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Phenotypic manipulation provides insights into the function of a sexually selected trait in a freshwater crustacean species complex

Rickey D. Cothran [a,*], Amber Kuzmic [b,1], Gary A. Wellborn [b,1], Rick A. Relyea [a]

[a] Department of Biological Sciences and Pymatuning Laboratory of Ecology, University of Pittsburgh
[b] Department of Zoology and Biological Station, University of Oklahoma

Although sexual selection is a fundamental driver of evolution, our understanding of its causes and evolutionary dynamics is often constrained by poor knowledge of mechanisms of trait function. A powerful method for assessing mechanisms of sexual selection is to combine phenotypic manipulation of male sexual traits with manipulation of behavioural interactions associated with sexual selection. We performed such experiments to assess the function of posterior gnathopods (large, sexually dimorphic claws) in mating and reproduction of *Hyalella* amphipods. Removal of male gnathopods reduced pairing success and shortened pairing duration. Patterns of pairing success were similar in both the absence and presence of competing males, and results were consistent across three species that live in ecologically distinct habitats. Collectively, our findings demonstrate that gnathopods in *Hyalella* function during the interactions between males and females that lead to pair formation, and such functions are relatively conserved within the *Hyalella* species complex. Finally, our findings for gnathopod function in *Hyalella* contrast markedly with a recent study of gnathopod function in another amphipod, *Gammarus pulex*, indicating that enlarged gnathopods may have diverse functions among mate-guarding amphipod species.

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An understanding of the evolution and maintenance of a sexually selected trait rests on an understanding of its function. Darwin (1871) recognized two mechanisms by which sexually selected traits may evolve. Intrasexual selection (most often in the form of male–male competition) affects traits that increase an individual's success in obtaining mates through contests between individuals of the same sex. Intersexual selection (most often in the form of female choice) affects traits that attract the opposite sex. Intersexual interactions, however, can be antagonistic (i.e. when a sexual trait increases the fitness of one sex at the expense of the other; Parker 1970, 1979; Trivers 1972). In these cases, sexual conflict leads to the evolution of offence traits in males and defence traits in females. While there is substantial empirical support for each type of selection, debate continues over their relative importance and the contexts in which they are employed (Møller & Jennions 2001; Cameron et al. 2003; Arnqvist & Rowe 2005; Kotiaho & Puurtinen 2007). Many traits, moreover, have manifold utility, functioning,

for example, as both armaments and ornaments (Berglund et al. 1996; Hunt et al. 2009). Although the form of traits can provide insights into their function, understanding sexual trait function requires experimental analysis (e.g. Sorenson & Derrickson 1994; Díaz & Thiel 2003; Hume et al. 2005).

Many amphipod crustaceans exhibit sexual dimorphism in the size of gnathopods, prehensile appendages lying between the anterior feeding appendages and thoracic walking legs (Fig. 1). Amphipods have two pairs of gnathopods, and in most species only the posterior gnathopod is sexually dimorphic (Conlan 1991). In *Hyalella* amphipods, the group examined in this study, the most distal segments of the posterior gnathopod, the propodus and dactyl, are greatly enlarged in males, being about 15-fold larger than those of females (Conlan 1991; Wellborn 2000; Fig. 1).

Among amphipods, variation in gnathopod dimorphism occurs largely at the level of superfamilies, and there is a strong association between presence of gnathopod dimorphism and mating behaviour among taxonomic groups (Conlan 1991). Gnathopod dimorphism is common within superfamilies in which males guard mates prior to the female's moult, but is absent within superfamilies in which males employ only mate-searching tactics to achieve matings (Conlan 1991), suggesting that enlarged male gnathopods function during the precopulatory mate-guarding phase of mating. Many mate-guarding amphipods exhibit precopulatory contact

* Correspondence: R. D. Cothran, Department of Biological Sciences, University of Pittsburgh, 101 Clapp Hall, 4249 Fifth Avenue, Pittsburgh, PA 15260, U.S.A.
  E-mail address: rdc28@pitt.edu (R.D. Cothran).
[1] A. Kuzmic and G. A. Wellborn are at the Department of Zoology, University of Oklahoma, 730 Van Vleet Oval, Norman, OK 73019, U.S.A.
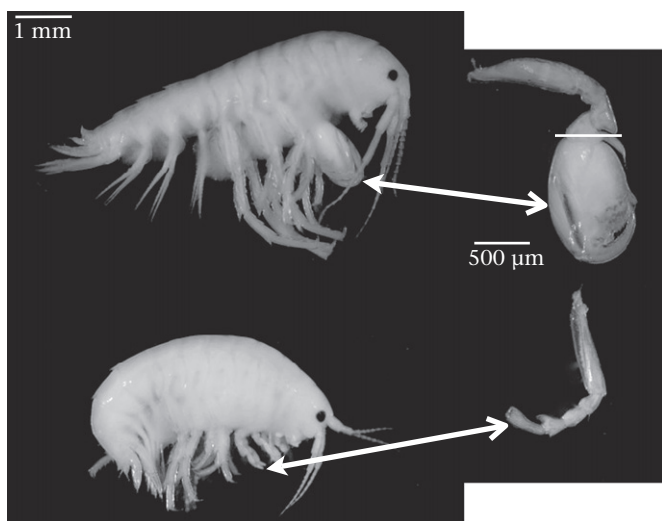
Figure 1. Male and female *Hyalella* amphipod, showing gnathopods. Male (top) and female (bottom) large-ecomorph (OK-L species). The double arrow points to the posterior gnathopod. The horizontal line indicates the point of dissection.

