

Research Paper

High prevalence of multiple paternity in the invasive crayfish species, *Procambarus clarkii*

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Abstract

Reproductive strategy is a central feature of the ecology of invasive species as it determines the potential for population increase and range expansion. The red swamp crayfish, *Procambarus clarkii*, has invaded many countries and caused serious problems in freshwater ecosystems. However, little is known about the effects of environmental conditions on crayfish paternity and offspring traits in the wild. We studied these reproductive characteristics of *P. clarkii* in wild populations from two different habitats (ponds and ditches) in three locations with different environmental conditions in China. Genotyping of 1,436 offspring and 30 mothers of 30 broods was conducted by using four microsatellites. An analysis of genotyping results revealed that gravid females were the exclusive mother of the progeny they tended. Twenty-nine of 30 mothers had mated with multiple (2-4) males, each of which contributed differently to the number of offspring in a brood. The average number of fathers per brood and the number of offspring per brood were similar ($P > 0.05$) among six sampling sites, indicating that in *P. clarkii* multiple paternity and offspring number per brood are independent of environmental conditions studied. Indirect benefits from increasing the genetic diversity of broods, male and sperm competition, and cryptic female choice are a possible explanation for the high level multiple paternity and different contribution of fathers to offspring in this species.

Key words: Decapod, allochthonous species, microsatellite, mating system, multiple paternity
Running title: Multiple paternity in red swamp crayfish

1. Introduction

Reproductive strategy is a central feature of the ecology of invasive species as it determines the potential for population increase and range expansion [1]. Sexual reproduction and high fecundity levels are some of the characteristics most frequently cited when compiling criteria promoting invasion [2]. The recent development and application of polymorphic DNA markers (e.g. microsatellites and SNPs) [3, 4] and data

analysis tools [5] to parentage studies in natural populations has initiated several important paradigm shifts in the field of reproductive biology [6]. Among the most prominent of these shifts is the realization that females of most animal species, even those believed to be socially monogamous, copulate routinely with multiple males and often produce broods composed of both full and half-sibs [7]. Recent studies

have shown that multiple paternity is frequent in both invertebrates [8] and vertebrates [9]. The general evolutionary significance of multiple paternity and the factors which cause it to vary in frequency among related taxa continue to be debated vigorously [10].

The red swamp crayfish, *Procambarus clarkii* (Girard 1852), native to south-central United States and north-eastern Mexico, has been introduced to Europe, Africa, central and South America and Southeast Asia [11]. This species was introduced to Nanjing, China from Japan in 1929 [12] and has rapidly spread to most provinces of China and has established dense populations [13]. *Procambarus clarkii* has caused serious problems to local fish, crustaceans, aquatic plants and freshwater ecosystems [13]. Extensive studies on reproduction [14, 15] and population dynamics [15-17] have been carried out. The red swamp crayfish reaches maturity in approximately three months, and in warm climates it may produce two generations per year [18]. Mating period of *P. clarkii* varies according to the hydrographic period and the environmental conditions, recruitment, as well as male/female maturation [14]. Size at reproduction is related to water temperature, population density, and the length of the hydroperiod. The number of eggs per female is size dependent, with reproductive output per unit of weight being highest in large females from permanent water bodies [14]. Large and healthy females typically produce over 200 viable young [19]. It is generally believed that some life history traits, such as rapid growth, high fecundity, polytrophism, resistance to diseases, pollution and extreme environmental conditions, make *P. clarkii* an invincible invasive species [20]. We applied four polymorphic microsatellite markers to investigate the patterns of multiple paternity in *P. clarkii* in six sampling sites (i.e. two different freshwater habitats in three locations in China) with different environmental conditions. The study of multiple paternity and effects of environmental factors on multiple paternity will help to understand the mechanisms underlying the successful invasion of the red swamp crayfish.

