

Influence of female moulting status on pairing decisions and size-assortative mating in amphipods

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Abstract

Precopulatory mate guarding is a common strategy, which has evolved in species where the female receptivity (and thus egg fertilization) is predictable, but also limited to a short period. Although males are larger than females in many amphipods, the largest males pair with the largest females, leading to a positive size-assortative pairing. Size-assortative pairing has received much attention but how moulting physiology could affect pairing decisions has rarely been studied. Here, we tested the hypothesis that the size-assortative pairing in the freshwater amphipod *Gammarus pulex* is closely related to the female moult cycle. We characterized moulting status by observing the new cuticle formation then tested the influence of the moulting status on pairing decision. Overall, female moult stage influences the variation and intensity of size-assortative pairing. Whereas individuals tended to pair at random as soon as the females become receptive (early beginning of the premoult stage), size-assortative pairing was stronger as females were closer to the moult. Thus, moulting and pairing decision could not be dissociated and moulting should be controlled for when examining the behavioural ecology of mate choice decisions in crustaceans.

Introduction

Males engage in a precopulatory mate guarding (PCMG) when female receptivity is predictable, but limited to a short period during her reproductive cycle (Yamamura, 1987). In the absence of synchronization of female reproduction, the operational sex ratio is male biased, and PCMG may evolve as a male competitive strategy (Parker, 1974). A number of crustacean species display PCMG or amplexus (Strong, 1973) in which the male carries a female beneath his ventral surface for several days before she becomes receptive. In amphipods and isopods, although males may be larger than females, precopula pairs show positive size-assortative pairing with large males pairing with large females and smaller males with smaller females.

Three main types of hypotheses have been proposed to explain size-assortative pairing (Crespi, 1989; Hume *et al.*, 2002), most of them are solely relying on the effect of animal body size. First, the 'microhabitat segregation' and 'spatial covariation' hypotheses suggest that the environment can affect the spatial distribution of the individuals according to their size, hence favouring size-assortative pairing at the habitat scale (Birkhead & Clarkson, 1980; Ward & Porter, 1993). Second, the 'mechanical constraints' and 'loading con-

straints' hypotheses suggest that the simple differences in the size of body parts and the energetic costs imposed by guarding (Sparkes, Keogh & Pary, 1996; Plaistow, Bollache & Cézilly, 2003) is an obstacle for small males to hold and transport large females. Such passive mechanisms could prevent unequal pairings (Adams & Greenwood, 1983; Williams, 2007). Third, whereas the predictions of the two first classes of hypotheses have never been wholly met both in the field and in the laboratory (Elwood, Gibson & Neil, 1987; Elwood & Dick, 1990; Bollache, Gambade & Cézilly, 2000; Williams, 2007), strong evidence exists for major role of sexual selection in the evolution of size-assortative mating (Jormalainen, 1998, 2007; Bollache & Cézilly, 2004a; Wellborn & Cothran, 2007). Given the preference of males for large, fecund females, males may compete by attempting to be the first to take a large female. The 'timing hypothesis' proposes that large males are better able to afford the costs of carrying a large female for a long time and should pair early in the female moult cycle (Elwood & Dick, 1990; Hume *et al.*, 2002). However, males may also compete directly for the access to females ('takeover hypothesis', Ward, 1983) and the contests between males are expected to be more frequent as the female is closer to the moult (Dick & Elwood, 1990). Large males are better able to both make takeovers when single and to resist takeover attempts when

paired; the size advantage being more important as the female time to the moult decreases and as female size increases.

There is growing evidence that female physiology (especially time to the moult) influences pairing decisions and male propensity to mate (Thomas *et al.*, 1998; Bollache & Cézilly, 2004b; Galipaud *et al.*, 2011). The physiological state of crustaceans varies over time and is strongly linked to their moult stage (Chang, 1995; Carvalho & Phan, 1998). For example, levels of ecdysone and vitellogenin fluctuate during the moult cycle and are involved in pairing decision (Dunham, 1978; Ducruet, 1982; Subramoniam, 2000). However, females do not reproduce at each successive moult as noticed for many species (Jormalainen & Merilaita, 1995; Souty-Grosset *et al.*, 1998) and even during the breeding period, egg-depositing moults (with reproduction) may alternate with growth moults (without reproduction). The types of moult are expected to affect differentially the pairing outcomes (Sparkes, Keogh & Haskins, 2000), as well as size-assortative mating. This latter point has not specifically been investigated in crustaceans.

