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Sperm precedence in a novel context: mating in a sessile marine invertebrate with dispersing sperm

John D. D. Bishop[1]*, Andrew J. Pemberton[1,2] and Leslie R. Noble[2]

[1]*Marine Biological Association of the UK, Citadel Hill, Plymouth, Devon PL1 2PB, UK*
[2]*Department of Zoology, University of Aberdeen, Tillydrone Avenue, Aberdeen AB24 2TZ, UK*

The compound ascidian *Diplosoma listerianum* releases aquatic sperm which are dispersed passively to potential mates as individual gametes prior to storage of sperm, internal fertilization and brooding of embryos. The storage of exogenous sperm enables *D. listerianum* to produce a lengthy series of progeny following a brief period of mating. Molecular paternity analysis following sequential mating of colonies in laboratory culture revealed a consistent pattern with a clear initial bias in paternity towards the first of two acting males. The sites of sperm storage and fertilization and the morphology of the ovary in *D. listerianum* suggest that this bias reflects first-in–first-out use of individual stored gametes. The proportion of second-male paternity subsequently increased with time within the progeny arrays. This may have reflected the ageing or passive loss of first-male sperm. It is also possible that the modular nature of the organism contributed to this temporal trend: any recently budded colony modules maturing in the interval between matings would have been available exclusively to second-male sperm as virgin zooids. Two sets of mating trials were run. In the first, the collection of progeny suffered an interruption of 13 days and each male gained a larger proportion of recorded paternity within the progeny analysed when mating first rather than when mating second. In one mating combination, the first male obtained almost 100% of recorded paternity. In the second set of trials, with different clonal combinations, the complete sequence of progeny was collected and the estimated overall proportion of second-male paternity (P_2) was consistently > 0.5. Taken as a whole, the results suggest that the overall P_2-value can vary widely within the population studied. Proposed mechanisms of mating-order effects in species with copulatory mating include several which can have no counterpart in indirect aquatic mating since they involve the active removal, sealing off, volumetric displacement or incapacitation of first-male ejaculates. It is nevertheless clear that mating-order effects can be pronounced during the type of non-copulatory mating examined here, which is widespread in marine invertebrates.

Keywords: *Diplosoma listerianum*; ascidian; sperm competition; sperm precedence

1. INTRODUCTION

When a female pairs with more than one male during a sufficiently short interval, sperm of different origin will compete within her reproductive tract to fertilize the available ova (Parker 1970). The outcome of this competition is commonly influenced by the order in which the males mate. Such sperm precedence effects have been documented in a wide range of animal taxa in which sperm is transferred directly between mates prior to internal fertilization (Smith 1984; Birkhead & Møller 1998). This process generally involves copulation, with the deposition of spermatozoa internally as part of an ejaculate or in a spermatophore. Mating order may have similar significance when spermatophores are deposited on an external surface to be subsequently transferred to the female tract (Achmann *et al*. 1992; Halliday 1998).

However, not all animals mate by directly transferring numerous spermatozoa to the female in an ejaculate or a spermatophore. Those with external fertilization are not considered here but, in addition, a diverse array of sessile marine invertebrates mate at a distance, by releasing sperm which are transported by water movements to a conspecific individual where they fertilize eggs which have been retained rather than spawned (reviewed by Ryland & Bishop 1993). Although in some cases water-borne sperm are packaged as spermatophores or spermatozeugmata, most species with this pattern of mating release dispersing, unpackaged sperm, which arrive at the female as individual gametes. Insemination by more than one male may be commonplace in this type of mating (Yund & McCartney 1994; Yund 1995; Bishop & Pemberton 1997). However, it cannot be assumed that sperm precedence will occur. Mating-order effects in species with direct pairing may be attributed to mechanisms such as the stratification of successive ejaculates or the sealing off, incapacitation, active removal or volumetric displacement of the first ejaculate during mating by the second male (for example, Parker 1984; Diesel 1990; Harshman & Prout 1994; Birkhead & Parker 1997; Simmons & Siva-Jothy 1998). These processes can have no exact counterpart during indirect aquatic mating, since here no intromittent organ is involved and no ejaculate is transferred. Nevertheless, the possibility of sperm precedence in sessile marine invertebrates is of considerable interest because the pattern of sperm competition in a species may exert a strong influence on its reproductive behaviour, morphology and physiology through sexual selection (Parker 1970; Smith 1984; Birkhead & Møller 1992, 1998).

