A primary sexual trait involved in courtship in insects: Male genital lobe morphology affects the chance to copulate in *Drosophila pachea*

running title: *D. pachea* mate competition analysis

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Abstract

Male genitalia are thought to ensure transfer of sperm through direct physical contact with female during copulation. Such primary sexual traits were also observed to affect pre-copulatory female mate-choice in some Vertebrates species, but whether this also occurs in insects is unknown. Males of the fruitfly *Drosophila pachea* have a pair of asymmetric external genital lobes, which are primary sexual structures and stabilize the copulatory complex of female and male genitalia. We tested for a pre-copulatory courtship role of these lobes with a *D. pachea* stock where males have variable lobe lengths. In 111 mate competition experiments with a single female and two males, females preferentially engaged into a first copulation with males that had a longer left lobe. Courtship durations increased with female age and when two males courted the female simultaneously, compared to experiments with only one courting male. In 54 additional experiments with both males having partially amputated left lobes, we observed a similar but weaker effect of left lobe length on copulation success. We conclude that left lobe length affects male mating success before genital contact. Our results suggest that primary male sexual traits in insects can serve as a signal for pre-copulatory mate-choice.

keywords: *Drosophila pachea*, primary sexual trait, mating behaviour, mate-competition experiments, genitalia

Introduction

Males and females exhibit different reproduction strategies due to a higher limitation of larger female gametes compared to smaller and more abundant male gametes (Bateman 1948). This implies male-male intrasexual mate-competition for siring the limited female gametes. In turn, females may optimize reproduction by choosing males that confer survival and fecundity benefits to her and to the offspring. This dynamics has been formalized into genetic models (Fisher 1930; Lande 1981) involving female preferences for particular male characters and male mate competition, and can contribute to the rapid evolution of female preferences and male sexual attributes.

Across animals with internal fertilization, genitalia are usually the most rapidly evolving organs (Eberhard 1985). Several hypotheses propose that the evolution of genitalia is based on sexual selection at different levels of reproduction, including competition of sperm from different males inside the female (sperm competition) (Birkhead and Pizzari 2002), female controlled storage and usage of sperm to fertilize eggs (cryptic female choice) (Thornhill 1983; Eberhard 1985, 2010), or due to a sexually antagonistic conflict between male and female over such fertilization decisions (Arnqvist 1998; Chapman et al. 2003). Male courtship is well known as a behaviour to attract females before copulation begins, but has also been reported to occur during or even after copulation (Eberhard 1991, 1994). It is thought to be a widespread and key aspect of copulation in the cryptic female choice scenario, where males stimulate the female to utilize his own sperm (Thornhill 1983; Eberhard 1985). A variety of insect species were reported where males had evolved elaborated male genital structures that are used during copulatory courtship to stimulate the female through tapping or other physical stimuli (Eberhard 1991, 1994).

Traditionally, sexual traits are categorized as primary or secondary. Genital structures are considered “primary” when they are directly used for the transfer of gametes during copulation or when they contribute to the complexing of female and male copulatory organs (Eberhard 1985).

Other traits that differ between sexes and that are linked to reproduction are considered “secondary” sexual traits. Secondary sexual traits can be involved in pre-copulatory mate-choice via long range signalling. Pre-copulatory mate-choice signals can be visual, for example in birds through discrimination of feather length, colouration or specific plumage ornaments (Andersson 1982; von Schantz et al. 1989; Hill 1991; Norris 1993; Petrie 1994), auditory through animal courtship songs (Hasselquist et al. 1996; Welch et al. 1998) or olfactory through pheromone release, for example in insects (Birch 1970; Sreng 1993). In some cases, secondary sexual traits were found to correlate with so-called “direct” female benefits on fecundity, parental care, or female survival (Andersson and Simmons 2006). In other cases, they were “indirectly” related to genetic quality of male gametes and survival of the progeny (von Schantz et al. 1989; Hill 1991; Norris 1993; Petrie 1994; Hasselquist et al. 1996; Welch et al. 1998; Andersson and Simmons 2006). As a result, secondary sexual traits are thought to evolve rapidly due to sexual selection associated with pre-copulatory events.

For primary sexual traits, sexual selection is thought to act mainly during copulation through direct physical contacts of female and male genitalia (Eberhard 1985, 1994). However, primary genitalia could also be possibly involved in pre-copulatory mate choice. Supposing that a particular male genital trait-variance enhances or favours female fecundity, it might become preferred by the female during pre-copulatory courtship. This preference could rely on direct benefits to reduce energy investment or predation risk caused by unsuccessful copulation attempts, or on indirect male genetic quality to be inherited into the offspring generation. A few examples of primary genitalia used in pre-copulatory courtship are known in vertebrates: male genital displays are reported in certain primates (Ploog and MacLean 1963; Wickler 1966) and in lizards (Bohme 1983). Furthermore, females of some live-bearing fish species were reported to prefer to mate with males with large gonopodia (Langerhans et al. 2005; Kahn et al. 2010). These cases involve visual stimuli and large male genital organs. In invertebrates, which usually harbour smaller genital

organs, no case of primary sexual trait influencing reproduction before copulation has been reported to our knowledge. Nevertheless, pedipalp drumming of the male wolf spider *Lycosa rabida* has a communicative function in courtship (Rovner 1967). The pedipalps are modified legs that serve as sensory appendages and are highly modified in males for an intromittent function to transfer the spermatophore to the female (Robinson 1982). In this sense, pedipalps can be regarded as primary sexual traits, although they are "secondary genitalia" in terms of their appendage homology. In some insect courtship descriptions, males were reported to present their distal abdomen to the female, such as abdominal flexion displays in praying mantid species (Quesnel 1967; Liske and Davis 1984, 1987), genitalia curling in the moth *Phlogophora meticulosa* (Birch 1970) or abdomen curving in the cockroach *Oxyhaloa deusta* (Lizée et al. 2017). In the latter two examples, such abdomen postures were associated with male pheromone release, but it is not clear whether these particular primary sexual traits influence mating success.

