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Efficiency of gamete usage in nature: sperm storage, fertilization and polyspermy

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Gamete production for both males and females can be energetically expensive such that selection should maximize fertilization opportunities while minimizing fertilization costs. In laboratory studies of *Drosophila* reproduction, however, the failure of eggs to yield adult progeny can be quite high, suggesting that female control over gamete utilization is surprisingly inefficient. We examined gamete utilization in *D. pseudoobscura* from nature and compared our observations to those for laboratory populations. In natural populations 100% of oviposited eggs effectively produce adult progeny, and fertilization is exclusively monospermic, indicating that in nature, *D. pseudoobscura* females maintain a very strict control over their reproduction such that gamete usage is extremely efficient. The potential reasons for the inefficient gamete utilization in the laboratory, as well as the potential impact on laboratory studies of sperm competition, sexual conflict, and the evolution of reproductive barriers are discussed. Furthermore, in this sperm-heteromorphic species, our observations show definitively that in nature, as well as in the laboratory, only the long sperm morph participates in fertilization.

Keywords: *Drosophila*; sperm heteromorphism; polyspermy; gamete utilization; sexual conflict

1. INTRODUCTION

Sperm storage and use in insects have recently received considerable attention as critical processes influencing the reproductive success of both sexes. Processes occurring within the female reproductive tract have been implicated in intraspecific sexual conflict and sperm competition (Eberhard 1996; Rice 1996, 1998; Price *et al.* 1999; Knowles & Markow 2001), as well as in reproductive isolation (Hewitt *et al.* 1989; Howard & Gregory 1993; Wade *et al.* 1994; Markow 1997; Price *et al.* 2001). Little is known about the nature of reproductive tract processes that control sperm utilization and how these processes interact to mediate both male and female reproductive success. It is clear, however, that gamete production can represent a considerable energetic investment for both females and males. For example, in *Drosophila*, female longevity can decrease due to the cost of producing eggs (Partridge *et al.* 1987*a*). Sperm production also is costly (Pitnick 1993, 1996; Pitnick & Markow 1994*a*,*b*), may decrease male longevity (Prowse & Partridge 1997), and can result in conditions in which females are sperm limited (Pitnick 1993). Given the costs to both males and females, mechanisms preventing gamete wastage, i.e. injudicious release of gametes, are expected to be under strong selection.

In laboratory populations of various *Drosophila* species, however, considerable gamete wastage occurs. The proportion of eggs laid by inseminated females that fail to hatch ranges from 1% to greater than 50% in *D. melanogaster* (Robertson & Sang 1944*a*,*b*; McMillan 1969; Trevitt *et al.* 1988; Armstrong & Bass 1989) to over 90% in *D. pachea* (Pitnick 1993). In some members of the *obscura* group, productivity can be as little as half that of fecundity. For example, 50% of eggs oviposited by *D. persimilis* and *D. affinis* (Snook 1998) and 25% of eggs in *D. pseudoobscura* (Turner & Anderson 1983; Snook 1998) fail to produce progeny. Examination of oviposited eggs reveals that, in some *obscura* group species, a large proportion of eggs are unfertilized (Snook & Karr 1998). For example, the percentage of unfertilized eggs found in six *obscura* group species ranged from 47% in *D. persimilis* (similar to the percentage of eggs that fail to produce adult progeny; Snook (1998)), to 10% in *D. pseudoobscura* (Snook & Karr 1998). Thus, *obscura* group females may experience a high level of gamete wastage due to the lack of fertilization and/or developmental mortality.

Polyspermy, the fertilization of an egg by more than one sperm, may represent another source of gamete wastage. In many taxa, such as mammals and ascidians (Hunter 1976; Lambert 2000), when polyspermy is observed it is associated with developmental failure (Gilbert 1997) and thus represents a form of gamete wastage; polyspermic eggs die at the same time that excess sperm are utilized. Polyspermy may also exacerbate conditions of sperm limitation. Given these costs, the female reproductive tract may have evolved many mechanisms for avoiding polyspermy (Hunter 1996; Suarez 2001). Recent literature on the evolution of polyspermy avoidance has suggested that selection for avoiding polyspermy results from a sexual conflict between male ejaculates and the female reproductive tract over the control of fertilization (Rice 1996, 1998; Howard *et al.* 1998; Frank 2000).

