COMMENTS

CRITERIA FOR DEMONSTRATING POSTCOPULATORY FEMALE CHOICE

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The recent discussion of Birkhead (1998) may cause confusion regarding the nature of cryptic female choice and how to test for its occurrence. Here I attempt to (1) clarify the distinction between cryptic female choice and ''sperm choice,'' the primary focus of Birkhead's discussion; (2) present more appropriate criteria for demonstrating cryptic female choice; (3) describe errors resulting from an either-or approach to male and female effects on sperm usage; and (4) discuss the usefulness of the kinds of indirect evidence that are often crucial in testing evolutionary hypotheses. I will treat each of these points in turn.

Inconsistent Use of Terms

Although the term cryptic female choice appears in Birkhead's title, most of the text is dedicated to discussing what he calls ''sperm choice.'' Unfortunately he uses this term inconsistently. At first, Birkhead defines sperm choice broadly, as ''the postcopulatory ability of females to favor the sperm of one conspecific male over another, that is, sperm choice'' (his abstract), a definition that would seem to make sperm choice synonymous with ''cryptic female choice'' sensu Thornhill (1983) and Eberhard (1985, 1996) (I will assume throughout, as has been customary in previous discussions, that ''postcopulatory'' refers to events following initiation of copulation, and thus includes processes that occur during as well as following copulation). Overlap between sperm choice and cryptic female choice is emphasized in Birkhead's first paragraph, where he claims that sperm choice is ''a necessary component of postcopulatory female choice.'' But then later Birkhead uses a much narrower definition of sperm choice that includes only a subset of the ways in which females may be able to bias paternity after copulation has begun: ''The simultaneous recognition of and discrimination between sperm of different males, either on the basis of the males' phenotype or that of their sperm constitutes sperm choice and is the focus of this review'' (p. 1213). This results in his proposing very narrow criteria for demonstrating what is usually treated as a broader phenomenon. Finally, Birkhead returns in his discussion to the broader definition in apparently equating sperm choice with cryptic female choice (p. 1217), which implies (misleadingly) that the same narrow criteria apply to both.

The narrow definition of sperm choice is incompatible with the broader definition because ''simultaneous recognition of and discrimination between sperm of different males'' is in no way necessary for many of the female processes that can affect the ''postcopulatory ability of females to favor the sperm of one conspecific male over another.'' These additional processes (which are summarized with lists of concrete examples in Eberhard 1996) include, among others, refraining from discarding the current male's sperm from her body, allowing intromission or spermatophore attachment to last long enough for maximal amounts of sperm and other seminal products to be transferred, discarding or digesting sperm from previous copulations, transporting sperm stored from previous copulations to sites where the current male can remove or otherwise inactivate them, failing to reject subsequent sexual advances of other males, allowing larger or smaller amounts of sperm from future copulations to be transferred and to be retained, rapidly maturing immature eggs, ovulating, and promptly ovipositing following copulation. (This last was the context in which the phrase ''cryptic female choice'' was first used [Thornhill 1983]). These processes can all have direct effects on sperm. A female that fails to ovulate within the survival period of sperm in her reproductive tract will have killed those sperm just as surely as if she flooded them with phagocytes. Birkhead mentions the existence of some of these processes and states that their effects on paternity appear ''to be well established'' (p. 1213). But he seems not to have realized that they too can affect the ''postcopulatory ability of females to favor the sperm of one conspecific male over another'' that he was attempting to evaluate.

The effect of this switching between broad and narrow definitions of sperm choice confuses the issue of establishing criteria for demonstrating postcopulatory female choice in general, as Birkhead claims to have done in his title and abstract. Birkhead was discussing criteria for a certain restricted subset of female mechanisms, not for postcopulatory female choice in general, as he implies. Birkhead's criteria are thus inappropriate for evaluating the existence of cryptic female choice in the wide sense in which this phrase has been used by other authors since its invention (e.g., Thornhill 1983; Eberhard 1985, 1996; Sakaluk and Eggert 1996; Dickinson 1997; Peretti 1997; Telford and Jennions 1998; Johnson et al. 1999; Tadler 2000).

Mistaken Criteria

Even if one uses Birkhead's narrow definition of sperm choice, his discussion of criteria is overly restrictive for two reasons. He emphasizes that unless the effects of sperm competition are controlled for, sperm choice cannot be demonstrated (e.g., p. 1213), and he proposes particular experi-

