

Territory defense as a condition-dependent component of male reproductive success in *Drosophila serrata*

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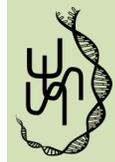
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Sexual selection arises from both intrasexual competition and mate choice. With respect to the evolution of male traits, there is a vast literature documenting the existence of female choice and male–male competition, and both have been shown to occur in many species. Despite numerous studies of these two components of male reproductive success in isolation, few have investigated whether and how they interact to determine total sexual selection. To address this, we investigate male territoriality in *Drosophila serrata*, a species in which female preference for male sexual pheromones (cuticular hydrocarbons or CHCs) have been extensively studied. We demonstrate that territoriality occurs, that it involves direct male–male aggressive interactions, and that it contributes to variation in male mating success. Results from a phenotypic manipulation also indicate that territorial success is condition-dependent, although a genetic manipulation of condition, involving three generations of full-sib inbreeding, failed to find a significant effect. Finally, selection assays also suggest that territorial success depends on male body size but not on CHCs, whereas the opposite is true for mating success.

KEY WORDS: Cuticular hydrocarbons, defense, mate choice, sexual selection, territoriality.

Sexual selection arises from variation in reproductive success which is itself the product of intrasexual competition and mate choice. In most species, successful reproduction requires males to succeed at several stages (Andersson 1994; Whitlock and Agrawal 2009). First, he must locate a female (or attract her to him), potentially competing indirectly with other males in doing so (i.e., scramble competition). He may then have to convince or otherwise coerce her to mate, potentially competing directly with other males (i.e., contest competition). Finally, prior to fertilization, his sperm may face competition with that of rival males, as well as cryptic female choice. Sexual selection research has tended to focus on the diverse and elaborate traits that have evolved in males because of the advantage they provide to one of these stages, often because such traits are presumed costly to other components of fitness (e.g., viability; Andersson 1994; Rowe and Houle 1996; but see Kotiaho 2001). Less attention has been given to quantifying how these different stages combine, and potentially interact, to determine overall variation in reproductive success and total sexual selection on traits (Hunt et al. 2009).

Here, we investigate territory defense as a potential component of male reproductive success. Gaining access to female gametes is essential to reproducing and to achieve this males of some species compete with other males to acquire and defend resources that are required by females (Emlen and Oring 1977; Andersson 1994). The idea is that, instead of fighting over females directly, males compete to control territories containing one or more resources that are needed by females, thereby gaining access to females that are attracted to the resource(s). This has been referred to as a “resource defense mating system” (Proctor et al. 2012), although in line with past *Drosophila* research we refer to it simply as territory defense or territoriality. Variation in territory defense contributes to variation in mating success in some species (e.g., green frog *Rana clamitans*, Wells 1977; small heath butterfly *Coenonympha pamphilus*, Wickman 1985; red deer *Cervus elaphus*, Carranza et al. 1990), and territorial behavior has been well studied with regard to the evolution of traits such as weaponry and other status signals (Møller 1987; Emlen and Nijhout 2000).



Territoriality and other forms of male–male competition are also known to occur alongside female mate preferences in many taxa (Bradbury and Davies 1987; Berglund et al. 1996; Qvarnström and Forsgren 1998; Wong and Candolin 2005). However, limited attention has been given to quantify the resulting sexual selection that each generates on its own, nor to how these combine to determine total sexual selection (Hunt et al. 2009).

To begin to address this, we study territory defense in the fruit fly, *Drosophila serrata*. Although male territoriality has previously been demonstrated in *D. melanogaster*, *D. simulans* (Jacobs 1960; Dow and von Schilcher 1975; Hoffmann 1987), and the lekking Hawaiian *Drosophila* (Spieth 1974; Boake 1989), it has not been studied in *D. serrata*. *Drosophila serrata* is a useful species in which to investigate territoriality because it is a model system for the study of another component of reproductive success: male mating success arising from female mate choice. Female mate choice in this species targets a suite of long-chain, nonvolatile cuticular hydrocarbons (CHCs) that act as contact pheromones in males (Chenoweth and Blows 2003, 2005; Howard et al. 2003; Rundle et al. 2009). The evolutionary genetics of male displays (CHCs), female preferences, and the resulting sexual selection has been extensively studied via a series of behavioral assays, evolution experiments, and quantitative genetic studies (Hine et al. 2002, 2004, 2011; Blows et al. 2004; Petfield et al. 2005; Delcourt et al. 2010, 2012; Delcourt and Rundle 2011; McGuigan et al. 2011a; Sztepanacz and Rundle 2012). CHCs are also known to be important to other aspects of communication in other *Drosophila* (Ferveur 2005; Kent et al. 2008), including male–male competition (Wang et al. 2011). Studying territoriality in a system in which mate choice is well understood allows the traits underlying each of these components to be compared and joint selection to be quantified. It may also ultimately provide a more comprehensive understanding of the relative importance of different sources of variation in male reproductive success.

The condition dependence of territoriality is also of interest. Traits enhancing reproductive success will be subject to persistent directional selection favoring individuals that allocate as much as they can afford to trait expression (Andersson 1982; Nur and Hasson 1984). If the marginal cost of trait exaggeration is greater for low-condition individuals than for high-condition individuals, theory suggests that heightened condition dependence should evolve such that trait expression becomes more tightly linked to available metabolic resources (Getty 1998, 2006; Proulx et al. 2002). As condition dependence evolves, variance in genome-wide genetic quality is captured in the expression of the trait, potentially maintaining variance in the face of persistent directional selection (Rowe and Houle 1996). There are many studies demonstrating the condition dependence of particular traits, often chosen because they are targets of female mate preferences (Cotton et al. 2004; Delcourt and Rundle 2011). However, the

condition dependence of overall male reproductive success has received limited attention, as has territorial success as a component of this. The condition dependence of reproductive success is important as it may contribute to adaptation and the purging of deleterious mutations (Whitlock and Agrawal 2009). Given the significant time and energy that is often invested in acquiring and defending a territory (Grantner and Taborsky 1998; Kelly 2008), a male's success at doing so is likely to be a function of his condition.

