RESEARCH PAPER

Microscale Laser Surgery Demonstrates the Grasping Function of the Male Sex Combs in *Drosophila melanogaster and Drosophila bipectinata*

Jorge L. Hurtado-Gonzales, Wesley Gallaher, Alexandra Warner & Michal Polak

Department of Biological Sciences, University of Cincinnati, Cincinnati, OH, USA

Abstract

Male secondary sexual traits of animals are richly diversified in form and complexity, yet there are many species in which their precise function remains unknown. Within the genus *Drosophila,* species belonging to the *melanogaster* and *obscura* species groups have evolved a remarkable variety of sex combs, male-limited secondary sexual traits located on the tarsi of both front legs. Information concerning sex comb function is minimal or absent, except for *D. melanogaster*, where previous studies indicate that the sex combs are used for grasping the female prior to copulation. These studies, however, do not unambiguously demonstrate comb function, because it has not been possible to ascribe observed behavioral outcomes of the various comb manipulations to changes in the combs *per se*. We used microscale laser surgery to manipulate comb size in *D. melanogaster* and *D. bipectinata,* and tested the hypothesis that the sex combs function as grasping devices in courtship, making them essential for copulation to ensue. Results of high-resolution behavioral analysis in small observation arenas demonstrated that in both species in which sex combs were surgically eliminated, males were unable to grasp, mount or copulate. The combless foretarsi of these altered males slipped off the end (*D. melanogaster*) and sides (*D. bipectinata*) of the female abdomen when courting males attempted to grasp. In most cases, males whose sex combs were reduced but not completely removed exhibited similar copulation probabilities as surgical control males, a result we demonstrated in observation chambers as well as under more ecologically realistic conditions inside population cages where males and females interacted on the surface of fruit substrates. Thus, the sex combs in *D. melanogaster* and *D. bipectinata* are grasping devices, essential for mounting and copulation.

Introduction

Secondary sexual traits, such as bright colors, extravagant plumes, antlers and elaborate courtship displays, evolve in response to competition for reproductive opportunities (Darwin 1871; Andersson 1994; Arnqvist & Rowe 2005) and may function to promote fitness before, during or after mating (Eberhard 1985; Andersson 1994; Birkhead & Pizzari 2002; Chapman et al. 2003). In many cases, divergence of secondary sexual traits is conspicuous among closely related species (Ryan et al. 1992; Barraclough et al. 1995;

Owens & Bennett 1997; Prum 1997; Seehausen & van Alphen 1999), and even among populations within species (Young et al. 1994; Endler & Houde 1995; Uy & Borgia 2000), indicating that sexual selection can fuel relatively rapid evolutionary change compared to traits not subject to sexual selection (Lande 1981; Panhuis et al. 2001; Emlen 2008).

In *Drosophila*, males of the *melanogaster* and *obscura* species groups exhibit prominent secondary sexual traits known as sex combs (Lakovaara & Saura 1982; Lemeunier et al. 1986). The sex combs are comprised of stout black bristles, or 'teeth', that develop on the

front foretarsal segments, and which make contact with the female during courtship and mating (Spieth 1952). In some species, such as *D. bipectinata*, sex comb size is condition dependent (Polak & Starmer 2005), and significantly heritable (Polak et al. 2004; Ahuja & Singh 2008), evidence for the trait's potential to undergo evolutionary diversification (Polak et al. 2004; Barmina & Kopp 2007).

Sex combs show dramatic structural differences in the number of teeth, axial orientation, and number of rows among species (Kopp & True 2002; Kopp 2011). Thus, it is not surprising that sex combs serve different functions during courtship and copulation (Spieth 1952) and that they can experience different patterns of sexual selection (e.g., in direction and intensity). For example, in a natural population of *D. simulans* in Arizona, copulating males had significantly fewer sex comb teeth compared to single males, revealing sexual selection for reducing comb size in this population (Markow et al. 1996). In contrast, significant sexual selection for increasing comb size was detected in Australian populations of *D. bipectinata* (Polak et al. 2004; Polak & Simmons 2009), but this effect has not been consistently found throughout the species' range (Polak & Taylor 2007).

Sex combs are microscopic structures (e.g., in *D. melanogaster* each tooth ≈ 40 μm in length and 8 μm in width), and thus their experimental manipulation poses challenges. Previous studies that have examined the function of the sex combs have attempted various removal techniques: amputation of foreleg segments (*D. persimilis* and *D. pseudoobscura*: Spieth 1952; *D. simulans* and *D. mauritiana*: Coyne 1985), breaking off the sex comb teeth with fine forceps (*D. simulans* and *D. melanogaster*: Cook 1977), or by genetic modification (*D. melanogaster*: Ng & Kopp 2008). However, these methods are not always ideal because they can lead to injury or to accompanying alterations to traits other than the sex comb. Thus, additional methods for studying comb function should employ fine-scale, precision techniques that would alter comb size without inflicting collateral damage, so that any observed outcomes of the manipulation can be ascribed to changes in the comb *per se*.

Additionally, the quantification of copulation behaviors of treated and control males will be important to identify the precise function of the comb. Most previous studies have not monitored behavior of treatment males (but see Cook 1977). For example, the work of Spieth (1952) and Coyne (1985) placed different groups of experimental males into copulation arenas with test females (i.e., 5 males of a given treatment with 10 females per vial) for 6–24 h. Rates

of insemination were then measured by dissecting female reproductive tracts and inspecting them for the presence or absence of sperm. Whereas insemination success of experimental combless males was significantly reduced relative to males with intact sex combs, the approach of these studies did not elucidate the precise cause of this effect. Moreover, a recent study in which the sex combs of *D. melanogaster* males were genetically ablated, behavioral measurements mainly were designed to verify that the genetic manipulations did not alter normal behavior of experimental males (Ng & Kopp 2008).

The purpose of the present study is to solidify our knowledge of comb function in *D. melanogaster* and *D. bipectinata*. We employ a precision laser surgical technique (Polak & Rashed 2010) to experimentally remove and reduce the size of the sex comb by ablating individual teeth one at a time. In *D. melanogaster*, males express a single row of teeth situated on the first tarsal segment (the metatarsus) of the foreleg (Fig. 1a; Bock & Wheeler 1972). Previous observations of the copulation behavior of unmanipulated males in *D. melanogaster* suggest that males are required to couple the genitalia before mounting and that the combs are involved in grasping the female genitalia (oviscape) prior to genital coupling (Cook 1975). In contrast, the sex comb in *D. bipectinata* occurs as two major rows of teeth, which we refer to as C1 and C2 (Fig. 1b), located on the first tarsal segment, in addition to a third group of fewer teeth on the second tarsal segment (Bock 1971). The grasping function of the sex combs in *D. bipectinata* has been likewise inferred from observation of intact males; in contrast to *D. melanogaster*, males grasp the female's lateral abdominal flanks with their foretarsi prior to genitalia coupling (Cooperman et al. 2007).