The *Drosophila nannoptera* species group is a promising model to study the evolution of primary sexual traits, especially with respect to the evolution of left-right asymmetry. Distinct directional left-right asymmetries and one-sided mating positions evolved repeatedly in the four described species of this group (Lang et al. 2014; Acurio et al. 2019). Male *D. pachea* have an asymmetric aedeagus (intromission organ) and a pair of asymmetric external lobes with the left lobe being approximately 1.5 times longer than the right lobe (Pitnick and Heed 1994; Lang and Orgogozo 2012; Acurio et al. 2019) (Figure 1), *D. acanthoptera* has an asymmetric aedeagus (Vilela and Bächli 1990; Acurio et al. 2019) and *D. wassermani* has a pair of asymmetric anal plates (cerci) (Pitnick and Heed 1994). In contrast, no asymmetries have been described for the fourth species *D. nannoptera* (Vilela and Bächli 1990; Acurio et al. 2019). These particular genital traits have likely evolved during the past 3-6 Ma (Lang et al. 2014). *D. pachea* and *D. nannoptera* both mate in a right-sided copulation position (Acurio et al. 2019). *D. pachea* males rest on top of the female abdomen with the antero-posterior midline shifted about 6-8 degrees to the right side of the

female midline (Lang and Orgogozo 2012; Rhebergen et al. 2016), while *D. nannoptera* tilts down the females right abdomen (Acurio et al. 2019). In *D. pachea*, male and female genitalia form an asymmetric complex during copulation and the asymmetric lobes stabilize this genital complex (Rhebergen et al. 2016). Furthermore, *D. pachea* and *D. nannoptera* are among the *Drosophila* species that produce the longest (giant) sperm (Pitnick et al. 1995, 1999), and their ejaculates contain in average about 40 and 80 sperm cells, respectively (Pitnick and Markow 1994). Thus, a particular right-sided mating position could potentially be associated with optimal transfer of giant sperm during copulation (Acurio et al. 2019).

The aim of this study was to test if the asymmetric genital lobes of *D. pachea* would have an effect on pre-copulatory mate-choice, in addition to their copulatory role in stabilizing the complex of male and female genitalia. Previously, we found that males originating from one of our *D. pachea* laboratory stocks possess short and rather symmetric lobes (Lang and Orgogozo 2012) (Figure 1), while others have the typical size asymmetry. This variable development, especially affecting the left genital lobe within the same fly stock enabled us to test if lobe length might have an effect on pre-copulatory courtship or mate competition. We selected *D. pachea* males with short left lobes and produced a stock with an increased variance of left lobe length. Next, we tested sibling males of this selection stock in mate-competition assays for their success to engage first into copulation with a single female. We also tested whether lobe length or copulation success in our assay would be affected by male courtship vigour and if lobe length affects sperm allocation into female storage organs. Finally, we surgically shortened the length of the left lobe in males that had developed long left lobes, to further test whether left lobe length affects copulation success.

Material and Methods

Fly stock establishment and maintenance

Two *D. pachea* stocks 15090-1698.01 and 15090-1698.02 were retrieved from the Drosophila Species Stock Center. Flies were maintained in 25 x 95 mm plastic vials containing 10 mL of standard Drosophila medium (60 g/L brewer's yeast, 66.6 g/L cornmeal, 8.6 g/L agar, 5 g/L methyl-4-hydroxybenzoate and 2.5% $^{v}/_{v}$ ethanol) and a ~ 10 x 50 mm piece of bench protection sheet (Bench guard). As *D. pachea* requires 7-dehydrocholesterol for proper development (Heed and Kircher 1965; Warren et al. 2001; Lang et al. 2012), we mixed the medium of each vial with 40 µL of 5 mg/mL 7-dehydrocholesterol, dissolved in ethanol (standard *D. pachea* food). Flies were kept at 25°C inside incubators (Velp) with a self-made light installation for a 12 h light: 12h dark photo-periodic cycle combined with a 30-min linear illumination change between light (1080 lumen) and dark (0 lumen). We used males of stock 15090-1698.02 to generate a new stock with increased proportions of males with the lobe length aberrant phenotype. For this, we chose 3 males with apparently symmetric (aberrant) genital lobes and crossed them with 3-4 sibling virgin females. We repeated the selection with the progeny for a total of 36 generations. Then, we removed males with clearly visible asymmetric (wild-type) lobes from the progeny for another 14 generations to derive the final stock (selection stock).

Virgin fly selection

Virgin flies at 0-1 d after emerging from the pupa were CO_2 anaesthetised on a CO_2-pad (INJECT+MATIC Sleeper) under a stereo-microscope Stemi 2000 (Zeiss), separated according to sex and maintained in groups of 20-30 individuals. Males and females were raised until reaching sexual maturity, about 14 days for males and 4 days for females at 25° C (Pitnick 1993). This allowed us to use virgin individuals in each mating experiment. Males were anaesthetised on the

CO_2 pad (see above), sorted according to lobe morphology (asymmetric and symmetric lobes) and isolated into single vials at least two days before each mating experiment took place.

Mate-competition assay

Two *D. pachea* males and a single *D. pachea* female were put together into a white, cylindrical mating cell (Additional datafile 1: Figure S1) with a diameter of 20 mm, a depth of 4 mm and a transparent 1 mm Plexiglas top-cover. Optionally, mating cells were concave with a diameter of 20 mm and a depth of 4 mm at the center (Additional datafile 2: experiments 1-16). Flies were transferred without CO_2 anaesthesia using a fly aspirator: a 7 mm diameter silicone tube closed at the tip with cotton and a 1000 µL wide bore (3 mm) micro-tip. Males were CO_2 anaesthetised under a binocular to visually select two males of the selection stock that differed in lobe length (Additional datafile 1: Figure S2). This selection was done to increase the average pairwise difference of lobe lengths between the two males. Movie recordings were started as soon as the chamber was immediately put under the camera (see below).

All mating experiments were carried out inside a temperature and humidity controlled climate chamber at 25° C ± 0.1° C and 80% ± 5% or 60% ± 5% (experiments 1-15) humidity. We used digital cameras MIRAZOOM MZ902 (OWL) or DigiMicro Profi (DNT) to record copulation and courtship behaviour. The MIRAZOOM MZ902 (OWL) camera was mounted on a modified microscope stand (191348, Conrad) equipped with a 8-cm LED white light illumination ring (EB-AE-COB-Cover, YM E-Bright) and a platform to hold four individual cylindrical mating cells (Additional datafile 1: Figure S1). Experiment 16 was filmed with the OWL camera and a concave shaped mating cell that was put on a flat plastic cover on top of the microscope stand. For experiments 1-15, we used concave shaped mating cells that were put onto the stand of the DigiMicro Profi (DNT) camera. Data was acquired with the programs Cheese (version 3.18.1) (https://wiki.gnome.org/Apps/Cheese) or GUVCVIEW (version 0.9.9) GTK UVC (experiments 1-

15) on an ubuntu linux operating system in webm or mkv format. Up to four experiments were recorded in a single movie, which was split after recording and converted into mp4 format with Avidemux 2.6 (http://www.avidemux.org/) to obtain a single movie per experiment.

