

# Reproductive interference via interspecific pairing in an amphipod species complex

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**Abstract** Many species experience reproductive interference defined as interspecific mating interactions that reduce the fitness of individuals in at least one of the species. We examined reproductive interference among three cryptic amphipod species that co-occur in the vegetated zone of lakes. Because these animals form precopulatory pairs and males commonly have indiscriminant mating behavior, we predicted that (1) there would be interspecific pairing, (2) interspecific pairing would be more common when conspecifics were rare, and (3) interspecific pairing would be more common in species combinations where males are larger than females. Using color phenotypes to distinguish species, a novel discovery reported here, we conducted experiments to evaluate patterns of interspecific pairing. In a no-choice experiment (i.e., amphipods had access to mates of only one species), we observed a low rate (5 %) of interspecific pairing and 80 % of these cases involved males that were larger than females. In a second experiment where individuals had access to conspecific mates at varying relative abundance, only 0.04 % of the pairs observed were between heterospecifics, suggesting that the interspecific pairing observed in the no-choice experiment was an artifact of not having access to conspecific mates. Our results suggest that at least one sex has

sufficient species-recognition abilities to circumvent the formation of interspecific pairs; therefore, reproductive interference may be minimal in these species.

**Keywords** Cryptic species · Interbreeding · Reproductive interference · Sperm storage · *Hyalella*

## Introduction

The application of molecular genetic studies to natural populations has revealed substantial hidden biological diversity across a variety of ecosystems (Pfenninger and Schwenk 2007). The co-occurrence of species that are morphologically indistinguishable has challenged our ideas about mechanisms of species coexistence. Consequently, there has been a flurry of studies on how ecological and neutral processes affect the ability of similar species to coexist (Siepielski and McPeck 2010). In addition to ecological interactions, the presence of phenotypically similar species in the same area may increase the likelihood of interspecific sexual interactions. In such cases, reproductive interference—defined as interspecific mating interactions that reduce the fitness of individuals in at least one of the species involved (Gröning and Hochkirch 2008)—may occur. Although reproductive interference appears to be common in nature, its role in structuring communities has received little attention (Gröning and Hochkirch 2008; Burdfield-Steel and Shuker 2011; *but see* McLain and Shure 1987).

Several processes may explain the seemingly paradoxical ubiquity of reproductive interference. First, a male's fitness is often determined by the number of fertilizations acquired over his lifetime (Bateman 1948). This emphasis on quantity over quality may explain why males harass heterospecific females (Burdfield-Steel and Shuker 2011). Also, in many populations only a small proportion of males mate successfully (Andersson 1994). However, unattractive

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or less competitive males may persist in mating attempts. If heterospecific females are easier to court, easier to subdue, or are perceived as more valuable than conspecific females, then low-quality males may pursue females of the wrong species. Even in the more choosy sex, mating between heterospecifics may occur if mate availability is low or if heterospecifics are more attractive than conspecifics (Pfennig 1998; Wirtz 1999; Hochkirch et al. 2007; Burdfield-Steel and Shuker 2011). Finally, in closely related species mating barriers may be insufficient resulting in misguided mating attempts (Gröning and Hochkirch 2008; Thiel 2011). Consequently, when similar species occupy the same space they risk reproductive interference.

Reproductive interference can decrease species diversity via sexual exclusion, i.e. the extirpation of a species from a habitat due to interspecific mating interactions that reduce fitness. Whether sexual exclusion occurs will depend on the local ecology and evolutionary history of interacting species (Gröning and Hochkirch 2008). Species diversity may be maintained if the most negatively affected species can occupy alternative habitats with fewer heterospecifics or move reproductive activities to times when heterospecifics do not reproduce. These two mechanisms in effect result in allopatry at a very local scale by reducing or eliminating encounters among heterospecifics. High reproductive capacities in the species most negatively affected by reproductive interference may also reduce encounters with heterospecifics via a dilution effect that allows the species to persist. In addition to these ecological mechanisms, reproductive interference may be alleviated by the evolution of more efficient species recognition via reproductive character displacement (Brown and Wilson 1956). Ultimately, whether species diversity is maintained in the face of reproductive interference will depend on the ecological context in which mating takes place and whether other factors (e.g., competitive asymmetries or differences in the ability to deal with natural enemies) work in favor or against it.

Here, we address reproductive interference among cryptic amphipod species that co-occur in the littoral zone of lakes. We take advantage of a new discovery reported here: these once cryptic amphipod species are distinguishable via color differences. In the first experiment, we employed a no-choice, interbreeding design (i.e. individuals only had access to one species as a potential mate) that included all possible combinations of species. In the second experiment, we used a choice design where each focal species was housed with varying proportions of heterospecifics while keeping total amphipod density and sex ratio constant. We predicted that the negative effects of reproductive interference (i.e., incidences of heterospecific pairing) would be most pronounced when the relative abundance of heterospecifics was high. We additionally predicted that reproductive interference would be most likely in species combinations where females are smaller than

males because larger males may more easily overcome female resistance to pairing.