mate guarding, during which males are positioned dorsally to females and use their anterior gnathopods (not the enlarged posterior gnathopods) to grasp and hold females until the female moults (Borowsky 1984). Immediately after the female moult, eggs pass into an external brood chamber, the marsupium, where they are fertilized (Wellborn & Cothran 2007a). Because female receptivity to fertilization is restricted to a brief period after the female's moult (Jormalainen 1998), amphipod mating systems characteristically have a male-biased operational sex ratio (Emlen & Oring 1977).

Darwin (1871) highlighted gnathopod dimorphism in amphipods in his discussion of sexual traits in Crustacea, and, in an explanation evocative of sexual conflict, he hypothesized that the enlarged gnathopods allow males to 'seize' females. Interestingly, empirical studies have confirmed conflict between the sexes over precopulatory pairing duration in amphipods and isopods, which share many components of their mating biology with amphipods, and it is possible that gnathopods mediate conflict resolution (Jormalainen & Merilaita 1995; Cothran 2008a). However, despite Darwin's early attention to the issue, little is known about the function of enlarged gnathopods in male amphipods. Posterior gnathopods have been suggested to act as weapons in male–male competition for access to females (Darwin 1871; Strong 1973; Borowsky 1984; Mattson & Cedhagen 1989) and as ornaments used to convey chemical and tactile cues during courtship (Dick & Elwood 1989; Mattson & Cedhagen 1989). Finally, it should be noted that ecological differences between the sexes may also contribute to gnathopod dimorphism (Darwin 1871; Shine 1989). In *Hyalella* amphipods, studies of field mating patterns have found that larger posterior gnathopods increase success in forming precopulatory pairs, and relative gnathopod size (after adjusting for allometry with body size) is under sexual selection in some species (Wellborn 1995, 2000; Wellborn & Bartholf 2005). Nevertheless, the functional role of posterior gnathopods during mating in *Hyalella* remains unclear.

In the most rigorous study of gnathopod function to date, Hume et al. (2005) removed the posterior gnathopods to experimentally evaluate their function in *Gammarus pulex*. Gnathopod removal did not influence the ability of males to pair with females, even when intact male competitors were present. However, gnathopod removal entirely prevented copulation and fertilization because posterior gnathopods were necessary to maintain proper positioning during the multiple bouts of copulation necessary for females to extrude eggs (Hume et al. 2005). Given the great diversity of amphipods (e.g. over 1870 freshwater species; Väinölä et al. 2008), it is not clear whether the gnathopod function found in *G. pulex* applies across amphipod taxa.

In this study, we evaluated gnathopod function in *Hyalella* amphipods, a group that is distantly related to *Gammarus* (*Gammarus* and *Hyalella* are in the superfamilies Gammaridea and Talitroidea, respectively). Specifically, we used a combination of gnathopod-removal treatments and manipulations of behavioural interactions to assess the contribution of male–male and male–female interactions in shaping gnathopod-based mating biases.

STUDY SPECIES AND GENERAL METHODS

Hyalella amphipods are common in freshwater habitats throughout much of the New World (Bousfield 1996; Väinölä et al. 2008). In North America, *Hyalella* form a diverse species complex, including many species identified through genetic studies, but currently lacking taxonomic descriptions (Witt et al. 2006; Wellborn & Broughton 2008). One emerging pattern in this North American diversity is the existence of multiple geographically widespread species that fall within one of two ecomorphs defined by body size and life history traits (Wellborn 1994; Wellborn et al. 2005). In regions where this group has been studied, these two ecomorphs, each represented by multiple species (Wellborn & Broughton 2008), sort among permanent aquatic habitats based on the intensity of predation by *Lepomis* sunfish (Strong 1972; Wellborn 1994; Wellborn et al. 2005). A small-bodied ecomorph is found in habitats where amphipods experience substantial predation from *Lepomis*, whereas, a large-bodied ecomorph is found in fishless habitats or in habitats where predation by fish is less intense.