Our goals are to establish whether *D. serrata* males will defend a territory, and if so, whether their success at doing so is a condition-dependent component of their reproductive success. Although an individual's condition is affected by both their genetic quality and the environment they have experienced, most studies of condition dependence address the latter. However, many signaler-receiver coevolution models, including good-genes theories (Rowe and Houle 1996; Tomkins et al. 2004), focus on genetic variation in condition and we therefore test for condition dependence using both an environmental and a genetic manipulation. Although condition dependence theory is widely interpreted as predicting concordant effects of genetic and environmental stress on the expression of condition-dependent traits (because both act to reduce an individual's condition), this has received little empirical attention (Whitlock and Agrawal 2009). Finally, we also quantify and compare sexual selection generated from territoriality and mating success on a suite of traits known to be important in female mate choice (male CHCs) and on male body size, a trait implicated in territorial defense in other *Drosophila* (Hoffmann 1987).

Materials and Methods

Our assays used a previously described, outbred, laboratory-adapted stock population of *D. serrata* (Rundle et al. 2006; Delcourt et al. 2009, 2010; Delcourt and Rundle 2011).

TERRITORIALITY AND ITS CONSEQUENCES FOR MALE REPRODUCTIVE SUCCESS

To test for territoriality, we conducted replicate assays in which two males competed for a single food resource. In an attempt to provide a phenotypic marker by which to differentiate the two males, we also used a population that was fixed for a homozygous recessive mutation resulting in an orange-eyed phenotype. However, this marker varies in its visibility in response to unknown factors and because eye color proved difficult to score in the virgins we collected for use in this assay, we decided at that time to additionally mark males by clipping a small piece from opposite wings (left wing for wild type, right wing for mutant males). Use of the orange-eyed marker was discontinued in all subsequent assays. In this first assay, the ability to control the

food resource did not differ on average between the left clipped, wild-type males and right-clipped, orange-eyed males (two-sided one-sample *t*-test of the “territoriality difference score,” as described below: $t_{99} = 1.33$, $P = 0.188$), indicating little effect of either of the markers.

Adult flies for use in the assay were collected upon eclosion from their pupae and separated by sex as virgins within 24 h using light CO₂ anesthesia. Males were housed at a density of 10 individuals/vial for 24 h prior to wing clipping, and then for two days further after clipping. Territorial interactions were examined after aspirating pairs of males (one wild type and one mutant) into each of 100 arenas, modified from Hoffmann (1987). The arenas were constructed from two petri dishes (100 × 15 mm), one inverted over the other. A small hole (covered with tape) was made in the top dish to allow addition of the flies, and a food cup (diameter = 20 mm, height = 1.2 mm), containing 5 mL of standard medium with a small ball of yeast paste (approximately 5 mm diameter) on top, was placed in the center of each arena. The bottom was lined with tap water-saturated filter paper to maintain humidity. Males were given 24 h to interact and territorial success was then scored via spot counts, taken at 30 min intervals across 2.5 h, in which the position of each male relative to the food was recorded (i.e., on/off). Using the same protocol, two additional series of observations were made for arenas containing either a single wild-type male or a pair of previously mated wild-type females (20 replicates each).

In addition to the territorial assays, to gain direct insight into the frequency and nature of male–male interactions involved in establishing territories, 10 replicate arenas of the same design were set up with pairs of three-day-old males. Observations began immediately and continued uninterrupted for 7 h. For each replicate, we recorded the number and type of male–male interaction, along with the position of each male when these occurred. An interaction was defined as starting with the approach of one male by the other, followed by a series of behaviors of varying levels of aggression, and ending with one male either retreating or being chased away. During each interaction, we recorded the occurrence of specific behaviors following Hoffmann (1987) and Chen et al. (2002), including (in approximate order of increasing intensity) wing threats, fencing (with their forelegs), chasing, lunging, holding, and tussling.

To assess whether variation in territory defense affects male mating success, pairs of three-day-old wild-type virgin males were again competed in each of 90 arenas as described above. Territorial success was recorded via spot counts every 30 min from 13:00 to 16:00, and again from 10:00 to 12:00 the following day. Four replicates were discarded at this point because one or both males had behaved abnormally, showing little movement and no territorial defense. A single virgin female was then aspirated into each arena, followed by two 3 h mating observation periods from

13:00 to 16:00 on the same day and 09:00 to 12:00 on the following morning. Observations involved continuously scanning all replicates such that each arena was checked approximately every 5 min. The identity of the successful male was recorded whenever a mating was observed, with each mating counted only once.

Statistical analyses

To test for territorial behavior, we assessed whether the probability a male being on the food source depended on the status (i.e., on or off) of the other. To provide a single overall test, we calculated a χ^2 statistic that compared the observed and expected (under independence) frequencies for the joint position of the two males (i.e., both males on, both males off, wild type only on, orange-eyed only on) using the combined data across all five observation periods from all replicate arenas. Because a given arena contributes multiple, nonindependent datapoints (i.e., one from each of the observation periods), the observed χ^2 value was compared to a distribution of expected values generated from 10,000 randomizations performed under the null hypothesis of independence. In each iteration, the occupancy status of one male within a particular replicate (i.e., arena) was randomly reassigned across all observation periods for that replicate, independent of the status of the second male but preserving the number of times it was observed on or off the resource (i.e., the overall occupancy rate). This method was similarly used to assess independence in the assays using two females. Given a significant overall effect, standard χ^2 tests of independence (1 df) were subsequently performed for each observation period separately, with Fisher’s exact tests used for the assay with females due to the smaller sample size.