## Methods

### Cryptic *Hyalella* amphipod species

Amphipods in the genus *Hyalella* are common grazers and detritivores in permanent freshwater habitats (Bousfield 1996). Until recently, most North American representatives of the genus were considered a single, widely distributed species, *Hyalella azteca*; however, the use of genetic markers has uncovered a tremendous amount of cryptic species diversity within the group (Witt and Hebert 2000; Wellborn and Cothran 2004; Witt et al. 2006; Wellborn and Broughton 2008).

Within this species complex, up to three undescribed species often co-occur in the littoral zone of glacial lakes in the northern USA (species A, B, and C in Wellborn and Cothran 2004; 2007a). These three species likely represent at least two independent evolutionary transitions from a large ecomorph form that is associated with habitats with weak size-selective predation by fish to a small ecomorph form that is found in habitats with fish (Wellborn and Broughton 2008). Although the three species are separated by substantial amounts of molecular evolution (COI nucleotide divergence: 17–19 % and amino acid divergence: 7–8 %), evidence from mtDNA sequence suggests that species B and C share a more recent common ancestor than either does with species A. These three species are reliably distinguished by examining PCR products based on species-specific priming sites within the mitochondrial gene cytochrome c oxidase subunit 1 (COI), as detailed in Wellborn and Cothran (2004). Analysis of nuclear genetic variation based on allozymes demonstrated that each of the three species is distinct with respect to these nuclear markers and show no evidence of hybridization (Wellborn and Cothran 2004). The three species also have distinct COI haplotypes (Wellborn and Broughton 2008).

These three species also differ in distributions across microhabitats, life history and morphological traits that may affect patterns of reproductive interference (Wellborn and Cothran 2004, 2007a). Although all three species co-occur on a local scale, the relative abundance of each species changes depending on the microhabitat sampled in the vegetated littoral zone of lakes (Wellborn and Cothran 2007a). In shallow, nearshore vegetation, species B dominates the amphipod assemblage. Species A and species C are more abundant than species B in the deeper water of the littoral zone. In this deeper water, species A is most common in vegetation near the surface of the water and species C on vegetation in the middle of the water column. These species also differ in body size,

which may affect patterns of reproductive interference. The negative consequences of reproductive interference may be most severe when the unselective sex has more physical power and greater fighting ability than the choosy sex (Yamamura and Jormalainen 1996; Jormalainen 1998). Physical power and fighting ability often depend on body size (Parker 1974; Thornhill and Alcock 1983). Species B males are 18 and 12 % larger than species A and species C females, respectively (Wellborn and Cothran 2004). On the other hand, species A and C males are smaller than or equal in size to females of all three species. Therefore, reproductive interference by species B males may be most likely in this group.

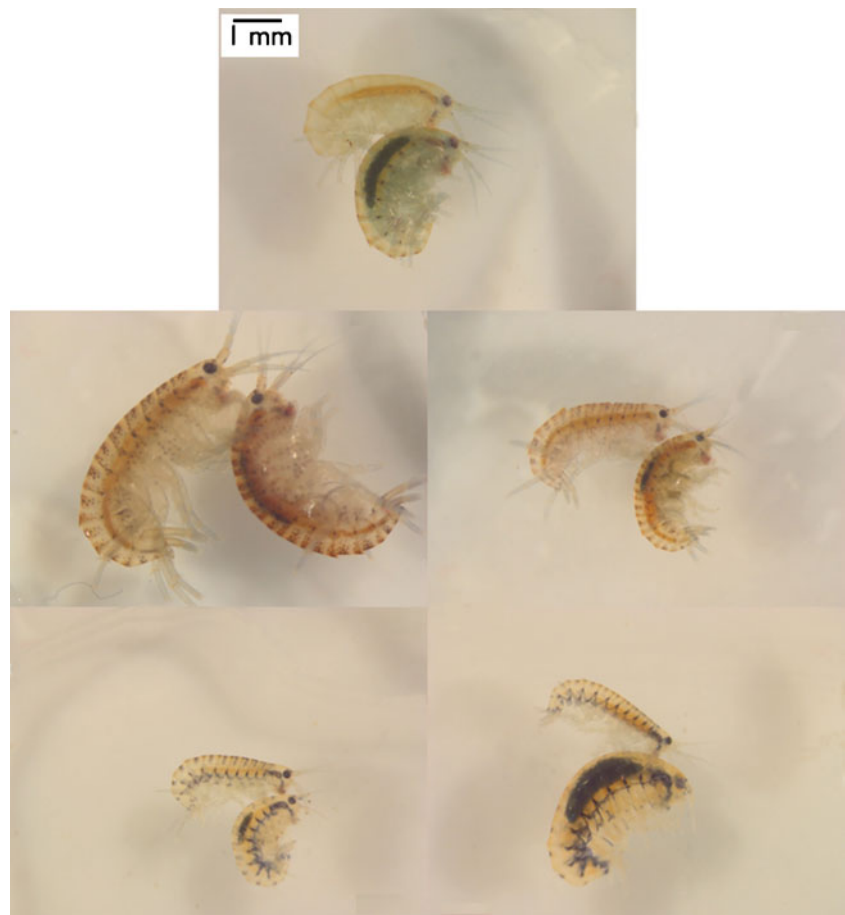
Amphipods in the genus *Hyaella* share a common mating biology that is tightly linked to the female molt cycle. All three species are reproductively active from late spring through early fall. At each reproductive molt, females produce a new clutch of eggs that are fertilized as they enter a ventral brood pouch called the marsupium (Strong 1972; Sutcliffe 1992; Wellborn and Cothran 2007b). These eggs can only be fertilized for a short period after the female molts and males employ precopulatory mate guarding behavior as a time investment strategy (Ridley 1983). Within species, the sexes disagree over how long they should pair with males favoring longer pairing

