

## Reproductive behavior of intersexes of an intertidal amphipod *Corophium volutator*

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**Abstract.** Intersexes are common in crustaceans. Typically, these intersexes are sterile or function as females, but prior evidence from laboratory experiments suggests that intersexes of a key species of gammaridean amphipod, *Corophium volutator*, might function as males. We observed that intersexes of *C. volutator* behaved as males by crawling (mate-searching) on a mudflat during ebb tides and pairing in burrows with female amphipods. In the laboratory, intersexes and males did not differ in aspects of crawling such as movement rate and measures of burrow investigation. Intersexuality was costly in that intersexes crawled less often than males on a mudflat, formed fewer pairs with females than males, and remained in tandem less often with receptive females than males. The use of PCR-based identification methods failed to identify the presence of transovarial, feminizing, microsporidian parasites as a major cause of intersexuality in this species in that infected females did not produce broods that contained more intersexes than broods produced by uninfected females. Because intersexes may be mistaken as females, the percentage of functional males in amphipod populations may be underestimated: an important consideration given male limitation in populations of *C. volutator*. The occurrence of intersexes has significant implications for studies on the evolution and ecology of sex ratios, and the use of crustaceans as indicators of environmental quality.

*Additional key words:* Amphipoda, mate-searching, microsporidia, sex ratios

The phenomenon of intersex, defined as having both male and female secondary sexual characteristics, is widespread among crustaceans (Ford & Fernandes 2005). Intersexual development has been found to be associated directly with the presence of endoparasites (bacteria: Rigaud & Juchault 1998; microsporidia: Kelly et al. 2004; Rodgers-Gray et al. 2004; Ford et al. 2006) and correlated with the presence of endocrine-disrupting pollutants (Ford et al. 2006). Recently, the various forms of intersex development have been documented, because intersexes may serve as indicators of environmental quality (Depledge & Billingham 1999; Ford et al. 2004a). Intersexes might also influence the size of crustacean populations by causing population increases in cases where they function as members of the limiting sex (Forbes et al. 2006) or declines in cases where costly intersex forms occur at the expense of “normal” animals (Ford et al. 2007).

Among gammaridean amphipods, intersexes usually function as females (Ford & Fernandes 2005). Costs of intersexuality for female-like forms include reduced pairing success with males (Dunn et al. 1990) and smaller brood sizes (Ford et al. 2003). For male-like intersexes, little is known about the costs of intersexuality, although costs may include a reduction in their ability to fertilize eggs of mates (McCurdy et al. 2004).

We investigated the causes and consequences of intersexuality in the amphipod *Corophium volutator* PALLAS 1766. This species is common to mudflats in the Bay of Fundy/Gulf of Maine and northern Europe (Fish & Mills 1979; Hicklin & Smith 1984). These amphipods are a key component of mudflat communities in that they serve as the main prey item for migratory shorebirds (Hicklin & Smith 1984), fish (McCurdy et al. 2005), and infaunal predators (McCurdy & Moran 2004). Their U-shaped burrows also influence the structure of mudflats by preventing erosion (Pender et al. 1994). Additionally, members of *C. volutator* are frequently used as a bioindicator in toxicity studies (e.g., Depledge & Billingham

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1999; Kirkpatrick et al. 2006; Krang & Dahstrom 2006). Earlier work suggested that intersexes of this species can function as males in that they are able to fertilize eggs of females in the laboratory (McCurdy et al. 2004) and they do not carry broods (Barbeau & Grecian 2003). However, we do not know how often intersexes act as males and whether they are competitive with males. The ability of intersexes to function as males in natural populations of amphipods may be important because of concerns that males are the limiting sex due to highly synchronous and limited receptivity of females (McCurdy et al. 2000a; Forbes et al. 2006). Determining how effective intersexes are in reproductive competitions is also important given the possibility that intersexual forms might be “poor-condition” animals; such intersexes might be especially common in polluted environments (Ford et al. 2006).