2. Methods

Selection of study sites and measuring parameters of water quality

Three locations were selected for collecting crayfish samples taking into account the different environmental conditions. In each location, samples were collected in two different habitats (pond and ditch), therefore there are a total of 6 sampling sites (Table 1). These three locations are Jiangning (31°15'16" N,

118°7'49" E) in Jiangsu Province, Huzhou (30°22' 29" N, 119°44'38" E) and Tongxiang (30°38'18" N, 120°17'38" E) in Zhejiang Province, China. The climate in three locations is slightly different [21]. In Jiangning (JN) the annual average temperature is 15.3°C and annual precipitation of is 1106.5 mm. In Tongxiang (TX), the annual average temperature is 15.8°C and the annual average rainfall is 1193 mm. Huzhou's (HZ) annual average temperature is 16.9°C and an annual average rainfall of is 1126 mm. In all of these locations, water bodies (i.e. rivers, ponds, ditches and reservoirs) are very abundant. Ponds are connected to rivers and ditches only during the rainy season. Ditches are connected to rivers and flowing water is abundant during raining and agricultural seasons due to the high level of water. Ditches are connected to rivers, and water in ditches is flowing during raining and agricultural seasons. Food resources (e.g. detritus, water plants, macroinvertebrates and snails) are more abundant in ponds than in ditches, and density of crayfish is higher in ponds than in ditches [22]. Water quality parameters (pH, chemical oxygen demand, dissolved oxygen, total nitrogen and total phosphorus) of ponds and ditches from the three locations were measured according to guidelines of State Environmental Protection Administration, China [23]. Statistical analysis of the difference of water quality parameters among six sampling sites (two habitats from each of three locations) was conducted using F-test for analyses of variance (one way ANOVA) with the SAS program (SAS Institute) due to the fact that multiple comparisons are required.

Collection of samples

Samples from the studied populations of wild red swamp crayfish were collected, from May to September 2005, at the two habitats in each of the three locations (see Table 1) using baited nets. All crayfish individuals were sexed as previously described [24]. We measured the total body length of females (from tip of the rostrum to tip of the telson) and counted the total number of hatchlings per ovigerous female. Statistical analysis of the difference of body length of females and the total number of hatchlings per ovigerous female in six sampling sites (two habitats from each of three locations) was conducted using one way ANOVA with the SAS program.

We collected a small part of the third pleopod of each adult female bearing hatchlings and all the hatchlings, and stored them in absolute ethanol. The details of sample number and sampling locations are listed in Table 2.

Table 1 Parameters of water quality in ponds and ditches from three sampling locations

Location	Jiangning		Huzhou		Tongxiang	
Site	Pond	Ditch	Pond	Ditch	Pond	Ditch
pH	6.96 ± 0.03 ^a	7.07 ± 0.03 ^a	6.76 ± 0.04 ^a	7.14 ± 0.14 ^a	6.88 ± 0.08 ^a	7.07 ± 0.07 ^a
COD (mg/ml)	14.76 ± 0.32 ^b	5.29 ± 0.18 ^{cd}	5.73 ± 0.09 ^c	3.51 ± 0.13 ^e	27.41 ± 0.63 ^a	4.63 ± 0.21 ^d
DO (mg/ml)	4.80 ± 0.10 ^e	11.59 ± 0.21 ^b	7.63 ± 0.17 ^d	12.20 ± 0.20 ^a	3.73 ± 0.03 ^f	10.69 ± 0.11 ^c
TN (mg/ml)	1.31 ± 0.05 ^b	0.51 ± 0.02 ^a	0.68 ± 0.01 ^c	0.42 ± 0.10 ^{de}	1.51 ± 0.04 ^a	0.35 ± 0.03 ^e
TP (mg/ml)	0.75 ± 0.01 ^b	0.11 ± 0.01 ^d	0.20 ± 0.01 ^c	0.09 ± 0.00 ^e	0.86 ± 0.10 ^a	0.07 ± 0.01 ^e

COD: Chemical oxygen demand; DO: Dissolved oxygen; TN: Total nitrogen; TP: Total phosphorus; M: Male and F: Female; Significant differences ($P < 0.05$) are shown using different letters in rows.