Previous studies have demonstrated that both male body size and relative posterior gnathopod size influence male mating success (Wellborn 1995, 2000; Wellborn & Bartholf 2005). In large-ecomorphs, male pairing success increases monotonically with both traits. In small-ecomorphs, the relationship between body size and mating success is weaker, and larger size-specific gnathopod size is only important in determining mating success for smaller males (Wellborn 1995, 2000; Wellborn & Bartholf 2005). Furthermore, in large-ecomorph *Hyalella* the mating advantage for larger males is probably driven by male–female interactions because removal of male–male competitive interactions does not decrease size-based mating biases (Cothran 2008b). Currently, however, we do not understand the mechanisms responsible for mating biases associated with posterior gnathopod size in *Hyalella*.

In the current study, one large-ecomorph species was examined. This species, referred to as OK-L in Wellborn & Broughton (2008), was collected from Lake Thunderbird, Cleveland County, Oklahoma, U.S.A., and is common in Oklahoma. The life history and relationships between body and posterior gnathopod size and mating success have been well studied in this large-ecomorph species (e.g. Wellborn & Bartholf 2005; Wellborn et al. 2005). Two small-ecomorph species were included in the study, referred to as Species B and Species C in Wellborn & Cothran (2004, 2007b). These small-ecomorph species were collected from Lake LeBoeuf, Erie County, Pennsylvania, U.S.A., a natural lake with a well-vegetated littoral zone composed primarily of *Potamogeton* sp. and *Myriophyllum* sp. The lake has a diverse fish assemblage, including *Lepomis* spp. that prey heavily on *Hyalella* amphipods (Wellborn 1994).

Although Species B individuals have a larger mean body size than Species C individuals, their adult size distributions overlap and they are otherwise very similar in phenotype, with no known

unambiguous characters distinguishing them (Wellborn & Cothran 2004). Therefore, we relied on two procedures to ensure that species were not mixed in experimental trials. First, because species segregate based on both a distance from shore gradient and a depth gradient (Wellborn & Cothran 2007b), we focused collection efforts spatially in habitats where each of the species is dominant. For Species C, we collected in areas several metres from shore and approximately 1 m below the surface. Collection efforts for Species B were focused very near the shore (Wellborn & Cothran 2007b). Second, at the conclusion of experiment 1, for some trials (approximately half of the trials for each species) we tested whether an incorrectly identified species had been included in the trial by determining whether the male that failed to pair was able to fertilize the female's eggs. We did this by allowing the unpaired male to mate with the female during the female's subsequent reproductive bout, and used the presence of fertilized eggs as an indicator that this male was the correct species. Unpaired males successfully fertilized eggs in 23 of 28 and 31 of 31 of trials for Species B and Species C, respectively. We conclude that species identifications were correct in at least the large majority of cases, but some misidentifications were possible, especially for Species B.

The goal of this study was to determine how posterior gnathopods contribute to male mating and reproductive success in *Hyalella* amphipods. In a series of three experiments, we used phenotypic manipulation to examine the role of gnathopods in both male–male competition for mates and in male–female interactions, providing insights into how this trait affects both pairing and fertilization success.

Phenotypic Manipulation of Males

The experiments used three manipulations of male appendages. In all cases, males were anaesthetized using a clove oil solution with an ethanol carrier (clove oil concentration of 2.97×10^{-4} ml/ml; Venarsky & Wilhelm 2006). In the first group of males, we removed the propodus and dactyl of the posterior gnathopod (hereafter, 'gnathopod-removed' males; Fig. 1). In a second group of males, we removed the propodus and dactyl of peraeopod 4 or 5 (hereafter, 'peraeopod-removed' males). Peraeopods, or walking legs, are serially homologous to gnathopods. In a third group of males, we conducted sham-operations in which amphipods were anaesthetized with clove oil and manipulated under the dissecting scope, but no appendage removal occurred (hereafter, 'intact' males). In gnathopod-removed and peraeopod-removed males, dissections were performed under a dissecting microscope using fine forceps, and the propodus and dactyl on both the right and left sides of each individual were removed. Wounds produced were similar because dissections were performed at the articulation of the propodus in each case. After surgery, males were transferred to individual cups, fed a 3:1 mix of alfalfa (Spring Valley, Springfield, MO, U.S.A.) and fish flakes (Tetramin®, TetraWerke, Melle, Germany), and given 24–72 h (72 h for experiment 1 and 24 h for experiments 2 and 3) to recuperate from surgery.

