Something Darwin didn’t know about barnacles: spermcast mating in a common stalked species

Marjan Barazandeh1,2, Corey S. Davis1, Christopher J. Neufeld1,2,†, David W. Coltman1 and A. Richard Palmer1,2

1Department of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada T6G 2E9
2Bamfield Marine Sciences Centre, Bamfield, British Columbia, Canada V0R 1B0

Most free-living barnacles are hermaphroditic, and eggs are presumed to be fertilized either by pseudo-copulation or self-fertilization. Although the common northeast Pacific intertidal gooseneck barnacle, Pollicipes polymerus, is believed only to cross-fertilize, some isolated individuals well outside penis range nonetheless bear fertilized eggs. They must therefore either self-fertilize or—contrary to all prior expectations about barnacle mating—obtain sperm from the water. To test these alternative hypotheses, we collected isolated individuals bearing egg masses, as well as isolated pairs where at least one parent carried egg masses. Using 16 single nucleotide polymorphism markers, we confirmed that a high percentage of eggs were fertilized with sperm captured from the water. Sperm capture occurred in 100 per cent of isolated individuals and, remarkably, even in 24 per cent of individuals that had an adjacent partner. Replicate subsamples of individual egg masses confirmed that eggs fertilized by captured sperm occurred throughout the egg mass. Sperm capture may therefore be a common supplement to pseudo-copulation in this species. These observations (i) overturn over a century of beliefs about what barnacles can (or cannot) do in terms of sperm transfer, (ii) raise doubts about prior claims of self-fertilization in barnacles, (iii) raise interesting questions about the capacity for sperm capture in other species (particularly those with short penises), and (iv) show, we believe for the first time, that spermcast mating can occur in an aquatic arthropod.

1. Introduction

Textbooks note two bizarre observations about typical stalked and acorn barnacles (Thoracica). First, unlike nearly all free-living arthropods, adults are sessile and typically glued to hard surfaces [1]. Second, they appear to be constrained by their evolutionary history to exchange sperm either by a form of copulation called pseudo-copulation (release of sperm into a partner’s mantle cavity) or, in hermaphrodites, by self-fertilization [2–4]. Ever since Darwin’s epic monographs [5,6], these beliefs about possible modes of barnacle mating have been strongly held [3,7]. Unfortunately, although pseudo-copulation is readily observable in many species [4,5], self-fertilization is almost always inferred from observations of isolated individuals bearing fertilized embryos [2,3,8–14].

Obligate pseudo-copulation imposes two significant constraints on mating success in a sessile species: (i) fertilization is limited to immediate neighbours, and (ii) individuals with no immediate neighbours cannot reproduce, except by self-fertilization. As a consequence, barnacles are famous for their long penises, including some of the longest in animals, relative to their body size [15,16].

Not all barnacles have long penises, however. Species of Pollicipes are unusual among thoracican barnacles because their penises are shorter than their feeding legs (figure 1a), not very extensible and vary little in size throughout the breeding season [4,5]. Pollicipes polymerus Sowerby 1833 is a hermaphroditic, stalked barnacle that inhabits wave-exposed intertidal shores of the northeast...
Figure 1. External body form and sperm leakage in the stalked barnacle Pollicipes polymerus. (a) Relaxed penis (arrow) and feeding legs of P. polymerus (soma wet mass = 0.785 g, rostro-carinal length = 19 mm) from a moderately wave-exposed shore near Bamfield, British Columbia, Canada. (b) Pollicipes polymerus leaking sperm in the field at low tide on Tatoosh Island, WA, USA.

Pacific, where it typically occurs in dense clusters or beds [17,18]. However, solitary individuals or small groups are not uncommon (M. Barazandeh 2009–2010, personal observation).

Spermcast mating—where sperm released into the water by males fertilize eggs retained in the body of a partner—has been reported in many sessile or sedentary marine invertebrates, including sponges, cnidarians, polychaetes, bivalved molluscs, entoprocts, ectoprocts, brachiopods, pterobranch hemichordates and colonial ascidians [19]. Despite being sessile, however, spermcast mating has never been considered possible in barnacles [19,20].

In typical hermaphroditic barnacles, a functional male searches for partners by random penis movements and then deposits sperm into the partner’s mantle cavity. A functional female can probably be fertilized by more than one male [3,21]. To copulate, though, a barnacle must be within penis range of at least one neighbour. Nonetheless, isolated individuals too distant to copulate sometimes bear developing embryos in many barnacle species in the field and laboratory, which has led many authors to conclude that self-fertilization must have occurred [2,3,8–14].