Predictions can be made about the role of the moulting cycle in size-assortative mating. If size-assortative pairing results from the constraints of carrying a female for males or from the spatial heterogeneity in the local environment, then the stage of moulting cycle should not have an effect. In this case, the variability in size-assortative pairing should be low and independent of where males and females are in their moulting cycle. If sexual selection indeed determines male choice ('timing and takeover hypotheses'), then the level of size-assortative pairing should vary according to female moult stage and be more pronounced late in the female moult cycle.

In the freshwater gammaridean amphipod *Gammarus pulex*, a pattern of positive size-assortative pairing during PCMG has been well described. The objective of the present work was to study the influence of female moult stage on the variation and intensity of size-assortative pairing in *G. pulex*. Both paired and unpaired individuals were collected in the field, sized and their position in the moult cycle was characterized by observing the new cuticle formation. The data allowed us to determine whether female moulting status influenced the individual's decision and therefore the outcome of pairing.

Materials and methods

Males and females of *G. pulex* were collected from the Suzon River at Val Suzon (in the north of Dijon, Burgundy, France) using a hand net and brought back to the laboratory. Individual females were maintained in the laboratory (temperature $15 \pm 1^\circ\text{C}$, light : dark cycle 12:12 h) in dishes (10 cm diameter, 8 cm height) filled with aerated dechlorinated ultraviolet-treated tap water and fed *ad libitum* with elm leaves. At the end of the assays, gammarids were anesthetized with CO_2 gas and then killed in 70% ethanol. Body size was measured by linear dimensions (height of the fourth coxal plate) using a Nikon SMZ 1500 stereoscopic microscope and Lucia G 4.91 software (Nikon, Tokyo, Japan).

Description of the moult cycle

To estimate the mean duration of the different phases of the moult cycle, as well as the duration of the whole cycle, the moulting status of each female ($n = 44$) was initially assessed by the observation of the dactylan claw (see below) and then the females were checked each morning until they moulted (M1) and again until the following moult (M2). The total duration of the moult cycle was measured as the interval between the two successive moults M1 and M2. Time between collection (i.e. assessment of the position in the moult cycle) and the first moult M1, relative to the total duration of the cycle, was used to estimate the duration of the different phases of the moult cycle. Hence, we obtained a percentage of the cycle that was already completed per individual and average values were computed to obtain mean duration of each moult stage.

The characterization of the moult cycle of *G. pulex* females followed the observation of a cyclic but constant and renewable phenomenon: the anatomical evolution of the dactylan claw and the dactylopodite shrinkage during a moult cycle. This has been previously described for other amphipods, *Orchestia sp.* and *Niphargus virei* (Charniaux-Cotton, 1957; Graf, 1968, 1986) and decapods (Drach, 1939; Drach & Tchernigovtzeff, 1967). For this purpose, the tip of the third right pereopod was carefully cut off using fine forceps and gently placed in a drop of a Ringer solution between a slide and a coverslip. Alternatively, the third left pereopod and the right and left fourth pereopods can be used for the dating of the same animal later in the moulting cycle. The claw of the pereopod was observed under a Nikon Eclipse E600 microscope ($\times 200$ magnification). Pictures were obtained using a Nikon Digital Camera DXM1200F and software ACT-1 (Nikon, Tokyo, Japan).

Field data: moult and size-assortative pairing

Pairs (amplexus) and unpaired males and females were randomly sampled in the field (River Suzon) and placed into tubes filled with water. Collected animals were examined within 3 h. The moult stage was determined for all animals. Females were checked for embryos in the ventral pouch (resulting from a previous reproductive event). The type of moult of females (growth moult or egg-depositing moult) was also determined by checking the presence of maturing black ovaries, dorsally visible through the cuticle (vitellogenesis). We examined 138 pairs, 60 unpaired males and 52 unpaired females.