durations (Cothran 2004; 2008a). In some *Hyaella* species, large male body size increases pairing success (Wellborn 1995; Wellborn and Bartholf 2005). Mating biases for body size are primarily due to male–female interactions, although whether females prefer large males or large males are better at coercing females into pairing remains an open question (Cothran 2008b). Males are indiscriminate in this group and sometimes pair with individuals of the wrong sex, developmental stage, and species, as well as with dead individuals, which has also been observed in *Gammarus palustris* amphipods (Borowsky and Borowsky 1987; RDC personal observation).

#### Congruence between color phenotypes and genetic markers

We assayed a random sample (taken from the no-choice interbreeding experiment described below) of each color phenotype (see Fig. 1) to determine if the color phenotypes corresponded to the three cryptic amphipod species known to co-occur in lakes. We examined the samples using PCR with genetic markers that produce species-specific size variants in gel electrophoresis, as described in Wellborn and Cothran (2004) and compared these molecular results to our assignments to color phenotypes.

**Fig. 1** Precopulatory pairs of species A (green, top panel), species B (brown, middle panels), and species C (striped, bottom panels) *Hyaella*. For species B and species C, we have provided pictures of two pairs to demonstrate within-species variation in body size



### No-choice interbreeding experiment

Both experiments were performed at the University of Pittsburgh's Pymatuning Laboratory of Ecology in NW Pennsylvania under laboratory conditions [14:10 day to night cycle; temperature (mean±SD) 22±0.2 °C]. The goal of this experiment was to assess whether heterospecifics would form precopulatory pairs in the absence of conspecifics. On 26 July 2009, we collected 48 precopulatory pairs of each *Hyaella* species at Lake Le Boeuf (Erie County, PA, USA). To collect pairs, we used small (125 by 100 mm), fine-mesh dip nets to sweep through macrophytes and gently emptied the contents into small white trays. To quickly collect pairs of all three species, we sampled each microhabitat (nearshore vegetation as well as surface vegetation and vegetation in the middle of the water column in the deeper water) in the littoral zone. Pairs were transferred immediately to the laboratory and animals were assigned to treatments.

Each pair was separated and females were assigned a conspecific male or a male of one of the two heterospecific species. Females were only included in the experiment if they were both not carrying recently fertilized embryos in their marsupium (ventral brood pouch) and had eggs developing in the ovaries (visible on the dorsal side of the animal). Amphipods are not known to store sperm so these criteria were used to assure that the female had not already mated and was fertile. One male and one female amphipod were transferred to a 30-ml plastic cup containing 25 ml of filtered water and periphyton, which provided food for amphipods. Sixteen replicates were established for each species combination for a total of 144 experimental units. Inspection of photographs after the study revealed one mismatched pair (a species C female in a conspecific treatment was paired with a species A male) and this experimental unit was discarded. Female *Hyaella* molt every ~10 days and produce a new clutch of eggs at each molt. Prior to the current study, it was assumed that females could not store sperm and needed to mate with a male at each molt to ensure fertilization of eggs. However, early results—i.e., from the first reproductive cycle—from our experiment (see below) suggested that paired females collected from the lake that were close to their molt had already mated with a conspecific male prior to being included in our experiment. Based on this information, to ensure that females only had access to sperm from males that we assigned them, females were isolated with the assigned male in the 30-ml plastic cup for two reproductive cycles (the one in progress when collected from the field [i.e., the eggs developing in the ovaries] and an additional cycle [i.e., the second clutch of eggs produced by the female]). After the females produced their second clutch of eggs, males were removed from arenas and photographed.

Females were allowed to brood their second clutch of eggs for 7 days, after which they were checked for developing embryos (indicating fertilization of eggs) and photographed. These photographs were inspected to double check species assignments in the experiment and to provide images that could be used to check for congruence between molecular markers and our assignments to color phenotypes. We initially checked the cups for pairs and dead females after 2 h and then daily thereafter until the end of the experiment. Dead females were not replaced in this experiment.

For females of each species, we used *G* tests to compare incidences of pairing, successful fertilization (defined as developing embryos in the female's marsupium), and female survival to the end of her second reproductive cycle among male species treatments. We observed a number of unexpected "successful" fertilizations between species during the first reproductive cycle (see results). These were the eggs the female was carrying in her ovaries when collected from the lake and, although not reported in the literature, it is possible that fertilization of these eggs was achieved by short-term storage of sperm received from the conspecific male the female was paired with when collected from the lake. To shed light on whether "successful" fertilizations were due to females having already mated with the conspecific male they were paired with when collected from the lake (indicated by them being closer to their molt), we also used a Kruskal–Wallis test, with adjusted pairwise comparisons, to compare the time elapsed, in days, before females oviposited their first clutch of eggs for each of the three species.