All attempts to observe fertilization in solitary P. polymerus in the laboratory have failed [14,22]. Even more curiously, although pseudo-copulation has been directly observed in the field or in the laboratory in many other thoracican barnacles, it has never been reported in P. polymerus despite multiple attempts to observe it [4,17]. Thus, pseudo-copulation is presumed to occur in this species based only on the presence of sperm in the mantle cavity [14,17].

Significantly, we observed occasional P. polymerus individuals in the field leaking sperm between their opercular plates during low tide (figure 1b). In addition, the occurrence of isolated yet fertilized individuals [14] raises three important questions about this species. (i) Can sperm leaked into the water fertilize eggs in distant individuals? (ii) Do fertilized eggs in isolated individuals arise from self-fertilization or from sperm captured from the water? (iii) When a barnacle has a single neighbour within copulation range, does it mate only with that neighbour or are some eggs fertilized by sperm captured from distant individuals?

2. Material and methods

(a) Barnacle sampling and measurements

Pollicipes polymerus were collected from shores of intermediate (Helby Island, 48.847° N, 125.168° W) and high (Seppings Island, 48.841° N, 125.209° W) wave exposure near the Bamfield Marine Sciences Centre in Barkley Sound, British Columbia (Canada) in August 2009 and August 2010. Maximum velocities of breaking waves at the exposed site are nearly twice as high as the intermediate site during the calm summer months of July and August [23].

We sampled and scored 599 individuals for body length, fertilization status, distance to a nearest neighbour and nearest-neighbour body length. Body length was measured as rostro-carinal opercular length: linear distance between the scutal plate anterior margin and tergal plate posterior margin parallel to the gape. Distances between neighbours were measured from the mid-point of the capitulum of each neighbour (where the scutal–tergal plate suture intersects the gape). Degree of isolation was defined as the ratio of nearest-neighbour distance to body (rostro-carinal) length of a given individual. We considered an individual fully isolated if it was more than two body lengths from its nearest neighbour (roughly three times the maximum reach of the extended penis; see below).

We collected 37 isolated individuals bearing embryo masses. To avoid potential problems posed by individuals with supple stalks, we sampled only individuals with short stalks, or wedged tightly into a crevice or between mussels. We did not sample individuals with juveniles attached to their stalks, even when juveniles were well below the minimum size of sexual maturity (approx. 4 mm) [17]. We also collected 34 individuals found as isolated pairs: adjacent individuals where both were more than two body lengths from any other conspecifics, where at least one individual carried embryo masses.

We removed the stalks of adult barnacles and the embryo masses, placed them individually into 70–95 per cent ethanol in labelled 1.5 ml microfuge tubes and stored them at −20°C until DNA extraction.

(b) Penis measurements

We measured relaxed and extended penis length in P. polymerus and in a common, sympatric acorn barnacle, Balanus glandula
Darwin 1854. Relaxed penis length was measured by freezing barnacles, thawing them in seawater, carefully extending and photographing the penis under a microscope (or the whole animal under water, as for figure 1a), and then digitally measuring length using ImageJ [24]. Extended penis length was measured using a gravity-fed pressure system for inflation [16] and used to calculate extension ratios (extended length per relaxed length).

(c) Marker development and genotyping
Numerous attempts to develop microsatellite markers for *P. polymerus* were not successful, so we developed single nucleotide polymorphism (SNP) markers to assess genetic relationships between embryo masses and potential parents [25]. We used a pool of five *P. polymerus* individuals to construct a genomic library, selected 30 contigs with the largest product sizes, and then amplified them in seven individuals. Twenty-two putative SNP loci, each with two alleles, were identified where the rare allele was observed in at least two of seven individuals and at least one heterozygote was observed. Among those 22, 16 SNP markers amplified successfully [25,26]. Both adults and embryo masses were genotyped using the ABI PRISM SNaPshot multiplex kit, and the results were scored using GeneMapper v. 4.0 (ABI) [25,27–29]. Although we were unable to genotype individual embryos, we tested how widespread non-parent alleles were among embryos within an egg mass for six isolated individuals by genotyping eight additional subsamples from different regions of the embryo lamellae.

An embryonic allele was called a non-parent allele if it was absent from the brooding parent in isolated individuals, or absent from both the brooding parent and the adjacent partner in isolated pairs.

3. Results

(a) Penis measurements
The penis of *P. polymerus* was significantly shorter and less extensible than that of *B. glandula*. The ratio of relaxed penis length to soft body length was 0.57 ± 0.025 (mean ± s.e., *n* = 10) for *P. polymerus* and 1.39 ± 0.059 (*n* = 10) for *B. glandula*, and measured extension ratios (extended length [16] per relaxed length) were, respectively, 1.21 ± 0.081 (*n* = 3) and 2.26 ± 0.102 (*n* = 10). Therefore, the penis of *P. polymerus* does not even extend to one body length (maximum extension of 0.69 body lengths), whereas a *B. glandula* penis can reach nearly three times its body length (maximum extension of 3.14 body lengths).