Pairing status and behaviour according to female and male moult stages were analyzed with a χ^2 test. Variation in size-assortative pairing was assessed overall using an analysis of covariance (ANCOVA) with the size of males as dependent variable and female size and moulting status as covariates. Pearson correlation tests were used to assess the magnitude of size-assortative pairing in each stage of the moulting cycle. All tests were performed using programs JMP[®] version 5 (SAS Institute, Cary, NC, USA).

Results

Moult cycle of *Gammarus pulex*

Females *G. pulex* with a size ranging from 1.68 to 2.58 (mean \pm SD, 2.10 ± 0.19 mm) for the coxal plate (between 8.45 and 11.90 mm for the body length) have a 30-day moult cycle at 15°C ($n = 44$, mean 30.2 ± 3.6 days, range 24 to 40 days). It is subdivided into five periods accordingly (Fig. 1; Table 1): (A) the early postmoult (0.5 day, 1.5%); (B) the late postmoult (1.5 days, 4.5%); (C) the intermoult (13 days, 44%); (D) the pre-moult (15 days, 50%), which is subdivided in four periods based on the genesis of the dactylian claw and the setae of the propodite; and finally (E) the exuviation. The relative durations of stages match those observed for crustaceans with short moulting cycles. The main features of the different stages are described below (Fig. 2).

The early postmoult period (A) is 12 h long. The cuticle is thin, soft and sticky (Fig. 2a) because the exuvial fluid persists at the cuticular surface. The epidermis is tight to the new cuticle. The animal has little colour. In the late postmoult period (B; Fig. 2b), the new exoskeleton is forming and the cuticle begins to harden, essentially by calcification.

The intermoult stage is almost half the moulting cycle (Fig. 2c). The integument thickens (lower arrows Fig. 2a–d) and acquires definitive characteristics (colour, thickness, rigidity). In gammarids (and other crustacean species with weak skeleton calcification), no specific criterion defines the boundary between B and C. This boundary depends on the valuation of the progressive thickening of cuticle. Here, we arbitrarily subdivided the stage C in early and late intermoult to account for the thickness and the hardening (concomitant processes) of the tegument.

The premoult is characterized by the apolysis, that is, the progressive separation of the epidermis from the old cuticle, simultaneously with the beginning of the secretion of the new skeleton. Premoult can be subdivided in four phases. In the

first stage in early premoult D0, the claw epidermis begins to separate from the cuticle, gradually from the distal end of the dactylian claw (upper arrows, Fig. 2d) to the more proximal regions of the propodite. Simultaneously, the setae epidermis withdraws from the old skeleton. Tissue retraction continues in the D1 stage (intermediate premoult) and is now visible in the propodite region. This stage is mainly characterized by the genesis of new setae. Invaginations appear around the matrix of the claw (left arrows, Fig. 2e) and setae. The secretion of the cuticle of the new claw continues; its cuticle becomes thicker and begins to refract, this refractiveness being accentuated at the end of the D1 stage (Fig. 2f). At the end of D1 and during the D2 stage (late premoult), the retraction of the new cuticle is maximal (Fig. 2g) and the thickness of the newly synthesized cuticle increases. This is particularly well visible at the level of the dactylopropodite; here, the cuticle is around 1/5 thickness of the old one at the end of the stage. The setae are clearly formed (arrows, Fig. 2g). At the latter premoult stage (D3), which is about 2 days, the main part of the new skeleton is synthesized (Fig. 2h) and the animal prepares to exuviate. The old cuticle has become thinner and more transparent because of calcium reabsorption (from this old cuticle) and is therefore less resistant.

The exuviation (E) or moult usually takes place at night. The dehiscence split occurs behind the cephalon and the animal exits from the exuviae within few minutes starting with the cephalon and the anterior part of the pereion.