To test for consistency (i.e., whether a male in possession of the food resource tended to remain so), we counted the number of times that ownership switched between observation periods for a given arena, comparing the observed mean number of switches to a distribution of expected values from a randomization procedure in which there was no consistency. A male was inferred as possessing the food resource when he was the sole occupant, and a switch was said to have occurred if possession switched between counts. The null distribution of expected number of switches was generated from 10,000 randomizations in which the observed joint position of the two males was shuffled among replicate arenas separately for each observation period. This procedure removed any correspondence across observation periods within a given replicate (i.e., any consistency) while still maintaining both the marginal and total occupancy rates. All randomizations were performed using the PopTools add-in for Microsoft Excel (Hood 2010).

To test whether territoriality was a component of male reproductive success, we calculated a “territoriality difference score” for each male pair as the score of the male that most frequently

occupied the resource minus the score of the other male. A “mating score” was then calculated as the difference in the number of matings obtained by the male with the higher territoriality score minus the number obtained by the male with the lower territoriality score. For cases in which the two males occupied the resource an equal number of times, the mating score was calculated as the difference in the number of matings between the right- and left-clipped male. (Results do not change qualitatively if calculated as left-clipped minus right-clipped.) Although the difference in territorial score is always ≥ 0 , the more successful male does not necessarily receive more matings, so the difference in mating success may be positive or negative. A positive difference is expected if success in holding a territory results in higher mating success, and we therefore used a one-sample *t*-test to test whether average mating score differed from the null hypothesis of zero. However, when differences in territorial success are small, differences in mating success might be similarly small and therefore difficult to detect. If holding a territory more often results in greater mating opportunities, then mating success score should increase with increasing territorial score. We therefore also tested for positive correlation between territorial and mating success scores.

CONDITION DEPENDENCE

We generated adult males of varying condition via both an environmental and a genetic manipulation. The environmental manipulation altered larval rearing density by allowing either 10 or 40 mated females (low vs high density, respectively) to oviposit onto 10 mL of standard yeast medium for 24 h (10 vials/treatment). The high/low density rearing environments should generate low/high-condition flies, respectively. The number of eclosing flies was counted and on day 12, 10 males from each replicate were collected, dried at 65°C for 24 h, and weighed to the nearest 10^{-6} g using a MX5 microbalance (Mettler-Toledo, Columbus).

The genetic manipulation used three generations of full-sibling inbreeding, the effects of which on male condition should be representative of segregating deleterious mutations in the stock population. Virgin male–female pairs were established in each of 285 vials, and from the offspring of each a single male and female sibling pair was collected and transferred to new vials for egg laying. This was repeated for two subsequent generations, resulting in a total of three generations of inbreeding (inbreeding coefficient, $f = 0.5$). At the F2 generation, a second male and female pair was also collected from each vial and mixed among vials to generate outbred control lines. This step was repeated at the F3 generation. Due to the loss of some lines at each generation, 90 inbred and 90 outbred “lines” were used in the subsequent competition trials. Flies from the inbred/outbred lines were considered low/high condition, respectively.

Virgins were collected upon eclosion as previously described, with males and females housed separately at a density of 5–7/vial.

To discriminate between competing males, high/low-condition flies had a small piece clipped from their left/right wing, respectively. Trials were conducted over three days, and all males in a given experiment were of the same age (five/four days in the environmental/genetic manipulations, respectively).

To manipulate the opportunity for a male’s territoriality to contribute to his mating success, experimental arenas contained either a single resource in the center or two resources placed 6 cm apart and centered in the middle of the arena. In both cases, the resource consisted of a food cup containing 5 mL of standard medium and a ball of yeast paste as in the previous assays. One low-condition male and one high-condition male were aspirated into each of 200/180 arenas for the environmental/genetic manipulation trials, respectively. For both experiments, half of the arenas contained a single resource (high opportunity for competition over territories) and the other half two resources (low opportunity for competition over territories). After approximately 16 h, both males were scored for territorial success by recording the position of each relative to the food source(s) (i.e., on vs off). Scores were taken every 0.5 h for a total of six counts.

After territorial scores were recorded, a single virgin female was aspirated into each arena and mating observations were made by scanning the arenas at approximately 5 min intervals. The identity of the successful male was recorded whenever a mating was observed, with each mating counted only once. Observations were made for 3 h (13:00–16:00) immediately after the females were added, and continued the following morning for a second 3 h (09:00–12:00). Both males were then frozen, subsequently dried at 65°C for 24 h, and weighed to the nearest 10^{-6} g.

Mean mass and time to emergence were compared between treatments using two-sample *t*-tests. The difference in territory and mating success between male pairs was calculated by subtracting the scores of low-condition males (i.e., high density or inbred males) from those of high-condition (i.e., low density or outbred) males. One-sample *t*-tests were used to assess whether this difference score was significantly different from zero, with a positive score indicative of an advantage for high-condition males. Two-sample *t*-tests were used to determine whether these difference scores differed between the two arena types (i.e., one vs. two food resources) representing low versus high opportunity for territoriality. Results were qualitatively unchanged if a generalized linear mixed model approach was used instead (online supplementary materials).

SELECTION ANALYSES

To estimate selection on CHCs and body size resulting from male–male territoriality and female mate choice, treatments were established that allowed successful and unsuccessful males to be identified for each of these components of reproductive success. First, to identify males that were successful or unsuccessful in defending a

territory, pairs of two-day-old virgin males were marked by wing clipping, allowed to recover for two days, and then aspirated into each of 300 experimental arenas. Arenas were identical to those used previously except that the yeast paste in the center of the food resource was covered with a wire mesh to prevent access by males. This design allowed males to detect the food so as to encourage territorial defense, but prevented them from consuming the live yeast as this may affect CHC expression (Gosden and Chenoweth 2011). Eighteen hours after being introduced, pairs were scored for territorial success via six spot counts made every 0.5 h in which the location of each male was recorded (i.e., on or off the food resource).

