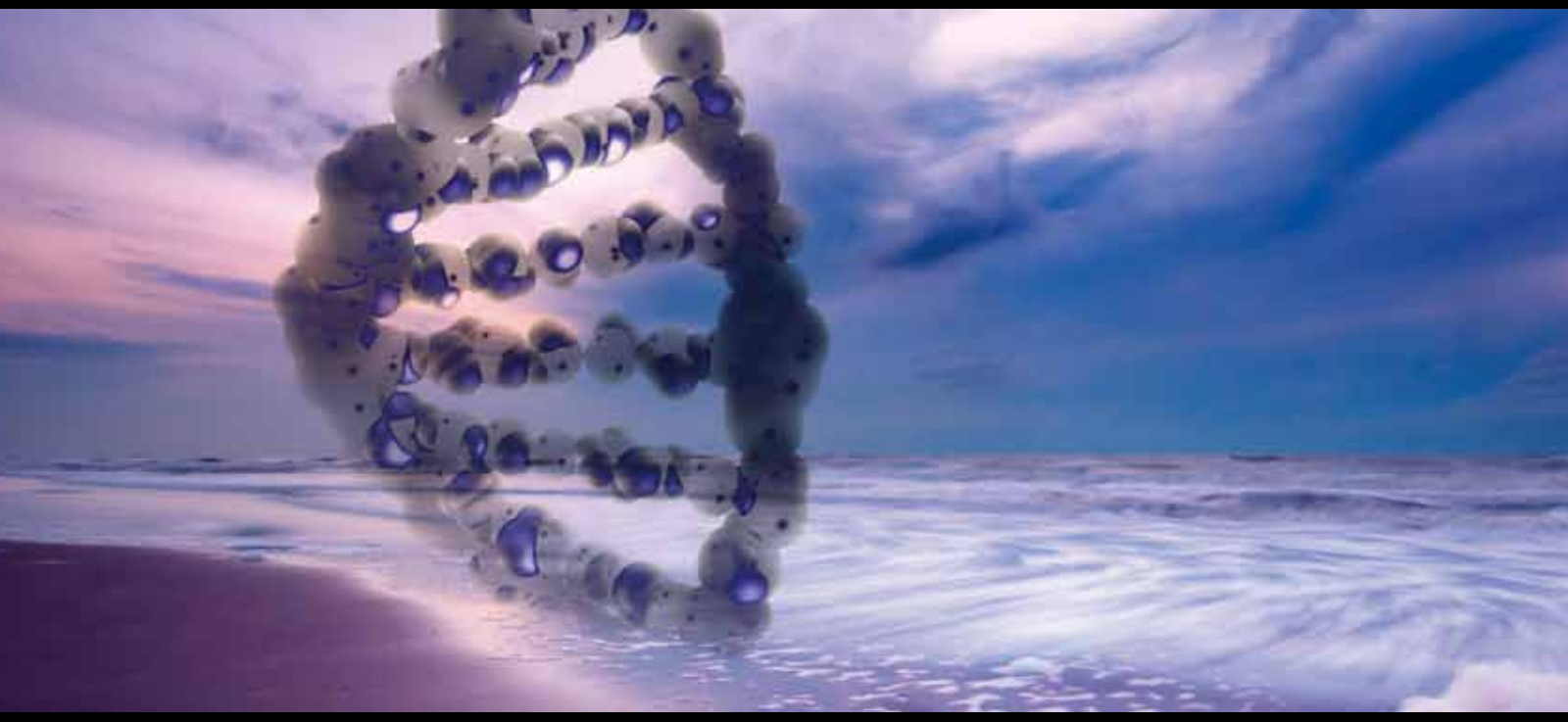
International Journal of Evolutionary Biology

The Evolution of Sex-Related Traits and Genes 2012

Guest Editors: Alberto Civetta, José M. Eirín-López, Rob Kulathinal, and Jeremy L. Marshall



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Editorial Board

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Editorial

The Evolution of Sex-Related Traits and Genes 2012

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The second special issue on the evolution of sex-related traits and genes brings together a wide variety of papers that explore issues dealing with diverse topics such as behavior, organismal defense systems, molecular evolution, speciation, and genomics. This broad diversity reflects the recent expansion of the field of reproductive biology and evolution. The review by R. S. Singh and S. Jagadeeshan provides a historical perspective that describes the breadth of work that has emanated from early protein electrophoresis studies to the genomics-based approaches that are more common today. The authors illustrate how the study of sex- and reproductive-related (SRR) genes has now impacted fields of evolution spanning from selection and speciation to gene birth and evolutionary developmental genetics.

A review article and a research article address questions related to mating preferences across two very different systems, *Tetrahymena* and *Drosophila*. S. S. Phadke et al. address an interesting question about the evolution of sex: does the evolution of more than one sex necessarily lead to the evolution of mating preferences? Using the ciliate species, *Tetrahymena thermophila*, which has up to seven "sexes," they tested the mating frequencies of four of the sexes to determine if they exhibited mating preferences. Their results suggest that mating is random among the four sexes, thus concluding that the evolution of multiple sexes does not necessarily lead to the evolution of mate preferences. They discuss their findings in the context of ciliate evolution as well as the evolution of sex in general. A review by A. J. Moehring and M. Laturney offers a clear insight into our current understanding of the genetic basis and evolution of sexual isolation between species. The review focuses on female mate preference as a premating behavioural barrier and highlights two interesting commonalities across a wide variety of *Drosophila* species: different genes control conspecific and heterospecific male choice and a preferential location of genes for heterospecific male rejection in areas of low recombination. Another paper in this issue deals with the question of reproductive isolation between species. J. L. Marshall and N. DiRienzo address a central question in evolutionary biology about whether the same genetic and developmental pathways contribute to reproductive isolation at both intraspecific and interspecific levels. The authors characterize a postmating, prezygotic phenotype, the ability of males to induce egglaying in females, between diverging populations, and species of crickets. Using mating assays and RNAi, the authors demonstrate similar decreases in female fecundity and the abundance of a particular female protein within populations as well as between species. While their results are suggestive of a connection between incompatibilities found within species and reproductive isolation between species, the authors also discuss alternative explanations and the need for future work.

Three papers address a topic not covered in our previous 2011 issue—the relationship between sex and organismal defense systems. In a review article focusing on whether or not different sexes evolve different defensive traits to avoid being harmed or eaten by a predator, G. Avila-Sakar and C. A. Romanow synthesize the literature on the hypothesis of male-biased herbivory in dioecious plant species. They outline shortcomings with the studies supporting the male-biased herbivory hypothesis and suggest a set of alternatives. Moreover, they outline a protocol that they suggest should be used to study plant-herbivore evolution in relation to

this important question. Two research articles from L. C. Harshman's lab address questions linked to the evolution of the immune system in *Drosophila*. In one paper, J. Ma et al. compare males and females for measures of metabolic rate and locomotion in populations that have been selected for survival under an infection regime with *Bacillus cereus*. The authors characterize the potential physiological costs associated with mounting an effective and elevated immune response by measuring respiration rates (per fly or adjusted by weight), in addition to behavioural responses as measured by overall fly movement. The authors find evidence of a male-biased response with only the males responding metabolically to selection on elevated immunity. In their other contribution, the authors embark on a selection for survival to infection experiment and identify two pleiotropic responses (i.e., an increase in egg production and delayed development time). Of particular interest to this issue is the identified relationship between reproductive fitness in the form of egglaying and immunity.

Three research articles use molecular biology and evolutionary genomics approaches to study diverse phenomena with respect to sex and evolution. R. L. Kanippayoor and A. J. Moehring address the functional and evolutionary consequences of the timing of protamine expression during the reorganization of the hereditary material in mature spermatozoa. Using transgenic *Drosophila*, they demonstrate that protamines are expressed from both alleles in diploid cells prior to meiosis, in contrast with the postmeiotic haploid expression of mammalian protamines. This work opens the door to future studies to ascertain the evolutionary benefits of diploid *versus* haploid expression of protamines, especially as it pertains to the fertilization success of sperm and an individual's fitness. By characterizing the evolution of the *Izumo* gene family in mammals, P. Grayson and A. Civetta explore how gene duplications can contribute to the adaptations on male reproductive traits. The *Izumo* gene family is comprised of four genes known to be expressed in sperm and possibly involved in sperm-egg interactions. The authors find contrasting patterns of molecular evolution which suggest a variety of evolutionary processes acting across different taxa. They conclude that such differing lineage-specific patterns of selection found in this gene family lend support to protein subfunctionalization as opposed to neofunctionalization or gene loss and represents species-specific adaptations to male fertility. In the final paper of this issue, to identify interlocus conflict in the genome, M. E. B. Hansen and R. J. Kulathinal generate and characterize male- and female-biased networks using the extensive genomic resources available in *Drosophila*. The authors integrate sex-specific expression data from modENCODE with known interaction data to identify putative direct and indirect interactions between male-biased genes and female-biased genes. Using this approach, the authors were able to demonstrate that a larger than expected fraction of the genome may potentially be involved in sexually antagonistic interactions at the molecular level.

This special issue on the evolution of sex-related traits and genes contains a wonderfully diverse sample of studies that addresses both old and new questions in evolutionary reproductive biology. The editors would like to thank the dedicated and generous contributions of all authors and reviewers for helping to make this issue so interesting.

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Research Article

Drosophila melanogaster Selection for Survival after Infection with *Bacillus cereus* Spores: Evolutionary Genetic and Phenotypic Investigations of Respiration and Movement

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Laboratory populations of *D. melanogaster* have been subjected to selection for survival after live spores of *B. cereus* were introduced as a pathogenic agent. The present study was designed to investigate correlated traits: respiration as a metabolic trait and movement as a behavioral trait. An underlying hypothesis was that the evolution of increased survival after *B. cereus* infection exerts a metabolic cost associated with elevated immunity and this would be detected by increased respiration rates. There was support for this hypothesis in the male response to selection, but not for selected-line females. Two phenotypic effects were also observed in the study. Females especially showed a marked increase in respiration after mating compared to the other assay stages regardless of whether respiration was measured per fly or adjusted by lean mass or dry weight. Given that mating stimulates egg production, it is feasible that elevated metabolism was needed to provision oocytes with yolk. Females also moved less than males, perhaps due to behaviors related to oviposition whereas elevated male activity might be due to behaviors associated with seeking females and courtship. Relatively low movement of females indicated that their elevated respiration after mating was not due to a change in locomotion.

1. Introduction

A tradeoff between immune function and reproduction has been observed in a range of studies. For example, an increase in reproductive effort is correlated with increased parasite incidence and disease [1, 2]. More generally, an increase in reproduction is associated with decreased immune system function [3, 4]. Two mechanisms have been proposed for this relationship. One is the Y model [5] in which there is an energetic competition between somatic function (immunity in the present study) and reproduction. The arms of the Y represent competition for energy between traits and the long axis represents resource input. Another potential mechanism for a tradeoff is a negative pleiotropic effect of hormones acting on two traits. An example is the effect of insulin signaling as a stimulator of reproduction and a suppressor of life span in *D. melanogaster* [6, 7]. In support of the role of hormones on insect life history tradeoffs, juvenile hormone (JH) has been shown to have negative pleiotropic effects on immunity and reproduction. In the flour beetle, *Tenebrio molitor*, mating causes a decrease in an immune system enzyme (phenoloxidase) by increasing the level of JH [8]. In the bee, *Apis mellifera*, a caste behavior was experimentally altered which increased JH and decreased both immune function and life span [9, 10].

Invertebrates are useful for investigating trade-offs between immune function and life history traits [11]. A study of the ground cricket, *Allonemobius socius*, documented that an elevated number of matings was correlated with decreased immune function and life span [12]. Male *D. melanogaster*

lose immune function when they mate more frequently [13]. The cost of immune function could also impact physiological or behavioral attributes which are the traits that are the focus of the research reported here.

Studies on vertebrates are also relevant to the present study as there has been more emphasis on measuring the energetic cost of mounting an immune response in this group of organisms. In the case of a study on a sparrow (*Parus caeruleus*), the energetic cost of the antibody reaction was approximately 8% to 13% of the baseline metabolic rate [14]. However, some studies on birds have failed to detect a cost of an immune response [15] and it has been argued that the energetic cost might be low [15, 16].

There have been fewer studies on invertebrates designed to measure the cost of immune responses. However, one study is noteworthy [17]. Using bumblebee workers (*Bombus terrestris*), two elicitors of immune responses were used: lipopolysaccharides (LPS) and microlatex beads that were about the same size as bacteria. Both induced immune responses that could have been in different pathways. The cost was evaluated in terms of starvation survival time, which was decreased from 1.5 to 1.7. Elevated lipid levels are associated with higher levels of stress resistance in selection experiments [18] and it is feasible that the immune responses in these bees depleted stored lipid for the additional metabolism needed for the immune responses. This could be the mechanism underlying decreased starvation resistance in *Bombus terrestris* as a result of induced immune responses.

The present study addresses the relationship between immune function and metabolism (respiration) or behavior (movement) in the context of a laboratory selection experiment on *D. melanogaster*. Lines of flies were selected for increased survival after *B. cereus* spore infection [19]. A key goal was to determine if there was a metabolic cost associated with elevated immunity in the selected lines. This cost would be inferred from increased respiration in the selected lines. Movement was also measured to determine if heightened immune function also altered activity. The questions addressed by this study are pertinent to understanding the cost of immune function, a fundamental issue in evolutionary biology [20].

The present study was conducted after 19 generations of selection on *D. melanogaster*. Selection resulted in a 3-log increase in the number of spores required for 50% mortality [19]. There were nine lines: three lines were selected by puncturing the live *B. cereus* into the body at a level of spore concentration that yielded approximately 50% *D. melanogaster* mortality, three lines in which the flies were punctured with sterile water (wound control lines), and three lines that were not punctured [19]. The latter were no-perturbation control lines.

In the present study, hypotheses related to a metabolic cost of immunity were tested. In terms of respiration, elevated CO_2 and O_2 rates in the untreated selected lines would be interpreted as a metabolic cost. Increased respiration (metabolism) results from increased use of endogenous resources and this would be a cost of immunity. We also hypothesized that movement could be affected by selection for *D. melanogaster* survival after infection with *B. cereus* spores or induction of the immune response by autoclaved spores. The inclusion of movement measurements in this study potentially allowed us to assess locomotion as a factor contributing to respiration rate differences between line types and treatments.

Table 1: Designations of lines, treatments, and assay stages.

	Identifiers and description
Line types	S: selected with live spores, CP: wound control lines punctured with sterile H_2O, CN: no perturbation
Treatments	AS: punctured with autoclaved (dead) spores, H_2O: punctured with sterile H_2O, NON: no treatment
Assay stages	Day 3: before perturbation, Day 5: punctured with autoclaved spores or punctured with sterile H_2O or remained unperturbed, Day 7: after mating

2. Materials and Methods

2.1. Drosophila melanogaster Lines and Treatments. The founding of the base population used for selection, the process of selection, and the response to selection are described in Ma et al. [19]. A brief description of selection follows. There were three selected lines which were independent subpopulations originated from the base population. At each generation of selection, 1000 females and 1000 males were exposed to spores of *B. cereus*, a species that is closely related to *B. anthracis*. Spores of *B. anthracis* have been used as bioterrorism agents and an ultimate goal of the selection experiment was to identify spore resistance genes in *D. melanogaster*. The spores were introduced by puncturing them into the thorax with a fine needle. The level of selection was adjusted incrementally to obtain 50% mortality each generation. After 19 generations, there was a 3-log increase in the number of spores required for 50% mortality. Three additional lines were established as wound control lines. Flies in these lines were punctured with sterile H_2O each generation. Three more lines were established; the flies in these lines were not perturbed (no treatment) each generation. In this study, there are line types (selected, wound control, and no-perturbation lines) treatments (punctured with autoclaved spores, punctured with water, and no treatment). The lines allow for assessment of genetic correlations and the treatments allow for assessment of phenotypic effects. The designation and description of lines and treatments and the successive stages of the respiration assay are shown in Table 1. "Line types" refers to the set of selected lines and the control lines (wound-control or no perturbation).

Prior to assays, flies from the selection and control lines were handled in a defined manner that is described below. Before conducting any of the assays, the selection experiment was relaxed (no selection) for two generations. To relax selection, all lines were reared without exposure to spores or wounding. It is important to emphasize that at this point there has been no explicit selection for two generations as all lines were reared in the same unperturbed manner. Each rearing vial was seeded with 100 eggs to standardize density. Flies

were raised on a cornmeal, molasses, torula yeast Drosophila food [19]. Prior to respiration or movement assays, flies were maintained in a room with 12-hour light-dark cycle at 25°C. In this environment, males and females from all lines were exposed to one of the following three conditions which are related to selection and control line environments: introduction of autoclaved (dead) spores with a tungsten needle (to induce an immune response) or punctured with a needle dipped in sterile H_2O (to induce a response to wounding) or left untreated. After three days in the room with a 12-hour light/dark cycle at 25°C, respiration assays were initiated. A movement study was conducted on the same flies after the respiration assay. The flies were 3–7 days old at the time of the respiration assays and 8 days old at the time of the movement assay.

2.2. Bacterial Culture and Spore Isolation. *Bacillus cereus* ATCC 10987 was used as a source of spores. Spore purification was conducted using a step gradient of Renografin [21]. Biomass for gradient purification was generated from a single colony of *B. cereus* grown in 25 mL of Difco sporulation medium and incubated at 37°C on a rotary shaker (150 rpm) until mid-log phase. This culture was expanded into 2 L of DSM from a 1 : 10 dilution, followed by incubation at 37°C on the rotary shaker for 48 hours. The pellet was resuspended in 200 mL of sterile water and stored overnight at 4°C. The pellet was resuspended in 200 mL of 4°C sterile water followed by the same centrifugation procedure. The pellet was again resuspended in 200 mL of sterile water and stored overnight at 4°C. After repeating the centrifuge-resuspension-centrifugation procedure, greater than 90% bright-field spores were observed by phase contrast microscopy. The spores and cell debris were harvested one final time and the pellet was resuspended in 20% Renografin then transferred to a 30 mL glass core tube with 15 mL of 50% Renografin. The spore suspension was centrifuged for 30 min at 4°C at 10,000 ×g. All layers containing vegetative cells were removed and the spore pellet retained. The pellet was resuspended in 10 mL of 4°C sterile water in an Oak Ridge tube. The spore suspension was centrifuged for 10 min at 10,000 ×g at 4°C. Trace amounts of Renografin were removed by 3 washes with 4°C sterile water as described above. The spore pellet was suspended in 2 ml 4°C sterile water. The concentration of spores was determined by serial dilution and spread plating.

Because of the need for increasing amounts of spores during selection, spores were prepared on two different occasions during selection (one preparation used for selection generations 1–11 and the second preparation used for selection generations 12–19). Each preparation was normalized for concentration and the normalized preparations resulted in very similar LD50s on the selected lines. The second preparation was the source of autoclaved spores used in this study.

2.3. Respiration. The experimental subjects for respiration assays consisted of four cohorts of flies which were all from generation 19. Temporally separated cohorts allowed for all the subjects to be assayed within the time available after selection generation 19. Temporal variation between cohorts was achieved by adjusting the four times of egg collection to create a timed-gap between cohorts. Respiration was measured on adult flies at three successive assay stages which are denoted as "day 3" (before treatment), "day 5" (after puncture with autoclaved spores or sterile H_2O or remained untreated), or "day 7" (after mating) (Table 1). This progression mimics the successive steps of the selection process. For example, 24 hours of mating was conducted prior to the last stage ("day 7" posteclosion, Table 1) in the respiration assay similar to the process of breeding in the selected and control lines to produce the next generation.

All respiration assays were conducted in a controlled manner. These assays were conducted during the 12-hour light period to eliminate behavior changes caused by a change of light. All flies used for the respiration assay were held in syringes that were otherwise empty when respiration measurements were taken. These flies were postprandial. There was no opportunity for artifacts arising from flies using dietary sources during the process of respiration measurement. The flies were only apart from food during the time that respiration was measured. The maximum time away from food was 19 minutes and thus there was no desiccation stress. The samples in one set (all permutations of lines, treatments and sex) were temporally randomized within the 12-hour light-cycle period. One set, (a replicate) of all sample types, was conducted in one 12-hour period. For each of the four cohorts (described in the first paragraph of this section), three replicates were run for each type of sample consisting of assay stages, line types, and sexes.

Respiration of the flies was measured using a parallel stop flow system and injecting air sampled from fly vials into a flow-through respirometry setup. Water-scrubbed ambient air (Drierite/soda lime) was used as the carrier gas in the flow-through system. A Nalgene carboy was used to remove short-term fluctuations in gas concentrations before the scrubber column. CO_2 was measured using an infrared gas analyzer (CA-10a, Sable Systems International, Las Vegas, NV, USA). O_2 was measured using a fuel cell-based oxygen analyzer (FC-10a, Sable Systems International, Las Vegas, NV, USA). The air flow was adjusted to 50 to 80 mL/min. Details about the mechanics, process, and calculations used for respiration can be found in Lighton [22].

For any one sample, respiration was measured using three flies that were aspirated into a 5 mL plastic syringe. The syringe had a three-way valve that closed the interior to outside air and gases accumulated. Before the respiration measurements, syringes were held within a PELT-5 environmental chamber at 25°C in the light for at least seven minutes. At the end of this period, each syringe was attached to a tubing port and opened to allow gas to be injected into the respiration instrumentation. A standard volume of almost all of the air in the syringe was introduced into the tubing leading to the CO_2 and O_2 monitors in succession. For a base line, a blank syringe with only scrubbed air was used for each set of flies subjected to respirometry. After respirometry, the ambient air within each syringe was flushed and replaced by the scrubbed air prior to introduction of the next set of flies.

The respiration measurements were normalized (divided) by the length of time that flies were in syringes.

Respiration rates were adjusted by lean mass or dry weight for each line, treatment, and sex. The mean values for respiration were divided by the mean values for lean mass or dry weight. The mean values and standard errors were derived from the three replicate lines of each type.

2.4. Movement. After each respiration rate measurement, the same flies were used for a movement assay. The flies were 8 days posteclosion which was one day older than the postmating assay stage. The number of movements of an individual fly was recorded every 10 minutes for 24 hours. There were two monitors (TriKinetics) with a total of 64 tubes to measure movement. For each measurement, an individual fly was aspirated into a glass capillary testing tube (5 mm in diameter and 65 mm in length). One end of the testing tube was partially covered with fly food so that flies would not be subjected to starvation, given the length of the assay. Moreover, the presence of food more closely replicates conditions the flies were exposed to in the selection experiment. A hole in the food plug allowed air to enter the tube. The other end of each tube was inserted into a placement site in the instrument. The location of each sample among and within the monitors was randomized to avoid bias potentially associated with position. Movement was recorded when a fly interfered with a laser beam projected through the set of tubes in a monitor. Two damp cotton balls were placed in a plastic bag surrounding each movement measurement device to maintain relatively high humidity. The assay was conducted at 25°C with a 12 : 12 light : dark cycle as this was the environment flies were held prior to assays.

2.5. Weight. Flies from generation selection generation 36 were used for weight measurements. As always was the case, selection was relaxed for two generations prior to weight measurements. After freezing for dry weight measurement, each fly was placed in an otherwise empty 2 mL eppendorf tube. The cap was left open and the flies were placed in a drying oven (70°C) with air blown throughout the chamber. The flies were left to dry for 24 hours. The weight was taken on individual flies and 10 flies were weighted for each treatment, sex, line, and assay stage. A Sartorius microbalance (M2P) was used for all fly weights. After the dry weight of flies was obtained, the flies were placed individually in 2 ml eppendorf tubes with 2 mL of ethyl ether. The caps were closed and the tubes were shaken for 24 hours to extract lipids. The flies were again dried and reweighed to obtain lean mass measurements. The conditions used for the weight measurements paralleled the conditions used for respiration assays.

2.6. Data Analysis. The data were analyzed by repeated measures ANOVA, ANOVA, and the Tukey-Kramer test using SAS version 9.3. Mixed model ANOVAs for each sex were employed with line types and treatments as fixed effects; the variations among lines of the same type were the random effects. Only CO_2 measures were used for statistical analysis.

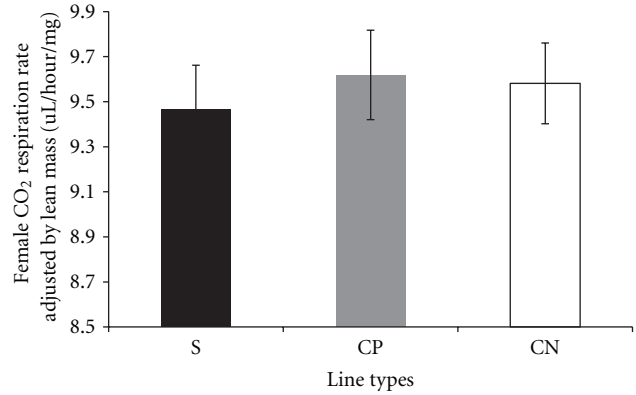

FIGURE 1: Average CO_2 respiration rates adjusted by lean mass for females from selected and control lines. S: selected lines, CP: wound control lines, CN: no perturbation control lines.

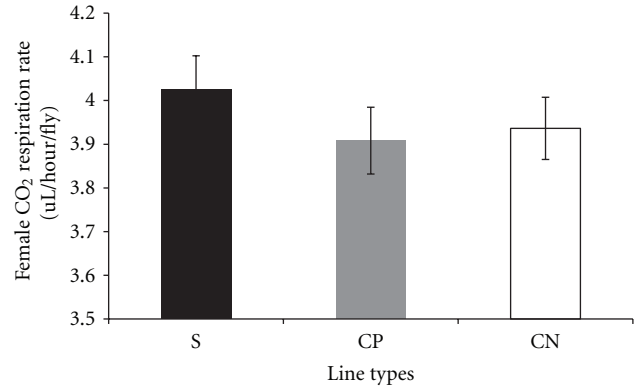

FIGURE 2: Average CO_2 respiration rates measured per fly for females from selected and control lines. S: selected lines, CP: wound control lines, CN: no perturbation control lines.

The O_2 values and trends were essentially the same as CO_2 as can be seen from the fact that respiration quotients are close to 1.00 for all treatments and lines.

3. Results

3.1. Statistical Analyses. Introducing line-to-line random effects improved the fit of the model in the analysis of CO_2 respiration rate adjusted by the lean mass or dry weight for both males and females, and CO_2 respiration rate measured per fly for females (see Table S1A in Supplementary Material available online at http://dx.doi.org/10.1155/2013/576452). Therefore, this source of variation was incorporated in the analysis. However, adding line-to-line random effects did not improve the fit of the model when respiration rate was measured per fly for males. All of the *P* values for respiration rates and weights and post hoc analyses of respiration rates are reported in supplementary tables (see Tables S1B, S1C and S1D in Supplementary Material).

3.2. Respiration

3.2.1. Line Types and Treatments. Carbon dioxide respiration rates adjusted by lean mass, measured per fly, and adjusted by

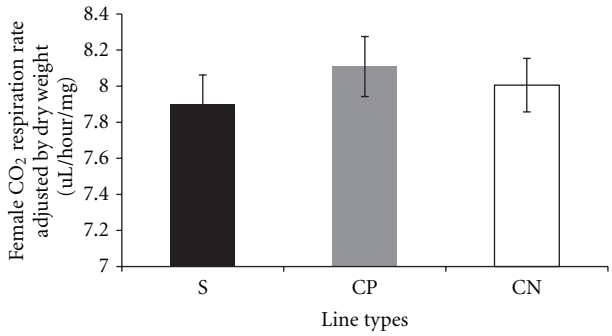

Figure 3: Average CO_2 respiration rates adjusted by dry weight for females from selected and control lines. S: selected lines, CP: wound control lines, CN: no perturbation control lines.

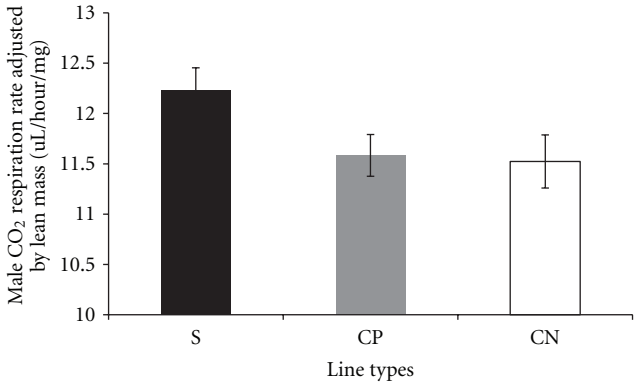

Figure 4: Average CO_2 respiration rates adjusted by lean mass for males from selected and control lines. S: selected lines, CP: wound control lines, CN: no perturbation control lines.

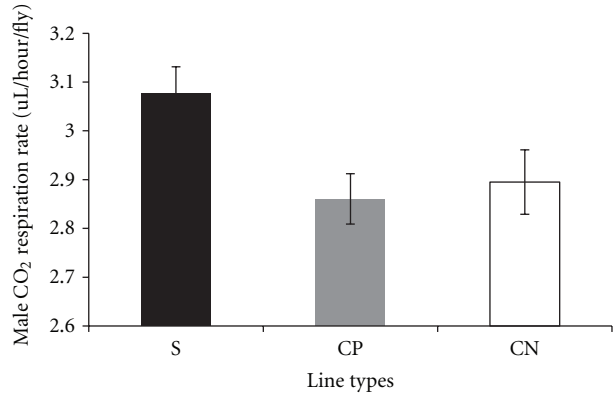

Figure 5: Average CO_2 respiration rates measured per fly for males from selected and control lines. S: selected lines, CP: wound control lines, CN: no perturbation control lines.

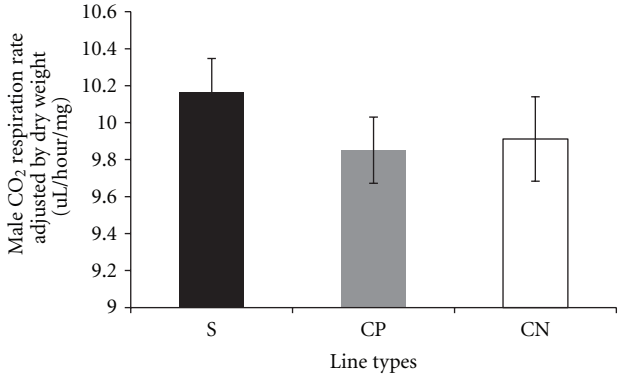

Figure 6: Average CO_2 respiration rates adjusted by dry weight for males from selected and control lines. S: selected lines, CP: wound control lines, CN: no perturbation control lines.

dry weight in the selected and control lines are presented in a series of figures (female: Figures 1, 2, and 3; male: Figures 4, 5, and 6). The numerical values for average respiration rates in the present study are presented in supplementary tables (see Tables S4A, S4B, and S4C). There were no statistically significant differences among line types or treatments for females or males when respiration was adjusted by lean mass or adjusted by dry weight. However, there was a sex by line type interaction when respiration rates were adjusted by lean mass ($P = 0.0513$). Underlying this interaction, males exhibited an elevated respiration rate in the selected lines (compared to females); male respiration rates were lower in the CP and CN lines whereas female rates were almost invariant among line types (Figure 19). For females, respiration rates per fly were not significantly different among line types or treatments. For males, there was a statistically significant difference among line types ($P = 0.0132$, see Table S1B). The pairwise post hoc statistical analysis based on comparisons of per fly measurements between line types indicated that the selected line males had higher level of respiration than the punctured control lines ($P = 0.0186$) and the no perturbation control lines ($P = 0.0494$). There was no statistically significant difference between the two types of control lines ($P = 0.9303$).

3.2.2. Assay Stages. There were differences in respiration rates among assay stages for females and males. The average CO_2 release rate of females was markedly higher after mating (day 7) and lowest at day 5 (females: Figures 7, 8, and 9). Males showed the same pattern as females except for the data determined per fly. In this case, the average CO_2 respiration rate was the lowest after mating (day 7), but the highest before treatment (day 3) (males: Figures 10, 11, and 12). Overall for females, there were statistically significant differences when respiration was adjusted by lean mass ($P < 0.0001$) or dry weight ($P < 0.0001$), or per fly ($P < 0.0001$). Overall for males, there were statistically significant differences when respiration was adjusted by lean mass ($P = 0.0005$) or dry weight ($P = 0.0046$), or measured per fly ($P < 0.0087$). Post hoc analysis by Tukey's method showed that there were statistically significant differences among assay stages. Post hoc comparisons for females when respiration was adjusted by lean mass indicated that respiration was the highest after mating (day 7) compared to day 5 ($P < 0.0001$) and day 7 compared to day 3 ($P < 0.0001$). The same pattern of statistically significant differences was observed when female respiration data was adjusted by dry weight. For female respiration per fly, the only statistically significant post hoc

difference was between day 7 after mating and day 5 after treatment ($P < 0.0001$). Post hoc comparisons for males when respiration was adjusted by lean mass indicated that respiration was the lowest at day 5 after treatment compared to day 3 ($P = 0.0044$) or day 7 ($P = 0.0011$). When male respiration rates were adjusted by dry weight, day 5 respiration rates were the lowest compared to day 3 ($P = 0.0250$) and day 7 ($P = 0.0067$). For male respiration rates measured per fly, the respiration rate was the lowest on day 7 after mating which was statistically significantly different than day 3 before treatment ($P = 0.0064$).

There were statistically significant interactions between sex and the three assay stages for CO_2 respiration rates. When respiration rates were measured per fly, there was a statistically significant interaction between sex and assay stages ($P < 0.0001$). For females when respiration was measured per fly, the highest CO_2 respiration rates were observed at the after-mating assay stage (day 7) while the lowest were at day 5 (Figure 13). On the other hand, for males measured per fly the highest CO_2 respiration rate was observed at the day 3 assay stage and the lowest was present at the postmating stage (Figure 13). There was also statistical support for an interaction between sex and assay stages when respiration rate data were adjusted by lean mass ($P = 0.0095$). Both females and males showed the same pattern, in which the postmating assay stage (day 7) had the highest CO_2 respiration rate while posttreatment assay stage (day 5) had the lowest. The increase in respiration rate on day 7 was greater for females than males (Figure 14) contributing to the significant interaction between assay stages and sexes.

Respiration rates (adjusted by lean mass or per fly) are shown for each line within a line type for females and males (see Figures S2A, S2B, S2C, and S2D). For females, the selected line means are not consistently higher for selected lines versus control-punctured and no perturbation control lines. For males, the line means of respiration rates are consistently higher for selected lines when measured per fly (S2D), but not when adjusted by lean mass (S2B).

3.2.3. Respiratory Quotients. Respiratory quotients were calculated from the data as described in Section 2. All RQ values were close to 1.0 (females Table 2(a), males Table 2(b)) which indicated that carbohydrates were used for respiration.

3.3. Movement. Movement for both sexes was investigated for all of the line types and treatments. The statistical analysis of female movement data indicated no significant line or treatment effects. The statistical analysis of male movement data indicated that there was no significant effect of line type or treatment effect. The overall analysis of the data set revealed a significant difference between sexes ($P = 0.011$); males moved more frequently than females (Figure 15).

3.4. Weight. Figure 16 shows the dry weight and lean mass data for each line type and sex. There was a statistically significant difference among line types for female lean mass ($P = 0.0004$) and female dry weight ($P < 0.0001$). There was a statistically significant difference among line types for

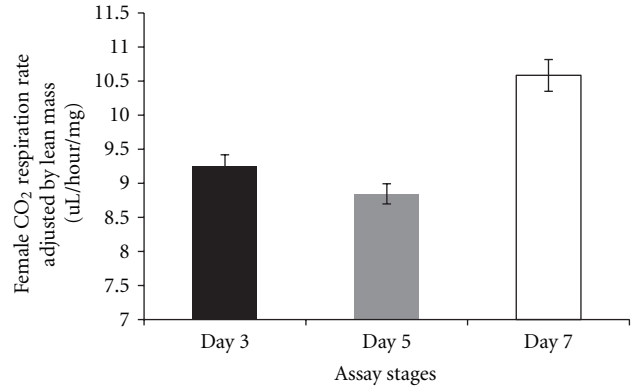

Figure 7: Average CO_2 respiration rates adjusted by lean mass for females at different assay stages. Day 3: before perturbations, Day 5: punctured with autoclaved spores or punctured with sterile H_2O or remained unperturbed, Day 7: after mating.

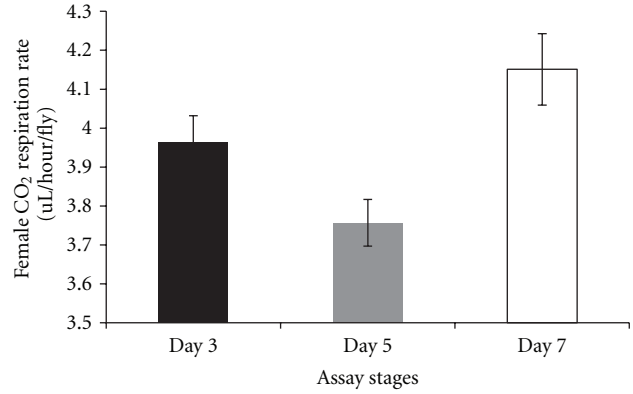

Figure 8: Average CO_2 respiration rates measured per fly for females at different assay stages. Day 3: before perturbations, Day 5: punctured with autoclaved spores or punctured with sterile H_2O or remained unperturbed, Day 7: after mating.

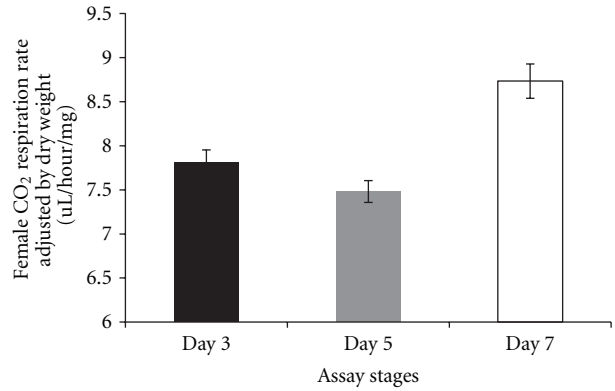

Figure 9: Average CO_2 respiration rates adjusted by dry weight for females at different assay stages. Day 3: before perturbations, Day 5: punctured with autoclaved spores or punctured with sterile H_2O or remained unperturbed, Day 7: after mating.

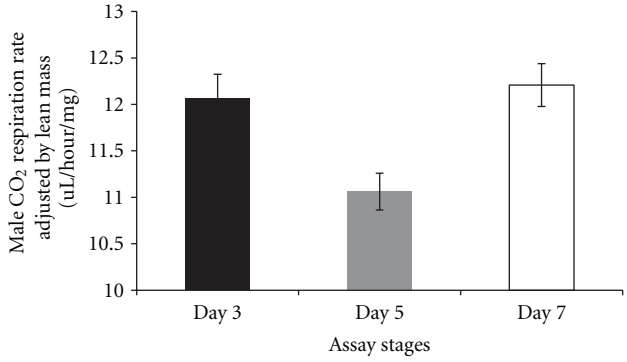

FIGURE 10: Average CO_2 respiration rates adjusted by lean mass for males at different assay stages. Day 3: before perturbations, Day 5: punctured with autoclaved spores or punctured with sterile H_2O or remained unperturbed, Day 7: after mating.

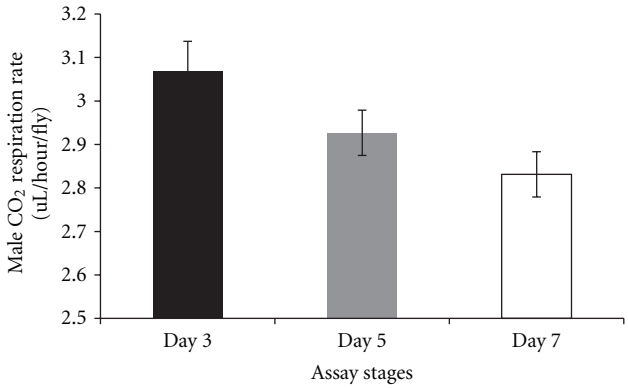

FIGURE 11: Average CO_2 respiration rates measured per fly for males at different assay stages. Day 3: before perturbations, Day 5: punctured with autoclaved spores or punctured with sterile H_2O or remained unperturbed, Day 7: after mating.

male dry weight ($P = 0.0334$), but not for lean mass ($P = 0.3050$). The difference between dry weight and lean mass is mostly due to neutral lipids (mainly triacylglycerides and diglycerides) that were extracted from dry weight flies to generate the flies that were used for lean mass measurements (see Section 4 for relevance to respiration rates).

A higher level of lipid was present in females than males. This was inferred by the observation that females exhibited a greater drop from dry weight to lean mass than males (see Figure S3). This contributed to a statistically significant interaction between weights (dry weight and lean mass) and sex ($P < 0.0001$). Both males and females lost weight (dry weight or lean mass) after mating (Figures 17 and 18). This was shown by the post hoc statistical analysis for females and males comparing the posttreatment assay stage (day 5) and postmating assay stage (day 7). For the female comparison of these two assay stages, the P value was $P < 0.0001$ for lean mass and $P = 0.0010$ for dry weight. For males the P values were $P < 0.0001$ for both lean mass and dry weight.