Blocks to polyspermy and whether polyspermy is detrimental have yet to be identified in *Drosophila* probably due to the rarity (less than 1% of fertilized eggs in *D. melanogaster*; Callaini & Riparbelli (1996)). In some *obscura* group species, however, a large proportion of eggs may be fertilized by more than one sperm despite similar fertilization constraints on the potential for polyspermy (Snook & Karr 1998). From laboratory females, 11% of

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D. subobscura and 5% of *D. pseudoobscura* eggs are polyspermic (R. R. Snook, unpublished data). Whereas polyspermy in this group, when observed, is typically dispermic, some eggs may contain as many as five sperm (Snook & Karr 1998). Embryos with multiple sperm can at least complete gastrulation (Snook & Karr 1998), raising questions as to whether polyspermy in the *obscura* group, and indeed other *Drosophila* species, is rare and costly.

Polyspermy in species of the *obscura* group may contribute to sperm limitation due to the unusual sperm production strategy employed by this group. Males exhibit sperm heteromorphism, or the simultaneous production within males of two types of sperm, long and short (Beatty & Sidhu 1970; Snook 1997). In the laboratory, *obscura* group males produce and transfer at least 50% short sperm within an ejaculate (Snook & Markow 2001). Although females store both sperm types, at least transiently (Beatty & Sidhu 1970; Snook *et al.* 1994; Bressac & Hauschteck-Jungen 1996; Snook & Markow 2001), only the long sperm morph has been observed to fertilize eggs in laboratory-reared flies (Snook *et al.* 1994; Snook & Karr 1998) and be stored by females for extended periods after mating (Snook & Markow 2001). Thus, given that at least 50% of gametes do not function in fertilization, selection should favour judicious use of the long, fertilizing sperm. However, the gamete wastage described above suggests that in the laboratory, at least, the expected efficiency of gamete utilization is not observed.

Attempts to understand the evolutionary significance of sperm heteromorphism through laboratory investigations (Snook & Markow 1996; Snook 1997, 1998) assume that observed sperm transfer, storage, and use reflects the processes found in natural populations. In those few cases where reproductive biology of wild and laboratory *Drosophila* have been compared, notable differences were observed (Partridge *et al.* 1987*b*; Markow 1988; Gromko & Markow 1993; Markow 2000). For example, copulation duration is 2–3 times longer in field populations of *D. melanogaster* and *D. simulans* compared with laboratory experiments (Gromko & Markow 1993). Also, force-mated teneral females produce progeny in 24% of laboratory matings, but in 65% of field matings (Markow 2000). Because teneral females cannot avoid these copulations, selection on post-copulatory mechanisms of fertilization control may be greater than originally assumed from laboratory studies (Rice 1996; Holland & Rice 1999).

The excessive and unpredicted gamete wastage seen in laboratory *D. pseudoobscura*, along with the inconsistencies reported between laboratory and field studies and the importance of gamete interactions for sexual selection and reproductive isolation led us to examine gamete wastage in wild *D. pseudoobscura*. We first compared levels of gamete wastage measured by unfertilized and polyspermic eggs oviposited by wild-caught females. Second, we compared the pattern of transfer and utilization of the long and short sperm morphs in wild and laboratory *D. pseudoobscura*.

2. MATERIAL AND METHODS

(a) *Laboratory and natural populations*

A laboratory culture of *D. pseudoobscura*, established from a multifemale line from Tempe, Arizona in 1990 and 1991, was mass reared on standard cornmeal–molasses–agar medium. Virgin males and females were separated at eclosion and stored separately in 8 dram culture vials, 10 flies per vial, until 5 days of age when they were mated for inclusion in different experiments. Fecundity and productivity data were gathered in 1991 and sperm production, transfer and storage data were collected during 1992.

Wild populations of *D. pseudoobscura* were aspirated from rotting fruit in Tempe, Arizona in 1998. Flies were directly transferred individually into culture vials and driven to the laboratory, 15 min away. Wild-caught individuals were used immediately in the different experiments outlined below.

(b) *Fertilization and sperm egg interactions*

Wild-caught females ($n = 25$) were immediately placed on egg-laying plates containing a small amount of yeast paste. Eggs were collected every 12 h for 2 days, fixed and processed for analysis. Eggs were examined to determine the proportion of fertilized eggs, what sperm type fertilized the eggs, and whether the eggs were polyspermic using the methods described in Snook & Karr (1998).