To estimate selection on CHCs and body size resulting from female mate choice, 300 binomial mating trials were conducted by introducing two males to a vial containing a single virgin female. Flies were introduced immediately prior to the observation period to limit the opportunity for males to establish territories, and there was also no live yeast in the vial for males to defend. Vials were observed continuously and the chosen and rejected males were identified from the first mating to occur. To minimize potential effects of prior experience, the two males in a particular replicate were taken from separate housing vials, and only replicates in which a first mating was observed within 1 h were included in the analysis. Although in theory male–male competition could still affect the outcome of such trials, several lines of evidence suggest that this assay primarily measures female choice in this species (see Delcourt et al. 2010).

For each treatment (i.e., territorial assays and female choice), both the losing (or rejected) male and the winning (or chosen) male had their CHCs extracted and quantified via gas chromatography as previously described (Delcourt et al. 2010), after which they were dried and weighed as previously described to provide an index of body size. Individual flies were treated as independent replicates because past work has shown that this has no discernible effect on the significance of individual selection gradients (Rundle et al. 2005). To minimize any confounding temporal effects (Gershman et al. 2014), extractions of males from each treatment (i.e., territoriality vs mate choice assays) were alternated throughout the day.

Individual CHC profiles were determined by integration of the area under nine previously identified peaks: (Z,Z)-5-9-C_{24:2}, (Z,Z)-5,9-C_{25:2}, (Z)-9-C_{25:1}, (Z)-9-C_{26:1}, 2-Me-C₂₆, (Z,Z)-5,9-C_{27:2}, 2-Me-C₂₈, (Z,Z)-5,9-C_{29:2}, and 2-Me-C₃₀ (Howard et al. 2003). To correct for technical error associated with quantifying absolute abundances, these values were converted to proportions and then logcontrast transformed as previously described (Delcourt et al. 2010), using (Z,Z)-5-9-C_{24:2} as the common divisor.

Prior to analysis, 13 multivariate outliers were identified and removed using the Mahalanobis distance technique implemented in JMP version 10.0 (SAS Institute Inc., Cary, NC). Standardized

sexual selection gradients (Lande and Arnold 1983) on the eight logcontrast CHCs and body size (dry weight) were estimated separately for each treatment (i.e., territoriality and female choice assays) using standard least squares multiple regression of relative success against standardized (mean = 0, standard deviation = 1) trait scores, with significance determined using logistic multiple regression, fit via maximum likelihood, because success scores are binomially distributed (Lande and Arnold 1983). To determine whether selection on these traits differed between treatment assay, we followed a sequential model building approach (Chenoweth et al. 2012). All of the data were combined in a single analysis using the multiple regression model described above, but with the inclusion of a fixed effect term representing the main effect of trial type (male–male territoriality vs female choice), along with nine terms representing the interaction of each trait with trial type. A likelihood ratio test (LRT) was used to compare the fit of this full model with a reduced one lacking the nine interaction terms, again implemented via logistic multiple regression. We focus on linear sexual selection because, even if a canonical rotation is used to condense all nonlinear selection onto the eigenvectors of γ (Blows and Brooks 2003), there is no evidence that nonlinear selection varies between the two assays (LRT, $\chi^2 = 8.16$, $df = 9$, $P = 0.518$).

Results

TERRITORIALITY

Significant nonindependence in occupancy of the food resource was detected between the two males overall (randomization, $P < 0.0001$). This nonindependence was also highly significant in separate tests of each observation period (Table S1), and in all cases involved a substantial overrepresentation of the situation in which one male was on the food and the other male was off it, and hence far fewer pairs than expected were either both on or both off of the food resource (Fig. 1). When placed singly into arenas, individual males were found on the resource in $70.8 \pm 18.7\%$ (mean \pm SD) of the observations, whereas in trials with pairs of males, both were found together on the resource in only $7.7 \pm 1.5\%$ (mean \pm SD) of the observations, substantially less than the 50.1% (i.e., $0.708^2 \times 100$) that would be expected if independent. In contrast, occupancy of the food resource by one female was independent of the location of the other female, both overall (randomization, $P = 0.710$; Fig. 1) and in separate tests of each observation period (Table S2). Furthermore, both females were found together on the food $68.8 \pm 10.3\%$ (mean \pm SD) of the time.

When territory ownership was examined across the five observation periods, an average of only 0.28 switches occurred in the identity of the male occupying the food source. This is significantly fewer than expected (mean = 1.17; randomization, $P < 0.0001$), indicating consistency in which male possessed

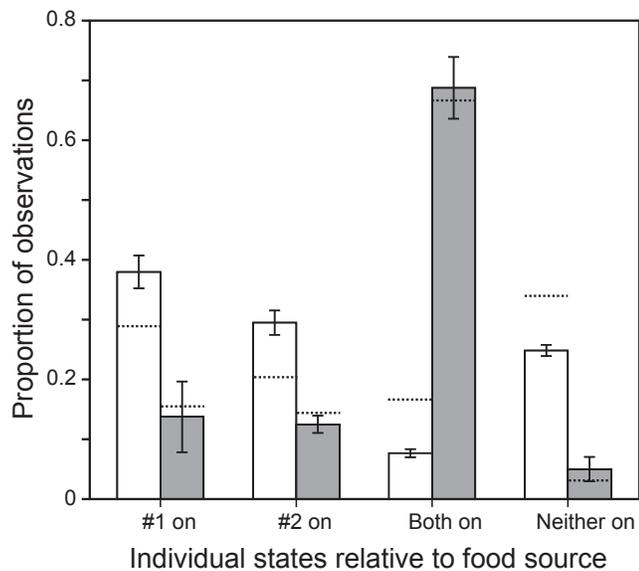


Figure 1. Average proportion of observations (\pm SE) in which pairs of males (white bars) or females (gray bars) were in each of four states relative to a yeast food resource during territorial assays. Dotted lines indicate expected values for each category under the null hypothesis of independence. For males, individual #1 corresponds to the left clipped (wild type) male, #2 to the right clipped (orange-eyed) male.

the resource. During continuous observations of male pairs in 10 replicate arenas, a total of 72 interactions were observed over 7 h. These interactions were initiated almost exclusively (71 out of 72 instances) on the food resource, and among replicate arenas their frequency was positively correlated with the total time that both males were observed together on the resource (Pearson's correlation: $N = 10$, $r = 0.88$, $P < 0.001$). These interactions usually involved a number of aggressive behaviors that ranged in intensity from wing threats to physical fights (e.g., foreleg fencing, lunging at one another, holding and tussling together; Table S3), and ended when one male retreated or was chased away by the other.