### Choice interbreeding experiment

The goal of this experiment was to assess whether reproductive interference occurred when each species had access to conspecifics and whether reproductive interference was more prevalent when the relative abundance of heterospecifics was high. On 28 July 2012, we collected all three amphipod species from Lake Le Boeuf. The following day, we set up amphipod populations ( $n=24$  females+16 males for a total of 40 individuals) in 1-l plastic containers (14 cm×14 cm×6 cm) filled with filtered water. Four pieces (each 5 cm long) of *Myriophyllum spicatum* served as a source of refuge and food for amphipods, which eat the periphyton that grows on these plants. Total amphipod density (~4,000 individuals per m<sup>2</sup>) and sex ratio (60 % female) were kept constant across treatments. For each amphipod species, we included a unispecific, low-heterospecific (20 % heterospecific), equally mixed, and high-heterospecific (80 % heterospecific) treatment. Heterospecific treatments included both males and females of the heterospecific species (see Table 1). We set up populations that included all pairwise combinations of

**Table 1** The number of conspecific and heterospecific amphipods added to experimental units for each treatment in the choice experiment

Treatment	Conspecifics		Heterospecifics		Total amphipods	
	Female	Male	Female	Male	Female	Male
Unispecific	24	16	0	0	24	16
Low-heterospecific	18	14	6	2	24	16
Equally mixed	12	8	12	8	24	16
High-heterospecific	6	2	18	14	24	16

species so that each species was tested against both heterospecifics. Each treatment was replicated six times for a total of 72 experimental units. We checked the experiment twice a day (between 8:00–11:00 and 18:00–23:00) for 8 days. At each check, we recorded the number and species identity of pairs and any dead individuals. Dead amphipods were replaced after each check.

The primary response variable in this experiment was heterospecific pairing across treatments, however, we only observed one heterospecific pair during the experiment (see results below). We also report results on female survival and conspecific pairing (proportion of females paired) in the unispecific treatment compared to heterospecific treatments. For each focal species, we ran separate models for each heterospecific species (i.e., a model for species A with species B and a separate model for species A with species C), because only one group of unispecific replicates ( $n=6$ ) was used in the experiment. Also, because the sex ratio was female-biased the maximum proportion of females that could be paired in an experimental unit at any given time was 0.67 (i.e., 16 out of 24). For both female survival and conspecific pairing, we used generalized linear models with a binomial probability distribution and a logit link function. Alpha was corrected for all pairwise comparisons using the sequential Bonferroni method (Holm 1979). Female survival was high and there were no strong patterns related to the presence of heterospecifics. These results are presented in an “ESM” file.

## Results

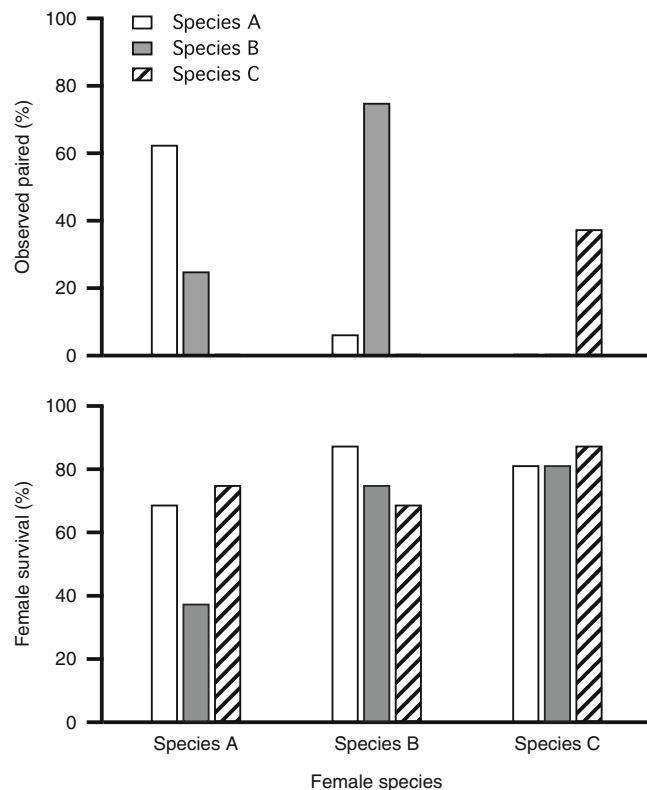
### Congruence between color phenotypes and genetic markers

The color phenotypes matched perfectly with the genetic markers that have been used to differentiate these *Hyalella* species (Wellborn and Cothran 2004). The green color phenotype is species A (15 out of 15 matches between color phenotype and the genetic marker for species A), the brown color phenotype is species B (19 of 19), and the striped color phenotype is species C (11 of 11). Hereafter, we refer to the three species as species A, B, and C for consistency with previous published work on this group (Wellborn and Cothran 2004, 2007a).