Table 2 Sampling location, environment, sample sizes, total body length (BL) of dams and inferred genetic paternity in 30 *Procambarus clarkii* broods

Location	Site	Dam's ID	BL (cm)	Number of offspring		Number of sires
				genotyped	total	
TX	ditch	M01	10.5	48	351	4
		M02	9.8	48	220	2
		M03	9.7	48	356	2
		M04	9.0	48	219	3
	pond	M05	10.5	46*	318	4
		M06	10.9	48	310	2
		M07	9.4	47*	236	2
		M08	11.6	48	331	4
		M09	10.1	48	220	3
		M10	10.2	48	316	3
JN	ditch	M11	8.7	48	209	2
		M12	11	48	308	2
		M13	11.2	48	325	4
	pond	M14	7.4	48	136	3
		M15	10.7	48	321	3
		M16	10.0	48	270	2
		M17	10.8	48	356	2
		M18	10.1	48	219	3
		M19	11.6	48	318	2
HZ	ditch	M20	11.5	48	330	2
		M21	7.4	48	136	3
		M22	10.7	47*	321	2
		M23	11.8	48	362	3
	pond	M24	10.4	48	256	2
		M25	10.0	48	219	4
		M26	11.9	48	318	1
		M27	11.8	48	361	2
		M28	8.6	48	188	2
		M29	7.5	48	136	4
		M30	11.0	48	321	2

TX : Tongxiang, Zhejiang province; JN: Jiangning, Jiangsu province and HZ: Huzhou, Zhejiang province. * In these broods, 48 offspring were genotyped, whereas genotypes of 1-2 offspring were not clear due to low quality of DNA.

DNA isolation and microsatellite genotyping

DNA of mothers and 48 hatchlings of each brood were isolated using a method that we developed [25] and were arrayed onto 96-well plates for PCR. Four microsatellites (*PCLG03*, *PCLG09*, *PCL02* and *PCL017*) described previously [26, 27] were selected for genotyping samples due to their high polymorphism, in-

dependent inheritance, ease of PCR and no appearance of null alleles. One primer of each pair was labelled with a fluorescent dye (6-Fam or Hex). Each microsatellite locus was amplified in a 25 μ L total reaction volume containing 1 \times PCR buffer (Finnzymes) with 1.5 mM MgCl₂, 40 ng genomic DNA, 200 nM of each primer, 200 μ M dNTPs and one unit DNA

polymerase (Finnzymes). The following program was used for PCR: 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 sec, 55 °C for 30 sec and 72 °C for 45 sec, and then a final extension at 72 °C for 5 min. The PCR products of 1 µL were detected on an ABI3730xl capillary DNA sequencer (Applied Biosystems). Fragment sizes of alleles were calculated against the size standard GS-500-ROX using GeneMapper (Applied Biosystems). A genotype table was exported for analysis of allele number, size range, and expected and observed heterozygosity using the software GDA [28]. Mean expected paternal exclusion probabilities were estimated across the four loci using the GERUD [29].

Parentage analysis

The genotypes of all mothers sampled were determined directly by genotyping the microsatellite loci, whereas the paternal alleles were inferred from offspring genotypes after the maternal alleles were accounted for. We determined the minimum number of fathers in each brood using GERUD version 1.0 [29]. This program reconstructs all possible multilocus genotypes of fathers and searches for the minimum number of fathers that can explain the offspring genotypes. If multiple solutions of father genotypes are obtained for a given minimum number of fathers, GERUD can rank them based on the Mendelian segregation of alleles and the allele frequencies in the population. The contributions of individual fathers can likewise be calculated in GERUD. Since technical limitations prevent the resolution of five or more males, the last two procedures can only be conducted for broods with up to four males. For each brood, we tested whether the male contributions departed from equality using χ^2 -tests.

Analysis of factors associated with the frequency of multiple paternity

We performed an ordinal logistic regression analysis to assess whether the number of detected fathers depends on the body size of females, and the association between the number of father and the number of hatchlings in broods, designating the number of detected fathers as the dependent variable. An ordinal scaling was chosen for the number of detected fathers since the paternity analysis did not allow differentiating between five or more fathering males (see above). The analysis was carried out with the Logistic Procedure of the SAS program (SAS Institute). We also conducted a regression analysis to assess the association between the total body length of the mother and the number of hatchlings in broods using the linear regression function with the program

JMP (SAS Institute).

We compared the average number of fathers and offspring in broods in six sampling sites (two habitats from each of three locations) using one way ANOVA with the SAS program. To further determine if habitat, location and their interaction affected the number of fathers in broods and the number of offspring in broods, we conducted statistical analysis (split-plot design) using the GLM procedure of the SAS program (SAS Institute):

$$Y_{ijk} = \mu + L_i + E_j + (LE)_{ij} + \varepsilon_{ijk}$$

Where, Y_{ijk} is the k th observation of the j th habitat in the i th location, μ is the mean value of in all broods, L_i is the mean value of the i th location, E_j is the mean value of the j th habitat, $(LE)_{ij}$ is the interaction between location and habitat and ε_{ijk} is the residual error.