We tested the hypothesis that the sex combs in *D. melanogaster* and *D. bipectinata* are necessary for copulation because they are required for grasping the female. We used non-competitive copulation trials to measure the effects of surgical treatments on copulation success and courtship behavior, evaluating the predictions that males without sex combs should be unable to grasp and copulate with test females. Our experimental design also incorporated partial excision of the sex comb (removal of all but the 5 most distal or proximal teeth in *D. melanogaster,* and removal of the first or second major segments of the comb in *D. bipectinata*) to assess the degree to which a less severe reduction of sex comb size would influence mating performance of males. These partial excisions reduced comb size beyond the range observed in intact males. We also tested the effects of both experimental
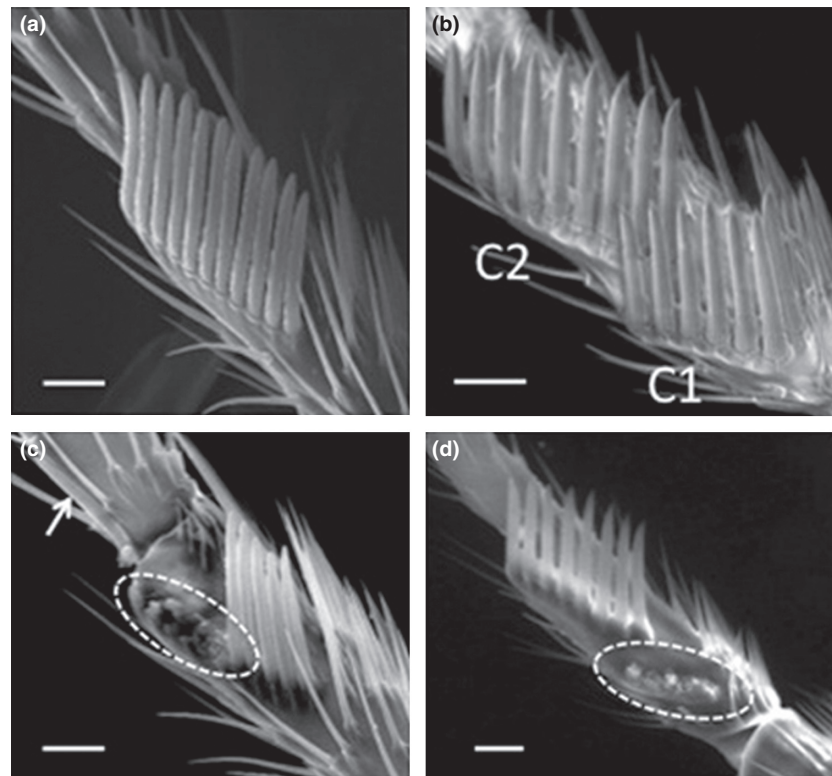
Fig. 1: Scanning electron micrographs (650X) of the sex combs of the studied species. Intact combs of *D. melanogaster* and *D. bipectinata* are shown in a and b, respectively. c: 'distal-cut' treatment in *D. melanogaster* resulting in the removal of all but 5 proximal teeth; the circled area encompasses the previous location of several distal teeth, and the arrow indicates an excised tooth fallen to the side. d: 'c1-cut' treatment in *D. bipectinata*, with the circled area encompassing the previous location of C1 (i.e., the proximal row of teeth). All scale bars 20 μm.

removal and reduction of the sex comb on copulation success in the presence of male–male competition within population cages wherein flies interact on the surface of fruits. This experiment was designed to simulate natural conditions more closely than the above non-competitive assays conducted in small observation chambers, which were used to study courtship behavior.

Methods

Experimental Flies

Stock populations of *D. melanogaster* and *D. bipectinata* were derived from 50 and 30 inseminated females (together with an equal number of males) caught from a wild population at Cape Tribulation, northeastern Queensland, Australia in 2005 and 2007, respectively. Stocks were mass cultured in 240-mL milk bottles containing 12 g of Instant *Drosophila* medium[©] (Carolina Supply Co., Burlington, NC, USA), and 53 ml of water with live yeast added, and under a 12 h light (25°C):12 h dark (23°C) photoperiod. Experimental male flies were collected as virgins within 6 h of eclosion, sorted under a light stream of humidified CO_2, and held in single-sex vials containing standard cornmeal-agar *Drosophila* medium in

groups of 10–15 until laser treatments were administered. Following laser treatments, males were held in single-sex cornmeal-agar vials in groups of 5–8 males. Males throughout were held in vials without yeast.

Laser Surgical Treatments

The sex combs of 24- to 36-h-old male *D. melanogaster* and *D. bipectinata* were ablated using the laser surgery protocol described in Polak & Rashed (2010). We produced full-cut males of both species by ablating all teeth at their base one at a time from both forelegs. Partial-cut male *D. melanogaster* consisted of removing all but the five most distal teeth (proximal-cut) or all but the five most proximal teeth (distal-cut) (Fig. 1c). In *D. bipectinata*, c1-cut and c2-cut treatments were administered by fully ablating either the first (C1) or second (C2) rows on both front legs, respectively (Fig. 1d). These surgical size reductions were beyond the range of intact males for both species: for the population of *D. melanogaster* used here, comb size of intact males ranges from 9 to 15 teeth per tarsus (N = 172), and for *D. bipectinata*, comb size ranges from 2 to 8 and 5 to 11 teeth per tarsus (N = 1118) in C1 and C2, respectively (and see Polak et al. 2004). All surgeries took ≈ 3 min each to complete. For each species, we used two control groups, as follows: (i)

sham control males were exposed to the laser light but not contacted by it (laser shots were delivered ≈ 20 µm away from the tarsal sections of the forelegs); (ii) surgical control males had four randomly selected non-sex comb bristles in proximity to the combs excised at their base from both forelegs. All males were used in copulation trials no sooner than 3 d post-treatment. There was no mortality in any experimental group prior to copulation trials. However, we excluded from our experiments control (N = 7) and treated (N = 3) males that had visible defects not related to the laser surgery (e.g., a bent or tattered wing) that potentially could have affected mating behaviors. All experiments were carried out between 0800 (fluorescent lights on) to 1100 h (23–24°C).

Non-Competitive Copulation Experiment

Non-competitive copulation trials involved pairing one male with one virgin female in individual observation chambers (chamber dimensions: 12.5 mm diameter, 6 mm depth; as in Grieshop & Polak 2012). A total of 85 male *D. melanogaster* (19 sham controls, 19 surgical controls, 14 distal-cut, 16 proximal-cut, and 17 full-cut males), and 95 male *D. bipectinata* (25 sham and 18 surgical controls, 11 c2-cut, 14 c1-cut,

and 27 full-cut males) were used. Male *D. melanogaster* and *D. bipectinata* were on average 6.21 (SD 1.54) and 8.77 (SD, 3.15) days old, respectively. On any given day, males across experimental categories of a given species were the same age. Females were 3–8 d old for *D. melanogaster* and 5–14 d old for *D. bipectinata*. As for males, females in all experimental categories were the same age on any given day of experimentation. Females of both species were collected as virgins and aged in sex-specific vials (15–20 females per vial) containing standard cornmeal-agar food, and active dry yeast sprinkled on the surface of the food.