(b) Fertilization/isolation measurements
The smallest fertilized *P. polymerus* we observed had a rostro-carinal length of 11 mm, and we found no fertilized individuals beyond 7.14 body lengths (approx. 14 cm) from a nearest neighbour (*n* = 63; figure 2). The proportions of fertilized individuals in clumps of barnacles did not differ significantly between the wave-exposed and intermediate site (*χ²* test, *p* = 0.47), so these data were pooled.

We confirmed results from an earlier study [14] that fertilization incidence declines with increasing distance to nearest neighbour in *P. polymerus* in the field (figure 2). Significantly, these results clearly reveal that some fertilized *P. polymerus* were well outside the extended penis range of any neighbour (i.e. beyond two body lengths).

Figure 2. Percentage of *Pollicipes polymerus* individuals fertilized (filled symbols) or bearing non-parent SNP alleles at two or more SNP loci (open symbols), as a function of isolation (number of body lengths to nearest neighbour). Numbers adjacent to points indicate sample sizes. Counts were pooled from the two sites sampled. The presence of non-parent SNP alleles indicates sperm capture.

Figure 3. Numbers of embryo masses with non-parent SNP alleles from 37 isolated individuals (grey bars) and 34 isolated pairs (black bars). A total of 16 SNP loci were scored per individual. The presence of non-parent SNP alleles indicates sperm capture.

(c) Genotyping results
Embryo masses from all 37 isolated individuals had non-parent alleles at one or more loci (figure 3), and all but one had non-parent alleles at two or more loci (figure 3; electronic supplementary material, table S1). All eight replicate subsamples of egg masses from four of the subsampled barnacles exhibited the same multilocus genotype as the original egg mass sample (electronic supplementary material, table S2). In the two remaining individuals, the multilocus genotype of all eight subsamples was identical to the original sample at 15 of 16 loci. In both cases, the allele that was not present in a subsample (in three of eight or all eight subsamples, respectively) was the rare allele. By scoring the genotypes of all individuals and egg masses at all loci, we estimated an average allelic scoring error rate (number of allelic mismatches per total number of alleles scored in all individuals) of 0.26 per cent, which is 100 times lower than the average rate of non-parent alleles observed in egg masses (27.4%; range 6.3–62.5%).

Among egg masses from 34 individuals from isolated pairs, eight (24%) also carried SNP alleles not present in either parent (figure 3). In these eight cases, on average 13.2 per cent of alleles were non-parent alleles (range 6.3–25.0%), roughly 50 times the genotyping error rate.
The average frequency of the dominant allele across all loci was 84 per cent (range 62–97%; electronic supplementary material, table S1). Among 120 pairs of 16 SNP loci, only two exhibited significant linkage disequilibrium (Pp190-1 and 190-2, Pp117-2 and 117-3; p ≤ 0.0004) [25].

4. Discussion

Although crustaceans are generally thought to copulate somehow [30] our observations confirm that a significant fraction of eggs of isolated 

**P. polymerus** were fertilized by sperm released by individuals beyond copulation range. Water is the most likely source for these non-parent alleles. Although we cannot rule out partial self-fertilization, our results reveal (i) that self-fertilization is not necessary for isolated individuals and (ii) that non-parent alleles are widespread throughout embryo masses. Even more remarkably, in isolated pairs where both partners were within penis range, many embryos throughout the embryo mass were still fertilized by sperm from the water in 24 per cent of individuals. Therefore—quite contrary to all prior expectations about mating in barnacles—

**P. polymerus** appear able to obtain sperm from the water in the field and do so even when an adjacent partner is available.

Our estimates of sperm capture rates are necessarily underestimates, perhaps by a large margin. Many instances of sperm capture would not have been detected because the overall frequency of the dominant allele exceeded 80 per cent at 11 of 16 SNP loci (electronic supplementary material, table S1). Unfortunately, we could not determine the number of males contributing to a given embryo mass because of (i) the high cost and difficulty of extracting enough DNA from individual embryos, especially early stage embryos, and (ii) the limited power of SNP loci to detect paternity. Significantly, however, we did observe the same multilocus genotype among all eight subsamples from four of the six subsampled egg masses. Furthermore, in each of the remaining two subsampled egg masses, only one of 16 SNP loci differed from the original genotype, and in both cases the allele missing from some subsamples was the rare SNP allele (electronic supplementary material, table S2).