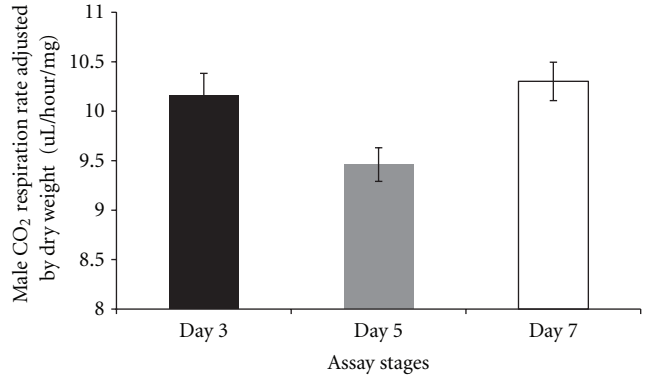

FIGURE 12: Average CO_2 respiration rates adjusted by dry weight for males at different assay stages. Day 3: before perturbations, Day 5: punctured with autoclaved spores or punctured with sterile H_2O or remained unperturbed, Day 7: after mating.

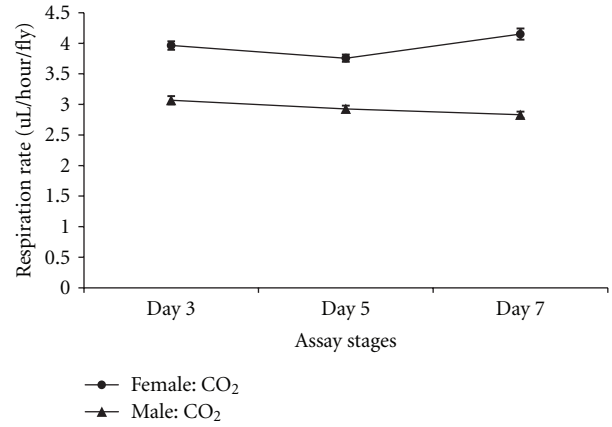

FIGURE 13: Line graph of average CO_2 respiration rates measured per fly for females and males at different assay stages. Day 3: before perturbations, Day 5: punctured with autoclaved spores or punctured with sterile H_2O or remained unperturbed, Day 7: after mating.

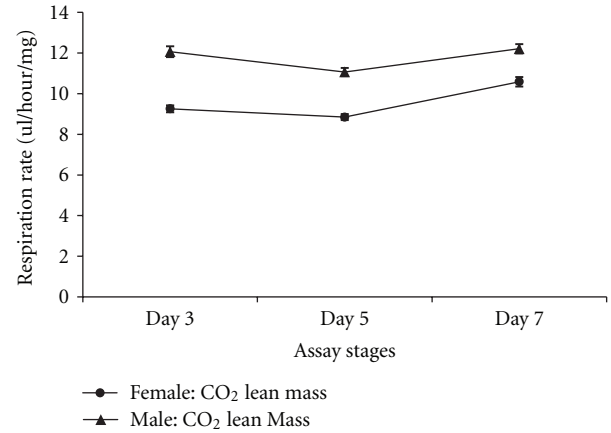

FIGURE 14: Line graph of average CO_2 respiration rates adjusted by lean mass for females and males at different assay stages. Day 3: before perturbations, Day 5: punctured with autoclaved spores or punctured with sterile H_2O or remained unperturbed, Day 7: after mating.

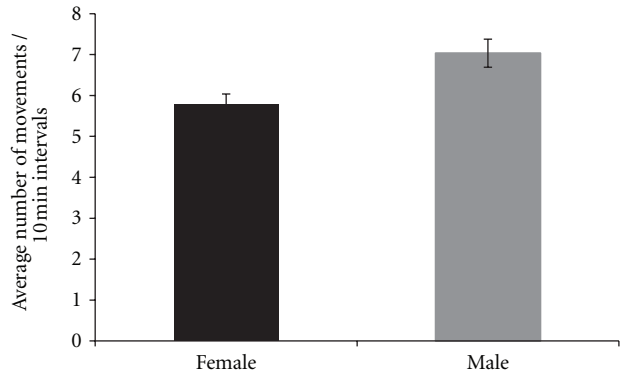

Figure 15: Average number of movements of females and males in 10-minute intervals detected when an individual interrupted the path of a laser beam in a movement monitor. Each assay was conducted for 24 hours.

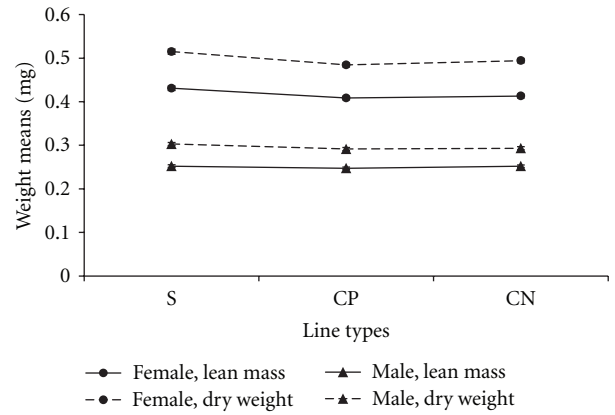

Figure 16: Weight (dry weight, lean mass) means for females and males from different line types. S: selected lines, CP: wound control lines, CN: no perturbation control lines.

4. Discussion

4.1. Overview. There were a number of traits investigated in this study that were associated with sex differences. There was mixed evidence that selected-line males exhibited an indirect response to selection that resulted in a higher level of CO_2 respiration rates (O_2 respiration rates exhibited a parallel pattern). There was consistently statistically significant support for this relationship when respiration rate was measured per fly. When respiration rate was adjusted by lean mass, the level of support for an interaction between sex and line types approached statistical significance; the P value is reported in the first paragraph of Section 2.3. Overall, there was evidence that selected-line males exhibited higher respiration rates (Figures 4, 5, and 19) whereas selected-line females did not (Figures 1, 2, and 19), but the data and statistical analyses did not uniformly support this observation as discussed detail in the following section of the Discussion. There were marked differences in respiration rate between assay stages. For females, when respiration was measured per fly or adjusted by lean mass or dry weight, respiration rates were the lowest at day 5 and markedly higher after mating (day 7). The high rate of female respiration after mating could be due to an increase in the synthesis of protein and lipid needed to provision the oocytes with yolk (vitellogenesis). Finally, there was a difference between males and females in movement. Hypothetically, males were more active as a result of mate-seeking behaviors versus females who were relatively stationary reflecting oviposition-related behaviors.

4.2. Respiration Rates. The hypothesis underlying this study was that evolved resistance to *B. cereus* spores was due to a physiologically costly immune system response to selection that would be reflected in elevated respiration rates. Elevated respiration was observed in selected-line males when the respiration rate was determined per fly. Also, there was an indication of an elevated male respiration rate from the interaction between sex and lines types when data was adjusted by lean mass. Respiration was elevated in selected-line males compared to the other line types whereas this was

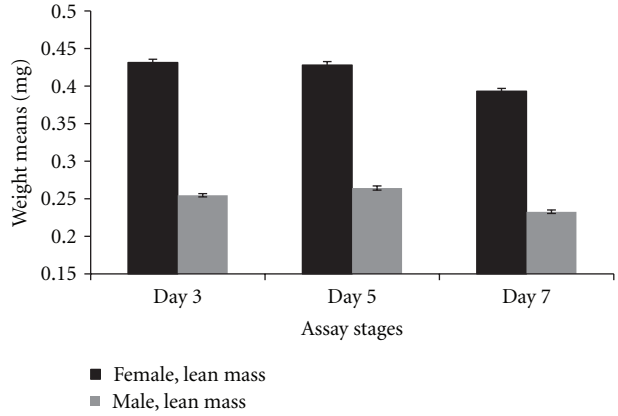

Figure 17: Weight (lean mass) means for females and males at different assay stages. Day 3—before perturbations, Day 5: punctured with autoclaved spores or punctured with sterile H_2O or remained unperturbed, Day 7: after mating.

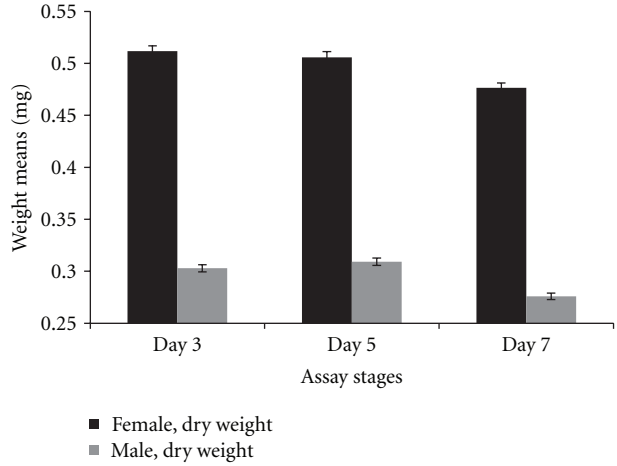

Figure 18: Weight (dry weight) means for females and males at different assay stages. Day 3: before perturbations, Day 5: punctured with autoclaved spores or punctured with sterile H_2O or remained unperturbed, Day 7: after mating.

Table 2: RQ values (a) female, (b) male.

(a)

Assay stages	Lines	Treatments	Mean RQ	S.E.
Day 3	CP	AS	1.02	0.022
Day3	CP	NON	1.04	0.025
Day 3	CP	H_2O	1.11	0.042
Day 3	CN	AS	1.06	0.029
Day 3	CN	NON	1.02	0.026
Day 3	CN	H_2O	1.00	0.023
Day 3	S	AS	1.04	0.027
Day 3	S	NON	1.05	0.027
Day 3	S	H_2O	1.07	0.025
Day 5	CP	AS	1.04	0.029
Day 5	CP	NON	0.97	0.022
Day 5	CP	H_2O	1.04	0.035
Day 5	CN	AS	1.03	0.027
Day 5	CN	NON	0.99	0.023
Day 5	CN	H_2O	0.98	0.019
Day 5	S	AS	1.00	0.033
Day 5	S	NON	1.03	0.032
Day 5	S	H_2O	1.04	0.021
Day 7	CP	AS	1.03	0.031
Day 7	CP	NON	1.03	0.031
Day 7	CP	H_2O	1.03	0.029
Day 7	CN	AS	1.05	0.027
Day 7	CN	NON	1.02	0.034
Day 7	CN	H_2O	1.00	0.035
Day 7	S	AS	0.99	0.028
Day 7	S	NON	0.99	0.024
Day 7	S	H_2O	1.03	0.026

(b)

Assay stages	Lines	Treatments	Mean RQ	S.E.
Day 3	CI	AS	1.16	0.042
Day 3	CI	NON	1.10	0.030
Day 3	CI	H_2O	1.11	0.028
Day 3	CN	AS	1.08	0.032
Day 3	CN	NON	1.13	0.032
Day 3	CN	H_2O	1.08	0.033
Day 3	S	AS	1.11	0.031
Day 3	S	NON	1.09	0.035
Day 3	S	H_2O	1.11	0.032
Day 5	CI	AS	1.10	0.040
Day 5	CI	NON	1.11	0.045
Day 5	CI	H_2O	1.08	0.040
Day 5	CN	AS	1.10	0.032
Day 5	CN	NON	1.05	0.032
Day 5	CN	H_2O	1.06	0.028
Day 5	S	AS	1.13	0.037
Day 5	S	NON	1.11	0.045
Day 5	S	H_2O	1.12	0.030
Day 7	CI	AS	1.17	0.058
Day 7	CI	NON	1.10	0.046
Day 7	CI	H_2O	1.06	0.038
Day 7	CN	AS	1.07	0.034
Day 7	CN	NON	1.10	0.033
Day 7	CN	H_2O	1.10	0.047
Day 7	S	AS	1.07	0.035
Day 7	S	NON	1.13	0.056
Day 7	S	H_2O	1.10	0.032

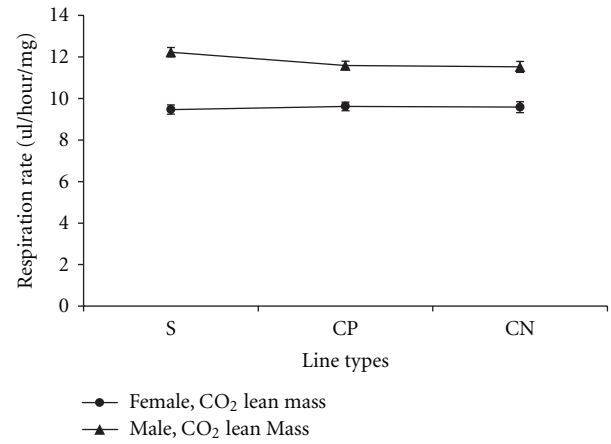

Figure 19: Line graph of average CO_2 respiration rates adjusted by lean mass for females and males among different line types. S: selected lines, CP: wound control lines, CN: no perturbation control lines.

not observed for females (Figure 19). The observations and data analyses described above in this paragraph suggested a *D. melanogaster* cost associated with selection for survival after *B. cereus* spore infection. On the other hand, there were no main-effect differences in male respiration rates adjusted by lean mass. Post hoc tests were nevertheless conducted and there were no statistically significant pairwise differences between line types in male respiration rate adjusted by lean mass. Thus, the evidence for an increase in male respiration rates in the selected lines was mixed. Elevated respiration necessarily must be due to oxygen use by the mitochondria and the corresponding production of CO_2 as part of the process of generating energy rich ATP for use in cellular work. If male respiration rates were elevated in the selected lines, then it could represent a metabolic cost.

The energy source in the selection experiment was obtained by calculating the respiratory ratio (RQ). RQs were always approximately 1.0. The compounds metabolized for energy were carbohydrates. This is typical for Drosophila. There was nothing about the treatments or the response to selection that drove flies to use a different energy compound for metabolism in the present study.

A difference between sexes in the pattern of respiration at different assay stages was observed in the present study (Figure 13: per fly, Figure 14: adjusted by lean mass). The most consistent and pronounced effect was elevated respiration

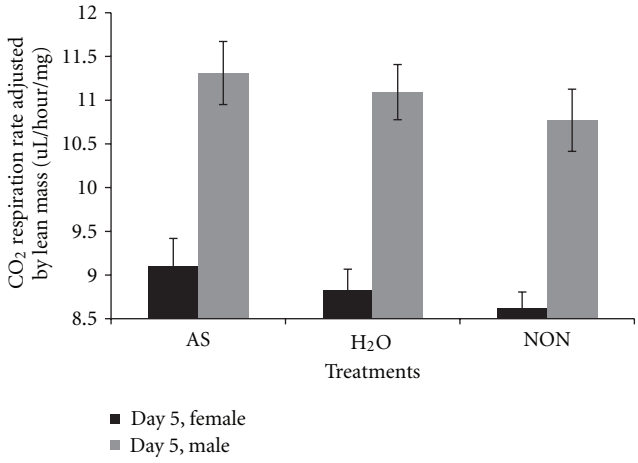

Figure 20: CO_2 respiration rate on day 5 (after treatment) adjusted by lean mass for different treatments and each sex. AS: autoclaved spores, H_2O: injected with sterile water as a wound control, NON: no perturbation.

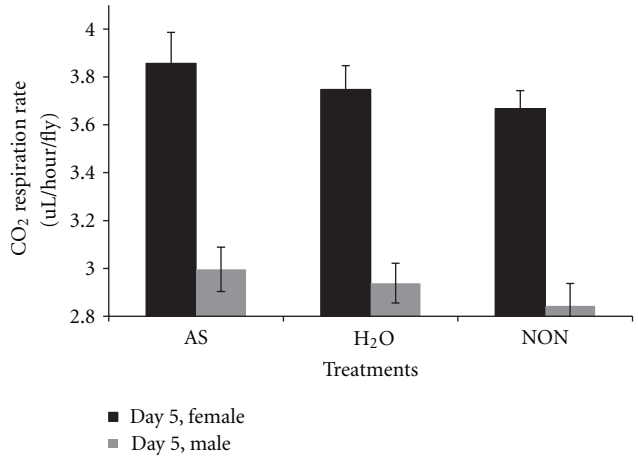

Figure 21: CO_2 respiration rate on day 5 measured per fly for different treatments and each sex. AS: autoclaved spores, H_2O: injected with sterile water as a wound control, NON: no perturbation.

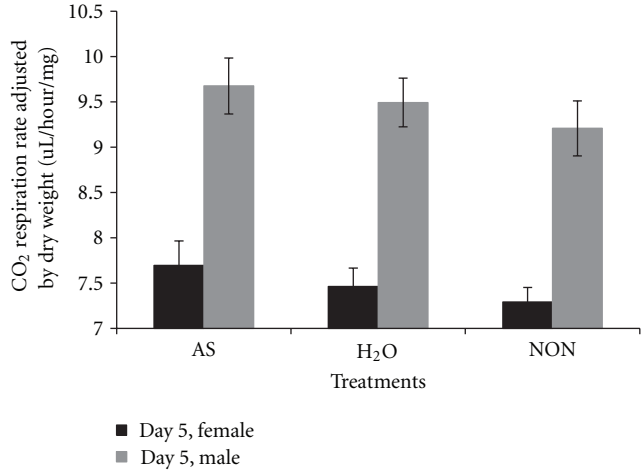

Figure 22: CO_2 respiration rate on day 5 adjusted by dry weight for different treatments and each sex. AS: autoclaved spores, H_2O: injected with sterile water as a wound control, NON: no perturbation.

on day 7 after mating in females (lean mass, dry weight, or per fly). This could be due to females expending substantial energy to provision oocytes with yolk. Insulin signaling plays a critical role in the hormonal control of vitellogenesis [23]. Elevated insulin signaling could be a factor in the relationship between female mating and respiration rates observed in the present study. Differences in the sexes in the pattern of respiration rates at different assay stages might reflect fundamental differences between the physiology and reproductive biology of *D. melanogaster* females and males. The two sexes differ in many ways including their genome-wide patterns of gene expression [24].

There were several issues concerning respiration rates that are pertinent to interpreting the results of the present study. One issue is whether to adjust respiration rate by dry weight as it includes stored lipid which is nonrespiring mass. Thus, biased data can be generated after adjusting by dry weight as illustrated by the following. As a general observation, *D. melanogaster* selected in the laboratory for stress resistance typically stores higher levels of lipid [18]. In previous studies, when respiration rate was measured for selection line flies, it appeared as though lower respiration rate was an indirect response to selection. However, when respiration rate was corrected for nonmetabolic mass (stored lipid) or reported per fly, then there was no reduction in respiration rate in the selected lines [18, 25]. In the present study, there were no differences among line types in lean mass, but there were in dry weight which suggests that adjusting respiration rates by dry weights could be biased by differences in the amount of stored lipid among line types. Another issue was whether there was evidence for a phenotypic response after flies were challenged by autoclaved spores. There was a trend indicating that male and female respiration rate was slightly elevated after flies were challenged by autoclaved spores relative to the other treatments even though there were no statistically significant differences (Figures 20, 21, and 22). Assuming that there was a small response to the spores, that was consistent over 19 generations of selection, the overall response to spores could have been amplified in the selected lines. Thus, a cumulative process could underlie any elevation in male respiration rate in the selected lines compared to the other lines. A final issue was whether day effects (age) could account for any of the respiration rate results. At the age (day 5) that treatments were administered, there was a set of flies that were not treated with autoclaved spores and were not wounded with a needle dipped in water. These untreated flies (NON) were controls for a day effect from day 3 to day 5. There was a statistically significant difference between day 3 and day 5 in respiration rate in flies adjusted by lean mass ($P = 0.0044$) whereby respiration rates dropped (Figure 14). However, it can be seen (Figure 20) that the untreated flies (NON) markedly dropped on day 5 indicating a day effect that was perhaps largely responsible for the reduced respiration rates in the other lines on this day. Two days later all flies were

mated and there was no set of flies that remained unmated that could act as a control for day effects. In wild-type flies, respiration rate changes slowly as a function of age and the change is a gradual decrease [26, 27]. This is opposite of the response observed in the present study. Thus, the elevated respiration rates observed after mating on day 7 is not likely to be due to a day effect.

4.3. Movement. Movement was measured in the present study in an assay that soon followed the respiration measurements. There was a phenotypic difference between sexes, but no difference among lines or treatments. The absence of male movement differences between the selected and control lines indicates that increased movement could not have been responsible for increased respiration in selected line males as potentially documented in the present study. This inference is strongly supported by the experimental design as the movement assays were conducted on the same flies as used for respiration. Relatively elevated male *D. melanogaster* movement has been observed in another study [28]. In the present study there was a general trend across lines for males to move more than females. It is possible that males are more active in order to find mates and court, whereas females are relatively stationary as oviposition-related behaviors might require less movement than mate-seeking and related behaviors.

Acknowledgments

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Research Article

Sex-Biased Networks and Nodes of Sexually Antagonistic Conflict in *Drosophila*

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Sexual antagonism, or conflict, can occur when males and females harbor opposing reproductive strategies. The large fraction of sex-biased genes in genomes present considerable opportunities for conflict to occur, suggesting that sexual antagonism may potentially be a general phenomenon at the molecular level. Here, we employ a novel strategy to identify potential nodes of sexual conflict in *Drosophila melanogaster* by coupling male, female, and sex-unbiased networks derived from genome-wide expression data with available genetic and protein interaction data. We find that sex-biased networks comprise a large fraction (~1/3) of the total interaction network with the male network possessing nearly twice the number of nodes (genes) relative to the female network. However, there are far less edges or interaction partners among male relative to female subnetworks as seen in their power law distributions. We further identified 598 sex-unbiased genes that can act as indirect nodes of interlocus sexual conflict as well as 271 direct nodal pairs of potential conflict between male- and female-biased genes. The pervasiveness of such potentially conflicting nodes may explain the rapid evolution of sex-biased as well as non-sex-biased genes via this molecular mechanism of sexual selection even among taxa such as *Drosophila* that are nominally sexually dimorphic.

1. Introduction

The cooccurrence of distinct morphs—male and female—in sexually reproducing taxa continues to fascinate and perplex developmental and evolutionary biologists alike. Ranging from the subtle to the dramatic, sexually dimorphic traits are presumed to be the product of dynamically evolving genetic architectures that rapidly respond to evolutionary pressures such as sexual selection [1] (for more recent overviews, see [2]). Recent genome-wide analyses have demonstrated that sexual dimorphism is also prevalent at the level of the genome with the majority of genes expressing a male- or female-bias across a range of developmental stages [3–7]. This emerging molecular view reveals that a large fraction of the genome can be expressed in either male or female states.

Like traits, genes can possess alternative strategies depending on the sex they are expressed in. A gene that is expressed in males may provide an important and critical role in his reproductive success while the same gene, when expressed in females, may impart a similarly important but different role in her survival. Thus, fitness effects from the same locus, under different context-dependent states, may be in conflict. This particular type of antagonism, in which a single gene is expressed differently depending on the sex, has been termed, intralocus sexual conflict. Over the last two decades, intralocus sexual conflict has become an integral component of sexual selection theory providing an alternative explanation to such phenomena as the rapid evolution of reproductive traits possessing divergent functions in both males and females [8] and speciation [9–11].

Genes, however, do not work in isolation. Genetic pathways and networks demonstrate a substantive interconnectability of genes to each other and offer a direct link to competing interests. The extent of *interlocus* sexual conflict across a genome largely depends on how extensive is the linkage between male- and female-specific gene networks. In a genetic network, genes refer to epistatic interactions between different loci or nodes via edges at either a domain

level (e.g., protein-protein interaction) or genetic level (e.g., transcription factor binding to regulatory domains). The emergence of genome-wide tools and resources to elucidate molecular interactions at both the genetic and protein levels has greatly increased our catalog of genome-wide interactions, making it possible to finally address interlocus (or intermolecular) conflict systematically across the entire genome [12–15].

In this paper, we generate male and female networks using transcriptome and interactome data from *D. melanogaster* to characterize network differences among male-, female-biased, and sex-unbiased genes so that we can identify potential nodes of interlocus sexual conflict, across the genome. Specifically, we describe and characterize two types of sexual conflict at the molecular level: (1) indirect conflict, which refers to sex-unbiased genes that interact with both male and female genes, and (2) direct conflict, which refers to male and female nodes that directly interact with each other. Our findings provide a first step in understanding antagonistic conflict at the molecular level, genome-wide.

2. Methods

2.1. Sex-Specific Expression in D. melanogaster. All expression data were obtained from the modENCODE project [16]. Specifically, separate genome-wide RNA-seq data from whole body tissue samples of two males and two females were downloaded from the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/), dataset identification GSE28078, which provided BAM files using *D. melanogaster* Genomic Assembly Release 5 as the reference genome. The *D. melanogaster* Annotation Release 5.39 gene models used were obtained from FlyBase (http://www.flybase.org/), provided as GFF files.

2.2. Estimating Sex Bias. The sex-bias per gene is calculated as follows. Consider a set of k samples of coverage data, indexed by $i = 1, \ldots, k$. Let N_{ig} be the total read depth of in sample i over some region g. Likewise, define $N_i = \sum_g N_{ig}$ to be the total read depth over all regions (the entire genome), and $N = \sum_i N_i$ to be the total reads across all samples and all regions. The sample weight of sample i is $w_i = N_i/N$. The sample average expression of a region g is defined as $\overline{N}_g = \sum_i w_i N_{ig}$. The average total expression per sample is $\widehat{N} = N/k$. The sex-bias of region g is defined on a log base 2 scale,

$$b_g \equiv \log_2 \left[\frac{\overline{N}_g^{\mathrm{m}}}{\overline{N}_g^{\mathrm{f}}} \right] - B, \tag{1}$$

where the superscripts refer to male and female, and $B = \log_2[\widehat{N}^{\mathrm{m}} / \widehat{N}^{\mathrm{f}}]$ is the global average read bias between the male and female samples. g derives from the total CDS (one CDS isoform was randomly chosen per gene).

For each gene, we estimated sex-bias from the annotated coding sequence (CDS). Genes with zero expression across all samples were discarded, yielding 13,643 genes with a sex-bias value; 12,453 of those genes with a sex-bias value were contained in the DroID network (see below). The bias thresholds are $b > 2$ for male-biased (four-fold higher in males than females), $b < -2$ for female-biased (four-fold higher in females than males), and $-2 \leq b \leq 2$ for sex-unbiased nodes. Sex-bias values were capped at $b_g = -10$ and $b_g = 10$ (i.e., representing over a 1000-fold difference in expression), in order to retain non-infinite sex-bias ratios. The Kendall tau correlation coefficient between the sex-bias defined here and the sex-bias defined from a meta-analysis from the Sebida sex-bias database [17] is $\tau = 0.711$.

2.3. Genetic Interactions in D. melanogaster. The gene-gene interaction network (GGIN) was downloaded from the DroID metadataset version v2012_04 [12], comprising a total of 15,254 genes and 514,325 gene-gene interactions. When assigning interactions between genes, we used two approaches based on permissive and a more conserved, or strict, criteria. The permissive approach used all 13 of the available interaction datasets from DroID—from protein-protein interactions to genetic interactions—allowing for a larger sample of genes. These included the files shown in Table 4.

A more strict approach, which formed the basis of our results, identified interaction pairs solely from six empirically-driven physical interactions (e.g., protein-interaction data derived from six experiments including yeast-two-hybrid and transcription-factor CHIP-seq analyses from the modENCODE project). These files are listed in the left column of Table 4.

2.4. Identifying Putative Nodes of Sexual Conflict. In order to significantly reduce the number of false positives among putative nodes of conflict, we took an ultra-conservative approach in defining indirect (unbiased node interacting with sex-biased node) and direct (male node interacting with female node) nodes of conflict. All sex-biased nodes that may potentially be involved in sexual conflict require a very stringent 32-fold expression difference between sexes. To be labeled as a male node, a gene has to be expressed, on average, 32 times greater in males relative to females. Sex-unbiased genes remain defined as any gene that has less than a two-fold expression difference between males and females.

3. Results and Discussion

3.1. Male and Female Genetic Networks. In nearly all sexual taxa surveyed, reproductive traits and genes are consistently rank among the most rapidly evolving functional classes (see [2]). Most reproductive genes are sex-specific and play a role in maintaining and promoting the divergence of sexually dimorphic traits over time. Sexual antagonism, or conflict, can provide an evolutionary and molecular mechanism to explain the rapid divergence of reproductive genes on a genome-wide scale. The goal of this present study was to identify interaction targets, using a genomics approach, that may potentially be in conflict with each other. To accomplish this goal, we first generated male, female, and sex unbiased networks in *D. melanogaster* by combining sex-specific gene expression with available curated interaction data.

In total, 12,453 genes (12,628 from the permissive set), representing over three quarters of all known *D. melanogaster* genes, were used in this analysis and 237,954 (403,518 using the permissive criterion) interaction partners were identified. Table 1 provides a summary of all interaction subnetworks. 1,327 male nodes interacted with another male node (the male-male subnetwork), representing over 10% of the total number of assessed genes. Similarly, 1,348 female nodes interacted with at least another female node (the female-female subnetwork). However, unlike the male network with 1,248 male nodes that included interactions not involving other male nodes, the female network only contained 319 non-female-interacting nodes. This may indicate that the female network contains a much larger fraction of shared subnetworks (e.g., female-unbiased, female-female, and female-male) that are more interconnected relative to the male network. Overall, the sex-unbiased network comprised of a much larger fraction of genes (Table 1); however, this high proportion is partially due to the use a very conservative sex-bias stringency.

The bin counts on the number of edges per node of the subnetworks, shown in Figure 1, display the often quoted power law behavior of genetic interaction networks, at least on the high degree tails (right tail). Nodes of lower edge/node degree deviate from this scale-free pattern, resulting in a complex network containing at least two distribution behaviors. This observation highlights that care must be taken when fitting degree distributions of genetic interaction networks to power laws. Here, we used a lower cutoff for the degree and only used nodes with number of edges greater than or equal to the cutoff when computing the power law fit. The specific cutoff values, which vary across the subnetwork types, were determined by visual inspection of the distributions, and tend to accord with the average edges per node values in Table 1. If the entire data set is fit to a power law without regard to whether a power law is appropriate over the entire range of node degrees, the resulting best fit power law exponents are problematic to interpret. For example, the female subnetwork shown in Figure 1 (red) displays a peak in the distribution around 5 edges per node. At higher degree values the distribution is approximately a power law with exponent, −3.01 (Table 1). If a cutoff was not used, the resulting power law exponent would be much smaller, −1.52 (data not shown). In some contrast, the male subnetwork, shown in Figure 1 (blue), has a monotonic distribution but tends to deviate from power law behavior at the smallest degree bin size (the distribution flattens out at lower degree values). Beyond the first bin, the best fit power law exponent for the male subnetwork is −2.75. If all the data were used in the power law fit, the resulting best fit exponent is −2.15 (data not shown), which is lower than the power law tail value, as in the female case. The impact of fitting the entire data set, even among very low degree nodes, in the power law fit has the greatest impact on the female subnetwork, and overstates the shallowness of female subnetworks compared to male subnetworks. In fact, both female and male subnetworks have similar power laws in their high degree tails, but differ mainly in the distribution on the low degree end.

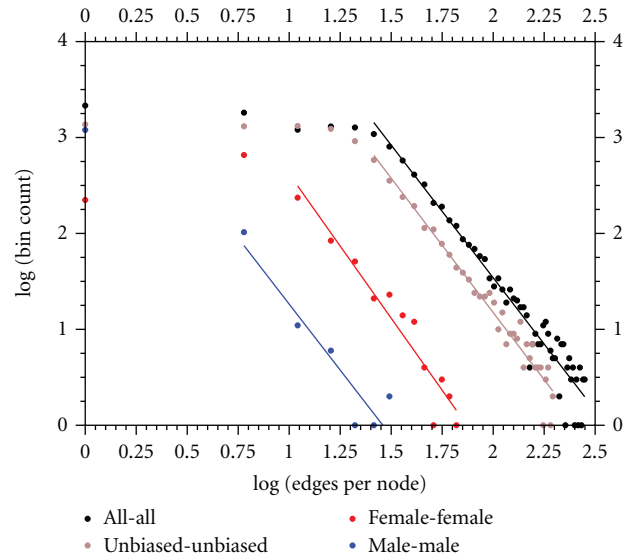


Figure 1: Frequency distribution of the number of edges per node for various classes of interactions. A log-log plot of the edges per node bin counts is shown for four (sub)networks, as indicated in the legend. The bin size is 5, and the bin counts are unnormalized. All interactions from the entire network (black) and those among the sex-unbiased node subnetwork (brown) display power law tails for degree above: 25. The female subnetwork (red) has a power law tail for degree above: 5, while the male subnetwork (blue) is approximately a power law in its entirety. Only the male subnetwork displays a monotonic distribution.

Malone et al. [18] recently generated male and female genetic networks in *D. melanogaster*, based on coexpression correlations. In total, they identified 4,104 female-biased genes and 2,694 male-biased genes using a more quantitative approach (our very conservative approach identifies a smaller gene set among male and female networks). Almost 60% of male nodes from our study matched the Malone et al. male network (1,494 out of 2,575). Similarly, there was a nearly 70% concordance among our study's identified females nodes compared to Malone et al.'s female network (1,012 out of 1,446). These overlaps are surprising since each study used very different approaches to assign interaction. Both our studies, report that the female and male subnetworks display a different overall structure. However, while Malone et al. 2012 base their conclusions on different power law exponents for the female and male subnetworks (−1.06 and −1.35, resp.), we find that the difference is not in the power law behavior on the high degree end (which are qualitatively, if not quantitatively similar) but in the deviation from power law behavior at the low degree end. Female subnetworks have a large cluster of genes with: 5–10 interaction partners, while for genes in the male subnetwork, the most frequent number of interaction partners is unity.

This difference between male and female networks, in terms of the identity of their interaction partners, is most easily observed as the total number of interactions that both networks harbor. While the number of female-female interactions, or edges, is an order of magnitude higher than

Table 1: Characteristics of sex-biased and unbiased networks. Each of the sex-unbiased, male-, and female-biased networks are classified into subnetworks by the expression bias of the interacting nodes. The letter codes A, U, F, and M refer to the node types "All", "Unbiased", "Female-biased", and "Male-biased", respectively. The number of nodes and the number of edges refer to first letter (boldface) of the network type. For example, the pair **X**-Y refers to all nodes of type X that have an edge to a node of type Y. The summary statistics reported are: (1) the total number of nodes (of boldface type), (2) the total number of unique edges connecting those nodes, (3) the overall ratio of edges per node, computed by dividing the second column by the first column, (4) the mean number of edges per node, (5) the standard deviation in the number of edges per node about the mean, (6) the 25th percentile of the number of edges per node, (7) the 75th percentile of the number of edges per node, (8) the exponent of the best fit power law to the degree distribution, and (9) the degree lower limit cutoff for a node to be included in the power law fit. Reported values are for the strict DroID network that only include interactions based on physical, experimental evidence; values in parenthesis refer to the permissive network that include all evidence types in the DroID database version "2012_04". The power law exponent is fit to the tail of the edges per node binned distribution (bin size = 5); edge per node values below the cutoff indicated in the last column are not used in the power law fit.

Network	Nodes	Edges	Edges/nodes	Mean edges/node	StD edges/node	25th%	75th%	Exponent	Cutoff
A-A	12,453 (12,628)	237,954 (403,518)	19.1 (32.0)	38.1 (63.7)	235.0 (244.7)	6 (9)	31 (59)	−2.77 (−2.13)	25 (25)
U-A	8,432 (8,517)	219,401 (370,642)	26.0 (43.5)	39.2 (65.6)	233.9 (244.1)	7 (11)	33 (61)	−2.79 (−2.17)	25 (25)
U-U	8,302 (8,429)	124,730 (214,508)	15.0 (25.4)	36.6 (61.3)	219.9 (229.3)	6 (10)	30 (57)	−2.80 (−2.19)	25 (25)
U-F	7,378 (7,680)	68,794 (108,317)	9.3 (14.1)	31.1 (51.6)	197.5 (206.0)	5 (7)	26 (47)	−3.13 (−2.46)	5 (5)
U-M	6,024 (6,802)	25,877 (47,817)	4.3 (7.0)	27.3 (44.7)	183.5 (190.3)	3 (5)	24 (40)	−2.61 (−2.66)	5 (5)
F-A	1,446 (1,452)	85,430 (137,027)	59.0 (94.4)	28.6 (46.7)	192.8 (199.4)	3 (5)	25 (41)	−2.59 (−1.54)	25 (25)
F-U	1,430 (1,440)	68,794 (108,317)	48.1 (75.2)	29.2 (47.5)	196.5 (202.9)	3 (5)	25 (42)	−2.67 (−1.73)	20 (20)
F-F	1,348 (1,365)	9,270 (16,956)	6.9 (12.4)	28.7 (46.9)	193.9 (200.3)	4 (5)	24 (41)	−3.01 (−1.92)	10 (10)
F-M	1,127 (1,244)	7,366 (11,754)	6.5 (9.4)	28.2 (45.9)	191.7 (198.0)	3 (5)	24 (40)	−2.09 (−2.23)	5 (5)
M-A	2,575 (2,659)	35,160 (63,737)	13.7 (24.0)	27.5 (44.9)	188.3 (194.7)	3 (4)	23 (39)	−2.55 (−1.79)	15 (15)
M-U	2,423 (2,547)	25,877 (47,817)	10.7 (18.8)	26.7 (43.7)	184.8 (191.2)	3 (4)	23 (38)	−2.83 (−1.98)	15 (15)
M-F	1,622 (1,832)	7,366 (11,754)	4.5 (6.4)	26.1 (42.5)	182.1 (188.2)	3 (4)	22 (37)	−2.90 (−2.42)	5 (5)
M-M	1,327 (1,636)	1,917 (4,166)	1.4 (2.5)	25.5 (41.4)	180.0 (185.7)	3 (4)	22 (36)	−2.75 (−2.18)	5 (5)

the number of female nodes, male-male nodes have a much smaller number of partners (Table 1). This pattern can also be seen in the frequency distribution of the number of edges (Figure 1). The male degree distribution peaks at its lowest value (a single edge), while the female distribution peaks at 28 edges, more similar to the distribution of all genes without regard to their sex-bias. In other words, male-biased genes appear to be far less interconnected with each other than similar nodes from the female network.

The less interactive nature of male networks (male-any) and subnetworks (male-male) is supported by evolutionary analyses that characterize new gene formation. These genome-wide analyses find that *de novo* genes are expressed often exclusively in males, and preferentially in the testis [19, 20]. It is possible that these genes generally become immediately coopted into male gametogenesis and fertility functions without embedding themselves into the existing male network. In contrast, female genes are rarely found among new genes and often have functions that overlap with embryogenesis and early development [21]. Furthermore, male-driven sexual selection may provide higher selective pressure to maintain and fix these male genes in populations, relative to female genes [22]. Thus, the interconnectivity of the female versus male networks may be the result of a combination of developmental system constraints and historical selective pressures.

3.2. Genome-Wide Sexual Conflict. By annotating the male and female networks using the unprecedented resources of *Drosophila melanogaster*, we are able, for the first time, to identify putative interacting nodes of conflict, across the genome. To understand the potential extent of genome-wide sexual conflict, we characterized sex-unbiased nodes (i.e., not already part of the reproductive network) that had connections to both highly male and highly female nodes (indirect conflict) as well as candidate nodes under direct conflict, that is, male-female edges (see Figure 2).

Our results supports the contention that sexual conflict has an enormous pool of indirect and direct targets to act extensively upon in the genome. We identified 598 sex-unbiased genes that can potentially act as indirect nodes of interlocus sexual antagonism in addition to 271 direct nodal pairs of potential conflict between male- and female-biased genes. (FlyBase accession lists for indirect and direct, permissive and strict, and male and female networks, are found in Supplementary Files, available on line at http://dx.doi.org/10.1155/2013/545392). A cursory GO analysis of these potentially conflicted sex-unbiased genes identified a variety of development and morphogenesis functional classes that were significantly overrepresented among the sex-unbiased, indirect candidate nodes (Table 2). Since developmental genes are generally more pleiotropic than other genes [23, 24], they may be indirectly involved in various male and female functions including testis and ovary development. It is also possible that different tissue types and developmental stages harbor different interactions. For future studies, it would be important to ensure that interaction datasets are derived from the same tissues and

Table 2: Significant gene ontology (GO) categories for sex-unbiased nodes that interact with at least one male-biased gene and at least one female-biased gene (indirect nodes of sexual conflict). Interactions using the strict criterion were used and only significant (Bonferroni corrected P-values ≤ 0.05) gene ontology classes (Biological Process "Fat" as computed in DAVID) that contain at least 10% of the gene set, are listed.

Rank	Gene ontology function (BP)	Gene ontology ID
1	Post-embryonic morphogenesis	GO: 0009886
2	Imaginal disc development	GO: 0007444
3	metamorphosis	GO: 0007552
4	Pattern specification process	GO: 0009653
5	Post-embryonic development	GO: 0009791
6	Regulation of RNA metabolic process	GO: 0009887
7	Instar larval or pupal development	GO:0002165
8	Regionalization	GO: 0003002
9	Regulation of transcription, DNA-dependent	GO: 0006355
10	Regulation of transcription	GO: 0045449
11	Reproductive cellular process	GO: 0048610
12	Gamete generation	GO: 0007276
13	Sexual reproduction	GO: 0019953
14	Reproductive process in a multicellular organism	GO: 0048609
15	Multicellular organism reproduction	GO: 0032504

Table 3: Significant gene ontology (GO) categories for female-biased genes that interact directly with male-biased genes (direct nodes of sexual conflict). Interactions using the strict criterion were used and only significant (Bonferroni corrected P values ≤ 0.05) gene ontology classes (Biological Process "Fat" as computed in DAVID) that contain at least 10% of the gene set, are listed. Male-biased nodes of direct conflict did not harbor any significant GO terms.

