Secondary sexual trait size reveals competitive fertilization success in *Drosophila bipectinata* Duda

Michal Polak\textsuperscript{a} and Leigh W. Simmons\textsuperscript{b}
\textsuperscript{a}Department of Biological Sciences, University of Cincinnati, Cincinnati OH 45221-0006 USA and \textsuperscript{b}Centre for Evolutionary Biology, School of Animal Biology (M092), University of Western Australia, Crawley WA 6009, Australia

The evolution of male secondary sexual traits traditionally has been ascribed to precopulatory sexual selection. In contrast, the importance of postcopulatory sexual selection for the evolution of secondary sexual traits is uncertain, and what little evidence exists for this process to contribute to the evolution of such traits is mixed. Here we test the hypothesis in *Drosophila bipectinata* Duda that the male sex comb, a rapidly evolving secondary sexual trait, is under positive postcopulatory sexual selection. We extracted replicate genetic lines exhibiting relatively large and small sex comb size from a natural population. Males from these lines were subjected to an assay of competitive fertilization ability, measured as *P*\textsubscript{2}, the proportion of a female’s clutch of eggs fertilized by the second male to mate. Males with the largest sex combs sired more offspring than less ornamented individuals, demonstrating for the first time in any *Drosophila* species that postcopulatory sexual selection favors increasing sex comb size. This study identifies a postcopulatory selective mechanism that may be contributing to the evolutionary diversification of a secondary sexual trait. Key words: competitive fertilization success, *Drosophila bipectinata*, ejaculate quality, *P*\textsubscript{2}, postcopulatory sexual selection, sex comb size. [Behav Ecol 20:753–760 (2009)]

Sexual selection is differential reproductive success arising from competition for mates and fertilizations (Darwin 1871; Andersson 1994). An important consequence of this selection can be rapid diversification of reproductive traits of both males and females (Andersson 1994; Eberhard 1996; Panhuis et al. 2006). Indeed, often the most dramatic differences seen among closely related polygynous species of animals, such as birds, fishes, and insects, are features of their mating systems and secondary sexual traits, underscoring not only that sexual selection can drive the diversification of phenotypes but also that it can contribute to the build up of reproductive barriers among populations, and ultimately, to speciation (West-Eberhard 1983; Dominey 1984; Barraclough et al. 1995; Müller and Cuervo 1998; Arntz et al. 2000). Whereas Darwin (1871) recognized sexual selection to be a powerful force in the evolution of morphological and behavioral gender differences, he placed exclusive emphasis on precopulatory mechanisms, which, from today’s perspective, is a relatively restricted view (Andersson and Simmons 2006).

A growing awareness over the past few decades of the evolutionary importance of postcopulatory mechanisms has greatly broadened the theoretical and empirical bases of sexual selection research (Parker 1970a; Eberhard 1996; Möller 1998; Simmons 2001; Birkhead and Pizzari 2002). It is now recognized that in animals with a polyandrous mating system (wherein females mate with 2 or more males during a reproductive episode), sexual selection has the potential to occur postintromission (or postspawning) and that the resultant competitive interactions among overlapping ejaculates may be a powerful engine driving the evolution of a wide variety of interacting adaptations observable at the genetic, biochemical, and morphological levels (Birkhead and Pizzari 2002; Panhuis et al. 2006). For example, in several insect groups, including flies, butterflies, and beetles, genital morphology is on average about twice as divergent in taxa in which females are polyandrous compared with taxa in which females mate only once (Arntz 1998). In *Drosophila melanogaster*, where females also are polyandrous, the male seminal fluid transferred to females during copulation contains an assortment of molecules (so-called accessory gland proteins or Acps) that can influence various aspects of male and female behavior, physiology, and reproductive function (Ram and Wolfner 2007). Recent molecular analyses indicate that interacting reproductive proteins in both sexes are evolving rapidly, in *Drosophila* as well as in a variety of other species, and that this evolution may be influenced by postcopulatory sexual selection (Swanson et al. 2001; Swanson and Vacquier 2002; Mueller et al. 2005).