Average mating success score, representing the difference in the number of matings received by territory winners and losers, was not significantly positive (mean = 0.01; one-sample t -test: $t_{85} = 0.05$, $P = 0.951$). However, consistent with territorial defense being a component of male reproductive success, there was a significant positive association between territorial success and subsequent mating success (Pearson's correlation, $N = 86$, $r = 0.265$, $P = 0.014$; Fig. 2), with males that were most successful at defending a territory acquiring almost two more matings on average than the males that were the least successful.

CONDITION DEPENDENCE

High versus low density rearing vials produced an average of 638.3 ± 21.9 (SE) and 167.4 ± 8.6 offspring, respectively,

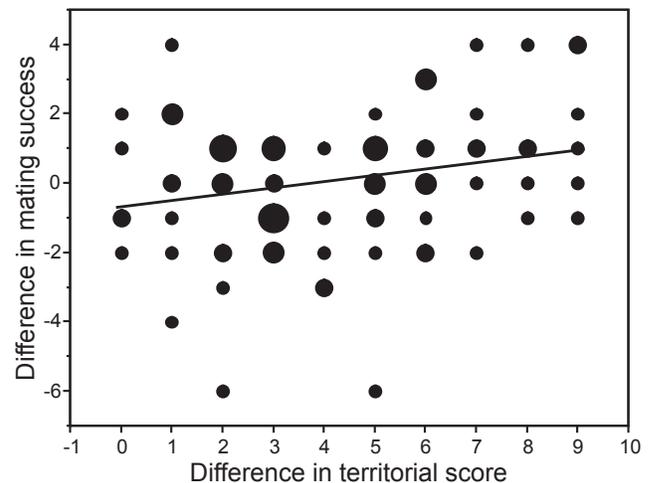


Figure 2. Difference in mating success (number of matings for the territory "winner" - number for the territory "loser") as a function of difference in territorial success (score territory "winner" - score territory "loser"; $r = 0.265$, $P = 0.014$). Circles are proportional to the number of contributing datapoints (smallest: $N = 1$; largest: $N = 6$).

yielding a relative viability of 3.63 (95% bootstrap confidence interval: 3.23–4.09). Flies reared under low density emerged nearly two days earlier and weighed almost 50% more on average than flies reared under high density (emergence time: $t_{18} = 9.48$, $P < 0.001$; mass: $t_{198} = 26.45$, $P < 0.001$; Fig. S1).

In the presence of a single food resource (i.e., high competition for territories), high-condition males were significantly more successful than low-condition males in acquiring a territory (one-sample t -test: $t_{99} = 2.63$, $P = 0.010$), generating a positive difference in their territorial success scores (Fig. 3A). By contrast, this advantage of high-condition males over low-condition males was absent in the arenas with two food resources and hence reduced competition for territories (one-sample t -test: $t_{99} = 0.87$, $P = 0.387$). In addition to having greater territorial success, high-condition males also acquired more matings than low-condition males when competition for territories was high (i.e., in the presence of a single food resource; one-sample t -test: $t_{99} = 3.20$, $P = 0.002$), again generating a positive difference in their mating success scores (Fig. 3B). High-condition males also tended to have a mating advantage when competition for territories was low (i.e., in the presence of two food resources), suggesting that mating success itself may be condition-dependent, independent of territorial success. However, this advantage was reduced compared to the single territory treatment and was nonsignificant (one-sample t -test: $t_{99} = 1.93$, $P = 0.056$; Fig. 3B). That the mating advantage of high-condition males over low-condition males was reduced and no longer significant when competition for territories was low is consistent with territoriality being a key component of mating success, although this difference in mating advantage