mental protocols in which such controls are included. But several female processes that can be influential after copulation has begun are not likely to be confused with competition among sperm if one observes animals directly. These include sperm dumping, interruption of copulation before maximum numbers of sperm are transferred, production by the female of physical plugs that promote retention of the sperm of the current male or prevent access of sperm from subsequent males to her reproductive tract, and female removal or degradation of such plugs (for concrete illustrations and references, see Eberhard 1996; Eberhard and Huber 1998). Thus Birkhead's criteria and the experimental protocols he proposes are inappropriate for testing the existence of several possible mechanisms of sperm choice, even in its sensu strictu meaning. In many cases research on other topics, such as functional morphology, may be more useful in resolving doubts. A concrete example comes from the yellow dung fly, *Scathophaga stercoriaria,* which Birkhead judged not to be a convincing case for female effects. Recent morphological data, however, have provided strong evidence for a female postcopulatory role in sperm usage that has proven persuasive even to former skeptics (Simmons et al. 1999; for further recent evidence supporting this female role in *S. stercoriaria,* see Otronen et al. 1997; Hosken and Ward 2000. The question of whether females systematically bias their effects in favor of particular types of males is still under investigation (Eberhard 1996; Ward 1998; Parker et al. 1999).

Second, Birkhead argues that in order to demonstrate postcopulatory female effects on sperm usage, one must demonstrate a significant effect on sperm usage patterns (P_2 values) that is due to differences among females. This criterion reduces to the unconvincing proposition that female effects are only important if there is variation in choice among females. For instance, if all pea hens have an equally strong preference for males with long tails, is one to conclude that there is no female choice on tail length (see Jennions and Petrie 2000)? A more reasonable position is that demonstration that the differences among females affect P_2 values is indeed evidence of a female effect (e.g., Price 1997; Wilson et al. 1997), but that such a demonstration is not a necessary condition for concluding that a female bias occurs.

A hypothetical experiment of a type proposed by Birkhead can serve to illustrate in more detail the inadequacy of the female variation criterion. Let us say that known numbers of sperm from two males (say 50% from male A and 50% from male B) were artificially introduced into a series of 10 females (a technique cited by Birkhead as appropriate to control for sperm competition effects in the search for female choice effects), and that male A fathered exactly 80% of the offspring of each of the females. Setting aside for the moment the fact that this experimental procedure is woefully inadequate to test for possible female-imposed biases (it eliminates most or all of the stimuli normally associated with copulation that might trigger differences in female responses such as differential transport, ovulation, etc.), what should one conclude? There is no variance in P_2 attributable to females, so the conclusion, using Birkhead's criteria, is that sperm choice is not occurring; the pattern of sperm use in this species is apparently to be explained by sperm competition (assuming there is no biased abortion). The sperm of male A are better at fertilizing eggs than are the sperm of male B.

But this is only a partial answer to the question of why the males and females of this species have the traits that they do. The competition between sperm occurs in a female environment, and it is likely that the degree of superiority of male A's sperm is influenced by their interactions with and adjustments to many different female-determined conditions. If, for instance, the female ducts are long and transport time to storage or fertilization sites is long, then long-lived sperm that could swim long distances might be favored over short-lived sprinters. A conservative list of other such female traits that could influence the outcome of sperm competition sensu strictu and that are known to occur in nature (for examples and references, see Eberhard 1996; Gomendio et al. 1998) includes the viscosity of the contents of the female's reproductive tract, the pH of the contents, the direction in which these contents flow, the temperature, the presence or absence of materials in the tract that adhere to sperm and slow their movements, nutrients, cells such as phagocytes that can kill sperm, sperm activating factors, folds or cracks where sperm can shelter, contractions of the ducts that facilitate (or inhibit) transport, and the properties of the egg membrane and the materials surrounding it that affect the sperm's motility and ability to adhere. Sperm that are competitively superior in one environment are unlikely to be superior in all others. Changes in these female-imposed environmental conditions, whether induced by the male or whether occurring in past or future evolutionary time, could change the outcome of competition between sperm from males A and B. Indeed, female-imposed bias in the past is a likely explanation for the traits currently found in the sperm of male A. Wiley and Poston (1996) made a similar point regarding the inevitable female influence on eventual winners in the context of precopulatory competition among males. For instance, male-male battles at leks will only have reproductive consequences for the males if females behave in particular ways: if they visit leks, and if they allow males to copulate with them there and not elsewhere.

It Takes Two to Tango: A Misleading Dichotomy between Sperm Competition and Female Choice

This last point can be placed in a more general context. Birkhead's line of reasoning is that ''the most plausible way to demonstrate the occurrence of female sperm choice is to control for both differential abortion and sperm competition'' (p. 1213). He mentions that different combinations of sperm competition via sperm interactions and female sperm choice can occur (p. 1213), but the implication of this type of analysis is that male effects can occur in the absence of female effects, and vice versa. Neither extreme is possible, however; sperm competition sensu strictu is between male products that are inside the female's body. Its outcome must always depend on interactions between the sperm and the female environment in which they find themselves. If one type of sperm wins out, it is because that type is better adjusted to perform under the particular conditions present in conspecific females (e.g., Eady 2000). In an analogy with sporting events, the male competition occurs on playing fields whose characteristics are determined by females. A common temptation has been to treat a female's traits as an unstudied ''given''

when discussing interactions among ejaculates, and to neglect to ask why it is that she has one particular set of traits instead of another.