We used a combination of field and laboratory studies to investigate the reproductive behavior of intersexes in comparison with males. By collecting amphipods in the field, we assessed whether intersexes behaved as males (crawling in search of mates) or as females (remaining burrowed in the mud) (Boates & Smith 1989; Boates et al. 1995; Forbes et al. 1996). We collected pairs of burrowed amphipods, as well as core samples from the substrate to determine whether intersex amphipods were paired with males or females, and whether intersexes differed in size from other types of amphipods. We compared crawling and investigative activity of males and intersexes in the laboratory as it relates to access to receptive females. Finally, we tested female amphipods for the presence of feminizing microsporidia and reared their broods to maturity to determine whether vertically transmitted parasites were associated with production of intersexes (these parasites are transmitted through ova and feminize males to avoid what would otherwise be an evolutionary “dead end” in males: Dunn et al. 2001; Mautner et al. 2007). If intersexual development is caused primarily by incomplete feminization by microsporidia (Rodgers-Gray et al. 2004), we expected that intersexes would occur more commonly in broods produced by females infected with these parasites.

## Methods

### Intersex identification

In the Bay of Fundy, intersexes make up 0–8.5% of populations of *Corophium volutator*, and their antennal morphology is intermediate to males and females (Barbeau & Grecian 2003). Intersexes possess

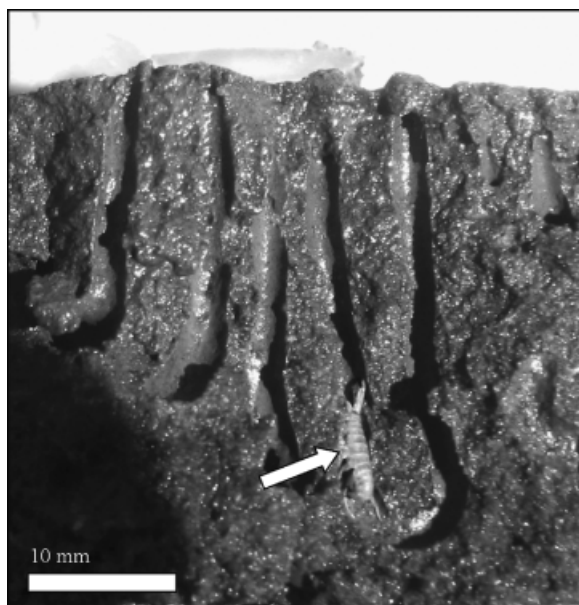
oostegites (characteristic of females) and one or two penial papillae (found on males) (Schneider et al. 1994; McCurdy et al. 2004). Internally, penial papillae are associated with the presence of testes, and the absence of a penial papilla from one side of an intersex is not associated with a developed ovary. Following McCurdy et al. (2004), we classified intersexes as Type I (those with non-setose oostegites) or Type II (those with oostegites bearing setae). Type I intersexes were the more common form, although both forms were able to fertilize eggs of females in the laboratory.

### Mate searching and pairing

To observe the behavior of intersex versus male and female amphipods, we collected crawling and burrowed amphipods from the mudflat at Blomidon, Nova Scotia, Canada (45°13'N; 64°22'W). This mudflat was chosen for study because it supports high densities of *C. volutator* (>20,000 m<sup>-2</sup>; Forbes et al. 1996), and the reproductive behavior of males as well as development of intersexes have been studied there previously (Boates & Smith 1989; Boates et al. 1995; Forbes et al. 1996; McCurdy et al. 2004). Various macroparasites and predatory shorebirds known to influence mate-searching behavior of amphipods are also absent from this mudflat (Boates et al. 1995; McCurdy et al. 1999, 2000b), simplifying the interpretation of amphipod behavior.

As stated above, male amphipods frequently emerge to crawl on the surface of mudflats for a few minutes on each ebb tide (Boates & Smith 1989; Fig. 1). This crawling activity is associated with mate-searching by males; females construct U-shaped burrows and rarely emerge to crawl on the surface at low tide (Boates et al. 1995; Forbes et al. 1996). To investigate the crawling (mate searching) activity of intersexes versus males and females, we collected samples from seven stations spaced 200 m apart on the mudflat parallel to the direction of tidal flow on eight visits to Blomidon July 7–20, 2005 (except on the first and last dates of sampling, when five and three stations were visited, respectively). On each visit, we collected all amphipods that emerged to crawl over a 5-min period immediately following exposure within two 0.25-m<sup>2</sup> plots placed randomly at each station (McCurdy et al. 2000b; *n* = 100 plots). As in previous studies (Boates & Smith 1989; Forbes et al. 1996), few amphipods emerged to crawl after the first few minutes of exposure.