### No-choice interbreeding experiment

#### Frequency of interspecific pairing

Pairing was more common within species than between species (Fig. 2a). For species A, pairing was observed in 63 % of trials with conspecific males, 25 % of trials with species B males, and 0 % of the trials with species C males ( $G=18.784$ ,  $P<0.001$ ,  $df=2$ ,  $n=16$  for all three male species treatments). For species B, pairing was observed in 75 % of trials with conspecific males, 6 % of trials with species A males, and 0 % of trials with species C males ( $G=30.596$ ,  $P<0.001$ ,  $df=2$ ,  $n=16$  for all three species). For species C, pairing was observed in 38 % of trial with conspecifics, but 0 % of trials with species A



**Fig. 2** Results from the no-choice experiment showing the frequency of pairing and female survival for females housed with conspecific and heterospecific males. Female survival is the percentage of females that survived to the end of their second reproductive cycle

or B males ( $G=15.71$ ,  $P<0.001$ ,  $df=2$ ,  $n=16$  for species A and species B and 15 for species C).

#### Success of fertilization

Fertilization was more successful within species than between species. For species A, 75 % and 73 % of crosses with conspecific males were successful for the first and second female reproductive cycle respectively, but none of the crosses with heterospecific males produced developing embryos (first reproductive cycle:  $G=35.989$ ,  $P<0.001$ ,  $df=2$ ,  $n=16$  for all three species; second reproductive cycle:  $G=21.271$ ,  $P<0.001$ ,  $df=2$ ,  $n=11$  for species A and species C and 7 for species B).

For species B, 94 and 80 % of crosses with conspecifics were successful for the first and second female reproductive cycle respectively, but only 6 % of the trials with species C males were successful, and none of the crosses with species A males were successful. All of the successful fertilizations with species C males occurred during the first female reproductive cycle (first reproductive cycle:  $G=46.143$ ,  $P<0.001$ ,  $df=2$ ,  $n=16$  for all three species; second reproductive cycle:  $G=26.547$ ,  $P<0.001$ ,  $df=2$ , species A  $n=14$ , species B  $n=10$ , species C  $n=9$ ).

For species C, all of the crosses with conspecific males were successful. When crossed with species A males, 75 % of the crosses were successful during the first female reproductive cycle but none of the crosses were successful during the second reproductive cycle. Similarly, when crossed with species B males, 63 % of the crosses were successful during the first female reproductive cycle but none of the crosses were successful during the second female reproductive cycle (first reproductive cycle:  $G=9.489$ ,  $P=0.009$ ,  $df=2$ ,  $n=16$  for species A and species B and 15 for species C; second reproductive cycle:  $G=45.829$ ,  $P<0.001$ ,  $df=2$ , species A  $n=11$ , species B  $n=13$ , species C  $n=12$ ).

It is possible that “successful” fertilizations between species during the first female reproductive cycle were the result of females having already mated with the conspecific male they were paired with when collected from the lake. To further explore this possibility, we compared the time that elapsed between setting up pairings for the first female reproductive cycle and when the female oviposited. The three species differed in the time that elapsed between setting up the experiment and oviposition (Kruskal–Wallis test statistic = 53.598,  $df=2$ ,  $P<0.001$ ). Species C females oviposited 1.5 day earlier than both species A and species B females (species A (mean±SD): 3.61±1.57, species B: 3.56±1.25, species C: 2.13±0.46; both  $P<0.001$ ). Time to oviposition did not differ between species A and species B females ( $P=1.0$ ). All of the species C females that produced developing embryos when housed with a heterospecific male oviposited within 2 days of the onset of the experiment. Also, the lone species B female

that produced developing embryos with a heterospecific male (species C) was also within 2 days of her molt when the experiment was initiated.

#### Female survival in the presence of conspecific vs. heterospecific males

Female survival ranged from 38 % to 88 % across treatments (Fig. 2b). For species B, female survival over the course of the experiment was similar when housed with conspecific and heterospecific males ( $G=1.748$ ,  $P=0.417$ ,  $df=2$ ,  $n=16$  for all three male species treatments). This was also the case for species C females ( $G=0.22$ ,  $P=0.896$ ,  $df=2$ ,  $n=16$  for species A and species B male treatments and 15 for the species C male treatment). However, for species A females there was a marginally non-significant effect of male species on female survival ( $G=5.404$ ,  $P=0.067$ ,  $df=2$ ,  $n=16$  for all three species). When housed with species B males, the survival of species A females fell to 38 % compared to 69 % with conspecific males and 75 % with species C males (Fig. 2b).