We waited for a copulation to take place between one of the males and the female (see below). The mating cell was shortly recovered from the climate chamber at about 5-10 min after copulation start and the non-copulating male (male 1) was removed from the cell with an aspirator and transferred into a 2-mL reaction tube filled with 70% ethanol. The copulating male (male 2) and the female were removed from the mating cell after copulation had ended and were also isolated into single 2-mL reaction tubes filled with 70% ethanol. Optionally, the female was kept alive for 12-24 h in a vial containing 5-10 mL grape juice agar (24 gr/L agar, 26 gr/L sucrose, 120 mg/L Tegosept, 20% $^{v}/_{v}$ grape juice, and 1.5% $^{v}/_{v}$ ethanol). The presence of eggs on the plate was systematically checked but never observed. Females were finally sacrificed to prepare spermathecae.

Our aim was to observe at least 50 experiments, where both males courted the female simultaneously and where copulation was observed. In total, we carried out 98 and 89 experiments with females of stocks 15090-1698.01 and 15090-1698.02, respectively (Additional datafile 1: Table S1). We removed 31 experiments from the analysis because either copulation was not observed until 1 h after the experiment started (20 experiments), flies escaped, got injured or died inside the mating cell (4 experiments) or the dissections of male genitalia failed (11 experiments, see below, Additional datafile 1: Table S1). We included 4 experiments into data analysis where one genital lobe broke during dissection but the other was intact. We observed that copulation ended upon removal of the non-copulating male in 3 / 76 experiments with females of stock 15090-1698.01 and in 4 / 76 copulations with females of stock 15090-1698.02. However, these experiments were included into data analysis for evaluation of the males to engage into a copulation. In total, we thus analysed 76 and 76 experiments with females of stocks 15090-1698.01

and 15090-1698.02, respectively (Additional file 1: Table S1).

Analysis of courtship and copulation behaviour

Videos were analysed with the OpenShot Video Editor software, version 1.4.3 (https://www.openshot.org) to annotate the relative timing of courtship and copulation in our experiments (Additional datafile 1: Figure S3). Data was manually entered into spreadsheets. We annotated the beginning of male courtship as the start of at least three consecutive male courtship behaviours according to Spieth (1952) (Spieth 1952), such as male touching the female abdomen with the forelegs, wing vibration, male following the female, and male touching the female ovipositor or ground next to it with the proboscis (mouth parts). The beginning of copulation was recorded as the moment when the male mounted the female abdomen. However, we only scored a copulation start if the male remained mounted for at least 15 sec. The end of copulation was considered as the moment when male and female genitalia were separated and the male had completely descended with the forelegs from the female abdomen. Male “licking” behaviour was estimated as the periods that one male spent with the head being close to the female ovipositor and intimately following it, which was often accompanied by the male proboscis (mouth-parts) touching the female oviscapt or the bottom of the mating cell next to it. In particular, licking behaviour was quantified in 104/111 experiments where both males courted the female simultaneously. We excluded 7 movies from the analysis because the males changed positions very fast so that they could not be unambiguously distinguished.

Movie-recording failed during copulation of 4 / 76 experiments with females of stock 15090-1698.02 and corresponding experiments were excluded from copulation duration analysis. We also observed that copulation ended prematurely upon male 1 removal in 7 / 152 experiments and included them into estimation of copulation duration. We found average copulation durations of 31.93 ± 7.96 min (N = 76, mean ± standard deviation (sd)) with females of stock 15090-1698.01

and 27.05 ± 7.59 min (N = 72, mean ± standard deviation) with females of stock 15090-1698.02, which were comparable to previous analyses of *D. pachea* copulation durations (Jefferson 1977; Pitnick and Markow 1994; Lang and Orgogozo 2012; Rhebergen et al. 2016). Thus, the removal of the non-copulating male sporadically affected copulation, but this had no significant effect on mean copulation durations.

Statistic analyses was performed with R version 3.6 (R Core Team 2014). Generalized linear model fits and logistic regression were calculated with the function glm and the gaussian(identity) and binomial(logit) link functions, respectively. Confidence intervals were estimated based on generalized linear models with the function confint.

Dissections

Adults were dissected in water with forceps (Forceps Dumont #5, Fine Science Tool) inside a transparent 25 mm round dissection dish under a stereo-microscope (Zeiss Stemi 2000). Male genitalia were isolated by piercing the abdomen with the forceps between the genital arch and the A6 abdominal segment and thereby separating the genitalia from the abdomen. We also mounted the left anterior leg of each dissected male in glycerol. Female spermatheca were recovered after opening the ventral abdomen with forceps and removal of the gut and ovaries. The spermathecae were isolated and separated according to left and right sides and immediately examined using a microscope (see below). All dissected tissues were stored in 200 µL storage solution (glycerol:acetate:ethanol, 1:1:3) at 4° C.

For imaging, male genitalia were transferred into a dissection dish filled with pure glycerol and examined with a VHX2000 (Keyence) microscope equipped with a 100-1000x VH-Z100W (Keyence) zoom objective at 300-400 fold magnification. Genitalia were oriented to be visible in posterior view. The left and right lateral spines, as well as the dorsal edge of the genital arch were aligned to be visible in the same focal plane. In some preparations, the genital arch broke and lobes

were aligned without adjusting the position of the dorsal genital arch. The experiment was discarded from analysis in cases where lobes could not be aligned. Male lobe lengths were measured on acquired images as the distance between the base of each lateral spine and the tip of each lobe (Figure 1C,D) using ImageJ version 1.50d (https://imagej.nih.gov/ij). Male legs were put on a flat glycerol surface with the inner side of the tibia facing to the camera. Legs were imaged at 200 fold magnification with the VHX 2000 microscope (Keyence) as described above. Female spermathecae were arranged on the bottom of the transparent plastic dissection dish, filled with water. Images were acquired using transmission light in lateral view with the Keyence VHX2000 microscope at 400-500 fold magnification. Sperm was directly visible inside the transparent spermathecae. Sperm filling levels were annotated for each spermatheca separately to match three different categories: 0, 1/6 or 1/3 of its total volume. Then, the average filling level for both spermathecae was calculated for each female.

Epandrial lobe surgery

Epandrial lobe surgery was done on 5-6 day-old *D. pachea* adult males of the selection stock following Rhebergen et al. (2016). Males were anaesthetised on a CO_2 pad (see above) and then further immobilized with a small copper wire, which was slightly pressed onto the male abdomen. The left epandrial lobe was shortened to various lengths with micro dissection scissors (SuperFine Vannas Scissors, World Precision Instruments). Flies were let to recover for at least 7 days on standard *D. pachea* food at 25° C in groups. No mortality difference was observed between males that underwent lobe surgery and non-treated males, similar to what was observed by Rhebergen et al. (2016).