EXPERIMENT 1: MALE–MALE INTERACTIONS

Methods

In experiment 1, we asked how gnathopods affect male mating success in the presence versus absence of male–male competition for three species of *Hyalella* amphipods. The experiment included two tether treatments crossed with two appendage-removal treatments. To remove male–male interactions, we tethered males to opposite sides of a 100 mm diametre petri dish. Males were tethered to small pebbles using superglue and a single ˜15 mm

strand of nylon thread (Cothran 2008b). This allowed males to move and interact with females locally, within the 7.5 mm radius around the pebble they were anchored to (males were unable to drag the pebble). Tethering males both removed any potential differences in scramble competitive abilities, by equalizing search area, and eliminated direct interactions between males. In a sham-tether control, a strand of nylon thread was glued to each male, but they were not anchoured to pebbles, allowing males in these trials to move and interact freely, as confirmed by observations during trials. Appendage-removal treatments allowed evaluation of the importance of posterior gnathopods in mating success. In one treatment, a gnathopod-removed male competed against an intact male, and in the second treatment a peraeopod-removed male competed against an intact male. The latter treatment was used to test the effect of surgery independent of gnathopod removal. The day after tethering, a single female carrying mid-stage embryos in her marsupium was added to each petri dish. Females at this stage are a few days away from pairing and thus had ample opportunity to sample males before pairing. Amphipods were fed a small amount of a 3:1 mix of alfalfa and fish flakes. We distributed food such that tethered males had access to it.

We recorded which male was paired with the female every 4 h between 0900 and 2100 hours. For the tethered treatment, we noted the position (side of the dish) of the paired male, and thus were blind to the identity of the paired male during observations. For the untethered treatment, we examined the unpaired male under a dissecting microscope for presence of gnathopods or peraeopods. A trial was ended when a pair separated and the female had oviposited, indicated by the presence of early-stage embryos in her marsupium. Cases in which one of the two males died were excluded from analyses. If males moulted or escaped their tether without any subsequent pairing, we reapplied the tether or affixed a new thread (untethered treatment) to the male and added a new female. Finally, if a female died or oviposited before pairing was observed, we added a new mid-moult female. After each trial, amphipods were preserved in 70% ethanol. Because male mating success is influenced by both relative gnathopod size and body size in *Hyalella* (Wellborn 1995; Wellborn & Bartholf 2005), we measured head length (an index of body size; Edwards & Cowell 1992) for all amphipods, and measured gnathopod width for males with intact gnathopods to assess whether appendage-removal treatments differed in body size (using independent samples *t* tests) or gnathopod size (after accounting for body size using ANCOVA with head length as a covariate). For each species, we found no significant differences between appendage-removal treatments in male body size (all $P > 0.28$) or gnathopod size (all $P > 0.76$), indicating no biases existed in the assignment of individuals to treatments.

We used a logit model to test for effects of species (Species B, Species C, and large-ecomorph species), tethering (yes or no) and appendage removal (gnathopod-removed or peraeopod-removed) on pairing patterns (whether the intact or appendage-removed male paired). Given the number of explanatory variables (including all possible interactions) we used a backward elimination approach. The analysis started with the full model (all explanatory variables and interactions) and at each step the term with the lowest explanatory power (highest *P* value assuming removal did not result in a significant change ($P > 0.10$) in *G* (i.e. fit)) was removed from the model. This process continued until only terms with high explanatory power ($P < 0.10$) remained in the model (Quinn & Keough 2002, page 364).

We predicted that the type of appendage removed would have an effect on pairing patterns. Specifically, gnathopod removal would decrease pairing success but peraeopod removal would have no effect (indicated by a significant main effect of appendage

removal). We also predicted that if gnathopods are only used in securing and maintaining paired positions in competition with other males, that the advantage of having this appendage would breakdown in treatments where males were tethered to opposite ends of the arena (indicated by a significant tether treatment main effect). Moreover, the effect of tethering should only manifest in treatments where gnathopods were removed (indicated by an appendage-removal-by-tether treatment interaction). On the other hand, if gnathopods are also important in interactions between males and females, then intact males should have a pairing advantage over gnathopod-removed males regardless of tethering treatment, whereas they would have no such advantage when competing against peraeopod-removed males (indicated by a significant appendage-removal effect, but no effect of tether treatment or the appendage-removal-by-tether treatment interaction). Finally, if the effects of appendage-removal and tether treatments were species specific, we expected significant species-by-treatment interactions.

Results

The percentage of pairing did not differ among species or between tethering treatments, but was affected by the manipulation of the male appendages (Table 1, Fig. 2). Gnathopod removal decreased pairing rates by 80%, 45% and 81% in Species B, Species C, and the large-ecomorph species, respectively. The tether treatment, which restricted male search area and removed male–male interactions, had no significant effect on pairing patterns. Pairing patterns were also similar across the three species, and no interaction effects were evident (Table 1).

EXPERIMENT 2: MALE–FEMALE PAIRING AND FERTILIZATION INTERACTIONS OVER THE FEMALE REPRODUCTIVE CYCLE

Methods

In experiment 2, we assessed mating behaviour over an entire female moult and reproductive cycle, providing insights into how gnathopod removal affects pairing, mating and fertilization success. The experiment examined behaviour of the same large-ecomorph species (OK-L species) used in experiment 1. Amphipods used in this experiment were collected from Cowan Creek, Marshall County, Oklahoma, U.S.A. Before the experiment, amphipods were maintained in aerated plastic tubs containing water and vegetation from their source habitat. Diets were supplemented weekly with fish food (Vitakraft® pond menu multivitamin, Bound Brook, NJ, U.S.A.; or Tetramin®, TetraWerke, Melle, Germany).