To assess pre-copulatory pairing by male, female, and intersex amphipods, we collected pairs of amphipods at low tide from the substrate at each station.



**Fig. 1.** Burrows of the intertidal amphipod, *Corophium volutator*. Males frequently emerge from U-shaped burrows to crawl (mate search) on the surface of the mudflat during ebb tides. Females (one is shown by an arrow) usually remain burrowed in the substrate. Males and females ultimately form pairs in burrows and copulation takes place following female molt.

Pairs of amphipods were obtained by removing sections of mud and breaking them open carefully to remove amphipods directly from their burrows (male–female pairing occurs before female receptivity, which follows female molt; McCurdy et al. 2000a). Core samples were also collected at all stations on each visit; these samples are representative of the amphipod population (Boates & Smith 1989; McCurdy et al. 1999, 2000b; Forbes et al. 2006). Using this design, crawling and pairing activity by various sexes of amphipods could be compared with sex ratios in the population. Core samples were  $86.5\text{ cm}^2 \times 10\text{ cm}$  deep and were passed through a  $500\text{-}\mu\text{m}$  wire sieve to retain adult amphipods (Crewe et al. 2001). The sizes of amphipods were measured as the length from the tip of the rostrum to the end of the telson (Boates & Smith 1989). The number of embryos and their developmental stages were assessed following criteria of Schneider et al. (1994).

To investigate aspects of mate-searching behavior by intersex and male amphipods, we collected crawling amphipods at the Blomidon mudflat and transported them back to the laboratory at Acadia University, Wolfville, Nova Scotia. We chose to track movements of amphipods in the laboratory because high densities of overlapping, U-shaped burrows make it difficult to track and recover individuals

in the field (Forbes et al. 1996). Amphipods were housed in the laboratory in seawater at  $18^\circ\text{C}$  and anesthetized in mineral water to be sexed and measured before the study (for details on this procedure, see McCurdy et al. 2004). Only adult amphipods ( $>5\text{ mm}$ ; McCurdy et al. 2000a) were retained for use in our study.

Males and intersexes were placed individually onto a round pan (diameter = 30 cm) lined with mud that contained 55 artificial burrows (burrows were made by aligning a plastic template over the pan and poking holes 5 cm deep in the mud using a dissection probe). Movements by each amphipod were then recorded by a video camera for 5 min (for details on this design, see McCurdy et al. 1999). Movement paths of 60 intersexes and 48 males were then traced to measure the total distance each amphipod moved, the numbers of burrows they probed with their antennae, the numbers of burrows they entered (defined as positioning the body vertically and moving downward into a burrow), and the numbers of burrows passed over (not probed or entered). To reduce the possibility that amphipods might follow paths of prior test subjects (an important consideration given the role of olfaction in mate location in this species; Krang & Dahstrom 2006), we washed and dried the artificial mudflat before the introduction of each new subject.

### Competition between males and intersexes

To explore competition between intersexes and males, we collected amphipods from Blomidon in July 2005 and housed them individually in  $20 \times 150\text{-mm}$  culture tubes. Females were checked daily for molt (signaling onset of receptivity) and each newly molted female was placed immediately in a separate glass aquarium ( $6.25\text{ cm} \times 3.50\text{ cm} \times 1.25\text{ cm}$ ) lined with mud and filled with seawater. A male and an intersex amphipod were then added to the aquarium, and the three amphipods were video-recorded for 90 min ( $n = 16$  sets of amphipods). We then subsampled 45 min of each recording by combining three 15-min recording periods (one at the start of the experiment, a second  $\sim 45$  min after the start, and a third  $\sim 75$  min after the start; data were pooled for analysis). From each recording, we observed the number of contacts between the receptive female and the intersex, and between the female and the male (as well as determining whether the number of contacts initiated by males vs. intersexes differed; initiators were defined as amphipods that moved toward others, with the end result being direct contact). We also recorded the total time spent by each

female in tandem with the intersex and the male. Copulatory behavior was noted (females typically release eggs into their brood pouches within seconds of the onset of copulation; McCurdy 1999).