Pairing behaviour and variation in size-assortative pairing according to female moulting cycle stages

Pairing behaviour of females collected in the field was significantly affected by their position in the moult cycle ($\chi^2_1 = 127.12$, $P < 0.0001$). The great majority of paired females were found in premoult stages but unpaired females were primarily in intermoult stages, respectively (Table 2, Fig. 3). In contrast,

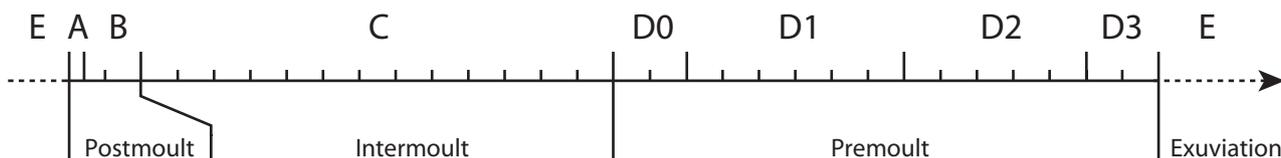


Figure 1 Relative duration in days of the successive stages of the moult cycle of *Gammarus pulex* adult females (1 unit = 1 day).

Table 1 Duration of moulting stages in *Gammarus pulex* females given as the time left to the moult and as the percentage of the total moult cycle

Moult stage	<i>n</i>	Time to moult (days)			% total moult cycle		
		Max	Mean \pm SD	Min	Min	Mean \pm SD	Max
C	13	29	21.46 ± 3.48	17	8.00	32.31 ± 12.04	48.48
D0	5	16	15.00 ± 0.71	14	38.46	44.93 ± 5.55	51.61
D1	8	13	10.25 ± 1.28	9	58.33	64.54 ± 3.83	72.73
D2	14	7	4.93 ± 1.07	3	76.67	83.84 ± 3.83	90.32
D3	4	3	1.75 ± 0.96	1	88.89	93.68 ± 3.54	96.43