We collected 60 precopulatory pairs from the stock population and assigned 20 pairs to each of the three male appendage-removal
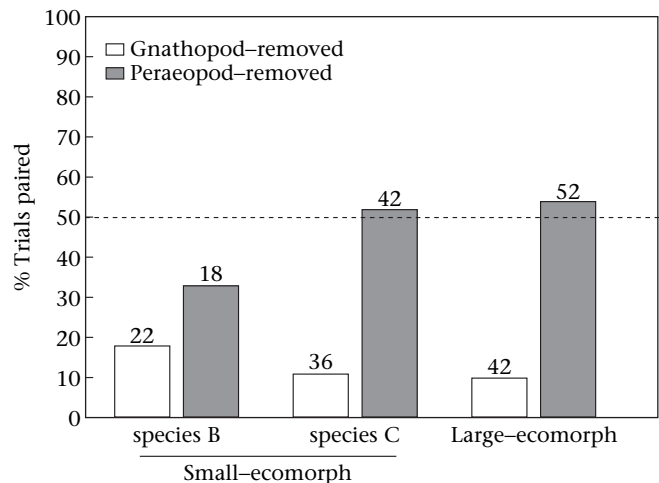
Table 1
Results of backward elimination procedure testing the effect of appendage-removal treatment (AR), tethering and species identity on whether a removed-treatment male or a control male paired in experiment 1

Variable	ΔG	df	P
AR	**37.38**	**1**	**<0.001**
Tethered[7]	0.2	1	0.66
Species[5]	2.47	2	0.29
AR*tethered[2]	0.46	1	0.5
AR*species[4]	3.08	2	0.22
Tethered*species[6]	1.87	2	0.39
AR*tethered*species[3]	2.56	2	0.28

ΔG = change in the log likelihood statistic when a term was removed. Superscripts refer to the step in the backward elimination procedure at which a variable was removed from the model. Significant effects are indicated in bold.

Figure 2. Percentage of trials in which a male *Hyalella* amphipod with a removed appendage paired with a female in competition against an intact male. The dashed line is the percentage of cases expected if appendage removal has no effect on pair formation (i.e. both appendage-removed males and intact males pair in 50% of cases). For each treatment, sample sizes are provided above bars. Results of the tether treatments were pooled because tethering had no effect on pairing patterns.

treatments. Pairs were first separated by placement on blotting paper, and in some cases were gently teased apart, and kept individually in glass jars (66 mm diameter) filled with 75 ml of well water and sediment. Females remained isolated over a few days as they moulted and passed a clutch of eggs into their marsupium. These unfertilized eggs disintegrate in about 2 days, and the absence of embryos in the marsupium was confirmed by inspection under a dissecting microscope. Because amphipods do not store sperm, this procedure assured that all viable embryos observed during a trial were fertilized by the focal male. Trials began with the reintroduction of the male into the jar containing the female. Jars were checked daily for pairing. Beginning approximately 5 days after the male was introduced, each female was examined daily under a dissecting microscope for the presence of developing embryos, an indication of successful fertilization. It is possible for fertilization to occur even when pairing is not observed because males may pair for only a brief time or might fertilize eggs as they are passed into the marsupium, although the latter behaviour has not been directly observed in *Hyalella* (personal observation).

We used Pearson's chi-square to test for an effect of removal treatments on the proportion of trials where pairing was observed and the proportion of females carrying developing embryos. Additionally, we used an ANOVA to test whether appendage removal affected pairing duration. Our analysis of pairing duration included replicates in which pairing was not observed but fertilization was successful, and these replicates were assigned a pairing duration of 0 day.

Results

Appendage removal affected the proportion of replicates in which pairing was observed ($\chi^2_2 = 23.105$, $P < 0.001$; Fig. 3a). Fewer gnathopod-removed males formed pairs than did either intact males ($\chi^2_1 = 10.99$, $P < 0.001$) or peraeopod-removed males ($\chi^2_1 = 17.14$, $P < 0.001$). Intact and peraeopod-removed control treatments did not differ in frequency of pair formation ($\chi^2_1 = 2.11$, $P = 0.15$).