Postcopulatory sexual selection includes both sperm competition and cryptic female choice (Parker 1970b; Eberhard 1996; Simmons 2001). Either mechanism can produce pronounced variation in paternity among males, which may or may not correlate with precopulatory mating success (Birkhead and Pizzari 2002). Thus, understanding patterns of precopulatory mating success, the traditional arena of sexual selection studies, may generally not portray a realistic picture of the net selective landscape characterizing a particular trait (Danielsson 2000; Demary and Lewis 2007). In some cases, pre- and postcopulatory episodes of selection may act synergistically (Birkhead and Pizzari 2002). In flour beetles (*Tribolium castaneum*), for example, males that are more attractive in precopulatory sexual selection because of long-range olfactory attractiveness to females also enjoy higher paternity share (Lewis and Austad 1994 and see Hosken et al. 2008). Such synergism may arise if enhanced expression of an ornamental trait reveals underlying genetic quality (Johnstone 1995), which could then translate to superior resource acquisition ability and thus ejaculate quality (Rowe and Houle...
1996; Tomkins et al. 2004). Indeed, in guppies (Poecilia reticulata), males advertising high levels of orange pigmentation are both more attractive to females and more successful in sperm competition (Evans et al. 2003).

In other cases, pre- and postcopulatory mechanisms may be independent or even antagonistic. Thus, detection of selection operating (or not) at one level may provide an incomplete or even misleading picture of the strength and direction (or lack thereof) of sexual selection overall. Antagonism between these processes may occur because of underlying physiological trade-offs among different male reproductive traits (Simmons and Emlen 2006; Demary and Lewis 2007) or as a result of sexual conflict (Arnqvist and Rowe 2005). In water striders, Gerris lacustris, and soay rams, Ovis aries, males that are most successful in precopulatory sexual selection sire a lower percentage of offspring because their high mating success renders them sperm depleted (Danielsson 2000; Preston et al. 2001), whereas in fireflies, Photinus greeni, males that produce the most attractive bioluminescent courtship displays sire a lower percentage of offspring; possibly because of energetic trade-offs between courtship display and investment into ejaculates (Demary and Lewis 2007).

In the present study, we used Drosophila bipectinata Duda to test for a relationship between the expression of a secondary sexual trait, the male sex comb, and competitive fertilization success. In this species, the sex comb is comprised of stout black bristles, or “teeth,” arranged in 2 major oblique rows on the first tarsal segment of the front legs of males. Sex comb size in *D. bipectinata* (as the number of teeth) is significantly heritable and condition dependent (Polak et al. 2004; Polak and Starmer 2005). The sex comb is present in many *Drosophila* species, but it is not widespread within this genus. Rather, the sex comb occurs only in members of the *melanogaster* and obscura species groups of the subgenus Sophophora (Kopp and True 2002). Despite its relatively restricted phylogenetic distribution within *Drosophila*, the sex comb nevertheless is a rapidly evolving morphological innovation, exhibiting pronounced variation in both sizes, position, shape, and color, among closely related species (Bock 1971; Kopp and True 2002; Barmina and Kopp 2007). The microevolutionary mechanisms responsible for this rapid diversification, however, remain obscure.

The species we studied here, *D. bipectinata*, is a member of the ananassae subgroup of the melanogaster species group. It is distributed in the Australian and Oriental biogeographic zones (Bock 1978) and is itself undergoing sex comb diversification; recent work has revealed significant variation in sex comb size (as tooth number) among biogeographic populations throughout the species’ range and between it and its sister taxa (Bock 1971; Polak et al. 2004; Matsuda et al. 2005; Mishra and Singh 2006). Whereas several studies of precopulatory sexual selection have been conducted in different populations of *D. bipectinata* (Polak et al. 2004; Polak and Taylor 2007), as well as 2 other sex comb-bearing *Drosophila* species (*Drosophila simulans* and *Drosophila pseudoobscura*) (Markow et al. 1996), no consistent pattern of precopulatory sexual selection operating on sex comb size has emerged, engendering uncertainty over the role of precopulatory sexual selection in the evolution of sex comb size in these systems. The mating system of *D. bipectinata* is scramble competition; males chase and court females of orange pigmentation and engage in agonistic interactions with competitors to position themselves behind the female where they court her vigorously in an attempt to induce her to mate. A recent field study on *D. bipectinata* in Taiwan revealed that sex comb size did not predict male copulatory success (Polak, Hsu, unpublished data), despite sample sizes being sufficiently large to detect such an effect previously reported by Polak et al. (2004) for another population (Cape Tribulation, Australia) of this species. Here we test the hypothesis that sex comb size is under postcopulatory sexual selection in this Taiwanese population. Our study used genetic lines recently extracted from nature, which permitted the joint assessment of phenotypic (i.e., male ornament and body size) and genotypic (i.e., line of origin) influences on competitive fertilization success in relation to a condition-dependent secondary sexual trait.