Results

Genital lobe lengths differ between *D. pachea* stocks

In our laboratory stock 15090-1698.01, *D. pachea* males display a characteristic left-right size asymmetry of genital lobes with the left lobe being consistently larger than the right lobe (Figure 1A,C, Figure 2 A, Table 1, Additional datafile 3). Similarly, in stock 15090-1698.02, most males reveal a larger left lobes, but a few individuals are observed with particularly small lobes that are rather symmetric in length (Figure 1b-d, Figure 2b). To create a stock containing a larger proportion of males with shorter left lobes, we selected 3 males of stock 15090-1698.02 with particularly short left lobes (Lang and Orgogozo 2012) and crossed them with 3-4 sibling virgin females. We repeated this selection in the offspring generation for a total of 36 generations (equivalent to approximately three years). Then, we changed the selection procedure and just removed males that had the typical lobe asymmetric lobe length ratio for another 12 generations (Material and Methods). In the final stock (selection stock) we observed an increased variance of left lobe length compared to the source stock 15090-1698.02 (Levene's test: selection stock / 15090-1698.02: DF = 1/147, F = 11.506, P = 0.0008913) (Figure 2C, Additional datafile 3). In contrast, the right lobe length differed only marginally among stocks (Table 1) and the variances of right lobe lengths were not significantly different (Levene's test, DF = 1/147, F = 0.5164, P = 0.4735).

Females tend to copulate first with males with longer lobes

To test whether lobe size might affect male mating success before copulation, we set-up a mate competition assay. We introduced a single female of wild-type stock 15090-1698.01 or 15090-1698.02 and two sibling males of the selection stock that visually differed in lobe length when inspected with a binocular microscope (Material and Methods) (Additional datafile 1: Figures S1 and S2, Additional datafile 2). Then, we video-recorded their behaviour. Once a copulation was

observed, the male that did not copulate was removed from the chamber about 5-10 min after copulation start with the other male. Both males courted the female simultaneously in the majority of experiments (111/152) (Additional datafile 1: Table S1), while courtship of a single male was observed to a lesser extend (41/152). No courtship was identified in 4 experiments (all with females of stock 15090-1698.01). In experiments where both males courted simultaneously, females engaged into a copulation more often with the male that had a longer left lobe (binomial test, N = 111, P = 0.00224) and left lobe length was positively associated with copulation success in experiments with females of stock 15090-1698.01 and stock 165090-1698.02 (Table 2), while right lobe length was not associated. To test for potential effect of body size, we measured the length of the left foreleg tibia in all males. Left lobe length tended to be loosely but not significantly associated with tibia length (GLM: left lobe length ~ tibia length: DF = 1/205, F = 3.2241, P = 0.07403). We found that females did not preferentially engage into copulation with the males displaying the larger tibia (binomial test, N = 103, P = 0.5546).

Courtship activity affects copulation success

Regardless of the stock for the female used in our experiments, courtship durations appeared to be longer when both males courted the female simultaneously compared to experiments where only one male courted (Mann-Whitney-Wilcoxon tests, females 15090-1698.01, W = 385, N = 25/51, P = 0.0053; females 15090-1698.02, W = 219, N = 16/60, P = 0.0009) (Figure 3A). We also observed that courtship duration was strikingly increased in females that were older than 11 days (after emerging from the pupa) compared to younger females (Additional datafile 1: Figure S4) (GLM, courtship duration ~ female age (<12 days/older): DF = 1/109, F = 14.9604, P = 0.0001872). We further examined experiments where both males courted the female simultaneously and tested whether courtship intensity or vigour of each male would correlate with the chance to copulate first with the female (male copulation success). For this, we estimated the contribution to courtship of

either male in experiments by quantifying one particular male courtship behaviour: we counted the frequency and duration of each male being closely following and/or touching the female ovipositor with the proboscis (mouth-parts), a behaviour denoted as “licking” (Spieth 1952) (Additional datafile 4). This measurement also enabled us to evaluate if one male might systematically be close to the female abdomen and potentially block physical contact between the other courting male and the female. In 104 analysed experiments, licking behaviour was observed throughout *D. pachea* courtship from shortly after courtship start (Materials and Methods) to the start of copulation (Additional datafile 1: Figure S3). Both males reached at least once the licking position in all experiments, with a median of 4 and a range of 1-36 times (Table 3). This indicates that the female had physical contact with the proboscis of both males before copulation started (Additional datafile 1: Figure S3). We calculated a licking index for each male as the ratio of the sum licking duration of the male over total courtship duration of both males (Additional datafile 1: Figure S3) to compare the relative courtship activity of both males in each experiment. The copulating male revealed a higher licking index than the non-copulating male (Figure 3b) and male copulation success was significantly affected by the licking index (GLM, copulation ~ licking index, females of both stocks, χ^2 deviance = 22.46/288.35, P = 0.000002151) (Table 2), indicating that courtship vigour positively affects the chance of a male to engage first into copulation. Licking index was not significantly affected by lobe length (GLM, licking index ~ left lobe length + right lobe length; left: DF = 1/202, F = 1.0855, P = 0.2987, right: DF = 1/201, F = 0.2094, P = 0.6477), nor by male tibia length (GLM, licking index ~ tibia length: DF = 1/193, F = 1.9043 , P = 0.1692). Altogether, our results suggest that male body size does not affect courtship vigour and that genital lobe length and courtship activity independently affect the chance of a male to copulate.

Lobe length does not affect sperm amount in female sperm storage organs

To test whether lobe length influences the amount of sperm deposited into the female after

copulation, we dissected a random subset of females from experiments where copulation occurred. We prepared the female paired sperm storage organs (spermathecae) at 12 h - 24 h after copulation (54 females from stock 15090-1698.01 and 24 females from stock 15090-1698.02, additional file 2) and examined the presence of sperm. *D. pachea* has giant sperm (Pitnick and Markow 1994), which makes direct sperm counts difficult. Therefore, we determined an apparent average spermathecae filling level per female based on visual inspection, similar to Jefferson (1977) (Jefferson 1977) (Material and Methods). Neither right lobe length, left lobe length, sum licking duration, copulation duration, female age (days after emerging from the pupa) significantly affected sperm content in the spermathecae (Table 4). However, male adult age had a strong negative effect. Copulating males with an age lower or equal to the median adult age of 16 d revealed a higher female post-copulatory spermathecae filling level (median: 0.250, CI: 0.22 - 0.28, N = 41) compared to older males (median: 0.167, CI: 0.11 - 0.17, N = 37) (GLM spermathecae filling level ~ male age, DF = 1/77, F = 31.3110, , P = 0.0000003322). Given that *D. pachea* males become fertile about 13 d after emerging from the pupa (Pitnick 1993), our results indicate an optimal period of male ejaculate mass at the beginning of their reproductive period. With our sample size of 78 inseminated females, we did not detect a significant effect of lobe length on the amount of ejaculate transfer into the female (Table 4).