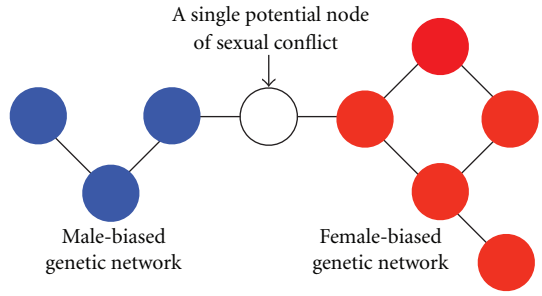
Rank	Gene ontology function (BP)	Gene ontology ID
1	Notch signaling pathway	GO: 0007219
2	Sexual reproduction	GO: 0019953
3	Female gamete generation	GO: 0007292
4	Reproductive cellular process	GO: 0048610
5	Gamete generation	GO: 0007276
6	Reproductive process in a multicellular organism	GO: 0048609
7	Multicellular organism reproduction	GO: 0032504
8	Cell fate commitment	GO: 0045165
9	Cell fate specification	GO: 0001708
10	Sensory organ development	GO: 0007423
11	Oogenesis	GO: 0048477
12	Cell fate determination	GO: 0001709
13	Reproductive developmental process	GO: 0003006

development timepoint as their sex-based expression experimental counterparts. Among the direct male-female conflict candidates, there was a lack of statistically significant gene ontology classes across male genes. However, female-biased genes involved in a direct interaction with a male node contained a range of GO terms with female gametogenesis and reproduction featured prominently (Table 3).

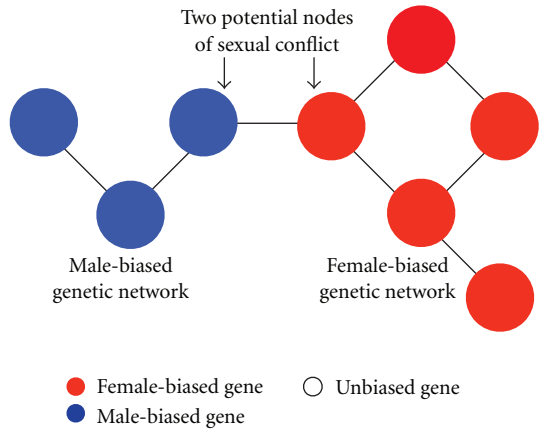
Innocenti and Morrow [25] used a different genome-wide approach to characterize potential nodes of conflict in flies by combining fitness levels of various lines with their gene expression levels, [25]. Specifically, they sampled gene expression levels in males and females across a sample of hemiclonal lines with opposing fitnesses between sexes. Their results identified putative genes involved in sexual conflict (and not the particular gene-pair interactions, as in our work). Overall, their screen found that 8% of all genes may be involved in sexual conflict. We looked for any significant overlap between our putatively conflicted genes and those genes identified by Innocenti and Morrow [25]. There was no significant overlap between our indirect conflict genes, for either the strict or permissive network, and those listed in Innocenti and Morrow [25] (hypergeometric test, two-tailed P value ≥ 0.5). On the contrary, there was a significant lack of overlap between our candidate genes for both the strict and permissive network, and those found in their survey (hypergeometric test, one-tailed P value ≤ 0.01). This suggests that there are other classes of epistatic interactions that have the potential to harbor conflict dynamics.

Table 4

FRIEDMANPERRIMON_COAP.txt	RNA_GENE.txt
CURAGEN_YTH.txt	FLY_GENETIC_INTERACTIONS.txt
HYBRIGENICS_YTH.txt	F LY_GENE_ATTR.txt
DPIM_COAPCOMPLEX.txt	HUMAN_INTEROLOGS.txt
FLY_OTHER_PHYSICAL.txt	YEAST_INTEROLOGS.txt
TF_GENE.txt	WORM INTEROLOGS.txt
	confidence_correlation.txt



(a) Indirect sexual conflict



(b) Direct sexual conflict

Figure 2: Identifying potential nodes of interlocus sexual conflict from male and female gene networks. Genes (nodes) are classified as male-biased (blue), female-biased (red), and sex-unbiased (open) according to their relative expression levels in each of the sexes. (a) Unbiased genes that are connected to both male- and female-biased genes are potential "indirect" nodes of interest for sexual antagonism at that locus. In other words, indirect sexual conflict can occur in sex-unbiased genes that have edges to both male biased and female biased genes. (b) A sex-biased gene directly connected to a biased gene from the opposite sex provides potential "direct" nodes of interest for sexual antagonism at that locus. In other words, direct sexual conflict can occur on male-biased genes that directly interact with female-biased genes, and vice versa.

From these two complementary studies, it appears that the genome provides a potentially large arena to precipitate an extensive evolutionary arms race between the sexes. However, while intuitively appealing, sexual conflict represents just one theoretical perspective to explain such sexual selection phenomena as rapidly evolving reproductive traits and genes, exaggerated sexual characters, and hybrid incompatibility [26]. Catalyzed by large variances in reproductive success, sexual selection can also be explained by alternative coevolutionary processes. Civetta and Singh suggest that sexual traits (and by extension, genes) can evolve rapidly under a process of sexual coadaptation that would harbor a different evolutionary dynamic including greater intraspecific variation [27]. Further work using population and interspecific variation may shed light on these alternative hypotheses.

4. Concluding Remarks

The recent availability of genome-wide datasets has unveiled an unprecedented opportunity for biologists to explore the genomic landscape in a systematic manner. By combining whole genome transcriptomes from males and females with genome-wide genetic interactions, we can begin to understand the genetic architecture of sexual dimorphism. With male and female networks identified in *D. melanogaster*, we are well on our way to understanding their evolution, and the evolution of potentially conflicted genes across populations and in other fly species. From a network perspective, it may be more difficult for a female-biased gene to evolve rapidly because of her greater number of interacting partners (i.e., greater selective constraints). Accordingly, we see evidence of lower levels of female-specific gene divergence compared to male-specific gene divergence in multiple studies from protein gel electrophoresis [28] to genomic [22, 29]. Applying a network approach can help move evolutionary genetics from out of its "beanbag" stage [30] and provide us with a new way to understand rapidly evolving gene networks and reproductive systems as a whole.

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Review Article

Divergence in Defence against Herbivores between Males and Females of Dioecious Plant Species

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Defensive traits may evolve differently between sexes in dioecious plant species. Our current understanding of this process hinges on a partial view of the evolution of resistance traits that may result in male-biased herbivory in dioecious populations. Here, we present a critical summary of the current state of the knowledge of herbivory in dioecious species and propose alternative evolutionary scenarios that have been neglected. These scenarios consider the potential evolutionary and functional determinants of sexual dimorphism in patterns of resource allocation to reproduction, growth, and defence. We review the evidence upon which two previous reviews of sex-biased herbivory have concluded that male-biased herbivory is a rule for dioecious species, and we caution readers about a series of shortcomings of many of these studies. Lastly, we propose a minimal standard protocol that should be followed in any studies that intend to elucidate the (co)evolution of interactions between dioecious plants and their herbivores.

1. Introduction

Sexual systems in angiosperms range from hermaphroditism (monomorphic populations of plants with bisexual flowers) to dioecy (dimorphic populations of male and female individuals) and include almost all imaginable combinations and gradations (Table 1; [1, 2] and references therein). Such remarkable diversity of sexual systems has perplexed naturalists and evolutionary biologists for a long time [3–6]. The evolution of dioecy from a hermaphroditic ancestor has been particularly difficult to understand because the invasion and maintenance of unisexual mutants in a population of hermaphrodites require that the loss of fitness resulting from the loss of one sexual function be compensated by increased fitness gains through the remaining sexual function of the unisexual mutant [7, 8]. This requirement seems very restrictive, and therefore considerable effort has been devoted towards understanding the conditions under which dioecy can evolve [5, 8–18]. In contrast, the evolution of sexually dimorphic traits following the evolution of dioecy (successful establishment of only two reciprocal unisexual morphs in a population) has received less attention. Consequently, our current understanding of the evolution of sex-related traits ultimately leading to morphological or physiological differences between unisexual morphs (i.e., sexual dimorphism or secondary sexual traits) is still limited, despite recent advances and excellent syntheses on the topic [5, 6, 19–21]. This paper focuses on one set of traits subject to becoming sexually dimorphic upon the evolution of dioecy: those traits that provide plants with defence against herbivores.

1.1. Herbivory and the Evolution of Dioecy. Sex-biased herbivory may be one of the selective pressures conducive to the evolution of dioecy, and it can also be a consequence of sex-specific selection on patterns of resource allocation in dioecious species. Considering only the gynodioecy pathway of the evolution of dioecy, we can think of three possible scenarios regarding the role of herbivory in each of the two steps involved in this pathway (Figure 1). The first step in the gynodioecy pathway to dioecy is the successful establishment of females (male-sterile mutants) in a population of hermaphrodites, thus resulting in a gynodioecious population. As mentioned above, this step requires that females compensate for the fitness loss incurred with the

Table 1: Terminology for flowers and sexual systems.

Term	Description
Flowers	
Pistillate	Unisexual flower with functional pistils only (female flower; may have vestigial, sterile stamens (staminodia))
Staminate	Unisexual flower with functional stamens only (male flower; may have vestigial, sterile pistils (pistilodia))
Bisexual, perfect	Bisexual flower with both functional pistils and stamens
Sexual system	
Monomorphic	One kind of plant (floral morph) in the population
Hermaphrodite	Most commonly applied to plants with bisexual flowers, but all monomorphic populations consist of hermaphrodite individuals
Monoecious	Pistillate and staminate flowers on same plant
Gynomonecious	Both bisexual and pistillate flowers on same plant
Andromonoecious	Both bisexual and staminate flowers on same plant
Trimonecious	Bisexual, pistillate, and staminate flowers on same plant
Dimorphic	Two kinds of plants (floral morphs) in the population
Dioecious	One morph male (with staminate flowers only); the other female (with pistillate flowers only)
Gynodioecious	One morph female, the other hermaphrodite (with either bisexual flowers or both pistillate and staminate flowers)
Androdioecious	One morph male, the other hermaphrodite (as above)
Trimorphic	Three floral morphs in the population
Trioecious	Males, females, and hermaphrodites

Modified from Dellaporta and Calderon-Urrea 1993.

loss of the male function. The reallocation of resources freed from the male function towards defence may contribute towards fitness compensation if increased defence results in greater fitness for the females [9]. Increased defence may result in lower herbivore damage on females than on hermaphrodites (Figure 1, path B). However, this is not the only possibility. Defence may be achieved through resistance: traits that reduce the rate of herbivore attack such as low nutritional content of tissues (particularly, N content), secondary metabolites, trichomes, cutin, waxy cuticles, lignin, and volatiles that attract natural enemies of herbivores [22]; and also through tolerance: traits that mitigate the negative effects of damage on fitness, including higher or lower growth rates, mobilization of stored resources, and activation of apical meristems [23]. If females reallocate resources to tolerance traits, they could be the morph with greater herbivore damage (Figure 1, path C).

The second step in the gynodioecy pathway to dioecy is the successful establishment of male individuals (female-sterile mutants) in a gynodioecious population followed by the loss of the hermaphroditic morph, thus resulting in a dioecious population. Upon the evolution of two unisexual morphs, defensive traits may evolve differently in each sex and eventually become sex linked [84–86]. The particular way in which defensive traits diverge between sexes will depend on the costs and benefits derived from the specific pattern of resource allocation to growth, reproduction, and defence in each sex. Currently, it is thought that females generally evolve greater resistance than males (see the following; Figure 1, path b).

This paper focuses on the origin of sex-biased herbivory in dioecious species. Therefore, we will not delve into morph-biased herbivory in gynodioecious species, which would be the topic of a different essay. However, we do recognize that sex-biased herbivory—indicative of sexual dimorphism in resistance against herbivores—is likely related to morph-biased herbivory in the ancestral gynodioecious population from which it evolved (Figure 1, path B-b). Thus, male-biased herbivory may have its origin in a gynodioecious population where hermaphrodites (functionally, the male morph) bear greater levels of herbivory than females.

1.2. Male-Biased Herbivory. The above view for the origin of greater resistance against herbivores in females is based directly on the principle of allocation: resources freed from the male function are used for the female function, growth, and defence. In contrast to this view, the finding of male-biased herbivory in dioecious populations has been explained on the basis of sex-specific selection of resistance traits, where the main difference between sexes that drives the sex-specific selection is the cost of reproduction. In this alternative view, female individuals of dioecious species are expected to have lower herbivory levels than males because the higher cost of reproduction of females confers a selective advantage to females with traits that reduce herbivore attack [66]. The logic of this argument is as follows: since females invest more in reproduction than males, they are left with a smaller pool of resources for growth and therefore must grow more slowly than males [20, 87]. According to

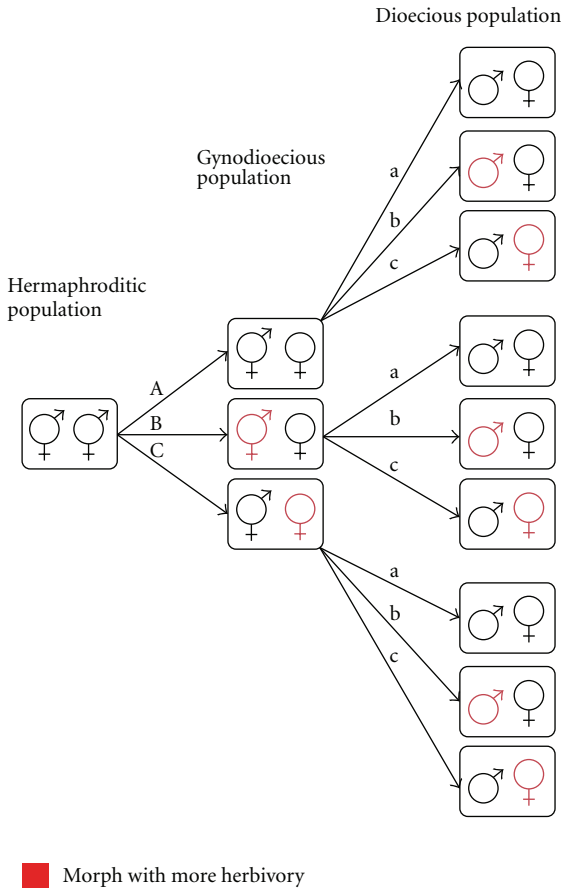

Figure 1: Possible scenarios of the inception of morph- or sex-biased herbivory in the evolution of dioecy via the gynodioecy pathway. Symbols represent hermaphrodite, male, or female morphs in a population (rectangle). Arrows represent evolutionary pathways between populations with different sexual systems. The first step in the pathway is the transition from a hermaphroditic to a gynodioecious population by the successful invasion of females (left-most set of arrows). The second step in the pathway is the transition from a gynodioecious to a dioecious population following the successful invasion of males and the disappearance of hermaphrodites (right-most arrows). Letters indicate different evolutionary paths.

the resource availability hypothesis, the fitness cost of losing tissue to herbivores is greater for plants that grow more slowly, thus favouring the evolution of increased defence against herbivores in slow-growing plants [88]. Since females tend to grow more slowly, they should be better defended against herbivores than males [88]. Consequently, dioecious species should experience male-biased herbivory. We must note that the argument for greater defence levels in females is usually understood in terms of resistance, but it could also be interpreted in terms of tolerance, in which case the predictions of sex-biased herbivory would be the opposite (Figure 1, path c), as developed below.

The first review of the empirical studies on the topic of sex-biased herbivory concluded that "males are more likely than females to be preferentially used by herbivores" and suggested that male-biased herbivory was widespread among dioecious species [72]. The authors, however, recognized that sex-biased herbivory was by no means a unanimous finding across all the dioecious species examined to that date, and that the relative susceptibility of each sex to herbivory could be influenced, among other factors, by fluctuations in ecological tradeoffs between functions (rather than evolutionary changes in patterns of allocation), such as phenological changes in resource allocation to reproduction and growth [75, 89]. Therefore, the life stage at which damage measurements are taken can determine whether a study concludes that herbivory is sex biased or not.

In addition, Ågren et al. cautioned against publication bias, whereby studies that found differences between genders could be more likely to be published than those that did not; and taxonomic bias, an overabundance of studies from certain genera or families. In this instance, the taxonomic bias is correlated with an ecological bias for studies of temperate species, despite dioecy being more prevalent in tropical ecosystems [90]. Ågren et al. called for future studies that (1) examine the causes of differential palatability between males and females, (2) measure the fitness consequences of natural levels of herbivory in both sexes, and (3) determine whether herbivore pressure can actually cause adaptive changes in tissue palatability. In addition, they urged for broadening

the taxonomical scope of the studies. In spite of such encouragement, there still is a paucity of studies that address these issues.

More recently, sex-biased herbivory in dioecious species was tested by means of a meta-analysis of 33 studies encompassing 30 species, 19 of which were previously included in Ågren et al.'s 1999 review [73]. The authors tested for publication bias and found it to be minimal. However, they did not emphasize other shortcomings of the dataset and concluded that male-biased herbivory in dioecious species is a rule.

Here we propose alternative evolutionary scenarios that could result in female-biased herbivory or lack of intersexual differences in herbivory levels. We invite the reader to reconsider the evidence for male-biased herbivory in dioecious plants and recommend a standard protocol for evolutionary-ecological studies of sex-biased herbivory in dioecious species that addresses the shortcomings listed below. We contend that taking for granted the generality of male-biased herbivory in dioecious species is hampering our progress in this field.

2. Critique of Theory: Evolutionary Scenarios

While male-biased herbivory has been explained as a consequence of sex-specific selection of resistance determined by the cost of reproduction of each sex, few of the reviewed studies (Table 2) are actually placed within such evolutionary context. J. Lovett-Doust and L. Lovett-Doust [44] were the first to argue, citing Charnov [91], that an evolutionary divergence between sexes in resource allocation patterns could result in sex-biased resistance against herbivory. Danell et al. [57] based their expectation of male-biased herbivory on sexual selection: it may be more advantageous for males to invest less in resistance and more in reproduction when exposed to a cyclic herbivore compared to a noncyclic herbivore because males will lose fitness only once every four years, and their reproductive output during years of little or no herbivory will more than compensate for the fitness lost to herbivores on the heavy herbivory years. The above explanation would hold only to the extent that there are no carry-over effects from one year to another.

More recently, McCall [83] cited Bierzychudek and Eckhart [92] and Delph [87] in support of the claim that the reproductive output of females is more limited by resources than that of males. However, while there may be ample evidence that reproductive allocation is generally greater for the female function, it is possible that, upon the separation of sexes, physiological mechanisms involved in resource acquisition and allocation evolve in such way as to minimize the differences in reproductive effort between sexes. For example, in *Ilex glabra*, sexes do not differ in total reproductive biomass produced in a growing season because the greater unitary investment in pistillate flowers and fruit development is negated by the sevenfold greater flower production in males [75]. Given the importance of the tenet of greater female reproductive allocation for the expectation of male-biased herbivory, all studies of sex-biased herbivory should test for intersexual differences in reproductive allocation or provide a reference to an empirical study that demonstrates such differences for the species in question. In the measurement of reproductive allocation, particular attention must be paid to obtaining reliable estimates of male reproductive output (pollen production), which presents its own logistical difficulties. In addition, resource expenditure in pollinator attraction needs to be considered, as this is another expenditure related to reproduction that may differ between sexes.

In essence, the presumed chain of evolutionary events that lead to male-biased herbivory in dioecious plants stems from the reallocation of those resources freed upon the loss of a sexual function in unisexual mutants towards defence. In most studies, defence has been equated to resistance. However, defence may also occur through tolerance [22, 93]. In comparison to resistance traits, tolerance traits have been more elusive. The capacity to store and mobilize carbohydrates, the presence of meristems and the capacity to activate them in response to damage have been proposed as tolerance traits [23]. Growth rate has also been proposed to influence tolerance, although it is controversial whether high or low growth rates favour tolerance [94–98]. A recent model shows that plants with low growth rates are more tolerant to herbivore damage [99]. This model also shows that plants that can change their growth rate positively in response to damage will tolerate damage better than those with a different response in growth rate. The activation of meristems and mobilization of resources in response to the loss of tissue are two well-documented responses [100, 101] that could contribute to increased plant growth rates in response to damage. Thus, according to this model, if females grow more slowly than males because of their greater allocation to reproduction, then females should be more tolerant to herbivory than males. This response was observed in *Urtica dioica* subjected to clipping of the stem apex [102]. Whether this prediction necessarily implies that females should be less resistant than males is not clear at this point since there is increasing evidence that these two modes of defence are not necessarily mutually exclusive [99, 103, 104].

There is one other possibility that has not been emphasized enough in the proposed models of the evolution of defence in dioecious species: while one possible consequence of a greater allocation of resources to reproduction in females is reduced allocation to growth, it is also possible that the main reduction in allocation is to defence. In this case, there would be no detectable detriment to growth. Consequently, female plants would suffer more damage (if they are less resistant; Figure 1, path c) or greater fitness losses (if they are less tolerant) compared to males. Greater damage in females could lead to fewer, more spaced reproductive events or greater interannual variability in reproductive output either directly through a decrease in the availability of resources for reproduction or indirectly through a decrease in pollinator visitation rates due to a lack of resources needed for floral display or nectar production [75, 105–108]. Fewer resources available for reproduction could also pose a selective pressure to become choosier about their mates, which may lead to increased fruit or seed abortion [75, 109, 110].

Table 2: List of dioecious species of angiosperms studied for dimorphic herbivore damage, and information on assessment of reproductive allocation, growth rate and resistance.

Species	Damage	Sex with greatest		Resistance	Reference	Review
		Reproductive allocation	Growth rate			
Alismatales						
Araceae						
Arisaema triphyllum	M	nm	nm	nd (N, C:N, leaf total phenolics)	[24]	3
		F	F (total dry mass)		[25]	
Arecales						
Arecaceae						
Chamaedorea alternans (= *C. tepejilote*)	nd	nm	nm	nm	[26]	1
		F			[27]	
			M (leaf production)		[28]	
Asterales						
Asteraceae						
Baccharis concinna	nd	nm	nm	nm	[29]	
		nm	nd (leaf production) M (shoot length)	nm	[30]	3
B. dracunculifolia	nd	nm	nm		[31]	3
			nd (shoot length)		[32, 33]	
B. halimifolia	M, F, nd, depends on herbivore	nd (flowers/shoot)	M	F (resin)	[34]	1
Brassiclaes						
Capparaceae						
Forchhammeria pallida	nd	nm	nm	nm	[35]	3
Caryophyllaceae						
Silene dioica	M	M (during flowering)	F	nm	[36]	1
	F	nm	nd (length of infected shoots)	nm	[37]	3
Chenopodiaceae						
Atriplex canescens	F	nm	nm	nm	[38]	3
	M	nm	nm; nd (height, width, fresh weight in spring), F (FW in winter)	nm	[39]	4
		(F)			[40]; the species includes hermaphrodites	
A. vesicaria	F	nm	nm	nm	[41, 42]	1
Nyctaginaceae						
Neea psychotrioides	M	M	nd (stem production)	nm	[43]	3
Polygonaceae						
Rumex acetosa	M	?	?	?	T. Elmqvist unpublished data	1

Table 2: Continued.

Species	Damage	Sex with greatest Reproductive allocation	Growth rate	Resistance	Reference	Review
R. acetosella	M, F, nd	nd	nm	nm	[44]	1
			F (ramet production)		[45]	
Fagales						
Myricaceae						
Myrica gale	M, nd	nm	nm	nm	L. Ericson unpublished data	1
	nd	nm	nm	F (1-digestibility), nd (phenolics, p-glycosides, tannins)	[46]	
Laurales						
Lauraceae						
Lindera benzoin	nd	nm	nd, M, depending on year	nm	[47]	1
		M (flowers/shoot), F (N and biomass)	M (plant volume)	F (phenolics on leaves, but nd on stems)	[48]	
Malpighiales						
Salicaceae						
Populus tremula	M	nm	nm	M (phenolics), nd (p-glycosides, tannins, digestibility)	[46]	1
Salix caprea	M,	nm	nm	F (1-digestibility), nd (phenolics, p-glycosides, tannins)	[46]	1
	nd		nd		[49]	
	nd	nm	nm	nm	[50]	4
S. cinerea	M, nd, varies by year	nm	nm	nm	[51]	1
			nd		[52]	
				nd (phenolic glycosides)	[53]	
S. eleagnos	M	nm	nm	nm	[50]	3
S. fragilis	nd	nm	nm	nm	[50]	4
S. lanata	nd	nm	nm	nm	[54]	3
S. lasiolepis	M (4 of 5 spp. of sawflies)		M (shoot length)	F (phenols, marginally significant)	[55]	1
	nd (miners, gallers)	nm	nm	nm	[56]	1
S. myrsinifolia-phylicifolia	M (at high plant density)	nm	nm	nm	[36]	1
	M	nm	nd (new shoots)	nm	[57]	1
	M (in high productivity habitat; decreases at higher herbivore pressure)	nm	nd (biomass)	nm	[49]	1
S. pentandra	M	nm	nm	F (phenolics) M (1-digestibility)	[46]	1
S. purpurea	nd	nm	nm	nm	[50]	4
S. sericea	M marginal	nm	nm	nm	[58]	3

Table 2: Continued.

Species	Damage	Sex with greatest Reproductive allocation	Growth rate	Resistance	Reference	Review
			nd	nd	[53]	
S. viminalis	nd	nm	nd (regrowth after pruning)	nm	[59]	3
	nd	nm	nm	nm	[50]	4
S. x *rubens*	nd	nm	nm	nm	[50]	4
Pandanales						
Pandanaceae						
Freycinetia arborea	M	nm	nm	nm	[60]	2
F. reineckei	M	nm	nm	nm	[60]	2, 3
Rosales						
Eleagnaceae						
Hippophae rhamnoides	M ?	?	?	?	L. Ericson unpublished data	1
			M		[61]	
Rhamnaceae						
Rhamnus alpinus	nd			M (anthraquinones)	[62]	3
		F	nd if age < 10 y M if age > 10 y		[63]	
Rosaceae						
Rubus chamaemorus	M, nd	nm	nm	nd	[64]	1
		F but varies with fruit set			[65]	
Urticaceae						
Urtica dioica	M ?	?	?	?	T. Elmqvist unpublished data	1
Sapindales						
Sapindaceae						
Acer negundo	M	nm	M (growth rings)	nd (astringency, total phenols, nitrogen, toughness), F (index of defence)	[66]	1
			variable: F near streams; M away from streams		[67]	
			nd		[68]	
			F		[69]	
Pistacia lentiscus	nd	nm	nm	nd (N)	[70]	1
Simaroubaceae						
Simarouba glauca	M	nm	nm	two flavonoid compounds on female flowers not present in male flowers	[71]	3

F: female, M: male, nd: no statistically significant intersexual differences, nm: not measured, CT: condensed tannins, TNC: total non-structural carbohydrates, N: nitrogen content (herbivores usually attracted to greater concentrations).
1: Ågren et al. 1999 [72], Table 2.
2: Ågren et al. 1999 [72], Table 3.
3: Cornelissen and Stiling 2005 [73].
4: Not mentioned in any of 1–3 above.

Evolutionary changes in the rate of resource acquisition in female individuals may occur through increased photosynthetic rates, canopy area, rates of mineral nutrient uptake, as well as greater branching of roots, and enhancement of mycorrhizal associations [19]. A greater rate of resource acquisition in females would decrease the relative differences in costs of reproduction between sexes: the sex with the greater resource demand for reproduction would have an increased capacity to garner resources. Under such scenario, the life-time cost of reproduction at the individual level would be equal between sexes, thus eliminating the source of inequalities in the patterns of allocation between males and females. In summary, nothing dictates that there is only one evolutionary pathway regarding changes in the patterns of resource allocation among reproduction, growth, and defence following the evolution of unisexuality (Figure 1).

Alternatively, a stage in which female individuals have heavier damage levels because of resource limitation for resistance may be a transient evolutionary stage prior to the invasion of mutants whose greater defence levels are attained at the expense of growth. In this case, we should observe female-biased herbivory in younger dioecious lineages and male-biased herbivory in those lineages in which there has been enough time for selection to reshape the patterns of resource allocation to reproduction, defence, and growth. We should be able to test this by means of relative dating of lineages with male- or female-biased herbivory.

Similarly, as long as there has not been selection on prereproductive growth rates following the evolution of unisexuality, we should not see differences in growth rates or other physiological vegetative traits between males and females before their first reproductive event. It is difficult to test this prediction without reliable morphological or genetic markers that allow juveniles to be sexed so that their performance can be compared on the basis of sex. Some sex-linked markers may be, effectively, sex-related traits expressed before the onset of reproduction. Whether the presence of these markers implies the existence of sex chromosomes is still an area in need of further investigation [84, 85, 111–113].

In short, without fitness gain curves for each sex, it is difficult to predict accurately which sex should evolve greater resistance against herbivores and whether we should expect or not male-biased herbivory in dioecious species [91]. In fact, we need fitness surfaces in order to include the effects of reductions in leaf area caused by herbivory. Moreover, the fitness surfaces should account for the short- and long-term responses of plants in terms of changes in photosynthetic rates, reallocation of resources to shoot or root, activation of meristems, and delays in phenology or shortening of life span brought about by herbivore damage [95, 99].

It has not escaped our attention that the evolution of defence in gynodioecious species can be approached from a similar perspective to the one presented above for dioecious species [12]. It is important to consider that some of gynodioecious species may be in evolutionary transition to dioecy while others are not [112, 114]. Another important difference with dioecious species is that, in gynodioecious plants, the morph that performs the male function—the hermaphrodites—may have a greater cost of reproduction because of the expenditure of resources on two sexual functions. Does this mean that hermaphrodites would be less resistant (Figure 1, path C) or grow more slowly and evolve greater resistance (Figure 1, path B)? Clearly, making predictions with respect to gender dimorphism in defensive traits for bisexual conditions along the gradation from monoecy to dioecy is not straight forward.

3. Critique of Datasets Used to Conclude Male-Biased Herbivory

The collection of studies cited in the reviews of herbivory in dioecious species [72, 73] has, as a group, important shortcomings that weaken the conclusion of male-biased herbivory as a generality in dioecious species. The main shortcomings are (1) the taxonomic bias of the sample of species studied; and (2) failure to test for or provide references of empirical studies of intersexual differences in (a) resistance traits—the purported cause of the intersexual differences in herbivory levels; (b) growth rates—the purported cause of the intersexual differences in resistance to herbivore attack; and (c) reproductive effort—the purported cause of the aforementioned intersexual differences in growth rates. These deficiencies had been pointed out earlier [72, 89], but judging by statements included in the introduction or discussion of many of the papers published after the 1999 review, such caveats have not been considered to their full extent, and many authors take for granted either the generality of male-biased herbivory in dioecious species or its expectation without reference to any theoretical context.

3.1. Taxonomic Bias. Cornelissen and Stiling's meta-analysis of sex-biased herbivory includes 30 species, 28 of which are angiosperms. Focusing only on angiosperms, 13 of the 28 species were not considered previously in Ågren et al.'s review (Table 2). These 30 species represent a total of 20 genera, 18 families, and 10 orders. Nine of those species belong to the same genus: *Salix*. Adding to those *Populus tremula*, the species in the Salicaceae represent one-third of all species considered for the meta-analysis. Such distribution contrasts greatly with the taxonomic distribution of dioecy in 14,620 species, 959 genera, 157 families, and 36 orders [90]. Of the four dioecious genera (consisting of solely dioecious species) with most species (400), only *Salix* has been studied. *Pandanus*, *Diospyros*, and *Litsea*, with 700, 500+, and 400 species, respectively ([115]; S. Renner, University of Munich, unpublished data) have not been studied for sex-biased herbivory yet. Clearly, we need to direct our research efforts to the most understudied orders and families if we want to arrive at generalizations regarding the biology of dioecious species, and particularly the influence of herbivores in their ecology and evolution.

In addition to this taxonomic bias, a critical reexamination of that list of species casts serious doubt on the conclusion that male-biased herbivory is a rule in dioecious species: only 13 of those species were reported invariably to have male-biased herbivory. This list includes

three *Salix*, two *Freycinetia*, and two species for which evidence of male-biased herbivory has not been published: *Hippophae rhamnoides* and *Rumex acetosa*. (In fact, male-biased herbivory in *Myrica gale*—not included in these 13 species—is also anecdotal.) Greater herbivore damage on females is reported for four species, while the rest show either no intersexual differences (16), or variation in the result, depending on different factors (species of herbivore, kind of herbivore, tissue damaged, time of year, phenological or ontogenetic stage, etc.). Moreover, it is possible that the results for those 13 species would show variation with either population or site, had these factors been studied.

3.2. Differential Growth between Sexes. Perhaps the most serious problem with several studies of herbivory and dioecy has been the failure to make the connection between sex-biased herbivore damage and intersexual differences in growth rate, precisely because the latter is the purported cause of the former. Of the 30 species of angiosperms in Table 2, either growth rate or a surrogate variable for growth (e.g., shoot length) was measured only in 21 species. Males grew faster in six species, females in two, no difference between sexes was detected in six species, and three species showed variable results. It must be noted that the same number of species shows no difference between genders as those that show greater growth rate in males. Considering solely the 13 species that invariably showed male-biased herbivory, only two show a greater growth rate in males: *Acer negundo* and *Hippophae rhamnoides*. However, as the evidence for male-biased herbivory in *H. rhamnoides* is anecdotal, we are left with only one species for which growth rate was measured in the same study as herbivore damage: *Acer negundo*.

3.3. Differential Reproductive Effort between Sexes. Only 12 of the 30 species listed were assessed for intersexual differences in reproductive allocation in terms of reproductive effort (the proportion of biomass or other currency devoted to reproductive structures relative to the total biomass or expenditure in the selected currency of an individual). Reproductive effort was greater in females of 10 species and in males for the other two species. In some species, reproductive effort was measured during flowering, but allocation to fruit production was not considered (e.g., *Silene dioica*). In those cases, we are left with an incomplete picture of reproductive allocation, and we can only join the authors in speculating whether species of the same genus have similar patterns of reproductive allocation.

The only species that have been assessed for foliar damage, growth rate, and reproductive allocation in the same study are *C. tepejilote*, *B. halimifolia*, *I. glabra, N. psychotrioides*, and *R. alpinus* (Table 2). These studies clearly made the chain of causal connections from sex bias in reproductive allocation all the way to sex bias in some resistance traits (except for *C. tepejilote* and *I. glabra*), and, as a consequence of the latter, sex bias in levels of damage. The study on *R. acetosella* at least established the connection between damage and growth [44]. The study of *R. alpinus* went even further, comparing these attributes between pre- and postreproductive plants, and thus emphasizing that the root of the differences in growth rates, resistance traits, and leaf damage is in the patterns of reproductive resource expenditures [63, 116].

In some species, reproductive allocation, growth rate, and/or resistance were reported after the initial publication of sex-biased herbivory. However, even with these studies, the number of species for which we have a more complete picture of the causal links amongst these attributes remains low: nine more species (*C. alternans, B. dracunculifolia. A. canescens, R. acetosella, S. caprea, S. cinerea, S. lasiolepis, S. sericeae,* and *H. rhamnoides* [?]; Table 2) now have published data for damage and growth rate, bringing the number of species in this situation to 15. Two more species, for a total of three, now have data on damage, growth rate, and reproductive allocation (*C. alternans, S. dioica,* and *N. psychotrioides*). Two more species now have data on reproductive allocation, growth rate, and resistance apart from herbivore damage, for a total of four species with all four variables measured (*A. triphyllum, B. halimifolia, L. benzoin*, and *R. alpinus*).

In summary, the majority of studies on the topic of sex-biased herbivory have neglected the purported causal connections between bias in reproductive allocation, differential growth rate, resistance, and herbivore damage. Also, some authors seemed to confuse theoretical expectations with empirical evidence of greater female reproductive allocation: while Lloyd and Webb [20] argue convincingly for the expectation of greater reproductive effort in females, they provided empirical evidence only for *Rumex acetosella*, citing Putwain and Harper 1972 [117]. Therefore, Lloyd and Webb's excellent paper cannot be cited as solid empirical evidence of greater reproductive effort in females. Lastly, anecdotal evidence should be taken with great caution and always flagged as such until data are published (see entries marked "?" in Table 2).

Using the search terms herbiv* and dioec* for entries between January 1998 and May 2012 on the Web of Science, we found nine studies encompassing 14 species that were not included in either of the previous reviews of the topic. Of these, only the study on the three species of *Chamaedorea* palms measured reproductive allocation, growth rate, resistance, and herbivore damage (Table 3; N.B.: one of these species had been studied before: *C. alternans* = *C. tepejilote*). Only one other study measured damage and reproductive allocation (*Sclerocarya birrea*, Table 3). Similarly, growth rate was assessed in only one other species (*Salix arctica*). The taxonomic breadth of the studies of herbivory in dioecious species increased only by one family (in an unplaced order of the Euasterids I). The general lack of consistency in the level of detail and the variables that have been measured in all these studies could be addressed if researchers interested in this topic followed a minimally standardized protocol.

Table 3: Studies of defence on dioecious species published after 2004, or published earlier but not mentioned in Ågren et al or Cornelissen and Stiling's reviews.

Species	Damage	Sex with greatest Reproductive allocation	Growth rate	Resistance	Herbivores	Reference
Arecales						
Arecaceae						
Chamaedorea alternans (= C. tepejilote)	M	F	F	F	Chrysomelid beetles	[74]
C. pinnatifrons	M	F	M	F	Chrysomelid beetles	[74]
C. ernesti-augusti	M	F	M	F	Chrysomelid beetles	[74]
Aquifoliales						
Anacardiaceae						
Ilex glabra	nd; marginally F after flowering	nd	nd	nm	lepidopteran larvae and leaf spot (fungal pathogens)	[75]
Sapindales						
Anacardiaceae						
Sclerocarya birrea	F	nd (wood/reproductive shoot)	nm	nd (wood density, branch breakability)	Elephants	[76]
Spondias purpurea	F	nm	nm	M (N, TNC)	Cerambycid beetle	[77]
Malpighiales						
Salicales						
Salix discolor	nd	nm	nm	M (mortality of herbivore)	Leaf galler	[78]
S. polaris	nm	F	nd	nd (phenolics, CT)	Reindeer	[79]
S. arctica	nd	nm	nd	nm	Muskox	[80]
S. planifolia	nd	F	nm	nm	Insects	[81]
Laurales						
Lauraceae						
Lindera obtusiloba	nd	nm	nm	nm	Unspecified	[82]
L. praecox	nd	nm	nm	nm	Unspecified	[82]
L. umbellata	nd	nm	nm	nm	Unspecified	[82]
L. erythrocarpa	nd	nm	nm	nm	Unspecified	[82]
Unplaced (Euasterids I)						
Hydrophyllaceae						
Nemophila menziesii	nd	nm	nm	nm	Larvae of lepidoptera (2 spp.) and coleoptera (1 sp.)	[83]

F: female, M: male, nd: no statistically significant intersexual differences, nm: not measured, CT: condensed tannins, TNC: total non-structural carbohydrates, N: nitrogen content (herbivores usually attracted to greater concentrations).

Table 4: Total number of species, number of dioecious species, proportion of dioecious species, and estimated 2% of dioecious species in the top 30 most species-rich families with a proportion of dioecious species greater than 0.5 (from unpublished data from S. Renner, University of Munich).

Family	Total species	Dioecious species	Proportion of dioecious species	2% of dioecious species
Arecaceae	815	778	0.955	16
Pandanaceae	777	777	1.000	16
Lauraceae	1123	776	0.691	16
Menispermaceae	577	577	1.000	12
Ebenaceae	487	487	1.000	10
Anacardiaceae	594	439	0.739	9
Salicaceae	436	435	0.998	9
Myristicaceae	367	365	0.995	7
Clusiaceae	590	365	0.619	7
Restionaceae	387	364	0.941	7
Aquifoliaceae	400	300	0.750	6
Smilacaceae	215	205	0.953	4
Cucurbitaceae	390	197	0.505	4
Flacourtiaceae	209	192	0.919	4
Burseraceae	234	175	0.748	4
Cecropiaceae	184	174	0.946	3
Thymelaeaceae	236	119	0.504	2
Vitaceae	155	118	0.761	2
Loranthaceae	147	114	0.776	2
Meliaceae	181	105	0.580	2
Theaceae	155	94	0.606	2
Proteaceae	84	84	1.000	2
Hydrocharitaceae	123	75	0.610	2
Monimiaceae	108	74	0.685	1
Rhamnaceae	140	71	0.507	1
Nepenthaceae	70	70	1.000	1
Siparunaceae	93	68	0.731	1
Myricaceae	52	51	0.981	1
Chloranthaceae	57	51	0.895	1
Casuarinaceae	96	51	0.531	1
Total	9482	7751		155

4. Future Directions: Standardized Protocol and Broadening of Species Studied

New studies must clearly allude to the theoretical framework from which the prediction of sex-biased herbivory levels (resistance) stems—resource allocation theory, in particular, sex allocation. The claim that male-biased herbivory is expected because it has been reported as a pattern, whether implicit or explicit, lacks heuristic value because it does not address the causes of such pattern. Moreover, a plethora of factors may modify the expected pattern, as shown above.