**MATERIALS AND METHODS**

**Base culture and genetic lines**

A base culture of *D. bipectinata* was initiated with 280 females and an equal number of males captured in the field at fruit baits at the Center of Academic Activities, Academia Sinica, Taipei, Taiwan, 15–26 September 2006. The fly population was mass cultured in 28 plastic (45 ml) vials, with a food substrate composed of 2.0 g Instant Drosophila medium (Carolina Biological, Burlington, NC), 6.6 ml water, and 1.5 ml banana-live yeast slurry applied to the surface of the medium. Flies were cultured at 25 ± 1 °C and a 12:12 h light:dark cycle in a walk-in environmental room. After 3 generations of mass culture, 50 lines (families) were initiated, each with a single virgin female–male pair selected at random from the mass culture. Each pair was successively transferred to 2 culture vials, after which the males (sires) were characterized in respect to body size and comb size (see below). Emerging progeny from both vials were pooled and used to propagate each genetic line in a vial with exactly 10 randomly sampled individuals of each sex. Flies were allowed to oviposit for 24 h and discarded. These vials yielded the *F* 2 progeny. This same procedure was followed to produce the next 2 generations of progeny. The sperm competition experiment used *F* 3 (Block 1) and *F* 4 (Block 2) flies.

**Phenotypic traits**

Males were killed with ether fumes, and their foretarsi carefully pulled free from the body and placed onto double-sided transparent tape on a microscope slide. The numbers of teeth in sex comb segments 1 and 2 on each tarsus were counted under an Olympus SZX12 stereomicroscope against a white background; tooth counts are highly repeatable (Polak et al. 2004). Comb size is defined as the number of teeth summed across C1 and C2 (Figure 1) and averaged across body sides; these segments constitute the major components of the sex comb and are positively genetically correlated (Polak et al. 2004). Thorax length, as an estimate of body size, was measured using an ocular micrometer.

**Choosing test lines**

Sex comb size of the sires was used as a guide to select lines for characterization. Ten lines initiated by sires with the highest residual comb size, and 10 lines from sires with lowest residual comb size were selected. Thorax length and sex comb size of 5 randomly selected male progeny from the *F* 2 and *F* 3 generations for the 20 lines were determined. Analysis of covariance (ANCOVA) with line and generation (*F* 2 and *F* 3) as factors, and thorax length as covariate, was then used to test for comb size differences among lines and the stability of these differences across *F* 2 and *F* 3 generations. Preliminary runs showed that line, generation × line, and generation × covariate were nonsignificant (all *Ps >* 0.2). From these among 20 lines, the 4 lines exhibiting the highest and the 4 exhibiting the lowest body size-specific sex comb size were selected for sperm competition studies. Thus, there were 2 categories of lines: high (large comb lines) and low (small comb lines).
Radiation dosage and male sterility

