Female freshwater crayfish adjust egg and clutch size in relation to multiple male traits

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Females may invest more in reproduction if they acquire mates of high phenotypic quality, because offspring sired by preferred partners may be fitter than offspring sired by non-preferred ones. In this study, we tested the differential maternal allocation hypothesis in the freshwater crayfish, *Austropotamobius italicus*, by means of a pairing experiment aimed at evaluating the effects of specific male traits (body size, chelae size and chelae asymmetry) on female primary reproductive effort. Our results showed that females laid larger but fewer eggs for relatively small-sized, large-clawed males, and smaller but more numerous eggs for relatively large-sized, small-clawed males. Chelae asymmetry had no effects on female reproductive investment. While the ultimate consequences of this pattern of female allocation remain unclear, females were nevertheless able to adjust their primary reproductive effort in relation to mate characteristics in a species where inter-male competition and sexual coercion may mask or obscure their sexual preferences. In addition, our results suggest that female allocation may differentially affect male characters, thus promoting a trade-off between the expression of different male traits.

**Keywords:** differential allocation; egg size; male–male competition; maternal effects; sexual coercion; trade-off

1. INTRODUCTION

In sexually reproducing organisms, an important fraction of individual breeding success may be determined by processes occurring after gamete release, one of which is cryptic female choice, a female-controlled process that selectively favours paternity by males with a particular trait over paternity of males that lack the trait when a female has copulated with both male types (Eberhard 1996). Cryptic female choice may occur as differential maternal allocation (Burley 1988), a form of maternal effect by which females finely modulate their parental investment according to the characteristics of their current mate, specifically its attractiveness, and the likelihood of finding a better mate in the future (Sheldon 2000). Differential allocation could arise since attractive mates transmit genes that will increase the fitness of their offspring either because they will be more viable (Trivers 1972; Zahavi 1975) or sexy (Fisher 1930; Lande 1981). Its main function is to confer an extra benefit to high-quality offspring, which may result in a larger long-term contribution to fitness (Sheldon 2000).

The target of differential allocation may be the primary as well as secondary reproductive effort. To date, females have been shown to allocate their primary reproductive effort (egg number, size or quality) differentially in several taxa according to the attractiveness of their mates (Burley 1988; Gil et al. 1999; Reyer et al. 1999; Cunningham & Russell 2000; Sheldon 2000; Kolm 2001; Saino et al. 2002a; Kotiaho et al. 2003). However, to the best of our knowledge, no study has addressed whether females differentially allocate resources to reproduction according to multiple male traits.

Here, we provide an experimental test of the differential allocation hypothesis in a freshwater crayfish, by analysing the effects of multiple male traits that may be the target of sexual selection (specifically, body size, chelae size and chelae asymmetry) on female primary reproductive effort (egg number and size). Differential maternal investment should occur frequently in species where females benefit indirectly from their mate choice and where pre-copulatory female choice may be overcome by sexual coercion (Møller & Thornhill 1998). Crayfish thus offer an ideal opportunity to study maternal investment, due to their promiscuous breeding behaviour and prolonged uniparental care. Based on current knowledge on crayfish sexual behaviour (see §1a), females paired with large-bodied, large- and symmetric-clawed males were predicted to produce larger eggs and/or lay larger clutches.

(a) Study species and rationale

Our model organism is the freshwater crayfish *Austropotamobius italicus* (Faxon 1914; Crustacea: Decapoda), a species endemic to Italy (Grandjean et al. 2000). In crayfish, males are larger, possess larger chelae than females, and use them to threaten and attack opponents during inter-male conflicts, as well as to secure and position females prior to and during copulation; thus, body and chelae size of males may be the target of sexual selection (Snedden 1990; Gherardi et al. 1999; Reyer et al. 2003). However, to the best of our knowledge, no study has addressed whether females differentially allocate resources to reproduction according to multiple male traits.

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et al. (2000). A. italicus, like the closely related A. pallipes, is a long-lived (maximum lifespan 10–13 years), k-selected crayfish which reproduces once a year, in October–November (Matthews & Reynolds 1995). Mating consists of short exchanges of tactile and olfactory signals, after which males grasp females by their claws, turn them on the back, and release spermatoophores, which are attached to the thoracic sternites of females (Acquistapace et al. 2002). Fertilization is therefore external, and spawning occurs a few days or weeks after mating. Eggs developing under the female’s abdomen are guarded through the winter and subsequent spring, a period during which females do not eat. After hatching (May–June), the young live attached to the female’s pleopods for a month. Throughout this period, besides providing protection, females continuously fan and groom the eggs and hatchlings (Reynolds 2002). Therefore, owing to this strong reproductive investment, it is likely that females play an active role in mating, either before or after copulation. When exposed to tethered males, females rejected the smallest males and those with a missing cheliped (Villanelli & Gherardi 1998), but the effects of such pre-mating preferences on male realized reproductive success are unknown. In other freshwater decapods, females may also select mates on the basis of chela size (Stein 1976; Berrill & Arsenault 1984; Snedden 1990). On the other hand, males copulate repeatedly and indiscriminately with receptive females, without regard to female body size (Villanelli & Gherardi 1998). Sexual coercion may indeed occur, setting the scene for the evolution of cryptic female choice.