### Intersex production in broods

To assess whether feminizing parasites of amphipods were associated with the occurrence of intersexes, we collected females carrying broods of newly fertilized eggs from the mudflat at Blomidon in June 2003 (Stage "A" embryos: McCurdy et al. 2000a). These females were then housed individually in the lab in 250-mL cups lined with 0.5 mL autoclaved mud containing artificial seawater (Instant Ocean, 18‰). After releasing their broods, females were removed and killed in 99% ethanol, and screened for the presence of microsporidia (described below). Broods from these females were then raised in 250-mL cups placed within environmental chambers (15°C; day:night cycle = 16 h:8 h; water containing 0.05% Liquifry fish food was changed weekly). After 3 months, broods reached sexual maturity and amphipods were sexed using secondary sexual characteristics (described previously). The survival rates of broods produced by infected and uninfected females were  $72 \pm 4\%$  and  $74 \pm 4\%$ , respectively.

Each of the 85 adult females that released broods in the laboratory was screened for the presence of microsporidian parasites using PCR (Mautner et al. 2007). The use of PCR provides a sensitive method to detect parasites that might otherwise be missed (a concern when studying the production of intersexes, which could result from low intensities of infection by feminizing parasites; Kelly et al. 2004). Because we were interested in testing females for the presence of feminizing microsporidia, we first dissected out ovaries from females to reduce the likelihood of detecting microsporidia that might be present in other tissues (feminizing parasites are found in ovaries and are transmitted through eggs: Dunn et al. 2001). Following protocols summarized in Mautner et al. (2007), we obtained genomic DNA from amphipod ovaries using a 2-h CTAB buffer/proteinase K buffer digestion at 65°C, followed by phenol–chloroform extraction (Sambrook et al. 1989). To confirm successful extraction, samples were first amplified with invertebrate cytochrome *c* oxidase subunit I primers (LCO1490 5'-ggtaacaaatcataagatattgg-3', HCO2198 5'-taaacttcagggtgacacaaaaaatca-3': Folmer et al. 1994) and were scored as positive if a characteristic 710-bp fragment was observed (the size of the band observed in *C. volutator* and other amphipods: Ironside et al. 2003; Mautner et al. 2007). For samples that yielded

positive results, we then ran and scored PCR reactions for microsporidian-specific primers 18sf (5'-gttgattctgcctgacgt-3') and 964r (5'-cgcgttgagtcacaaattaagccgcaca-3') to amplify a component of microsporidian DNA, if present (for details on PCR reaction mixtures, cycle parameters, and a discussion on the role of annealing temperatures, see Terry et al. 2003; Mautner et al. 2007). Samples were scored blindly and were considered to be positive for the presence of microsporidia if a single 900-bp band was observable on 1.5% agarose gels stained with ethidium bromide.

### Statistical analysis

We used log-likelihood tests of independence to compare the frequencies of males, females, and intersexes that crawled on ebb tides, and to compare the rates that males and intersexes paired with females (Zar 1999). To analyze whether multiple measures of behavior based on trail length, number of artificial burrows probed, number of burrows not probed, and number of burrows passed over differed between males and intersexes, a multiple analysis of variance (MANOVA) was used (McCurdy et al. 1999). A MANOVA was chosen over separate univariate tests because the response variables were correlated and amphipod body size was included as a covariate in the model. Paired *t* tests were used to compare behavioral measures of males and intersexes placed in competition for receptive females in the laboratory. To investigate whether the number of intersexes produced in broods was related to infection status of the mother, total brood size, and the interaction of these terms, we constructed generalized linear models that included numbers of intersexes per brood and total brood size as continuous variables, infection status as a categorical variable, and body sizes of mothers as a covariate. In all cases, assumptions of various parametric tests (e.g., normality and homogeneity of variance of residual values) were assessed and found to be met (following Zar 1999).