Here we report on an investigation of sperm precedence in a compound ascidian which mates by transmitting independent spermatozoa between colonies through the intervening seawater prior to true internal

*Author for correspondence (jbis@mba.ac.uk).

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fertilization. Molecular paternity analysis following the sequential mating of colonies in laboratory culture indicates that marked mating-order effects can indeed occur in this context. However, two features of the species complicate the observed pattern. First, the storage of exogenous sperm means that a lengthy succession of progeny can be produced following a brief period of mating; mating advantage does not remain constant between early- and late-produced young. Second, the species buds to produce a modular colony of similar, small individuals (zooids). The paternity of the total progeny brooded by a colony reflects independent events in the separate female reproductive tracts of numerous zooids; these zooids may have differed from one another in developmental phase and, thus, receptivity during the critical periods of sperm availability.

2. MATERIAL AND METHODS

(a) *Biology of* Diplosoma listerianum

A colony of *Diplosoma listerianum* is founded by the settlement and metamorphosis of a motile, sexually produced tadpole larva. Vegetative budding produces a colony consisting of a single layer of zooids, each *ca.* 1.5 mm long, within the outer colony covering or tunic, which is attached to a solid surface. Zooids are monomorphic: all feed and, when mature, have both male and female gonads (for example, Berrill 1950). Sperm are released by individual zooids in discrete emissions of a few thousand gametes, leave the colony via an exhalant opening in the tunic and rapidly disperse (Bishop & Ryland 1991; Bishop 1998).

Released sperm presumably enter another colony with the inhalant current via the branchial basket (the filtering apparatus) of a zooid, although this has yet to be demonstrated. Fertilizing sperm pass up the fertilization duct (oviduct) of a zooid and enter the lumen of the ovary (Burighel *et al.* 1986; Burighel & Martinucci 1994*a*; Bishop & Sommerfeldt 1996). They may then be stored in the ovarian lumen for several weeks prior to fertilization (Bishop & Ryland 1991; Burighel & Martinucci 1994*a*; Bishop & Sommerfeldt 1996; Bishop 1998). Self sperm re-entering the colony of their origin may pass into the fertilization duct of a zooid but are generally blocked and phagocytosed a fraction of the way along the duct (Bishop 1996). Exogenous sperm from some sources may be blocked by the same compatibility mechanism (Bishop 1996). Simultaneous mating with two compatible males can produce broods of mixed paternity (Bishop *et al.* 1996).

Colonies grown in reproductive isolation do not generally produce fully grown oocytes (Ryland & Bishop 1990), although some genotypes habitually or occasionally produce ova in reproductive isolation (Bishop & Ryland 1991). Vitellogenic egg growth is typically triggered by exposure to a compatible colony and the first ovulations take place at least nine days later. A given zooid will then ovulate a single ovum at intervals of seven to 12 days (Ryland & Bishop 1990). Ovulation is not synchronized between zooids. The storage of sperm by each zooid enables outcrossed zygotes to be produced from several successive ovulations over a period of weeks (Bishop & Ryland 1991; Bishop 1998). Sperm are stored in the lumen of blind-ended peduncular extensions of the ovary, which penetrate the follicle cell layers surrounding developing oocytes (Burighel & Martinucci 1994*b*; Bishop & Sommerfeldt 1996). However, sperm do not pass through the ovarian epithelium into the oocyte cytoplasm until around the time of ovulation (Burighel & Martinucci 1994*b*). The ovum/zygote does not enter the oviduct but is deposited directly into the colonial tunic (Burighel & Martinucci 1994*b*) where the embryo develops to an advanced stage before being released as a swimming tadpole larva after *ca.* 13 days (Ryland & Bishop 1990). The delays involved in oocyte growth and subsequent brooding of embryos mean that the release of young typically does not commence until more than three weeks after the exposure of virgin *D. listerianum* to sperm.