Clearly, we need to increase the taxonomic breadth of the studies of herbivory in dioecious species. There are several ways to achieve greater taxonomic representation. We could direct our attention to those families with the greatest number of dioecious species or those with the greatest proportion of dioecious species. The first alternative will miss families with low species richness that may have a high proportion of dioecious species. The second method will miss families with high species richness but low proportion of dioecious species. One possible compromise is to focus our studies on the families with the greatest number of dioecious species among those with a large proportion of dioecious species, for instance, 50% or more (Table 4). So far, we have studied only 2% of the dioecious species in the most studied family (Salicaceae). If we took that as a target, we would have to study about 155 species for the 30 most dioecious species-rich families of angiosperms. However, by this method we would include only one species per family for many families, thus failing to achieve adequate representation of those families. In addition, we must consider that the conditions that determine sex-biased herbivory can change with habitat, and therefore some species may need to be studied in several habitats.

In addition to the taxonomic bias, there is a preponderance of studies of woody plants. While this is understandable

because most dioecious species are woody, we should strive for representation of the herbaceous component. With increased research on herbaceous dioecious species, we can address the influence of life history traits on the evolution of dioecy and defence.

Lastly, we propose that all studies aimed at assessing whether herbivory levels differ between sexes and whether these differences are a consequence of differential growth rates (in turn resulting from differential allocation to reproduction) should conduct, at least, the following measurements and observations: (1) levels of herbivory, measured as precisely as possible (preferably for more than one growing season in perennials); (2) species of herbivores responsible for most of the damage; (3) growth rates, measured either as RGR for whole individuals or from increments in branch length or leaf production; (4) reproductive allocation, measured both as the number of reproductive structures (flowers and pollen production for males, flowers, and fruits for females), and also as reproductive effort (the proportion of individual or shoot biomass allocated to reproduction, and when possible N and P allocation to reproductive structures); and finally (5) the most important resistance characters that could be influencing the levels of herbivory and measure them quantitatively. In addition, these studies could add an experimental component in which plants are damaged at least at the highest rate seen in the surveys of natural damage, so as to measure tolerance to herbivory as well as resistance [75, 102]. Ideally, damage should be performed by placing natural herbivores on the plants because mechanical damage does not necessarily elicit the same physiological responses as herbivore damage [118]. Also, these studies should consider that resistance and tolerance may vary both with ontogeny and with respect to other reproductive phenology because the acquisition and expenditure of resources vary at different stages of development and life history [119–121]. We must reiterate that other authors have emphasized the need to address several of the points outlined above. It is our hope that future studies take these recommendations seriously so that we have to assume and speculate less, and we have empirical data to further our understanding of the evolution of defence in dioecious species.

5. Conclusions

The study of the evolution of sex-biased herbivory is hampered by the notion that male-biased herbivory in dioecious species is a rule. We have shown that the evidence used to support this conclusion has important shortcomings. We have presented other possible evolutionary outcomes with regards to sex-biased herbivory in the transition from hermaphroditic populations to dioecious ones. We have also discussed how these different outcomes can be predicted under different theoretical assumptions. Therefore, future studies of herbivory in dioecious species should be based on a clear theoretical framework. In particular, we urge that all new studies of herbivory in dioecious species include assessments of reproductive allocation, growth rates, and resistance traits deemed to differ between sexes and, therefore, determine sex-biased herbivory. In addition, tolerance should also be considered as a potentially important defence mode that can vary between sexes. In this manner, we should be able to explain better the results of any given study. The advancement of our knowledge about sex-related defence in plants should help us gain a better understanding of the evolution of sex-related traits in general.

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Research Article

Noncompetitive Gametic Isolation between Sibling Species of Cricket: A Hypothesized Link between Within-Population Incompatibility and Reproductive Isolation between Species

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Postmating, prezygotic phenotypes are a common mechanism of reproductive isolation. Here, we describe the dynamics of a noncompetitive gametic isolation phenotype (namely, the ability of a male to induce a female to lay eggs) in a group of recently diverged crickets that are primarily isolated from each other by this phenotype. We not only show that heterospecific males are less able to induce females to lay eggs but that there are male by female incompatibilities in this phenotype that occur within populations. We also identify a protein in the female reproductive tract that correlates with the number of eggs that she was induced to lay. Functional genetic tests using RNAi confirm that the function of this protein is linked to egg-laying induction. Moreover, the dysfunction of this protein appears to underlie both within-population incompatibilities and between-species divergence—thus suggesting a common genetic pathway underlies both. However, this is only correlative evidence and further research is needed to assess whether or not the same mutations in the same genes underlie variation at both levels.

1. Introduction

The link between intraspecific variation and interspecific divergence has been of general interest to evolutionary biologists since Darwin (e.g., [1–3]). More recently, researchers have been asking the question, does variation within the same genetic/physiological pathways, in the same genes, and, or at the same nucleotide position underlie both intraspecific and interspecific variation in a phenotype? These are important questions whose answers can provide insights into how reproductive isolation evolves.

For example, is the evolution of reproductive isolation so idiosyncratic that within-population variation and between-species divergence in the same phenotype are the by-product of different genes or pathways, thus yielding little predictability beyond the importance of the given phenotype? Or are there really genes, or pathways, that matter and are consistently involved in phenotypic variation at all levels (e.g., [4]). These two outcomes clearly represent the ends of a continuum and research is likely to find systems scattered across the whole spectrum. However, one of the goals of evolutionary biologists is to identify general patterns and, in this case, the goal should be to identify those kinds of phenotypes where a particular answer is likely.

To begin to add a data point to this discussion for a particular phenotype, we assessed the likelihood that a single genetic pathway may underlie a postmating, prezygotic phenotype that varies both within populations and between species. In particular, we examined a case of two cricket species (*Allonemobius socius* and *A. sp. nov.* Tex) that have diverged rapidly over the past 30,000 years [5–7] and are only isolated from one another by postmating, prezygotic phenotypes, including the reduced ability of heterospecific males to induce females to lay eggs [5]. Moreover, based on preliminary data and past experiments (e.g., [8, 9]), it appears that within a population there is a significant variation in the ability of individual males to induce females to lay eggs.

Given these data, we did more extensive tests to determine the degree of within-population and between-species variation in this phenotype. Moreover, we identified and

tested (with RNAi) a protein in the female reproductive tract that is correlated with this postmating, prezygotic phenotype. We found that the ability of a male to induce a female to lay eggs does vary within populations and between species, that a chemosensory protein in the female reproductive tract is directly correlated with this phenotype, and that failure to induce a female to lay eggs results in the same dysfunction of the female chemosensory protein whether the male is conpopulation or heterospecific. Overall, these data suggest that the same genetic and physiological pathways underlie both within-population incompatibilities and reproductive isolation between species. However, this is just a hypothesis that needs further testing to determine if the same mutations and genes influence both levels of variation.

2. Materials and Methods

2.1. A Noncompetitive Gametic Isolation Phenotype between Sibling Species. For the sibling species *A. socius* and *A. sp. nov.* Tex, previous research suggested that heterospecific males are less able to induce females to lay egg relative to their conspecific counterparts [5]. To further test this finding, we conducted crosses between a series of populations of each species. Specifically, we collected individuals from three populations of *A. socius* (AR30, collected in SW Arkansas; TX30/146, collected near Mt. Vernon, Texas; TX30/198, collected near New Boston, Texas) and three populations of *A. sp. nov.* Tex (TXIII2, near Taylor, Texas; TXIII3, near Hearne, Texas; TXIV6, near Lufkin, Texas).

From these collections, juveniles were allozyme genotyped for species identification following Marshall [5] and reared to adulthood in sex-specific cages (following [10]). Virgin adults, 10–14 days posteclosion, were used in no-choice mating experiments following standard protocols for *Allonemobius* (see [9]). Females were either mated once with a conspecific or heterospecific male. In the case of the conspecific mating trials, the males were from a different population (i.e., heteropopulation). Following successful copulation, males were removed and females were allowed to oviposit for four days. The number of eggs laid by each female was counted after this four-day period. The resulting data for each cross-type were analyzed and the difference between con- and heterospecific egg laying for each population was assessed with a t-test.

In addition to the above crosses, we also conducted a set of con- and heterospecific crosses where we measured the length of time the male spermatophore (i.e., the spherical protein structure that contains the sperm and seminal fluid proteins that are passed to the female during copulation; see [11]) was attached to the female. Spermatophore attachment time in this case was estimated as copulation time plus the length of time the spermatophore was attached to the female following successful copulation [9]. These crosses used populations of *A. socius* from Georgia (GA985/22, near Cornelia, Georgia) and Missouri (Fenton, Missouri) and *A. sp. nov.* Tex from near Caddo Mills, Texas (TX30/87).

These latter crosses were conducted and analyzed as above; however, spermatophore attachment time was used as a covariant. Additionally, following the egg-laying period, all females were frozen at -80°C and their reproductive tracts subsequently checked for the presence of the ejaculate. Also, all females were checked for sterility (i.e., no/few eggs present in the abdomen and no/few eggs laid) and the occurrence of egg reabsorption (a form of physiological senescence which is indicated by eggs turning brown within the female). If a female was sterile or reabsorbing her eggs then she was removed from the analysis. This approach yielded a scenario where successful copulations resulting in limited egg laying could be ascribed to an unsuccessful male-female interaction rather than sterility or reproductive senescence. The purpose of these crosses was to determine if there is a relationship between spermatophore attachment time and the number of eggs laid by a female.

Lastly, we conducted a set of crosses using *A. sp. nov.* Tex (TX30/87) where we not only assessed the spermatophore attachment time but also the DNA concentration within the female reproductive tract following a single, successful copulation. The purpose of this experiment was to determine if there is a relationship between spermatophore attachment time and the amount of DNA in the female reproductive tract—a proxy for the amount of male ejaculate. If a positive relationship is found, then it can be assumed that longer attachment times result in a greater amount of ejaculate being transfered to the female—which may affect after copulation physiologies like egg laying.

2.2. Identification of a Protein Linked to Induction of Egg Laying. The interaction of the male ejaculate and female reproductive tract determines the success of a particular copulation and ultimately determines if after copulation physiologies are turned on. To begin to assess this male versus female dynamics, we did single, no-choice conspecific matings as outlined above using *A. socius* from GA985/22. After a successful copulation, females were given four days to lay eggs before being frozen at -80°C and their resulting laid eggs being counted. Next, the female reproductive tract (which includes the spermatheca and spermathecal duct) from each mated female was dissected out, placed in a 1.5 mL microcentrifuge tube with 20 μL water, ground with a pestle and sonicated, and centrifuged at 15,000 rpms. The resulting supernatant from each male versus female interaction sample was assessed for protein concentration with a NanoDrop. Samples from each individual (50 μg each) were run on a NuPAGE 4–12% Bis-Tris Gel with samples being arranged from the fewest eggs laid to the most eggs laid. After running and staining the gel, it was imaged and the relative abundance of particular proteins was assessed. Relative abundance was calculated by comparing the abundances of proteins of interest to a protein that was invariable across all samples. For each protein band on the gel, we determined whether or not the abundance of that protein correlated with the number of eggs laid by females. This protocol was repeated for heterospecific matings using male *A. sp. nov.* Tex from Terrell, Texas (TX20/RA).

From these gels, proteins of interest were identified with MS/MS following the protocols outlined in Marshall et al. [7, 11]. The resulting peptide data from MS/MS analyses

Table 1: Con- and heterospecific mating crosses.

Comparison	Female species	Female population	Male population								
			Conspecific mating			Heterospecific mating			Statistics		
			Male	*N*	Number of eggs laid (SD)	Male	*N*	Number of eggs laid (SD)	Percentage of reduction	*t*-test	$P_{\text{one tailed}}$
1	*A. socius*	AR30	TX198	14	116.1 (94.0)	TXIV6	19	1.4 (3.6)	98.8	5.19	<0.0001
2	*A. socius*	TX30/146	SC95/172	20	143.6 (61.6)	TXIII2	22	35.7 (53.1)	75.1	6.11	<0.0001
3	*A. socius*	TX198	TX30/146	9	70.6 (41.1)	TXIII2	28	13.5 (19.2)	80.9	5.74	<0.0001
4	*A. sp. nov.* Tex	TXIII2	TXIV6	22	142.4 (63.1)	TX198	48	58.5 (41.0)	58.9	6.66	<0.0001
5	*A. sp. nov.* Tex	TXIII3	TXIII2	25	89.5 (68.2)	TX30/146	17	43.4 (43.2)	51.5	2.47	0.0091
6	*A. sp. nov.* Tex	TXIV6	TXIII3	14	81.4 (74.8)	AR30	16	39.4 (38.0)	51.6	1.97	0.0293

were compared with our 454 EST library from the female reproductive tract of *A. socius*. For peptides that matched a sequence from our 454 library, we used BLASTp in NCBI to determine a possible identification of the proteins of interest.

2.3. A Functional Genetic Test of a Female Reproductive Tract Protein. For the one protein whose abundance was correlated with patterns of egg laying, we conducted an RNAi experiment to test the function of this protein and determine if protein knockdown resulted in a phenotype consistent with the original correlative pattern. To accomplish this, we followed established RNAi protocols for *Allonemobius* [11]. In brief, we used female *A. socius* from GA985/22 and male *A. socius* and *A. sp. nov.* Tex from GA985/22 and TX20/RA, respectively. We generated dsRNA from a PCR template (~500 bp in length) using RNA polymerase and primers with a T7 promoter (T7 region is underlined; F primer, CSP1F, TAATACGACTCACTATAGGGAGAGAGCAGGTAGACACCTTCAT; R primer, CSP1R, TAATACGACTCACTATAGGGAGAGGAGGGTGTAAAAGGCTAAT). After cleaning the dsRNA product, we injected 1μL of 1μg/μL dsRNA into the abdomen of a set of virgin females (that were <10 days posteclosion). As a control, we injected a separate set of virgin females with 1 μL of saline. For the first six days postinjection we randomly sampled females from both treatments, dissected out their reproductive tracts, and ran the resulting protein samples on a protein gel to assess protein knockdown in the dsRNA treatment. Our focus was on the protein-level knockdown, rather than transcript-level knockdown, as it is the protein-level phenotype that was correlated with patterns of egg laying.

Following confirmation of successful protein-level knockdown, we conducted mating trials with the remaining females. Specifically, we conducted single, no-choice mating trials using both sets of females with some being mated to a conspecific male and others to a heterospecific male. Following successful copulation and spermatophore transfer, each female was given four days to lay eggs before being frozen and her eggs were counted. As above described, we determined the reproductive status of each female to remove the effects of sterility and senescence. The resulting egg-laying data were analyzed for the effects of RNAi knockdown.

3. Results

3.1. A Noncompetitive Gametic Isolation Phenotype between Sibling Species. For both species, successful heterospecific copulations ($N = 150$) result in females laying significantly fewer eggs compared with conspecific (but heteropopulation; $N = 104$) copulations (Table 1). Heterospecific copulations, relative to conspecific copulations, result in an average reduction of 75% to 99% in the number of eggs laid by a female *A. socius* and a 51% to 59% average reduction for females of *A. sp. nov.* Tex (Table 1). This pattern of heterospecific males being less able to induce a female to lay eggs is also seen when we control for the length of time the spermatophore is attached to the female (Figure 1(a)). In general, conspecific and heterospecific matings appear to have similar spermatophore attachment times—a finding also found for *A. socius* and *A. fasciatus* [9].

Interestingly, the standard deviation for all cross-types was large, indicating that individual crosses could result in no to hundreds of eggs being laid (Table 1). Part of this variation can be explained by the length of time the spermatophore was attached to the female (Figure 1(a), conpopulation cross). The "normal induction" line (a term used to signify that females were induced to lay eggs) shows a significant relationship between the total attachment time of the spermatophore and the number of eggs laid per day by a female for a conpopulation cross ($r = 0.89$; $F_{1,3} = 11.53$; $P = 0.0426$; Figure 1(a)). This relationship is consistent for distant populations of *A. socius* (Figures 1(a) and 1(b); populations from Georgia and Missouri) and for both species (Figures 1(a), 1(b), and 1(c)) and is likely driven by longer spermatophore attachment times resulting in a greater ejaculate transfer (Figure 1(d)).

However, the length of time the spermatophore is attached to the female cannot explain the large variance seen within conpopulation and conspecific crosses (Table 1 and Figures 1(a), 1(b), and 1(c)). Indeed, the "reduced induction" line (a term used to specify a cross where a male does not appear to have induced a female to lay her

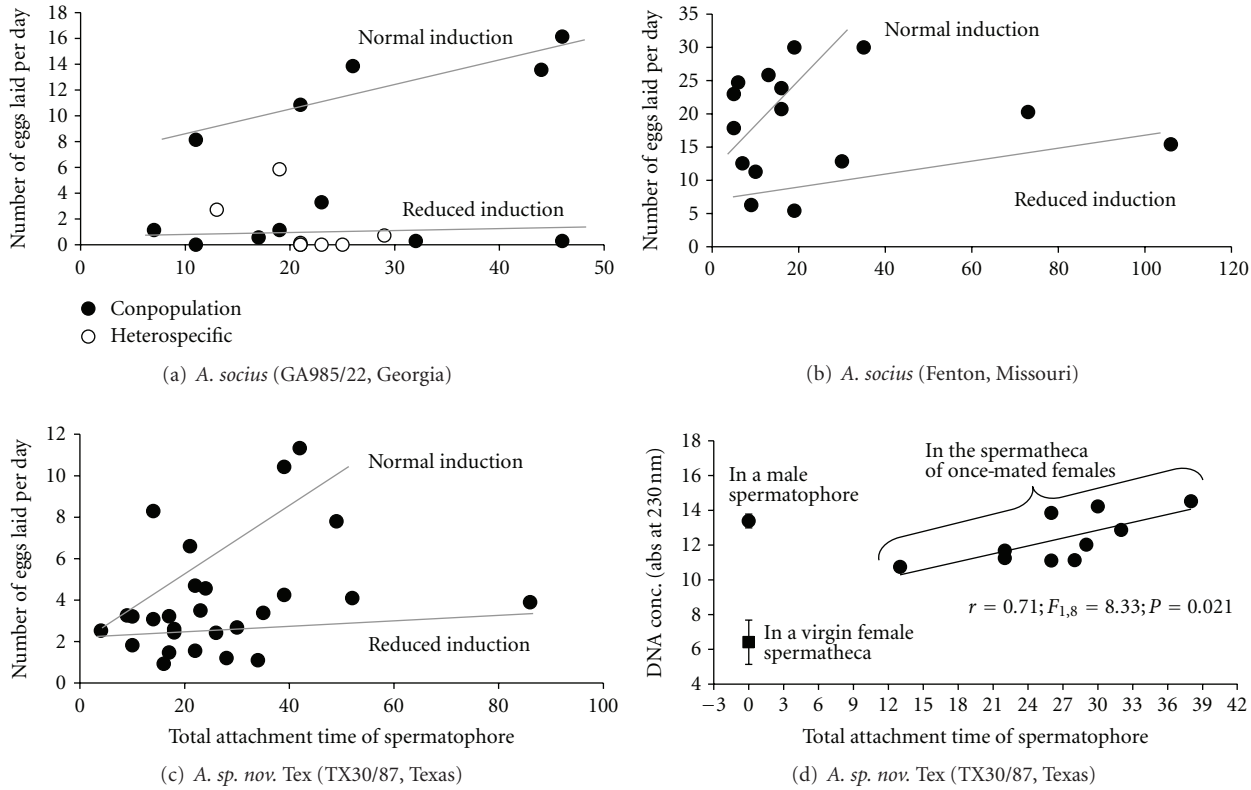
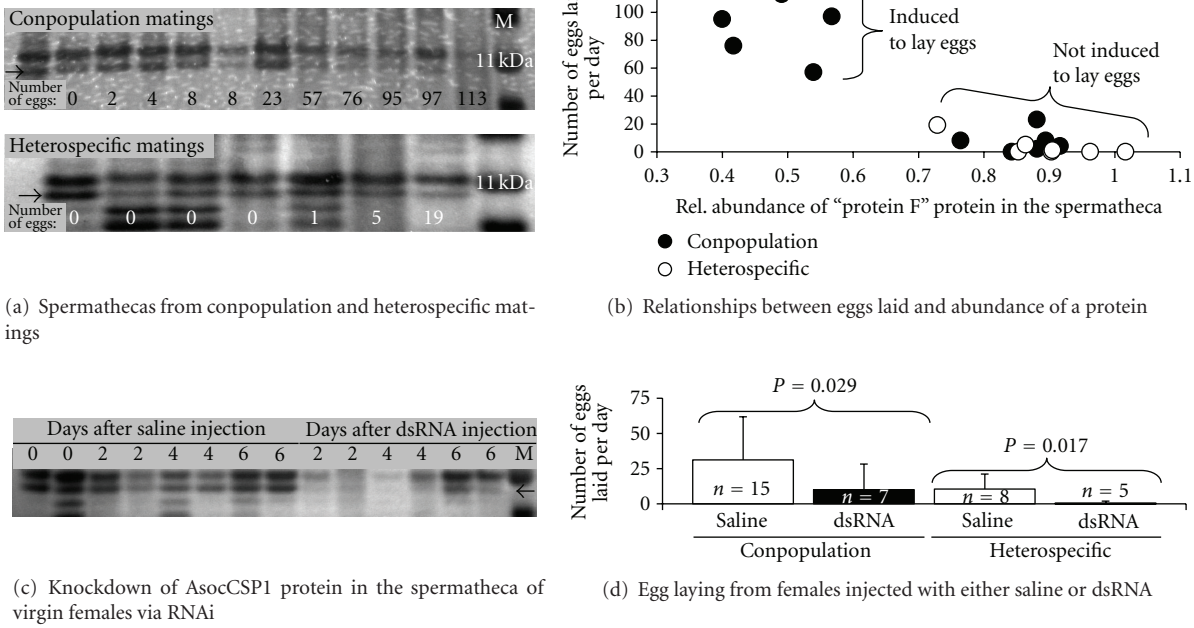


Figure 1: The relationship between spermatophore attachment time and egg laying for two populations of *A. socius* ((a) Georgia, USA; (b) Missouri, USA) and one population of *A. sp. nov.* Tex ((c) Texas). For each panel two patterns of egg laying are shown—the "normal induction" and "reduced induction" lines. Panel (d) shows the amount of ejaculate (measured by DNA concentration) that is passed to females during copulation. There are also two baseline measurements including the amount of DNA in a virgin female reproductive tract and in a male spermatophore.

normal complement of eggs despite the successful transfer of ejaculate) shows that even if the spermatophore is attached for relatively long periods of time, a female may not be induced to lay eggs (Figures 1(a), 1(b), and 1(c)). This within-species pattern occurs in both species and suggests that a male versus female interaction underlies normal- or reduced-induction of egg laying. Moreover, at the phenotypic level, patterns of egg laying from heterospecific copulations resemble those of unsuccessful (i.e., reduced induction) conpopulation copulations (Figure 1(a)).

3.2. Identification of a Protein Linked to Induction of Egg Laying. When running protein samples from the female reproductive tracts of mated females in the order of the fewest to the most eggs laid, we found that one protein (for the now called protein F) appeared to correlate with this pattern in both conspecific and heterospecific matings (Figure 2(a); see the arrow; $N = 11$). If we look at the relative abundance of protein F and patterns of egg laying, we find that when protein F is at reduced levels, females are induced to lay eggs, while the reverse is the case when protein F is at high levels (Figure 2(b)—data from Figure 2(a)). This pattern suggests that a successful copulation (i.e., one that will ultimately lead to egg laying) triggers protein F to be degraded or cross-linked or leave the female reproductive tract. Interestingly, heterospecific copulations yield patterns of protein F abundance that resemble those of conpopulation copulations where the female was not induced to lay eggs (Figure 2(b); $N = 7$). These data allow us to hypothesize that the same genetic and/or physiological pathway underlies both within-population incompatibility and reproductive isolation between species.

So, what is protein F? Using MS/MS, we identified two peptides of protein F that matched an EST in our female reproductive tract library (Table 2). We sequenced the underlying gene from cDNA derived from the female reproductive tract (NCBI accession number KC020194) and blasted the resulting sequence in NCBI and found that it matched a chemosensory protein in other insects (Table 2). Chemosensory proteins (abbreviated as CSPs) are small (~15 kDa) proteins that can reversibly bind small molecules/ligands and can be used to transport molecules from cell to cell or tissue to tissue within the body of insects [12]. Given these data, we propose to rename protein F as AsocCSP1. Interestingly, identifying this protein as a CSP suggests that the reduced abundance of this protein in the female reproductive tract following the successful induction of egg laying is likely the result of this protein leaving the female tract (and carrying the egg-laying stimulus) as opposed to being degraded or cross-linked.



(a) Spermathecas from conpopulation and heterospecific matings

(b) Relationships between eggs laid and abundance of a protein

(c) Knockdown of AsocCSP1 protein in the spermatheca of virgin females via RNAi

(d) Egg laying from females injected with either saline or dsRNA

Figure 2: Patterns of variation and an RNAi experiment on protein F. (a) The abundance of protein F in the female reproductive tract following either a conpopulation (top) or heterospecific (bottom) copulation. (b) The measured relative abundance of protein F relative to patterns of egg laying for conpopulation and heterospecific copulations. (c) A gel showing that RNAi can knock down the abundance of AsocCSP1 (a.k.a. protein F). (d) An RNAi experiment showing patterns of egg laying in conspecific and heterospecific matings.

Table 2: The identification of protein F with MS/MS and BLAST.

MS/MS Analyses				BLASTp info			
Peptides matched	Delta mass (Da)	MS/MS Ion score ($P < 0.05$*)	Hit number	Description	Organism	Accession number	E value
YDPQNLYAQAHPELFQ	−0.23	52*	1	Insect pheromone-binding family	*Anopheles gambiae*	XP_317405	$1E-11$
QPQWEQIQK	−1.23	28					
			2	Chemosensory protein precursor	*Locusta migratoria*	AAO16790	$9E-11$

3.3. A Functional Genetic Test of a Female Reproductive Tract Protein. To functionally test the correlation between AsocCSP1 abundance and egg laying in females, we conducted an RNAi experiment. Specifically, we wanted to test the prediction that if you knock down the abundance of AsocCSP1, that is, the protein that is hypothesized to carry the egg-laying stimulus out of the female reproductive tract, females would lay fewer eggs. To test this predication, we needed to knock down the abundance of the AsocCSP1 protein in the female reproductive tract. Using RNAi, we were able to successfully knock down the abundance of AsocCSP1 in the female reproductive tract of virgin females (Figure 2(c)). Given this success, we then mated both saline- and dsRNA-injected females with either a conpopulation or heterospecific male. We found that females injected with dsRNA laid significantly fewer eggs than females injected with saline when mated to a conspecific male ($P = 0.029$, Figure 2(d)). This pattern was also found when injected females were mated to heterospecific males ($P = 0.017$, Figure 2(d)). Additionally, we recovered the pattern that heterospecific males are less able to induce females to lay eggs (Figure 2(d)). Overall, this RNAi experiment supports the hypothesis that the AsocCSP1 protein is needed to successfully transmit the egg-laying induction signal from the female reproductive tract to a target elsewhere in the female's body.

4. Discussion

Our mating experiments showed that heterospecific males of both species of *Allonemobius* are less able to induce females to lay eggs and that this reduced ability is likely the by-product of a male versus female incompatibility rather than incomplete ejaculate transfer (i.e., short spermatophore attachment times). These data are consistent with previous findings between *A. socius* and *A. fasciatus* [8], suggesting that this noncompetitive gametic isolation phenotype is common in this species complex. The finding that this form of postmating, prezygotic isolation is common in this species complex is not trivial. Indeed, besides conspecific sperm

precedence, this mechanism of reproductive isolation is the only barrier to gene flow that is present in both the *A. socius-A. fasciatus* and *A. socius-A. sp. nov.* Tex contact zones. Therefore, given that this entire complex of crickets likely diverged over the past 30,000 years [5, 6], we have the opportunity to understand the evolution and genetic basis of reproductive isolation by studying this phenotype in this cricket complex.

Additionally, one of the most common postmating, prezygotic phenotypes is the ability of a male to induce a female to lay eggs (with over 29,000 publications indexed for "egg laying or oviposition" in Web of Science). Examples of this phenotype acting as a mechanism to reproductively isolate species can be found across the phylogeny of insects (e.g., green lacewings [13, 14], crickets [8, 15], walking sticks [16], beetles [17–19], wasps [20], and flies [21–23]). Therefore, our understanding of this phenotype in *Allonemobius* could shed light on a common mechanism of reproductive isolation in insects.

The importance of this postmating, prezygotic phenotype as a mechanism of reproductive isolation between species has overshadowed the occurrence of this phenotype within populations. For decades, mating experiments with these species have overlooked the fact that some matings just do not result in egg laying—writing it off as just something that happens (e.g., [24]). However, based on the more detailed analyses conducted here, it is clear that this pattern is the result of an incompatible male versus female interaction between the male ejaculate and female reproductive tract. This result is intriguing as it provides a hypothetical link between male-female incompatibilities within populations to divergence between species. In all, it was this hypothesis that prompted our search for a potential common genetic pathway.

While the same phenotype was found both within populations and between species, it is important to remember that "similar phenotypes that vary within and between species may or may not be caused by the same genetic mechanisms" [4]. In this case, our findings are consistent with a common genetic/physiological pathway underlying this phenotype at both levels. It is important to remember, though, that this is only a hypothesis as we have yet to identify the exact mutations that result in this variation. However, our working hypothesis on how this genetic pathway functions is a two-step process. Step one, is a master on-off switch that results in the female being induced to lay eggs or not. More than likely, this is a male versus female allelic interaction between genes whereby the correct interaction turns on the after copulation egg-laying switch, while a dysfunctional interaction leaves the switch in the off position or only partially turned on—as if on a dimmer switch. Such a mechanism would explain variation at both the within-population and between-species levels. However, once the egg-laying switch is flipped on, then the amount of ejaculate (or a specific ejaculate substance) influences the number of eggs a female lays (as seen in Figure 1). Therefore, this second step is a dose-dependent step in which longer spermatophore attachment times, and thus greater ejaculate transfer, can result in more eggs being laid by the female.

While our work provides justification for further testing this hypothesis, we still do not know if it is variation in the same genes (i.e., the actual male and female genes that interact) or mutations at the same nucleotide positions in those genes. Our next step is to identify the male and female genes that interact to initiate egg laying in these species and determine if mutations in the same genes and nucleotide positions account for our observed patterns. While the answers are still a few years out, our research points to a few G-protein coupled receptors in the female reproductive tract that interact with a set of peptides in the male ejaculate to initiate egg laying.

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Review Article

Sex and Speciation: Drosophila Reproductive Tract Proteins— Twenty Five Years Later

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The protein electrophoresis revolution, nearly fifty years ago, provided the first glimpse into the nature of molecular genetic variation within and between species and showed that the amount of genetic differences between newly arisen species was minimal. Twenty years later, 2D electrophoresis showed that, in contrast to general gene-enzyme variation, reproductive tract proteins were less polymorphic within species but highly diverged between species. The 2D results were interesting and revolutionary, but somewhat uninterpretable because, at the time, rapid evolution and selective sweeps were not yet part of the common vocabulary of evolutionary biologists. Since then, genomic studies of sex and reproduction-related (SRR) genes have grown rapidly into a large area of research in evolutionary biology and are shedding light on a number of phenomena. Here we review some of the major and current fields of research that have greatly contributed to our understanding of the evolutionary dynamics and importance of SRR genes and genetic systems in understanding reproductive biology and speciation.

1. Introduction

Science aims to provide simple and general explanations for natural phenomena, and all sciences must deal with the problem of heterogeneity. Variation and heterogeneity are the hallmarks of biological diversity and capture the attention of anyone interested in trying to unravel the mysteries of the biological world. Explaining biological diversity was indeed the problem for which Darwin provided a simple but revolutionary solution [1]. Variation and adaptation are the first two words that come to mind in relation to living organisms, and it was Darwin's genius that in using these two observations he was able to formulate the theory of natural selection to explain the diversity that we see reflected in the millions of different kinds of organisms or species on this planet. Given its spectacular success in providing a causal explanation for organismic change (evolution within lineages), it is equally remarkable that Darwin was unable to provide a causal mechanism of speciation (evolution between lineages). Such a causal theory had to wait nearly a century after the publication of Darwin's *Origin of Species* [1] and it materialized only after the evolutionary synthesis of the 1940s after population genetics had developed a theoretical framework [2, 3].

2. Speciation Genetics: Mendelian versus Molecular Approach

Diversity is a problem in biology in two ways: the most obvious of which is that diversity needs to be explained; the other is that it can thwart our efforts in elucidating precise and simple explanations for the complexity of biological phenomena. As a consequence, we trade precision for generality [4] and a rich theoretical base has been developed, which at first appears to ignore diversity. Population genetics is a good example as it developed a significantly elaborate theory of how genes change in evolution without prior knowledge of the material/chemical basis of the gene or of genetic variation. This is also true of speciation as well. The

allopatric theory of speciation relied on geographic isolation and differentiation of populations [3] and cumulative effects of gene differences and gene incompatibility [2, 5] without specifying anything about the nature of the genes involved. It was therefore surprising that when gel electrophoresis made its debut in scientific methods, genetic differences between closely related species turned out to be minimal [6]. Much of this had to do with the fact that the genes being investigated by molecular biologists were of the general cell metabolism category (i.e., allozymes) and had no direct relation to reproductive biology—the "crime scene" of reproductive isolation. On the other hand, the Mendelian approach to studying speciation focused on the right phenotype—hybrid dysfunction— and the breakdown in the sexual machinery of hybrids. The Mendelian approach therefore eventually became more successful in speciation research by uncovering regions of chromosomes and discrete genes with large effects that played a role in causing hybrid sterility/inviability [7].

These so-called candidate "speciation genes" provided a glimpse into the nature and the variety of genes that effect postzygotic reproductive isolation, but by the virtue of deliberately being chosen as "large effect" genes (for the ease of mapping), they may or may not represent the pool of genetic variation that is the basis of speciation. It is for this reason that another, parallel approach to investigating the nature of genetic variation affecting reproductive isolation was needed. To understand the genetic basis of reproductive isolation, a more systematic methodology was needed to screen genes and genetic variation associated with the reproductive system. A systematic genomic/proteomic approach was essential because not all genes in the reproductive system would affect reproductive isolation; many are indeed essential for development and reproductive biology. We needed to find and target the genes and proteins in the reproductive system that matter—genes with minor or large effects that are most likely involved in the early stages of species isolation.

It was indeed this realization that led us to the idea of investigating genetic variation in the reproductive tracts of *Drosophila*. The idea of using 2D electrophoresis to examine reproductive proteins seemed exciting but there was some hesitation due to the technical difficulties associated with the technique. Mike Coulthart, a graduate student at that time, was up to the task as he had what was needed: technical precision, patience, and perseverance. He investigated the levels of genetic variation and genetic differentiation, respectively, within and between sibling species of the *Drosophila melanogaster* group.

The 2D results were surprising and somewhat uninterpretable at the time. By separating over 250 protein spots from the reproductive tract and comparing them between species, Mike found little genetic variation within species but high genetic divergence between species [8–10]. Under the neutral theory we expected parity between the levels of within-species and between-species variation, which was indeed the case in the massive amount of data that had accumulated using one-dimensional gel electrophoresis [6, 11]. The 2D results were therefore interesting and revolutionary given the dominant framework of neutrality and the expected constant and slow rate of evolution. These novel data raised many interesting questions but received little attention. This was mainly because reproductive tract proteins were considered essential to the organism and therefore not expected to evolve rapidly.

The unusual nature of these results called for more investigations and research on sex and reproduction-related (SRR) molecules began. The ensuing series of experiments involving 2D electrophoresis showed that (1) nonenzymatic, abundant proteins were generally less polymorphic than enzymes; (2) reproductive tissue proteins were more diverged between species than nonreproductive tissue proteins, such as those of the brain [12]; (3) testes and ovary proteins showed higher levels of species divergence than nonreproductive proteins [13]; (4) reproductive proteins (and reproductive morphological traits) showed more gene expression breakdown in species hybrids than nonreproductive proteins [13–16]. These data and particularly Civetta and Singh's [13, 14] research, which showed that sex and reproduction-related (SRR) genes evolve faster than nonreproductive genes, unveiled the importance of studying the evolution of SRR molecules in speciation research [14, 17–20].

3. DNA Sequence Variation and Rates of Evolution

While the average rates of evolutionary change per gene may be small, genes can evolve rapidly depending upon the environmental conditions and the selection pressure. The dynamics of selection acting on each locus will determine its rate and pattern of evolution. Some groups of genes may evolve rapidly by virtue of their functions as is the case with the immune response genes in mammals [21]. Immune response genes are an example of a coevolutionary system where evolution of immunity or resistance in hosts is countered by the evolution of virulence in pathogens and/or parasites. Immune system genes and virulence genes are locked in an antagonistic coevolutionary arms race and are expected to evolve rapidly [22]. Sexual system genes provide another example of a coupled coevolutionary system, in this case involving the interactions between males and females of the same species.

3.1. Rapid Evolution of SRR Genes. Advances in molecular technology particularly DNA amplification and sequencing propelled SRR gene research and a remarkable trend of pervasive rapid SRR gene evolution emerged at several levels. Some sex determining genes, assumed to be conserved due to their important functions during early development, were shown to evolve rapidly [23]. When genes expressed in testis, ovary, and nonreproductive tissues were screened for rates of evolution it became clear that a substantial proportion of these genes evolved more rapidly than genes expressed in nonreproductive tissues [24, 25]. A divergence trend of testis > ovary > somatic genes emerged suggesting male and female SRR genes evolve under different selective pressures [26]. Microarray and computational methods

using entire tissue-specific transcriptomes showed that testis that expressed SRR genes were more likely to break down in species hybrids [27, 28]. Rapid SRR gene evolution was also found in gametes. Sperm proteins were shown to evolve rapidly and divergently in invertebrates and mammals [29–32]. Proteins in the seminal fluid of *Drosophila* were also found to be evolving rapidly and were shown to confer specific physiological and behavioural modifications in the female [33–37]. These studies not only indicate that sexual reproduction provides an opportunity for exerting constant selection pressure generation after generation but also that differences in the evolutionary dynamics of male and female reproductive systems are presumably due to intersexual selection pressure arising from male-female interaction in each generation. The relationship between rapid evolution of SRR genes and reproductive isolation is attested by the fact that the known candidate "speciation genes" are either SRR genes or autosomal genes that, via incompatible interactions with genes on the X chromosome, affect hybrid dysfunction (reviewed in [38–41]). In addition, genome-wide evidence of rapid evolution of SRR genes provided a mechanistic framework to discuss the nature of genetic changes that may occur during speciation [14, 17, 42].

3.2. Evolution of New SRR Genes. The discovery of *jingwei* [43] and *Sdic* [44] opened up investigations into the origin of new SRR genes marking yet another important step into understanding the evolutionary dynamics of genetic systems [45–48]. Novel genes arise through a variety of molecular mechanisms, including being derived from previously noncoding DNA [49] and may be important in functional diversification. What is extremely interesting is that the majority of novel genes or gene copies that have evolved novel functions have also evolved testis-specific expression. Interestingly, *Odysseus* (*OdsH*), a hybrid sterility gene, evolved as a duplicate of the neuron expressed *unc-4* gene and has taken up a testicular expression and role [50, 51]. Another example is *ms(3)K81,* a gene that evolved by duplication and retroposition from a previously ubiquitously expressed copy to acquire a male-germline-specific expression and function [52]. *ms(3)K81* is only found in the 9 species of the melanogaster subgroup and in its new functional role is crucial for zygote viability [52]. Accessory gland proteins (*Acps*) are a prime example of male-specific genes in *Drosophila* that have taken up a variety of reproduction-related functions and have important physiological effects in the female reproductive system [34–37, 53–56]. Retrotransposition is another important means of gene copying and shuffling that can be important in the evolution of new functions [45, 46]. Interestingly more genes moving from the X chromosome to autosomes have been retained (active) than genes moving in the opposite direction [7, 47, 57–61]. Again, testis-specific expression and rapid evolution appear to be common amongst retroposed genes. Thus for some yet unexplained reason, it appears that the testes act as cauldrons of retaining, if not manufacturing, new/refugee genes. In fact, it turns out that not only the evolution of new genes but also gene loss (loss of orthology) is also elevated in male-biased genes as compared to female-biased genes in *Drosophila* species [27]. The genetic machinery of the sexual system shows faster rates of turnover and it points to the role of sexual selection acting preferentially through the males (male-driven sexual selection) [62, 63]. The evolution of new genes and novel functions can be a potent driving force of reproductive isolation as exemplified by *Odysseus* (*OdsH*).