(c) *Egg and progeny production*

To assess whether field-caught females exhibit the same level of gamete wastage as laboratory females, wild-caught females were brought back to the laboratory, placed in yeasted food vials and measured for fecundity and productivity. Females were transferred to new vials every day for four consecutive days. After flies were transferred to new vials, we immediately counted the number of eggs oviposited in the prior vial and saved all vials for subsequent adult progeny counts to determine the total number of eggs and offspring produced.

Fecundity and productivity in either singly or multiply mated laboratory-reared females were also determined. For singly mated females, we placed one virgin female in a yeasted food vial with two virgin mature males. Once copulation began, the non-copulating male was aspirated from the vial and, after copulation, the mating male was also removed. The same design was employed for multiply mated females, except that females were given twice daily opportunities to remate, 2 h in the morning and 2 h in the afternoon (periodic interaction design; Pyle & Gromko (1978)), for five consecutive days. In both singly and multiply mated treatments, flies were transferred to new vials and the number of eggs oviposited in the prior vial was immediately counted. All vials were saved for subsequent adult progeny counts. We performed two replicates for each treatment and utilized *t*-tests to determine if replicates were homogeneous. Data for individual females were discarded if females in the multiple mating group did not remate or if females in either treatment failed to produce progeny, since copulations were observed and timed.

To accurately compare fecundity and productivity between wild-caught and laboratory-reared females we performed the following analysis. Data from laboratory-reared females were measured for life, and mating schedules were controlled. Clearly this is not the case for wild-caught females. Thus, data used for singly mated laboratory females were the cumulative number of eggs oviposited and the subsequent progeny produced for the fourth to eighth consecutive days after females were mated. Data used for multiply mated laboratory females were the same as singly mated females except that the fourth to eighth days were those after the *last* remating in multiply mated females. These times were chosen in laboratory females based on what we observed in dissections of wild-caught females and on sperm

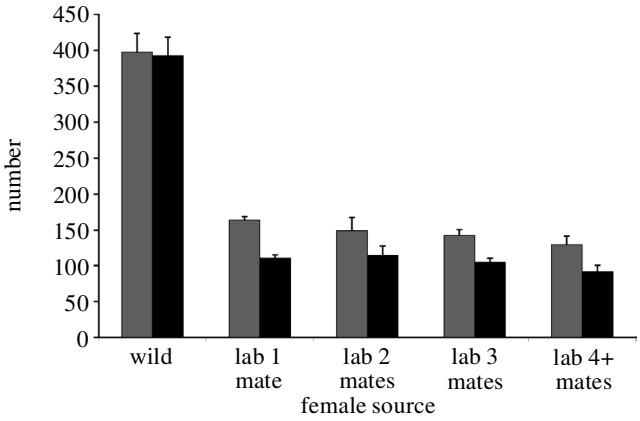

Figure 1. The mean number of eggs and progeny (+ s.e.) produced by wild-caught (wild) females compared with laboratory females having one, two, three, or four or more mates. The mean progeny-to-egg ratio + s.e. are: wild = 98.7 + 0.003; lab 1 = 69.0 + 0.02; lab 2 = 78.0 + 0.03; lab 3 = 74.0 + 0.03; lab 4+ = 71.0 + 0.05. Grey bars, eggs; black bars, progeny.

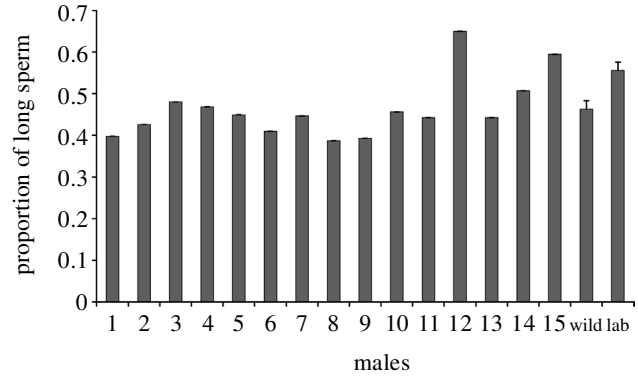

Figure 2. The proportion of long sperm transferred to virgin laboratory females by 15 wild-caught males, the mean proportion + s.e. of wild-caught males (wild) compared with the mean proportion + s.e. from laboratory males (lab).

movement, remating and oviposition in laboratory-based females. The majority of wild-caught females did not appear to have recently mated (most uteri were devoid of sperm; see § 3b and figure 3*a*) and after mating in the laboratory females appear to require 24–48 h before oviposition begins (Snook 1998) and typically do not remate until oviposition starts.