2. MATERIAL AND METHODS

(a) Subjects and housing conditions
Sexually mature A. italicus (carapace > 30 mm) were collected from a stream in the northern Apennines (N. Italy) during September–October 2004. Sexes were held separately under a natural light: dark cycle in opaque plastic jars (80×60×60 cm) filled with 150 l of recirculating, filtered water (20 individuals per jar), provided with a gravel substratum and shelters, and fed with dry crayfish food twice a week. Water temperature ranged from 18 °C in October to 14–12 °C in November and December. Carapace length and right and left chela length were measured using a digital calliper (accuracy ± 0.01 mm); as a measure of chela length we used the length of the longest chela. Individuals with one or both missing chelipeds were discarded. The occurrence of regenerated chelipeds was noted, and chela asymmetry was calculated as the absolute difference in length of the chela and expressed as a percentage of the longer of the two chelae. At the end of experiments, all crayfish were returned to the stream of origin.

(b) Experimental design
One hundred and two males and 102 females were used in pairwise-designed mating trials. We established dyads of males with contrasting characteristics (large versus small, 16 dyads; large-clawed versus small-clawed, 17 dyads; symmetric-clawed versus asymmetric-clawed, 18 dyads), and paired them with a receptive female, as assessed by the presence of whitish patches deriving from glair gland maturation along abdominal sternites. Females were selected to be as similar as possible (±2 mm in carapace length) within each dyad of males, in order to evaluate the effects of male traits on female reproductive output irrespectively of female traits. Large and small males were separated and sorted by carapace length; those belonging to the larger half of the distribution were assigned to large male pairs, whereas those belonging to the smaller half were assigned to small male pairs. Dyads were then formed by pairing the smallest male of the small male pairs with the smallest male of the large male pairs, and so on by increasing size. Large and small males thus differed in carapace length, but also in chela length, due to the growth of all body parts with age (see electronic supplementary material). Large- and small-clawed males were assigned to dyads by selecting individuals of similar carapace length (±2 mm) and chela asymmetry but differing by at least 15% in chela size (see electronic supplementary material). Finally, symmetric- and asymmetric-clawed males were assigned to dyads by selecting individuals of similar carapace length (±2 mm), but differing in the presence of a regenerated cheliped (see electronic supplementary material). Individual females in a dyad were randomly assigned to males (see above), with the only constraint that the largest female of a female dyad had a carapace length equal to (±1 mm) or smaller than the smallest male of the dyad (see electronic supplementary material). In this way, we avoided pairing males with females that were larger than them, in order to maximize mating probabilities.

Mating trials were conducted in the evenings from 2–25 November. Male pairs belonging to a given dyad were tested simultaneously, in order to control for within-dyad variation in potential confounding effects due to, e.g. date of experiment, time of day and water temperature. Ten minutes before a trial started, pair members were placed separately in a 15 l plastic aquarium, provided with a gravel substratum and an opaque plastic sheet, forming two habituation chambers. The sheet was then removed and the animals allowed to interact for 30 min. Pairs were observed under dim red light; if copulation and spermatoaphore deposition did not occur during the trial, a pair was placed individually in a rectangular plastic mesh cage within a 150 l plastic jar (see above) and checked daily until insemination occurred (for a maximum of 30 days). After insemination, females were placed into 150 l spawning jars (see above) and checked for spawning every second day in order to minimize disturbance. Clutches were carefully removed from the female abdomen the day after spawning was completed. Eggs were counted and the wet weight was recorded for the entire clutch and for 10 randomly selected eggs from each clutch (Sartorius R200D balance, accuracy ± 0.01 mg), after drying off excess water with a paper cloth. Egg weight was highly repeatable within each female (0.91, F1.61.2 = 103.9, p < 0.0001). The diameters of the same 10 eggs were measured under a stereomicroscope (±0.06 mm) to calculate egg volume (the volume of a sphere whose diameter was equal to the mean value of the two measurements per egg). Egg measurements were averaged for each female. Mean egg weight and egg volume were strongly correlated (r = 0.99), so we used egg weight to estimate egg size. Measurements were taken blind of male or female traits.