4. Evolution of Sex-Biased Genes: Role of Sexual Selection versus Selection in relation to Sex

Sexual selection, strictly speaking, is only a small part of the total selection pressure that the organism is exposed to in relation to sex. Classical theories of sexual selection apply only to those traits and genes that are influenced, directly or indirectly, by female choice. On the contrary, selection in relation to sex, or what has been called "sexual selection in the broad sense" [64], applies to all aspects of reproductive biology—from soliciting mates, courting, and mating, to production of offspring. A large proportion of the genome is involved in the development and maintenance of reproductive systems and a significant proportion of genes (~30%) in the *Drosophila* genome shows sex-biased gene expression, most of which is reproductive tissue specific [65, 66]. This raises the possibility of an unexpected level of conflict between natural and sexual selection. Current studies of sexual selection have expanded to different aspects of reproductive biology and constitute a major area of research in evolutionary biology. A few examples are discussed below.

4.1. Sexual Dimorphism and Sex-Biased Gene Expression. Sexual dimorphism is common and often dramatic amongst animal taxa. Despite being "genetically" identical (with the exception of the Y-chromosome), males and females are expected to differ in genes associated with primary sexual characteristics such as ovary, testes, and copulatory organs. Traditionally thought to be associated to few genes on the sex chromosome, it turns out that the breadth and complexity of sexual interaction between the two sexes has become so elaborate that a large number of genes controlling a variety of traits have become associated in a sex-specific manner (sex-biased and sex-enriched genes) expanding the level of sexual dimorphism [66, 67]. In the *Drosophila* genome a substantial proportion of genes show sex-biased expression [65, 66]. Male-biased genes are underrepresented on the X chromosome and female-biased genes are enriched on the X chromosome. These genes are expressed in a tissue-specific manner (e.g., somatic tissues, ovary, and testis) and they even show sex-specific elevated levels of movement between sex chromosome and autosomes [59, 60, 67–72]. In the last decade, several theories including sexual antagonism, dosage compensation, meiotic sex chromosome inactivation, and meiotic drive have been proposed to explain the paucity of male-biased gene on the X chromosome and the driving force responsible for the evolution of sex chromosomes, their gene content, and expression patterns [58, 67, 72–74]. Meiotic sex chromosome inactivation (MSCI) pioneered first by Lifschytz and Lindsley [75] and then shown at a genomic

scale [58, 72] appeared to be convincing in *Drosophila*; however, recent evidence shows otherwise and is currently under debate [74, 76–79]. While explaining the relocation and expression pattern of sex-biased genes will remain a prominent research area, it is noteworthy that, with respect to rates of evolution, genes with sex-biased expression and particularly male-biased sex genes show unusually high rates of evolution [67, 80, 81]. It is not surprising then that the sexes differ in their rates of evolution of sterility and inviability during speciation as pointed out by Haldane [82].

4.2. Evolution of Egg-Sperm Interaction. Sea urchins have traditionally been a model organism for development biology and reproductive biology but have recently received considerable attention from an evolutionary standpoint—particularly in the evolution of reproductive isolation to explain speciation in the sea [83, 84]. In most internally fertilizing animals, specific courtship behaviours mediate male and female interactions ensuring species-specific copulation and fertilization. In contrast, in externally fertilizing organisms with little or no such mating behaviours (e.g., sea urchins) gametes are shed into the sea where species-specific fertilization occurs. This requires the evolution of elaborate molecular mechanisms that ensure specific-specific fertilization. Several molecules on the surface of gametes that mediate various stages of sperm-egg interaction have been characterized [85–87]. Studies on two important proteins exemplify the evolutionary dynamics of gametic molecules in externally fertilizing marine organisms (see [83] for a recent review). Studies on the abalone sperm molecule *lysin* and its egg receptor *VERL* demonstrate the fact that male and female gametic proteins coevolve species-specific structures and affinities to maintain species-specific interactions and avoid cross-fertilization [83, 84, 88–90]. The sperm molecule *bindin* and its egg receptor are another classic example from sea urchins [83, 84, 88, 91]. While the evolutionary dynamics of the egg receptor for *bindin* remains obscure, the sperm protein *bindin* evolves rapidly and divergently in some genera but not in others [83, 84]. However, *bindin* does appear to have some involvement in reproductive isolation since its divergence correlates with the degree of gametic incompatibility between but not with time since species divergence [92]. In all likelihood, other molecules must be involved and there is a need for further characterization of such gametic and other sex and reproduction-related molecules in broadcast spawners. Once such molecules are identified, it will be interesting to correlate the evolution of egg-sperm interacting molecules to the patterns of hybrid incompatibility in these organisms. External fertilizing systems may provide a unique opportunity to assess the relative roles and genetic consequences of sexual selection and conflict in driving divergence of reproductive molecules and speciation. While research into reproductive molecules is at its inception in externally fertilizing systems, the sperm proteome of *Drosophila* has opened up exciting venues of research in reproductive biology [93–95]. As with other male-biased genes, sperm genes are underrepresented on the X chromosome and are nonrandomly clustered in the genome. While certain groups of sperm proteins, such as binding factors, do evolve rapidly, overall, the sperm proteome does not appear to be evolving fast, there is little evidence of positive selection, and there is widespread functional and structural constraint [94]. This is in stark contrast to seminal fluid proteins that evolve fast and are under selection. The contrasting evolutionary patterns of the two groups of male ejaculate proteins are interesting and are indicative of the complexity of reproductive processes, where crucial sperm-egg interacting proteins are sheltered but seminal fluids that accompany them interact with the general environment of the female reproductive tract and proteins therein are under strong selection. Future research on sperm-egg interacting proteins promises to increase our knowledge about the functional evolution of the male and female fertilization machinery and, more broadly, the evolutionary origins of sexual reproduction.

4.3. SRR Genes and the Evolution of Hybrid Sterility. Haldane's rule (of speciation) points to the preferential appearance of hybrid sterility and and/or inviability in the heterogametic sex [82]. In flies and mammals, it is the males who are affected while in moths and birds it is the females [39, 96, 97]. The genetics of hybrid sterility and inviability have been of intense focus in speciation studies and a great deal of effort has been made in mapping and characterizing genes involved in hybrid "breakdown" [38, 39, 50, 98–106]. The evolution of hybrid sterility/inviability is explained by the Bateson-Dobzhansky-Muller incompatibility model which states that incompatibility is the result of independent evolution of genes in isolated populations [7, 106]. Haerty and Singh [27] showed that the genes showing breakdown in the hybrids are preferentially sex-related genes and that these genes evolve faster both in sequence and gene expression [27, 28, 107]. In the light of this it is interesting to note that all but a handful of the so-called "speciation genes" and hybrid-sterility genes are characterized by high sequence divergence; they are often sex-related genes or somatic genes that affect the sexual system [7, 38, 40, 41, 108].

The effects of genes are prone to change in response to incorporation of new mutants and during the course of speciation earlier mutations would have fewer interactions than older mutations (cascade effect). Thus it's entirely likely that the large effect "speciation genes" may have started as small-effect minor genes and have become elaborate in their genic interactions later through species-specific adaptation and evolution. In this scenario there is no conflict between the role of minor and major genes. Thus the effect of cascade evolution is not only that speciation would occur rapidly and precipitously but also that speciation genes would evolve in their average effect from being minor to major genes. So while in reality speciation may occur in an incremental manner through a combination of many minor genes, in post-speciation genetic investigations genes would often appear as major genes. This is an interesting scenario and we must find a way to approach this problem experimentally.

4.4. SRR Genes and the Evolution of Development. SRR genes provide new opportunities to mount comparative studies of the role of selection versus developmental constraints in evolution. For example, SRR genes have provided an excellent example of testing alternative explanations for Von Baer's third law. Von Baer observed that earlier stages of ontogeny were more conserved than later stages [109]. This was later interpreted to be the result of selection against changes in earlier stages of development, which could have cascading, deleterious developmental repercussions. Darwin on the other hand explained the conservation of morphology in earlier stages as being due to lack of opportunity for natural selection to act. Since natural selection results from changes in the environment, it follows that earlier, sessile stages that have not fully developed will have little opportunity to experience variation in the environment. Darwin further pointed out that secondary/sex-specific sexual traits appear when they are needed and this can be seen in the secondary sexual traits in animals. Two recent studies [28, 107] explored the relationship between expression level over ontogeny and rates of divergence and found support for both selection against deleterious cascading effects and Darwin's hypothesis: genes expressed during early stages show reduced divergence. However, the more rapid divergence of later, adult stages, is dominated by genes expressed in adult males, which are, as noted above, presumably diverging under the effect of directional (sexual) selection. As a result of these observations, Artieri et al. [107] proposed a model of divergence involving two factors playing dominant roles during different periods of development: conservation early and opportunity late. More of such studies should shed light on the relationship between evolution and development [110–113] as well as on the broader aspects of speciation and macroevolution beyond reproductive isolation.

5. Sexual Conflict, Sexual Arms Race, and Sexual Selection

While SRR research has provided a useful, complementary approach to study speciation, there is a need to explain the evolutionary forces driving pervasive rapid evolution of male and female reproductive genes. Initially rapid SRR gene evolution invoked the role of sexual selection by female choice but recent developments on Parker's [114] original theses revived the role of sexual conflict in explaining evolutionary changes in sexual systems [115]. Sexual dimorphism in higher organisms leads to sex-specific life styles, reproductive behaviours, and investments in sexual interactions. It is expected that these differences will lead to conflicts of interest between males and females and this conflict may work as a stimulus for "retaliatory" evolutionary changes known as "sexual arms races" [115–117]. A sexual arms race would require action and reaction on the part of both partners and thus it provides a test of the role of female choice, male-driven sexual selection, and of sexual arms race theories. Increasing empirical evidence suggests that sexual conflict may be pervasive [90, 115, 118–123] but much remains to be done particularly at the level of genes to substantiate how sexual conflict and sexual selection affect male and female genetic systems differently. Genomics provides the means to explore the molecular consequences of sexual arms races and associated sexual selection theories. Intersexual interactions, be they mutualistic or antagonistic, have the potential to drive population divergence in a self-accelerating manner and this may be one of the reasons why origins of diversity and speciation are much higher in higher organisms. Evo-devo studies will also help to answer the perennial question: during evolution and speciation what comes first—reproductive isolation or adaptive radiation?

6. Conclusion

Since its inception 25 years ago, SRR gene research has rapidly evolved into a large coherent field in evolutionary biology, particularly influencing reproductive biology and speciation. The focus on SRR system studies has provided valuable mechanistic frameworks that directly relate to theories of how speciation occurs. It has emphasized the role of sexual selection in evolution, propelling research on how sexual selection and sexual conflict work at the population level. The genomics era has revolutionized SRR gene research and resulted in the characterization of rates of evolution and patterns of gene expression in reproductive transcriptomes. Rapid evolution is now commonly associated with reproductive genes but, in the future, work will be needed to understand the functions of rapidly evolving SRR genes and details of why they evolve rapidly and how their rapid evolution affects the rest of organismal biology. It will call for an integrated approach, unifying disparate fields of science, particularly biochemistry, genetics, ecology, and molecular biology. A key issue will be to explore the relationship between changes in gene sequence, gene expression, protein syntheses, and protein function in reproductive systems. Fundamental behavioural and ecological studies will be essential in explaining the nature of molecular changes associated with the reproductive systems. Selection in relation to sex, encompassing sexual selection in the strict sense, and in the broad sense is a large and growing area of research in evolutionary biology. Investigating the molecular consequences of sexual interaction and their role in speciation stands to open one of the most important areas of research bearing on the biology of sexual reproduction.

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Research Article

Drosophila melanogaster Selection for Survival of *Bacillus cereus* Infection: Life History Trait Indirect Responses

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To study evolved resistance/tolerance in an insect model, we carried out an experimental evolution study using *D. melanogaster* and the opportunistic pathogen *B. cereus* as the agent of selection. The selected lines evolved a 3.0- to 3.3-log increase in the concentration of spores required for 50% mortality after 18–24 generations of selection. In the absence of any treatment, selected lines evolved an increase in egg production and delayed development time. The latter response could be interpreted as a cost of evolution. Alternatively, delayed development might have been a target of selection resulting in increased adult fat body function including production of antimicrobial peptides, and, incidentally, yolk production for oocytes and eggs. When treated with autoclaved spores, the egg production difference between selected and control lines was abolished, and this response was consistent with the hypothesis of a cost of an induced immune response. Treatment with autoclaved spores also reduced life span in some cases and elicited early-age mortality in the selected and wound-control lines both of which were consistent with the hypothesis of a cost associated with induction of immune responses. In general, assays on egg production yielded key outcomes including the negative effect of autoclaved spores on egg production.

1. Introduction

Genetic selection in the laboratory provides a powerful tool for evolutionary analysis of complex traits [1]. It has been used to study many phenomena at different levels of biological organization including life histories, physiology, demography and population dynamics, behavior, form, sex, whole-genome evolution, altruism, and speciation [2]. Selection results in amplification of genetic differences between selected and control lines which is the basis of phenotypic differentiation. Often, correlated (indirect) responses to selection are of particular interest in these experiments as they can suggest tradeoffs between traits. For example, selection for increased *D. melanogaster* life span and late-age reproduction resulted in decreased early-age reproduction [3, 4]. The nature of tradeoffs between traits is an important topic in life history evolution [5].

In the present study, the insect model *D. melanogaster* has been used in selection experiments for increased survival after bacterial infection. A previous study of responses in a laboratory selection experiment using *Pseudomonas aeruginosa* has examined the impact of *D. melanogaster* resistance on life history traits [6]. This study showed considerable costs in life span and larval survival as correlated responses to selection. While fly survival increased from 15% to 70% within ten generations in the selected lines, adult and larval viability decreased markedly relative to the control lines. In this selection experiment, microarray data indicated that a greater number of cellular immunity genes changed expression in the selected lines compared to the number of humoral immunity genes that changed, suggesting the relative importance of cellular immunity for resistance to *P. aeruginosa*.

A series of laboratory selection studies has also been conducted in which *Drosophila* evolved resistance against parasitoids [7, 8]. After 5 generations of selection the encapsulation frequency, important against parasitoids, increased

from 5% to 60% in response to *A. tabida* and 0.5% to 45% in response to *L. boulardi*. Increased resistance to parasitoids was accompanied by a correlated evolutionary response in a number of traits, including doubling of the number of circulating haemocytes, smaller adult size, lower fecundity, reduced larval competitive ability, and increased pupal susceptibility [9, 10].

One area of tremendous interest is the selection response of host organisms to infectious or zoonotic diseases. These diseases have significant impact on human and animal health, and understanding the evolutionary underpinnings of responses in humans may provide keys to alternative methods of prevention or intervention [11–16].

To further understand the evolutionary implications of infectious disease resistance, and the potential for novel interventions, we have exploited *D. melanogaster* to study the effects of selection for resistance/tolerance to a spore-forming bacterial species (*Bacillus cereus*) which is closely related to the pathogenic spore-forming bioterrorism agent *Bacillus anthracis*. Because the spore is the most frequently encountered form of this organism (natural or otherwise), we used the spore form as a basis for selection. A strong response to selection for resistance to infection by spores was obtained, observed by a 3.3-log change in the number of spores required for approximately 50% mortality, within 24 generations. Here, we now demonstrate that life history traits were also affected as a consequence of selection and introduction of autoclaved spores. In wound-control and selected lines, exposure to autoclaved spores decreased life span. Selection was strongly positively correlated with egg production. When treated with autoclaved (dead) spores, the large difference between selected and control lines was abolished suggesting a cost of activating an immune response. There was a difference between untreated selected and control lines in progeny development time; the selected line progeny developed relatively slowly. After a series of matings were conducted to separate female and male effects, it was documented that exposure of selected and wound-control line males to autoclaved (dead) spores resulted in relatively rapid progeny development time. Finally, the selected and wound-control lines evolved heightened early-age mortality in response to the autoclaved spore treatment, which was interpreted as being consistent with the hypothesis of a cost associated with inducing an immune response.

2. Materials and Methods

2.1. Fly Populations. The procedure for establishing the base population as well as subpopulations used for selected and control lines in the present study was described in Schwasinger-Schmidt et al. [17]. Briefly, a large base population was maintained at approximately 10,000 individuals in an overlapping generation regime for approximately two years before being subdivided into 9 subpopulations (lines) for 5 generations in a similar population maintenance regime to that designed for the selected and control lines. Each rearing vial was seeded with 100 eggs to standardize density during the 5 discrete generations before the initiation of the selection experiment. In this artificial laboratory selection experiment, there were 9 lines which were separate outbred populations that evolved independently: selected lines, wound-control lines (punctured only with sterile H_2O), and no-treatment lines. There were three replicates of each line type; replicates lines were independent populations that were subject to essentially the same conditions. The different sets of lines (selected, wound-control, and no perturbation) are referred to as "line types" in the present study. In *D. melanogaster* experimental evolution studies, "lines" are commonly used as a term to describe selected and control populations (e.g., see Rose 1984 and many subsequent *Drosophila* laboratory selection experiments).

2.2. Selection and Control Lines. Selection was conducted in a specific manner. *B. cereus* spores were used for selection. Spores were introduced into adults of the selected lines using a tungsten needle dipped into spores suspended in H_2O. A concentration of spores that killed approximately 50% of the females and males was determined before the first generation of selection (2×10^6 per mL). Every generation of selection the goal was to attain approximately 50% mortality after introduction of live spores. Each generation of selection, one thousand virgin females and the same number of virgin males were infected for each of three selected lines. After three days at room temperature, the number of survivors was determined for each selected line (S1, S2, and S3). Typically, there were 500 surviving females and 500 males per line. Surviving males and females within each line were counted and randomly placed in bottles to mate at a density of approximately 80 flies per bottles. Flies were kept in bottles for 24 hours to mate and lay eggs. The next day, eggs were collected from the bottles to initiate the following generation. Approximately, one hundred eggs were collected for each vial used to propagate the next generation. The vials with eggs were placed in 18°C for development. A temporal synopsis of the selected and control lines, and treatments, is presented in Figure 1.

There were two types of control lines. For the wound-control lines (CP for control punctured), females and males were punctured with sterile water without spores. Another set of control lines (CN) was used, and, in this case, there was no perturbation (no infection, no puncture).

The number of breeders used for each generation was the same for each set of matched lines of each type (selected, wound-control, and no perturbation). For example, the number of survivors after selection in selected line S2 was matched to the numbers of the control-punctured line CP2 and the no-perturbation line CN2. There were approximately 1000 flies (500 females and 500 males) used as breeders for each line each generation.

2.3. Treatments. All flies used from all lines were subjected to one of three treatments prior to life history assays. The treatments were similar, or the same, as used for selection and in the control lines (Figure 1). The autoclaved spore treatment (AS) was designed to induce an immune response with dead spores; it was analogous to administration of live spores. Live spores could not be used before assays at the level used for selection as they would cause excessive mortality.

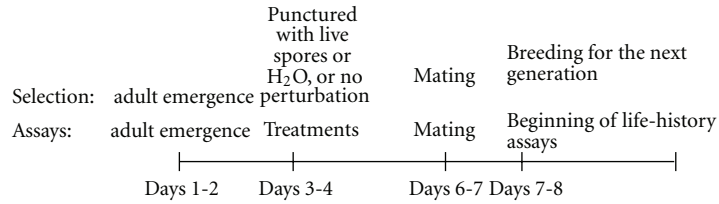

Figure 1: Timeline of the selection experiment and life-history assays. The life-history assays were designed to be conducted in parallel with the process used for the selected and control lines. Days were recorded as days posteclosion.

The treatment with sterile H_2O was the same as used for the wound-control lines. Also, no treatment (NON) was the same condition as used for the no perturbation control lines.

2.4. Bacterial Culture and Spore Isolation. *Bacillus cereus* ATCC 10987 was used for spore purification using a step gradient of Renografin [18]. A single colony of *B. cereus* was inoculated in 25 mL of Difco Sporulation Medium (DSM) and incubated at 37°C on a rotary shaker (150 rpm) until mid-log phase. Inoculation of this culture into 2 L of DSM generated a 1 : 10 dilution which was followed by incubation at 37°C on the rotary shaker for 48 hours. The culture was centrifuged for 10 min at 10,000 ×g, and the supernatant gently discarded. The pellet was resuspended in 200 mL of 4°C sterile water followed by the same centrifugation procedure. The pellet was again resuspended in 200 mL of sterile water and stored overnight at 4°C. After repeating the centrifuge-resuspension-centrifuge procedure, greater than 90% of bright-field spores were observed under phase contrast microscopy. The pellet was resuspended in 20% Renografin and the suspension transferred to a 30 mL glass core tube with 15 mL of 50% Renografin. The spore suspension was centrifuged for 30 min at 4°C at 10,000 ×g. All layers containing vegetative cells were removed and the spore pellet retained. The pellet was resuspended in 10 mL of 4°C sterile water in an Oak Ridge tube. The spore suspension was centrifuged for 10 min at 10,000 ×g at 4°C. Trace amounts of Renografin were removed, by three washes with 4°C sterile water as described immediately above. The spore pellet was suspended in 2 mL of 4°C sterile water. The concentration of spores was determined by serial dilution and spread-plating.

Spores were isolated twice during the portion of the selection experiment presented in this study. They were isolated the first time for selection generations 1–11. Also, they were isolated a second time for selection generations 12–24. Each preparation had very similar effects on mortality.

2.5. Life History Assays. Conditions were standardized prior to life history assays. There was no selection for two generations prior to life history assays to minimize the impact of any effects that carry over from generation to generation such as maternal effects. There were nine lines and each was subject to three conditions prior to a life history assay (see Treatments section). Life span and egg production assays were conducted on flies two generations after relaxing selection at generation 18. Development time was conducted on flies derived from selection generation 24 after two generations of relaxed selection.

The age of flies assayed for life histories was designed to conform to the conditions of the selection experiment (Figure 1). The start of the life-history assays corresponded to the age of the flies used for the start of selection which was 7–9 days old (days posteclosion). Virgin flies (days 1-2) were collected from the breeding vials. At 3–5 days of adult life, the virgin flies were treated (punctured with autoclaved spores, punctured with water, and untreated). After three days, the level of survival was tabulated. Then, males and females were combined and allowed to mate and lay eggs for 24 hours before life-history assays were initiated.

2.6. Life Span. Life span, and all other life-history assays, were conducted with populations of flies held under standard conditions (25°C, 12 : 12 L : D). The cages used for the life span assay were made out of quart-size plastic containers. The lid had mesh inserted for ventilation. There was a grommet in the side of each container with a tube allowing for replacement of used food vials with fresh food vials every three days. A rubber patch was sewn into the opposite side of each container, and it had a slash in it to allow insertion of a Pasteur pipette. The pipette was used to aspirate dead flies from the bottom of the cage allowing them to be removed and recorded every three days. Each cage initially received 30 flies of the same sex that had been allowed to mate for 24 hours prior to the assay. There were four replicate cages for each sex and treatment for all lines. The cages were monitored until all flies were dead.

2.7. Egg and Progeny Production. Egg production was recorded for all lines and treatment combinations. Twenty mated females (males were discarded after the 24-hour mating period) from each line and treatment were placed individually in vials at 25°C. Females were transferred to new vials each day for 49 days at which time almost all of the eggs were produced.

For determination of adult progeny numbers, after eggs were counted, the replicate vials for all lines and treatments were placed at 18°C. Emergent progeny was counted from all vials until all adults emerged.

2.8. Progeny Development Time. The design for development time was more complicated than for life span or fecundity as additional combinations of matings were used. For each line, five different F_1 crosses were employed to parse out

Table 1: Mating design for the progeny development time assay.

	Mating for progeny development time
One sex treated with autoclaved spores	A treated females × untreated males
	B treated males × untreated females
One sex types treated with sterile H_2O	C treated females × untreated males
	D treated males × untreated females
No treatment	E untreated females × untreated males

All line types (selected—S, puncture control—CP, and no perturbation—CN) were used for crosses A, B, C, D, and E. The average emergence (eclosion) time of progeny was determined for each cross (A–E).

male and female treatment effects on progeny development time (Table 1). For example, females treated with autoclaved spores were mated to untreated males which allows for assessment of the effect of female treatment on progeny development time. The reciprocal mating allows for assessment of the effect of the autoclaved spore treatment on males with progeny development time as the outcome. These were the first two matings. Similarly, the effect of puncturing females or males with sterile H_2O was evaluated by reciprocal crosses with progeny development time as the outcome. These were the third and fourth matings. The fifth mating allowed for assessment of progeny development time when neither male nor female was treated. There were six replicates of each of the five different F_1 matings. The five matings were used for all lines to investigate progeny development time. Approximately 100 eggs were collected from each cross after the 24-hour mating period and placed in each vial to control larval density. All of the vials (matings, lines) were randomized with respect to the order that matings were initiated. The time of first emergence of adults was t_0 for the progeny development time assay. The counting period of emerged progeny continued well beyond the time when no additional adults eclosed.

2.9. Survival after Administration of Autoclaved Spores. There was a three-day period after treatments, when survival was monitored before the beginning of life-history assays. In Figure 1, this period is shown as occurring after the administration of treatments and before mating (posteclosion ages 3-4 to 6-7). Mortality during this period was considered separately from life span or other life-history assays. This data was tabulated and statistically analyzed.

2.10. Statistical Analysis. The data was analyzed by ANOVA using SAS version 9.3 (SAS, 2009). The data was treated as continuous. A mixed model analysis of variance was used with line types and treatments as fixed effects. Random effects were derived from variation among the three replicate lines of the same type. Variation among lines of the same type was nested within fixed effects for the analysis. For each sex, all lines and treatments were analyzed with one ANOVA for every life history trait. Any significant, or nonsignificant, interaction terms were derived from an analysis using the full model. Residuals were examined by QQ-plots and histograms in order to detect deviations from normality.

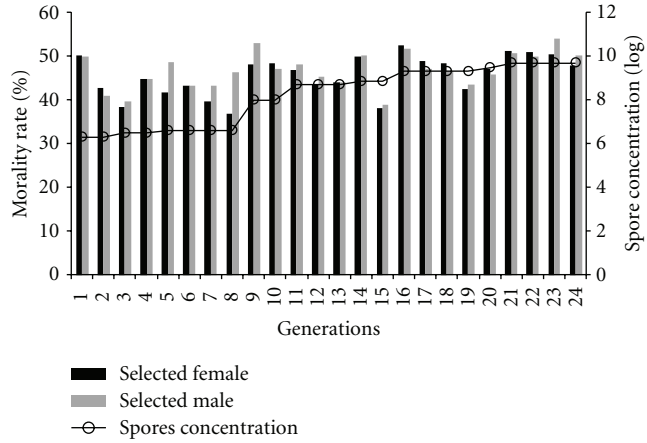

Figure 2: Direct response to selection for survival after *B. cereus* live spore infection. After 24 generations of approximately 50% mortality of females and males, the concentration of spores required for this level of mortality increased by 3.3log.

For life span, the average survival time, median survival time, first quartile survival (25% mortality), and time to third quartile survival (75% mortality) of females or males were used for statistical analysis. For development time, the average progeny eclosion time was used for statistical analysis. Total egg and progeny numbers were used for statistical analysis of reproduction. Any statistically significant interactions between treatments and lines were explicitly described in the present study in terms of the pattern of the data.

3. Results

3.1. Statistical Analyses. The statistical analysis was conducted using mixed model ANOVAs. The degrees of freedom and F values are presented in supplemental tables (see S1a—life span, S1b—egg and progeny production, and S1c—progeny developmental time in Supplementary material available online at doi:10.1155/2012/935970). Examination of QQ-plots and histograms for all of the data indicated no major deviations from normality.

3.2. Direct Response to Selection. In order to exert steady selective pressure across multiple generations, the spore concentration used for selection at each generation was increased to attain 50% mortality. As shown in Figure 2, this

approach led to a steady incremental response to selection over 24 generations, producing a 3.3-log increase in spore concentration necessary for 50% mortality. The response to selection was almost log-linear. In some cases, there was an increase in survival in the next generation after relatively strong selection in the previous generations (generations 8 to 9 and generations 15 to 16). This might have resulted from selecting to greater degree in generations 8 and 15, hypothetically resulting in a genetically more resistance subset of the population, which could have responded in the next generation by elevated survival in generations 9 and 16, respectively.

For the S1 selected line, a late generation of selection (generation 36) was tested for resistance by introducing the spore concentration (2×10^6 per mL) used for the first generation of selection into 100 females and 100 males. There was no mortality after the standard three-day observation period. This result complements the observation of an incremental response over 24 generations and provides further evidence of a direct response to selection.

3.3. Indirect Responses to Selection. The *P* values for all of the line types (selected, wound-control, and no perturbation) and treatments (autoclaved spores introduction by puncturing, punctured with H_2O, and no treatment) are presented in Table 1.

For reporting results in the following text, there typically are separate subheadings for untreated samples and samples treated with autoclaved spores. However, the sterile H_2O treatment was sometimes also included. Untreated lines represent the consequences of evolution, whereas lines treated with autoclaved spores hypothetically represent the consequences of eliciting an induced immune response. In the parlance of McKean and Lazaro [19], untreated lines are analogous to "standing defense," and autoclaved spore-treated lines were designed to be analogous to "deployment."

3.4. Life Span

3.4.1. Untreated Lines. The average percent survival (life span) of untreated selected and control-line females was determined. Overall, there were no line effects for females or for males. The survival curves for both sexes when untreated are presented in supplemental figures (S2a and b).

3.4.2. Autoclaved Spore—Treated Lines. The average percent survival (life span) of the autoclaved spore treatment applied to selected and control females is presented in Figure 3(a). The average percent survival (life span) of the autoclaved spore treatment applied to selected and control males is presented in Figure 3(b). The average survival time after treatments is presented in Table 2(a) (females) and Table 2(b) (males). Overall, there was a statistically significant effect of treatments on females ($P < 0.0001$) and males ($P = 0.0003$). Treatment with autoclaved spores reduced mean female life span in the selected lines (27.65 days) and wound-control lines (27.92 days) compared to the no-perturbation lines (31.35 days). The decrease in selected female life span was approximately 10%. Autoclaved spores reduced mean

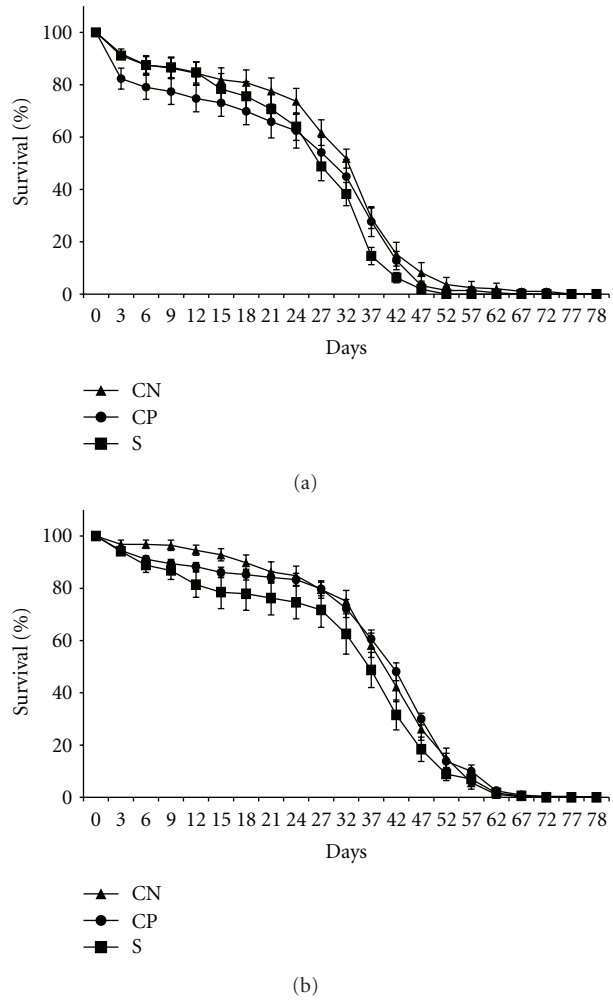

Figure 3: (a) Average percentage survival of adult females from the selected and control lines treated with autoclaved spores. The mean was determined from the replicate lines of the same type: S—selected lines, CP—lines punctured with H_2O (wound control), and CN—no perturbation lines. (b) Average percentage survival of adult males from the selected and control lines treated with autoclaved spores. The mean was determined from the replicate lines of the same type: S—selected lines, CP—lines punctured with H_2O (wound control), and CN—no perturbation lines.

male survival in the selected lines (35.17 days), compared to wound-control (40.10 days) and no-perturbation (40.50) lines. Autoclaved spores decreased the survival of selected males by 14.5%. In neither females nor males was there a statistically significant interaction between treatments and line types. In general, the administration of autoclaved spores reduced life span in the wound-control lines and for selected line males. This observation is consistent with the hypothesis of the cost of an induced immune response (deployment).

3.5. Egg and Progeny Production

3.5.1. Untreated Lines—Egg Production. Total average egg production number is shown in Table 3 for lines and per female per day in Figure 4(a). Overall, for total egg production, there were statistically significant line ($P = 0.0215$),

Table 2: *P* values from comparisons (average survival of females, average survival of males, total egg production, total progeny production, and average progeny development time) of treatments within lines of the same type and comparisons of line types for each treatment.

Overall effects	Lines/treatments	Comparisons	*P* value of female life span	*P* value of male life span	*P* value of egg production	*P* value of progeny production	*P* value of progeny development time
Lines			0.4444	0.5330	0.0215	0.0961	0.1457
Treatments			<0.0001	0.0003	0.0039	0.0005	0.0051
Lines* treatments			0.1027	0.1603	0.0280	0.1535	0.1790
	Lines						
	No perturbation control (CN)	Treatment[a]	0.1969	0.6687	0.4517	0.0022	0.8001
	Control punctured (CP)	Treatment[a]	<0.0001	0.0027	0.3270	0.1776	0.1773
	Selected (S)	Treatment[a]	0.4262	0.0019	0.0001	0.0354	0.0012
	Treatments						
	AS	Lines[b]	0.6967	0.1663	0.6729	0.0462	
	H_2O	Lines[b]	0.2074	0.2502	0.1727	0.0288	
	NON	Lines[b]	0.2717	0.8843	<0.0001	0.9560	
	A[c]	Lines[b]					0.0088
	B[c]	Lines[b]					0.8619
	C[c]	Lines[b]					0.3675
	D[c]	Lines[b]					0.4857
	E[c]	Lines[b]					0.0098

[a]Treatments: punctured with autoclaved spores (AS), punctured with sterile water (H_2O), and no treatment (NON).
[b]Line types: selected (S), punctured with H_2O each generation (CP), and no perturbation each generation (CN).
[c]A–E were F_1 matings for the progeny development time assay (see Table 1).

Table 3: Average survival time of female and male flies, average total number of eggs, and average progeny production: lines and treatments.

Lines	Treatments	Mean (S.E.) female survival	Mean (S.E.) male survival	Mean (S.E.) egg number	Mean (S.E.) progeny number
No perturbation control (CN)	Autoclaved spores	31.35 (1.84)	40.50 (1.63)	577 (45.43)	185 (18.7)
No perturbation control (CN)	H_2O	35.37 (0.84)	42.88 (0.64)	639 (47.48)	274 (17.6)
No perturbation control (CN)	NON	35.65 (0.84)	42.22 (1.64)	561 (37.49)	214 (18.1)
Control punctured (CP)	Autoclaved spores	27.92 (2.16)	40.10 (1.19)	636 (46.17)	225 (18.9)
Control punctured (CP)	H_2O	37.17 (1.24)	47.65 (1.62)	699 (38.21)	262 (17.3)
Control punctured (CP)	NON	38.20 (1.24)	40.65 (1.83)	737 (50.09)	219 (17.5)
Selected (S)	Autoclaved spores	27.65 (1.68)	35.17 (2.83)	588 (55.27)	161 (17.5)
Selected (S)	H_2O	29.37 (3.51)	43.44 (1.93)	763 (45.48)	210 (18.0)
Selected (S)	NON	30.64 (3.10)	41.57 (1.86)	874 (55.66)	222 (17.3)

Treatments: punctured with autoclaved spores (AS), punctured with sterile water (H_2O), and no treatment (NON).
Line types: selected (S), punctured with H_2O each generation (CP), and no perturbation each generation (CN).

treatment ($P = 0.0039$), and line by treatment interaction ($P = 0.0280$) effects (Table 2). A major difference between line types was observed when there was no treatment ($P < 0.0001$). Selected lines produced a markedly high number of eggs (874), wound-control lines were intermediate (737), and no-perturbation lines produced the lowest number eggs (561) (Table 3). The selected line produced 19% more eggs than the wound-control lines and 56% more eggs than the no-perturbation lines. The wound-control lines produced 31% more eggs than the no-perturbation lines.

3.5.2. Autoclaved Spore Treatment—Egg Production. Egg production for autoclaved spore-treated females (and males) for all line types is presented in Table 3 and Figure 4(b). The effect of treatment with autoclaved spores was to markedly reduce average egg production in the selected lines (588 eggs) and in the wound-control lines (636 eggs) (Table 3). The effect of treatment was statistically significant for the selected lines ($P = 0.0001$), but not the wound-control lines. There was no reduction of average total egg production in the no-perturbation control lines as a result of treatment

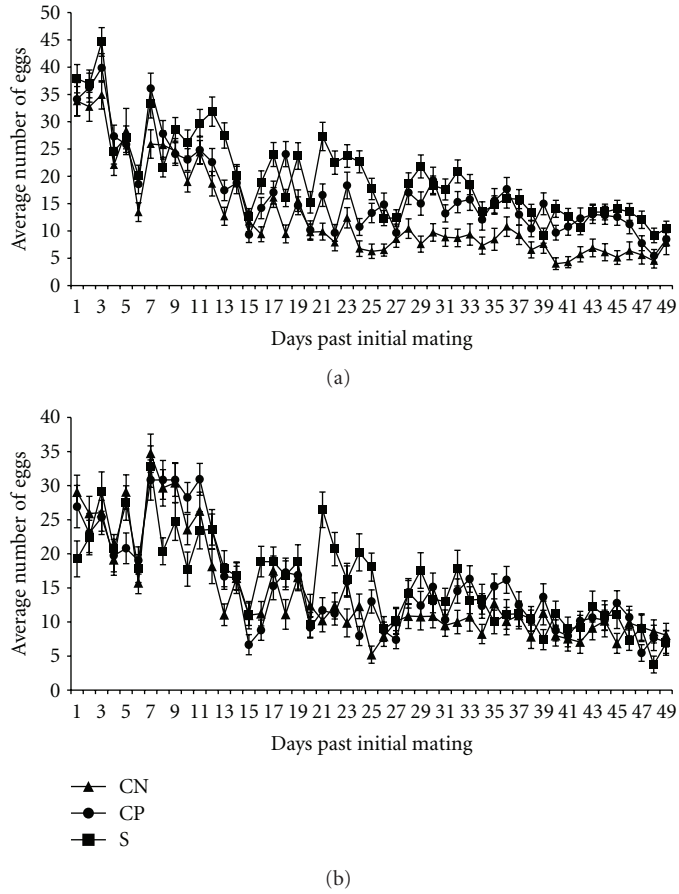

Figure 4: (a) Average daily egg production among line types when there was no treatment of adult females and males. The mean was determined from the replicate lines of the same type: S—selected lines, CP—lines punctured with H_2O, and CN—no perturbation lines. (b) Average daily egg production among lines when adult females and males from the same line were treated with autoclaved spores. The mean was determined from the replicate lines of the same type: S—selected lines, CP—lines punctured with H_2O, and CN—no perturbation lines.

with autoclaved spores (Table 3). The statistically significant interaction resulted from the decrease in egg production in the selected and wound-control lines, but not the no-perturbation lines. This reduction in total egg production after treatment with autoclaved spores, especially acute in the selected lines, is consistent with the hypothesis of a cost associated with induction of an immune response (deployment).

3.5.3. Untreated Lines—Progeny Production. Table 3 presents the progeny number for lines and treatments. There were only marginal statistically significant differences in progeny production among line types ($P = 0.0961$, Table 2). In dramatic contrast to egg production, the selected lines did not produce significantly more progeny (Table 2). Progeny production is dependent on the number of sperm transferred to females and stored after the 24-hour mating period, and this did not differ appreciably among line types.

3.5.4. Autoclaved Spore and Sterile H_2O Treatments—Progeny Production. Table 3 presents the progeny numbers after treatment with autoclaved spores or sterile H_2O. The treatment effects were statistically significant overall ($P = 0.0005$, Table 2). Autoclaved spore treatment resulted in the fewest number of progeny (CN and S) and punctured with sterile H_2O resulted in the greatest number (CN and CP) (Table 3). There were no statistically significant interactions between line types and treatments.

3.6. Progeny Development Time. Progeny development time represents egg to adult emergence time. As described in the materials and methods and Table 4, four different crosses allowed us to separate the progeny development time effects of treatment (autoclaved spores or sterile H_2O) on progenitor females or males. The fifth mating allowed us to evaluate an absence of treatments (mating pattern E in Table 4). All adults from all lines were subjected to each of the five crosses (A–E in Table 4).