Males to be used as donors of “defensive sperm” were sterilized with a sublethal dose of gamma radiation from a $^{60}$Co source. Irradiated (IR) sperm are able to fertilize eggs, but the zygote dies and fails to hatch as a result of lethal mutations (Simmons 2001). To select the appropriate dosage, groups of males were treated with a dosage series: 100, 150, and 200 gray (Gy). Radiation was administered while flies were anesthetized with nitrogen in a plastic vial loosely stoppered with cotton. Fifteen randomly selected males from each of these groups were each mated once to a 7-day-old virgin female. A control group of 8 males was handled in an identical manner but was not IR prior to mating. The singly mated females were individually held for 24 h in plastic shell vials containing an oviposition substrate (banana–yeast–grape juice–agar substrate). The number of eggs deposited over 24 h, and the hatch rate (number of eggs that produced first instar larvae divided by the total number of eggs laid) were determined. The average number of eggs (± standard deviation [SD]) deposited by females mated to males exposed to the different dosages was similar (0 Gy, 19.3 ± 8.5, n = 7; 100 Gy, 25.5 ± 12.1, n = 15; 150 Gy, 28.2 ± 13.7, n = 15; and 200 Gy, 17.4 ± 5.5, n = 15). Hatch rate was zero for all females fertilized by males exposed to 200 Gy. One egg hatched in the 150 Gy category. Five eggs hatched across 4 females at the 100 Gy dose. For females mated to control, unirradiated males, 74% (SD, 42.5; n = 7; range = 0–100%) eggs hatched. Because the dosage we desired for the sperm competition experiment was one that just yields a negligible hatch rate (Simmons 2001), we selected the 150 Gy dosage for the sperm competition study. In a subsequent test, 8 females were doubly mated to males IR with 150 Gy. These doubly mated females were transferred to fresh food vials and allowed to oviposit for 24 h, and the hatch rate was determined; only one egg hatched. The average number of eggs laid by these females was 33.6 eggs (SD, 23.6; n = 8; range = 6–64).

Sperm competitive ability

First matings

The sperm competition experiment was conducted as 2 time blocks in immediate succession. The source of females for both blocks was the general, mass-cultured base population from which the test lines had been extracted. Adult males for irradiation were also collected from the general mass culture on the day they emerged and held in single-sex groups in vials with food substrate for 6 days (Block 1) and 2 days (Block 2). Males were then exposed to a 150-Gy dose of radiation, as described above. Two days after radiation treatment, IR males in each block were paired individually with 110 virgin females in food vials; females were aspermatic first into individually numbered vials, and males subsequently were aspermatic individually into these vials. Vials with pairs of flies were lined up along a desktop and monitored continuously: vials were scanned in successive order by 2 observers between 0845 and 1300 h at room temperature (range: 23.2–24.3 °C). The onset and end times of all copulations were recorded; one copulation occurred per female. The difference between the onset and termination of copulation defined the copulation duration. In Blocks 1 and 2, 71 and 83 females mated with an IR male, respectively. All IR males that copulated were preserved in alcohol for later comb and body size determination (see above). Females that mated to an IR male were placed individually into vials with oviposition substrate and transferred daily to a fresh vial until their second mating. All eggs deposited by females between their first and second matings were counted; these values are referred to as “pre-$P_2$ eggs” and reflect reproductive rates of females over this time period.

Second matings

The second males to mate came from the 8 genetic test lines (4 small comb and 4 large comb lines). An equal number of virgin males were harvested from 3 replicate culture vials per line on the day they emerged, gently mixed while anesthetized under a light stream of CO$_2$, and randomly assigned to groups of 15. These groups were aged in food vials until use. Males were mated to females when they were 3–7 days old; males from the different lines used on any given day were the same age.

Second matings were administered as follows. An equal number of males from each of the 8 test lines were aspirated into numbered agar vials lined up along a desktop. The males from the different lines were randomly interdigitated along the desktop. The previously mated females were then loaded individually into the vials, and the time of this event recorded. Beginning and end times of all copulations were recorded, and the latency (time elapsed from the introduction of the female to the onset of copulation) and copulation duration were ascertained for all copulations. Females were transferred to fresh oviposition vials no more than 4 h after their second copulation and allowed to oviposit for 24 h. Vials were vacated, and all eggs counted. Vials were checked every 12 h for 2 subsequent days for hatched larvae, and all larvae were counted.