## Results

### Mate searching and pairing

Based on core samples of *Corophium volutator* taken from the substrate at Blomidon, intersexes made up 3.48% of the amphipod population ( $n = 3276$ ). Compared with counts of males, females, and intersexes removed from the substrate, intersexes were much more likely to be present among crawling

amphipods on the surface of the mudflat than female amphipods, but crawled at lower rates than male amphipods ( $G = 1959.2$ ,  $df = 2$ ,  $p < 0.001$ ; based on the densities of various types of amphipods found on the surface vs. in the substrate, crawling rates per ebb tide were 11.4% for intersexes, 0.5% for females, and 28.7% for males). Crawling males and intersexes also assessed burrows by poking them with their second antennae and many attempted to enter burrows. Crawling intersexes were smaller than crawling males (mean  $\pm$  SE length intersexes =  $6.0 \pm 0.1$ ; males =  $7.3 \pm 0.1$ ;  $t = 5.3$ ,  $df = 1472$ ,  $p < 0.0001$ ), although there was no overall difference in size between intersex and male amphipods in the population, based on core samples taken from the substrate (mean  $\pm$  SE length intersexes =  $6.9 \pm 0.3$  mm; males =  $6.8 \pm 0.3$  mm;  $t = 0.8$ ,  $df = 96$ ,  $p = 0.79$ ). Of the 230 intersexes observed in our study, 97% were Type I form (i.e., they possessed oostegites that lacked setae). Of the six Type II forms observed in our study, we found at least one egg in the brood pouches of three of these intersexes (all of the ovigerous intersexes also had only one penial papilla and were found in the substrate).

Pairs of amphipods collected from burrows in the substrate ( $n = 199$  pairs) consisted of a male and a female (74.9%), two females (22.6%), or an intersex and a female (2.5%). Intersexes and males only paired with females and were never observed in same-sex pairings or paired with each other. Among the pairs collected, females were less likely to be paired with intersexes versus males than expected, based on numbers of intersex and male amphipods found in substrate core samples ( $G = 11.72$ ,  $df = 1$ ,  $p < 0.001$ ; intersexes comprised 15.3% of the pool of male-like amphipods in the substrate, but only 3.3% of the pool of male-like amphipods paired with females). When comparing the number of males versus intersexes paired with females to numbers of these amphipods found crawling on the surface, males were not represented in pairings more than intersexes ( $G = 3.3$ ,  $df = 1$ ,  $p = 0.07$ ). Intersexes from pairs were significantly smaller than males (mean  $\pm$  SE length intersexes =  $6.0 \pm 0.1$  mm; males =  $7.1 \pm 0.2$  mm;  $t = 2.4$ ,  $df = 152$ ,  $p = 0.02$ ). Intersexes from pairs did not differ significantly in size from those collected from core samples of the substrate ( $t = 1.1$ ,  $df = 18$ ,  $p = 0.27$ ), although males found in pairs were significantly larger than males collected from core samples ( $t = 2.3$ ,  $df = 230$ ,  $p = 0.02$ ). Females paired with males and intersexes did not differ in size ( $t = 1.2$ ,  $df = 152$ ,  $p = 0.24$ ) and females found paired with males versus intersexes did not differ in their level of receptivity (i.e., those close to receptivity that were carrying late-stage/

no embryos were as likely to be found with males as intersexes;  $G = 0.1$ ,  $df = 1$ ,  $p = 0.72$ ).

In the laboratory, we found no significant differences between intersexes and males in the distance they crawled (mean  $\pm$  SE intersexes =  $27.1 \pm 3.3$  cm; males =  $37.1 \pm 3.7$  cm), number of artificial burrows they probed with their antennae (mean  $\pm$  SE intersexes =  $0.4 \pm 0.1$ ; males =  $0.7 \pm 0.1$ ), number of burrows they entered (mean  $\pm$  SE intersexes =  $0.8 \pm 1$ ; males =  $1.0 \pm 0.1$ ), and number of burrows they passed over and did not probe (mean  $\pm$  SE intersexes =  $1.1 \pm 0.2$ ; males =  $1.7 \pm 0.3$ ) (Wilk's  $\lambda = 0.94$ ,  $n = 104$ ,  $p = 0.43$ ).