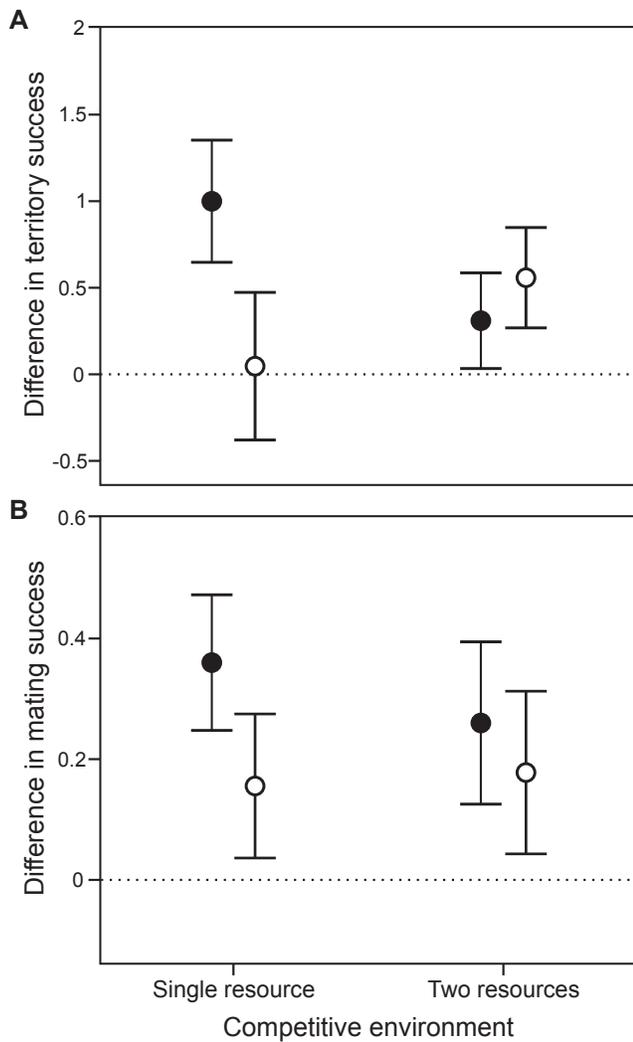


Figure 3. Average difference (\pm SE) between high- and low-condition males in (A) territorial success and (B) number of matings for treatment environments containing either one or two food resources (corresponding to high vs low opportunity for territoriality). High- and low-condition males were generated from either an environment (filled circles) or genetic (open circles) manipulation.

between one versus two resource treatments was not significant (two-sample t -test: $t_{198} = 0.57$, $P = 0.569$).

With respect to the genetic manipulation, inbred males were significantly smaller than outbred control males (mean \pm SD; outbred: 0.282 ± 0.024 mg; inbred: 0.268 ± 0.027 mg; $t_{335} = 4.90$, $P < 0.001$), although this difference in average mass ($\sim 5\%$) was much smaller than that observed in the previous larval density manipulation ($\sim 50\%$). Also in contrast to results from the density manipulation, outbred control males showed no significant advantage in either territorial success (one-sample t -test: $t_{89} = 0.052$, $P = 0.959$; Fig. 3A) or mating success (one-sample t -test: $t_{89} = 1.305$, $P = 0.195$; Fig. 3B) compared to inbred males in the single territory treatment. Outbred males similarly

had no advantage in either measure when multiple territories were present (one-sample t -tests, territorial success: $t_{89} = 1.687$, $P = 0.095$, Fig. 3A; mating success: $t_{89} = 1.321$, $P = 0.190$; Fig. 3B), and the difference between treatments was also not significant for territorial success (two-sample t -test: $t_{156,82} = 0.992$, $P = 0.323$) or mating success ($t_{178} = 0.124$, $P = 0.902$).

SELECTION ANALYSES

Sexual selection on male CHCs and body size (mass) differed significantly between the territoriality and mate choice trials (LRT, $\chi^2 = 25.0$, $df = 9$, $P = 0.003$). In the territoriality trials, sexual selection was significant overall (LRT, $\chi^2 = 18.4$, $df = 9$, $P = 0.031$). However, examination of the individual selection gradients revealed a significant effect on mating success of only increasing male body size and not any of the logcontrast CHCs, although one approached significance (Table 1). Sexual selection was also significant overall in the mate choice trials (LRT, $\chi^2 = 17.4$, $df = 9$, $P = 0.043$), but in contrast to the territoriality results, individual selection gradients were significant on two logcontrast CHCs while there was no effect of male body size (Table 1). Consistent with these results, there was also a significant positive association between body size and territorial success in both the genetic and environmental manipulations testing condition dependence (online supplementary materials).

Discussion

Despite a large body of sexual selection research, we lack an integrated understanding of how selection arising from different components of male reproductive success combine, and potentially interact, to affect trait evolution (Hunt et al. 2009). In addition, manipulative tests of condition dependence of territoriality are uncommon. Here, we used a laboratory population of *D. serrata*, a model system for the study of female preference for male sexual display pheromones, to investigate male territoriality within the context of these outstanding issues. First, we demonstrated that males (but not females) will defend a food resource, that this often involves direct, aggressive interactions between them, and that variation in their success in doing so is positively correlated with their subsequent mating success. Second, we showed that a male's success in defending a territory was affected by an environmental but not a genetic manipulation of condition. Third, in line with past studies, we showed that CHCs were under significant directional selection arising from female choice in this species (Chenoweth and Blows 2003, 2005; Howard et al. 2003; Rundle et al. 2009; Delcourt et al. 2010; Sztepanacz and Rundle 2012), but body size was not. In contrast, territorial success did not generate directional selection on CHCs but did generate selection for increased male body mass. We discuss these results in detail below.

Table 1. Standardized linear selection gradients (β) on body mass and logcontrast cuticular hydrocarbons from assays of male–male territoriality and female mate choice.

Trait	Male–male territoriality		Female choice	
	β	<i>P</i>	β	<i>P</i>
Body mass	0.146	0.003	0.035	0.439
(Z,Z)-5,9-C _{25:2}	0.010	0.892	−0.095	0.210
(Z)-9-C _{25:1}	0.031	0.598	0.010	0.867
(Z)-9-C _{26:1}	−0.032	0.586	−0.012	0.814
2-Me-C ₂₆	−0.175	0.111	0.128	0.297
(Z,Z)-5,9-C _{27:2}	−0.034	0.623	−0.018	0.771
2-Me-C ₂₈	0.192	0.194	−0.121	0.432
(Z,Z)-5,9-C _{29:2}	−0.032	0.587	0.142	0.007
2-Me-C ₃₀	−0.156	0.066	0.160	0.031

Bold denotes significant gradients ($P < 0.05$).

TERRITORIALITY AS A COMPONENT OF MALE REPRODUCTIVE SUCCESS

Drosophila serrata males showed behaviors consistent with a resource defense mating system. In particular, given an environment with a discrete food/egg-laying resource, single males tended to occupy this resource at the exclusion of another male and the identity of the territory owner tended to remain relatively constant over time. Our continuous observations of pairs of males revealed that aggressive encounters were initiated almost exclusively (71 out of 72 occurrences) when both males were present on the food resource. Although aggressive male–male interactions have been noted previously in *D. serrata* (Hoikkala et al. 2000), their role in territoriality had not been examined. Similar behavior has been observed in *D. melanogaster*, *D. simulans* (Jacobs 1960; Dow and von Schilcher 1975; Hoffmann 1987), and the Hawaiian *Drosophila* (Spieth 1974; Boake 1989).