3 Results

Water quality parameters in ponds and ditches at three sampling locations

Five parameters (pH, chemical oxygen demand, dissolved oxygen, total nitrogen and total phosphorus) related to water quality were measured (Table 1). Besides pH values that showed no significant difference (one way ANOVA, $F = 1.23$, d.f. = 5, $P > 0.05$) in six sampling sites (i.e. ditches and ponds at each of the three locations), all other parameters of water quality: chemical oxygen demand (COD), dissolved oxygen (DO), total nitrogen (TN) and total phosphorus (TP) differed significantly among six sampling sites (one way ANOVA, d.f. = 5, $F = 272.2 \sim 2707.6$, $P < 0.05$). These differences suggest different environmental conditions in six sampling sites. Water quality was poorest in the pond in Tongxiang (TX), whereas the best quality of water was obtained in the ditch in Huzhou (HZ).

The number of offspring per brood and parentage analysis

We collected samples of mothers and offspring from 30 broods of red swamp crayfish and amplified 30 adult females and 1,440 of their offspring using four polymorphic microsatellites. The average offspring number per brood was 276.3 ± 12.9 (Table 2). There were no significant differences of number of offspring/brood among the six sampling sites (one way ANOVA, d.f. = 5, $F = 0.526$, $P > 0.05$) (Table 3).

With the exception of four offspring, all samples were successfully genotyped with the four microsatellites. In these 30 mothers, the allele number ranged from 5 for the locus *PCLG03* to 10 for the locus *PCLG10* with an average of 7.5 alleles/locus. The average expected heterozygosity of these markers was

0.77, whereas the observed heterozygosity was 0.63. Paternity exclusion probability for all four loci combined was 0.959. Alleles at all four loci in each brood were inherited in simple Mendelian way. All 1,436 offspring from the 30 broods showed alleles consistent with maternal genotypes.

Multiple paternity was found in 29 of 30 broods and six broods had a minimum of four different fathers (Table 2). In all 29 multiple sired broods, male parental contributions deviated significantly from equality (goodness-of-fit χ^2 -tests, $P < 0.05$; Fig. 1).

Table 3 Multiple comparisons of the body length of mothers, number of offspring and number of fathers in 30 *Procambarus clarkii* broods among habitats within location *

Location	Habitat	N	Body length of mothers (cm)		No. of offspring		No. of sirs	
			Mean	SD	Mean	SD	Mean	SD
HZ	ditch	5	10.36	1.75	281.0	89.7	2.4	0.5
	pond	6	10.13	1.79	257.2	88.9	2.5	1.2
NJ	ditch	4	9.57	1.84	244.5	88.6	2.8	1.0
	pond	5	10.64	0.64	296.8	53.2	2.4	0.5
TX	ditch	4	9.75	0.61	286.5	77.4	2.8	1.0
	pond	6	10.45	0.75	288.5	47.6	3.0	0.9

* Differences among environments within location were not significant.