Non-parent alleles were therefore widespread throughout individual embryo masses and were not simply an artefact of PCR amplification of a few stray captured sperm. This observation further reduces the likelihood that these embryo masses arose primarily from self-fertilization. We are also confident in our non-parent embryo counts because our use of multiple loci decreased the chance that parents and egg masses would appear identical when truly different [31]. In addition, our allele-scoring error rate was very low (0.26%), so our counts of non-parent alleles were not inflated by genotyping errors. Finally, because embryos from all isolated individuals had one or more non-parent alleles, **P. polymerus** are clearly able to obtain sperm from the water, even if it is not the primary mode of mating in this species.

Three alternative hypotheses for how these isolated individuals might have been fertilized can be dismissed. First, fertilization by an adjacent individual that subsequently died is highly unlikely. **Pollicipes pollynomus** eggs require around 25 days to hatch after fertilization [32]. Stalk remnants or footprints of a dead barnacle, which are readily visible, typically require longer than this to degrade, and we observed no such remnants near any barnacle we sampled. Second, we took great care to avoid sampling individuals that might have been able to reposition themselves with their flexible stalk. Third, although **P. polymerus** is reportedly a hermaphrodite with no dwarf males [1], we nonetheless avoided sampling any barnacles with attached ‘juveniles’ on their stalks, no matter how small.

**Pollicipes pollynomus** therefore appear to be capable of sperm-cast mating, a type of mating seen in at least nine other phyla of sessile or sedentary marine invertebrates [19], but never before reported for barnacles. At present, we cannot say how prevalent sperm-cast mating is compared with pseudo-copulation in this species, because we sampled only isolated individuals or isolated pairs—pseudo-copulation may still be an important mode of sperm transfer in dense clumps. Nonetheless, we can conclude that sperm-cast mating can and does occur. We also do not yet know whether the primary mode of sperm release is (i) leakage of sperm at low tide (figure 1b), (ii) active ejection of sperm when immersed or (iii) leakage of sperm from mated individuals because pseudo-copulation is ‘loppy’. For example, in barnacle pseudo-copulation, sperm are deposited into the mantle cavity [1] and subsequent feeding or respiratory movements would be likely to cause some leakage from a recently mated individual. Given the high overall densities of **P. polymerus**—well over 1000 m⁻² in suitable habitat [14,17]—free sperm may therefore be widely available in the water. In addition, because **P. polymerus** feed by extending feeding legs into the backwash after waves break [17], sperm leaked out at low tide (figure 1b) could readily be captured from the backwash of the first waves to reach that height on the shore on an incoming tide.

Even though mating systems vary considerably among stalked barnacles [1,33], capture of sperm from the water adds a wholly new potential mode to the array. We do recognize that **P. polymerus** is somewhat unusual: it has a relatively short and inextensible penis (see §3e), and it lives in an extreme physical environment (wave-swept rocky shores [17,18]), so sperm capture may be more likely in this species than in others. Nonetheless, our observation of sperm capture is significant for three reasons. First, it challenges the widely held belief that some form of copulation is obligatory when crustaceans mate [30]. Second, it raises doubts about prior reports of self-fertilization in isolated barnacles [2,8–14], because sperm-cast mating was not considered a possibility. Third, if sperm-cast mating occurs—even occasionally—in other thoracican barnacle species, some rethinking of barnacle reproductive biology may be required. Depending on the prevalence of sperm capture in other species, population genetic [12,34] and sex allocation [3,21] models for barnacles may need to be revised because functional females (i) may be fertilized by many more males than previously thought, (ii) will not be restricted to mating with immediate neighbours, and (iii) can still mate even if no neighbours are within copulation range. We are currently testing whether sperm-cast mating occurs in two acorn barnacles (B. glansula Darwin, 1854 and *Chthamalus dalli* Pilsbry, 1916). In another acorn barnacle, *Tetraclita rubescens* Darwin, 1854, individuals appear to mate reciprocally with their nearest neighbour, occasionally even at distances of multiple body lengths [35]. But only 17 out of 130 broods (13%) were fertilized by more than one father—even when many potential mates were nearby—and 14 of these were fertilized by only two...
fathers. So capture of sperm from the water may not occur in T. rubescens.

Finally, much of interest remains to be learned about sperm cast mating in barnacles. Three obvious questions arise for P. polymerus. (i) How prevalent is sperm cast mating compared with pseudo-copulation under normal conditions? (ii) How are sperm released and captured? (iii) Is behaviour, limb form, sperm form [36] or ejaculate form modified to increase the success of sperm capture? Intriguing questions are also raised about how widespread this surprising ability is in other barnacles. For example, several species have relatively short penises (e.g. shorter than the feeding legs), including stalked barnacles such as Scalpellum [5], and acorn barnacles such as Pachyseris and Octoneis [6]. Clearly, further work is needed to test how often sperm cast mating supplements pseudo-copulation in other species.

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