Table 1. *Mating sequences of cultured clones of* D. listerianum *with estimates of the overall P_2-values for each mating sequence in experiment 2*

(The values in parentheses exclude the progeny collected in the 16–19 days interval; see the first paragraph of § 3)

acting female	first male (day 0)	second male (day 8)	number of replicates	estimated P_2
experiment 1				
E	F	35	3	—
	35	F	3	—
	F	—	2	—
	35	—	2	—
F	E	36	3	—
	36	E	3	—
	E	—	2	—
	36	—	2	—
experiment 2				
A	E	I	2	0.51
	I	E	2	0.67
	E	—	1	—
	I	—	1	—
F	E	I	2	0.73
	I	E	2	0.82
	E	—	1	—
	I	—	1	—
H	E	I	2	0.73
	I	E	2	0.57
	E	—	1	—
	I	—	1	—
J	E	I	2	0.68 (0.70)
	I	E	2	0.56 (0.60)
	E	—	1	—
	I	—	1	—

When *D. listerianum* is cultured on glass microscope slides (Ryland & Bishop 1990) colonies can be cut up and the pieces (ramets) allowed to re-attach to separate substrates, resulting in the clonal propagation of the genetic individual. In this way a single genotype can be replicated as virgin ramets within and between experimental treatments.

The sequences of matings for investigating sperm precedence are indicated in table 1. Two sets of trials were undertaken, which are referred to below as experiments 1 and 2.

(b) *Experiment 1*

Four laboratory clones of *D. listerianum* designated as E, F, 35 and 36 were used. Clones E and F, the acting females whose progeny were collected and analysed, are fully sexually compatible with one another and were mated reciprocally. As the alternative males, clone 35 was mated with clone E and clone 36 with clone F, an arrangement necessitated by the incompatibility of the reverse pairings. Clones 35 and 36 are full siblings, the laboratory-bred progeny of clones B (acting female) and C

(male). Clones B, C, E and F originated as unrelated larvae from a wild population as detailed by Bishop *et al.* (1996).

Each pairwise mating was initiated by placing two microscope slides bearing the different *D. listerianum* clones, each with virgin ramets totalling 75 ± 10 zooids, in the same 800 ml culture tank, so that the ascidians were exposed to each other's released sperm. Mating was terminated after three days by separating the slides into different tanks containing clean seawater. Second-male mating was initiated five days after the termination (and, thus, eight days after the initiation) of first-male mating. This interval was anticipated to be the maximum delay between males which still allowed second-male sperm to be present during the fertilization of the first oocytes that developed as a result of exposure to the first male. Second-male sperm were thus expected to have at least a theoretical possibility of fathering the earliest progeny.

The progeny were collected as larvae or metamorphs from each slide of clones E and F on six occasions at four-day intervals between days 21 and 44 after the initiation of first-male mating. New young were not collected during days 45–57, but progeny subsequently released during days 58–64 were collected as a single batch from each maternal replicate. Each of the offspring for paternity analysis was preserved individually, frozen (-20°C) in 8 µl of 0.1 M ethylenediaminetetra acetic acid (EDTA) (pH 8).

(c) *Experiment 2*

Clones E and I were used as the acting males, with clones A, F, H and J as the females (table 1). These clones are compatible in all of the combinations adopted. The mating protocols followed those of experiment 1, although larger ramets (*ca.* 200 zooids) of clone H were used because earlier trials had indicated relatively low fecundity in the planned pairings. The progeny were collected without interruption at four-day intervals between days 16 and 67 after the initiation of first-male mating and preserved as in experiment 1.

(d) *Paternity analysis*

The progeny were subjected to the same protocols for randomly amplified polymorphic DNA (RAPD) analysis as described by Bishop *et al.* (1996), except that the DNA extraction in experiment 2 was by Chelex resin, following Sommerfeldt & Bishop (1999), rather than phenol–chloroform. The inheritance of all markers was elucidated in the progeny from single-male matings and additional progeny from single-male matings were included in polymerase chain reaction runs from which paternity data were collected, in order to confirm the behaviour of marker bands. The RAPDs protocol was found to give repeatable results as in previous studies (for example, Bishop *et al.* 1996; Sommerfeldt & Bishop 1999).