Duration of pairing differed between appendage-removal treatments ($F_{2,47} = 12.056$, $P < 0.001$; Fig. 3b). Gnathopod-removed males paired for about a 1.5 days less than intact and peraeopod-
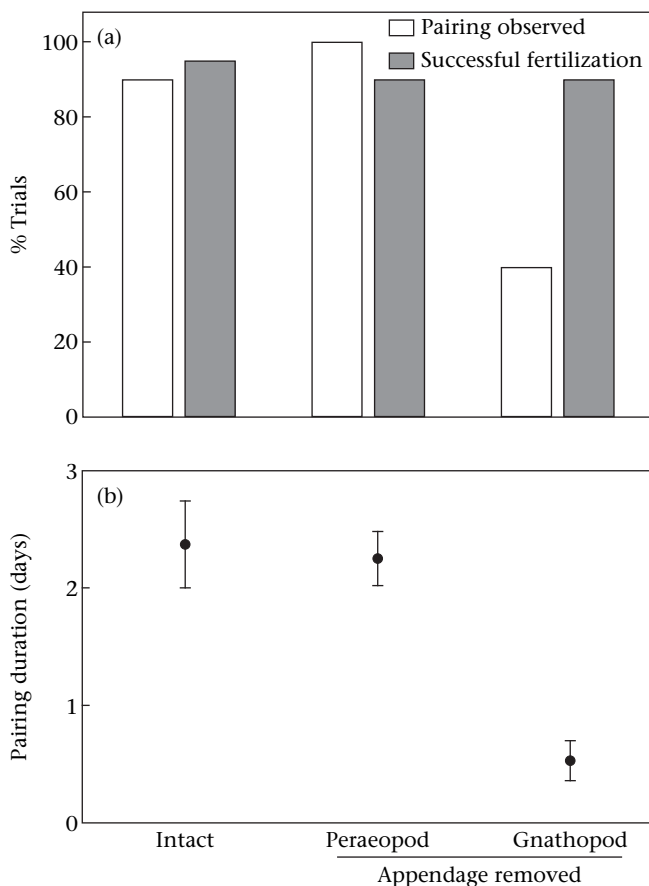
Figure 3. Effects of gnathopod removal on pairing duration and fertilization success in the absence of mate competition. (a) Percentages of trials where pairing was observed and fertilization was successful for each of the appendage-removal treatments ($N = 20$ for each group). (b) Mean $\pm$ SE pairing duration (in days) for intact males ($N = 19$), peraeopod-removed males ($N = 16$) and gnathopod-removed males ($N = 15$).

removed males (Tukey contrast: $P < 0.005$ in both comparisons). Pairing duration did not differ between intact and peraeopod-removed treatments (Tukey contrast: $P = 0.953$).

Despite low rates of pairing observed by gnathopod-removed males, nearly all males successfully fertilized eggs, regardless of appendage-removal treatment or whether they paired with females. Treatments did not differ in the number of females carrying developing embryos ($\chi^2_2 = 0.641$, $P = 0.726$; Fig. 3a).

EXPERIMENT 3: MALE–FEMALE BEHAVIOURAL OBSERVATIONS

Methods

The goal of experiment 3 was to examine the effect of male gnathopod removal on mating behaviours that lead to pair formation. For this experiment we examined the large-ecomorph *Hyalella* species (OK-L species). Collection information and laboratory maintenance of the stock population were identical to that for experiment 2. For each male appendage-removal treatment, we observed behavioural interactions between a single male and female over a 10 min period. Females used in trials were taken from precopulatory pairs to assure their receptivity to pairing, and then placed in 60 mm diameter dishes containing conditioned well water. After 5 min, a male was added and all encounters (defined as physical contact between the male and female) were recorded, along with the presence of pairing and time elapsed before pair

formation. Twenty replicates were performed for each of the three appendage-removal treatments. Because no new encounters can occur after pair formation, encounter rates were calculated as the number of separate encounters divided by the time elapsed before pair formation, or by the observation period (10 min) if no pair formed. We used ANOVA to test for differences between treatments in mean encounter rates (log transformed) and mean time until pairing, and used Pearson's chi-square to test for treatment differences in the frequency of trials in which pairing occurred.