This problem of an either-or approach is exemplified in Birkhead's criticism of the possibility that female sperm choice occurs in the beetle *Chelymorpha alternans* based on the length of the male's long thin genitalic flagellum. He cites the alternative explanation that ''males with a longer flagellum may simply be more efficient at getting their sperm to the best place to achieve fertilization'' (p. 1214). However, females of this beetle have a very long, highly convoluted and complex spermathecal duct, thus Birkhead's proposal is not an alternative, but rather a statement regarding payoffs for different male behavioral and morphological strategies once the female has set the playing field (in this case the tortuous female duct).

Questions about which sex controls processes that result in differences in paternity will have different answers, depending on the level of analysis (Eberhard 1998). Differences in the lengths of male organs in *C. alternans* and presumably their ability to deposit sperm at particular sites in the female do explain some of the variance in paternity in this beetle. At a deeper level, the reason why the lengths of male organs affect paternity this way can be explained by the details of the design of the female. Another recent example emphasizes the interactive nature of male and female effects in the now classic context of sperm removal by male damselflies. In at least some species, the male's chances of removing the sperm of previous males are influenced by female ejection of sperm from deep within her spermatheca where his genitalia cannot reach, and this ejection is, in turn, influenced by male copulatory stimulation of the female (Cordoba-Aguilar 1999). Female traits lurk behind many explanations based on male traits and, of course, vice versa. An ''either-or'' approach is not appropriate for studying male and female traits involved in sexual interactions. By setting up a contrast between sperm competition and postcopulatory female choice, Birkhead focuses attention in a way that tends to perpetuate this kind of erroneous dichotomy.

What Constitutes ''Sound'' Evidence?

A final point of difference concerns the scientific value of different types of data. Birkhead's use of the loaded phrase ''sound evidence'' (p. 1212) in introducing his criteria implies that other types of evidence presented previously regarding cryptic female choice (e.g., ch. 5–7 of Eberhard 1996, where predictions of general patterns derived from theoretical considerations are checked against the data from many different groups) are somehow ''unsound,'' and implies that a phenomenon is of dubious importance unless one has directly observed or demonstrated experimentally that it occurs at present. But one need not blow up the side of a mountain experimentally to deduce that landslides are powerful forces of nature: the importance of landslides can also be deduced from observing their consequences. It is worth remembering that in *The Origin of Species,* Darwin did not directly ''demonstrate'' the creation of a single species, nor did he experimentally prove that natural selection caused changes in any given species. Rather he amassed data and compared their

patterns with the expected consequences of evolution by natural selection. Darwin's technique of checking predictions of hypotheses against general trends in biological data continues to be valid (e.g., Brandon 1996), and arguments derived from this technique should not be ignored unless one presents direct, specific demonstrations of errors of fact or interpretation. This does not mean that experimental studies of sperm precedence will not be useful for understanding phenomena related to sperm usage—they are likely to be fascinating. But this is not the only type of test that is useful and, as shown above, incautious interpretation of such data can even lead to serious mistakes in evaluating the importance of postcopulatory or cryptic female choice.

In summary, Birkhead's inconsistent use of the term sperm choice led him to draw inappropriately general conclusions regarding postcopulatory female choice on the basis of data related to only a narrow a range of possible female effects. These conclusions are not appropriately applied to the more general interpretation of cryptic female choice that has been used throughout the history of this term, as implied in his title. Birkhead's statement (his first paragraph) that sperm choice is ''a necessary component of postcopulatory female choice'' is simply wrong, no matter which of his definitions is used, because it fails to take into account many postcopulatory female processes. By presenting sperm competition and sperm choice as ''alternatives,'' Birkhead confused the issues of the evolution of male and female effects on paternity and how they can be established. Differences among females are not necessary for female choice to occur, as his criteria imply. Because fertilization results from an *interaction* between the two sexes, the effects of both sexes are inextricably interrelated, and female traits are not appropriately taken as static ''givens'' in these interactions. Birkhead is correct in proposing that further studies of the mechanisms determining sperm usage promise to provide interesting and exciting results, but his position that direct observation of current biases are necessary to provide ''sound'' evidence for the existence of postcopulatory female choice is unjustified.