After partial amputation left lobe length still affects male copulation success

Other, undetected characters might co-vary with left lobe length and could possibly account for the higher success of males with longer lobes. To test this, we artificially shortened the left lobes of two sibling males of the selection stock that had developed long left lobes. Males were anaesthetised and selected by visual inspection of their genitalia using a stereomicroscope in order to keep the specimen alive. Partially amputated lobes ranged in length from lacking at least their most distal tip to more reduced stumps whose length approximated the corresponding right lobe. We

shortened the left lobes of both males by making wounds and amputation sections of similar sizes in order to be able to neglect possible effects of the caused wound on the male's chance to achieve a copulation. Both amputated males also lacked the bristles on most distal tip of the left lobe, which may be involved in male courtship or copulation performance. We introduced two left-lobe-manipulated males with a virgin female of stock 15090-1698.01 and monitored them as in the previous assay. Similar to experiments with unmodified males, we found that left lobe length was associated with copulation success in a logistic regression analysis, although the effect was weaker (54 experiments, Table 2). Our results indicate that the left lobe length increases the chance of *D. pachea* males to copulate with a female when left lobes have been partially amputated.

Discussion

Left lobe length affects the chance of a male to copulate

We observed a tendency of females to copulate first with the male that had the longer left lobe when two males courted simultaneously in our experiments. These results suggest that a long left lobe might not only stabilize an asymmetric genital complex during copulation (Rhebergen et al. 2016), but also increases the chance of a male to copulate first. Other factors, unrelated to lobe length, can also bias copulation. For example, females might prefer to copulate with males of larger body size. We found that left lobe length was slightly but not significantly correlated with tibia length, an approximation for overall body size. However, the chance for a male to engage into copulation was not dependent on the pairwise tibia size difference of the two males added to each experiment. The mutation(s) associated with lobe length variation in this stock are unknown. It is possible that one or several alleles associated with lobe length still segregate in this stock, even after 50 generations of inbreeding. If such segregating mutations have pleiotropic effects on other inconspicuous male characters (morphological, physiological or behavioural), these traits are

expected to covary together with lobe length and the effect of lobe length that we detected might actually be due to other co-varying factors. In experiments with artificially introduced lobe length variation (through surgery), males were taken from the selection stock but had the expected wild-type lobe asymmetry. Left lobe length difference was then randomly and artificially introduced in those males, so that the length difference would not be expected to co-vary with the relative phenotypic expression of the supposed underlying mutation. In such experiments, we still observed an effect of lobe length on copulation success in our logistic regression analysis, although the effect was less pronounced. Thus, we conclude that the length of the left lobe appears to affect copulation success directly, and not through the collateral effect of a hypothetical pleiotropic mutation affecting both lobe length and another trait.

Some examples of asymmetric male sexual characters are known, such as differentially sized claws of fiddler crabs (Uca), where males use their larger claw during courtship to attract females with a waving movement (Perez et al. n.d.; Oliveira and Custódio 1998; Jordão et al. 2007; Cummings et al. 2008). Asymmetric antlers of the fallow deer *Duma duma* were also associated with male fighting and displaying (Alvarez 1995) and a polymorphic leg asymmetry in the dance fly *Empis jaschhoforum* was suggested to alter courtship display and male attractiveness to females (Daugeron et al. 2011). Those asymmetries are found in secondary sexual characters, which are well visible and are used in visual signalling.

D. pachea male genital lobes are located externally during copulation and are not intromittent organs. Nevertheless, these lobes can be considered as genital structures and as primary sexual traits by following Eberhard's (1985) broad definition of genitalia because they contact the female abdomen and stabilize the complex of female and male genitalia during copulation (Rhebergen et al. 2016). Our results thus indicate that in *D. pachea* a primary sexual trait is also used as a sexual courtship signal. To our knowledge, this is the first time that a primary sexual trait is found to be involved in pre-copulatory courtship in insects. Future work should focus on how

lobe length might affect pre-copulatory courtship signalling. We did not observe direct contact of the female with male genitalia prior to the male mounting attempts at copulation start.

The pre-copulatory signal mediated by the left lobe could be visual or vibratory. During courtship of *D. melanogaster* and closely related species, males perform abdomen shakes ("quivers") that generate substrate-borne vibratory signals (Fabre et al. 2012). Perhaps such quivering also occurs in *D. pachea* and the frequency or amplitude of such signals could be affected by the length of the left lobe, thus producing varying vibration signals. Whether left lobe length affects mating via its visual impact on the female could be tested with blind females, or in the dark. Our experiment was not suitable to test the importance of the right genital lobe length, since the absolute variation in right lobe length was relatively low (Figure 2C). We cannot exclude that the right lobe is also selected by the females, but our present data does not allow us to draw any conclusion on this point.

Potential female benefits gained from mate-choice for males with long left lobes

Total courtship durations were increased in experiments where both males courted the female simultaneously compared to experiments with only one male courting. This indicates that females potentially require more time to accept one of the males for copulation in cases where two males court simultaneously. Alternatively, longer courtship durations might rely on male-male competition causing mutual disturbances in courtship display. Since we did not find any effect of left lobe length on male licking activity, left lobe length has probably little or no effect on male-male competition. Otherwise, the presence or behaviour of the future copulating male would potentially reduce courtship vigour of the non-copulating male and this would be correlated with left lobe length. Thus, the copulation bias towards males with the longer left lobe potentially reflects female mate-choice.

Female mate choice on male sexual traits was suggested to rely at least in part on the way a sexual trait is displayed or moved (Byers et al. 2010). This implies that a certain quantity but also accuracy of locomotor activity would matter in courtship and might better reflect overall male quality and “truth in advertising” than a morphological character or ornament alone. Independent of left lobe length, the licking index also affected the chance of a male to engage first into copulation with the female. Left lobe length might therefore influence female mate choice through enhancing the quality of courtship signals while the licking index reflects overall male courtship vigour. Female mate choice is hypothesized to be based on direct benefits, affecting fecundity and survival of the female, but also on indirect benefits that relate to genetic quality, fecundity and survival of the progeny (Kirkpatrick and Ryan 1991; Byers et al. 2010). One possible direct benefit to mate with a male that has a long left lobe could potentially be an increased efficiency of sperm or ejaculate transfer during copulation. Males of *D. pachea* produce giant sperm and previous estimates revealed that a *D. pachea* male transmits only about 44 ± 6 sperm cells per copulation (Pitnick and Markow 1994), while the maximum female sperm storage capacity in the spermathecae was estimated to be much higher and to be about 304 sperm cells (Pitnick and Markow 1994). Wild-caught *D. pachea* females were found to contain sperm from at least 3-4 males based on spermathecae filling levels (Pitnick and Markow 1994). As asymmetric lobes stabilize the genital complex (Rhebergen et al. 2016), they might have a positive effect on the amount of sperm transferred per copulation. However, we have not observed any effect of lobe length on sperm presence in the spermathecae after copulation. We note that our quantification method was not precise and that sample size was limited. Accurate direct sperm counts requires a radio-labeling methods (Pitnick and Markow 1994), which was not applicable in our experimental setup. In future approaches, improved sperm counts should be applied to test for a correlation between lobe length, female-male copulation complex stability and sperm quantity in female spermathecae. Ideally, the

ejaculate quality is expected to be correlated with left lobe length, in terms of sperm cell number and sired offspring per copulation.