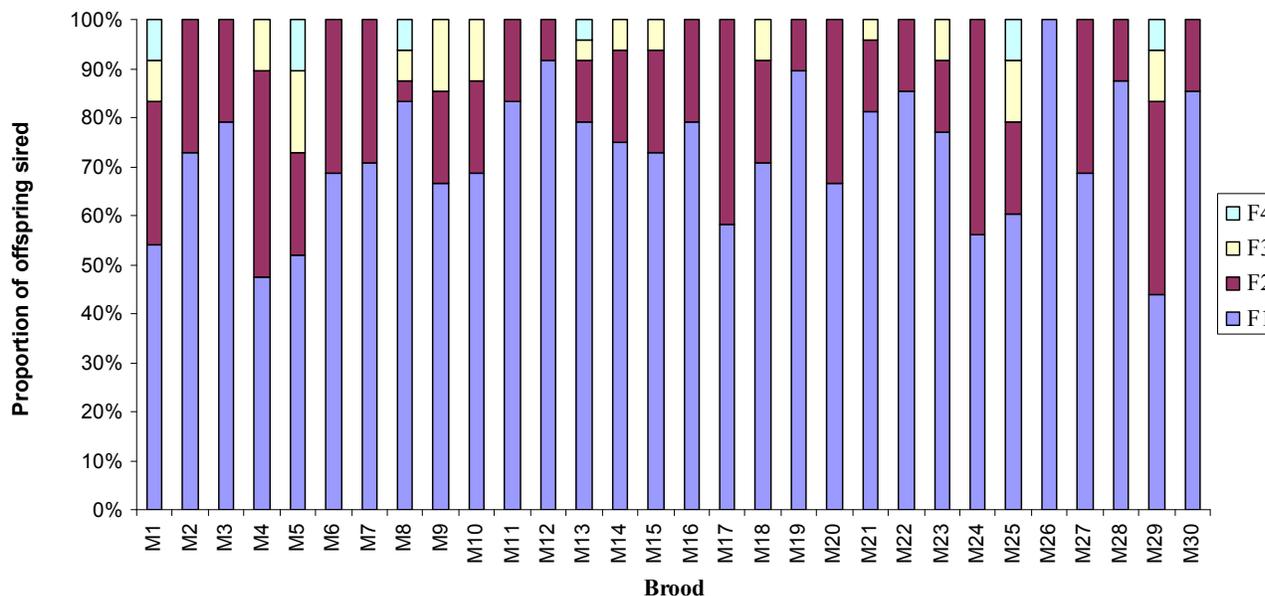


Fig 1 Relative contribution of fathers to broods sired by multiple paternity in 30 broods of red swamp crayfish (*Procambarus clarkii*). F1: the first father; F2: the second father; F3: the third father and F4: the fourth father.

The average body length of mothers collected in the six sampling sites (one way ANOVA, d.f. = 5, $F = 0.416$, $P > 0.05$) was similar (Table 3). The body size of the mothers was significantly associated with the number of offspring ($r^2 = 0.77$; d.f. = 29; $P < 0.01$) (Fig. 2), whereas the number of offspring in broods was not correlated with the number of fathers sired in broods ($\chi^2 = 0.77$; d.f. = 1; $P > 0.05$) (Fig. 3). The ordinal logistic regression analysis revealed that the body size of mothers was not associated with the number of males sired in broods ($\chi^2 = 1.07$; d.f. = 1; $P > 0.05$) (Fig. 4). Multiple paternity appeared in all three locations studied (Table 2 and Figure 1). The average number of fathers and the average number of offspring in broods were not statistically different (one way ANOVA, d.f. = 5, $F = 0.344-0.526$, $P > 0.05$) among six sampling sites (Table 3). The effects of location, habitat and the interaction between them on the number of fathers in broods and the number of offspring in broods were not statistically significant (Split-plot design, d.f. for locations = 2, d.f. for habitats = 1, d.f. for interaction between habitat and locations = 2, $P > 0.05$).

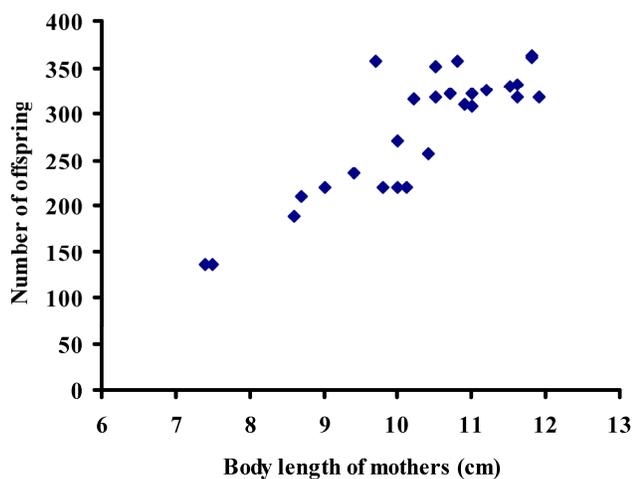


Fig. 2 The number of offspring per brood plotted against female body size in 30 broods of red swamp crayfish (*Procambarus clarkii*)