In the first block, the previously mated females were exposed to second males 3, 5, and 7 days after their first mating. On these days, 11/71 (15%), 7/60 (12%), and 4/53 (8%) females remated. All second males were preserved in alcohol for later characterization. The time (in days) elapsed between a female’s first and second copulation is referred to as the “intercopulation interval.” In the second block, 83 previously mated females were paired with test males in vials 7 days after the first mating, of which 33 (40%) females remated. Because the intercopulation interval did not significantly affect $P_2$ values (see Calculation and analysis of P$_2$), for the sake of efficiency, we exposed females to second males on day 7 only (i.e., days 3 and 5 used in Block 1 were eliminated in Block 2). Thus, the total sample size was 55 across the blocks. The frequency at which large and small comb second males mated was analyzed using a chi-square test for each block separately. We also evaluated the effect of comb size, male body size, and pre-$P_2$ eggs on latency to copulation for second males using multiple
regression. Copulation latency was square-root transformed; the distribution of residuals was reasonably close to normal (Wilk–Shapiro $W = 0.95, P = 0.015$) and distributed evenly across predicted values of latency.

**Calculation and analysis of $P_2$**

$P_2$, the proportion of offspring sired by the second male to mate, was calculated as the number of eggs that hatched divided by the total number of eggs deposited by a given female (Boorman and Parker 1976; Simmons 2001). $P_2$ data were arcsine square-root transformed prior to analysis.

In some cases, the second male to mate failed to fertilize any eggs so that some $P_2$ values equaled 0. We analyzed the relationship between the probability of this outcome and traits of interest using logistic regression; factors were Block (1 and 2) and Line Category (Large and Small comb size), and the covariates were pre-$P_2$ eggs, first male’s (male 1) comb size, male 1 thorax length, male 2 comb size, and male 2 thorax length.

The $P_2$ data were analyzed with a generalized linear model (GLM, SAS Institute 2001). We included a total of 13 independent terms that we expected could explain variation in $P_2$. The factors were Block (1 or 2), Line Category (high or low), Line (1–4), and the Intercopulation Interval (3, 5, and 7 days); the Block × Line Category interaction was also entered. Line was nested within Line Category and treated as a fixed factor. Continuous variables were comb size of males 1 and 2, thorax length of males 1 and 2, copulation duration of males 1 and 2, pre-$P_2$ eggs, and the interaction between comb size of males 1 and 2. We sequentially eliminated from the model terms that did not explain a significant or near-significant ($P < 0.1$) portion of the variation in the dependent variable (in fact, none of the $P$ values for the removed terms were $< 0.2$). We settled on a final, reduced model consisting of 3 terms (Line Category, Line nested within Line Category, and sex comb size of male 2). As a final check, we entered into this model the difference in comb sizes between the 2 males and verified that this term was not significant ($P = 0.82$). The difference in the overall explanatory power ($r^2$) of the initial full model and the final reduced model was 0.04. The residuals of the final analysis were normally distributed in both Line Categories and all Lines (Shapiro–Wilk tests, all $P > 0.1$) and in the overall data set ($W = 0.98, P = 0.6$). Residual variances were homogeneous across Line Categories (Levene’s test, $F_{11.93} = 0.01, P > 0.9$) and Lines ($F_{19.94} = 0.66, P = 0.7$). In a separate model, interactions between the covariate and Line Category ($F_{1.39} = 1.25, P = 0.27$) and Line (Line Category) ($F_{5.39} = 0.92, P = 0.49$) were not significant, satisfying the homogeneity of slopes assumption of ANCOVA.

**RESULTS**

**Comb size variation**

ANCOVA, with line and generation as factors and thorax length as covariate, revealed significant differences in sex comb size among the 20 genetic lines (Table 1). The effect of generation and the line × generation interaction were not significant, demonstrating stability in the difference in ornament size across consecutive generations. Four high and 4 low genetic lines (Figure 2) were selected for use in the sperm competition experiment.