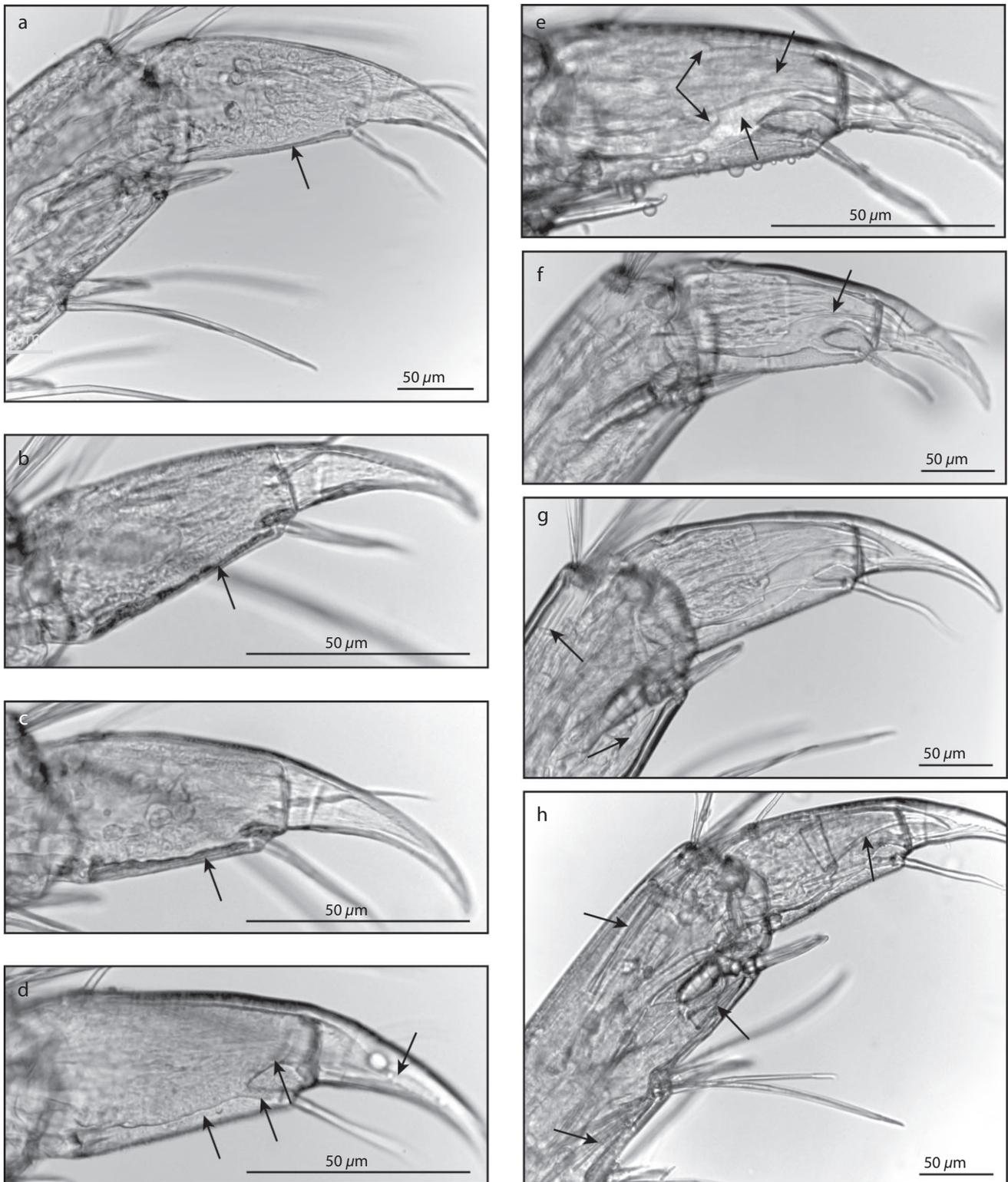
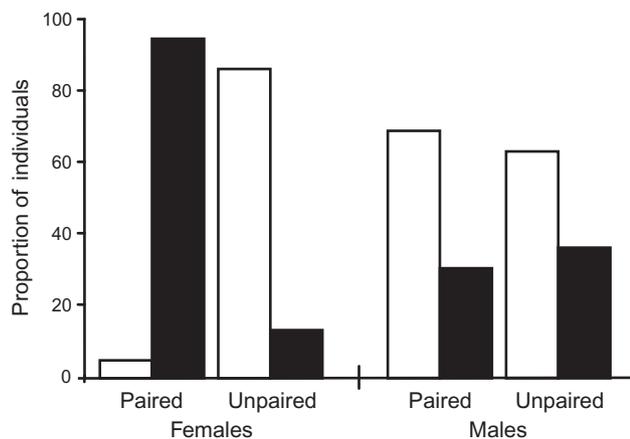


Figure 2 Stages of the moult cycle of *Gammarus pulex* based on the observation of the cuticle of the propodite and dactylopodite and characterized by the progressive retraction of the tissues from the old cuticle and the synthesis of the new cuticle. (a) Early postmoult, (b) late postmoult, (c) intermoult, (d-g) premoult, (h) late premoult. Arrows indicate the main features characterizing each moulting cycle stage (described in the text).

Table 2 Variation in size-assortative pairing in *Gammarus pulex* collected in the field according to female's moult cycle

Female Moult stage	Assortative pairing			Mean size \pm SE	
	<i>r</i>	<i>P</i> *	<i>n</i>	Females	Males
C–D0	0.42	0.0409	24	1.95 \pm 0.20	2.69 \pm 0.31
D1	0.28	0.0950	35	1.88 \pm 0.19	2.49 \pm 0.32
D2	0.27	0.1118	34	1.93 \pm 0.14	2.53 \pm 0.27
D3	0.44	0.0027	45	1.90 \pm 0.23	2.55 \pm 0.33
All stages	0.38	<0.0001	138	1.91 \pm 0.20	2.56 \pm 0.32

*Significant values are shown in bold.