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DISTINGUISHING BETWEEN FEMALE SPERM CHOICE VERSUS MALE SPERM COMPETITION: A COMMENT ON BIRKHEAD

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Sperm competition has received a lot of attention from behavioral ecologists in the past decade (Birkhead and Møller 1998). In the strict sense, it can be defined as the competition among spermatozoa from different males for the fertilization of a set of ova (Parker 1970). Sperm competition can thus be seen as the postcopulatory form of competition among males for access to females and might therefore be an important mechanism for sexual selection (Andersson 1994; Møller 1998). Although there is obviously a lot at stake for males (in terms of reproductive success), it has become clear that females also play an important role. Female behavior and reproductive physiology are shaped by selection to reduce the fitness costs to females of male adaptations to sperm competition (Rice 1996) and to increase the benefits of having eggs fertilized by particular males (Keller and Reeve 1995). Among evolutionary biologists, interest arose in the possibility that females can exert postcopulatory choice on who

fathers their offspring (e.g., Eberhard 1996). If sperm competition occurs, that is, if sperm from different males is present within the female reproductive tract, females might be able to discriminate between and differentially utilize the sperm of different males, a process referred to as ''sperm choice'' (Birkhead 1998; Olsson et al. 1999). Just as competition among males for access to females is more obvious than subtle female choice for a particular male, sperm competition seems more obvious and easier to study than female sperm choice.

Birkhead (1998) proposes that three criteria need to be fulfilled to unequivocally demonstrate female sperm choice and he describes the types of experiments needed. This is an important contribution, because—as Birkhead's review of studies shows—the current evidence for sperm choice is limited and a clarification of the criteria needed to demonstrate it is clearly valuable. However, we believe that the proposed

TABLE 1. Representation of the experimental design suggested in Birkhead 1998 to evaluate variance in P2 due to (a) males and (b) females.

(a)

Male pair	$M_1M_{1'}$	$M_2M_{2'}$	$M_3M_{3'}$	...	$M_nM_{n'}$
Females	F_{11}	F_{21}	F_{31}	...	F_{n1}
	F_{12}	F_{22}	F_{32}	...	F_{n2}
	F_{13}	F_{23}	F_{33}	...	F_{n3}
	...	...	...	...	...
	F_{1m}	F_{2m}	F_{3m}	...	F_{nm}

(b)

Female	F_1	F_2	F_3	...	F_n
Male pairs	$M_{11}M_{11'}$	$M_{21}M_{21'}$	$M_{31}M_{31'}$	...	$M_{n1}M_{n1'}$
	$M_{12}M_{12'}$	$M_{22}M_{22'}$	$M_{32}M_{32'}$	...	$M_{n2}M_{n2'}$
	$M_{13}M_{13'}$	$M_{23}M_{23'}$	$M_{33}M_{33'}$	...	$M_{n3}M_{n3'}$
	...	...	...	...	...
	$M_{1m}M_{1m'}$	$M_{2m}M_{2m'}$	$M_{3m}M_{3m'}$	...	$M_{nm}M_{nm'}$

experiments cannot provide conclusive evidence for or against sperm choice. In this commentary, we explain why.

The three criteria for demonstrating female sperm choice proposed in Birkhead (1998) are based on experiments where two males copulate with a single female and they can be summarized as follows: (1) it is necessary that there is variance in P_2 values (the proportion of offspring sired by the second male when two males copulate with a female); (2) one has to demonstrate that some of this variance is attributable to males; and (3) that some of this variance is attributable to females. Birkhead (1998) then describes two experiments aimed at attributing variance in P_2 values to males and females, which we will now discuss in some detail.

Variance Attributable to Males

The aim of the first experiment suggested by Birkhead (1998) is to attribute some of the variance in P_2 values to males. In this experiment *several pairs of males are allowed to inseminate several different females* (Table 1a), a design used in a study on *Tribolium castaneum* by Lewis and Austad (1990). In Table 1a, *n* is the number of male pairs, and *m* the number of females inseminated by each male pair. Such an experiment would lead to $m \times n$ P_2-values (we assume that each male pair copulates with an equal number of females, but this is not important for our argument). Birkhead suggests that a repeatability analysis be performed, for which the values obtained from different females are seen as repeated measurements for each male pair. A high repeatability would thus indicate that the variance within the same pair of males is smaller than the variance among the male pairs. In other words, a significant proportion of the variance in P_2 would be attributable to *male pairs*. However, the fact that P_2-values in this experiment are repeatable or, as Lewis and Austad (1990) show with a nested ANOVA, that some of the variance that is attributable to *male pairs* does not provide *any* information about the mechanism behind it. It is easy to see that a high repeatability can be a consequence of sperm competition, female sperm choice or both, a point made clear by Lewis and Austad (1990, p. 356). Suppose, for example, that in the first male pair the ejaculate of M_1 is more competitive than the ejaculate of M_1', whereas in the second pair

M_2 has a less competitive ejaculate than M_2'. An outcome where P_2 values for M_1M_1' are consistently lower than P_2 values for M_2M_2' can then be expected as a result of sperm competition. Alternatively, imagine that all males have ejaculates of similar quality (i.e., are equally sperm competitive), but M_1 is strongly preferred by all females over M_1', whereas M_2 is less attractive than M_2'. A similar outcome in terms of P_2-values as the one described above can now be expected, if female sperm choice occurs. Note that we assume that all females rate attractiveness in the same way, and thus exert sperm choice in the same way. As Birkhead points out, this is the type of sperm choice that would be important in relation to sexual selection. If females rate males according to compatibility, no repeatability and thus no variance attributable to males is expected, despite female sperm choice.