### Competition between males and intersexes

In the laboratory experiment where sets of amphipods containing an intersex, a male, and a receptive female were observed, the mean number of contacts between females and males, and between females and intersexes, did not differ significantly (although females did tend to contact males more than intersexes; mean  $\pm$  SE males =  $43.4 \pm 9.9$ ; intersexes =  $31.3 \pm 5.3$ ; paired  $t = 1.97$ ,  $df = 15$ ,  $p = 0.07$ ). When examining these interactions further, females initiated more contacts with males than intersexes (mean  $\pm$  SE males =  $24.2 \pm 5.7$ ; intersexes =  $17.8 \pm 5.0$ ; paired  $t = 3.00$ ,  $df = 15$ ,  $p < 0.01$ ), whereas there was no difference in the mean number of contacts toward females initiated by males versus intersexes (males: mean  $\pm$  SE males =  $19.4 \pm 5.8$ ; intersexes =  $13.4 \pm 3.2$ ; paired  $t = 0.98$ ,  $df = 15$ ,  $p = 0.34$ ). Females also spent more time in tandem with males than intersexes (mean  $\pm$  SE males =  $566.9 \pm 89.4$  s; intersexes =  $358.3 \pm 64.4$  s; paired  $t = 2.1$ ,  $df = 15$ ,  $p = 0.05$ ). Of the four successful copulatory events observed, three involved females and males, and one involved a female and an intersex. The successful mate (male or intersex) in all cases was the larger of the two competitors.

### Intersex production in broods

Overall, 12 of 85 females produced broods that contained at least one intersex and 2.7% ( $n = 1314$ ) of offspring produced by females in the laboratory were intersexes (mean  $\pm$  SE% intersexes per brood =  $2.3 \pm 0.7$ ). Ovaries from 46% of mothers tested positive for the presence of microsporidia using PCR (samples from all females were positive for COI, indicating successful DNA amplification). Both infected and uninfected females produced intersexes within broods. In the generalized linear model, the number of intersexes increased with brood size (model  $R^2 = 0.11$ ,  $n = 85$ ,  $p = 0.02$ ; brood size:

$p = 0.04$ ) and was related to infection status (controlling for brood size, infected mothers produced fewer intersexes in broods;  $p = 0.04$ ). The interaction term was also significant (brood size  $\times$  female infection status;  $p = 0.02$ ) and a *post hoc* examination of this interaction found that uninfected mothers with larger broods produced more intersexes ( $R^2 = 0.09$ ,  $n = 46$ ,  $p = 0.05$ ), whereas the relationship between brood size and numbers of intersexes produced was not significant for infected females ( $R^2 = 0.01$ ,  $n = 39$ ,  $p = 0.56$ ). There was no relationship between the sex ratio of broods (proportion of females vs. males) and numbers of intersexes found in broods ( $R^2 = 0.004$ ,  $n = 85$ ,  $p = 0.55$ ).

## Discussion

### Behavior of intersexes

We observed that intersexes of the amphipod *Corophium volutator* tended to behave as males in a natural population and in the laboratory. Specifically, intersexes at Blomidon crawled frequently on the surface of a mudflat during ebb tides and at much higher rates than females ( $23 \times$  higher). Frequent crawling is consistent with male mate-searching observed in this species (Boates et al. 1995). Intersexes also exhibited male-like behaviors (Forbes et al. 1996) in that they frequently searched burrows using their antennae and entered burrows. Our laboratory analysis of crawling activity of intersexes and males also suggested that crawling was associated with mate searching, in that intersexes were as likely to probe and enter artificial burrows in the laboratory as males. While behaviors observed on an artificial mudflat are only a proxy for mate searching and mate acquisition in nature, the use of a laboratory experiment allowed us to control factors such as female receptivity and male-male competition, both of which can influence mate-searching behavior (Forbes et al. 1996; below). Intersexes at Blomidon also paired only with females, which was the same pattern observed for males in our study and for males of this species observed in other studies (Boates & Smith 1989; Forbes et al. 1996).