(d) *Determination of sperm transfer and sperm storage*

Individual field-caught males were transferred directly to vials, each with a 5 day old virgin laboratory-raised female. Following the end of copulation, the female reproductive tract was dissected to determine the number of each sperm type transferred by field-caught males as previously described for laboratory males (Snook *et al.* 1994). These values were compared with those obtained from laboratory-reared males that had been treated similarly (reported in Snook *et al.* (1994)).

Following collection, wild-caught females were immediately dissected in the laboratory to determine the number and proportion of sperm types stored in the different sperm storage organs, using the methods previously described for laboratory-reared females (Snook *et al.* 1994). Values from wild-caught females were compared with those obtained from laboratory-reared females that had been mated 48 h earlier (Snook *et al.* 1994). This time was chosen for comparison because: (i) only 1 out of 11 wild-caught females appeared to have mated recently (see figure 3*a*) and (ii) laboratory-reared females begin oviposition *ca*. 24–48 h following mating.

3. RESULTS

(a) *Fertilization and progeny production*

We examined 161 eggs oviposited by wild-caught females. One hundred per cent of those eggs were fertilized, compared to 91% of eggs laid by laboratory-reared females (Snook & Karr 1998). Only one of the 161 eggs, less than 1% of the total number examined, was polyspermic (dispermic) in comparison with an average of 5% in previous laboratory studies (Snook & Karr 1998; R. R. Snook & T. L. Karr, unpublished data). Furthermore, of the 161 eggs, all were fertilized by only the long sperm morph.

The developmental success of eggs of wild-caught females is nearly 100% (figure 1), consistent with the observation that 100% of eggs from field-caught females contain sperm. We have no knowledge of the number of times the wild-caught females had mated but previous studies indicate that females in nature carry sperm from up to four males (Anderson 1974; Cobbs 1977). Thus, we compared progeny-to-egg ratios of wild-caught females (that could have from one to at least four mates; $n = 16$) with laboratory-raised females that had either one ($n = 52$), two ($n = 9$), three ($n = 27$), or four plus ($n = 13$) mates (figure 1). Wild-caught females oviposited significantly more eggs across the same time than laboratory-raised females mated with one to four mates (figure 1; $F = 74.86$, d.f. = 4,112, $p < 0.0001$). Females from natural populations also produced significantly more progeny than laboratory females of any mating treatment (figure 1; $F = 119.95$, d.f. = 4,112, $p < 0.0001$). Not only do laboratory-reared females lay fewer eggs, but up to 25% of their eggs do not produce progeny (figure 1).

(b) *Sperm transfer and storage*

The observation that only long sperm fertilize eggs raises the possibility that wild males transfer a higher proportion of long sperm, explaining the higher fertilization/developmental success of eggs from wild-caught females. Contrary to our prediction, wild-caught ($n = 15$) males transferred a significantly lower proportion of long sperm to virgin females compared with laboratory-reared ($n = 12$) males (figure 2; $t = 2.727$, d.f. = 25, $p = 0.012$; all proportion analyses were arcsine transformed for analysis but are graphed normally). Out of 15 wild-caught males, 13 transferred a lower proportion of long fertilizing sperm than the mean proportion transferred by laboratory-reared males (figure 2).

We also compared laboratory-reared and wild-caught females for both the number and proportion of each sperm type in three locations of the female reproductive tract (the uterus and the two sperm storage organ types, the ventral receptacle and paired spermathecae; figure 3). Three females had large sperm masses in the uterus, only one female appeared to have recently mated with no sub-