The paternity markers used in experiment 1 were as follows: primer OPR-13, a 500 bp amplification fragment (also used by Bishop 1998) which is homozygous in clone E, but absent in clones F, 35 and 36; and primer OPY-04, a 560 bp fragment which is homozygous in clone F but absent in clones E, 35 and 36. Where available, six progeny were analysed from each four-day collection interval for each of the maternal replicates of clones E and F involved in two-male mating sequences.

The paternity markers used in experiment 2 depended on the maternal clone involved. For clone F as the acting female, the OPR-13 500 bp fragment, which is homozygous in clone E but absent in F and I, was used as in experiment 1. This band was heterozygous in clone H; with clone H as the female, the *absence* of the OPR-13 500 bp band therefore characterized an expected proportion of 0.5 of clone I progeny. Two additional bands (905 and 915 bp) from primer OPR-13 were heterozygous in clone E but absent in both clones I and H. Together these gave a probability of 0.75 of detecting clone E paternity from the presence of one or both bands. For clones A and J as the brood parent, primer OPY-14 yielded a 970 bp fragment which is heterozygous in clone E and a slightly $>$ 1500 bp fragment which is heterozygous in clone I, both of which were absent in the opposing sperm source and in the maternal clones and, therefore, characterized an expected proportion of 0.5 of the progeny sired by the respective sperm sources. Where available, six progeny per collection interval per replicate ramet were analysed; on a few occasions slightly more than six were analysed.

As expected, for the broods of clones A, H and J, in which heterozygous markers were used, a substantial number of the progeny analysed lacked a diagnostic RAPD fragment and could not be attributed to either father; they are thus not plotted in figure 3. For clone H broods, the paternity attributed to clone E (probability of detection 0.75) was reduced by one-third to make it comparable with the recorded clone I paternity (probability of detection 0.5). The probability of detecting the paternity of clones E and I was equal within broods of clones A (both probabilities = 0.5), F (1.0) and J (0.5) and the raw counts were thus used.

To estimate the P_2-values (the proportion of paternity attributed to the second male) for the entire progeny arrays in experiment 2, the total progeny counts during each collection interval were multiplied by the proportion of paternity attributable to the first and second males in the progeny analysed from that interval and the results summed over the intervals. For clone H broods, the paternity attributed to clone E was reduced by one-third as above.

3. RESULTS

The release of progeny generally commenced during or after the collection interval 21–24 days (experiment 1) or 20–23 days (experiment 2; figure 1) since the initial exposure to first-male sperm, implying that second-male sperm would typically have arrived in the female tract before the first fertilizations occurred. However, in experiment 2 clone J already had a number of vitellogenic oocytes at the time of first exposure and subsequently released larvae earlier, between days 16 and 19 (figure 1). It is expected that the earliest progeny in these arrays would have been fertilized prior to the arrival of second-male sperm in the female tract, leading to the calculation of alternative P_2-values in table 1.

The paternity inferred from the RAPD analysis in experiment 1 (figure 2) showed a marked bias in favour of the first male during the early collection intervals. In general, this bias was subsequently eroded, with the second male achieving an increasing proportion of fertilizations between days 21 and 44. The second male dominated paternity in the final (days 58–64) collection in three out of four mating sequences. The exception was with clone E as the acting female and clone F as the first male; here, the second male (clone 35) was unrepresented in the 12 progeny analysed from days 58–64. In this regard, it is also notable that, in the reverse mating order, clone F as the second male achieved unusually good early representation in the progeny from days 21–44.