Reproductive behavior by intersexes of this species is consistent with evidence for male function observed in the laboratory (McCurdy et al. 2004), where intersexes were able to fertilize eggs of females. Further evidence for male function of intersexes comes from the observation that nearly all intersexes observed in our study were non-ovigerous (98.7%), whereas 48% of all females collected in our study were carrying broods. Barbeau & Grecian (2003) and

McCurdy et al. (2004) found no ovigerous intersexes in other collections of *C. volutator*. Further, no ovigerous intersexes were found among hundreds observed at four mudflat sites in 1996 and 1997 (D.G. McCurdy, unpubl. data), and Kopec (2005) found only one ovigerous intersex (a Type II form and taken in a core sample) among 105 crawling and burrowed intersexes he collected at Blomidon in 2004.

The reproductive success of intersexes is likely lower than that of actual males. Intersexes at Blomidon crawled at lower rates than male amphipods ( $2.5 \times$  less than males, based on frequencies of intersexes and males in the substrate) and they were less likely to be paired with females than males, suggesting that they might obtain fewer mates in nature. In the laboratory, intersexes were approached less frequently by females than competing males and spent less time than males in tandem with receptive females. Although costs of intersexual development are not well known for male-like amphipods (Ford et al. 2004b), Dunn et al. (1990) observed that female-like intersexes of another amphipod, *Gammarus duebeni* LILJEBORG 1852, were less likely to pair with males than "normal" females (male intersexes are rare in populations of *G. duebeni* and intermediate forms are usually sterile: Dunn et al. 1994). In the amphipod *Echinogammarus marinus* LEACH 1815, Ford et al. (2003) also observed that female-like intersexes carried smaller broods than normal females, but in *Gammarus fossarum* KOCH 1835, female-like intersexes were as likely to pair as normal females and produced broods of similar sizes (Ladewig et al. 2007). Among male-like intersexes, McCurdy et al. (2004) found that females that mated with intersexes of *C. volutator* produced smaller broods than those that mated with males, suggesting additional, direct costs of intersexual development.

A large body size was associated with greater reproductive activity by males, but not intersexes of *C. volutator*. Specifically, males that crawled in search of mates and those found to be paired with females tended to be larger than average-sized males. Crawling and paired males were also larger than intersexes involved in these activities at Blomidon. For males, having a large body size might enable them to mate search more effectively and compete successfully for access to receptive females (Forbes et al. 1996). It is not clear why this relationship would differ for intersexes, although one possibility is that rates of growth and maturation differ between males and intersexes, leading to differences in schedules of reproduction (males and females differ in rates of maturation; Watkin 1941). Mate preferences of females could also play a role in the behavior of males and

intersexes (females did initiate fewer contacts with intersexes than males in the laboratory), although females of *C. volutator* are generally thought to exhibit low selectivity of mates due to male-limitation caused by synchronous and limited receptivity in females (Forbes et al. 2006). In fact, females frequently attempt to pull mate-searching males into their burrows (Forbes et al. 1996).

### Intersex production in broods

We found no link between the presence of feminizing microsporidia in the amphipod *C. volutator* and production of intersexes, despite the fact that microsporidia are common at Blomidon and are associated with feminization of male amphipods (Mautner et al. 2007; infected females produced broods containing  $83 \pm 5\%$  daughters vs.  $70 \pm 4\%$  daughters found in broods of uninfected females). When controlling for brood size, intersexes appeared to be as common, if not more common, in broods of females that were not infected by microsporidia than in broods of infected females. In other species of amphipods, positive links between microsporidian infection and intersex development have been found, but vary spatially. For example, Dunn et al. (1993) observed no difference in intersex production between females of the amphipod *G. duebeni* infected with microsporidia and uninfected controls. However, Rodgers-Gray et al. (2004) observed that 89% of intersexes of *G. duebeni* were infected with microsporidia at another site. Ford et al. (2006) also found an association between microsporidia and intersexes in *E. marinus*, but only at polluted sites. We discounted protandric hermaphroditism as a cause of intersexuality in *C. volutator* because we have never observed this mode of development in the field or laboratory, and it has not been found in amphipods (reviewed by Ford et al. 2003). Intersexuality caused by hybridization among species of amphipods also seems to be unlikely in that breeding experiments in other amphipods have ruled this out (Ginsburger-Vogel 1991).