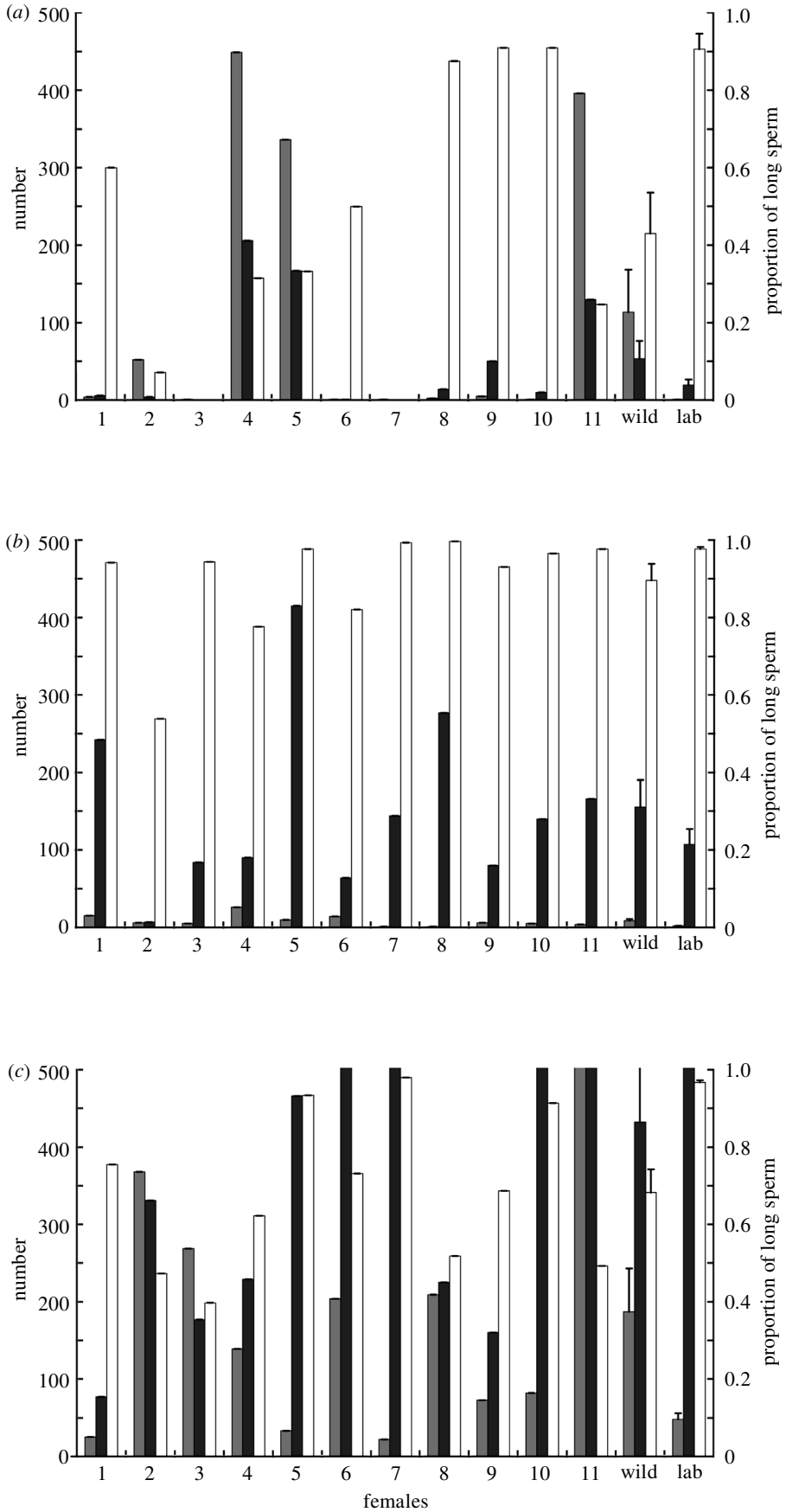

Figure 3. The number of short and long sperm (left ordinate) and the proportion of long sperm (right ordinate) found in the uterus (*a*), ventral receptacle (*b*), and spermathecae (*c*) of 11 wild-caught females. The mean + s.e. of wild-caught females is compared with the mean + s.e. of laboratory females. Grey bars, short sperm; black bars, long sperm; white bars, proportion long sperm.

sequent oviposition (female 5) based on sperm movement within the uterus. Wild-caught females exhibited considerable variation in the total number of each sperm type found in the uterus (figure 3*a*); however, they had a lower proportion of long sperm, thus, a significantly higher proportion of short sperm compared with laboratory-reared females ($t = 4.537$, d.f. = 20, $p < 0.001$). The lower proportion of long sperm found in the uteri of field-caught females (figure 3*a*) probably reflects the proportions of the two sperm types that wild-caught males transfer to females (figure 2) and perhaps less wastage of fertilizing sperm. The uterus is not a sperm storage organ and the number and type of sperm present in this organ reflects either what males have recently transferred to females, or what is being released from the sperm storage organs during oviposition. The proportion of long sperm in the ventral receptacle of wild-caught females was significantly lower than that of laboratory-raised females (figure 3*b*; $t = 2.2563$, d.f. = 24, $p = 0.02$). The number of short sperm ($t = 3.357$, d.f. = 24, $p = 0.003$) but not long sperm ($t = 1.271$, d.f. = 24, $p = 0.216$) was greater, however, in wild-caught females (figure 3*b*). Differences also existed in the proportion of long sperm and total numbers of short and long sperm in the spermathecae (figure 3*c*). Wild-caught females had a lower proportion of long sperm ($t = 6.140$, d.f. = 24, $p < 0.001$), and both fewer long ($t = 7.332$, d.f. = 24, $p < 0.001$) and short ($t = 2.875$, d.f. = 24, $p = 0.008$) sperm in the spermathecae (figure 3*c*).