**Figure 3** Proportion of males and females of *Gammarus pulex* sampled in the field, according to their pairing status (paired or unpaired) and their moulting status: intermoult (open bars) or premoult (solid bars).

the moult stage of males had no effect on the probability of pairing ($\chi^2_1 = 0.61$, $P = 0.4$; Fig. 3). The proportion of paired (90.5%) versus unpaired (84.6%) females carrying eggs or embryos in the ventral pouch did not differ ($\chi^2_1 = 1.12$, $P = 0.3$). However, all females that were collected as paired in the field, all ($n = 139$ paired females) were in vitellogenesis compared to only 30% of the unpaired females ($n = 52$ unpaired females; $\chi^2_1 = 117.74$, $P < 0.0001$).

We found an overall size-assortative pairing between male size and female size (ANCOVA, $F_{1,130} = 20.99$, $P < 0.0001$) but the size of males did not change with female moult stage ($F_{3,133} = 1.55$, $P = 0.21$). The interaction term was not significant and was removed. This might be explained by the fact that for a given size of female the male size will be the same. Indeed, although males and females tend to be larger in the late C–D0 sample (Table 2), body size does not differ among individuals found in the four distinct samples according to the position of the female in the moulting cycle (ANOVA; males: $F_{3,134} = 2.01$, $P = 0.12$; females: $F_{3,134} = 0.69$, $P = 0.56$). However, the intensity of size-assortative pairing varied according to the position of the female in the moulting cycle (Table 2). In late intermoult/early premoult (late C–D0), there was a slightly significant relationship, whereas in premoult stages (D1 and

D2), no significant size-assortative pairing was detected. One strong significant positive size-assortative pairing was detected at the end of the premoult stage (D3).

Discussion

Among the hypotheses put forward to explain size-assortative mating in crustaceans, only those related to active mate choice with regards to precopula duration (and thus female moult) are likely to have a major role (Dick & Elwood, 1990; Elwood & Dick, 1990; Hume *et al.*, 2002). Our study provides evidence that knowing an individual's position in their moulting cycle is necessary for understanding the pairing decision for both males and females of *G. pulex*. This is directly related to (1) female time left to the moult and (2) female vitellogenesis status (albeit only in females approaching an egg-depositing moult). In *G. pulex*, as in most female amphipods, copulation and ovulation happens shortly after the moult. Thus, ovarian and behavioural cycles are coordinated and may share a physiological (hormonal) control mechanism (Borowsky, 1980). Female moulting (secretion of a new cuticle) and vitellogenesis (the main event of the female reproductive cycle) may be closely related and could affect the behavioural decision of the individuals. The mechanisms by which male gammarids evaluate the female status are unclear. Female chemical cues may play an important role (Dahl, Emanuelson & Mecklenburg, 1970; Hammoud, Compte & Ducruet, 1975; Thiel, 2011), which could also happen in our particular system. Males might detect the impending female's moult through the release of the moulting hormone (ecdysone/20-OH-ecdysone), but other molecules produced during maturation of the oocytes (vitellogenin) may also play a role (Blanchet-Tournier, 1982; Sutcliffe, 1992; Subramoniam, 2000).

It is worth noting that not all females collected in the field either unpaired or in precopula were carrying eggs or embryos in their ventral pouch from a reproduction event after their previous moult. Thus, a succession of growth and egg-depositing moults occurs during the breeding period in *G. pulex*. Whereas males are likely to be unable to discriminate between females in growth or egg-depositing moults in the isopod *Lirceus fontinalis* (Sparkes *et al.*, 2000), here, in *G. pulex*, all females engaged in a growth moult were found unpaired. However, most of them were determined to be in intermoult so it was difficult to determine on the ability of *G. pulex* males to discriminate and to avoid females in growth moult. The influence of the type of moult on pairing outcomes clearly deserves further investigation.