Flies were kept in the dark until recording. After 5–10 min of being moved into the light, pairs were aspirated into chambers, and given 60 s to acclimate. All behavioral activities up to 10 min or until copulation ended were recorded using a Hitachi KP-D50 video camera. The camera was positioned above the chamber, and recordings were made through an Olympus SZX12 stereomicroscope, permitting high-resolution analysis of courtship behaviors. Video signals were streamed directly onto a Power Mac G5 hard drive for frame-by-frame analysis. From the videos and with no knowledge of the treatments to which males belonged, we extracted frequencies of specific male courtship behaviors (Table 1). Courtship latency was the time elapsed between the beginning of the record-

Table 1: Descriptions of courtship behaviors in male *D. melanogaster* and *D. bipectinata* quantified in the present study (from Spieth 1952; Cook 1975; Crossley 1986; Cooperman et al. 2007)

Behavior	*D. melanogaster*	*D. bipectinata*
Wing vibrations	A bout of wing vibrations occurs when a male extends and vibrates one or both wings in close proximity to the female. Wing extends often to right angle with the body. May occur as male moves around the female while facing her	Occurs in *D. bipectinata*, but was not quantified in the present study
Grasp attempt	Male approaches female from behind, extends his foretarsi forward and toward her, but achieves little if any sustained traction	
Lunge		Male briskly moves his body toward the female while extending his foretarsi toward the female, generally from behind her
Grasp	Grasp attempt leads to the male gaining a hold of the female's distal end, often her extruded genitalia. At the same time male curls his abdomen downward and partly forward, but disengages before genital contact is made	Upon lunging, male gains hold of the female by pressing his foretarsi against the lateral sides (flanks) of the female's abdomen. At the same time male curls his abdomen downward and partly forward, but disengages before genital contact is made
Grasp-abdominal curl	Male grasps a female by her genitalia and his abdomen is bent downward and forward. Male probes with the tip of his abdomen, aligning his genitalia with that of the female for copulation. Persistent probing, genital contact and copulation attempts are typically made	Male grasps the female along her abdominal flanks, pulls toward her and often slightly on top of her distal abdomen; bends his abdomen downward and forward. Male probes with the tip of his abdomen, aligning his genitalia with that of the female. Persistent probing, genital contact, and copulation attempts are typically made

ing session to when any aspect of courtship was first seen. Following behavioral recordings on a given morning, male and female thorax lengths were measured, and male sex comb treatments were verified under a stereomicroscope. For *D. melanogaster* and *D. bipectinata*, 2 and 5 females were damaged or escaped, respectively, so female thorax length could not be determined for these females.

The frequencies of successful copulations were contrasted between experimental categories using contingency table analysis (Zar 2010). All courtship behaviors of males for both species are expressed throughout as rates (Cook 1975). Rate of a behavior was calculated as the frequency at which the behavior occurred divided by the time (in minutes) over which the behavior was tallied (either the time elapsed from the beginning of the observation trial to the onset of copulation, or 10 min of observation for cases where copulation did not occur).

Rates of behaviors were $\log_{10}(y + 0.01)$-transformed to improve fit to the normal, and analyzed with analysis of covariance (ANCOVA) (Neter et al. 1990). For *D. melanogaster*, we quantified grasp attempts, grasps, and grasp-abdominal curls, and for *D. bipectinata*, we quantified lunges, grasps, and grasp-abdominal curls (Table 1). Treatment category was entered as the categorical factor, and male and female thorax lengths (mm) were entered as co-variables. Preliminary analyses indicated that there was no significant effect of male thorax length on the rate of any behaviors for either species (all p values > 0.30), thus male thorax length was excluded from all analyses. Female thorax length was negatively related only with rate of male gasp-attempts ($\hat{b}$, SE = −2.60, 1.607, t = −2.43, p = 0.017), so female thorax length was entered as a co-variable in the analysis. For consistency, female thorax length was entered as a co-variable for all the above behaviors for both species. To aid interpretability, back-transformed least-squares means are presented with associated 95% confidence intervals (note, these limits are asymmetrical around the mean) (Sokal & Rohlf 1981). To assess whether surgical treatment had a harmful effect on male sexual motivation, we examined the effect of treatment on courtship latency in both species, and the rate at which males performed wing vibration bouts in *D. melanogaster*.

Competitive Copulation Trials in Population Cages

Competitive copulation trials were performed in population cages designed to measure copulation success under conditions where multiple males competed for access to females on fruit substrates. We used non-virgin females, given that in nature females encountered by courting males are likely to be already inseminated (Polak et al. 2004). Prior to the copulation trial, 2-d-old virgin females were aspirated into 8 fluid dram polystyrene vials (15–20 per vial) lined with cornmeal-agar substrate containing an equal number of virgin males, and allowed to interact and mate with males for 24 hours. Females were then sorted and held in groups of 20 in single-sex vials for an additional 4 d (females were 7 d old when they were used in a given copulation trial).

Two copulation trials, each on a separate day, were conducted for the two species. A copulation trial consisted of documenting copulations occurring in a population cage, built of clear acrylic (L × W × D: 24 cm × 11.5 cm × 11.5 cm), the ends of which were sealed with mesh to permit air flow through the cage, and contained three flat squares of freshly cut papaya (*c.* 110 g each). For *D. melanogaster*, females (N = 50) were aspirated into the cage at 0600 h on the day of the trial and allowed to acclimate for 1 h before loading experimental males (10 sham controls, 10 surgical controls, 10 full-cut, 10 proximal-cut, and 10 distal-cut). For *D. bipectinata*, females were aspirated into cages the day before the experiment at 1700 h and allowed to acclimate overnight. Forty males (5–9 from each of the categories, that is, sham control, surgical control, full-cut, c1-cut, and c2-cut) and females (N = 40) were introduced the next day at 0700 h.

For both species, upon introducing the males, a dim fluorescent light was turned on first 2 m from the cage, and 1 h into the trial full overhead lights were turned on. Each cage was kept under constant observation for 2 h from introducing the males, or until *c.* 50% of flies copulated; copulating pairs were removed from the cage using an aspirator as they formed. Copulation latency for each pair was recorded as the time elapsed between the time males were loaded into the cage and the onset of copulation. Immediately following the trial, all remaining flies were aspirated from the cage. We measured thorax length of each fly and determined the identity of each male in respect to its surgical treatment under a stereomicroscope.

The effects of copulation success on surgical treatments were evaluated using multiple logistic regression (Hosmer & Lemeshow 1989) with copulation success as the binary response variable (1 = yes, 0 = no), and block (cages 1 and 2) and surgical treatment (5 levels, see above) as categorical factors, and female and male thorax lengths as covariates.