3.6.1. Untreated Adult Females and Males. Figure 5 presents the cumulative percentage progeny emergence per time period for all lines types when there was no adult fly treatment. The progeny development time for selected line flies was slowest (Table 4). Statistically significant effects on progeny development were observed when there was no adult treatment ($P = 0.0098$, Table 2). This observation is

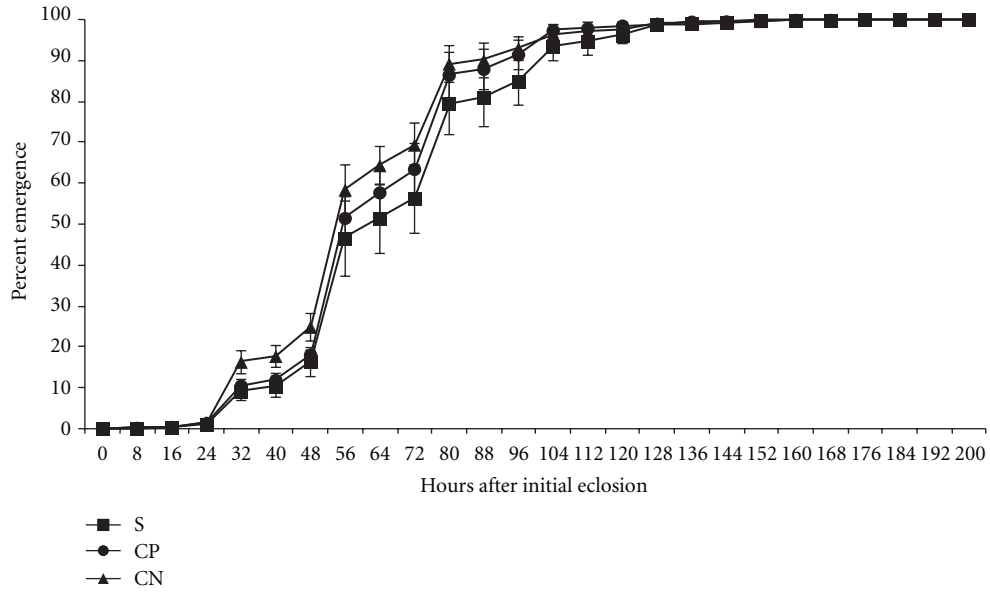

Figure 5: Average adult progeny emergence (eclosion) for line types when females and males were untreated. Line types: S—selected lines, CP—lines punctured with H_2O each generation, and CN—no perturbation lines.

Table 4: Average progeny emergence (eclosion) time from each of the following crosses applied to each line type.

Lines	Treatments	Mean (hours)	S.E.
No perturbation control (CN)	A	63.78	2.08
No perturbation control (CN)	B	63.17	2.78
No perturbation control (CN)	C	65.31	2.82
No perturbation control (CN)	D	64.14	2.58
No perturbation control (CN)	E	62.96	2.02
Control punctured (CP)	A	67.63	3.22
Control punctured (CP)	B	64.53	2.43
Control punctured (CP)	C	68.34	2.74
Control punctured (CP)	D	64.20	2.67
Control punctured (CP)	E	66.32	2.08
Selected (S)	A	71.60	3.61
Selected (S)	B	63.75	3.21
Selected (S)	C	68.45	3.50
Selected (S)	D	66.80	2.84
Selected (S)	E	70.68	3.08

Treatments: females punctured with autoclaved spores and mated with untreated males (A), males punctured with autoclaved spores and mated with untreated females (B), females punctured with sterile water and mated with untreated males (C), males punctured with sterile water and mated with untreated females (D), and untreated females mated with untreated males (E).

Line types: selected (S), punctured with H_2O each generation (CP), no perturbation each generation (CN).

consistent with the hypothesis of an evolved cost (standing defense) which is manifest as delayed progeny development time. However, an alternate hypothesis is presented in the discussion.

3.6.2. Treatment of Adult Females and Males. There was a statistically significant sex-dependent effect of adult treatment on progeny development time ($P = 0.0051$, Table 2). When females were treated with autoclaved spores, or sterile H_2O, progeny development time was similar to that observed when neither sex was treated (Figures 6(a) and 6(b)). The rank order of progeny development time was selected lines slowest, wound-control lines intermediate, and no-perturbation lines fastest. The grand mean value for male treatments (autoclaved spores and sterile H_2O, treatments B and D in Table 4) was 64.4. This value was similar to 66.7 which was the mean for untreated females and males (mating E in Table 4). Thus, when males were treated, the adult progeny emergence time dropped below the level of the untreated lines (Figures 6(a) and 6(b)). This male effect was more pronounced in the selected lines ($P = 0.0012$), again emphasizing the impact of evolution for resistance in these lines.

The number of adult progeny emerging was tabulated for each population and treatment to evaluate if there was a density effect on progeny development time. Comparing the number of progeny from treated males (treatments B and D) with treated females (treatments A and C), there was a 0.9% difference. The effect of treated males on progeny development time was not due to larval density as inferred from the number of adult progeny that eclosed.

3.7. Three-Day Survival after Administration of Autoclaved Spores. The selected lines and wound-control lines evolved an increase in mortality in the three-day period after autoclaved spores were introduced (Figure 7). Importantly, this three-day period occurred before the start of life-history assays (Figure 1). The no-perturbation control lines exhibited 2.11% mortality after introduction of the autoclaved

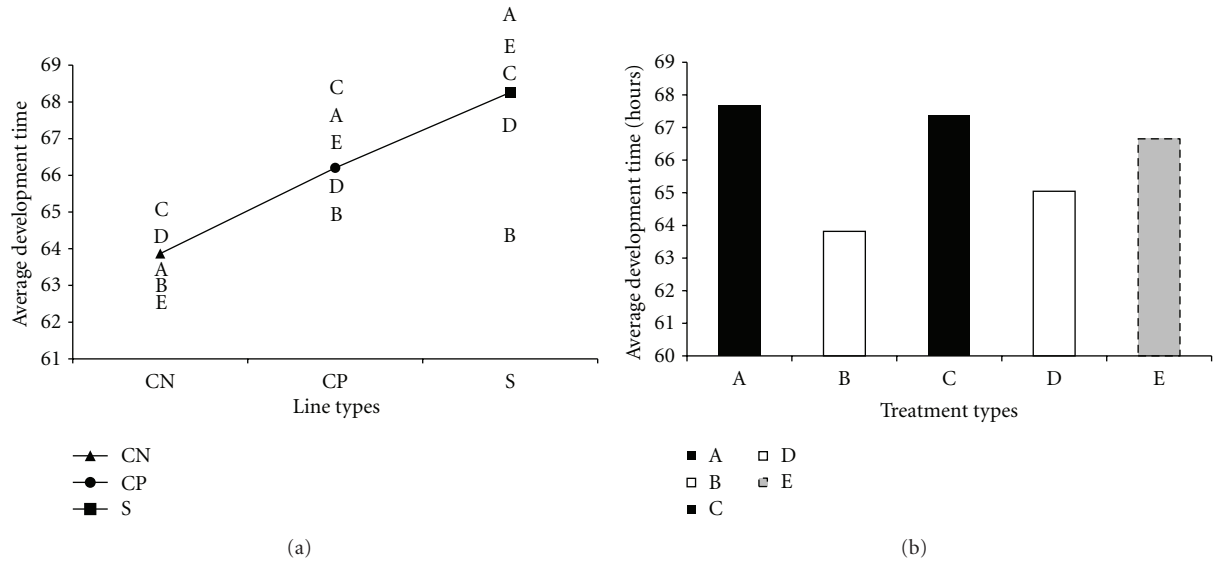

Figure 6: (a) Line graph of progeny development time from different F_1 matings shown for each line type. Line types: S—selected lines, CP—lines punctured with H_2O each generation, and CN—no perturbation lines. The F_1 treatments are described in Table 1 and reiterated here: A—only females treated with autoclaved spores, B—only males treated with autoclaved spores, C—only females treated with sterile H_2O, D—only males treated with sterile H_2O, and E—both sexes untreated. (b) Bar graph of overall means of progeny development time for each F_1 mating. The F_1 matings are described in Table 1 and reiterated here: A—only females treated with autoclaved spores, B—only males treated with autoclaved spores, C—only females treated with sterile H_2O, D—only males treated with sterile H_2O, and E—both sexes untreated.

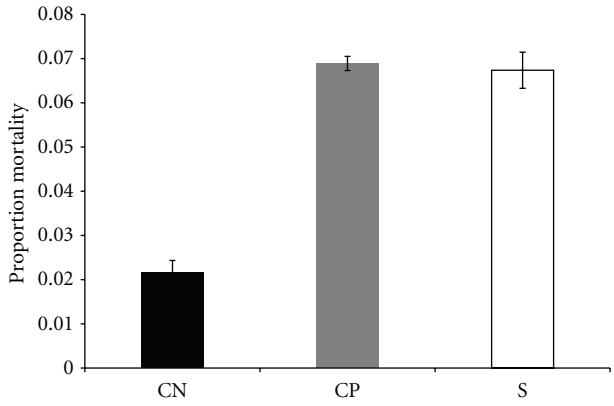

Figure 7: Average proportion mortality after treatment with autoclaved spores. The average was obtained by pooling all of the variates for all lines of the same type. S—selected lines, CP—lines punctured with H_2O (wound control), CN—no perturbation lines.

spores, whereas the selected lines (6.74% mortality) and wound-control lines (6.89% mortality) showed a marked increase in relative mortality after introduction of autoclaved spores. This difference was statistically significant ($P = 0.0003$). The increase in mortality of the selected lines compared to no-perturbation lines was 3.19-fold and 3.27-fold for the selected and wound-control lines, respectively. It is important to emphasize that there was almost no mortality after the flies were only wounded (sterile H_2O treatment). Thus, the effect shown in Figure 7 was not an evolved response to mortality from wounding. In general, there was an early-age spike of mortality in the selected lines and wound-control lines after autoclaved spore administration. This observation is consistent with the hypothesis that there was cost of inducing an immune response (deployment).

4. Discussion

Our work here has established that *D. melanogaster* can evolve high levels of resistance to *B. cereus* spores. Specifically, a 3.3-log change in the number of spores required for 50% mortality over 24 generations was documented. After a substantial direct response to selection, we investigated life history trait indirect responses to selection which was the focus of this study. Extrapolating from McKean and Lazzaro [19], selection responses *per se* represent the "standing defense" which can exert a cost. Moreover, when an inducer of the immune response was introduced, autoclaved spores in the present study, then "deployment" is the cost of maintaining the immune system in an activated state under conditions where its function is unnecessary and indeed detrimental, and this can also exert a cost [19]. This is in many ways analogous to the detrimental effects of inflammation on multiple systems in humans (e.g., metabolic disorders, IBD, and arthritis) as illustrated by the inflammatory bowel diseases [20]. In the present selection experiment, we could compare the cost of selection under noninducing conditions and differentiate this cost from that observed when a putative inducer (autoclaved spores) was introduced into flies.

There were six principle indirect responses in the present study. The first was that life span was reduced after

introduction of autoclaved spores into males in the selected lines, and females and males in the wound-control lines. This observation is consistent with the hypothesis of a cost associated with induction of an elevated immune response. Second, egg production was markedly elevated in untreated selected lines and to a lesser degree in untreated wound-control lines. There might have been increased titers of one or more hormones that could have a mutually stimulatory effect on reproduction and the immune response. Or, selection for increased adult fat body could underlie this indirect response as described below in the paragraph starting with "An alternative perspective…." Third, the relatively high egg production in the untreated selected lines, and to lesser degree in the wound-control lines, was abolished after introduction of autoclaved spores. The observation is consistent with the hypothesis of a cost of induced immunity in these lines. Fourth, untreated selected lines exhibited relatively slow development time. This observation is consistent with the hypothesis of an evolved cost associated with a high degree of resistance to live *B. cereus* spores (standing defense). However, there is an alternate adaptive hypothesis that could explain delayed development (see the paragraph below "An alternative perspective…"). Fifth, the progeny of selected line males exposed to autoclaved spores, then mated to untreated females, developed relatively rapidly. The biological interpretation of this observation is not obvious, but it might reveal a novel effect of male accessory gland secretions. Sixth, a pathological outcome (decreased early-age survival) was observed in the selected and wound-control lines after treatment with autoclaved spores. This observation is consistent with the hypothesis of a deleterious effect associated with induction of the immune system (deployment). This was an intriguing evolutionary observation as it suggests how the selection response for elevated immunity could eventually be constrained. Overall, there were multiple instances in which deployment was associated with a cost, and one case in which standing defense was potentially associated with a cost.

Evolution of resistance to bacterial spores has been observed in *Aedes aegypti* populations when *Bacillus thuringiensis* subspecies *israelensis* was the pathogen [21]. In this instance, resistance likely evolved due to changes in toxin-gut receptor interactions as observed in the diamond back moth [22]. In the present study, the level of resistance to *B. cereus* spores was many-fold greater than previously observed for selection on *A. aegypti* using *B. thuringiensis* spores. The direct response to selection in the present study was unprecedented for insect resistance to *Bacillus spores*.

The absence of a negative effect on life span in the selected lines in the present study differs from the selection experiment on *D. melanogaster* for resistance to *P. aeruginosa* [6], in which life span was negatively affected. There were appreciable differences between the selection process in Ye et al. [6] and the present study that could account for the different outcomes. This includes the use of vegetative cells versus spores, use of a gram-negative versus gram-positive pathogen, the size of the selected and control populations, and using approximately 50% mortality level for every generation of selection in the present study. Another possible explanation might be that *P. aeruginosa* is a more virulent pathogen and could exert a greater effect on life span than did *B. cereus* spores in the present study.

Generally, a negative relationship between reproduction and immunity has been observed [19, 23]. For example, decreased reproductive activity is associated with increased immunity in the cricket and in *Drosophila* [24, 25]. Thus, the major increase in egg production in the untreated selected lines in the present study was an unexpected outcome. The endocrine system of *D. melanogaster* might provide insight into understanding this phenomenon. In females, the evolution of elevated juvenile hormone (JH) could stimulate egg production, but elevated JH would also be expected to suppress immunity [26]. It is possible that male accessory gland proteins and peptides could have evolved to stimulate female egg production as there are a number of peptides in the male ejaculate that have this effect [27]. Almost all of the male ejaculate effect on female egg production is a result of the action of the sex-peptide [28]. However, the sex-peptide also stimulates JH production [28], and this would tend to suppress immunity. Importantly, the elevated egg production response in the selected lines was manifest for most of the reproductive life time. Thus, it does not seem likely that elevated egg production was a male effect resulting from mating for 24 hours relatively early in life. Another endocrine candidate is insulin, insulin-like signaling (ISS). ISS mutations in *Drosophila* result in sterility or extremely low levels of oocyte production [29, 30]. ISS in *D. melanogaster* is known to be the key hormone in the endocrine control of vitellogenic oocyte development [31], and ISS mediates the signals from nutrients to upregulate egg production [32]. However, the effect of elevated insulin signaling on *D. melanogaster* is to suppress innate immunity in this species [33]. At present, it is not clear which hormone in adult *D. melanogaster* could have caused a positive correlation between evolved elevated survival after *B. cereus* infection and high levels of egg production in the untreated selected lines.

An alternative perspective, perhaps involving hormones, might provide an explanation for the strong positive correlation between the evolution of a high degree of resistance to spores and elevated egg production. Specifically, part of the evolved response to spore infection might have been a delayed development rate which was observed in the selected lines in the present study (Figure 5, Table 4). If this delay resulted in an increase in adult fat body tissue, then there would be more of the tissue that principally secretes antimicrobial peptides. It is known that an acceleration of development time results in lower levels of fat in *D. melanogaster* larvae and adults [34, 35], and, conversely, a delay in development is expected to increase adult fat content. Hormones could mediate delayed development if juvenile hormone titers were relatively high in larval and pupal stages, and relatively low in the adult stage to avoid suppression of the immune response in the life stage at which selection occurred. The hypothesis of delayed development time as an adaptation could explain the positive correlation between egg production and the response to selection. Moreover, the interpretation of the delay in development as a

standing defense cost of selection would be replaced by delay of development as an adaptive response.

Introduction of autoclaved spores abolished the relatively high egg production in the selected and wound-control lines; after the autoclaved spore treatment, these lines did not produce more eggs than the no-perturbation lines. This result is consistent with the hypothesis of a cost of inducing (deployment) of the immune response.

There was a striking difference between the results for egg production in the present study versus the results for progeny production. Progeny production is a much different trait. For example, the total number of progeny is limited by the number of sperm stored after a mating. Lifetime egg production does not have this kind of constraint and the numbers can be much greater.

Two examples of changes in progeny development time were observed in the present study. The selected lines exhibited delayed development when the F_1 generation was untreated. This observation is consistent with the hypothesis of an evolved (standing) cost manifest in progeny development. In the context of a species that develops rapidly, delayed development could be interpreted as a cost. However, delayed development time could be an adaptation as described in a paragraph above ("An alternative perspective…"). The implications of observing accelerated development after treatment of adult males with autoclaved spores or sterile H_2O (Figures 6(a) and 6(b)) are less clear. It might be the case that the effect of male seminal fluids is normally to delay the development time of progeny. In this scenario, when males are impacted by wounding or spores, then the normal male effect might be blocked. However, an effect of male seminal fluids on progeny development time is not established; the present study may suggest a novel function for male seminal fluids.

An interesting evolutionary observation in the present study was that introduction of autoclaved spores into the selected and wound-control lines resulted in elevated early-age mortality. This effect was observed during the three-day period when mortality was monitored after introduction of autoclaved spores, puncture with H_2O, or no treatment before life-history assays were conducted (Figure 1). In two line types, selected and wound-control, exposure to dead spores resulted in relatively high mortality. One hypothesis is that the wound-control lines evolved a similar response to the selected lines because wounding each generation activated immune responses in these lines. If these responses were costly each generation, then the wound-control lines might have evolved indirect responses that were similar to the selected lines. Another hypothesis is that the short-term mortality response of the selected lines was entirely due to wounding. However, there was no increase in mortality in either line type after treatment with sterile H_2O which is the wounding alone treatment. In general, the evolved higher mortality in selected and wound-control lines in response to autoclaved spores suggests one way in which selection for immunity can be constrained which is through counter-tending negative effects that oppose the direct response to selection.

In this study, the effect of exposure to autoclaved spores is consistent with the hypothesis of a cost of induced immunity (deployment) on life history traits. This was observed for life span, egg production, and early-age mortality. Overall, the observation of an inducible cost resulting from the introduction of autoclaved spores is consistent with the hypothesis that the selected and wound-control lines have evolved to become hyper-inducible in response to autoclaved spores. The evolution of inducible responses apparently is a general response to selection in experimental evolution studies [36, 37] and perhaps in natural populations.

An extension of the studies described here will provide insight into the evolution of *B. cereus* spore infection resistance and/or tolerance in *D. melanogaster*. Whole-genome mapping of the responses to selection is underway as is a whole-genome transcriptome study. Through these approaches, and by other means to investigate resistance/tolerance, there is potential to increase our understanding of mechanisms underlying the dramatic response of *D. melanogaster* to selection by *Bacillus cereus* spores. Novel mechanisms of resistance/tolerance may emerge from these studies.

Acknowledgments

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Research Article

Is Evolution of Mating Preferences Inevitable? Random Mating in the Multisex System of *Tetrahymena thermophila*

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Ciliate mating systems are highly diversified, providing unique opportunities to study sexual differentiation and its implications for mating dynamics. Many species of ciliates have multiple (>2) sexes. More sexes may mean more choice and an opportunity for evolution of preferential mating. We asked if the multiple sexes of the ciliate *Tetrahymena thermophila* mate preferentially among each other. We quantified pairing frequencies among four sexes of *T. thermophila* using experiments that allowed the sexes to compete as mating partners. We found that all sexes mated equally frequently among each other, that is, we found no evidence of preferential mating with respect to sex. This suggests that the "mate choice" in this ciliate is binary, between whether to form a pair or not and, in this regard, sex facilitates only self-/non-self-distinction. Thus, presence of multiple sexes does not necessarily result in the evolution of mating bias, which could decrease the maximum amount of mating that would otherwise be possible in a population. Our result of random mating verifies a key assumption in the theoretical model of sex ratio evolution in *T. thermophila*. Investigation into molecular differences between the sexes will be necessary to reveal the mechanistic basis of random mating among them.

1. Introduction

Mating is random when two individuals in a population are just as likely as any other two individuals to mate. Evolution of mating preferences requires that potential mates are differentially attractive. Thus, random mating is expected if there is little variance in the perceived "quality" of mates. In natural populations, mating is rarely random [1].

Nonrandom mating results when individuals tend to choose mates with a specific phenotype and the associated genotype(s) among compatible mates. Dynamics of nonrandom mating have been studied in sexually dimorphic species in which size, sound, and color often describe the most preferred phenotype [2]. Among the microbial eukaryotes, mate-preference has been demonstrated in the yeast *Saccharomyces cerevisiae*, in which the highest amount of pheromone produced defines the most preferred phenotype for the cells of either sex [3]. It is largely unclear how mates are chosen in other unicellular organisms. Often, unicellular species have more than two sexes, raising an obvious yet previously unanswered question: do more sexes mean more choice, thereby making evolution of mate preference among the sexes inevitable? In other words, is selective mating observed when there is an opportunity to choose between many compatible sexes? For instance, the multiple sexes of a species could form a hierarchy from the best phenotype (the most preferred sex) to the least preferred one. Alternatively, the multiple sexes could be grouped such that sexes within a group mate more frequently with each other than those between groups, resulting in pronounced mating preferences between groups.

The ciliate *Tetrahymena thermophila* has seven, self-incompatible mating types (sexes). Pair formation between cells of any two sexes initiates mating (conjugation) and subsequent genetic exchange. Although each of the seven sexes identified in *T. thermophila* readily forms pairs with the other six sexes [4] the frequency of pair formation between sexes, that is, the degree of selective mating, has not

been quantified when more than two compatible sexes are simultaneously present in a population offering a choice of mate.

In *T. thermophila*, cells of different sexes engage in physical interactions (costimulation), which last for up to 2 hours prior to pair formation. Costimulation by one compatible sex does not block pair formation with any other sex. Also, the extent of costimulation by one compatible sex does not affect the efficiency (total amount and the "speed") of pairing with another compatible sex [5]. Although this shows that costimulation is not sex-specific, the exact molecular interactions that occur during costimulation are still a mystery. Also, molecular differences between the seven sexes are unknown. Current speculation is that a unique glycoprotein ligand-receptor pair may characterize each sex and that the interaction between a sex-specific ligand carried by one partner with its receptor displayed on the surface of the other partner may lead to mating pair formation in *T. thermophila* [6–8]. Under this model, affinity between the ligand and receptor may determine how likely a sex is chosen as a mate, that is, pairs would more often be formed with the sex whose ligand shares the strongest affinity for the receptor. This would result in nonrandom mating frequencies for the various sexes in a population. Another way in which the molecular affinities may affect the pairing frequencies is through mating kinetics, which affect the rate of pair formation [9]. A sex whose ligand and receptor share the strongest affinity may begin pairing with the other sexes early, resulting in nonrandom mating if limited time is available to find a partner; however, given enough time, the initial differences in pairing frequencies may disappear. Mating kinetics has been previously documented to contribute to nonrandom mating in yeast [10, 11].

Here, we tested the null hypothesis that the sexes of *T. thermophila* mate randomly with respect to sex of a partner. Our experimental design allowed individuals a limited amount of time to choose between two compatible sexes (or not to mate at all), and we recorded the number of times each compatible sex was chosen as a partner. Under the conditions tested, we found that this species mates randomly with respect to sex.

2. Materials and Methods

2.1. Strains. We obtained the strains CU427.4, CU428.2, and CU438.1 (Table 1) from the *Tetrahymena* stock center (Cornell University). Each strain carries a different dominant drug resistance marker in the germline nucleus. These strains have a sensitive phenotype in all drugs because their somatic nuclei contain the drug sensitive allele (Table 1). All strains have the *mat*-2 allele at the sex determination locus [12, 14, 15]. We performed a genomic exclusion (GE) cross of each strain (following the methods in [13, 16]) with the strain A*III, which does not contribute its genome to the progeny. We obtained 4 progeny, with mating types II, IV, V, and VII, from each parental strain (Figure 1(a), Table 1). Each progeny inherits the *mat*-2 allele and expresses one of the seven sexes according to the sex determination pattern of *mat*-2 [6]. The progeny of a strain carry the same drug resistance marker as their parent strain, but in contrast to the parental strain, the progeny express the drug resistant phenotype.

2.2. Culture Media. Stocks of all strains were maintained frozen under liquid nitrogen for the entire duration of the study. Frozen stocks were thawed, and cells were grown to log phase for 48 hours prior, to use in the experiments. We used 2% w/v Proteose Peptone (PP) to grow cells asexually. 1% PP was used for isolation of mating pairs. This medium, unlike 2% PP, buffers the pairs against osmotic shock, allowing completion of mating and subsequent asexual growth [16]. To induce mating, all strains were starved in 2% bacterized peptone (BP). To make 2% BP, an overnight culture of *Klebsiella pneumoniae* grown in 2% PP was diluted 1 : 50 with sterile water. In this medium, ciliates grow asexually by feeding on the bacteria and starve upon exhausting the bacteria in about 48 hours [16]. We used 2% BP, instead of conventional starvation media (e.g., 10 mM Tris), to mimic starvation in the natural environment. Also, 2%BP is the least likely to modify the molecular interactions and influence mating propensities between different sexes. The starved cells were washed and all matings were performed in autoclaved distilled water.

2.3. Identification of Sex. Mating type (MT) tests are used to identify mating type (sex) of a new progeny cell produced as a result of a cross [16]. Self-incompatibility, which is the inability of cells to form pairs with other cells of the same sex, is a key property used in MT tests. When mixed separately with a culture of each of the seven sexes, a clonal culture forms pairs with all but one of the seven mating type tester strains. Absence of pairing is interpreted as evidence that the progeny culture has the same sex as the tester strain. We used this protocol to determine the sex of each progeny strain generated by genomic exclusion (Table 1).

2.4. Drug-Resistance. *Pmr* is a dominant structural mutation in the coding region of small subunit of the rDNA, and it confers resistance to paromomycin (30 ng/μL) [17]. *Chx* is a dominant mutation, which causes structural modification of large subunit of rDNA, and confers resistance to cycloheximide (15 ng/μL) [18, 19]. The dominant mutant allele *Mpr* is mapped to chromosome 2R, and confers resistance against 6-methylpurine(25 ng/μL), which is a structural analog of adenine, and disrupts DNA synthesis in sensitive cells [20]. Since all drugs are lethal at the respective concentrations, the sensitive phenotype manifests as the presence of dead cells. A resistant phenotype is indicated by the presence of log-phase cells after 72 hours of exposure to a single drug [12] or 48 hours of exposure to two drugs applied simultaneously (this study).

We verified the stability of drug resistance markers in the parental as well as the progeny strains listed in Table 1. Parental strains obtained from the stock center carry resistance alleles in their germline nucleus, but sensitive alleles in the somatic nucleus [15]. Because alleles in the

Table 1: *T. thermophila* strains. All strains are whole-genome homozygotes and carry the *mat-2* allele at the sex determination locus [12].

Parental strain[a]	Progeny strain[b]	Mating type (sex)	Drug resistance marker in germline (in soma)[c]
CU438.1		IV	Pm-r (Pm-s)
	CU438.1-2	II	Pm-r (Pm-r)
	CU438.1-4	IV	Pm-r (Pm-r)
	CU438.1-5	V	Pm-r (Pm-r)
	CU438.1-7	VII	Pm-r (Pm-r)
CU428.2		VII	Mp-r (Mp-s)
	CU428.2-2	II	Mp-r (Mp-r)
	CU428.2-4	IV	Mp-r (Mp-r)
	CU428.2-5	V	Mp-r (Mp-r)
	CU428.2-7	VII	Mp-r (Mp-r)
CU427.4		VI	Cy-r (Cy-s)
	CU427.4-2	II	Cy-r (Cy-r)
	CU427.4-4	IV	Cy-r (Cy-r)
	CU427.4-5	V	Cy-r (Cy-r)
	CU427.4-7	VII	Cy-r (Cy-r)

[a]Strains obtained originally from the *Tetrahymena* stock center are derived from the inbred strain B upon mutagenesis (P. Bruns, pers. comm.). These strains were used to construct drug resistant progeny strains of various mating types.
[b]Progeny strains were generated using genomic exclusion [13]. All progeny strains show resistance to the respective drug owing to the resistance alleles they inherited from the germline of their parental strain.
[c]Drugs are abbreviated: Pm: paromomycin, Mp: 6-methylpurine, Cy: cyclohexamide. Resistant phenotypes are indicated by "-r" and sensitive phenotypes by "-s".

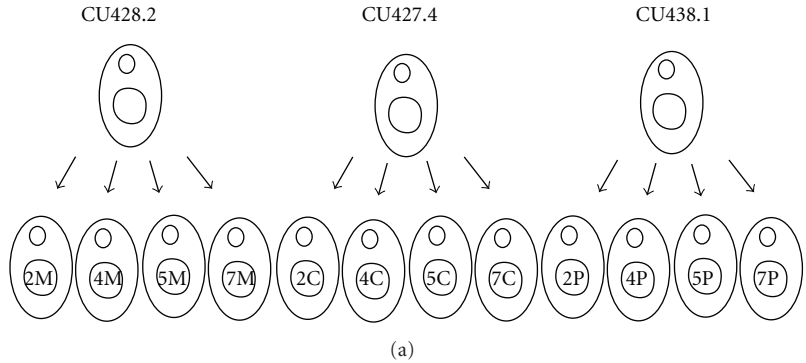
somatic nucleus determine phenotypes, each parental strain is expected to show sensitivity to all drugs, including the one for which they carry resistance alleles in the germline. Progeny strains carry resistance alleles in their germline as well as somatic nucleus. Each progeny strain is expected to be resistant to only one drug, characteristic of the resistance allele in the germline nucleus of its parental strain. From a clonal culture of each strain (parental or progeny), we isolated 48 single cells and grew them asexually for 48 hours. Each of the 48 cultures was exposed separately to the three drugs, and scored for resistant phenotype. This allowed us to determine the frequency with which cells spontaneously acquired or lost resistance to one or more of the drugs.

We also tested whether resistance markers affect the efficiency of each other. Efficiency of a resistance marker is calculated as the frequency of observing a resistant phenotype when expected. We performed all pairwise crosses between the 12 progeny strains (Table 1) to construct strains heterozygous for every pairwise combination of the markers. We picked 48 pairs from each cross, let their cultures grow asexually for 72 hours, and identified which cultures are sexually immature. Immaturity confirms that the pair mated successfully and exchanged the resistance markers (see below). We scored for dual drug resistance of the immature cultures. The extent of association between confirmed immaturity and the presence of dual drug resistance provides the efficiency of drug resistance markers in the presence of each other.

2.5. Mate Choice Assay. We used mate choice assays to quantify pairing frequency between the sexes II, IV, V, and VII. Every assay was performed between three sexes, each sex carrying a different drug-resistant genotype (Figure 1(b)). To create such a triplet of sexes, we used one progeny strain of each parent (Table 1, Figure 1). Thus, drug resistance allowed identification of morphologically indistinguishable sexes and quantification of the number of pairs formed between each of the three sexes.

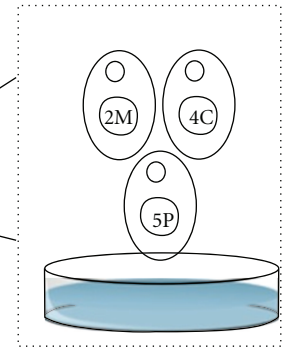
Starvation is necessary to induce mating between sexes. At the beginning of every assay, we starved three sexes (i.e., the respective progeny strains) separately and adjusted the density of each to 2×10^5 cells/mL. We mixed about equal numbers of the three sexes (6.6×10^3 cells per sex) to a total density of 2×10^5 cells in 1 mL (0.33 mL per sex). We define *T0* as the time at which we mix the sexes. Pairs start forming at ~3 hours (*T3*) after mixing. A pair takes between 10 and 12 hours to complete mating and then separates [21]. Before separation, the two sexes involved in a pair reciprocally exchange haploid genomes, including the drug-resistance markers. After separation, the partners (now called progeny) have dual drug-resistance, characteristic of the sexes involved in the pair. After 24 hours (*T24*), mostly single cells are observed, indicating separation of all pairs.

For every triplet, the mate choice assay was performed under a strict competition regime by providing limited time for choosing a mate. Thus, we picked 96 mating pairs early during mating process (*T6.5*). We put each pair in an individual drop of 1% PP medium, and grew them asexually for 48 hours. We replicated each drop-culture into 2% PP medium containing each drug separately and into the pairwise combinations of the three drugs. If the pair mated successfully, its culture will have dual drug resistance characteristic of the two sexes in the pair and is expected



(a)

Triplet	Trial	Drug		
		M	C	P
2, 4, 5	1.1	2	4	5
	1.2	5	2	4
	1.3	4	5	2
2, 4, 7	2.1	2	4	7
	2.2	7	2	4
	2.3	4	7	2
2, 5, 7	3.1	2	5	7
	3.2	7	2	5
	3.3	5	7	2
4, 5, 7	4.1	4	5	7
	4.2	7	4	5
	4.3	5	7	4



(b)

Figure 1: Experimental design. (a) Strain construction: three parental strains obtained from the *Tetrahymena* stock center (Table 1) were individually subjected to a genomic exclusion cross with the strain A*III [13] to construct an array of drug resistant progeny strains of sexes II, IV, V, and VII (shown here as Arabic numbers for convenience). Drugs are represented by M: 6-methylpurine; C: cyclohexamide; P: paromomycin. These 12 progeny strains (Table 1) were used to set up experimental trials. (b) Mate choice assay: for each triplet of sexes, separate trials were conducted, in which the resistant genetic backgrounds alternated between the three sexes. Thus, each trial contained a unique combination of progeny strains that were allowed to compete for mating partners.

to grow in the respective drug combination. If mating was unsuccessful, its culture will grow in the presence of two of the individual drugs, but not in any pairwise combination of drugs. This allowed us to identify sexes involved in successful as well as unsuccessful pairing. We performed four replicates of every trial. For every triplet, we also repeated the mate choice assay giving the cells unlimited time to choose a mating partner by testing the dual-drug resistance of single cells isolated at *T24* (data not shown).

In addition to the drug resistance markers, we used immaturity tests to verify whether the isolated pairs successfully completed mating [16]. Mating partners in a pair separate after about 10 to 12 hours. If genetic exchange was successful, the separated partners are now progeny cells, which are sexually immature and show no pairing with any of the seven mating types until they reach sexual maturity (~100 asexual divisions). In an unsuccessful mating, the separated partners retain their sexual maturity. Hence, soon after separation, they are able to form pairs with a compatible mating type. We tested cultures of each pair for immaturity by mating tests with the parental strains within 5 days after picking mating pairs.

2.6. Differential Viability of Strains. Significant differences in viability of mating pairs between the progeny strains would skew our estimates of pairing frequencies. Thus, we conducted a separate experiment to test how frequently mating pairs between progeny strains died before scoring for dual resistance. We made every pairwise cross (total 36 crosses) between the 12 progeny strains (Table 1), and

Table 2: Pairing frequencies within triplets of sexes were analyzed by ANOVA. The F ratios and P values refer to the effect of sex.

Triplet[a]	F ratio	P value
2, 4, 5	0.7209	0.5403
2, 4, 7	0.3372	0.7323
2, 5, 7	4.8167	0.0861
4, 5, 7	0.3385	0.7315

[a]Sexes are indicated as Arabic numbers instead of Roman numerals for convenience.

isolated 46 pairs at 6.5 hours after mixing the two progeny strains. We scored the survivorship as the proportion of viable pairs in 1% PP at 48 hours after-isolation. This procedure was replicated twice. We used these viability data to correct the estimates of the pairing frequencies in mate choice trials. We divided the number of pairs observed between two sexes in a mate choice trial by the viability estimate for the corresponding pair of progeny strains (Table S1 available online at doi:10.1155/2012/201921). We then normalized the corrected numbers to 96: the total number of pairs isolated in each mate choice trial. This gave us an estimate of the actual number of pairs that likely formed between the two sexes. We used these normalized estimates of pairing frequencies in the analyses of mating biases.

2.7. Statistical Analysis. In every trial conducted on a triplet of sexes, three types of pairs can be formed between the three sexes (Figure 1(b)). Under random mating, we expect 1/3 of total pairs to be of each type. We used goodness-of-fit tests to determine if mating was random within a trial. Also, we used mixed-effect ANOVA to analyze if mating was random in a triplet (i.e., pooling the data for all trials conducted for a given triplet). The experimental design (Figure 1(b)) contained one fixed factor (sex) and one random factor (drug resistant genotype). All statistical analyses were performed in *R*.

3. Results

Our aim was to investigate whether preferential mating occurs among sexes, when more than two sexes are present in a population. Hence, we quantified biases in pair formation between four (of seven) sexes of *T. thermophila*. We created experimental populations containing three sexes each and used drug resistance markers to identify how often the various sexes formed mating pairs.

3.1. Drug-Resistant Alleles Serve as Reliable Genetic Markers. We determined the reliability of the drug resistance alleles as genetic markers and found that they are stable against spontaneous mutations. The frequency of mutations conferring a loss or gain of resistance was below the limit of detection for all strains listed in Table 1 (data not shown), that is, we found no such spontaneous mutations. We also found the efficiency of the markers in conferring resistance to be 100% (data not shown) because only those strains that were immature showed dual drug resistance. Thus, the three resistance alleles are reliable genetic markers in the genetic backgrounds used in our experiments. While we did not explicitly test the neutrality of these markers towards fitness (growth rate) of the strains, at no step in our mate choice assays was there an opportunity for fertility selection. We did not, however, observe any obvious differences between growth rates of the progeny strains with different markers.

3.2. Viability of Mating Pairs Is Contingent upon the Strains Forming the Pairs. We measured viability of mating pairs as the proportion of total pairs that survived and grew 48 hours after isolation in growth medium. We found that some mating pairs did die more frequently than others (one-way ANOVA, $P < 2.2e - 16$). This indicates that viability of the pairs is contingent upon the progeny strains forming the pairs (Table S1). We could not associate the observed mortality with the presence of any particular resistance marker, but rather conclude that viability is a property of the specific genetic backgrounds of the strains.

3.3. Mating Is Random with respect to Sex. Each experimental trial (Figure 1(b)) contained three sexes in equal proportion, which ensures equal mating opportunity for each sex. Thus, under random mating, the three types of pairs that could be formed among the three sexes should be represented at an equal frequency of ~0.33. We tested this null hypothesis for every trial using a goodness-of-fit G-test and found evidence of nonrandom mating ($P < 0.05$) in many trials but without a strong bias towards any particular sex (data not shown). The three sexes within a trial, however, had different genetic backgrounds. Hence, the apparent deviations from random mating may not represent mating preference with respect to sex of an individual but instead reflect an effect of genetic backgrounds on pairing frequencies in a trial. The genetic backgrounds used in our experiments represent a randomly chosen subset of the available drug resistant backgrounds; therefore, any effect of the genetic background on pairing frequencies may be specific to the strains we used, and must be isolated from the effect of the sexes.