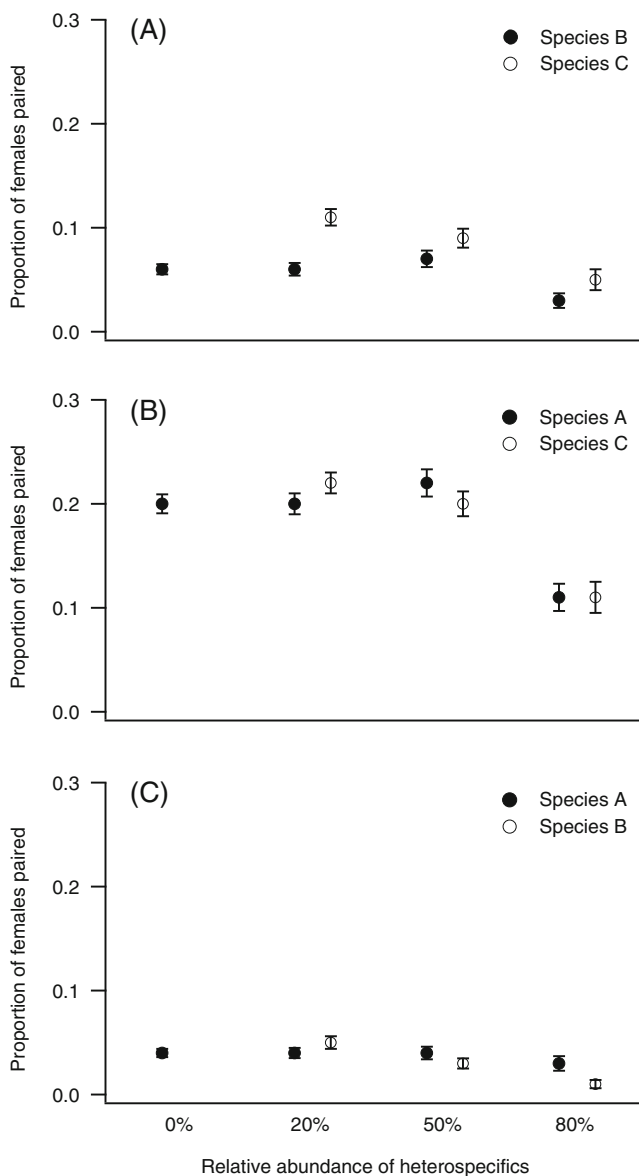
#### Choice interbreeding experiment

Over the 8-day observation period we recorded 2,592 observations of precopulatory pairs (species A: 610 pairs; species B: 1686 pairs; species C: 296 pairs) and only one (0.04 %) was a female paired with a heterospecific male (species C male paired with a species B female).

#### Frequency of pairing with conspecific males with increasing heterospecific relative abundance

The proportion of females observed in precopulatory pairs with a conspecific male was sensitive to the presence of heterospecifics and these patterns differed depending on the focal species (Fig. 3). For species A, the presence of species B affected pairing frequency ( $G=14.113$ ,  $P=0.003$ ,  $df=3$ ; Fig. 3a). Relative to the unispecific treatment, species A pairing frequency was reduced but only when species B was at high relative abundance ( $P=0.008$ ; all other pairwise comparisons  $P\geq 0.304$ ). Species C also affected species A pairing frequency ( $G=41.172$ ,  $P<0.001$ ,  $df=3$ ). Relative to the unispecific treatment, more species A pairs were observed when species C was present at low to medium relative abundance (both  $P\leq 0.008$ ). Pairing frequency did not differ between the unispecific and high species C relative abundance treatments ( $P=0.355$ ).

For species B, the presence of species A affected pairing frequency ( $G=33.829$ ,  $P<0.001$ ,  $df=3$ ; Fig. 3b). Relative to the unispecific treatment, species B pairing frequency was reduced only when species A was at high relative abundance ( $P<0.001$ ; all other pairwise comparisons  $P\geq$



**Fig. 3** Results from the choice experiment showing the proportion of females in conspecific pairs across heterospecific relative abundance treatments. **a** Species A females, **b** species B females, and **c** species C females. Note, that the filled circle symbol is also used to represent the case where heterospecifics were absent. Markers are means $\pm$ 1 SE

0.664). The presence of species C also affected species B pairing frequency ( $G=31.613$ ,  $P<0.001$ ,  $df=3$ ). Similar to species A, species C only reduced species B pairing frequency when at high relative abundance ( $P<0.001$ , all other pairwise comparisons  $P\geq 0.361$ ).

For species C, pairing frequency was not affected by the presence of species A ( $G=3.419$ ,  $P=0.331$ ,  $df=3$ ; Fig. 3c). However, the presence of species B did affect species C pairing frequency ( $G=23.142$ ,  $P<0.001$ ,  $df=3$ ). Relative to the unispecific treatment, species C pairing frequency was reduced only when species B was at high relative abundance ( $P<0.001$ , all other pairwise comparisons  $P\geq 0.4$ ).

## Discussion

In this study, we used color phenotypes, reported here for the first time, to study interspecific mating interactions in what were once considered cryptic species. We discovered that *Hyalella* amphipods have species-recognition abilities that limit or prevent the negative consequences of forming precopulatory pairs with heterospecifics. In addition, *Hyalella* females rarely produced viable embryos when housed with heterospecific males, with the exception being females of species C during the first female reproductive cycle. None of the females produced developing embryos when paired with a heterospecific male during the second reproductive cycle suggesting that “successful” fertilization in heterospecific trials are most likely attributable to short-term sperm storage from mating with conspecific males prior to collection from the lake, and that these species are reproductively isolated.