Indirect paternal effects on the growth and survival of offspring could potentially play a role in *D. pachea* female mate-choice for males with long left lobes. It has been demonstrated that mate choice based on particular male sexual characters correlates with offspring survival in diverse species (von Schantz et al. 1989; Norris 1993; Petrie 1994; Hasselquist et al. 1996; Hoikkala et al. 1998; Welch et al. 1998). However, our experiment did not allow us to test for these effects because females were sacrificed after the mating experiment and no progeny was obtained. We assessed sperm filling levels instead of the amount of female progeny because *D. pachea* females rarely lay eggs after a single insemination and about four copulations are necessary for a female to start oviposition (Pitnick 1992). To test for indirect effects, future studies should ideally use progeny males from wild caught *D. pachea* females and test if subtle lobe length variation in those males would correlate with offspring survival in single couple crosses. A similar approach was used by Hoikkala et al. (1998) (Hoikkala et al. 1998), who found that *Drosophila montana* courtship song frequency of wild caught males correlated with the survival rate of the male's progeny.

Age of reproductive activities appear to be non-overlapping in sibling males and females

The amount of sperm present in the female spermathecae after a single copulation was negatively affected by the age of the copulating male. Similarly, it was previously found that the number of progeny in single couple crosses was dependent on male adult age in *D. pachea* (Jefferson 1977). Males in *D. pachea* need about 10-14 days at 25° C after emergence to become sexually mature (Jefferson 1977; Pitnick 1992, 1993). This is related to adult testis growth and production of giant sperm (Pitnick and Markow 1994). It was shown that the relatively long time to reach male sexual maturity impacts the proportion of sexually active *D. pachea* adults (operational sex ratio), which is female-biased (Pitnick 1993). Our findings suggest that males potentially have a

maximum fertility period at the beginning of their sexual maturity at approximately 13-16 days after emerging from the pupa. The detected reduction of transferred sperm mass in males older than 16 days implies an additional male reproductive limitation.

It was hypothesized that the delay in male sexual maturity might potentially lead to decreased sibling mating in *D. pachea* (Pitnick 1992). It was observed in *D. melanogaster* that sibling matings yielded fewer progeny compared to crosses with unrelated individuals (Jefferson 1977). In our study, the female was not a sibling of the two males. Courtship durations were shortest in experiments with “young” virgin females, between 6 and 11 days. Given that females reach sexual maturity at about 4 days after emerging from the pupa (Jefferson 1977; Pitnick 1993), our observation suggests that young females engage more quickly into copulation compared to older females. Overall, female and male peaks in reproductive abilities appear to be non-overlapping among siblings of opposite sex and similar age. Such an effect should be relevant in the wild if a cactus rot hosts sibling fly progeny, which is realistic given that a single female usually lays about 25-33 eggs per day under laboratory conditions (Pitnick 1993).

In conclusion, we found that the chance of a male to copulate first with a female was affected by the relative courtship activity and the left lobe length of each male. Our results indicate that long left lobes enhance the chance of *D. pachea* males to engage into copulation, which is expected to confer a reproductive advantage over males with short left genital lobes. Thus, the evolution of left-right asymmetric genitalia might not only relate to its function as a stabilizing device during copulation, but may also be used as a courtship signal in pre-copulatory mate choice. This study represents the first example of a primary sexual trait involved in pre-copulatory courtship in insects. The possibility that primary sexual traits may act as mating signals before copulation has rarely been investigated in insects. Our data suggests that this phenomenon may be more common that previously thought and may contribute to the rapid rate of genitalia evolution in animals with internal fertilization.

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Author Contributions

ML and BL designed the experiments. BL and DC recorded fly copulation and performed light microscopy analysis of *Drosophila* male genitalia. BL and ML analysed the data. ML, VCO and BL wrote the manuscript. All authors have read and approved the manuscript.

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Supporting Information

Additional datafile 1: Supplementary Figures and Tables, **Figure S1:** Camera device for movie recording, **Figure S2:** Pairwise lobe length differences of males used in the competition mating experiments, **Figure S3:** Courtship and copulation duration in competition mating experiments **Figure S4:** Courtship duration increases with female age, **Table S1:** Competition mating experiments used for courtship analysis.

Additional datafile 2: Measurements of mating behaviour, genital lobe lengths and spermathecae filling levels of individuals used in the competition mating experiments.

Additional datafile 3: Raw measurements of lobe lengths in different *D. pachea* stocks

Additional datafile 4: Dataset for courtship index analysis.

Tables

Table 1: lobe lengths in *D. pachea* stocks. †Confidence interval

stock	left lobe [µm], median (95% CI†)	right lobe [µm] median, (95% CI†)	n
15090-1698.01	206.7 (169.7 - 215.4)	147.6 (145.4 - 153.1)	50
15090-1698.02	204.7 (190.5 - 209.2)	145.4 (138.6 - 146.4)	50
selection stock	186.1 (171.8 - 185.0)	142.2 (138.7 - 144.2)	99

Table 2: Effects of lobe length and courtship behaviour on male copulation success. N Experiment: number of experiments, N licking index: number of experiments where the licking index was calculated. †GLM model: male copulation ~ licking index, ‡GLM model: male copulation ~ left lobe length + right lobe length; ANOVA, χ^2 : deviance / null residual)

Experiment	N Experiment / N licking Index	Copulating males [%] (binomial test: equal proportions)	Effect on copulation success, logistic regression		
		larger left lobe length	licking index†	left lobe length‡	right lobe length‡
all experiments with unmodified males	111/104	64.87 ***P* = 0.00224**	χ^2 = 22.46/288.35 **P = 0.000002151**	χ^2 = 15.71/415.83 **P = 0.00007395**	χ^2 = 0.07/415.83 P = 0.7878
Females 15090-1698.01 unmodified males	51/47	68.63 ***P* = 0.01097**	χ^2 = 14.22/130.31 **P = 0.0001631**	χ^2 = 9.54/209.32 **P = 0.00201**	χ^2 = 0.01/209.32 P =0.9247
Females 15090-1698.02 unmodified males	60/57	61.67 *P* = 0.09246	χ^2 = 9.60/158.04 **P = 0.001949**	χ^2 = 6.59/206.5 **P = 0.01026**	χ^2 = 0.24/206.5 P = 0.62693
Females 15090-1698.01 left lobe cut	54/---	57.41 *P* = 0.34090	---	χ^2 = 5.72/169.13 **P = 0.01673**	χ^2 = 0.03/169.13 P = 0.8387