Among estuarine invertebrates, evidence is emerging that endocrine-disrupting pollutants may also interfere with hormonal production and cause intersexes to occur in molluscs (Chesman & Langston 2006; Oehlmann et al. 2007) and fish (Campbell et al. 2006). Consistent with this possibility, Ford et al. (2004a) found that intersex amphipods, *E. marinus*, were more common at heavily polluted sites in the United Kingdom. While our use of a laboratory experiment throughout nearly all of the developmental period of amphipods suggests that such factors might

not play a major role in intersex production in *C. volutator* (the water quality, photoperiod, temperature, and food resources were held constant), future studies might benefit from a focus on early development of embryos because some pollutants can affect development of crustaceans as early as the first embryonic cleavage (Olmstead & LeBlanc 2007). Determining the role of pollutants as a cause of intersexuality remains an important goal if intersexes are to be used as biomarkers. Given recent efforts to use the burrowing behavior of *C. volutator* as an indicator of chronic toxicity (Scarlett et al. 2007), it will be important to elucidate the cause(s) of intersexuality and account for the consequences of these forms on behavior, especially if pollutants mediate intersexual development.

One possibility that should not be discounted is that intersexual development results from host responses to sex-distorting parasites (e.g., masculinization of infected males or females in response to feminizing parasites). Such a response need not be exclusive to other causative agents and could come in the form of an autosomal masculinizing gene that affects androgenic gland development (Rigaud & Juchault 1998) or be mediated through effects of microorganisms (Juchault et al. 1991). Adaptive host responses to parasitism that act to balance operational sex ratios would also explain why intersexes are observed commonly in populations that exhibit a high prevalence of feminizing parasites, account for heritability of intersexual development observed in amphipods (Ginsburger-Vogel 1991; Dunn et al. 1993), and could account for spatial and temporal variation in sex ratios (Jaenike 2007).

Regardless of the underlying causes of male-like intersexuality in crustaceans, all research that we are aware of indicates that intersexuality is associated with impaired function of the androgenic gland (Ford et al. 2005). In fact, male-like behavior similar to that we observed has been found in adult intersexes of crayfish subjected to androgenic gland ablation (crayfish intersexes fought with males and mated with females; Barki et al. 2006). We do not know whether microsporidia interfere with androgenic hormone production in *C. volutator*, but our data suggest that such interference, if it exists, does not result in greater likelihood of intersexuality. A direct connection between microsporidian infection and reductions in androgenic gland hormone production has been observed in the amphipod *G. duebeni* (Rodgers-Gray et al. 2004). Macroparasites (e.g., trematodes: Hastings 1981) are thought to interfere with androgenic gland development in amphipods, although such parasites are not present at Blomidon (McCurdy

et al. 1999, 2000b) and were not present in the laboratory population of amphipods we reared to maturity.

The prevalence of intersexes in our study (3.5%) was similar to that observed at Blomidon in another year (3.3%; McCurdy et al. 2004) and is within the range observed for this species and others elsewhere (Barbeau & Grecian 2003; Ford & Fernandes 2005). Although easily mistaken as females (based on morphological characteristics), our results indicate that intersexes in natural populations function mostly as males. Because males are the limiting sex in populations of *C. volutator* due to short and synchronous bouts of female receptivity and limited reproductive potential by males (McCurdy et al. 2000a; Forbes et al. 2006), intersexes in *C. volutator* might contribute significantly to population growth through male function. The use of models that incorporate intersex function, costs of intersexual development, and frequencies of intersexes (e.g., Ford et al. 2007) would enhance our understanding of the consequences of this phenomenon for individuals, populations of key invertebrates, and the structure of aquatic communities.

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