For every triplet of sexes, we had conducted three trials in which the drug resistant, genetic backgrounds alternated among the three sexes (Figure 1(b)). If sex of an individual was driving biases in pairing frequencies observed within a trial, we would expect a consistent bias towards a particular pair of sexes across the three trials irrespective of the associated genetic backgrounds. On the other hand, if the sexes exhibit no preferences, the pairing frequencies across the three trials will reflect random mating with respect to sex. To investigate any consistent effects of sex (with alternating genetic backgrounds) on pairing, we analyzed mating biases by pooling across the three trials conducted for a given triplet of sexes. A mixed effect ANOVA indicated no significant effect of the sex on the dynamics of pairing in each triplet of the sexes (Table 2, Figure 2). This suggests that mating was indeed random with respect to sex of an individual, and that the biases apparent in pairing frequencies in individual trials likely represent an effect of the genetic background rather

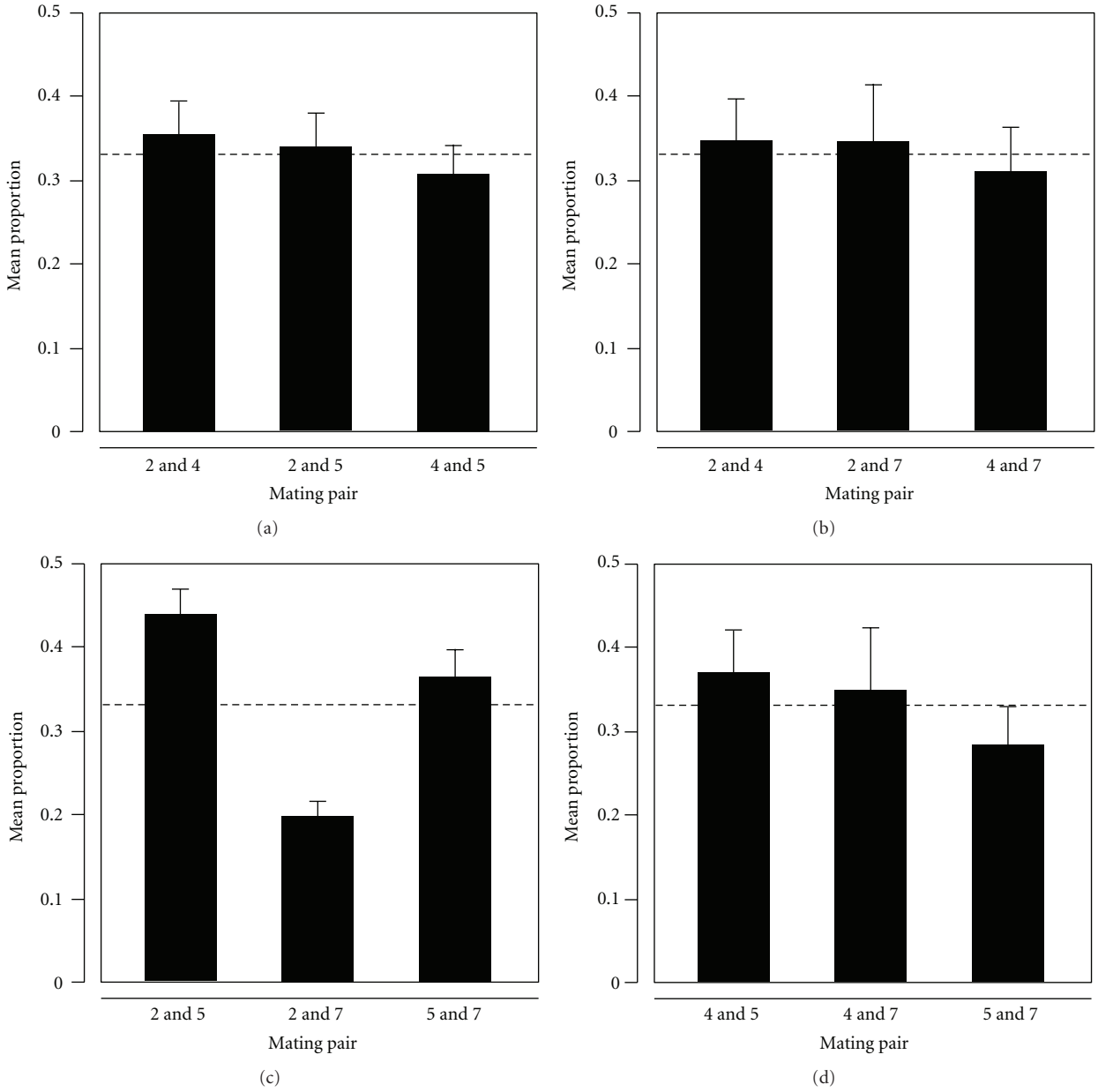


Figure 2: Random mating among sexes in *T. thermophila*. Bar charts show the relative frequency of pairs formed between sexes. Sexes are represented in Arabic numbers. Panels (a): triplet 2, 4, 5; (b): triplet 2, 4, 7; (c): triplet 2, 5, 7; (d): triplet 4, 5, 7. *y-axis* shows the mean proportion from 12 replicates for each pair type, averaged over the three trials for a given triplet (Figure 1(b)). A total of 1152 pairs ($N = 96$ pairs per trial X4 replicates per trial X3 trials per triplet) were analyzed for each triplet. Dashed line indicates the proportion of pairs (~0.33) expected under random mating. Error bars indicate standard error. All triplets show evidence of random mating with respect to sex (mixed-effect ANOVA $P > 0.05$, Table 2).

than preferences among the sexes. Nonpreferential mating, that is, random mating with respect to sex, was verified in assays conducted at *T24* (data not shown).

4. Discussion

Nonrandom mating is pervasive in nature, occurring in various ways, and resulting in differing evolutionary consequences [1]. In contrast, our results suggest that *T. thermophila* mate randomly among each other with respect to sex. This lack of mate preference was apparent even under strict competition for mates imposed by limited time to find a mating partner, and subsequently when time restriction was removed.

Sexes of *T. thermophila* are self-incompatible, that is, cells of the same sex never form mating pairs, but each of the seven sexes can pair with all the other (i.e., six) sexes. Thus, even though all sexes look morphologically alike, they are

somehow able to distinguish self from nonself. Our analysis suggests that beyond this basic distinction, most sexes of *T. thermophila* are unable to differentiate the various nonself sexes. Thus, the "choice" in this multisex system is binary, that is, it is about whether or not to form a mating pair rather than which of the available sexes to form the pair with. Our results are consistent with the previous finding that the sexes functionally substitute each other during costimulation—the stage immediately prior to mating pair formation [5]. Although it used a similar experimental design, the previous study did not allow quantification of mating preference of the four sexes. Our experimental design allowed us to verify that none of the sexes II, IV, V, and VII show bias for mating among each other when presented simultaneously with a choice between two compatible sexes. This could be possible if the ligands characterizing various sexes diverged to be sex-specific but still share a common receptor, enabling the sexes to replace each other functionally. Alternatively, random mating found in our study may reflect presence of a unique receptor for each sex-specific ligand and thus lack of competition between the sexes.

Neither the molecular differences between the sexes nor the molecular interactions responsible for pairing are known in *T. thermophila*. Interaction between a sex-specific ligand on the surface of one sex and the receptor on the surface of the other sex is hypothesized to be the underlying mechanism of pairing between sexes, but awaits empirical support [6]. If pairing indeed results from such molecular interactions, then we suggest that affinities between the sex-specific ligands and receptor(s) may be of equal strength, leading to random mating between the sexes. It is generally observed in other unicellular species that the affinities between sex-specific molecules rarely determine the intraspecific mating propensities. For instance, the unique pheromones secreted by the two mating types in the yeast *Saccharomyces cerevisiae* bind to specific receptors displayed by the opposite mating type. Variation in the amount of pheromone produced, but not in the affinity to the receptor was found to be the basis of nonrandom mating [3]. In the ciliate *Euplotes raikovi*, sex-specific pheromones and receptors characterize the multiple sexes, which show variable mating interactions among each other beyond self-/non-self-distinction. Biased mating among the multiple sexes is a function of amount of pheromone produced such that higher secretion translates into higher mating success for any given sex. Thus, factors other than molecular affinities largely determine mating propensities among sexes in a population of this species [22]. Also, affinities between sex-specific molecules play a minimal role in interspecific mating interactions. For instance, although recognition of opposite mating type occurs and interspecific hybrids sometimes form between the two closely related yeasts *S. cerevisiae* and *S. paradoxus,* mating takes place preferentially with conspecifics and is more efficient and frequent [10, 23]. The overall genetic background rather than species-specific pheromone-receptor affinities are largely responsible for mating selectively within species. Thus, unlike in anisogamous plants and animals, prezygotic reproductive barriers may rarely occur at the pheromone/receptor level [11] in isogamous unicellular eukaryotes, allowing extensive diversification of mating types within species.

The genetic background may also affect viability in *T. thermophila.* For example, pairing with the strain 5C always resulted in low viability (Table S1) for reasons that are yet unclear and may involve genetic incompatibilities, which could be investigated in future studies. Although mating is random with respect to sex, the effect of genetic background on viability implies that some sexes may contribute to the gene pool more than the others, contingent upon the genetic background they are associated with. The effect of a sex and its genetic background on mating propensities could be explored in future studies using a full factorial design involving six (instead of three) trials for every triplet of sexes (Figure 1(b)).

Natural populations of *T. thermophila* are likely patchy, thus due to self-incompatibility and the inability to switch sexes, finding a compatible mate may be difficult ([24–26], P. Doerder, pers. comm.). Random mating with respect to sex creates the highest possible opportunity for mating, contingent upon the sex ratio, the relative frequencies of multiple sexes, in the population. High frequency of sex has been documented in natural populations of *T. thermophila* [25–27]. Thus, when searching for mates is costly, random mating with respect to sex is likely to be advantageous by avoiding further delays in initiating mating. It is unclear, however, why self-incompatibility would be maintained under such a scenario, though it is possible that high levels of inbreeding depression select for the maintenance of self-incompatibility. Transitions to self-compatibility in *Arabidopsis* have been linked to mate-limiting conditions [28]. Mating between cells of the same sex and other selfing strategies, including autogamy, are also observed in many species of ciliates and may reflect ways to avoid the cost of finding a mate [7, 29]. Alternatively, presence of multiple sexes may compensate, at least partially, for the cost of self-incompatibility. Very few data exist to test the correlation between the number of sexes and the capacity to transition to self-compatibility in ciliates.

The demonstration of random mating with respect to sex verifies a key assumption of our model on sex ratio evolution in *T. thermophila* [30]. Although violating the assumption of random mating would not change the equilibria predicted in this model, it would change the approach (time and the trajectory) of the populations to those equilibria. We studied mating preferences of the four sexes specified by a single sex determining allele (*mat*-2). The analysis presented here does not rule out the possibility of finding nonrandom mating between sexes specified by different *mat* alleles, which may lead to assortative mating with respect to the genotypes at the *mat* locus in *T. thermophila*.

Discovery of mating bias among sexes in *T. thermophila* could have delivered insights into affinities between sexes at the molecular level and facilitated predictions about the structural differences between the sex-specific molecules, which are currently unknown. Our results emphasize the need to decipher the molecular machinery that enables random mating among multiple sexes.

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Review Article

The Genetic Basis of Female Mate Preference and Species Isolation in *Drosophila*

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The processes that underlie mate choice have long fascinated biologists. With the advent of increasingly refined genetic tools, we are now beginning to understand the genetic basis of how males and females discriminate among potential mates. One aspect of mate discrimination of particular interest is that which isolates one species from another. As behavioral isolation is thought to be the first step in speciation, and females are choosy more often than males in this regard, identifying the genetic variants that influence interspecies female mate choice can enhance our understanding of the process of speciation. Here, we review the literature on female mate choice in the most widely used model system for studies of species isolation *Drosophila*. Although females appear to use the same traits for both within- and between-species female mate choice, there seems to be a different genetic basis underlying these choices. Interestingly, most genomic regions that cause females to reject heterospecific males fall within areas of low recombination. Likely, candidate genes are those that act within the auditory or olfactory system, or within areas of the brain that process these systems.

1. Introduction

Sex has long been a popular topic of research among evolutionary biologists. Our personal fascination with the subject is related to the variation that is seen in sexual behavior. This includes the different roles that make up mating rituals, such as courtship traits or preference for the traits, and the variation of these behaviors observed both within and between species. Understanding the biological basis of mating behavior is not only interesting, it is also important for our understanding of evolution as it can shed light on how species boundaries are formed and maintained. Different mating behaviors of closely related species can act as an isolating barrier that stops gene flow between two interbreeding populations. This usually results from closely related species having diverse mating signals: one or both of the sexes fail to identify the other as a suitable mate [1–4]. For example, males of some species court conspecific females more often or with more vigor than heterospecific females [5] and females mate more readily with conspecific than heterospecific males [6, 7].

The impact of *Drosophila* in this area of research has been pronounced primarily because many obstacles can be bypassed in this system. First, the stereotypical mating behavior observed in this genus is relatively easy to score [8–10], there are genetic tools available to allow manipulation of the development and physiology of mating behavior [11], and there is relative ease in housing large numbers of individuals in a uniform environment. Second, many *Drosophila* sister species are only partially isolated in a lab setting, producing viable and fertile hybrids [2].

Females of most *Drosophila* species are usually the sex that determines whether copulation occurs [9]. Males preferentially court conspecific females with larger body sizes, which is a good indicator of female fecundity [12], and in some species (e.g., *D. virilis*) males are able to discriminate against heterospecific females [5]. However, it is more often found that males readily court heterospecific females [13], while females discriminate against heterospecifics males [6]. Females easily prevent unwanted copulations by flying away from the courting male orextruding her ovipositor [8]. Furthermore, mating behavior has been found to be cyclic

with alternating bouts of high mating activity and low mating activity; with the use of arrhythmic mutants, it has been shown that females determine when mating occurs [14]. Therefore, in order to understand what isolates species from each other, attention should be focused on female mating behavior.

2. The Evolution of Genes for Female Mating Behavior

The majority of research in behavioral isolation has been influenced by the Modern Synthesis [15], which is a general account of speciation and evolution. The tenants of the Modern Synthesis state that a population contains genetic variation which is apparent at both the gene level (with multiple alleles produced by random mutation) and at the chromosomal level (with different combinations of alleles within individuals produced by recombination). A population's gene frequencies and allele combinations can change over time through multiple processes, including natural selection and genetic drift. While the contribution of natural selection has been wellsupported, the impact of genetic drift has been debated within the literature. For example, a computer model exploring the development of behavioral isolation via sexual selection [16] and research that employed extreme bottlenecking [17] both showed that genetic drift can rapidly lead to some level of sexual isolation. On the other hand, speciation by genetic drift has been shown to be unlikely to occur [18, 19] because genes for mating behavior are most likely either pleiotropic and directly under natural selection, or are closely linked to genes that are under selection and therefore would not simply be fixed by a random process [20].

If a population is divided, the newlyformed subpopulations can potentially become genetically differentiated from each other. Over time, the genomes of each subpopulation can diverge from each other either due to the different distribution of alleles that made up the founder population, the different selective pressures on these alleles, and new genetic mutations that arise. As with other traits, the genetic variants that contribute to male and female mating behaviors may cause a difference in phenotype between individuals of the two subpopulations (for review, see [21]). Differences in mating signals can influence female mate choice, which can subsequently act to reduce gene flow between the groups if they come into contact.

Secondary contact between diverging populations can, however, produce hybrids between species in nature. If these hybrids have a relatively high fitness, it is possible that enough gene flow can occur between these two species to cause them to merge back into one. In contrast, if hybrids have a low fitness, a selective pressure to assortatively mate within both populations can act directly on the genes for mating behavior, favoring alleles that differentiate courtship behaviors and enhance preferences for traits that distinguish potential mates of the two groups. This phenomenon, known as reinforcement, has been observed in nature where two closely related species, for example, *Drosophila pseudoobscura* and *D. persimilis*, have partially overlapping regions. In response to the selective pressure to avoid heterospecific matings, populations from the sympatric region have a greater level of behavioral isolation compared to those from allopatric populations [22, 23]. However, reinforcement's role in speciation was historically disputed as alternative theories could explain the increased level of behavioral isolation [24], controlled experiments on the topic were generally lacking [25], and some experiments failed to support the theory of reinforcement. For example, the presence of reinforcement within *D. mojavensis* and *D. arizonensis* was tested with the use of two groups: one with the traditional rearing substrate of banana agar food, and the other with fermenting cactus—the natural food of these two species. Although behavioral isolation was still found between the species, and the general pattern of reinforcement was still present, the sympatric population was not significantly more behaviorally isolated than the allopatric population [26]. Additionally, reinforcement is not required for differences in mating behavior to arise. For example, a population of *Drosophila* was subdivided into three groups within the lab: one group remained on the ancestral food source, while two other subpopulations were reared on novel food sources for multiple generations. Afterwards, female mating preferences were tested and were found to be changed in parallel with population divergence [27].

Recently, however, strong empirical data in support of reinforcement has surfaced. Lab investigations have shown that reinforcement can strengthen behavioral isolation between two closely related species [28], and once the selective pressure for species discrimination is removed, the likelihood of interspecific mating has been found to increase [29]. A meta-analysis also found evidence to support the previouslyuntested predictions of reinforcement, such as concordant isolation asymmetries (because reinforcement potentially evolves due to unfit hybrids, pre- and postzygotic isolation should evolve in the same direction) and rare-female effect (females from the smaller population would encounter more heterospecific males and therefore have a stronger selective pressure to choose conspecific males) [30]. Thus, separated populations can evolve divergent behaviors, and these behaviors can potentially be enhanced when the populations are once again in contact.

3. Intraspecific Sexual Selection versus Interspecific Female Mate Choice

The relationship between within- and between-species mating preferences is not fully understood, but they are often considered extreme ends of the same continuum. With time, two populations of a species are thought to slowly slide from assortative mating to heterospecific discrimination, by sexual selection either directly acting on genes that influence mating behavior or indirectly acting on genes that enhance survival. Blows and Allan [1] argued that if species isolation was produced by sexual selection, then the traits involved in species isolation should be the same traits used by both sexes during within-species mate choice. To test this hypothesis, they investigated the behavioral isolation between *D. serrata*

and *D. birchii*. These two species have overlapping geographic regions along the east coast of Australia. Although morphologically very similar, there is strong behavioral isolation between the two species [31]. They showed that the two species have different cuticular hydrocarbon (CHC) profiles, which are used as sexual pheromones. By performing perfuming experiments, which transferred CHCs from one species onto another, these researchers determined that the same mechanism (olfaction sensation of CHCs) is used for within-species mate choice (sexual selection) and between-species female mate preference (behavioral isolation).

Although this shows that variation in the same trait can be used for both within- and between-species female mate choice, it does not necessarily mean that they have the same genetic basis. The assumption is that there is a set of genes that control female mating behavior in the ancestral population, and once the population is divided, those genes accumulate mutations in the new populations which cause changes in the behavior. The genes that control intraspecific behavioral variation, however, may not be the same genes that are important in interspecific behavioral variation. Although, for example, genes for olfactory system development used to detect different CHC profiles could be important for normal female mating behavior in both species, the genetic basis for the interpretation of variation in the CHC profile may vary between species.

Investigation into this question led to a series of studies that showed the relationship between interspecific hybridization and intraspecific receptivity. Carracedo et al. [32] proposed that if intraspecific and interspecific mating behaviors have the same genetic basis, females that are slower to accept conspecific males may also be more reluctant to accept heterospecific males. In other words, high level of general within-species receptivity would be selected against due to its pleiotropic effect on high interspecies hybridization. In the lab, when a high level of interspecies hybridization (reduced choosiness) in females was selected for, a decrease in time to start copulation with conspecific males was also found [33]. This was interpreted as a linked increase in intra- and interspecific receptivity, giving support to the notion that both types of mating behavior have the same genetic underpinning. However, when interspecific mating was directly tested by placing females in a choice assay with conspecific and heterospecific flies of the opposite sex, almost no heterospecific matings were observed, showing that selection for heterospecific mating is unlikely to influence within species mate choice in nature, where multiple mates are available [34]. The ultimate test of whether the genetic bases of intra- and interspecific mating behavior are under the same genetic control would be to determine and compare the genetic basis of both systems. Unfortunately, no gene has been identified to be involved with interspecific female mate choice. However, a few studies that have identified the regions that most likely contain genes that isolate species do not seem to overlap those regions that contain genes known to influence within-species mating behavior [6, 7, 35].

More unexpected results came from the female mating behavior of island populations. When migrants populate a new island, it is likely that the least choosy females will propagate the most offspring since the most choosy females may not find a high-quality male and therefore will not reproduce [36, 37]. Assuming that low intraspecific choosiness results in high hybridization rates, we would then expect isolated island species to have high levels of hybridization. Although we do find this relationship in the North American and Bogota strains of *D. pseudoobscura* [38], we see the opposite trend in many other species pairs [19]. For example, *D. mauritiana* and *D. sechellia* females, both from island populations, are more choosy against males from the closely related mainland species, *D. simulans*, than mainland females are against island males.

4. Genes for Interspecific Female Mating Behavior

Mating behaviors in *Drosophila* usually have a genetic basis (e.g., of an exception, see [39]). The genetic information that one inherits can predispose a female to behave a specific way: which partner she chooses to accept. These genetic factors can influence both behavioral variation within a species and behavioral differences between species. The latter of these two is critical for our understanding of the genetic basis of species isolation, as it is thought that these behavioral differences are the first barrier to arise in species isolation [40]. By identifying the genetic variants that cause interspecific differences in mating behavior, we can determine which mutations and alterations in the genetic material cause the differences in behavior between two isolated species, and thus may underlie the speciation process itself.

Despite its importance for species isolation, the genetic basis of behavioral isolation is not well understood. This is primarily due to the most commonly used method in genetics for locating genes that contribute to variation in a quantitative trait, namely, recombination mapping. This method necessitates crossing two divergent lines and producing fertile offspring. However, by definition, separate species usually do not produce either fertile or viable offspring. Second, identifying the genetic basis of a behavior requires the location of multiple genes with different effect sizes [41], necessitating a repeatable measure of the behavior, large sample sizes, and the availability of powerful genetic tools such as readily available single gene mutant lines [42].

Despite these obstacles, the genetic basis of mating behavior has been studied in different species of animals and plants. The genetic basis of floral scent production in *Petunia axillaris* (*Petunia*) has been found to play an important role in pollinator attraction and thus contributes to isolation between related species of plants [43]. Research on butterfly mating behavior has found a consistent relationship between wing color and mate preference [44] and both traits may be caused by the same gene (*wingless*) or multiple genes linked to *wingless* [45]. Male cichlids in Lake Victoria have divergent species-specific coloration which has been shown to be driven by female choice [46] and this interspecific female mating preference for conspecific coloration has been found to be heritable in cichlids, with only a few loci responsible [47]. Although butterfly and cichlid coloration

and preference have provided insight into the genetic basis of behavioral isolation, these systems are limited in that they do not have the powerful genetic tools that are available in *Drosophila*, a well-developed genetic model system.

Using mutagenesis studies, multiple genes have been identified in *D. melanogaster* that influence within-species mating behaviors for both males and females. Male behavior has traditionally taken the spotlight in genetic studies on mating behavior. Through mutagenesis studies, approximately 55 genes have been identified to influence within species male mating behavior, while only a handful of genes have been identified that act within a female to increase or reduce her receptivity (see Supplementary Table 1 available online at doi:10.1155/2012/328392).

These studies are of great importance as they provided crucial information into both the sensory system used in *Drosophila* mating and the types of genes that can influence the construction of mating behavior. However, these studies eliminate the gene's function in order to test whether it affects a behavior. While this demonstrates that the gene is important for creation of the behavior, it does not necessarily tell us anything about the naturally occurring genetic variation that contributes to the differences seen within or between species. For example, genes identified during mutagenesis for normal male mating behavior were not found to contribute to variation seen in courtship [48], did not contribute to variation between low and high mating male lines [41], and did not vary in expression in a natural population of *D. melanogaster* [49]. The genes important for normal female mating behavior were also not found to vary in expression between courted and naïve same-age virgin females [50]. The genes identified through mutagenesis consistently do not appear to influence the variation in mating behavior within a species, and, therefore, may also not contribute to the variation observed between species [51].

Although no individual genes for behavioral isolation have been identified, recombination mapping studies have located regions of the genome that influence behavioral isolation, which do not include genes identified through mutagenesis (see below). However, since the preliminary observations of interspecific female mating behavior do not resemble the expectations set out by prevailing theory, it is difficult to determine strong candidate genes for interspecific female receptivity within these regions [52, 53]. In order to identify which genes are candidates for influencing interspecific female mating behavior, we could first evaluate which signals females are basing their choice upon.

5. The Modes of *Drosophila* Male Signaling during Courtship

The variability we see in female preference, both within and between species, is most likely dictated by the integration of the auditory and olfactory systems [54]. To complicate investigation of these two systems, the amount that females of each species rely on one system over the other is most likely species specific [3, 4, 55, 56]. A gene for interspecific female preference is most likely going to be associated with the signaling pathway of the auditory system used to recognize differences in male courtship song characteristics [3, 57], the olfactory system used to recognize CHC pheromone profiles [1], or both systems via the organization of the part of the brain that receives and interprets signals from both pathways [54, 58]. This is because both modes of signaling are used during *Drosophila* courtship [8–10] and vary between species [1, 3, 56, 59]. A candidate region for such integration in the brain is the mushroom body, which receives signals from many sensory systems in *Drosophila* [60], including the olfaction system [61], and has been linked to sexual behavior [62, 63], specifically female receptivity [64].

There are two main elements to the courtship song—the sine song and the interpulse interval—and males of different species usually differ from each other on both accounts [53]. A female's ability to identify conspecific song over heterospecifics can lead to behavioral isolation [3]. For females in the melanogaster group of *Drosophila*, the most important element of courtship song is the interpulse interval (IPI) which differs among the males, and preference for variants of IPI seems to differ among females [65]. The most famous gene to influence courtship song is the *period* (*per*) gene. Mutations in this gene influence IPI [66], and transgenic *D. melanogaster* flies with *D. simulans per* produced *D. simulans*-typical rhythm [57]. Instead of a species difference reflecting a complex genetic basis, the species differences in song rhythm reflect just a small number of amino acid changes [57]. Females from this same transgenic line showed associated preference for the transgenic male's IPI [67], and a later study also showed evidence of assortative mating with a different *per*-transgenic line [68]. Although the genetic basis of this preference is not straightforward, it is clear that females may be using the variations in song between species in determining mate choice. Females can detect male song and male movement with use of the receptors in the antenna; neurons from the antenna project to the dorsal brain, which requires feminization in order for females to be receptive (for review, see [58]).

In addition to song, females also use pheromonal cues to distinguish mates. Each species of *Drosophila* has cuticular hydrocarbons (CHCs) on the outer surface of their body that act as a protective barrier to desiccation and most likely evolved as an adaptation to dry climates [69]. These compounds also are important in mating behavior [70] and are used during mate selection as pheromones that both allow males to distinguish females [71] and affect female receptivity [72]. The majority of CHCs are nonvolatile compounds that are detected by both males and females, most likely through touch (gustation) at close proximity, rather than smell at long distances [70]. Detection of the CHC profile occurs through a large family of odorant receptors that send information about the environment via odorant sensory neurons to the antennal lobe, which is analogous to the olfactory bulb in mammals (for a review, see [58]).

Billeter et al. [71] used a Gal4-UAS system to block the development of oenocytes, which are cells specialized to produce the cuticular hydrocarbons. Flies without working oenocytes (oe^-) were completely devoid of all CHCs but behave normally. However, female response towards oe^-

males was significantly altered: wildtype females were significantly less receptive to oe$^-$ males and oe$^-$ males took significantly longer to achieve copulation. Therefore, CHCs not only enhance within species female receptivity [71], but they can also potentially be used to deter females from heterospecific matings [1]. Furthermore, it has been shown that males' CHC profiles respond more easily to lab-induced natural and sexual selection than the females' CHC profile [73], indicating that the male profile could be a more likely avenue by which selection acts in nature.

Although there are more than 20 different CHC molecules on the cuticle of the fly, only the predominant hydrocarbons have received much examination and have been primarily studied within the melanogaster subgroup of *Drosophila* [74]. *D. simulans* and *D. mauritiana* have a monomorphic CHC profile, with the main hydrocarbon of both males and females being the same 23-carbon chain compound, *cis* 7-trisosene (7-T). However, *D. melanogaster* and *D. sechellia* are dimorphic: the males have large amounts of 7-T, but females lack this hydrocarbon and instead have large amounts of a 27-carbon molecule, *cis, cis*-7,11-heptacosadiene (7,11-HD) [75]. Most *Drosophila* species have males that predominately produce 7-T as their main CHC and also share multiple minor compounds as well. However, the ratio between the different CHCs is slightly altered between species, creating unique pheromone "blends" [70].

Through mutagenesis studies, genes have been identified to affect CHC production, such as *dsat1* and *dsat2* [76], *Enhancer of zest* [77], *Ddc* [78], *nerd* [79], *seven pentacosene*, and *smoq* [80], as well as some sex determination genes, such as *doublesex* [81]. However, only the genetic basis of the main CHC components (7-T and 7,11-HD) have been examined. Additionally, it is unclear if variation in these genes produces the variation that is seen in CHC production between populations of the same species, or variation in production between species [56, 82, 83].

From the research dedicated to identifying the genetic basis of CHC variation between species and courtship song variation between males of different species, we can comfortably deduce that different species have different CHC profiles, different courtship songs, and females preferentially mate with conspecific males based at least partially on both signals.

6. Genetic Basis of Female Behavioral Isolation for Different Species Pairs

To date, no individual genes have been identified as influencing intra- or interspecific female preference in *Drosophila*, although the trait has a clear heritable basis [8]. Due to the requirement of fertile hybrids for traditional recombination mapping, the majority of studies seeking to address this question have been done in *Drosophila* species other than *D. melanogaster* (Table 1), since *D. melanogaster* does not produce fertile offspring with any of its sibling species[2, 3, 6, 7, 59, 84]. The majority of studies that have examined the behavioral isolation between *D. melanogaster* and *D. simulans* have done so in a limited way, showing that specific chromosome arms influence behavioral isolation, and until recently these attempts have not come close to isolating individual genetic variants that affect behavioral isolation [85–87]. However, the genomes of 12 different species of *Drosophila* have now been sequenced [88], and recently the powerful genetic tools available in *D. melanogaster*, such as the Gal4-UAS system (used to manipulate gene expression) and transposon vectors (for use in mutagenesis studies), have now been modified for other species of *Drosophila* [89]. Despite the previous limitations, various genomic regions have been identified that contribute to behavioral isolation in multiple species of *Drosophila*, and the expansion of the available tools makes further refinement of these studies now possible.

6.1. D. pseudoobscura and D. persimilis. *Drosophila pseudoobscura* are found across much of Western North America and are located both in sympatry and in allopatry with *D. persimilis* [123]. The initial genetic basis of isolation between these species, termed basal isolation, was found to be caused by only two regions in the genome: one on the left arm of the X chromosome (which is homologous to the X in *D. melanogaster*) and one on the second chromosome (homologous to the right arm of chromosome 3, called 3R, in *D. melanogaster*), within an interspecific inversion that differentiates *D. pseudoobscura* and *D. persimilis* [84].

Female *D. pseudoobscura* from sympatric regions hybridize less with male *D. persimilis* than females from allopatric regions without *D. persimilis*, which has made this a model system for studying reinforcement [22]. Ortiz-Barrientos et al. [109] investigated the genetic basis of the increased discrimination of sympatric *D. pseudoobscura* females. By introgressing (crossing) pieces of the sympatric *D. pseudoobscura* genome into an allopatric *D. pseudoobscura* background, they mapped the increase in behavioral isolation to two alleles of strong effect, one on the right arm of the X chromosome (called *Coy-1*; homologous to 3L in *D. melanogaster*) and one on the fourth chromosome (called *Coy-2*; homologous to 2L in *D. melanogaster*). However, Barnwell and Noor [124] used six pairs of different inbred strains in a quantitative trait locus (QTL) mapping study to try to replicate the previous identification of *Coy-1* and *Coy-2*. They could not, and therefore determined that *Coy-1* and *Coy-2*, although they may be important, are not the primary loci causing increased behavioral isolation in sympatric versus allopatric populations. These alleles may be present at low frequencies in natural populations and therefore would not be present in most inbred laboratory lines.

Although they may not underlie species-wide discrimination, an examination of the two loci could still provide important insight into the genetic basis of reinforcement. To this end, each of the *D. pseudoobscura* sympatric and allopatric *Coy2* alleles was introgressed into a *D. persimilis* background (creating per*Coy2*sym and per*Coy2*allo lines) [110]. If the reinforced behavioral isolation was caused by an increased receptivity for *D. pseudoobscura* (conspecifics) by the *D. pseudoobscura* sympatric population, the expected results would be that per*Coy2*sym females are more likely to

Table 1: Summary of existing genetic analyses of *Drosophila* species pairs that are behaviorally isolated. The current mode of isolation, trait studied, experimental design (E D), and number of loci potentially affecting behavioral isolation are listed. E D's are chromosome substitution (C), deficiency complementation mapping (D), complementation mapping of single genes (G), homozygous for a mutation (H), introgression (I), microarray (M), quantitative trait locus mapping (Q), and recombination mapping (R).

Species pair	Isolation	Trait	E D	Number of loci
D. melanogaster (two "races")	Allopatric	Male prezygotic isolation [90–92]	C, I	≥5
		Female prezygotic isolation [90–93]	C, I, M	≥4
		Female pheromone production [94]	R	1
D. melanogaster and *D. simulans*	Sympatric	Female pheromone production [95]	D	≥5
D. simulans and *D. sechellia*	Allopatric	Female pheromone production [56, 74]	Q	≥11
		Male prezygotic isolation [59]	Q	≥1
		Male copulation duration [59]	Q	≥1
		Male genital morphology [96]	Q	≥7–11
		Male sex comb tooth number [96]	Q	≥4
		Male pheromone production [59, 97, 98]	Q, C	≥1–5
		Female prezygotic isolation [2]	C	≥2
		Male courtship song [99]	Q	≥6
D. simulans and *D. mauritiana*	Allopatric	Male prezygotic isolation [2, 4, 100]	C	≥2
		Male copulation duration [100]	C	≥3
		Male sex comb tooth number [101]	Q	≥2
		Male genital morphology [101, 102]	Q	≥9
		Female prezygotic isolation [2, 13, 103]	C	≥3
		Mau female discrimination [6]	Q	≥7
		Sim male trait [6]	Q	≥3
		Mau male trait [6]	Q	≥6
D. mauritiana and *D. sechellia*	Allopatric	Female pheromone production [104]	R	≥6
D. mojavensis (different populations)	Allopatric	Male courtship success [105]	Q	≥1
		Male copulation latency [105]	Q	≥3
D. mojavensis and *D. arizonae*	Sympatric	Male prezygotic isolation [106]	C	≥2
		Female prezygotic isolation [106]	C	≥2
D. heteroneura and *D. silvestris*	Sympatric	Male head shape [107, 108]	C	≥9-10
D. pseudoobscura and *D. persimilis*	Sympatric	Female prezygotic isolation and reinforcement [109, 110]	Q, I	≥4
		Male prezygotic isolation [111, 112]	C, R	≥3
		Male courtship song [113]	Q	≥2-3
		Female prezygotic isolation [84]	I	≥2
		Pheromone production [114]	C	≥2
D. virilis and *D. littoralis*	Sympatric	Male song production [115]	C	≥3
D. virilis and *D. lummei*	Sympatric	Male courtship song [116]	C	≥4
		Male pheromone productions [117]	C	≥5
		Female pheromone productions [117]	C	≥4
D. virilis and *D. a. texana*	Sympatric	Male prezygotic isolation [118]	Q	≥1
D. virilis and *D. novamexicana*	Sympatric	Male prezygotic isolation [118]	Q	≥1
D. auraria and *D. biauraria*	Sympatric	Male courtship song [55]	C	≥2
D. montana (different strains)	Sympatric	Male pheromone production [119]	Q	≥9
D. santomea and *D. yakuba*	Sympatric	Female prezygotic isolation [7]	Q	≥3
		Male trait [7]	Q	≥3
D. ananassae (different populations)	Sympatric	Assortative mating [120]	H	≥1
D. ananassae and *D. pallidosa*	Sympatric	Female prezygotic isolation [3, 121]	C, I, R	≥2
		Male song production [122]	C	≥2

mate with *D. pseudoobscura* than per*Coy2*allo, but instead they found the opposite: per*Coy2*sym females were less likely to mate with *D. pseudoobscura* than per*Coy2*allo. This suggests that an allele for reduced interspecific mating within a species (*Coy2*sym) can cause the same reduction in interspecific mating when placed within another species [110]. The explanation provided by Ortíz-Barrientos and Noor is that *Coy-2* may be a "One-Allele" mating locus. This theory suggests that one allele (*Coy-2*) can exist in both the sympatric population of *D. pseudoobscura* and in *D. persimilis* population, and aids in the reinforced behavioral isolation between these populations, but not in the basal behavioral isolation. In other words, the same allele causes females of both species to have an increased discrimination against heterospecifics. This is possible if, for example, the gene encodes for increased odor sensitivity or reduced dispersal [125]. This theory would explain why per*Coy2*sym females were less likely to mate with *D. pseudoobscura* than per*Coy2*allo.

6.2. D. ananassae and D. pallidosa. Drosophila ananassae and *D. pallidosa* are present in overlapping pan-tropical geographic regions. Males of both species court females of both species, but there is strong female interspecific female preference that reduces the gene flow between the two. The genetic basis of this behavior was first explored with female F_1 hybrids, which were found to prefer *D. ananassae* males over *D. pallidosa* males [3]. This suggests that *D. ananassae* genes for interspecific female choice must be dominant over those from *D. pallidosa*. The same study created introgression lines to locate the genomic regions responsible for this behavior. A region on the left arm of the second chromosome (homologous to 3R in *D. melanogaster*) near the *Delta* locus was identified to play a role in female species mate choice: females that were almost entirely *D. pallidosa* except for a small region near the *Delta* locus mated significantly more with *D. ananassae* males and significantly less with *D. pallidosa* males [3]. In other words, this locus both increased intraspecific mating in *D. ananassae* and decreased interspecific mating between *D. ananassae* females with heterospecific males. This region was later confirmed by a study that found 2L (3R in *D. melanogaster*) to be important for the willingness of *D. pallidosa* females to mate with *D. ananassae* males, and XL, 2L, and 3R (X, 3R, and 2L in *D. melanogaster*, resp.) for *D. ananassae* female's willingness to mate with *D. pallidosa* males. All of the identified regions had species specific inversions [121], suggesting that regions of the genome with reduced recombination between the species may be more likely to harbor behavioral isolation loci.

6.3. D. santomea and D. yakuba. Drosophila santomea and *D. yakuba* diverged approximately 400,000 years ago [126]. *D. yakuba* is wide-spread across Africa, including some of the islands off of the coast. On one of these islands, *D. santomea* are found [127]. Although this species pair has a small overlapping geographic region, no reinforcement has been observed [128]. Male courtship behavior may contribute to the behavioral isolation between these two species as *D. santomea* males do not court heterospecific females with any vigor. To investigate the genetic basis behind the female interspecific mating, a QTL map was created for female rejection of heterospecific males [7]. Three QTLs were identified for *D. santomea* female discrimination against *D. yakuba* males: two on the X chromosome (homologous to X in *D. melanogaster*) and one on the third chromosome (3R in *D. melanogaster*).

6.4. D. simulans and D. sechellia. Drosophila simulans is a cosmopolitan species, while its closely related sibling species *D. sechellia* is only found on the Seychelles Islands in the Indian Ocean. There is an asymmetrical behavioral isolation between *D. simulans* and *D. sechellia*: *D. simulans* females are less choosy against *D. sechellia* males than *D. sechellia* females are against *D. simulans* males [2]. Hybrids have an intermediate level of *D. simulans* rejection when paired with *D. simulans* males, suggesting an additive genetic basis. Further backcrossing of these F_1 hybrids to *D. simulans* males, and pairing the female offspring with *D. simulans* males, revealed no isolation, and therefore locating the genes for behavioral isolation in *D. sechellia* females is not possible with this technique. When the F_1 hybrids are backcrossed to *D. sechellia* males, and the resulting females were assayed with *D. simulans* males, the second and third chromosomes (2 and 3 in *D. melanogaster*) were found to have a moderate and strong effect, respectively [2].

6.5. D. simulans and D. mauritiana. D. simulans is a cosmopolitan species and *D. mauritiana* is only found on the island of Mauritius in the Indian Ocean. It is thought that *D. mauritiana* resulted from colonization by a recent common ancestor with *D. simulans* about 250,000 years ago [129]. Females of these species are almost identical, and the males are only distinguishable by the shape of their genital arch [130]. Asymmetrical species isolation is present, with *D. simulans* being the less choosy of the two courted females. Although *D. simulans* females are not choosy and readily mate with *D. mauritiana* males, matings between these two species are abnormally short and result in no or limited sperm transfer, decreasing the number of hybrid offspring [2].

The absence of heterospecific mating by *D. mauritiana* females is due to the rejection of males by these females, since females of both species are courted vigorously by males of both species [13]. Hybrids produced by *D. mauritiana* males and *D. simulans* females mate readily with *D. simulans* males, and thus the genes for interspecific mate discrimination in *D. mauritiana* females must be recessive [2, 13]. By backcrossing the hybrids to *D. mauritiana* males, Coyne was able to asses each *D. mauritiana* chromosome's effect on decreasing mating with *D. simulans* males [13], He found each of the main autosomes has very large effects with the effect of X being very small [13]. Further dissections of the second chromosome determined that each arm of the second chromosome contains at least one gene for reducing *D. mauritiana* female mating with *D. simulans* males (2R and 2L in *D. melanogaster*); this method of uncovering

recessive *D. mauritiana* genes also possibly removed *D. simulans* genes for conspecific mate preference—these genes may or may not be one in the same. When the same pairings were examined with a more refined map, seven QTL were identified that contribute to *D. mauritiana* discrimination against *D. simulans* males: two on the X chromosome, two on the second chromosome, and three on the third chromosome (X, 2, and 3 in *D. melanogaster*, resp.) [6].

6.6. D. simulans and D. melanogaster. *Drosophila melanogaster* and *D. simulans* are both cosmopolitan species found worldwide and have broad overlapping geographic distribution. Although both females show some behavioral isolation, *D. simulans* females are far more choosy [131, 132]: interspecific crosses with *D. melanogaster* females are produced with relative ease in the lab, but the reciprocal interspecific cross with *D. simulans* females very rarely occurs [133]. F_1 hybrids made from *D. melanogaster* females are all sterile females, and from the reciprocal cross are all sterile males. Due to the complete sterility of hybrids, the conventional method of QTL mapping is not possible as this would require an F_2 generation, typically through backcrossing to one of the parental species. Therefore, other methods used to determine the genetic basis of behavioral isolation between these two species have been employed.

Using chromosomal substitution, a genomic region was identified on the third chromosome for *D. melanogaster* female receptivity, and genomic regions on all three major chromosomes were identified for rejection of *D. simulans* males by *D. melanogaster* females [85]. Although there is some evidence that male *D. simulans* may have reduced courtship of interspecific females, and thus contribute to the behavioral isolation [132], there is no such evidence for discrimination by *D. melanogaster* males [134]. Therefore, the strong behavioral isolation demonstrated by *D. simulans* females is largely due to rejection of heterospecific (*D. melanogaster*) males.