4. DISCUSSION

Gamete use in natural *D. pseudoobscura* populations is clearly more efficient than in the laboratory. In contrast to observations on laboratory-raised flies (Snook & Karr 1998; R. R. Snook, unpublished data), 100% of eggs oviposited by wild-caught females are effectively fertilized and the frequency of polyspermy is nearly zero. Additionally, wild-caught females produce more eggs and have greater productivity compared with laboratory-raised females of various mating histories mimicking those of wild-caught females. Despite receiving and storing more short sperm relative to laboratory females, wild females exclusively used only long sperm to fertilize eggs. Laboratory-raised females have very few short sperm in storage 48 h after mating (Snook *et al.* 1994; Snook 1998). In the current study, wild-caught females have more short sperm but they are still not used in fertilization and presumably would disappear from storage as in laboratory-raised females (Snook & Markow 2001). Moreover, the fact that only long sperm have been found in polyspermic eggs in both laboratory and natural conditions emphasizes the fact that short sperm do not participate in fertilization.

The level of gamete wastage in laboratory-reared compared with wild-caught flies can be attributed to several potential causes: the oviposition of unfertilized and polyspermic eggs and reduced larval survival. We did not measure larval survival but, even if there was an effect, it would be minor. Our findings show that, irrespective of any differences in larval survival, wild-caught females oviposit significantly more eggs, of which more are fertilized and fertilized by a single sperm, and thus more progeny are produced relative to laboratory-raised females.

We hypothesize that two dietary factors may play a role in the observed differences in gamete wastage. First, the laboratory diet may be lacking a critical element, the absence of which compromises the capacity for normal fertilization and/or subsequent development of eggs produced under laboratory conditions. Second, laboratory females are typically stored in vials seeded with live yeast for several days prior to their being mated. A superabundance of dietary yeast results in the accumulation of a large number of mature oocytes by the time these females are mated (Soller *et al.* 1999; R. R. Snook, personal observation). Thus, after mating, when many mature oocytes are present, eggs may be released from the ovaries at a rate too rapid for normal female control over fertilization.

Oocyte senescence in laboratory-raised flies, perhaps not experienced by wild-caught females, may also contribute to the observed fecundity and productivity differences. Because virgin laboratory females typically have accumulated a large number of mature oocytes by the time they mate, some of their oocytes may have undergone some degree of senescence, potentially enhancing the probability of fertilization failure, polyspermy, or abnormal development. This explanation presumes that oocytes of wild-caught females rarely undergo senescence as a result, either of 'overproduction' of mature oocytes due to abundant food resources, or 'storage' of mature eggs as a result of limited mating and/or oviposition opportunities. However, the degree to which these factors occur in the wild is unclear for most *Drosophila* species. If females of some species experience, at certain spatial or temporal scales, an excess of dietary resources compared with oviposition sites, then the opportunity for oocyte senescence would be increased. Conversely, if nutrients were limited compared with oviposition sites, oocyte overproduction and the probability of a mature egg undergoing senescence would be less likely. Egg senescence (Liu & Keefe 2000), if it occurs in *Drosophila*, will probably only be found in stage 10–14 eggs since, at least in *D. melanogaster*, eggs may be resorbed only until they enter stage 10 (Soller *et al.* 1999). These hypotheses can be tested by employing examination of apoptotic mature eggs (Liu & Keefe 2000).