Non-significant terms (p > 0.05) were sequentially excluded from the model. For both species, the final model ended up including surgical treatment only. Copulation latency was $\log_{10}(y + 0.01)$-transformed, and differences among treatments were assessed with ANOVA; back-transformed means with 95% confidence intervals are presented. Preliminary analyses indicated that for both species the effects of male thorax length, female thorax length, and block on copulation latency were not significant, and so were excluded from reported models. All analyses were performed in JMP (ver. 9.0.2 SAS Institute 2011).

Results

Non-Competitive Copulation Trials: Copulation Success

When males were paired singly with virgin females, there was a strong effect of laser surgery treatment on the frequency of copulation in both species (*D. melanogaster*: $\chi^2 = 38.08$, df = 4, p < 0.0001; *D. bipectinata*: $\chi^2 = 52.51$, df = 4, p < 0.0001). Male *D. melanogaster* whose combs were fully ablated failed to achieve even a single copulation, compared to copulation probabilities of *c.* 70% for sham and surgical controls (Fig. 2a). Similarly, for *D. bipectinata*, although full ablation of the combs did not eliminate male ability to copulate, full-cut males nevertheless achieved a sharply reduced copulation probability compared to either control group (Fig. 2b).

Partial ablation of the combs had no statistical effect on copulation probability in either species (Fig. 2a, b); removal of the full-cut treatment from the above full contingency table analysis eliminated the statistically significant effect of surgical treatment (*D. melanogaster*: $\chi^2 = 1.99$, df = 3, p = 0.57; *D. bipectinata*: $\chi^2 = 6.07$, df = 3, p = 0.11). The lack of a statistical effect of partial ablation strengthens the conclusion that comb removal *per se* eliminates or sharply reduces (in the case of *D. bipectinata*) copulation, because it indicates that contacting the comb with the laser beam itself cannot be the cause of copulation failure exhibited by full-cut males. That laser contact itself has no ill effects is also indicated by the result that copulation probability did not differ between sham and surgical controls.

Non-Competitive Copulation Trials: Behavior

In *D. melanogaster*, there was a significant overall effect of treatment on grasp attempt rate ($F_{4, 79} = 7.73$, p < 0.0001), with full-cut males exhibiting the highest grasp attempt rate than any other experimental category (Fig. 3a). Despite this high attempt rate, there was not a single case in which a full-cut male succeeded to grasp a female (Fig. 3b) or to perform a grasp-abdominal curl (Fig. 3c). For these behaviors, the effect of treatment was likewise strongly significant ($F_{4, 79} = 43.03$, p < 0.0001; $F_{4, 79} = 41.06$, p < 0.0001, respectively). When the full-cut category was excluded from these analyses, mean rates of grasping ($F_{3, 63} = 1.40$; p = 0.25) and grasp-abdominal curling ($F_{3, 63} = 0.725$; p = 0.54) failed to differ among the remaining categories.

In *D. bipectinata*, there was a significant effect of treatment on lunge rate ($F_{4, 85} = 2.96$; p = 0.024), with full-cut males exhibiting a significantly great lunge rate than sham controls (Fig. 3d). The overall effect of treatment on grasp rate was strongly significant ($F_{4, 85} = 7.28$; p < 0.0001), with full-cut males performing fewer grasps per unit time than any other category (Fig. 3e). Likewise, the treatment effect on rate of grasp-abdominal curling was strongly significant ($F_{4, 85} = 6.25$; p = 0.0002), with full-cut males again performing the least number of these behaviors per unit time (Fig. 3f).

In *D. melanogaster*, surgical treatment did not significantly affect courtship latency ($F_{4,80} = 1.68$, p = 0.16) (Fig. 4a), or the rate at which males performed wing vibration bouts ($F_{4,80} = 0.774$, p = 0.54) (Fig. 4b). In *D. bipectinata*, surgical treatment likewise did not significantly affect courtship latency ($F_{4,80} = 0.550$, p = 0.70) (Fig. 4c). The data for courtship latency and wing vibrations suggest that the surgical treatment did not adversely affect male sexual motivation.

Competitive Copulation Trials: Copulation Success

For *D. melanogaster*, there was a strong significant effect of surgical treatment on copulation success in population cages where males competed for non-virgin females on fruit substrates (Likelihood ratio test: $\chi^2 = 46.41$, df = 4, p < 0.0001). As in the non-competitive environment, full-cut males achieved no copulations (Fig. 2c). In contrast to the non-competitive environment, however, proximal-cut males also showed a significant reduction in copulation probability; in fact, their copulation success was similar to that for the full-cut males (Fig. 2c). When just the full-cut category was removed, the significant effect of treatment persisted (Likelihood ratio test: $\chi^2 = 23.81$, df = 3, p < 0.0001), but when both the full-cut and proximal-cut groups were removed from the analysis, the significant treatment effect on copulation probability was lost (Likelihood ratio test: $\chi^2 = 2.26$, df = 2,
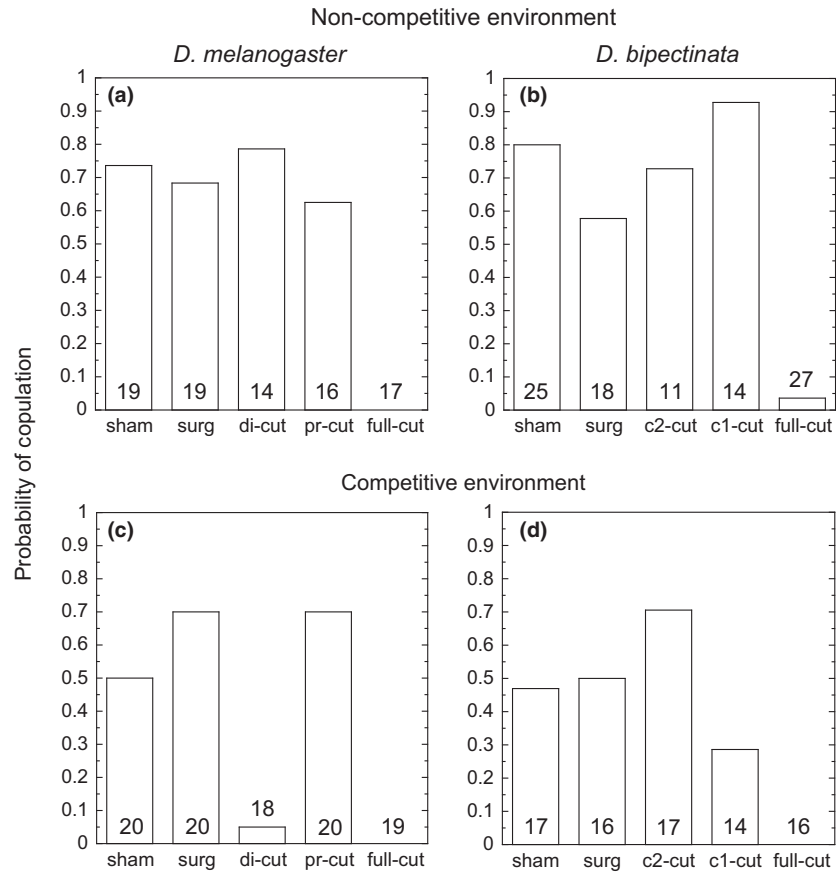
Non-competitive environment