Table 3: Courtship duration, courtship index and copulation duration in experiments where both males courted the female

female	male	n	total courtship duration [min]		sum licking duration [min]		No. licking periods		Licking Index		copulation duration [min]	
			median	range	median	range	median	range	median	range	median	range
15090-1698.01	non-copulating	47	3.23	0.30-33.80	0.63	0.02-7.68	4	1-22	0.23	0.016-0.60	---	---
	copulating	47			1.00	0.10-12.53	3	1-29	0.40	0.014-1.00	32.84	10.13-49.21
15090-1698.02	non-copulating	57	6.3	0.74-23.10	1.58	0.04-14.24	5	1-36	0.37	0.017-0.84	---	---
	copulating	57			2.80	0.14-14.58	5	1-35	0.45	0.038-0.96	27.7	2.72-50.21

Table 4: Effects of lobe length, sum licking duration, copulation duration and age on spermathecae filling levels. †GLM model, spermathecae filling level ~ predictor variable, gaussian(link=identity), F-test

Effect on approximate spermathecae sperm filling level †					
left lobe length	right lobe length	sum licking duration	copulation duration	female age	male age
DF = 1 / 76 F = 0.0256 P = 0.9096	DF = 1 / 76 F = 2.2775 P = 0.1310	DF = 1 / 47 F = 0.4541 P = 0.5038	DF = 1 / 77 F = 2.3555 P = 0.1383	DF = 1 / 77 F = 1.3021 P = 0.2525	**DF = 1 / 77** **F = 32.029** **P = 0.00000033221**

Figure Legends

Figure 1: *Drosophila pachea* male genital lobes. (A) Male of stock 15090-1698.02 in ventral view and with asymmetric lobes. (B) Male of the selection stock with apparently symmetric lobes. The scale bars correspond to 200 µm. (C) Posterior view of a dissected male terminalia of stock 15090-1698.02 with asymmetric lobes. (D) Posterior view of a dissected male terminalia of the selection stock; the scale bars correspond to 100 µm, dots indicate lobe length measurement points.

Figure 2: Genital lobe lengths differ between *D. pachea* stocks. Lengths of the left and right epandrial lobe lobes are presented for (A) stock 15090-1698.01, (B) stock 15090-1698.02, and (C) the selection stock. Sibling males of those used in the mating experiment are shown in panel (C). Each point represents one male. The variance of left lobe length is increased in the selection stock compared to the source stock 15090-1698.02 (Levene's test: selection stock / 15090-1698.02: DF = 1/147, F = 11.506, P = 0.0008913), while the variance of the right lobe length were not significantly different (Levene's test, DF = 1/147, F = 0.5164, P = 0.4735).

Figure 3: Courtship durations and relative courtship activity. (A) The total courtship duration was shorter in experiments with only one male displaying courtship signs (one, white box-plots) compared to experiments with both males courting simultaneously (both, grey box-plots) (Mann-Whitney-Wilcoxon test, female stock 15090-1698.01, W = 385, N = 25/51, P = 0.0053 ; females of stock 15090-1698.02, W = 219, N = 16/60, P = 0.0009). (B) The licking index (ratio of sum licking duration of a single male over total courtship duration of both males) of the copulating male (cop, grey box-plots) was higher than the licking index of the non-copulating male (non-cop, white box-plots) (GLM logistic regression, copulation ~ licking index: females of 15090-1698.01, χ^2 deviance = 14.22/130.31, P = 0.0001631; females of 15090-1698.02, χ^2 deviance = 9.60/158.04, *P* = 0.001949). horizontal bars indicate pairwise comparisons with P-values ** < 0.01. Each point represents one mating experiment in (A) and one male in (B).