Variance Attributable to Females

To attribute some of the variance in P_2 to females, Birkhead (1998) suggests a second experiment using "*several females each inseminated by several different pairs of males* in successive breeding cycles" (Table 1b). In Table 1b, n refers to the number of females used in the experiment, while m is the number of different male pairs per female (for simplicity, each female is assigned to the same number of male pairs). Birkhead states that "if individual females show significant repeatability in P_2-values this would be good evidence that females were responsible for some of the overall variance in P_2." This is of course true, but what does it mean? Why would one expect that if females are inseminated by several *different* pairs of males, the P_2-values for individual females would be repeatable? This is not clear.

Significant repeatabilities are not expected if females choose sperm of particular males based on male attractiveness, because the attractive males could be the first or the second in a pair.

The description of the second experiment could perhaps be interpreted in a different way, namely that several females are each paired with one pair of males for the first clutch, with a second pair of males for the second clutch, and so on (referring to Table 1b, this would mean that $M_{1i}M_{1i'} = M_{2i}M_{2i'} = M_{3i}M_{3i'} = \ldots = M_{ni}M_{ni'}$, where i = 1, 2, ... m). However, this does not make much difference in the above argumentation.

One can show that between female variance in P_2-values is neither a necessary, nor a sufficient condition for sperm choice. For example, imagine that sperm choice exists and that all males are equally sperm competitive. Further assume that males differ in attractiveness and that all females rate attractiveness in the same way. Then in the proposed experiments a significant proportion of the variance in P_2-values will be attributable to the male pair, but *none* of the variation will be attributable to the female. Alternately, assume that a significant proportion of the variance in P_2-values *can* be attributed to the female. This finding can be explained by a mechanism other than female choice. For example, females could differ in their reproductive tract in such a way that it affects fertilization success with respect to insemination order. For example, if sperm competition occurs by displacement of sperm from the sperm storage organ, P_2-values would

be high for a female with a small organ (the second male could remove all the sperm) and close to 50% for a female with a large organ (only a small fraction of the sperm from the first male would be removed). In such a case, the experiment would show a high repeatability of P_2-values for females, without the occurrence of sperm choice.

Sperm Choice and Genetic Compatibility

Females may base their choice of sperm on male attractiveness or on genetic (in)compatibility with the male. Evidence is accumulating that male and female genotype both affect fertilization success (Wedekind et al. 1996, Clark et al. 1999). If female sperm choice is a mechanism to avoid the negative effects of inbreeding (as the studies reviewed in Birkhead 1998 suggest), one could perform P_2-experiments using groups of related versus unrelated females. Such an example is provided by Wilson et al. (1997) and discussed in Birkhead (1998). A recent study on decorated field crickets (*Gryllodes supplicans*) used a different approach (Stockley 1999). In this study, each female of a sibling pair was allowed to copulate first with a sibling male, followed by either another sibling male or a nonsibling. The paternity obtained by the first sibling male (P_1 value) was lower when the second male was a nonsibling, providing some evidence for female sperm choice based on genetic similarity (Stockley 1999).

Conclusions

From the above it can be seen that which sex accounts for variance in P_2 should not be confused with the sex that controls whose sperm fertilizes the egg(s). Thus, the experiments and the three criteria posed by Birkhead (1998) do not allow discrimination between sperm competition and female sperm choice, nor do they unequivocally demonstrate the existence of sperm choice. Such a simple set of criteria, although extremely valuable in guiding the design of experiments, may simply not exist.

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CRITERIA FOR DEMONSTRATING FEMALE SPERM CHOICE

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In a recent paper, Birkhead (1998) considers the criteria necessary to demonstrate that females are able to coordinate their use of sperm to bias paternity in favor of some males over others. This was a timely and much needed exercise, given the recent development of cryptic female choice theory (Eberhard 1996) and proliferation of interest in postcopulatory sexual selection in general. However, we disagree with Birkhead regarding the methods of demonstrating sperm choice. In this paper, we analyze Birkhead's (1998) criteria and indicate why they are collectively neither sufficient nor

necessary to demonstrate female sperm choice. We suggest that statistical inferences of sperm choice require a pattern of variation different from that suggested by Birkhead. Furthermore, no matter how clear the patterns of variation, direct evidence of sperm choice will ultimately require either experimental manipulation of the putative criteria of choice or identification of the mechanisms that females employ to affect their choice.