Fig. 2: Probability of copulation for experimental groups in non-competitive and competitive environments for *D. melanogaster* (a and c) and *D. bipectinata* (b and d). Numbers within bars represent sample sizes. Sham = sham control, Surg = surgical control, di-cut = distal-cut, and pr-cut = proximal-cut.

p = 0.32). Among the males succeeding to mate, there was no significant difference in copulation latency among treatment categories ($F_{3,35}$ = 1.13, p = 0.35) (Fig. 5a).

In *D. bipectinata*, surgical treatments had a strong effect on male copulation success (Likelihood ratio test: χ^2 = 28.12, df = 4, p < 0.0001; Fig. 2d). This significant effect arose because none of the full-cut males copulated (Fig. 2d); when the full-cut group was removed from the analysis, the significant effect was lost (Likelihood ratio test: χ^2 = 2.41, df = 3, p = 0.49; Fig. 2d). Among the males of this species succeeding to mate, there was no significant difference in copulation latency among treatment categories ($F_{3,35}$ = 0.136, p = 0.94) (Fig. 5b).

Discussion

Our study demonstrates experimentally that the male sex combs in *D. melanogaster* and *D. bipectinata* are essential for copulation. We first showed that complete removal of the sex combs eliminated the ability of males to copulate with virgin females under non-competitive conditions. Analyses of the behaviors of individual males shed light on the causes of this effect, namely, that the sex combs in both species function as grasping apparati, although the combs are implemented in slightly different ways in the two species. For *D. melanogaster*, the grasping function of the combs has previously been described through observing the behavior of intact males (Spieth 1952; Cook 1975, 1977), and our results confirm these earlier reports. In intact males, the sex combs are used to grasp the female's terminal abdominal segments and ovipositor, permitting the male to grasp hold of the female and align the female's genitalia with his own prior to genital contact (Spieth 1952; Cook 1975, 1977). Upon genital coupling, the intact male moves forward and upward onto the female and mounts her. Combless (i.e., full-cut) *D. melanogaster* males in our experiment failed to grasp the female ovipositor because they were unable to achieve traction with the end of the female's abdomen and genitalia; the foretarsi of combless males slipped off the tip of the female. Consequently, combless males failed to perform full abdominal curls, and thus were not able to
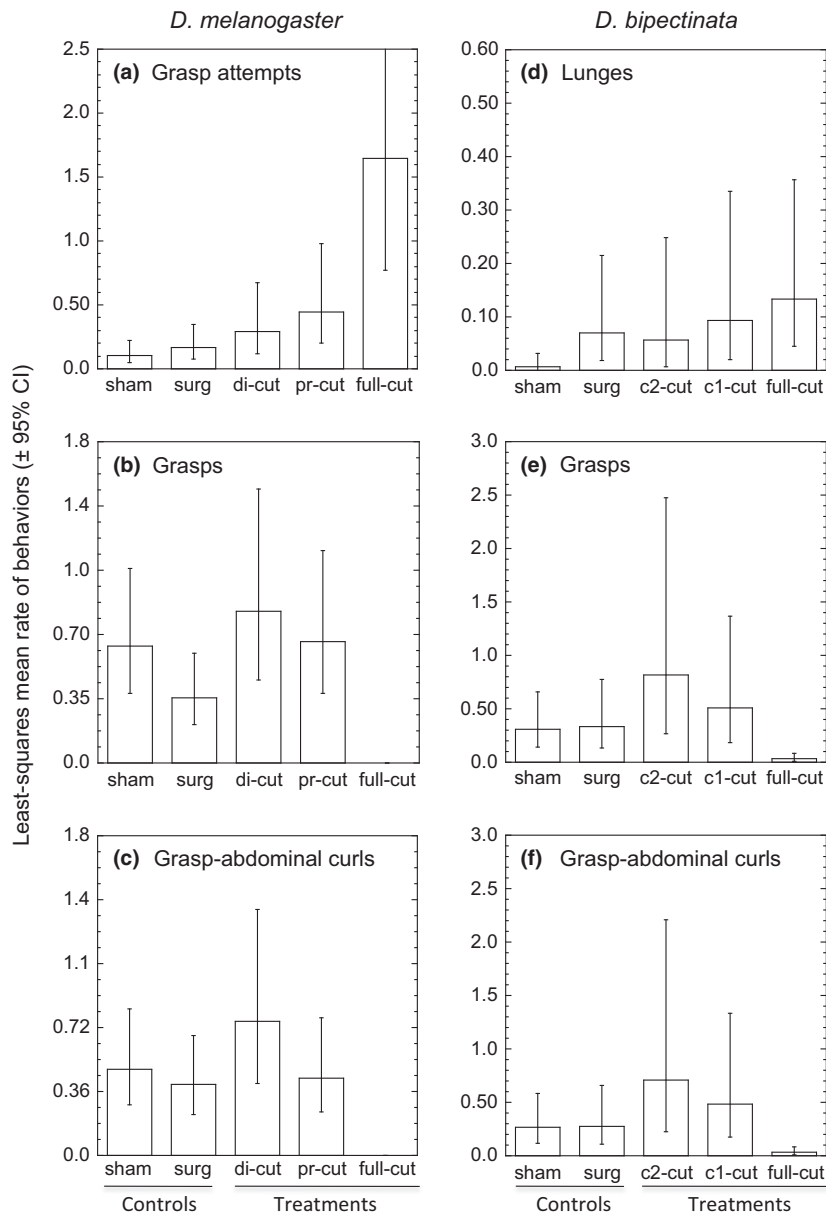
Fig. 3: Mean rate of mating behaviors expressed as frequency per minute for *D. melanogaster* (a, b and c) and *D. bipectinata* (d, e, and f). Data were $\log_{10}(y + 0.01)$-transformed prior to analysis; back-transformed means are presented to aid interpretability. Error bars are 95% confidence intervals. Sham = sham control, Surg = surgical control, di-cut = distal-cut, and pr-cut = proximal-cut.

achieve genital contact and copulation; in other words, removal of the combs interrupted the normal sequence of behaviors leading to copulation.