Size-assortative pairing may have a temporal pattern. Our results suggest that size-assortative pairing in the field varies with female moult stages and that precopula pairs do not always form a stable entity. Most of the females become receptive for the first time during the late intermoult/early premoult stages. At that time, any male encountered at random could detect and directly pair with any freshly receptive female, resulting in an absence of pattern of size assortment. However, it is widely accepted that large males have several advantages over smaller ones (Ward, 1983; Elwood & Dick, 1990; Bollache & Cézilly, 2004a; Franceschi *et al.*, 2010). Among

others, they are better able to pay the costs of carrying a large female, and hence, guard her for longer (Elwood & Dick, 1990; Hume *et al.*, 2002). Hence, according to the 'timing hypothesis', large males are expected to pair with large females at an earlier stage than smaller males. Here, in the earliest moult stage, we found a slightly significant positive size-assortative pairing. Males and females early paired tended to be larger as well (fitting the predictions of the 'timing hypothesis'), although this was not fully supported by the analyses.

Size-assortative pairing is expected to increase as female time to the moult decreases. Here, the degree of size assortment was stronger (and the most significant) as females were closer to the moult (late premoult stage, D3). Non-assortative or mismatched pairs that had formed when females were first receptive could be gradually replaced by pairs matched for size through two mechanisms. First, males that are already paired can hold and assess two females simultaneously and tend to retain the female of higher quality in terms of fecundity and proximity to the moult (Dick, 1992). In this case, the shorter the female time left to the moult, the more the pairs previously made between mismatched pairs are likely to be broken because a large paired male would preferentially leave a small female for a bigger one (Dick & Elwood, 1989; Dick, 1992; Murata & Wada, 2002; Wada *et al.*, 2011). Second, males can struggle for the access to a precopula female. Takeovers, where an unpaired male outcompetes a paired male in the access to a receptive female may account for positive size-assortative pairing because of the advantage of large males in both making takeovers and resisting takeover attempts (Ward, 1983; Dick & Elwood, 1990). The 'takeover hypothesis' would predict that paired males would on average be larger in size when females are in late moulting stages. However, our data do not support this hypothesis. Takeovers usually occur at a low frequency (Dick & Elwood, 1990) and their effect might be negligible in *G. pulex* (Franceschi *et al.*, 2010).

There is increasing evidence that the generation of size-assortative pairing is not entirely male determined and that female resistance also plays a role in the pairing process (Jormalainen, 2007; Wellborn & Cothran, 2007). Female resistance to pairing attempts are interpreted either as a form of female choice or as a way to shorten precopula duration to reduce the costs associated to guarding (Jormalainen, Merilaita & Riihimäki, 2001; Cothran, 2008). Female resistance could become more important through time, so that the more mismatched the initial pairings, the more rapidly the female may be able to dislodge the male (Jormalainen, 1998; Sutherland, Hogg & Waas, 2007). Only larger males may be able to keep hold on or subdue resisting females (Elwood *et al.*, 1987; Jormalainen, Merilaita & Hardling, 2000; Sutherland *et al.*, 2007). Consequently, under the scenario of female resistance, the size of females found paired should differ according to moult stage, with a pattern where only males hold small females and/or large males hold large females in the early premoult stages. We did not find any difference (or any tendency) in mean female size between the premoult stages. This pattern suggests that *G. pulex* females are at least indifferent to males. If the costs associated to precopula paid by females are weak, female resistance is expected to be low or negligible.

Accordingly, in *G. pulex*, early- and long-lasting precopula have been shown to confer on females some benefits by decreasing the intermoult duration (Galipaud *et al.*, 2011). As such, pairs are likely to be formed early in the moult cycle.

In summary, we propose a simple and useful tool for assessing female moult stage. Our results indicate that the physiological status of females (both moulting and reproductive status) influences the individual's decisions, and thus, the outcome of pairing in the amphipod *G. pulex*. The degree of size-assortative pairing is likely to vary across the female moult cycle, being stronger when females are closer to the moult. Size-assortative pairing may be overestimated in pooling data procedures without any consideration of the female moulting status. Moulting and pairing decision could not be dissociated, and moulting should be controlled for when examining the behavioural ecology of mate choice decisions in crustaceans.

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