Birkhead's (1998) paper makes three important contributions. First, it clarifies that the most controversial part of cryptic female choice is the operation of female sperm choice (rather than differential abortion, differential investment in offspring, etc.). Second, it points out that the few studies to have demonstrated female sperm choice have consistently revealed female biases against the sperm of closely related or otherwise genetically incompatible males. This observation merits further theoretical and empirical attention to address whether the same mechanisms that enable avoidance of self-fertilization and inbreeding can be used to choose sperm on the basis of relative male quality. Third, Birkhead (1998) outlines the criteria that are necessary and sufficient to demonstrate female sperm choice. Here we discuss the efficacy of Birkhead's (1998) criteria.

Before proceeding, however, it is crucial to define sperm choice because this will influence the evidence required to demonstrate its existence. Sperm choice is the form of cryptic female choice that deals with biases in the use of stored sperm within a female's genital tract. Birkhead (1998, p. 1213) adopted Eberhard's (1996) definition of cryptic female choice as ''a female-controlled process or structure that selectively favors paternity by conspecific males with a particular trait over that of others that lack the trait when the female has copulated with both types.'' We believe the emphasis on ''female control'' to be problematic because it implies that females exercise authority over sperm use and thus they alter sperm use for their own purposes. We prefer a more objective definition of cryptic female choice that excludes the concept of ''control'' and defines it simply as nonrandom paternity biases resulting from female morphology, physiology, or behavior that occur after coupling. This definition ascribes as sperm choice any biases in paternity owing to the way females handle sperm, regardless of the specific mechanism or evolutionary cause, and regardless of proximate control. To illustrate our concerns over the definition of cryptic female choice, consider a mechanism of differential sperm use by a *Calopteryx* damselfly. Córdoba-Aguilar (1999) demonstrated that horns on the distal tip of the male's penis are used to stimulate cuticular plates bearing mechanoreceptive sensilla within the female's genital tract. This stimulation results in the ejection of sperm stored from previous mates, and penis width positively correlates with the amount of sperm ejected. As a consequence, females presumably use more sperm from males with wider penises. Córdoba-Aguilar (1999) suggests that males exploit the female sensory system that controls egg fertilization, thus circumventing female control and not satisfying Eberhard's (1996) definition. Although Córdoba-Aguilar's interepretation may be correct, an alternative is that females use this capacity to favor the paternity of superior males, in which case Eberhard's definition is satisfied. Thus, Córdoba-Aguilar and Eberhard's definition is contingent on

speculation about the evolutionary history of the trait, whereas ours identifies female sperm choice as a proximate mechanism of sexual selection regardless of history. The only relevant consideration for our definition is whether a female-mediated process generates sexual selection on males. The cause of sperm choice, be it through benefits of sire discrimination (i.e., good genes or Fisherian models; Eberhard 1996), sexual conflict (Holland and Rice 1997, 1998), or male manipulation of pre-existing biases (Ryan 1990), is an important issue, but it should be held separate from demonstration of the existence of sperm choice.

BIRKHEAD'S PROTOCOL

Birkhead (1998) contends that three criteria are necessary to demonstrate female sperm choice: (1) overall variation in P_2; (2) variance in P_2 that is attributable to the males; and (3) variance in P_2 that is attributable to the females. He outlines two complementary experiments needed to test these criteria. The first experiment is that used by Lewis and Austad (1990), in which several pairs of males each inseminate a number of different females. Calculating the repeatability of P_2 within pairs of males mated to different females estimates the variance in P_2 that is attributable to males. The second experiment simply reverses the procedure so that individual females are each inseminated by multiple pairs of males during successive breeding cycles. Significant repeatability of P_2 in this experiment indicates variance in P_2 that is attributable to females. Birkhead concludes that variance attributable to females is needed to show that females are responsible for variation in paternity and variance attributable to males is necessary to show that female choice results in nonrandom male fertilization success.

How do we interpret positive results for Birkhead's (1998) three criteria? His first criterion, that there must be variation in paternity (or variation in P_2 in the classic sperm competition experiment), is a given and must exist for the subsequent two criteria to hold. The second criterion, variance attributable to males, means that males differ consistently (and thus nonrandomly) in fertilization success. This pattern of variance in paternity is, by definition, sexual selection on males. However, male-attributed variance in sperm use may be due to male sperm competition or female sperm choice that consistently targets certain males. These first two criteria are absolutely necessary, but are not sufficient to demonstrate sperm choice.

We hold that the third criterion, female-attributed variation in sperm use, is neither a necessary nor sufficient condition of any demonstration of sperm choice. A lack of variation in P_2 that is attributable to females does not mean that female sperm choice is absent. If all females in the population show the same pattern of sperm choice (e.g., an open-ended preference), directional sexual selection on males via female sperm choice could be taking place, but it would only contribute to variation in P_2 that is attributable to males. Furthermore, the presence of variation among females in P_2 does not imply the existence of female sperm choice. This pattern of variation simply means that females differ consistently in the extent to which they favor first mates over second mates, which may have nothing whatsoever to do with idiosyncratic

features of the males. Generally, female-attributed variance in P_2 should weaken sexual selection on males because it reduces the amount of residual variance able to be explained by male phenotype.