Cook (1977) and Ng & Kopp (2008) previously studied the function of sex combs in *D. melanogaster* using manipulative techniques. Cook (1977) removed the sex combs with the use of surgical forceps, while Ng & Kopp (2008) genetically 'feminized' males. In both cases, 'combless' males achieved significantly fewer copulations, although copulation success was not eliminated as it was in our study. The reasons for these differences are not totally clear. In Cook's (1975) study, approximately 7% of the combless

males were able to mate, perhaps because the process of breaking the combs off with forceps did not cleanly remove all of the teeth at their base, enabling some traction with the female's abdomen. Ng & Kopp (2008) showed that combless males were more often observed attempting to copulate than controls, their analysis did not shed light on the causes of the reduced mating success of combless males, nor on why a relatively high percentage of feminized males (*c.* 25% on average) were able to copulate. The reason for this latter observation may be that the genetic ablation technique restores bristle morphology to an ancestral, sexually monomorphic state (Ng & Kopp
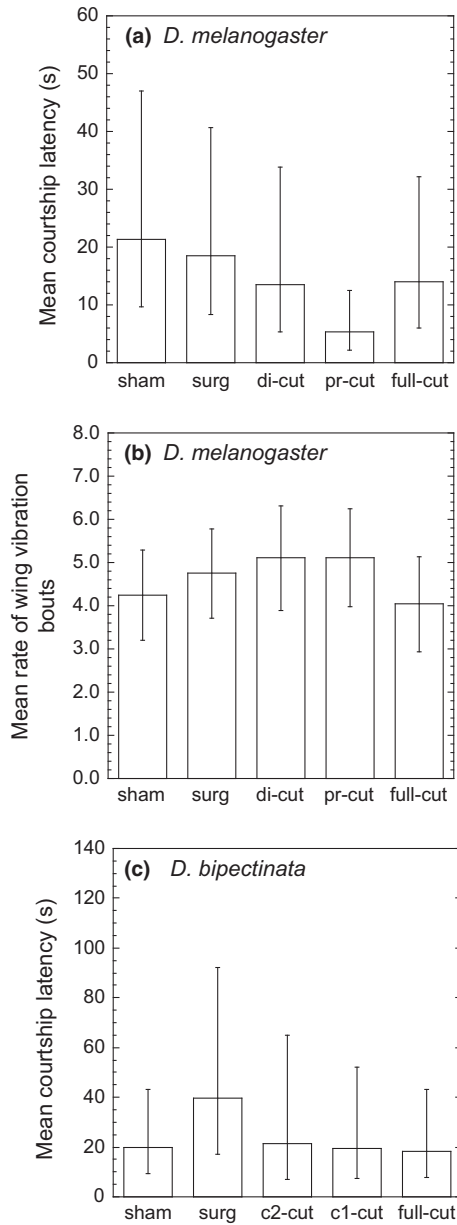
Fig. 4: Mean courtship latency and rate of wing vibration bouts for *D. melanogaster* (a, b), and mean courtship latency for *D. bipectinata* (c). Error bars represent 95% confidence intervals. Rate of wing vibration bouts were not $\log_{10}(y + 0.01)$-transformed prior to analysis. Sham = sham control, Surg = surgical control, di-cut = distal-cut, and pr-cut = proximal-cut.

Fig. 5: Mean copulation latency for *D. melanogaster* (a) and *D. bipectinata* (b) in population cages. Error bars are 95% confidence intervals. No error bar is provided for the 'di-cut' treatment because n = 1 in this case. Sham = sham control, Surg = surgical control, di-cut = distal-cut, and pr-cut = proximal-cut.

2008), so that the substitute female-like bristles on the foretarsi may have afforded males residual grasping power as in the case of Cook's study.

In *D. bipectinata*, intact males press their sex combs against the lateral flanks of the female's abdomen (as opposed to the tip of the abdomen and ovipositor in *D. melanogaster*), up to a third of the way along this
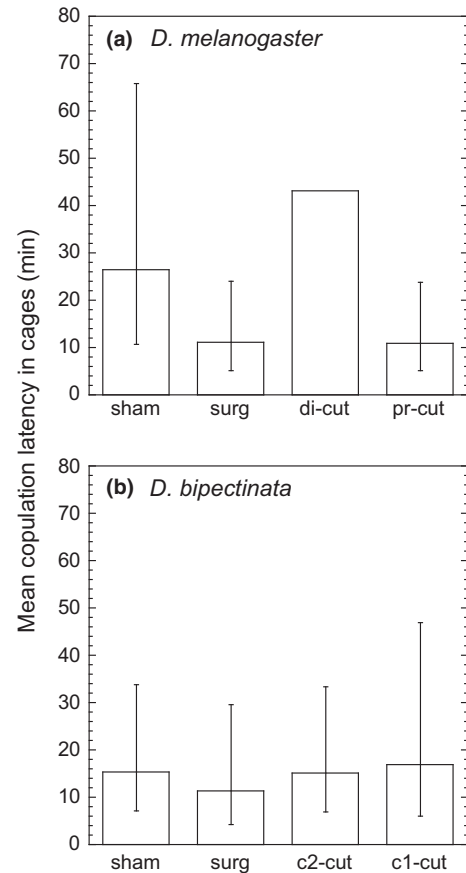
body segment. The male then mounts the female and bends his abdomen downward and forward in an attempt to achieve genital contact (Cooperman et al. 2007). In the present study, males experimentally rendered combless failed to grasp females because their foretarsi slipped off the sides of female's abdomen. For example, one combless male was observed to make 114 lunges but not a single grasp or grasp-abdomen curl in the 10 min observation period. Although combless males on average exhibited the lowest rates of grasping and grasp-abdominal curling relative to other groups owing to foretarsal slippage, they were nevertheless able at times to perform these behaviors. Follow-up inspection of these successful males verified that all their sex comb teeth had indeed been fully removed, indicating that in some cases males without their combs are able to exert sufficient pressure to gain traction against the female's abdomen. Yet, and as indicated above, males without their

combs never were seen to achieve the full sequence of behaviors leading to copulation.

Courtship latencies accompanied by data on the occurrence of wing vibrations (*D. melanogaster*) in mating arenas suggest that altered males did not differ in their motivation to court. Thus, the reduced copulation success of combless males was unlikely the consequence of collateral injury to males from the laser surgery. In addition, previous studies that have used this same laser technique to manipulate other morphological structures (*Drosophila* genital spines) (*D. bipectinata*, Polak & Rashed 2010; *D. ananassae*, Grieshop & Polak 2012) corroborate the conclusion that the laser surgical procedure has negligible effects on normal courtship behavior in *Drosophila*.

