity as well as offspring size by *P. clarkii* females mated with different size males.

To summarize, the present study highlighted the importance of BSD between sexes in mating and spawning success of *P. clarkii*. Therefore, the effectiveness of SMRT in controlling invasive *P. clarkii* populations may depend on the size of males. We recommend using sterile medium- and large-size males with high reproductive potential to satisfy the mutual mate choice in *P. clarkii* populations comprising various size females (Scalici and Gherardi 2007; Jin et al. 2019; Hamasaki et al. 2020).

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