We illustrate why among-female variation in P_2 is not the equivalent of sperm choice with an example of allometric variation in the size of the female's sperm-storage organs. In *Drosophila* there is a significant positive relationship between female size and the length of the primary sperm-storage organ (S. Pitnick, unpubl. data). A similar relationship has been found between spermathecal volume and body weight in the moth *Plodia interpunctella* (Gage 1998) and the dung fly, *Scathophaga stercoraria* (Hosken and Ward 2000). Suppose there is a positive correlation between sperm-storage organ length and P_2, which could occur if longer organs created greater stratification of ejaculates and thus reduced sperm mixing, resulting in higher P_2 values. This situation would generate among-female variation in P_2 that is not necessarily indicative of any special use of one kind of male's sperm over another. Females with longer sperm-storage organs would exhibit higher P_2 values irrespective of the males with which they mated.

To use a statistical approach to look for the possibility of variation among females in sperm choice, we need to study whether paternity varies consistently among females depending specifically on male identity or features of male phenotype. To this end, Birkhead (1998, p. 1214) recommends that reciprocal-order matings always be conducted ''to establish whether order effects are symmetrical for each male in a pair.'' That is, for each pair of males, the experiment must be repeated so that a given male precedes his competitor in one replicate, but is followed by his competitor in another replicate. Birkhead's intention is that this approach will permit quantification of female sperm choice while controlling for sperm competition, but we disagree. Unless individual male pairs are mated to multiple females (see below), any variation within male pairs due to mating order cannot be attributed to male- versus female-mediated processes.

Testing Female Sperm Choice

There are two criteria that together are necessary and sufficient for demonstrating female sperm choice: (1) nonrandom use of sperm, as Birkhead (1998) clearly stated in his second criterion; and (2) variation in sperm use that results from female-mediated processes, which was the goal of Birkhead's third criteria. The potential complexity of female-mediated sperm use (Eberhard 1996) may preclude the efficacy of any one approach for all systems, but we suggest that sperm choice may be inferred or directly demonstrated using any one of the following three approaches: (1) demonstrating significant male $\times$ female interaction effects on P_2; (2) testing the effect of experimentally manipulated perceived male quality on P_2; and (3) identifying the mechanism(s) of female-mediated paternity control. The last of these approaches is advocated and discussed by Eberhard (1996), Birkead (1998), Telford and Jennions (1998), and others and requires no further discussion here. The first two approaches, along with their strengths and limitations, are outlined below.

Experiment 1: Male $\times$ Female Interaction Effects

By combining Birkhead's (1998) two experiments into a single large experiment, male $\times$ female interaction can be measured, in addition to variance in P_2 that is attributable to females and to males. A significant female $\times$ male interaction suggests not only that females differentially handle sperm, but that differences in sperm use among females depend on male identity. Thus, a significant interaction supports female sperm choice in a fashion that the simple demonstration of female-attributed variation does not. The trick to establishing an interaction is to mate each pair of males to each of a series of females two or more times. This tends to limit this design to species in which sperm is fully used or expelled after each breeding episode. The simplest experiment, using just two pairs of males with each female and mating each pair to the female on only two successive breeding cycles, would require that P_2 be measured for four breeding cycles and a total of eight matings per female. In addition, the effect of female age on sperm choice or sperm competition would need to be assessed. Thus, this approach is intractable for many study organisms. However, three recent studies have elegantly overcome this obstacle by measuring consistency of sperm use within genetically similar females, rather than by repeated trials on individual females.

In the first study, Wilson et al. (1997) took advantage of familial relatedness to partition sources of variation in P_2 in the cowpea weevil, *Callosobruchus maculatus.* This work is described in detail by Birkhead (1998) and by Telford and Jennions (1998). In the second study (published after Birkhead's review), more sophisticated genetic crosses were used to examine variation in P_2 in the fruitfly, *Drosophila melanogaster.* By using females from lines rendered homozygous for X, second, and third chromosomes, Clark and Begun (1998) demonstrated the presence of polymorphic female genes affecting P_2. Consistent with our viewpoint, the authors recognized that genetic variation of this type is completely neutral in the absence of pleiotropy or interaction between variation in the two sexes. Clark et al. (1999, p. 217) thus performed pairwise P_2 experiments among six different isogenic lines and found a significant interaction effect, indicating that ''success of a particular male's sperm was found to depend on the genotype of the female with which he mates.'' This is suggestive of female sperm choice.