We also demonstrated a strong negative effect of full comb ablation on copulation success in population cages, thus strengthening the main conclusion of the study that copulation cannot occur without the presence of the comb. In these cages males competed for access to non-virgin females on fruit substrates, thus more closely simulating natural conditions (Polak et al. 2004) compared to the non-competitive trials where single males were paired with virgin females in small observation arenas. Not only was the intensity of sexual selection in the cages elevated because of male-male competition, but as non-virgin females were used, there was likely also an elevation in female resistance to mate and possibly female mate choice. The potential for female resistance is reflected particularly in *D. bipectinata*, where we observed a reduction in overall mating probability relative to the non-competitive mating assay (contrast Fig. 2b,d). Under the enhanced competitive conditions of the cages, there was an apparent strengthening of the negative effect of treatment (such that distal-cut males exhibited reduced copulation probability here but not in the non-competitive context) on copulation probability in *D. melanogaster* (and see Ng & Kopp 2008), but not in *D. bipectinata*. In *D. melanogaster*, reduction in comb size by way of ablating all but the 5 proximal-most teeth reduced copulation to near zero, in contrast to the non-competitive situation where comb reduction (in either direction) had no statistical effect on copulation success.

Our results indicate that the sex combs in both species are larger than necessary for the grasping and enabling genital coupling, suggesting that other selective forces are driving comb size evolution beyond the size needed for these mechanical acts. In this sense, sex comb evolution may parallel that of ornamental traits of many other animal species, where the selection pressure responsible for the origin of the trait is different from the form of selection that contributed to its subsequent sex-specific elaboration (via sexual selection) (Andersson 1994). For example, the male 'sword' in swordtail (*Xiphophorus*) fishes, which is the target of female choice, is a colored extension of the lower margin of the caudal fin (Basolo 1990; and see Arnqvist & Rowe 2005, pp. 74–75). Likewise, the elongated tail feathers of male barn swallows (*Hirundo rustica*), which have been shown to be the target of sexual selection (Møller 1988), are extensions of the outer tail feathers important for aerodyamic performance in both sexes (Thomas 1993; Balmford et al. 1994). In *Drosophila*, it is conceivable that the origins of the sex comb may be traced to natural selection, if, for example, it first served a grooming function. Alternative, combs may have originated through sexual conflict, which occurs when male and female evolutionary (fitness) interests diverge (Parker 1979; Arnqvist & Rowe 2005). Here, the original function of the combs could have been to enhance grasping and maintaining contact with inseminated females, coercing them to mate.

In general, if a trait that originates by natural selection comes to impose non-trivial costs to its bearers, such as in a changing environment, the subsequent elaboration of the trait could then be driven by sexual selection, either through the handicap ('good genes') or Fisherian processes (Fisher 1930; Zahavi 1977; Kirkpatrick & Ryan 1991; Tomkins et al. 2004). In *D. bipectinata*, sex comb size is both condition dependent and heritable (Polak et al. 2004; Polak & Starmer 2005; and see Ahuja & Singh 2008; Ahuja et al. 2011), predisposing it to sexual selection. Indeed, males with relatively large body-size specific comb size have been shown to have a mating advantage in the field (e.g., at Cape Tribulation, Australia: Polak et al. 2004), although sexual selection for increasing sex comb size is not always found in *D. bipectinata* (Polak & Taylor 2007) and other *Drosophila* species (Markow et al. 1996; Snook et al. 2013). Moreover, males with the largest sex combs in *D. bipectinata* have been shown to have an advantage in sperm competition (Polak & Simmons 2009), suggesting that sex comb size is also under indirect post-copulatory sexual selection (Birkhead & Pizzari 2002; Mautz et al. 2013; *cf.* Snook et al. 2013). Thus, although the origin the sex comb is unclear, and may be linked to natural selection and/ or sexual conflict, there is evidence that the elaboration of the trait beyond its grasping function results from sexual selection. Sexual selection may explain the observed incipient diversification of comb size among geographic populations of *D. bipectinata* and

related species (Crossley & Taylor 1985; Polak et al. 2004; Matsuda et al. 2005; Mishra & Singh 2006), and it may also have contributed to the rich diversity of sex comb shape and size more broadly within the genus.

Acknowledgements

Conflict of interest

The authors have no conflict of interest to declare.

Literature Cited

Ahuja, A. & Singh, R. S. 2008: Variation and evolution of male sex combs in *Drosophila*: nature of selection response and theories of genetic variation for sexual traits. Genetics **179**, 503—509.

Ahuja, A., De Vitto, S. & Singh, R. S. 2011: Condition dependence and the nature of genetic variation for male sex comb bristle number in *Drosophila melanogaster*. Genetica **139**, 505—510.

Andersson, M. 1994: Sexual Selection. Princeton Univ. Press, Princeton, NJ.

Arnqvist, G. & Rowe, L. 2005: Sexual Conflict. Princeton Univ. Press, Princeton, NJ.

Balmford, A., Jones, I. L. & Thomas, A. L. R. 1994: How to compensate for costly sexually selected tails: the origin of sexually dimorphic wings in long-tailed birds. Evolution **48**, 1062—1070.

Barmina, O. & Kopp, A. 2007: Sex-specific expression of a HOX gene associated with rapid morphological evolution. Dev. Biol. **311**, 277—286.

Barraclough, T. G., Harvey, P. H. & Nee, S. 1995: Sexual selection and taxonomic diversity in passerine birds. Proc. Biol. Sci. **259**, 211—215.

Basolo, A. L. 1990: Female preference predates the evolution of the sword in swordtail fish. Science **250**, 808—810.

Birkhead, T. R. & Pizzari, T. 2002: Postcopulatory sexual selection. Nat. Rev. **3**, 262—273.

Bock, I. R. 1971: Taxonomy of the *Drosophila bipectinata* species complex. Univ. Texas Publ. **7103**, 273—280.

Bock, I. R. & Wheeler, M. R. 1972: The *Drosophila melanogaster* species group. Univ. Texas Publ. **7213**, 1—102.

Chapman, T., Arnqvist, G., Bangham, J. & Rowe, L. 2003: Sexual conflict. Trends Ecol. Evol. **18**, 41—47.

Cook, R. M. 1975: Courtship of *Drosophila melanogaster*: rejection without extrusion. Behaviour **52**, 155—171.

Cook, R. M. 1977: Behavioral role of the sexcombs in *Drosophila melanogaster* and *Drosophila simulans*. Behav. Gen. **7**, 349—357.

Cooperman, A. F., Polak, M., Evans, C. S. & Taylor, P. W. 2007: Different sexual traits show covariation among genotypes: implications for sexual selection. Behav. Ecol. **18**, 311—317.

Coyne, J. A. 1985: Genetic studies of three sibling species of *Drosophila* with relationship to theories of speciation. Gen. Res. **46**, 169—192.

Crossley, S. A. 1986: Courtship sounds and behaviour in the four species of the *Drosophila bipectinata* complex. Anim. Behav. **34**, 1146—1159.

Crossley, S. & Taylor, I. 1985: Male sex-comb teeth number in four species of the *Drosophila bipectinata* complex and their hybrids. Experientia **41**, 103—104.

Darwin, C. 1871: The Descent of Man, and Selection in Relation to Sex. John Murray, London.

Eberhard, W. G. 1985: Sexual Selection and Animal Genitalia. Harvard Univ. Press, Cambridge, MA.