In contrast with the patterns observed in males, females showed no evidence of territorial behavior but were attracted to the food resource. Territorial defense is thus male specific and territory holders are likely to benefit from greater access to females. Consistent with this, variation in the ability of males to defend a territory was significantly correlated with subsequent mating success in a competitive setting involving initially virgin, but later mated (as the trials proceeded), females. Similar relationships have been demonstrated in *D. melanogaster* (Dow and von Schilcher 1975; Hoffmann and Cacoyianni 1989) and may relate to the function of the defended resource as a site for oviposition and/or to its importance to female fecundity, which has been shown to increase in proportion to the availability of live yeast (Linder and Rice 2005). There is some evidence in *D. melanogaster* that females discriminate against males they have already mated (Ödeen and Moray 2008), and if this occurs in *D. serrata* it may have caused us to underestimate the

benefits of territoriality (because once mated to the territorial holder, females would subsequently prefer the other male for their next mating). In *D. melanogaster*, territorially successful males do not always have a mating advantage, however, with the relationship between territorial success and mating success differing in response to competitive environment, whether females were virgin, and the population/genotype in question (Hoffmann and Cacoyianni 1989; Cabral et al. 2008). Such issues remain to be addressed in *D. serrata*.

Although *D. serrata* behavior has not been studied in nature, field observations in *D. melanogaster* suggest that the defense of discrete resources by males may be common (Partridge et al. 1987; Taylor and Kekic 1988; Hoffmann and Cacoyianni 1990). Females tend to use areas of decaying fruit, which are often ephemeral and unevenly distributed, as food and a substrate for oviposition (Atkinson and Shorrocks 1981; Hoffmann 1987). Territoriality may therefore also contribute importantly to variation in reproductive success under natural conditions in *Drosophila*, although field studies will be needed to test this.

CONDITION DEPENDENCE

Results from our larval density manipulation showed that high-condition males were more successful than low-condition males in acquiring and defending a food resource, and that this difference arose entirely from the situation in which resources were limiting (i.e., high opportunity for territoriality). In contrast, in the presence of two resources, low-condition males performed as well as high-condition males. These results suggest that territorial defense is sensitive to at least some aspect of male condition. High-condition males also had a significant mating advantage over low-condition males, which is not unexpected given the known condition dependence of CHC-based sexual displays that contribute to male attractiveness (Delcourt and Rundle 2011). The

joint condition dependence of both territoriality and mating success suggests that overall male reproductive success is likely to be condition-dependent, and this should cause natural and sexual selection to align (Whitlock and Agrawal 2009).

Although our earlier results indicated that territorial success was a component of reproductive success, the mating advantage of high-condition males over low-condition males in the condition assay was not significantly greater when territories were limiting. The inability to detect a significantly greater mating advantage when territoriality mattered may be explained by condition dependence of mating success on its own, which may have reduced the ability to detect an added effect of territoriality. Alternatively, the importance of territory defense to male reproductive success may depend on other, as yet unidentified, social and/or other environmental factors (Hoffmann and Cacoyianni 1989; Cabral et al. 2008).

In contrast to the results of our environmental manipulation, we found no significant effect of three generations of inbreeding on either male territorial defense or mating success, although the latter approached significance ($P = 0.073$). The lack of an effect may be explained by the comparatively small difference in mass ($\sim 5\%$) between the outbred and inbred males. The extent of inbreeding depression is known to vary considerably among species and environments (Lynch and Walsh 1997; Keller and Waller 2002), and may be reduced by the particularly homogeneous environment of the laboratory (Long et al. 2013). It is therefore possible that our inbreeding protocol had only a weak effect on genetic quality and hence condition. Inbreeding depression could also have been reduced if the stock itself was already inbred. This, however, is unlikely as the stock had been kept at a census size of > 1000 individuals/generation since previous quantitative genetic studies revealed significant additive genetic variance for male and female fitness, CHCs, and female preferences for them (Delcourt et al. 2009, 2010; Delcourt and Rundle 2011). Alternatively, in creating the inbred flies only 90 of 285 survived three generations of inbreeding, suggesting ample opportunity for the purging of low fitness genotypes which could have minimized the effect of the genetic manipulation.

Separate from explanations that focus on the absence of inbreeding depression, condition is likely multifaceted and different traits may be sensitive to different aspects of it. In particular, the expression of some traits may be more strongly linked to acquiring and processing particular nutrients as opposed to overall resource acquisition. If the mutational target of the former is small, such traits may be relatively insensitive to overall genetic quality but nevertheless be affected by an environmental manipulation that alters resource availability. Although environmental manipulations, including diet quality and density, have frequently been used as a proxy for deleterious alleles when interest concerns variation in genetic quality, empirical tests of this assumption are lacking

(Whitlock and Agrawal 2009). Similar to what was found here, Clark et al. (2012) showed that although high-condition males generated via a diet quality manipulation had a postcopulatory advantage with respect to sperm offense, no effect was detected when condition was manipulated by introducing known deleterious mutations. This is an important area for future study.

QUANTIFYING SELECTION

Empirical studies of sexual selection have tended to focus on either female choice or male–male competition, or have studied overall reproductive success without attempting to understand the contribution of different components (Hunt et al. 2009). We investigated the roles of male–male competition over territories and female mate choice in generating selection on CHCs and body size in *D. serrata*, traits previously implicated in one or the other of these components in *Drosophila*. Although the role of CHCs in mate recognition and courtship has been well characterized in *D. serrata*, the contribution of these signals to male–male aggression was unknown. With respect to male mating success, we found no effect of body size, but consistent with multiple previous studies of this species (Chenoweth and Blows 2003, 2005; Hine et al. 2004, 2011; Delcourt et al. 2010; Sztepanacz and Rundle 2012), we found significant directional selection on male CHCs arising from female choice.