(c) Statistical analyses
In order to investigate the effects of multiple male traits (namely, carapace length, chela length and chela
asymmetry) on latency to insemination (days elapsed between first contact and spermatophore deposition) and spawning (days elapsed between spermatophore deposition and spawning), and on measures of female reproductive output (egg weight, clutch size and clutch weight), we ran two separate multivariate analyses of variance models (Stevens 2002). In one model, latency to insemination and spawning were included as dependent variables, while in the other, the dependent variables were the measures of female reproductive output. In both models, male traits were included as covariates, and male dyad as a factor, which takes into account the pairwise nature of the experiment. The pairwise analysis allowed the evaluation of the multivariate effects of male traits on latencies and female reproductive output, as well as the evaluation of the relative effects of each male trait independently of the effect of other male traits. In addition, it had the advantage of directly taking into account the potentially confounding effects of the experimental setting (see §2b) and between-dyad variation in female traits (see also Saino et al. 2003 for a similar procedure). To improve normality, latency to insemination and clutch size were ln-transformed, while chelae asymmetry was $\sqrt[-1]{\text{arcsine}}$-transformed. Significance of multivariate tests was assessed by Wilks’ $\lambda$ (Stevens 2002). Latency to insemination can represent an index of pre-mating female preference, since females may resist advances by undesired males (P. Galeotti 2004, personal observation). Latency to spawn could be considered an index of female post-mating preference (a short latency could reduce the probability of being inseminated by a non-preferred male), or the result of variation in female post-mating investment (e.g. if females mating with favoured males take more time in producing larger eggs or clutches).

### 3. RESULTS

Nineteen out of 102 pairs failed to reproduce. Failures occurred either because the female died ($n=7$), spawning can also be fatal to females in the wild; P. A. Nardi 2004, personal observation), copulation did not occur within 30 days from pairing ($n=6$), or females did not spawn after mating ($n=6$). In addition, 15 pairs that reproduced successfully had to be excluded from the analyses because of the failure of their counterpart pair of a dyad. Thus, our results were based on 68 pairs (34 dyads). Overall, most matings (55%) occurred during trials (range: 0–12 days), and 81% of females spawned within 10 days after insemination. Mean clutch size was 96.7 eggs (95% C.I. 87.4–106.0, range 25–299) and mean egg weight was 14.2 mg (95% C.I. 13.7–14.8, range 9.2–19.0).

Male traits or dyad had no effect on the latency to insemination or spawning (multivariate and univariate tests, all $p>0.10$ and $>0.3$, respectively; see electronic supplementary material). Multivariate tests revealed that measures of female reproductive output were simultaneously affected by male carapace and chelae length, but not by chelae asymmetry (table 1). Specifically, the univariate analyses showed that egg weight increased with decreasing carapace length and increasing chelae length, clutch size showed the reverse pattern (table 1, figure 1). Finally, no effect of male traits on clutch weight was observed (table 1). All results were qualitatively unchanged whether the analyses were performed on untransformed variables (details not shown). Therefore, females laid smaller clutches but with larger eggs for relatively small-sized, large-clawed males.

### Table 1. Multivariate analysis of variance of female reproductive effort measures [egg weight, clutch size (ln-transformed), and clutch weight] in relation to male traits [carapace length, chelae length and asymmetry ($\sqrt[-1]{\text{arcsine}}$-transformed)]. The factor ‘dyad’ is included to take into account the pairwise nature of the experimental design (see §2 for details). Parameter estimates for continuous predictors are reported together with their standard errors.

<table>
<thead>
<tr>
<th></th>
<th>$F$</th>
<th>d.f.</th>
<th>$p$</th>
<th>estimate (s.e.)</th>
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<tr>
<td><strong>egg weight</strong></td>
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<td></td>
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<td>1.64</td>
<td>99, 87</td>
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<td>3, 29</td>
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<td>chelae length</td>
<td>4.19</td>
<td>3, 29</td>
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<td></td>
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<tr>
<td>chelae asymmetry$^b$</td>
<td>0.41</td>
<td>3, 29</td>
<td>0.75</td>
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<tr>
<td><strong>clutch size</strong></td>
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<td></td>
<td></td>
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<tr>
<td>dyad</td>
<td>1.86</td>
<td>33, 31</td>
<td>0.043</td>
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<td>carapace length</td>
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<td>1, 31</td>
<td>0.002</td>
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<tr>
<td>chelae length</td>
<td>7.03</td>
<td>1, 31</td>
<td>0.013</td>
<td>$0.279 (0.105)$</td>
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<tr>
<td>chelae asymmetry$^b$</td>
<td>0.01</td>
<td>1, 31</td>
<td>0.93</td>
<td>$0.002 (0.022)$</td>
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<tr>
<td><strong>clutch weight</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>dyad</td>
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<td>33, 31</td>
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<td>chelae length</td>
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<td>1, 31</td>
<td>0.011</td>
<td>$-0.043 (0.016)$</td>
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<tr>
<td>chelae asymmetry$^b$</td>
<td>1.27</td>
<td>1, 31</td>
<td>0.27</td>
<td>$-0.004 (0.003)$</td>
</tr>
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$^a$ approximate F-values for Wilks’ $\lambda$.