Results

Encounter rates between males and females differed among appendage-removal treatments ($F_{2,57} = 4.79$, $P = 0.012$; Fig. 4a). Tukey contrasts indicated gnathopod-removed males had higher encounter rates than peraeopod-removed males ($P = 0.011$), and this tended to be the case when compared to intact males as well ($P = 0.087$). Intact males did not differ from peraeopod-removed males ($P = 0.68$). Frequency of pair formation also differed between treatments ($\chi^2_2 = 17.368$, $P < 0.001$; Fig. 4b). No gnathopod-removed males formed mating pairs during the 10 min observation period, but both peraeopod-removed and intact males formed pairs in 11 of the 20 replicates in their respective treatments. Considering only replicates in which pairs formed, the minutes to pair formation did not differ between peraeopod-removed and intact male treatments (peraeopod-removed: mean$\pm$SD = 4.0$\pm$2.4; intact: 2.95$\pm$2.4; ANOVA: $F_{1,20} = 1.03$, $P = 0.32$).
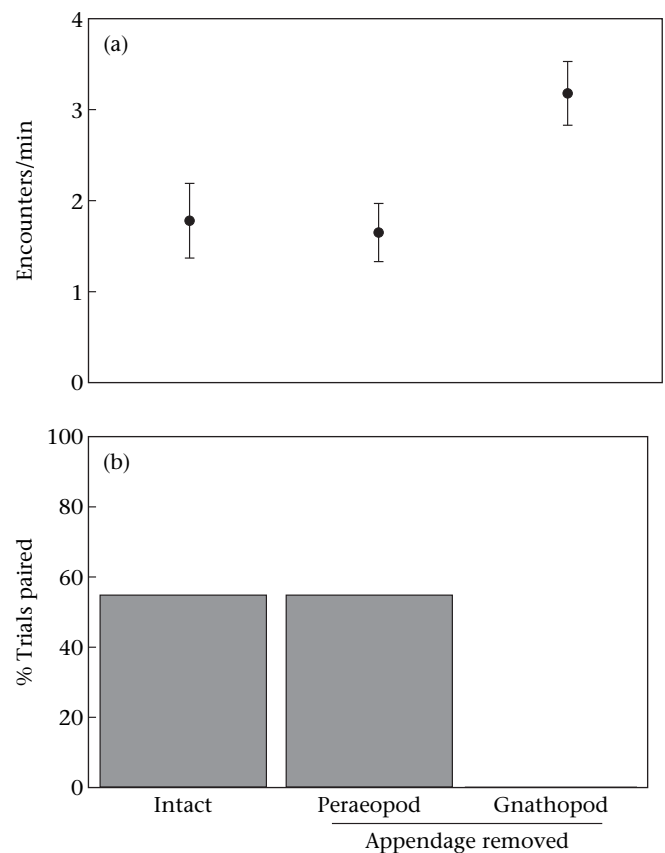

Figure 4. Effects of gnathopod removal on encounter rate and pairing success during short-term (10 min) behavioural observations. (a) Mean $\pm$ SE encounter rate between males and females for each treatment. (b) Percentages of trials that ended in pairing for intact males, peraeopod-removed males and gnathopod-removed males ($N = 20$ for each group).

DISCUSSION

Our results clarify some key questions concerning the functional role of enlarged posterior gnathopods during mating interactions in *Hyalella* amphipods. Phenotypic manipulation experiments yielded three primary findings. First, when male–male competition for mates was absent, gnathopod removal resulted in a substantially reduced frequency of pair formation, but this was not due to lower encounter rates. Second, when gnathopod-removed males did pair with females, they did so for a shorter duration than control males, suggesting that gnathopod-removed males form pairs closer to the end of the female's moult cycle. Third, male–male interactions and constraints on male search activity had no effect on pairing success of gnathopod-removed males relative to control males, implying that reduced pairing success of gnathopod-removed males was due to male–female interactions, and not to male–male interactions or male search behaviour. Fourth, we found virtually identical results across all three *Hyalella* species, suggesting that key elements of gnathopod function are shared within the genus. Finally, gnathopod-removed males and control males all had very high rates of successful fertilization, demonstrating that enlarged posterior gnathopods are not required for successful fertilization of eggs. This finding contrasts with the critical role of enlarged gnathopods in fertilization for *Gammarus pulex* (Hume et al. 2005), and suggests that enlarged gnathopods have diverse functions across amphipod species.

Darwin (1871) suggested that enlarged posterior gnathopods might be used by males to coerce females into pairing, and results of a previous study suggest sexual conflict over pairing occurs in *Hyalella* (Cothran 2008a). In that study, experimental reduction of female resistance behaviours increased pairing duration, implying sexual conflict over pairing duration with males preferring a longer pair duration, as is often expected under the male-biased operational sex ratio typical of mate-guarding amphipods (Cothran 2008a). Cothran (2008a) did not manipulate male phenotypes to assess whether a reduction in pairing duration is observed when females have an advantage in the conflict over pairing, but the current study may have imposed such a manipulation. If enlarged gnathopods have a coercive function in sexual conflict, our removal of posterior gnathopods would provide an advantage to females in the conflict over pairing duration. Under this reasoning, the reduced pairing durations for gnathopod-removed males in the present study may represent a shift in conflict resolution in favour of females. Together, the extended pairing durations with reduced female resistance in Cothran (2008a), and the reduced duration due to gnathopod removal suggest that natural pairing durations represent a compromise between male and female interests (Yamamura & Jormalainen 1996; Härdling & Smith 2005). Further exploration of this hypothesis requires an understanding of the mechanism by which gnathopods are used to coerce females. Although males do not use the enlarged gnathopods to hold females during the pairing phase, they might use them to subdue females during interactions that precede pair formation.