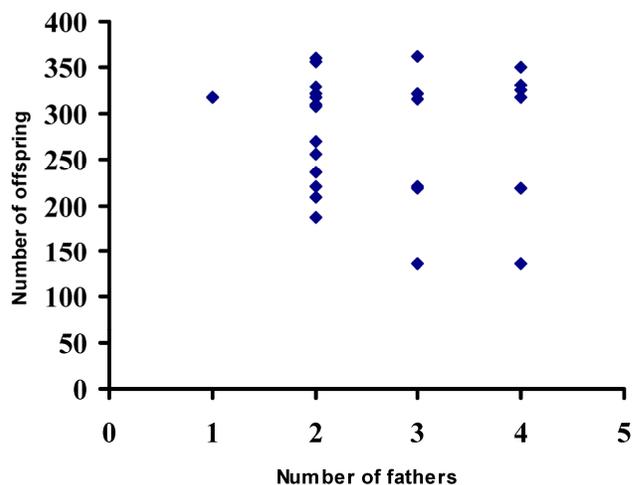


Fig. 3. The number of offspring in 30 broods plotted against the number of fathers in 30 broods of red swamp crayfish (*Procambarus clarkii*)

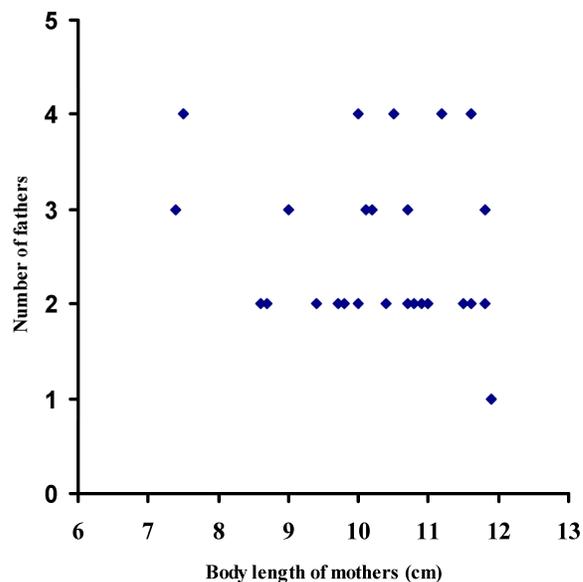


Fig. 4. The number of fathers detected in 30 broods plotted against female body length in 30 broods of red swamp crayfish (*Procambarus clarkii*)

4 Discussion

Little is known about the reproductive strategies of *P. clarkii* in different natural ecosystems, although reproductive performances [14, 16, 18] and population dynamics [15, 17] have been extensively studied recently. In this study, we presented the data on paternity analysis using polymorphic microsatellites that could provide new insights into the mating strategies of *P. clarkii* under different environmental conditions. Two major conclusions could be drawn from this study: (1) multiple paternity was common in different habitats and locations with significantly different environmental conditions. The average number of fathers per brood and the number of offspring per female were not statistically different in habitats and locations with dissimilar environmental conditions; (2) the contribution of each father to the number of offspring in each brood was significantly different. Most offspring were sired by one primary sire per brood; the gravid female was the exclusive mother of the progeny it tended.

Multiple paternity

We discovered that the majority (96.7%) of mothers had mated with multiple (2-4) males. In crayfish, during copulation, a spermatophore is transferred to an opening of the female's seminal receptacle. This leads to a sperm storage vessel [30] that, after mating, may be sealed by a sperm plug [11]. However, in *P. clarkii*, our study showed that even if sperm plugs were inserted, they were not effective to prevent paternity by other males. Sperm plugs may function to hold sperm in place [31], rather than block later insemination. We found that the frequency (96.7%) of multiple paternity was much higher than that (60.0%) reported for other crayfish species *Orconectes placidus* [32], the American lobster, *Homarus americanus* (13% of 108 brooders) [33], the Norway lobster, *Nephrops norvegicus* (54.6% of 11 brooders) [8] and the porcelain crab, *Petrolisthes cinctipes* (80% of 10 brooders) [34]. It is likely that multiple paternity supplies a chance for females to ensure fertilization of more eggs or to select high quality sperm for fertilization, thus improving the number and quality of offspring. A previous study [35] revealed that broods of adders (*Vipera berus*) and sand lizards (*Lacerta agilis*) with multiple sires have higher embryonic survival, fewer deformities and, in *L. agilis*, the offspring is heavier and present higher survival rates during the first year of life. Although there is dispute on whether polyandry increases the effective population size [36], it is generally believed that polyandry is likely to increase the effective population size due to genetic diversity [37, 38]. To ensure long-term success of sur-

vival, the maintenance of genetic variation is very important [37]. Reduced genetic variability as a result of genetic drift limits the opportunity for future expansion. Therefore, high frequency of multiple paternity in *P. clarkii* may be an important factor ensuring the production of high number and quality of offspring, and maintaining effective population size, and this in turn promotes its ability to expand to new habitats.