$^b$ variable $\sqrt[-1]{\text{arcsine}}$-transformed.

$^c$ variable ln-transformed.
4. DISCUSSION

The experiment clearly showed that females of a freshwater crayfish adjust their primary reproductive effort according to multiple male traits. Female crayfish produced larger eggs but smaller clutches when paired with relatively small-sized, large-clawed males, and larger clutches of smaller eggs for relatively large-sized, small-clawed males. This resulted in overall female investment (clutch weight) being unrelated to male chelae or carapace length. Chelae asymmetry, due to the presence of a newly regenerated cheliped, had no effect on female allocation to egg number or size, which contrasts with the reported female preference for males with both chelipeds (Villanelli & Gherardi 1998). Autotomized males or those with regenerated chelipeds may encounter difficulties in access to mates within competitive contexts, but females did not counterselect this handicap in their cryptic choice.

The lack of a significant negative correlation between egg and clutch size indicates that our results reflected only partly a trade-off between egg and clutch size (Bernardo 1996). This may suggest that females adopted two co-occurring strategies of maternal allocation depending on male traits, laying fewer but larger eggs for relatively small-sized and large-clawed males, and the reverse for relatively large-sized and small-clawed males. Fitness costs and benefits for females following one or the other strategy of maternal allocation will depend on: (i) the energetic costs of varying either clutch or egg size; and (ii) the relative importance of these two egg features and paternal genes for the fitness of the progeny. While the energetic costs of varying egg or clutch size are currently unknown in decapods, egg size may indeed be a critical trait influencing offspring fitness (reviewed in Bernardo 1996), particularly in organisms where parental care does not extend after hatching, including crustaceans and fish (Sinervo 1990; Clutton-Brock 1991; Williams 1994; Gimenez et al. 2004). In A. italicus, this maternal effect can be of crucial importance for early progeny performance, because offspring originating from large eggs may survive better soon after hatching, when they are unable to feed for a 10-day period (Reynolds 2002).

Therefore, assuming that large eggs are beneficial to the hatchlings, we may put forward two alternative explanations for our results.

First, this allocation pattern may reflect a female cryptic preference for small-sized and large-clawed males. A preference for relatively large-clawed males was expected, since these can be better at dominating rivals during aggressive intra-sexual encounters, and at turning and securing females during copulation (Snedden 1990). Therefore, all else being equal, large claws confer to their bearers a competitive advantage over other males, and females allocating more resources to eggs when paired with such males are likely to increase the fitness of their offspring, provided that this trait is heritable. On the other hand, female cryptic preference for small males may instead stem from the higher sperm expenditure of such males (Rubolini et al. in press). Thus, it may pay

Figure 1. Relationships between residual egg weight (a, b) or residual clutch size (ln-transformed; c, d) and residual male traits (carapace length and chelae length), after accounting for the effects of dyad and other male traits (carapace length, chelae length and asymmetry). Residual data points are symmetrical with respect to the origin, and pairs of symmetrical points correspond to data from a given dyad. Regression lines are shown, and significance tests are reported in table 1. See §2 for further details.
females to allocate differentially more resources to eggs when paired with small, younger males, because they may secure a higher egg fertilization rate and be more successful in sperm competition in cases of multiple matings. It remains, however, unclear why females laid smaller clutches for these males.

Alternatively, females may favour relatively large-sized and small-clawed males by laying larger clutches. In fact, if these males are of high quality, females can maximize their reproductive success because this allocation pattern may lead to an increased production of high-quality offspring. Under this scenario, females following the other strategy may allocate more resources to egg size for small-sized, large-clawed males in order to compensate for their lower quality (e.g. Saino et al. 2002b). However, this explanation seems unlikely, because there is no evidence that smaller chelae should indicate high-quality males, but rather the opposite (Snedsen 1990; Gherardi et al. 2000). In addition, several lines of evidence indicated that large males (i.e. carapace length > 45 mm, age > 6–7 years) in this species could be senescent individuals: they were sluggish and slower moving than small males in the wild (Nardi et al. 2004), and appeared to be sperm-limited (Rubolini et al. in press).

In conclusion, our experiment showed that females can finely tune the allocation of resources to different measures of primary reproductive effort in relation to multiple male traits, which enable them to exert post-mating mate preferences even when sexually coerced.

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REFERENCES