Another explanation for reduced pairing success of gnathopod-removed males is that enlarged posterior gnathopods are a target of female choice. Females may view gnathopod-removed males as poor-quality mates and preferentially mate with gnathopod-intact males. Female choice could also explain the short pairing durations observed in experiment 2 and complete lack of pairing in experiment 3 because females may refrain from pairing with these (perceived) low-quality males until options are limited near the end of the moult cycle (females cannot reabsorb eggs and do not store sperm).

In practise, it is exceedingly difficult to distinguish effects of male coercion from those of female choice in *Hyalella* and other mate-guarding amphipods because of the nature of their tactile interaction, sometimes referred to as a 'struggle' that precedes pairing (Wellborn & Cothran 2007a). The interaction sometimes results in pair formation, and sometimes does not. Given that both sexes may gain benefits and incur costs associated with the timing of pair formation and choice of mate, it may be naïve to view male coercion and female choice as mutually exclusive alternatives, rather both processes probably contribute importantly to mating behaviour and sexual selection in *Hyalella* (Wellborn & Cothran 2007a).

Prevention of male–male interactions in experiment 1 did not affect amphipod pairing patterns, suggesting that direct male–male competition does not contribute to effects of gnathopod removal on pairing success. This result is consistent with a previous study that examined the influence of male body size on pairing success in both the large- and small-ecomorphs (Cothran 2008b). Although male body size is often correlated with male mating success (Wellborn & Cothran 2007a), prevention of the opportunity for male–male competition, via tethering, in Cothran (2008b) had no effect on size-based pairing patterns. However, in both Cothran's (2008b) study and the current study amphipod densities were low compared to those found in nature. In our experiment, density was about 382 individuals/m² compared to the 750–12 000 individuals/m² typical of *Hyalella* populations (Wellborn 1994). Given the importance of density in determining the intensity of intrasexual (and intersexual) selection (Shuster & Wade 2003; Kokko & Rankin 2006), future studies should assess whether the functional role of posterior gnathopods is sensitive to changes in density.

In *Hyalella* and other mate-guarding amphipods we know little about the final phase of pairing, during which the female moults and passes eggs into the marsupium, where they are fertilized, a process that may last only several minutes in *Hyalella*, but has never been examined empirically. From the males' perspective, females have their highest value at this stage because male time investment is minimal (Jormalainen 1998). Females, however, may be least able to exercise mate choice at this stage because their integument is unlikely to be fully hardened following the moult. It follows that coercive fertilization attempts by nonguarding males and male–male competition for fertilization may be common during this period, especially in light of the high population densities often seen in *Hyalella* (Wellborn 1994). If such interactions occur, enlarged gnathopods are probably important in mediating the outcomes of these interactions. Rectifying our ignorance of this potentially critical final stage of mating will provide valuable insights on whether pairing success equals mating success in this system.

It must be noted that effects of our appendage-removal treatments on mating and reproduction may result directly from changes in an individual's ability to use the removed appendage, changes in how the individual is perceived by other individuals, and via changes in the manipulated individual's behaviour. For example, in our study gnathopod-removed males had higher encounter rates with females than peraeopod-removed and intact males. Thus, in addition to having direct effects on the ability of males to use their gnathopods, our manipulation also affected male behaviour. While our experiment was not designed specifically to tease apart these three possibilities, which are not mutually exclusive, our results clearly show that posterior gnathopods are critical for resolving intersexual interactions that precede pairing. Future studies that closely examine the mechanical and behavioural consequences of gnathopod removal during intersexual interactions are necessary to tease apart the specific mechanism by which gnathopod-removed males do not achieve pairing success.

Posterior gnathopod function in *Hyalella* contrasts sharply with gnathopod function in *Gammarus pulex* (Hume et al. 2005), a distantly related freshwater amphipod that also has enlarged,

sexually dimorphic posterior gnathopods. In a study of *G. pulex*, gnathopod removal had no effect on frequency of pair formation or on duration of pairing (Hume et al. 2005). Gnathopod-removed males were unable to successfully copulate, however, which resulted in very few females extruding eggs and a complete absence of fertilization success (Hume et al. 2005). *Gammarus pulex* males use enlarged gnathopods to grasp and position females for repeated bouts of copulation, and it is these bouts of copulation that precipitate extrusion of eggs that are then fertilized. Without gnathopods, eggs are not extruded, and no fertilization is possible (Hume et al. 2005). Effects of gnathopod removal in *Hyalella* were very different. We found gnathopod removal in *Hyalella* affected both the frequency and duration of pairing, but clearly had no impact on copulation and fertilization success. Based on available phylogenetic evidence, Conlan (1991) suggested that contact mate guarding in *Gammarus* and *Hyalella* evolved independently, providing a possible explanation for the distinctly different functions of gnathopods in these genera. Despite differences in gnathopod function, *G. pulex* and *Hyalella* are similar in that effects of gnathopod removal on pairing success were not affected by the presence or absence of male–male interactions.

Acknowledgments

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