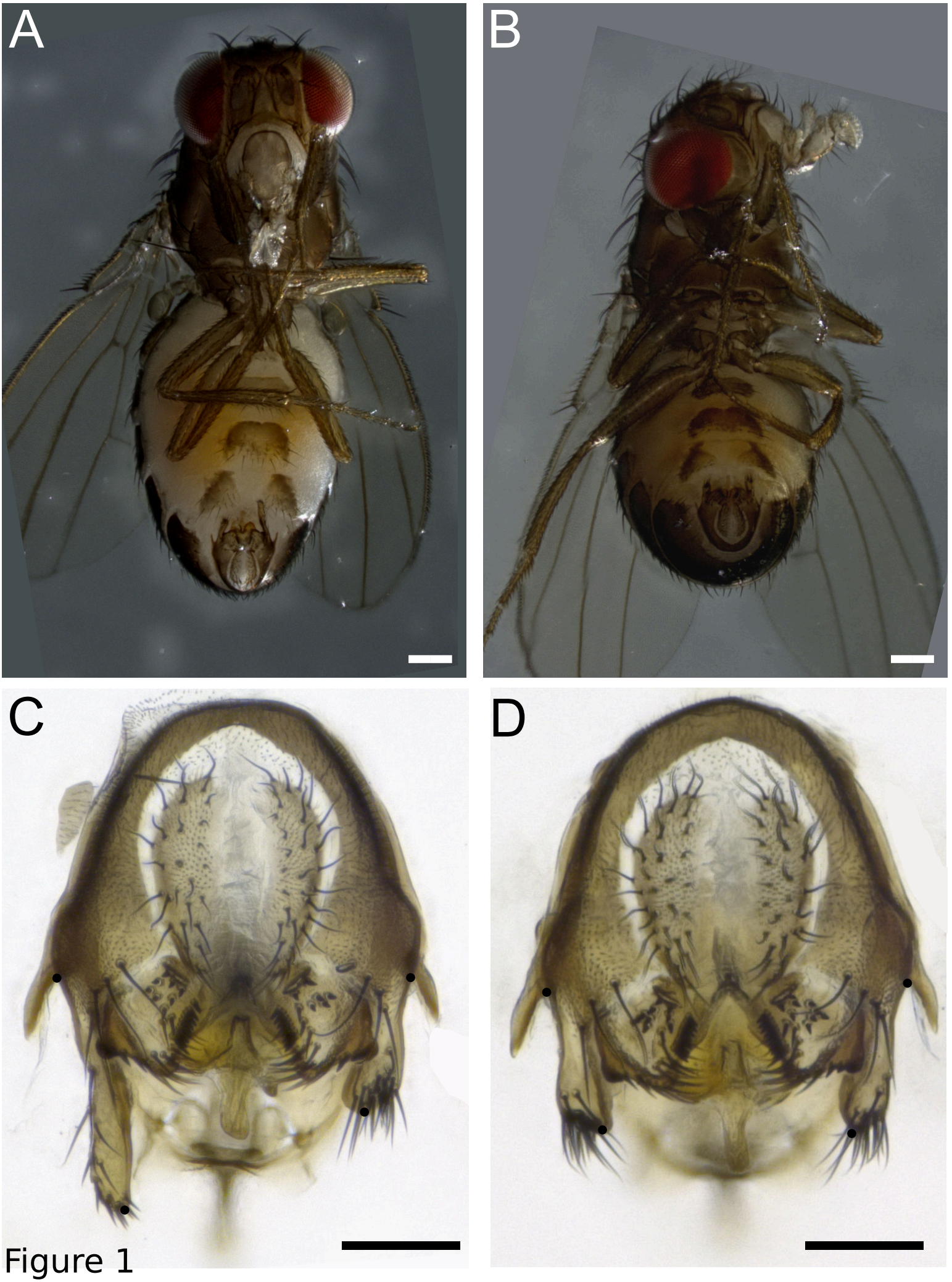

Figure 1

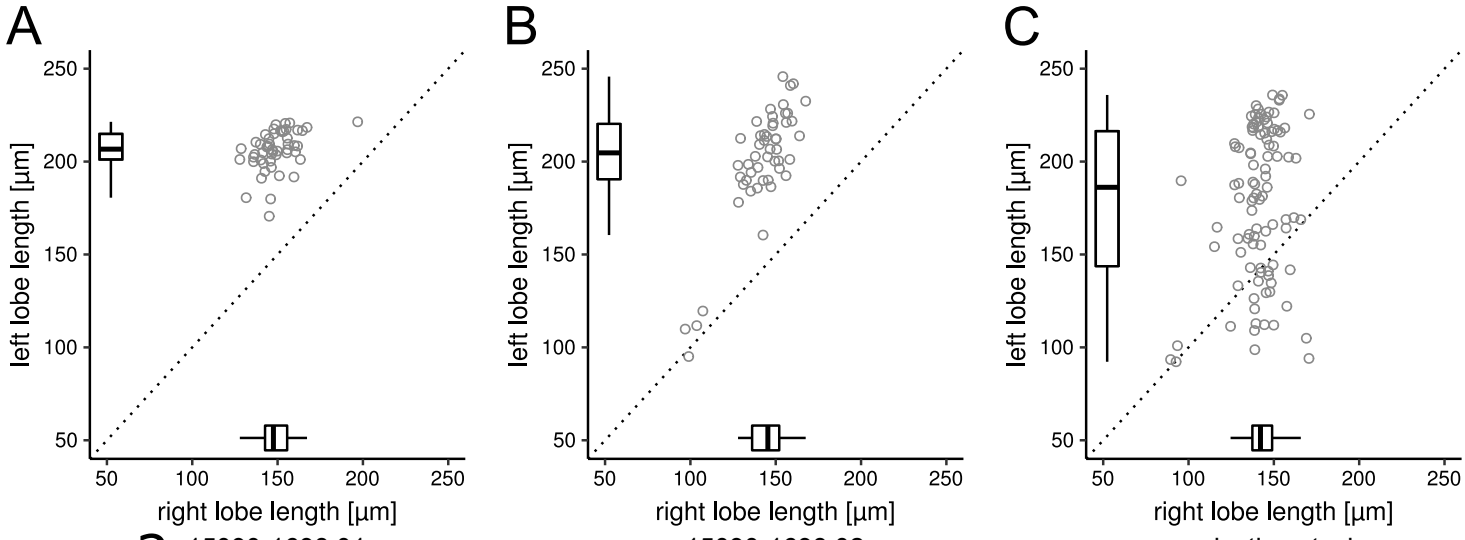
A
B
C
left lobe length [µm]
right lobe length [µm]
250
200
150
100
50
15090-1698.01
15090-1698.02
selection stock

Figure 2

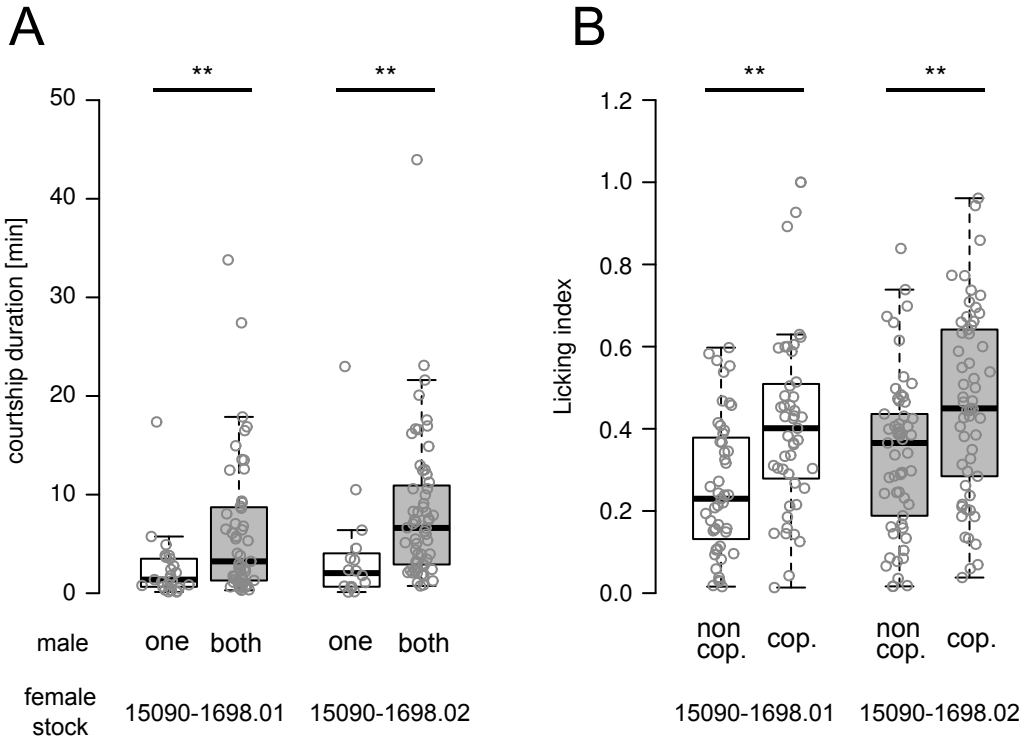
A
B
**
**
courtship duration [min]
50
40
30
20
10
0
male
one
both
one
both
female stock
15090-1698.01
15090-1698.02
Licking index
1.2
1.0
0.8
0.6
0.4
0.2
0.0
non cop.
cop.
non cop.
cop.
15090-1698.01
15090-1698.02

Figure 3