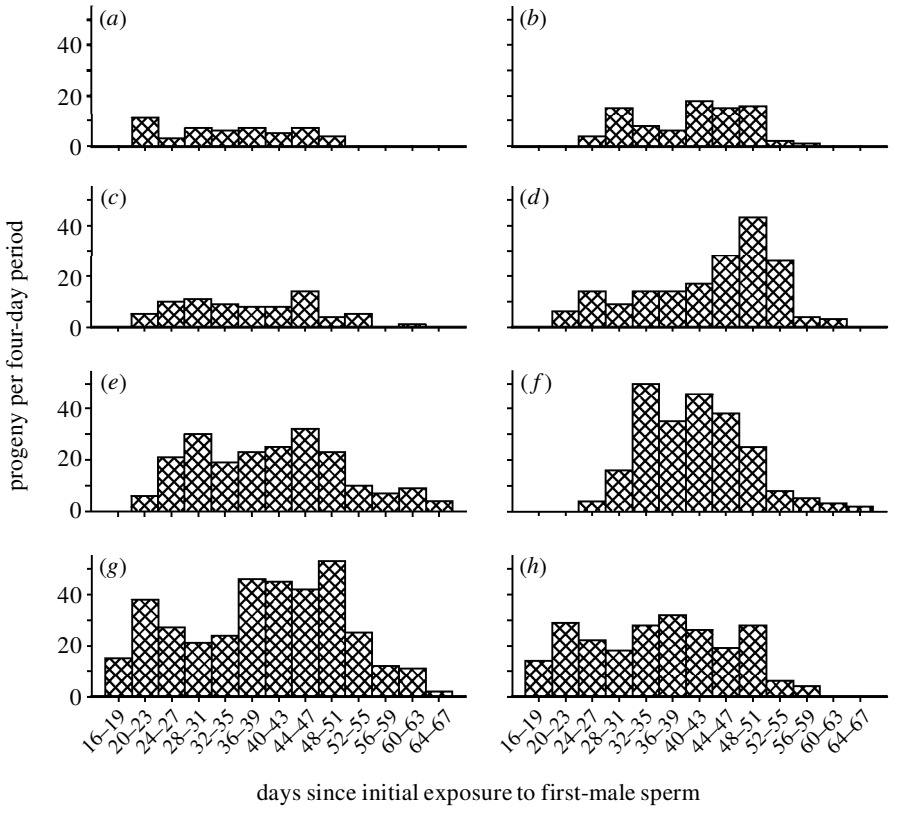

Figure 1. *Diplosoma listerianum*: the number of larvae released per four-day collection interval after sequential mating with two acting males in experiment 2. Each histogram shows the combined data for two replicate maternal ramets. Male E then male I with (*a*) female A, (*c*) female F, (*e*) female H and (*g*) female J. Male I then male E with (*b*) female A, (*d*) female F, (*f*) female H and (*h*) female J.