Emlen, D. J. 2008: The evolution of animal weapons. Ann. Rev. Ecol. Syst. **39**, 387—413.

Endler, J. A. & Houde, A. E. 1995: Geographic variation in female preferences for male traits in *Poecilia reticulata*. Evolution **49**, 456—468.

Fisher, R. A. 1930: The Genetical Theory of Natural Selection. Clarendon Press, Oxford.

Grieshop, K. & Polak, M. 2012: The precopulatory function of male genital spines in *Drosophila ananassae* [Doleschall] (Diptera: Drosophilidae) revealed by laser surgery. Evolution **66**, 2637—2645.

Hosmer, D. W. & Lemeshow, S. 1989: Applied Logistic Regression. John Wiley, New York.

Kirkpatrick, M. & Ryan, M. 1991: The evolution of mating preferences and the paradox of the lek. Nature **350**, 33—38.

Kopp, A. 2011: *Drosophila* sex combs as a model of evolutionary innovation. Evol. Dev. **13**, 504—522.

Kopp, A. & True, J. R. 2002: Evolution of male sexual characters in the oriental *Drosophila melanogaster* species group. Evol. Dev. **4**, 278—291.

Lakovaara, S. & Saura, A. 1982: Evolution and speciation in the *Drosophila obscura* group. In: The Genetics and Biology of Drosophila, Vol. **3b**. (Ashburner, M., Carson, H. L., Thompson, J. N. Jr, eds). Academic Press, London, pp. 1—59.

Lande, R. 1981: Models of speciation by sexual selection on polygenic traits. Proc. Natl Acad. Sci. USA **78**, 3721—3725.

Lemeunier, F., David, J., Tsacas, L. & Ashburner, M. 1986: The melanogaster species group. In: The Genetics and

Biology of Drosophila, Vol. **3e**. (Ashburner, M., Carson, H. L., Thompson, J. N. Jr, eds). Academic Press, London, pp. 147—256.

Markow, T. A., Bustoz, D. & Pitnick, S. 1996: Sexual selection and a secondary sexual character in two *Drosophila* species. Anim. Behav. **52**, 759—766.

Matsuda, M., Tomimura, Y. & Tobari, Y. N. 2005: Reproductive isolation among geographical populations of *Drosophila bipectinata* (Diptera: Drosophilidae) with recognition of three subspecies. Genetica **125**, 69—78.

Mautz, B. S., Møller, A. P. & Jennions, M. D. 2013: Do secondary sexual characters signal ejaculate quality? A meta-analysis. Biol. Rev. **88**, 669—682.

Mishra, P. K. & Singh, B. N. 2006: Unique phenotypes and variation in the sex comb patterns and their evolutionary implications in the *Drosophila bipectinata* species complex (Diptera: Drosophilidae). Eur. J. Entomol. **103**, 805—815.

Møller, A. P. 1988: Female choice selects for male sexual tail ornaments in the monogamous swallow. Nature **332**, 640—642.

Neter, J., Wasserman, W. & Kutner, M. H. 1990: Applier Linear Statistical Models. Irwin, Homewood, IL.

Ng, C. S. & Kopp, A. 2008: Sex combs are important for male mating success in *Drosophila melanogaster*. Behav. Gen. **38**, 195—201.

Owens, I. P. F. & Bennett, P. M. 1997: Variation in mating system among birds: ecological basis revealed by hierarchical comparative analysis of mate desertion. Proc. Biol. Sci. **264**, 1103—1110.

Panhuis, T. M., Butlin, R., Zuk, M. & Tregenza, T. 2001: Sexual selection and speciation. Trends Ecol. Evol. **16**, 364—371.

Parker, G. A. 1979: Sexual selection and sexual conflict. In: Sexual Selection and Reproductive Competition in Insects. (Blum, M. S., Blum, N. A., eds). Academic Press, New York, pp. 123—166.

Polak, M. & Rashed, A. 2010: Microscale laser surgery reveals adaptive function of male intromittent genitalia. Proc. Biol. Sci. **277**, 1371—1376.

Polak, M. & Simmons, L. W. 2009: Secondary sexual trait size reveals competitive fertilization success in *Drosophila bipectinata* Duda. Behav. Ecol. **20**, 753—760.

Polak, M. & Starmer, W. T. 2005: Environmental origins of sexually selected variation, and a critique of the fluctuating asymmetry-sexual selection hypothesis. Evolution **59**, 577—585.

Polak, M. & Taylor, P. W. 2007: A primary role of developmental instability in sexual selection. Proc. Biol. Sci. **274**, 3133—3140.

Polak, M., Wolf, L. L. & Starmer, W. T. 2004: Sexual selection for size and symmetry in a diversifying secondary sexual character in *Drosophila bipectinata* Duda (Diptera: Drosophilidae). Evolution **58**, 597—607.

Prum, R. O. 1997: Phylogenetic tests of alternative intersexual selection mechanisms: trait macroevolution in a polygynous clade (Aves: Pipridae). Am. Nat. **149**, 668—692.

Ryan, M. J., Perrill, S. A. & Wilczynski, W. 1992: Auditory tuning and call frequency predict population-based mating preferences in the cricket frog, *Acris crepitans*. Am. Nat. **139**, 1370—1383.

Seehausen, O. & van Alphen, J. M. 1999: Can sympatric speciation by disruptive sexual selection explain rapid evolution of cichlid diversity in Lake Victoria? Ecol. Lett. **2**, 262—271.

Snook, R. R., Gidaszewski, N. A., Chapman, T. & Simmons, L. W. 2013: Sexual selection and the evolution of secondary sexual traits: sex comb evolution in *Drosophila*. J. Evol. Biol. **26**, 912—918.

Sokal, R. R. & Rohlf, F. J. 1981: Biometry. W.H. Freeman, New York.

Spieth, H. T. 1952: Mating behavior within the genus *Drosophila* (Diptera). Bull. Am. Mus. Nat. Hist. **99**, 395—474.

Thomas, A. L. R. 1993: On the aerodynamics of birds' tails. Philos. Trans. R. Soc. Lond., B, Biol. Sci. **340**, 361—380.

Tomkins, J. L., Radwan, J., Kotiaho, J. S. & Tregenza, T. 2004: Genetic capture and resolving the lek paradox. Trends Ecol. Evol. **19**, 323—328.

Uy, J. A. C. & Borgia, G. 2000: Sexual selection drives rapid divergence in bowerbird display traits. Evolution **54**, 273—278.

Young, J. R., Hupp, J. W., Bradbury, J. W. & Braun, C. E. 1994: Phenotypic divergence of secondary sexual traits among sage grouse, *Centrocercus urophasianus*, populations. Anim. Behav. **47**, 1353—1362.

Zahavi, A. 1977: The cost of honesty (further remarks on the handicap principle). J. Theor. Biol. **67**, 603—605.

Zar, J. H. 2010: Biostatistical Analysis. Prentice Hall, New Jersey.