When amphipods were housed only with a heterospecific of the opposite sex, we found some evidence for asymmetric reproductive interference in the three *Hyalella* species. Interspecific pairing was observed between species B males and species A females in 25 % of the trials. The only other interspecific pairing was between a species A male and a species B female. Similar indiscriminate mating behavior has been found in other crustaceans although in some cases habitat preferences and the timing of reproduction prevent interspecific pairings (Mead and Gabouriaux 1977; Kolding and Fenchel 1979; Kolding 1986). In our study, however, when males and females of the three species had access to conspecifics only one of 2,592 pairs observed (i.e., 0.04 %) was between heterospecifics. These results suggest the cryptic species have species-recognition mechanisms that prevent reproductive interference in the form of heterospecific pairing.

Species recognition may manifest through male, female, or a combination of male and female discriminatory behavior. Traditionally, the avoidance of costly interspecific mating is attributed to choosy females rather than males (Gröning and Hochkirch 2008). Sex differences in discriminatory behavior are thought to be a consequence of females investing more in each reproductive event, and thereby yielding higher costs of a poor decision for females (Williams 1966; Trivers 1972; Wirtz 1999). Mounting evidence suggests this is a naïve assumption given that male ejaculates can be expensive and males are often choosy (Dewsbury 1982; Bonduriansky 2001; Wedell et al. 2002; Edward and Chapman 2011). Perhaps a more likely reason to expect females to prevent costly interspecific mating is that they are more likely to successfully mate than males. Variance in male mating success is often higher than in females and, consequently, a large portion of the male population may have limited access to conspecific females.

These males may be most likely to participate in heterospecific pairing attempts. In *Hyaella* amphipods, females have considerable control over pairing. Studies have demonstrated that female resistance behavior can shorten pairing duration and bias pairing success towards males with particular phenotypes (Wellborn and Bartholf 2005; Cothran 2008a). On the other hand, male amphipods are known for their indiscriminate nature in pairing with other males, juveniles, and even dead individuals (Borowsky and Borowsky 1987; Wellborn and Cothran 2007b). Therefore, it is likely that females prevented interspecific pairing in the choice experiment, although detailed behavioral observations are necessary to ascertain whether males attempt to mate with heterospecific females.

The no-choice experiment revealed asymmetric patterns of interspecific pairing, which was consistent with size differences between the two interacting individuals. Species B males are on average 18 % larger (comparing head length a measure of body size in amphipods; Edwards and Cowell 1992) than species A females, which is the greatest sex difference in size among the three species (Wellborn and Cothran 2004). The increased incidence of interspecific pairing in this dyad may therefore be due to forced pairing by species B males. Interspecific forced mating is observed in a variety of taxa, especially waterfowl, although it is widely regarded as rare in nature (Wirtz 1999). Such an antagonistic interaction has obvious fitness consequences in that females may be injured during struggles with heterospecific males in addition to any costs associated with prolonged pairing and wasted gametes (Gröning and Hochkirch 2008). However, species B males are also 11 % larger than species C females and we observed no interspecific pairings between this species combination. This suggests that size differences between the sexes are either not important in producing this pattern or that males are only able to overcome female resistance when a minimum size difference between the sexes is reached. More importantly, the addition of just a few conspecific females for species B males eliminates interspecific pairing with species A females. Furthermore, we found little evidence of heterospecifics interfering with the formation of conspecific pairs. The only exception was at high-heterospecific relative abundance where a reduction in pairing relative to the unispecific treatment was observed for most species combinations. However, it is difficult to interpret these results because pairing duration, and thus the frequency of observed pairs, may have been reduced due to lower conspecific densities (Jormalainen 1998). To distinguish these two possibilities one would need treatments where conspecific density is manipulated in the absence of heterospecifics. Collectively, these results suggest that males may be choosier than we suspect in this species complex or that female behavior (e.g., species differences in

the efficacy of female resistance) is critical in producing the species differences in interspecific pairing.

One possibility for the discordance in interspecific pairing between the choice and no-choice experiment is that mate choice by one or both sexes may change as the female molt approaches. Like many crustaceans, female *Hyaella* can only have their eggs fertilized for a short period after their molt. It has been proposed that this mating biology favors the evolution of plasticity in female choice. Females may transition from being choosy to indiscriminate as the molt approaches because a low-quality male is better than having a clutch of eggs go unfertilized (Janetos 1980; Jormalainen and Merilaita 1993). In our no-choice experiment, females that were offered only a heterospecific male may have paired with those males because they reached the phase of their molt cycle where they cease resisting male pairing attempts to assure the presence of a male at the molt. In cases where females were given a choice, females were not “forced” to pair with heterospecifics because even in the high heterospecific treatment females would have had access to conspecific males.