Other factors could have differed between our wild-caught and laboratory-raised populations that contributed to the low fecundity and productivity seen in the laboratory flies. These include: (i) genetic differences between the laboratory and wild populations, (ii) efficient sperm use may not have been selected for in laboratory populations because females live a long time, or (iii) pleiotropic side effects of inadvertent selection for early reproduction in laboratory flies. We argue that these are unlikely to explain the observed differences. First, the progeny-to-egg ratio reported in two studies of *D. pseudoobscura* using different strains that had been in the laboratory at different times, and thus were probably genetically different from each other, had similar ratios (Turner & Anderson 1993; Snook 1998). Second, previous work in other species demonstrates that multiple mating by laboratory females is costly (Chapman *et al.* 1995) so selection in the laboratory should still function to reduce gamete wastage. Third, while the ages of the wild-caught individuals were unknown, they were in excellent condition and the laboratory females we used were 5–15 days old and we would therefore not expect the pleiotropic effects of intense

selection for early reproduction in laboratory flies to be manifested at this age.

Wild-caught *D. pseudoobscura* males produce more short sperm than laboratory-raised males. The difference between wild and laboratory males in the ratio of sperm produced may indicate that there is an energetic cost to producing long sperm (Pitnick *et al.* 1995) in the wild not seen in the laboratory. One caveat is that data from laboratory males reflect the proportion and number transferred by virgin males. The mating status of males from nature was unknown but if males mate frequently and long sperm take longer to produce (Pitnick *et al.* 1995), then the number of long sperm transferred to wild-caught females may be limited. There is some evidence to support this interpretation; laboratory-raised *D. pseudoobscura* males that mated either daily or every other day for 15 days produced significantly fewer long, fertilizing sperm than virgin males of the same age (Snook 1998).

The reduced numbers of long, fertilizing sperm available to females in nature suggest that they may experience some degree of sperm limitation that would favour mechanisms to avoid gamete wastage. Additionally, whatever adaptive significance the short sperm have (Snook 1997), selection may operate more strongly in the wild leading to a greater number of them in both the ventral receptacle and spermathecae. Rice's (1998) suggestion that nonfunctional sperm may represent a mechanism by which males could continually coerce females, rather than the intermittent influence of accessory gland chemicals, does not appear to operate with respect to female remating behaviour in laboratory populations of *D. pseudoobscura*. Female remating latency in the laboratory is not influenced by the presence of short (or long) sperm in storage (Snook 1998). However, it may be fruitful to repeat this experiment in natural populations since wild-caught females receive and store more short sperm. Of course, with respect to female manipulation, short sperm may not be selected to influence remating behaviour but some other untested trait.

The gamete wastage seen in laboratory populations has important implications for interpretation of experiments designed to assess sperm competition and sexual conflict. When laboratory females produce many unfertilized eggs, their reproduction costs, for a given number of progeny, may be higher than in nature. Polyspermy may also represent a source of conflict that is greater in the laboratory than in nature. If we assume that polyspermy in this group is not detrimental, then females experience no cost, whereas males would—their sperm would be used at a faster rate with no gain in progeny and could compromise assessments of sperm competitive success if sperm number influences P_2. If females remate based on sperm numbers (Gromko & Markow 1993), however, then polyspermy would result in females remating faster and being exposed to more toxic male secretions (Chapman *et al.* 1995). In laboratory populations of *D. pseudoobscura* female remating behaviour does not appear to be related to sperm load (Snook 1998), so the latter cost to females may not occur.

Reproductive processes are obviously prime targets of selection that can act both cooperatively and antagonistically between males and females. One source of sexual cooperation and antagonism is gamete utilization, and selection has led to such phenomena as sperm competition (Birkhead & Møller 1998) and reproductive isolation through differential gamete usage (Howard 1999). The comparisons between laboratory and wild populations that we have made highlight the potential to reach different conclusions concerning the control of reproductive processes related to gamete utilization. Female *D. pseudoobscura* appear to maintain a tighter control over fecundity and productivity in natural populations than indicated by laboratory studies, consistent with a history of selection acting within the arena of the female reproductive tract. Wild male *D. pseudoobscura* will receive a benefit from this tighter control, however, they appear to experience different sperm production regimes with respect to sperm heteromorphism. As the evolutionary significance of sperm heteromorphism in this group remains a conundrum (Snook & Markow 1996; Snook 1998), the cost or benefit of this difference is unknown. Given the recent explosion of studies of sexual conflict (Holland and Rice 1999; Hosken *et al.* 2001; Pitnick *et al.* 2001), sperm competition (Birkhead & Møller 1998), and interspecific gamete interactions (Howard 1999), the described differences should be kept in mind when reaching conclusions about the nature of selection on reproductive processes.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.