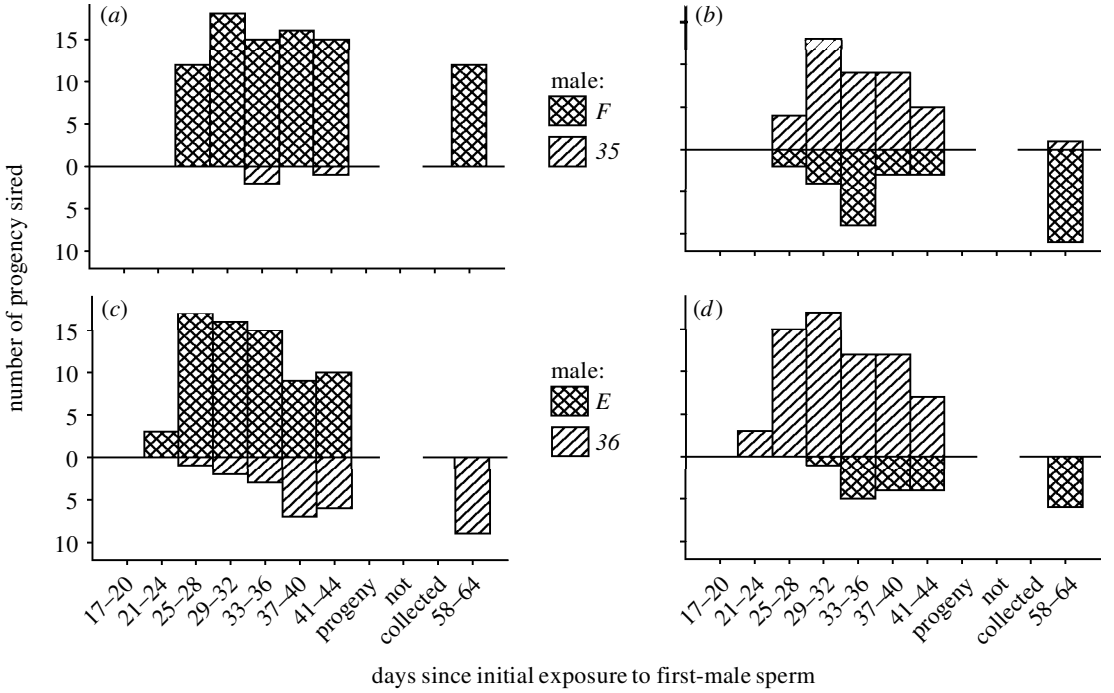

Figure 2. Experiment 1: the paternity of the progeny released after mating with two acting males at an interval of eight days shown relative to the time since initial exposure to first-male sperm. First-male paternity is shown above the line and second-male paternity is shown below the line. Progeny released between days 45 and 57 were not collected. Note that the final collection was over a seven-day interval, which is longer than the earlier collection intervals (four days). (*a*,*b*) Female E and (*c*,*d*) female F.