In contrast to the traits underlying mating success, competition over territories generated no significant directional selection on any of eight logcontrast CHCs (although 2-Me-C₃₀ did approach significance), but caused significant directional selection on body mass. That male size may matter to territoriality is not surprising as male–male aggressive interactions occur in *D. serrata* (Hoikkala et al. 2000), the outcome of which may be body size dependent. The lack of any effect of CHCs is somewhat more surprising as pheromones have been found to stimulate aggressive behavior in a variety of invertebrate and vertebrate species (Chamero et al. 2007). In *D. melanogaster* in particular, the pheromone 11-*cis*-vaccenyl acetate (cVA), along with several CHCs, has been identified in the regulation of aggression (Wang and Anderson 2010). Masculinization of female pheromones in this species causes males to stop courting and instead behave aggressively, further indicating that pheromonal cues are used by *D. melanogaster* males in recognizing conspecifics as competitors (Fernández et al. 2010). *Drosophila serrata* lacks cVA, however, and there is no evidence of pheromonal transfer during mating (S. Gershman and H. Rundle, unpubl. data).

The apparent absence of any shared traits associated with success in these different assays suggests that they are measuring different components of male reproductive success. However, an alternative possibility is that male–male interactions do contribute to the outcome of the mating trials, despite their being designed to minimize the opportunity for this to occur (i.e., no food resource

present and two males, previously unknown to one another, that are introduced immediately before the female that tends to mate quickly). If this was the case, an absence of net selection on body size could occur if female prefer smaller males and this counteracts the territorial advantage of larger males. Although we view this as unlikely, in the absence of no-choice trials that eliminate any opportunity for male–male interaction, we cannot rule it out.

Despite an apparent lack of any role for CHCs in the outcome of territorial interactions in *D. serrata*, territoriality could still alter selection on CHCs if these processes occur sequentially and the former (territoriality) limits the subsequent pool of males available for females to choose among (Wong and Candolin 2005). For example, if competitive success is indicative of higher quality males, females may maximize their fitness by also preferring such males as mates, and male traits indicative of competitive ability may become direct targets of female mate preferences (Andersson 1994; Berglund et al. 1996; Qvarnström and Forsgren 1998). Alternatively, more competitive males may not always be preferred by females (Moore and Moore 1999; Casalini et al. 2009), for example, if dominant males provide poor parental care, are not of higher genetic quality, or if the costs associated with mating with them outweigh the potential benefits (Qvarnstrom and Forsgren 1998).

In our case, territoriality generated selection on the CHC 2-Me-C₃₀ that approached significance and was opposite in sign to that arising from female mate choice (Table 1), suggesting that females may be attempting to avoid more competitive males. In contrast, territoriality also tended to favor heavier males, and if females tend to mate at their food/oviposition site, then this could act to remove the smallest males from the pool of individuals available to females during mate selection. Scoring males for the vector of directional selection gradients on CHCs (i.e., β from Table 1) to generate a single trait representing the multivariate combination of CHCs that best determines mating success (see McGuigan et al. 2011b), we found a positive, albeit weak, phenotypic correlation between mass and multivariate attractiveness that approached significance ($r = 0.082$, $P = 0.066$). This suggests that male competition over territories may not only act to remove the smallest males, but may also restrict female choice to those males that display attractive CHC blends, although ultimately it is the underlying genetic correlation that is of interest.

Results here indicate that male size and composition of CHCs separately influenced the outcome of male–male territorial competition and female mate choice, respectively. Female choice and male–male competition targeting different phenotypes has been observed in other species as well (e.g., Okada et al. 2014). Nevertheless, there are likely instances in which males express traits that influence the outcome of both (Berglund et al. 1996), and the extent and manner in which they interact may vary among

species and environments, and is likely to be influenced by the presence of dominance hierarchies, the extent of sexual conflict, and whether they occur simultaneously or sequentially (Moore et al. 2001; Hunt et al. 2009). Additional components of male reproductive success, such as search effort and sperm competitive ability, are less studied, and it remains important to examine all components of male reproductive success to gain a comprehensive understanding of sexual selection in a given system.

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DATA ARCHIVING

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Table S1. Total number of males observed (expected under null hypothesis of independence) in each of four states relative to a food/oviposition resource during male territorial assays, as determined in five sequential observation periods of 100 replicate arenas.

Table S2. Total number of females observed (expected under null hypothesis of independence) in each of four states relative to a food/oviposition resource during female territorial assays, as determined in four sequential observation periods of 20 replicate arenas.

Table S3. Behaviors observed during territorial assays of male *D. serrata* pairs.

Table S4. Results of generalized linear mixed models testing the effects of condition (“cond”; high vs low), the opportunity for territoriality (“oppo”; one vs two food resources), and their interaction on the territorial score and number of matings achieved by individual males (*F*-values, with significance in parentheses).

Table S5. Results (*F*-ratio, *P*-value) from the condition dependence assays of separate analyses of covariance modeling territorial score and mating success (number of matings) as a function of the fixed effect of opportunity for territoriality (Oppo) and the male body size (dry mass).

Figure S1. Tukey boxplots of (A) days to eclosion and (B) dry mass of males (B) reared under low and high larval density conditions (10 and 40 females ovipositing in a vial for 24 h, respectively).

Figure S2. Average (A) territorial score and (B) mating success of high (filled circles, solid line) and low (open circles, dashed lines) condition males as generated from an environmental (density) manipulation.

Figure S3. Average (A) territorial score and (B) mating success of high (filled circles, solid line) and low (open circles, dashed lines) condition males as generated from a genetic (inbreeding) manipulation.