**Sperm competition**

The total number of eggs laid by females used to calculate $P_2$ ranged from 8 to 64 ($\bar{x} \pm SD = 35.0 \pm 14.7, n = 22$) in Block 1 and 9 to 46 ($27.3 \pm 9.7, n = 33$) in Block 2. Across blocks, $P_2$ (untransformed) values ranged from 0 to 1 ($0.493 \pm 0.36, n = 55$; Figure 3).

In the first block, large and small comb second males mated with equal frequency: 38% and 39% ($\chi^2 = 0.0084$, degrees of freedom $df = 1, P > 0.9$) of males from large comb and small comb lines, respectively, mated with females that had previously been mated to an IR male. In the second block, a significantly larger fraction of large comb males (82%) than small comb males (60%) mated ($\chi^2 = 4.84, df = 1, P < 0.05$). Multiple regression revealed nonsignificant effects of comb size ($F_{1.51} = 0.017, P = 0.90$), male thorax length ($F_{1.51} = 0.10, P = 0.75$), and pre-$P_2$ eggs ($F_{1.51} = 0.00, P = 1.0$) on latency to the second mating.

The probability of whether a second male failed to fertilize any eggs was analyzed by multiple logistic regression, and this probability was found to be similar across the 2 blocks of the experiment; $P_2$ equaled 0 in 3/22 (14%) second matings in Block 1 and 4/33 (12%) in Block 2 ($\chi^2 = 0.8, df = 1, P = 0.25$). In contrast, there was an effect of comb size on this outcome, such that the probability of 100% fertilization failure decreased significantly as comb size increased ($\alpha$ standard error $\pm 0.38$, $\chi^2 = 4.5, P = 0.034$). All other factors/covariates in the logistic regression model were nonsignificant ($P > 0.10–0.91$).

### Table 1

Results of ANCOVA, with thorax length as covariate, showing the effects of genetic line and generation ($F_2$ and $F_3$) on sex comb size

<table>
<thead>
<tr>
<th>Source</th>
<th>Mean square</th>
<th>df</th>
<th>$F$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thorax length</td>
<td>3.60</td>
<td>1</td>
<td>0.74</td>
<td>0.39</td>
</tr>
<tr>
<td>Line</td>
<td>33.76</td>
<td>19</td>
<td>6.94</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Generation</td>
<td>1.36</td>
<td>1</td>
<td>0.28</td>
<td>0.60</td>
</tr>
<tr>
<td>Line × Generation</td>
<td>6.92</td>
<td>19</td>
<td>1.42</td>
<td>0.12</td>
</tr>
<tr>
<td>Error</td>
<td>4.87</td>
<td>159</td>
<td></td>
<td></td>
</tr>
</tbody>
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![Figure 2](image_url)  
**Figure 2** Least-squares mean (±1 standard error, $n = 10$ males/line) sex comb size identifying genetic lines subjected to tests of sperm competitive ability.
Copula duration did not significantly predict \( P_2 \) for either the first (\( b \pm SE = 2.61 \times 10^{-3} \pm 3.30 \times 10^{-5}, F_{1,52} = 0.0002, P = 0.94 \)) or second (\( 5.09 \times 10^{-3} \pm 3.79 \times 10^{-5}, F_{1,52} = 1.80, P = 0.19 \)) copulations. ANCOVA likewise revealed nonsignificant effects of Block (\( F_{1,42} = 1.94, P = 0.17 \)), Line Category (\( F_{1,41} = 2.46, P = 0.12 \)), or Line (\( F_{3,42} = 0.81, P = 0.50 \)) on the duration of the second copulation.

Results of the main ANCOVA revealed significant effects of the second male’s comb size on \( P_2 \) (Table 2). The leverage plot in Figure 4 illustrates the positive effect of comb size on \( P_2 \). The effects of Line Category and Line were nonsignificant (Table 2; Figure 5). This analysis was repeated on a data set from which all \( P_2 \) values (\( n = 7 \)) were excluded, to ascertain the degree to which our conclusion might be sensitivity to the inclusion of these data. The effects in the reanalysis were as follows: Line Category, \( F_{1,39} = 0.93, P = 0.34 \); Line (Line Category), \( F_{6,39} = 1.78, P = 0.13 \); and Male 2 comb size, \( F_{1,39} = 10.5, P = 0.002 \). Thus, the conclusion that increasing sex comb size reveals superior sperm competitive ability is supported by both analyses.