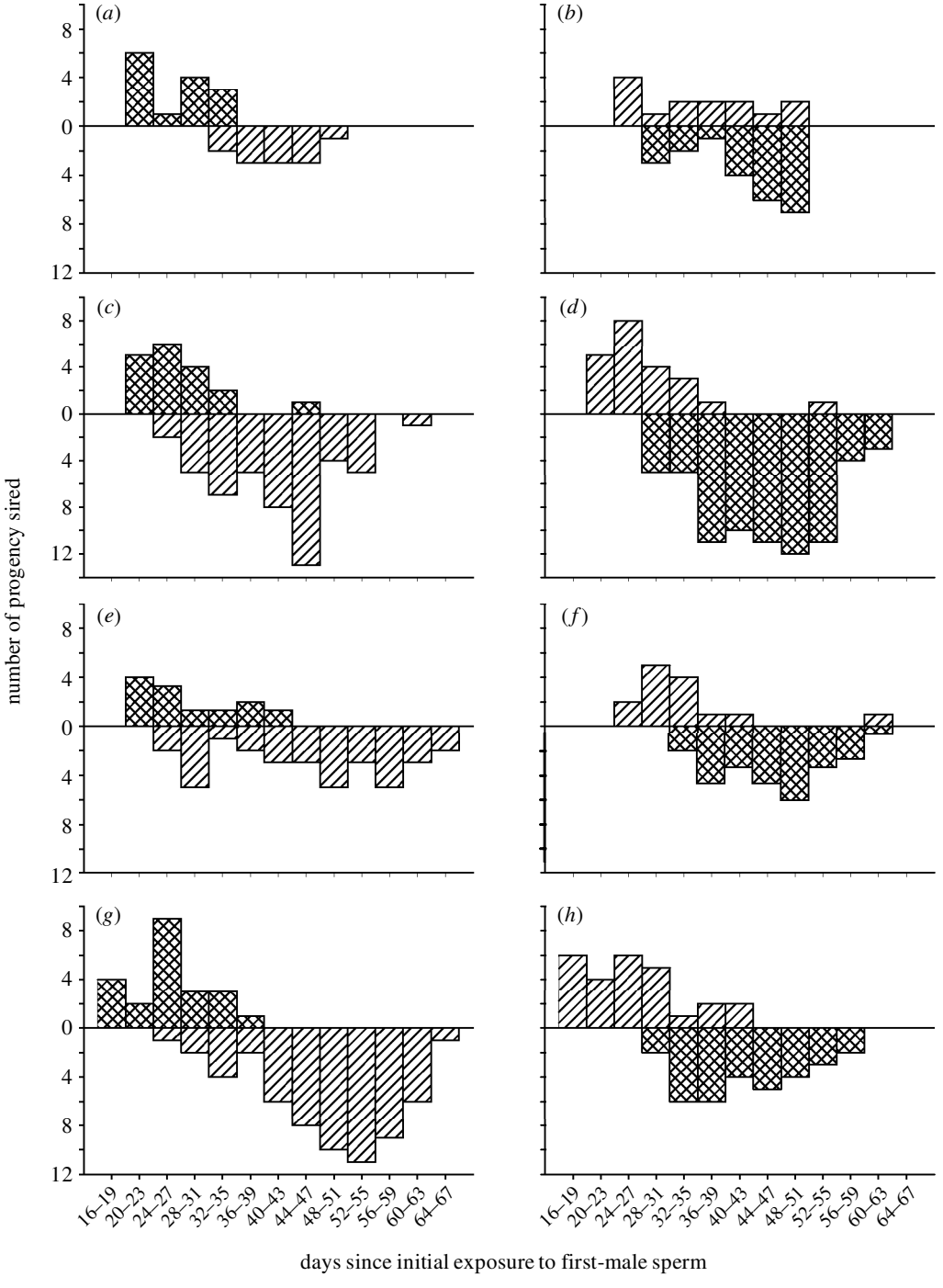

Figure 3. Experiment 2: the paternity of the progeny released after mating with two acting males at an interval of eight days shown relative to the time since initial exposure to first-male sperm. First-male paternity is shown above the line and second-male paternity is shown below the line. Clone E paternity double cross-hatching and clone I paternity single cross-hatching. (*a–h*) Same scheme as in figure 1.

All matings in experiment 2 showed an initial first-male advantage, which was replaced in later progeny by a preponderance of second-male paternity (figure 3). The estimates of the overall P_2-values for the entire progeny arrays were consistently in excess of 0.5 (table 1).

4. DISCUSSION

Although many of the mechanisms suggested to underlie sperm precedence in animals with copulatory sperm transfer involve intromission or interactions between ejaculates of competing males, the present results indicate that strong mating-order effects can exist in animals where sperm arrive individually at the female. In experiment 1 a pattern of first-male priority in the early progeny of *D. listerianum* gradually giving way to second-male advantage for later young was clear in matings with clone F as the acting female and clones E and 36 as competing sperm sources, and in these the ratios of first- and second-male paternity appeared very

similar between the reciprocal mating orders. However, the fairly high rates of larval release in the 58–64 days period in these matings suggest that a substantial number of progeny were missed during the preceding gap in collection. In experiment 1 matings with clone E as the acting female, clone F achieved a greater proportion of fertilizations than clone 35 in the respective male roles, most notably almost excluding clone 35 from the recorded paternity when clone F was the first male. Even with the gap in the collection of progeny between days 44 and 58, it appears that, with clone F as the first male, the overall P_2-value must have been considerably less than 0.5. The results suggest that clone F has a greater ability than clone 35 for obtaining fertilizations when sperm from both sources co-occur, at least with clone E as the acting female. This may partly reflect different levels of sperm production, although the three-day matings employed here should ensure the release and uptake of abundant sperm from each source and single-male matings with clone 35 produced numerous progeny (the total progeny per maternal replicate between days 21 and 44 did not differ significantly between singly and doubly mated ramets: Mann–Whitney U-test, $p = 0.392$ for clone E as the female and $p = 0.670$ for clone F as the female). Further experiments with quantified allocations of sperm from the competing sources would be desirable.

The succession of paternity between first and second males was consistent in experiment 2 and matched that seen in three of the four mating combinations in experiment 1: the initial first-male paternity was replaced in later progeny by a preponderance of second-male paternity (figure 3). The overall P_2-values calculated for the entire progeny arrays (table 1) would be associated with large (but undefined) error terms, but consistently exceeded 0.5, suggesting overall second-male advantage, constant across females, in this mating protocol with clones E and I as the acting males.