Skew contribution of males to the offspring

In this study, fathers in 29 of 30 broods contributed with different numbers of offspring to a brood, i.e. one of the inferred sires was assigned to a large proportion of offspring in each brood. Similar results have been reported in another crayfish species *Orconectes placidus* [32]. This kind of skewness may arise from many factors. One factor is the mating order. In some insect species where internal fertilization was similar to that in crayfish, fertilization success is biased toward the last-mating male [39], whereas in mammals, fertilization success is biased toward the first-mating male [40]. However, the current study is not able to answer the question about the order of sperm use and the length of time that sperm may survive stored in the female. To address this question, additional experiments need to be conducted. Another factor affecting the skewness of male contribution to the offspring is the body size of males and females. Under laboratory conditions, large body size is selected by both sexes [41]. However, in this study, we found that female size did not have an effect on the frequency of multiple paternity. Male and sperm competition are typical for many polyandrous mating systems [7, 42, 43] resulting in male reproductive skewness. Multiple paternity may supply the chance for females to select different males to copulate or select the best sperm for fertilization of their eggs, thus resulting in male reproductive skew and improving the fitness of offspring. Skew of multiple paternity is believed to be a major driving mechanism of evolution [44]. Therefore, in *P. clarkii* skewed male contribution to the offspring may be related to sperm competition and enhanced progeny fitness.

Effect of environmental conditions on multiple paternity

Many factors can greatly influence multiple paternity and reproductive performance. Previous studies have demonstrated that environmental conditions could influence multiple paternity [45] and reproductive performance of animals [6]. Some species can only reproduce in specific habitats, while others can reproduce in a wide range of environmental conditions. Successful reproduction is precondition of

alien species to survive and expand in new environments. In this study, in the three locations and two types of habitats, the environmental conditions were different, while the reproductive performance (i.e. the number of offspring) of the females and the frequency of multiple paternity were similar in different environmental conditions. In the pond in Tongxiang, the water quality was quite bad, *P. clarkii* could not only survive, but also reproduce successfully. Our results indicate that reproductive performance of females is independent of environmental conditions examined in this study. This independence may be related to their high ability to mate with several males (multiple paternity). However, previous studies showed variation in the reproductive performance of *P. clarkii* under different environmental conditions [14, 15]. In the future it would be interesting to further study the reproductive performance of females and males living in more diversified environments.

Several factors have been related to the incidence of multiple paternity: body size of females [33, 46], sperm number of males [47], population density and environmental conditions [48]. Laboratory experiments showed that both males and females favoured large sized individuals for mating in *P. clarkii* [41]. However, in this study, we demonstrated that the number of males sired was not associated with the body size of the females, suggesting that mating behaviour is somewhat different in the laboratory than in the field. In some species, sperm of males is limited. In this case, to ensure fertilization of all eggs, multiple paternity is required [42]. However, in this study, we found that the number of offspring per brood was not associated with the number of fathers per brood, suggesting that sperm limitation was not a factor causing multiple paternity. In the two habitats (ponds and ditches), the density of crayfish was quite different, while the frequency of multiple paternity and number of fathers/brood were almost the same, suggesting population density is not a factor promoting multiple paternity. We also showed that the number of fathers sired in the broods collected from different locations and habitats with different environmental conditions was similar, indicating that multiple paternity in this species is independent of environmental conditions. This allows females to produce large number and high quality offspring in different environments, which in turn permits this species to thrive in less favourable conditions. In the future, it is necessary to study paternity in more habitats to examine whether we can generalize our conclusion that the reproductive performances of females and multiple paternity in *P. clarkii* are independent of environmental conditions.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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