**DISCUSSION**

For the first time in any *Drosophila* species, we show that the expression of the male sex comb size is associated with enhanced competitive fertilization success. Thus, our results identify postcopulatory sexual selection as a previously unrecognized selective mechanism that may be contributing to ornament diversification in this group of insects. Our protocol involved extracting genetic lines from a natural population developing relatively large and small sex combs, rearing these lines under common environmental conditions in the laboratory, and subjecting randomly sampled males from these lines...
to an assay of competitive fertilization ability. The experimental design we employed thus had the advantages of permitting the joint assessment of the genotypic and phenotypic contributions to competitive fertilization success and, through the use of significantly divergent genetic lines, enhancing the sensitivity of our test for the effects of comb size on $P_o$. Moreover, by incorporating multiple lines from either extreme of the natural ornament size distribution, we avoided the possibility that any associations observed between ornament size and fertilization success could be driven by the unusual property of any one or a few naturally segregating genotypes.

We uncovered a significant positive relationship between the size of the male sex comb and fertilization success among males mated experimentally to nonvirgin females, demonstrating that sex comb size reveals superior "offensive" fertilization success in this Taiwanese population of $D. bipunctata$. Importantly, this relationship was strongly significant despite accounting statistically for variation in the genetic background (i.e., line of origin), suggesting that phenotypic variation in this sexual ornament per se predicts male fertilization success. Future work will assess the degree to which comb size might also predict defensive fertilization success.

The relationship we observed between ornament size and offensive fertilization success may be the result of either sperm competition and/or cryptic female choice (Thorhill 1983; Simmons 2001; Birkhead and Pizzari 2002). The former hypothesis would require that ornament size be positively correlated with components of offensive sperm competitive ability, for example, such as sperm numbers, sperm viability, or concentration of bioactive peptides in the ejaculate derived from males' accessory glands (Simmons 2001); indeed, some Acps in $Drosophila$ are known to mediate competitive fertilization success, a subset of which may function specifically in the enhancement of offensive sperm competitive ability (Ram and Wolfner 2007). Other studies have documented a link between the expression of a secondary sexual trait and different aspects of ejaculate quality. For example, in capercaillies, $Tetrao urogallus$, the amount of sperm in an ejaculate is positively correlated with the rate of male courtship display (Mjelstad 1991). Likewise, in field crickets, $Gryllus lineaticeps$, male chirp rate in the calling song is positively correlated with sperm number in the ejaculate, which translates to elevated female lifetime fecundity and fertility (Wagner and Reiser 2000; Wagner and Harper 2003). In guppies, $P. reticulata$, males with more carotenoid pigmentation displayed on their bodies produce faster swimming and more viable sperm than less attractive males (Locatello et al. 2006) and are competitively superior in gaining fertilizations (Evans et al. 2003). The mechanisms underlying these different effects in guppies are unknown, but perhaps the relationship between ornamental display and sperm quality is the result of a mutual dependency on dietary carotenoid intake (Blount et al. 2001) or more generally on overall phenotypic quality (i.e., body condition) of the males (Evans et al. 2003).

This latter explanation may well apply to the results presented here as sex comb size in $D. bipunctata$ is known to be significantly condition dependent (Polak and Starmer 2005), rendering the possibility of a resource-based link between comb size and ejaculate quality feasible. In theory, males of a given species that invest most into secondary sexual trait expression may be of highest phenotypic condition in the population as a result of their superior ability to acquire and assimilate resources for allocation to competing physiological functions (Rowe and Houle 1996; Bonduriansky and Rowe 2005). By extension, males sporting the most well-developed ornaments may also be those able to invest most into ejaculate quality and associated structures (e.g., testes; Möller and Erritzoe 1988; Sheldon 1994). In $D. bipunctata$, because the male sex comb is composed of rows of stout, melanized teeth (modified bristles), internally made up of structural protein (actin) filaments (Tilney et al. 2000), variation in ornament size may reveal individual feeding history, and their ability to accumulate body supplies of critical amino acids and other essential nutrients, thereby driving an association between ornament size and ejaculate quality (see also Amitin and Pitnick 2007).