Although the process of indirect mating in animals such as *D. listerianum* has many parallels with that of flowering plants, it does not seem appropriate to ascribe the initial first-male advantage in the ascidian to a race to penetrate the eggs analogous to a pollen-tube race in angiosperms. This is because compatible sperm move through the oviduct in a matter of hours but are subsequently stored in the ovary, potentially for weeks, prior to penetration of an ovum around the time of ovulation (Bishop & Ryland 1991; Burighel & Martinucci 1994*a*; Bishop & Sommerfeldt 1996; Bishop 1998); sperm arriving over a relatively long period might therefore compete for fertilizations. In the present experiments, egg penetration leading to the earliest progeny would typically have occurred in the presence of sperm from both sources. Given the storage of sperm in ovarian diverticula leading to oocytes (Burighel & Martinucci 1994*b*; Bishop & Sommerfeldt 1996), a more appropriate model for the pattern of initial mating advantage in *D. listerianum* may be sperm queuing: it is conceivable that the first sperm to enter a diverticulum occupy the most favourable storage sites (nearest to the oocyte?) for the subsequent penetration and fertilization of the grown ovum. This would be analogous to a first-in–first-out stratification during conduit-type storage of ejaculates or spermatophore contents, which is suggested to predispose some copulating animal species to first-male precedence (Austad 1984; Elgar 1998). In *D. listerianum* the relative positions of individual sperm rather than of fluid masses would be involved. The pattern of uptake of individual sperm and their movement to and penetration of oocytes/ova in other aquatic suspension-feeding taxa (Bryozoa and many sponges) (Fell 1989; Temkin 1996; reviewed by Ryland & Bishop 1993) suggests that first-male advantage, at least in early progeny, may be general in sessile marine invertebrates with dispersing sperm and fertilization of retained eggs. This contrasts with the general prevalence of second-male advantage in taxa with copulatory mating, although considerable inter- and intraspecific variation in P_2-values in this better-studied mating process has been documented (Smith 1984; Birkhead & Møller 1992, 1998).

The subsequent increase in the P_2-value in the later progeny of *D. listerianum* might reflect the depletion of first-male sperm through fertilizations, ageing or passive loss. The phagocytosis of sperm in the ovarian lumen reported by Burighel & Martinucci (1994*a*) apparently represents the demise of stored sperm, potentially clearing the way for later gametes. Changing values of P_2 with the time since last mating have also been reported in various copulatory insects (for example, Siva-Jothy & Tsubaki 1989; Eady 1994; Cooper *et al*. 1996; Lewis & Jutkiewicz 1998). In most cases this involved P_2 declining from high early values, a trend ascribed to the progressive mixing of ejaculates or depletion of second-male sperm after initial stratification favouring the second male. However, Eady (1994) reported a slight but significant increase in P_2 in a beetle species with overall second-male precedence. He suggested that this increase might be attributable to a pattern of accelerating loss of competitiveness during the ageing of stored sperm, giving younger (second-male) sperm an increasing advantage over time. A similar mechanism could contribute to the temporal trend seen in most *D. listerianum* mating sequences, although the prevalence of first-male clone F paternity even in the progeny from days 58–64 (figure 2) indicates that this process is not inevitable. Loss of sperm competitiveness during ageing is an attractive explanation for the overall second-male advantage recorded in experiment 2: first-male sperm would be in competition with younger (second-male) sperm, potentially curtailing first-male paternity, while second-male sperm would encounter no younger competitors as they aged, allowing continued fertilizations. (However, a separate explanation, as discussed above, is needed for the initial advantage of the first male in early fertilizations.) The sperm ageing mechanism would be predicted to produce a general last-male advantage in sequences of matings involving more than two males; this advantage would be expected to decline with decreasing mating intervals.

Additional factors may be suggested to contribute to an increasing P_2-value with the time since last mating in *D. listerianum*. If stored sperm are not mobile within the ovary, an increase in second-male paternity could arise if some young oocytes at the end of new ovarian diverticula become available for the first time only during the interval between matings. However, the dynamics of oocyte development and cycling within the ovary are not sufficiently

understood in this species to assess this idea critically. Another possibility stems from the modular, colonial nature of the study organism: depending on their maturity or the phase of their budding cycle, the zooids may have differed in receptivity during the successive periods of sperm availability. For instance, a small proportion of zooids might have been receptive to sperm only from the second male because they were still immature during first-male mating. The eight-day delay between mating periods would ensure that the progeny of these zooids would be released correspondingly later in the collection sequence. Such fertilizations by the second male in newly mature zooids would not involve direct competition with first-male sperm within the female tracts concerned.

It is thus apparent that sperm precedence has a role in the poorly understood process of indirect mating in sessile marine invertebrates, as well as in the more familiar procedure of direct sperm transfer. The modular construction of colonial forms and the attendant complexities of zooidal demography represent a potential influence on the pattern of realized paternity unmatched in unitary species.

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