In the third example, Ward (1998) predicted that female dung flies would choose the sperm of males with the same homozygous genotype at the phosphoglucomutase (*Pgm*) locus when temperature was stable to increase homozygosity in progeny. Females would prefer the sperm of males having a different genotype when temperature was variable to increase heterozygosity in progeny. P. I. Ward (unpubl. ms.) supported these predictions. It is worth noting, as emphasized by Ward (unpubl. ms.), that the amount of the variation in paternity that was due to female-mediated effects was small (yet significant) and would have been difficult to detect without first controlling for male-mediated processes (i.e., Ward statistically removed that variation explained by copulation duration, which is known to be under male control; Parker and Simmons 1994). Thus, this example thus also points out

the importance of controlling for male-mediated effects, as noted by Birkhead (1998).

Two potential shortcomings of the male $\times$ female interaction approach should be noted. First, this protocol will reveal sperm choice only when females differ in their preferences, not when females agree on male quality. Second, a male $\times$ female interaction may also be due to cryptic male choice, such as males tailoring ejaculate size or investment in sperm manipulation according to their perception of female quality. There have been few investigations into cryptic male choice, probably because of the concept that sperm are cheap and thus a male's ability to produce ejaculates does not limit his reproductive success. In fact, in Eberhard's (1996) book on cryptic female choice, cryptic male choice warrants only two paragraphs, which address the sex role-reversed pipefish, *Sygnathus typhle*. The operation of cryptic male choice may be widespread, however, and does not require sex role reversal. Male discrimination among potential female mates is known to occur (Brown 1990; Gwynne and Simmons 1990; Batorczak et al. 1994; Itzkowitz et al. 1998; Polak et al. 1998; Kvarnemo and Simmons 1999; Van Gossum et al. 1999), and because sperm and ejaculates may have substantive costs (Dewsbury 1982; Gwynne 1983; Pitnick and Markow 1994; Pitnick et al. 1995; Pitnick 1996), cryptic male choice may be common. Several investigations have found that males adjust the size of their ejaculate according to the age, size, or reproductive status of the female (Linley and Hinds 1975; Svärd and Wiklund 1986; Suter and Parkhill 1990; Cook and Gage 1995; Gage and Barnard 1996; Gage 1998) or according to the immediate perceived risk of sperm competition (Baker and Bellis 1989, 1993; Bellis et al. 1990; Gage 1991; Gage and Baker 1991; Wedell 1992; Simmons et al. 1993; Gage and Barnard 1996). Variation in P_2 that is attributable to females could be explained by cryptic male choice, and a significant female $\times$ male interaction in P_2 could theoretically represent variation in cryptic male choice.

Experiment 2: Manipulation of Perceived Male Quality

Our second experimental approach is useful when a male trait suspected of being subject to sperm choice has been indentified. By manipulating either the trait or the female's perception of the trait, effects on P_2 due to sperm choice may be distinguished from effects due to sperm competition.

For example, Lewis and Austad (1994) assessed male olfactory attractiveness in the flour beetle *Tribolium castaneum* by testing females in a two-choice trial in which a paper disk exposed to the pheromones of a single male was tested against a control disk and then the disk of another male. After determining relative male attractiveness, Lewis and Austad determined P_2 for each wild-type male by allowing him to inseminate a female that had been previously inseminated by a male with a genetic marker. They found a significant positive correlation between male olfactory attractiveness and P_2. This relationship could be the result of males with more attractive pheromones being better at displacing another male's sperm or transferring more sperm or it could reflect preferential use of sperm by females (Lewis and Austad 1994). These alternatives can be distinguished by measuring

P_2 while manipulating the female's perception of male attractiveness.

Female perception of relative male quality could be manipulated by pairing the female with the male in the presence of another male's pheromones. To examine effects on P_2, a fully factorial design could be used that tests attractive and unattractive males in the presence of pheromone from different attractive and unattractive males. Verifying that positive results are indeed due to female sperm choice would require a demonstration that the presence of another male's pheromones does not influence a male's ejaculatory characteristics.

CONCLUSION

It is a great challenge to experimentally discriminate between female- and male-mediated processes in determining nonrandom patterns of sperm use, just as it has been with nonrandom mating patterns (Andersson 1994). Partitioning variance, if done properly, has the potential to yield important inferences about the presence of sperm choice, but it is unlikely to provide sufficient information to unequivocally demonstrate sperm choice. Convincing demonstrations of sperm choice require a mixture of careful investigation of the mechanism of choice and experimentation on targets of choice. Just as Ryan's (1980) account of the process by which female túngara frogs sample males and Andersson's (1982) ability to alter the males preferred by females by manipulating their tail length provided some of the most convincing evidence of precopulatory mate choice, we believe that investigation of mechanisms and manipulation of choice criteria will produce the best tests of sperm choice.

One clear goal of Birkhead's (1998) attempt to establish explicit criteria for the demonstration of female sperm choice was to initiate discussion of this topic. We hope that this comment will help to continue the conversation.

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