The positive relationship between comb size and competitive fertilization success could also be a function of cryptic female choice. In $Drosophila$, the female influences insemination and sperm transfer from the uterus into her sperm storage organs via nervous system control (Arthur et al. 1998), so it is possible that males with larger combs stimulate the female via her peripheral nervous system in such a way as to induce her to retain sperm and in the event of successful insemination, to shunt more sperm into storage. As the male presses his foretarsi against the sides of the female’s abdomen during late stages of precopulatory courtship, the sex comb on either leg of the male directly contacts the female (Cooperman et al. 2007), at which point she may be receiving the requisite tactile cues concerning the size of the sex comb; males, however, do not actively brush or rub the female with the combs, as male $D. silvestris$ do with their foreleg cilia (Carson and Teramoto 1984). Alternatively, comb size could be correlated with some aspect of courtship performance which itself induces females to preferentially utilize sperm. Indeed, copulatory behavior in $Drosophila$ stimulates females to release previously stored sperm, thus increasing fertilization success of the current male (Snook and Hosken 2004). Either effect would represent a kind of male behavioral conditioning of a female physiological response to utilize sperm (Eberhard 1996), as has been documented, for example, in $T. castaneum$ beetles (Edvardsson and Arnqvist 2000). These authors showed that the intensity with which a male beetle rubs his legs on the lateral edges of the female’s wing cases is positively associated with the fertilization success of his ejaculate when in competition with that of a control male (see also Sirot et al. 2007).

In addition to finding a significant effect of comb size on paternity, we found that males with smaller combs suffered a higher probability of failure to fertilize any eggs laid by the female, although there was a total of only 7 cases in which this occurred across both blocks (i.e., where $P_o = 0$). Whereas the reason for these instances is unknown, it may be that they represent failed inseminations, which could similarly result from a female-mediated process, involving, for example, active ejection of the sperm of males with small sex combs. In some species of $Drosophila$, females expel sperm from the uterus after mating (Alonso-Pimentel et al. 1994), possibly as a means of biasing paternity in favor of particular males. In domestic jungle fowl ($Gallus gallus domesticus$), females can eject semen immediately after insemination through clausal contractions, a mechanism they apparently can use to bias paternity in favor of socially dominant males (Pizzari and Birkhead 2000).

In contrast, the available evidence suggests little, if any, precopulatory sexual selection for comb size in the studied population. Indeed, in the present study, we found that latency to copulation was unrelated to male sex comb size, a relationship predicted to be negative if females were more willing to accept more ornamented males as mates. Likewise, comb size was unrelated to the probability of mating in the first block. In the second block, however, we did find that a significantly larger fraction of males from large comb lines mated compared with males from small comb lines, representing a sole source of evidence (however indirect) for the existence of precopulatory sexual selection favoring increased ornament size.
In contrast, a large-scale field study (conducted at the same time and location where the population for the present study was collected) revealed a nonsignificant relationship between sex comb size on mating probability on natural fruit substrates in Taiwan (Polak M, Hsu Y, unpublished data). This lack of evidence for precopulatory sexual selection in the wild was not the result of low statistical power. A power analysis (Zar 1998) showed that this field study had 99% power to detect a 3% difference in sex comb size between mated and single individuals; an effect size of this particular magnitude was significant in a previous field study but in a different population in northeastern Australia (Polak et al. 2004). Thus, phenotypic variation in ornament size appears to be effectively neutral in respect to precopulatory sexual selection, at least in the Taiwanese population studied here. Thus, when taken together, these studies reveal a lack of consistency between pre- and postcopulatory sexual selection linked to secondary sexual trait size (and see Danielsson 2000; Pizzari et al. 2002; Demary and Lewis 2007) and emphasize the importance of evaluating the roles of both these processes to understand the net force of sexual selection that may be influencing secondary sexual traits.

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