Males may mistakenly pair with heterospecific females that are close to their molt if the cues they use to assess female quality are consistent across species. Males in other amphipod species and isopods, which share a similar mating biology, prefer females that are close to their molt, a preference that increases male fitness by lowering missed opportunity costs (Jormalainen 1998; Thiel 2011). The cues that males use to evaluate females appear to be either leaky or surface chemicals that are more concentrated near the female molt (Shuster 1981; Thompson and Manning 1981; Borowsky 1985). Molting hormones have been identified as an important cue that males use to assess female molt status in an isopod species (Sparkes et al. 2000). The arthropod molting hormone, 20-hydroxyecdysone (20HE), is shared by all three *Hyaella* species. Interestingly, detailed studies of the chemical ecology of the crab *Carcinus maenas* discovered that 20HE is not the chemical that attracts males to molting females (Hardege and Terschak 2011). Instead, males are attracted to the nucleotide pheromone uridine diphosphate (UDP) that is produced in the urine of molting females. However, UDP is also found in variety of taxa and thus unlikely to be a species-specific cue. If these chemicals are the primary cues males use to assess female quality, a female that is close to her molt may be considered high quality to males regardless of species. This possibility, combined with species differences in a female’s ability to resist or decreased female resistance to pairing near her molt (Jormalainen and Merilaita 1993, 1995), may explain the patterns of interspecific pairing we observed when males only had access to heterospecific females.

There is strong sexual conflict over pairing duration in *Hyaella* amphipods and females use behavioral resistance to reduce pairing duration (Cothran 2008a). Species C females



had a shorter time to molt when collected as pairs from the field and were less likely to be observed in precopula in both experiments than both species A and species B females, which is consistent with species C females having more efficient resistance capabilities than females of the other two species. Such patterns may be the result of differences in selection on female resistance among the three species. Unlike species B females, which live in shallow, nearshore habitats, species C females live in deeper water of lakes where predatory fish are abundant (Wellborn and Cothran 2007a). Fish preferentially consume paired amphipods and the increase in risk relative to being single is greater for females than males (Cothran 2004), therefore, females that enter into precopula too early substantially increase their risk of mortality. While species A females also live in deeper water where fish are present, they are smaller and less frequently consumed by these predators (Wellborn and Cothran 2007a). The green color of species A may also provide some crypsis against the aquatic vegetation on which they live (Fig. 1). Thus, species differences in the costs to females of lengthy precopula and the resulting variation in the strength of selection on female resistance may explain why species B males were more successful at taking species A females into precopula than species C females in the no-choice experiment.

Alternatively, patterns of reproductive interference in the no-choice experiment may be explained by phylogenetic patterns (Gröning and Hochkirch 2008). If this were the case, we would expect species A and B to be more closely related to each other than either is to species C. However, this is not the case. The cryptic species used in this study are separated by substantial molecular evolution that corresponds to a divergence time of approximately 9 million years (Witt and Hebert 2000; Wellborn and Cothran 2004; Wellborn and Broughton 2008) and it appears that species B and species C share a more recent common ancestor than either does with species A (Wellborn and Broughton 2008).

Serendipitously, we also found evidence consistent with short-term sperm storage in *Hyalella* amphipods. These animals were thought to be unable to store sperm (Strong 1973; Wellborn and Cothran 2007b). However, especially in species C, we observed several cases of viable embryos in females that were collected in the field as pairs, split from their original partner and not given access to a conspecific male (i.e., they were housed with a heterospecific male). In all of these cases, as well as the lone case where a species B female produced viable embryos without a conspecific male, females were within 2 days of their molt. In fact, all females that were within 2 days of their molt when collected from the field produced viable embryos despite the absence of a conspecific male. These cases of successful fertilization are unlikely to be due to interspecific mating because none of the females that were housed with only a heterospecific male during the first reproductive cycle for more than 2 days produced viable

embryos. Furthermore, none of the females produced viable embryos with heterospecific males during the second reproductive cycle when they only had access to the male that we assigned them. Therefore, the most likely explanation for this result is that sperm transfer from the female's original partner occurred before we initiated the experiment, but without these females ovipositing eggs into the marsupium, and with fertilization occurring at some point in the process. However, a rigorous test of the sperm storage hypothesis that demonstrates viable sperm being stored in the female's marsupium or reproductive tract is required before we can address its implications for sexual selection and sexual conflict in amphipods.

Our results show that interspecific pairing is unlikely in the *Hyalella* species complex. This conclusion suggests that at least one sex has well-developed species-recognition abilities that prevent costly interspecific pairing. However, this does not rule out the possibility that reproductive interference is important in these amphipods. Although interspecific mating interactions rarely lead to pairing they may still impose significant costs if interspecific pairing attempts by males decrease female fitness (Magurran and Seghers 1994; McLain and Pratt 1999; Sakurai and Kasuya 2008; Rossi et al. 2010; Gasparini et al. 2012). For example, resisting heterospecific male pairing attempts may be energetically costly and draw the attention of predators. Detailed behavioral observations will shed light on whether males do indeed pursue heterospecific females as mates when conspecific females are available. Although our study has focused on the importance of reproductive interference, we look forward to research that considers how other mechanisms, including both abiotic conditions and biotic interactions, affect species distributions over space and time. Such an approach has been implemented in understanding the exclusion of native gammarid amphipods by invasive gammarid species with great success (MacNeil et al. 2003; Bollache et al. 2007; Dick 2008; Kestrup et al. 2011).

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