To investigate whether there is genetic variation for *D. simulans* female preference, different lab strains of *D. simulans* females [86, 135] and *D. melanogaster* males [86] were compared for their rate of interspecific mating. Crossability, the ability for the parental strains or species to successfully produce offspring, varied among strains for both *D. melanogaster* males and *D. simulans* females [86, 87], but were still highly correlated [135]. When strains of *D. simulans* were crossed, the pure species F_1 females were then crossed *D. melanogaster* males and the crossability was compared to the two parental strains. Mixed results were found: while one study found that F_1 females always showed greater levels of hybridization [87], another study found that in most cases F_1 females showed significantly lower levels of hybridization [86], making it unclear whether increased discrimination within *D. simulans* against heterospecifics is dominant or not. Further inconsistencies include one study that found that X and the third chromosome act additively to contribute to the rejection of *D. melanogaster* males by *D. simulans* females [87], while another study found that the X and the left arm of the second chromosome influenced the trait [133]. These results may be due to the low genetic variability within inbred laboratory lines, and may support the hypothesis that the genetic basis of behavioral isolation varies among populations of the same species. Recently, the right arm of the third chromosome (3R) was mapped using deficiency mapping, revealing five regions (all in areas of low recombination) that contribute to the rejection behavior of *D. simulans* females towards a courting *D. melanogaster* male [35]. While a list of candidate genes in these regions was generated, fine mapping of these regions to the individual gene level remains.

6.7. M and Z Forms of D. melanogaster. *Drosophila melanogaster* are found all over the world, usually commensally with humans, and it was once thought that there was gene flow between populations, including those found spread across large continents [136]. However, a Zimbabwe population was found to have twice the amount of genetic variation compared to North American populations, with certain variants only present in Zimbabwe [137]. Females from these Zimbabwe lines (Z) show behavioral isolation against males from cosmopolitan regions (M): when they have the choice, Z females prefer to mate with Z males, but show no postzygotic isolation (hybrid sterility or inviability) when they are mated with M males. Females from cosmopolitan regions also show behavioral isolation with Z males, but it is weaker than that seen in Z females [90]. The genetic basis for this strong preference in Z females was mapped to all three major chromosomes, with the largest effect being contributed by the third chromosome [91]. With the use of recombinant lines and visible markers (dominant mutations to identify which homologous chromosome was inherited from which parental species), the genetic basis of the female preference in Z females for Z males was mapped to four regions: a region of large effect and a region of minor effect on the left arm of the third chromosome (3L) and a region that most likely houses two loci on the right arm of the third chromosome (3R) [92].

7. Conclusions

In the quest to identify the genetic basis of behavioral isolation, genomic regions have been mapped for interspecific female receptivity in a variety of species pairs. These efforts have yielded maps that vary in refinement from whole chromosomes, chromosomal arms, subchromosomal regions, to specific QTLs. Although the genetic basis of female discrimination may be species pair specific [135], one common attribute of these loci is their location in the genome: most of these loci fall within areas of low recombination, such as species inversion polymorphisms, regions near the centromere, and regions near the telomere. Behavioral isolation loci between *D. santomea* and *D. yakuba* were found near the centromere on 3R [7], and near the telomere for both the *D. simulans* and *D. mauritiana* species pair [6] and the M and Z forms of *D. melanogaster* [92]. Loci responsible for the behavioral isolation between *D. ananassae* and *D. pallidosa* [121], and the isolation between *D. pseudoobscura* and *D. persimilis* [84] all fell within interspecific inversion polymorphisms. Although this was

not true for the regions responsible for increased behavioral isolation caused by reinforcement in the latter species pair [109], these loci for reinforcement were not confirmed by further studies [124].

Inversions have also been shown to play a role in within-species assortative mating. Unlike other species of *Drosophila*, *D. ananassae* males have spontaneous meiotic recombination which contributes to the entire species having a high degree of inversion polymorphisms. One inversion, called "alpha," is a large paracentric inversion covering the majority of 2L (3R in *D. melanogaster*). To investigate whether this inversion could contribute to behavioral isolation within this species, Nanda and Singh [120] created karyotypically different strains homozygous for one of three naturally occurring inversions. Through mate choice assays, they found a preference for homogamic matings in all three populations.

Genomic rearrangements, centromeric, and telomeric areas can act as an island of low recombination between two potentially interbreeding populations, allowing for the creation and maintenance of population-specific gene complexes (genes inherited together). Over time, new mutations can occur within theses complexes and, due to reduced recombination [138], can create a population-typical phenotype if the complexes contain variants for local adaptation [139]. Therefore, even in the face of gene flow between the two groups, a new population identity can be created.

While it has been shown that similar sensory systems may be used for both intra- and interspecific mate discrimination, it is unknown whether these two levels of discriminatory behavior have the same genetic basis. Genomic regions identified as influencing species-specific female preference could contain genes that affect either the auditory or olfactory system, as both are used in mate discrimination, or the brain where this information is processed. If these genes could tolerate a genetic variant causing a slight change in function, selection could then act directly on a new allele, or on other genes within this genetic island, to cause different alleles to reach a high frequency in different populations, causing a slight difference in female mating preference between them. If mutations that occur within these regions cause a change in female preference by influencing assortative mating within species [120], these areas can influence behavioral isolation between species, and thus potentially induce a speciation event [84, 140].

The genetic basis of interspecific female preference is a significant component necessary for understanding the genetic basis of species isolation. While many broad-scale mapping studies have allowed for a solid understanding of the genetic architecture underlying female preference—the number and relative location of genomic regions contributing to female discrimination—to date, no individual genes for this trait have been identified. This limits the ability to assess the mechanism by which females process and evaluate heterospecific mating signals, and thus maintain species isolation. As the genetic tools available in *D. melanogaster* become more widely available in other systems, and as new mapping techniques are developed, refined genetic dissection of this trait is becoming more tenable. By identifying the genetic mutations that cause interspecific variation in mating behavior, we can start to understand the biological basis species isolation, and better our understanding on the definition of a species. Perhaps the most interesting aspect, however, is that we can finally begin to understand the molecular basis of sex.

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Research Article

Positive Selection and the Evolution of *izumo* Genes in Mammals

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Most genes linked to male reproductive function have been known to evolve rapidly among species and to show signatures of positive selection. Different male species-specific reproductive strategies have been proposed to underlie positive selection, such as sperm competitive advantage and control over females postmating physiology. However, an underexplored aspect potentially affecting male reproductive gene evolution in mammals is the effect of gene duplications. Here we analyze the molecular evolution of members of the *izumo* gene family in mammals, a family of four genes mostly expressed in the sperm with known and potential roles in sperm-egg fusion. We confirm a previously reported bout of selection for *izumo1* and establish that the bout of selection is restricted to the diversification of species of the superorder Laurasiatheria. None of the *izumo* genes showed evidence of positive selection in Glires (Rodentia and Lagomorpha), and in the case of the non-testes-specific *izumo4*, rapid evolution was driven by relaxed selection. We detected evidence of positive selection for *izumo3* among Primates. Interestingly, positively selected sites include several serine residues suggesting modifications in protein function and/or localization among Primates. Our results suggest that positive selection is driven by aspects related to species-specific adaptations to fertilization rather than sexual selection.

1. Introduction

Molecular evolutionary analysis of protein coding genes show that most genes evolve under purifying selection with few exceptions of rapid change between species driven by positive selection [1, 2]. One class of rapidly evolving genes are those linked to reproduction, and in many cases positive selection has been found to drive their evolution towards species-specific adaptations. Broad-sense sexual selection [3] can lead to rapid evolution of primary sexual traits and genes that function largely to improve reproductive success, thus sexual selection has been suggested as an explanation for the rapid adaptive diversification of reproductive proteins. In mammals, many male reproductive genes have shown evidence of rapid evolution driven by positive selection [4–6]. It has been more difficult, however, to link positive selection to sexual selection, and numerous hypotheses have been proposed to explain this difficulty [7, 8].

One possible explanation is that most genes in mammals are members of gene families that have experienced several rounds of gene duplication. After duplication, genes can follow different paths in terms of their evolutionary trajectory such as the loss or adoption of new functions in different branches within a phylogeny [9]. Also, the use of phylogenetic-based methods to test for selection might be affected by localized bouts of positive selection to specific phylogeny branches. For example, two semenogelin genes transferred in the male's ejaculate contribute to the formation of a copulatory plug in promiscuous species, but only SEMG2 has shown evidence of positive selection that associates with differences among proxies of sexual selection in both Rodents and Primates [10–12]. SEMG1 has shown no evidence of selection using phylogenetic-based tests, but a population genetics study has shown evidence of a selective sweep with low polymorphism in chimpanzees and high divergence driven by positive selection between humans and chimpanzees [13]. Moreover, gorillas, with a polygamous mating system, have lost SEMG1 gene function entirely [14]. These studies seem to suggest that sexual selection bouts have been phylogenetically restricted or perhaps "softened" for SEMG1 but not SEMG2.

The ADAM gene family contains a number of testes and sperm expressed genes of significant reproductive

importance in mammals. Glassey and Civetta [15] found that codon sites within the adhesion domains (regions often linked to cell-cell adhesion) of ADAM2 and ADAM32 in mammals were under positive selection driven by male/female interactions at the molecular level. Subsequent work by Finn and Civetta [16] associated positive selection at ADAM 2 and ADAM18 to the level of promiscuity in primate mating systems (a well-documented proxy of sexual selection). They also noted that the signal of positive selection seen at ADAM18 disappeared when the phylogeny was expanded to include mammals other than primates. This lends further support to the hypothesis that bouts of sexual selection are often restricted to specific phylogenetic groups [16].

The izumo sperm-egg fusion gene (*izumo1*) is a member of the immunoglobulin superfamily (IgSF) of proteins with an important role in sperm-egg fusion. Gene knockouts of *izumo1* produce sterile males whose sperm is unable to fuse to the egg membrane of zona pellucida free eggs [17]. Three other testes-expressed *izumo* genes (*izumo2*, *izumo3,* and *izumo4*) have been recently identified in mammals, with only *izumo4* being expressed in testes and nonreproductive tissues [18]. Although no formal tests of orthology and paralogy have been conducted, sequence comparisons across all *izumo* genes between human, mouse, rat, bull, and dog with the exception of *izumo3* (human, mouse, rat, and guinea pig) have shown that all four *izumo* genes have eight conserved cysteine residues within 144 amino acids with four α-helices hypothesized to exist between these residues [18]. This gene region has been named the izumo domain.

Here we first test orthology among *izumo* genes in mammals. We then explore whether positive selection is widespread across *izumo* genes in mammals or clade-specific, suggesting possible protein subfunctionalization. Our results show that *izumo* genes duplicated before the diversification of mammals and most likely prior to the diversification of vertebrates. Interestingly, most *izumo* genes show no evidence of positive selection and in one case clear relaxation of selective constraints (*izumo4* in Glires). The detected signals of positive selection within genes are either driven by ancient mammalian bouts of selection (*izumo1*) or more recent mammalian group diversification (*izumo3*) with no clear evidence to suggest any role for sexual selection during the diversification of this gene family in mammals.

2. Materials and Methods

2.1. Sequence Data Collection and Phylogenetic Reconstruction. We retrieved nucleotide and amino acid sequences from 23 (*izumo1*), 21 (*izumo2*), 13 (*izumo3*), and 20 (*izumo4*) mammalian species from Ensembl (see Supplementary Table 1 available online at doi:10.1155/2012/958164 for accession numbers). Sequences for all four genes were aligned using the global alignment algorithm ClustalW and visually inspected [19]. To maximize the number of informative sites during phylogenetic reconstructions, we limited the number of species included in the analyses to 16 for *izumo1* and 13 for *izumo2*, *izumo3*, and *izumo4*. To confirm that gene sequences labeled as members of the same *izumo* gene each formed a single monophyletic clade, the entire gene family phylogeny was reconstructed. Different phylogenetic tree models were tested based on protein alignments using the ProtTest 2.4 Server [20]. We choose the model with the lowest AIC (akaike information criterion) value as it takes into account both the likelihood of the model and the number of parameters contained within a model. Phylogenies for the individual *izumo* genes were reconstructed following the same protocol. Phylogenetic reconstructions were carried out using Maximum Likelihood in Mega 5.05 and the reliability of the tree branching assessed using 1,000 replicate bootstraps [21, 22].

2.2. Tests of Selection. Tests of selection were performed using codeml within Phylogenetic Analysis by Maximum Likelihood (PAML; v 4.4e) [23, 24]. For each *izumo* gene, the likelihoods of the M7 and M8 models were calculated to identify phylogenetic groups that had experienced positive selection. The M7 model assumes that ω values lie between 0 and 1 across the sequences, which is indicative of purifying selection or neutral evolution. The M8 model allows ω to exceed 1, which is characteristic of positive selection. To ensure that relaxed selection was not misinterpreted as positive selection, the likelihood of the null model M8a (where ω is fixed at 1) was also compared to that of M8 [5, 25]. When codons within an alignment were identified as being under positive selection, the Bayes Empirical Bayes (BEB) method was utilized to identify the specific codon sites under positive selection [26]. Codon sites with a posterior probability of positive selection higher than 90% were further evaluated based on the nature of the amino acid substitution occurring between species (e.g., cysteine residues influencing secondary structure and phosphorylation sites that can change the function or localization of a protein). We also mapped positively selected sites within protein domains described in the literature [18] or identified using the Motif Scan server [http://myhits.isb-sib.ch/cgi-bin/motif_scan], GenBank's protein site descriptions [http://www.ncbi.nlm.nih.gov/], and through the protein summary page on Ensembl [http://uswest.ensembl.org/index.html].

3. Results

3.1. Phylogenetic Relationship among izumo Gene Family Members. The JTT model of sequence evolution [27] with G, the shape parameter for the gamma distribution, and F, the amino acid frequencies, was identified as the best fit for phylogenetic reconstruction of the *izumo* gene family according to its AIC value (JTT+G+F: lnL = −14693.74; AIC = 29641.5). Results were similar when using a JTT+I+G+F model, where I is the proportion of invariant sites (Table 1). The phylogeny supports orthology of all mammalian *izumo* genes (Figure 1). The duplication events that gave rise to gene family members are ancestral to the diversification of mammals and most likely vertebrates as predicted *izumo* genes from zebrafish,

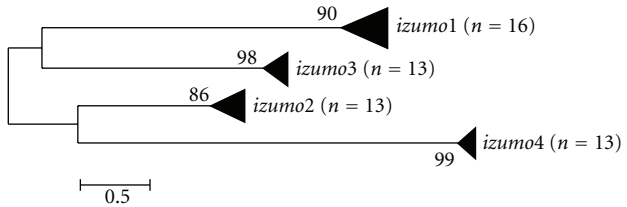

Figure 1: Molecular phylogeny of the *izumo* gene family emphasizing the formation of independent clades. *n* is the number of species included in each gene clade. The evolutionary history was inferred by using Maximum Likelihood and the JTT+G+F substitution model. The tree is drawn to scale, with branch lengths measured as the number of amino acid substitutions per site. The reliability of the tree branching was assessed using 1,000 replicate bootstraps. Bootstrap values lower than 50 are not shown.

Table 1: Phylogenetic model testing for amino acid sequence alignments. Only the top three fits are shown.

Genes	Models	AIC	lnL
All	JTT+G+F	29641.5	−14693.7
	JTT+I+G+F	29642.4	−14693.2
	JTT+G	29647.6	−14715.8
izumo1	JTT+I+G+F	13166.7	−6533.3
	JTT+G+F	13168.7	−6535.3
	JTT+I+G	13173.5	−6555.7
izumo2	JTT+I+G+F	6132.2	−3022.1
	JTT+G+F	6133.8	−3023.9
	JTT+I+F	6147.6	−3030.8
izumo3	JTT+G	5468.6	−2710.3
	JTT+I+G	5470.6	−2710.3
	JTT+G+F	5471.6	−2692.8
izumo4	JTT+I+G	4655.4	−2302.7
	JTT+G	4657.4	−2304.7
	HIVb+I+G	4663.7	−2306.9

anole lizard, and the living fossil coelacanth (Genbank: XP_002663229, Ensembl: ENSACAP00000018570, and Ensembl: ENSLACT00000025764, resp.) were orthologs to their mammalian counterparts instead of forming outgroups (data not shown).

We then tested organismal clades within each *izumo* gene phylogeny. ProtTest identified JTT+I+G+F as the best model for *izumo1* and *izumo2* (lnL = −6533.3; AIC = 13166.7 and lnL = −3022.1; AIC = 6132.2, resp.). Models JTT+G and JTT+I+G were found as the best fits for *izumo3* and *izumo4* (lnL = −2710.3; AIC= 5468.6 and lnL = −2302.7; AIC = 4655.4, resp.) (Table 1). All *izumo* genes show good support for Primates as a distinct clade (bootstrap values higher than 90%) but grouping within the Glires, which includes orders Lagomorpha and Rodentia, and within the superorder Laurasiatheria were weaker. The best bootstrap values for Glires (86%) and Laurasiatheria (89%) as distinct clades were for *izumo3* and *izumo1,* respectively (Figure 2). Particularly poor was the resolution of Glires and Laurasiatheria as two separate clades for *izumo4* (Figure 2).

3.2. Evidence of Selection for izumo Genes. A previous study had identified evidence of positive selection at *izumo1* using M8 *versus* M8a models within PAML in a phylogeny including human, rat, and mouse, plus a combination of either chimpanzee or macaque and dog or bull [10]. We replicated the analysis and confirmed previous results, but noted that the detection of positive selection was sensitive to the species included in the analysis as we failed to detect positive selection when the tree included both bull and chimpanzee ($2\Delta\ell_{\text{M8a-M8}} = 2.48; P = 0.12$). This PAML analysis was subsequently carried out for *izumo2–4* with all four possible species combinations for each gene. Positive selection was only identified once more: when dog, human, macaque, mouse, and rat were examined together for *izumo2* ($2\Delta\ell_{\text{M8a-M8}} = 10.29; P = 0.001$).

Tests of selection were subsequently performed with additional species that were found to cluster into the groups of Primates, Glires, or Laurasiatheria for each gene. Different combinations of species were utilized to include both the maximum number of sequences available per group, as well as the same species across all four genes. Among Primates, *izumo2* ($2\Delta\ell_{\text{M8a-M8}} = 6.10; P = 0.014$) and *izumo3* ($2\Delta\ell_{\text{M8a-M8}} = 8.91; P = 0.003$) showed evidence of positive selection but the signal of selection for *izumo2* was dependent on the species included in the analysis (Table 2). None of the *izumo* genes showed evidence of positive selection among Glires. *Izumo1* appeared more highly conserved than others based on comparisons of models' likelihoods, with *izumo4* showing evidence of relaxation of selective pressures (Table 2). Finally, we only found evidence of positive selection for *izumo1* among Laurasiatheria, and the results were consistent regardless of the species included in the analysis ($2\Delta\ell_{\text{M8a-M8}} = 30.22; P < 0.001$ and $2\Delta\ell_{\text{M8a-M8}} = 5.92; P = 0.015$, resp.) (Table 2).

Among *izumo1* sequences for species of the superorder Laurasiatheria, nine amino acid sites within the izumo domain (representing 6.4% of the domain), nine within the immunoglobulin domain (8.5%), and ten at the caboxi-end of the protein (21%), two of which fell within the 15 site transmembrane domain (13%), had posterior probabilities higher than 90% of being under positive selection (Figure 3). Among Primates, only two amino acids within *izumo2* had posterior probabilities higher than 90% of being under positive selection, with the two sites located within the izumo domain (data not shown) and the signal of selection being sensitive to the number of species included in the analysis. An estimate of ω per branch within the *izumo2* phylogeny showed that only when nine species of Primates were included, the branch leading to macaques had an ω higher than one ($\omega = 1.31$). All eleven positively selected sites at *izumo3* among Primates were located within the first half of the izumo domain (11%) (Figure 4). Five of the eleven amino acid sites, and four out of six with posterior probabilities higher than 95% of being under positive selection, included substitutions involving serine residues, suggesting modifications in protein function and/or localization. It is interesting to notice a pattern of preservation of the serine residues only between human, chimpanzee and gibbon, or gorilla and marmoset (Figure 4).

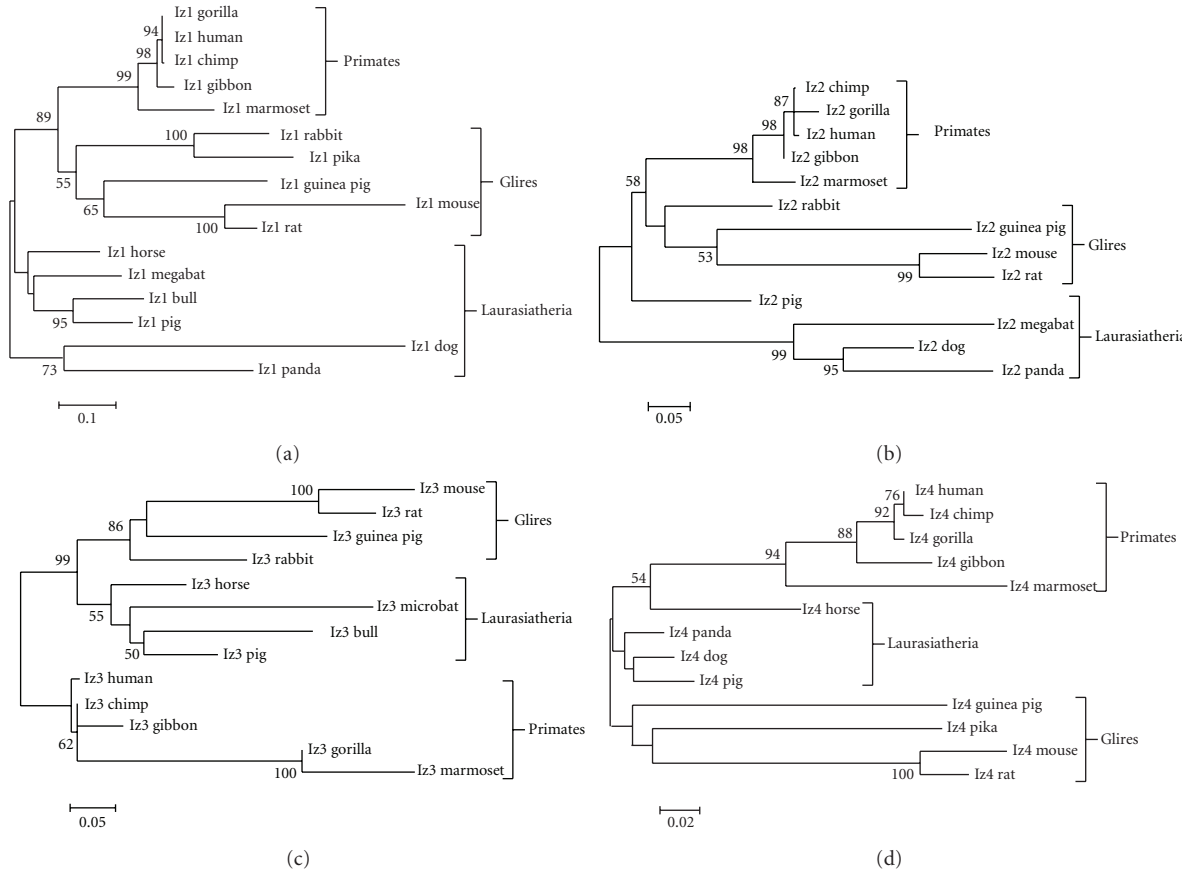

Figure 2: *Izumo* genes phylogenies. Molecular phylogenetic relationships of mammalian species within *izumo1* (a), *izumo2* (b), *izumo3* (c), and *izumo4* (d) emphasizing the formation of common clades between orthologs (superorder Laurasiatheria, clade (grandorder) Glires, and order Primates). The evolutionary history was inferred by using Maximum Likelihood and different JTT substitution models (see Table 1). The trees are drawn to scale, with branch lengths measured as the number of amino acid substitutions per site. The reliability of the tree branching was assessed using 1,000 replicate bootstraps. Bootstrap values lower than 50 are not shown.

4. Discussion

We have not found a consistent pattern of selection for a specific *izumo* gene across phylogenetic groups but rather signals that are gene and phylogenetic group specific. This is evident by the fact that two *izumo* genes have experienced positive selection among species of different well-supported phylogenetic groups. Positive selection at *izumo1* in mammals appears to be driven by selective bouts that have primarily affected the evolution of the wider group of species within the Laurasiatheria superorder. Species included in the analysis for which we detected positive selection diversified on average approximately 80 MyA with the closest relatives being pig and bull (60.5 MyA) [28]. Thus, positive selection is most likely the consequence of an ancient bout of selection and not linked to species-specific adaptations. On the other hand, *izumo3* showed evidence of selection among more recently diversified species of Primates with the most diverged pairs of New World monkey and Apes diversifying approximately 42.5 MyA [28]. While the bout of selection at *izumo3* is more likely linked to species-specific adaptations, the fact that positively selected sites include a large proportion of serine residues that group marmosets and gorillas away from human, chimpanzee and gibbon suggests that the selective bout is not linked to sexual selection. This is because both groups, although small in numbers, include species with very different mating systems such as gibbons (monogamous) and chimpanzee (polygynandrous) and gorilla (polygynous) and marmoset (polyandrous).

It is unclear what drives the different patterns of positive selection at *izumo1* and *izumo3*. It has been recently shown that the IZUMO1 protein forms homo-multimers that are likely essential for the formation of a sperm membrane multiprotein complex with a crucial role in fertilization [18]. IZUMO1 has also been shown to form complexes that are reproductively nonessential with another protein (ACE3) in mice [29]. Ellerman et al. [18] have found evidence suggesting that the transmembrane domain and/or the cytoplasmic tail of IZUMO1 functions in forming multimers while the izumo domain is required for the formation of homo-dimers. Little is known about the function of *izumo3* but its expression is independent of the presence of *izumo1*, and the IZUMO3 protein appears to form only homo-dimers [18]. Thus, it is not surprising that IZUMO1, with its central role in mammalian fertilization and its unique ability to form

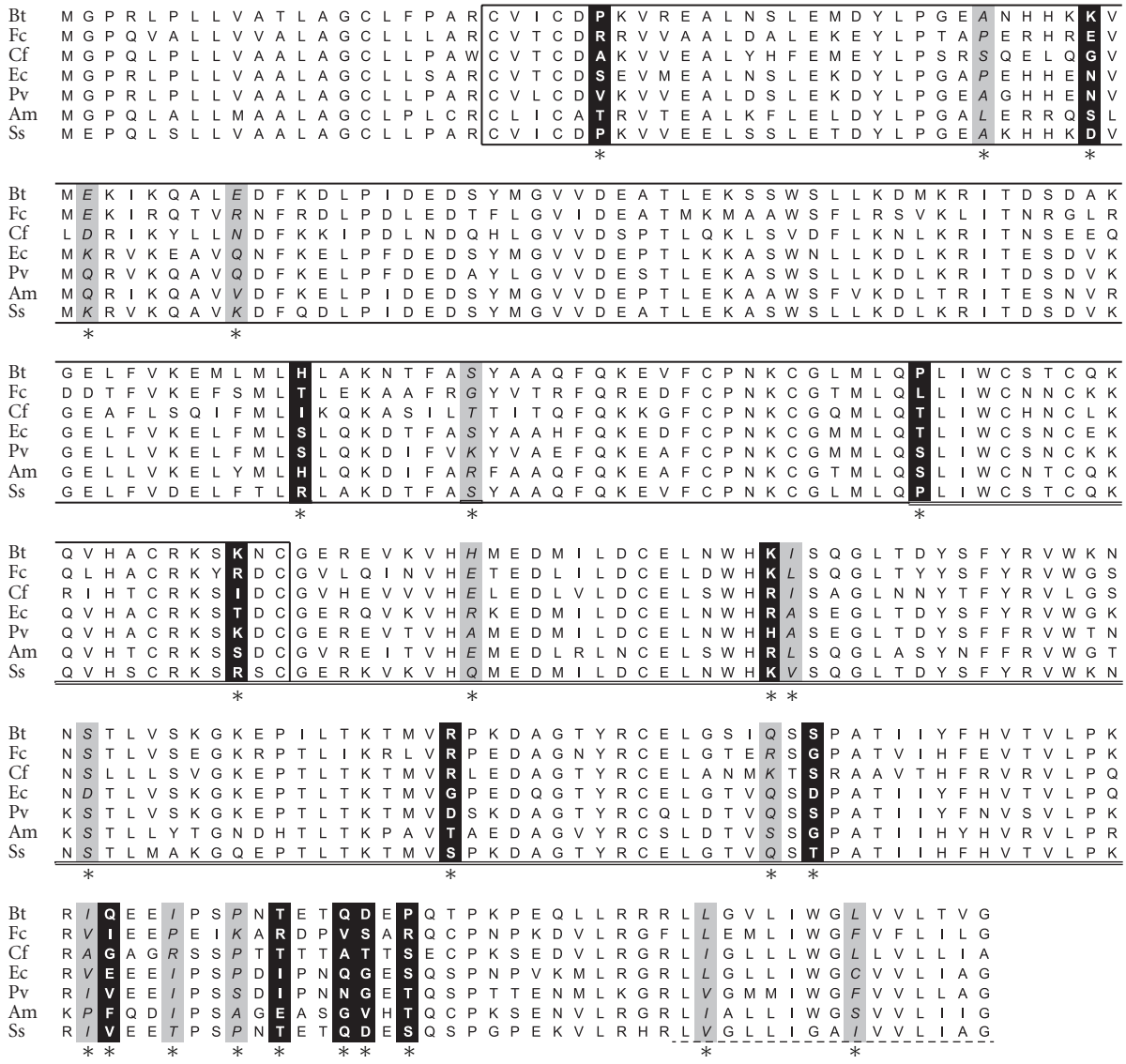

Figure 3: Amino acid sequence alignment of IZUMO1 for species of the superorder Laurasiatheria. Distribution of positively selected sites (see asterisks) with posterior probability higher than 90% (grey background/italic font) and 95% (black background/white font). The izumo domain is boxed (single black line), the immunoglobulin domain is double underlined, and the transmembrane domain is underlined by a dotted line. Two letters are used to indicate species: Bull/Cow (*Bos taurus*—Bt), Cat (*Felis catus*—Fc), Dog (*Canis lupus familiaris*—Cf), Horse (*Equus caballus*—Ec), Megabat (*Pteropus vampyrus*—Pv), Panda (*Ailuropoda melanoleuca*—Am), and Pig (*Sus scrofa*—Ss).

multimers, would be more conserved among closely-related species groups, whereas *izumo3*, with a more limited number of protein interactions, could have evolved towards different adaptations among more closely related species.

The differences in the locations of the positively selected sites between *izumo1* and *izumo3* are also interesting. The IZUMO1 protein has positively selected sites throughout an uncharacterized region and the izumo, immunoglobulin, and transmembrane domains. The transmembrane domain of IZUMO1 has been linked to the formation of multiprotein complexes, which have not been reported for any other IZUMO protein [18]. The IZUMO3 protein is only capable of forming homo-dimers, and all positively selected sites fell within the izumo domain, with no positively selected sites within the transmembrane domain or uncharacterized regions. This finding leads to two competing hypotheses. The ability to form both dimers and multimers is an ancient adaptively important function of *izumo* genes but all current members (except IZUMO1) have lost the ability to form multimers, or the ancestral *izumo* gene was only able to form dimers, and IZUMO1 gained the ability to form multimers sometime after the ancestral gene duplicated. The fact that positively selected sites within the transmembrane domain were only found in IZUMO1 for a group comparison that included the most distantly related mammalian species (Laurasiatheria), but no other species groups, lends indirect support to the idea that the multimer forming ability of *izumo1* is an ancient function. Thus, it is

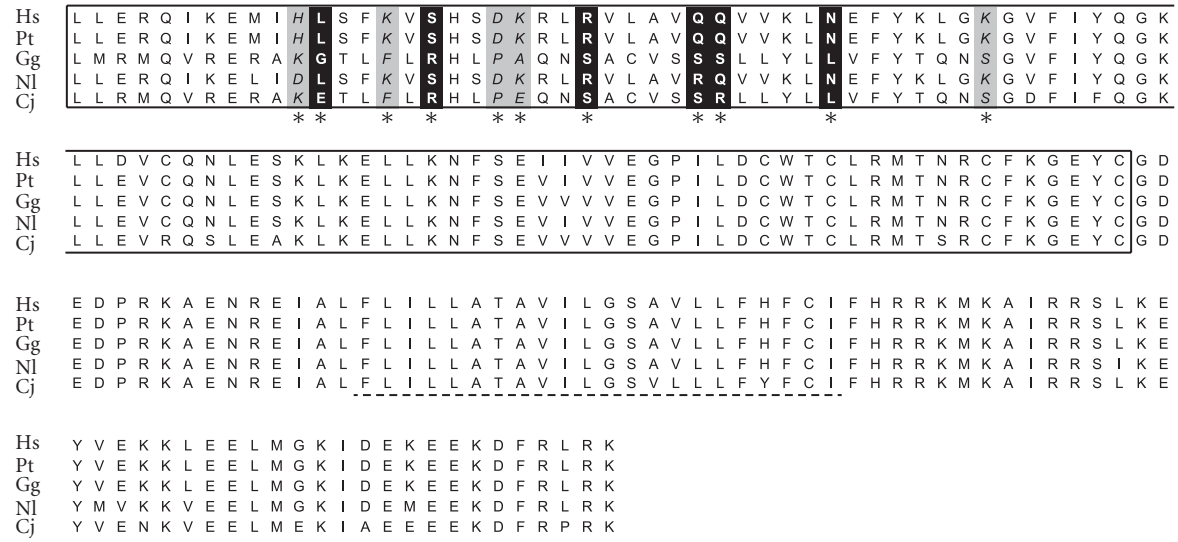

Figure 4: Amino acid sequence alignment of IZUMO3 for species of the order Primates. Positively selected sites and protein domains are indicated as in Figure 3. Two letters are used to indicate species: Human (*Homo sapiens*—Hs), Chimpanzee (*Pan troglodytes*—Pt), Gorilla (*Gorilla gorilla*—Gg), Gibbon (*Nomascus leucogenys*—Nl), and Marmoset (*Callithrix jacchus*—Cj).

Table 2: Evidence of positive selection at *izumo* genes within three major groups of mammals.

Group	Gene	n	p_{seq}	$2\Delta\ell$ (M8-M7)	p_1; ω	$2\Delta\ell$ (M8-M8a)
Primates	*izumo1*	9; 5	0.66; 0.99	1.94; 0.62		
	izumo2	9; 5	0.72; 0.81	6.79*; 0.15	0.04; 5.39	6.10*
	izumo3	5	0.73	9.03*	0.36; 2.36	8.91**
	izumo4	9; 5	0.73; 0.76	4.19; 3.43		
Glires	*izumo1*	7; 4	0.46; 0.86	1.33; 1.17		
	izumo2	6; 4	0.83; 0.99	4.33; 3.64		
	izumo3	4	0.91	4.82		
	izumo4	5; 4	0.76; 0.83	10.99**; 4.57	0.08; 1.48	0.94
Laurasiatheria	*izumo1*	7; 4	0.77; 0.82	39.75***; 7.03*	0.24; 2.57	30.22***; 5.92*
	izumo2	6; 4	0.48; 0.76	0.31; 0.88		
	izumo3	4	0.68	4.85		
	izumo4	6; 4	0.66; 0.79	0.00; 0.00		

n : number of species included; p_{seq}: proportion of sequence analyzed after removing gaps; $2\Delta\ell$: the difference in likelihood estimates from different models of selection (see methods); p_1: proportion of codon sites under positive selection; ω: d_N/d_S per codon for sites under positive selection. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

more likely that other *izumo* genes have lost this function. Moreover, nonmammalian orthologs have been found for *izumo1* in zebrafish and coelacanth and for *izumo2* in lizard. The zebrafish and coelacanth lineage is approximately 100 million years more ancient than the lizard lineage when comparing both to mammals [28], lending further indirect support to the hypothesis that the ability to form multimers is an ancestral state, although we must concede that this finding could be influenced by the current state of genomes functional annotations.

Given that all four *izumo* genes are expressed in sperm, the different patterns of selection among mammalian groups lends support to protein subfunctionalization rather than neofunctionalization or gene loss, but the expression of *izumo4* in tissues other than the testes and sperm should not be overlooked. The rapid evolution found to be driven by relaxed selection in the Glires might be indicative of an ongoing process of gene loss [30]. Species-specific functional assays should be carried out on these genes and proteins to determine how an ancestral function might have been partitioned among gene family members (subfunctionalization) or whether new functions might have arisen from a simpler ancestral protein (neofunctionalization).

Most *izumo* genes appear to have evolved among mammals under different levels of purifying selection. The fact that the divergence of several *izumo* genes cannot be linked to positive selection should serve as a cautionary message against a preconception that rapid divergence among testes-specific genes lends support to species-specific adaptations. Moreover, signals of positive selection at testes or sperm specific genes should not be directly associated with sexual selection as in many cases, particularly in mammals, such

signals might be driven by clade or species diversification in fertilization requirements, immune-related challenges, or even influenced by patterns of genome duplication and the gene's genomic environment [7, 8].

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Research Article

Allelic Expression of *Drosophila* Protamines during Spermatogenesis

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In typical somatic cells, DNA is tightly organized by histones that are necessary for its proper packaging into the nucleus. In sexually-reproducing animals, the haploid product of male meiosis must be further condensed to fit within sperm heads, thus requiring an even greater degree of packaging. This is accomplished in most organisms by replacing histones with protamines, which allows DNA to be compacted into the reduced space. In mammals, protamines are produced after meiosis is complete and are transcribed by the single allele present in the haploid genome that is to be packaged into the sperm head. Here, we present our findings that protamine expression occurs from both alleles in diploid cells, rather than haploid cells, in two species of *Drosophila*. The differential allelic expression of protamines likely influences the selective pressures that shape their evolution.

1. Introduction

Spermatogenesis is a highly orchestrated process that, when operating properly, results in functional and motile sperm. The maturation of spermatids into fully functional spermatozoa occurs in the final stages of spermatogenesis, known as spermiogenesis. Here, chromatin reorganization and an increased level of compaction are essential for proper packaging of nuclear material into the sperm heads [1, 2]. This packaging is necessary for proper sperm head morphology, sperm motility, protection against DNA damage, and the ability to penetrate an ovum [3–5].

Unlike somatic cells, where histones serve to condense DNA, most organisms use protamines to properly organize DNA into a more highly condensed state within the sperm head [6]. Protamines increase the ability of DNA to be packed more tightly by organizing the DNA in linear, side-by-side arrays, rather than by induced supercoiling, with further stability achieved through protamine cysteine-cysteine residue interactions [6, 7]. In mammals, transcription of protamines occurs in the haploid genome, after meiosis is complete [8]. Histones are first replaced by transition proteins TP1 and TP2, followed by protamines [9]. To date, it is unclear if the haploid expression of protamines occurs only in mammals, or if this allelic expression is consistent across all sexually-reproducing animals.

Extensive studies on the genes that encode for protamines have mostly been performed in vertebrates, particularly in mammalian models (reviewed in [10]). With respect to invertebrates, two genes have been identified and characterized in the fruit fly, *Drosophila melanogaster*: *Mst35Ba* and *Mst35Bb.* These genes encode for *Drosophila* protamine A (protA) and protamine B (protB), respectively [11]. Interestingly, *in situ* hybridization in *D. melanogaster* uncovered the presence of these protamine transcripts in primary spermatocytes (diploid cells), which have yet to undergo meiosis [11]. This raises the interesting possibility that insects may differ in temporal expression of protamine genes than in mammals. Furthermore, this has implications for the parental influence of protamines and their evolution: in haploid cells, only one parent contributes the genes coding for the protamines used to package the sperm head, while in diploid cells, both parental genomes may be used when transcribing protamines.

Here, we present our findings on protamine production in two related species of *Drosophila*: *D. simulans* and *D. mauritiana*. To determine the parental contribution towards protamines, and thus whether they are contributed by one

parent's genome (one allele) or both parent's genomes (two alleles), we use transgenic flies that produce a red fluorescent protein (RFP) or green fluorescent protein (GFP) attached to protB [12]. The sperm heads of these transgenic flies emit a red or green fluorescent signal due to the tagged protamines. By crossing a male possessing the transgene of one fluorophore (e.g., RFP) with a female carrying the transgene of the other fluorophore (e.g., GFP) and examining the sperm fluorescence of the male offspring, henceforth referred to as a transgenic hybrid, we can elucidate when protamine gene expression occurs. During *Drosophila* male meiosis, the synaptonemal complex is absent and chromosomes do not undergo recombination [13, 14], and thus the male offspring produced from these crosses cannot recombine the two separate transgenes onto a single chromosome in their sperm. Therefore, the sperm that is produced will only exhibit fluorescence due to either a GFP- or RFP-tagged protamine, but not both. If transcription occurs from a single allele, then we should observe a single fluorescent signal of either red or green. In contrast, dual expression of RFP or GFP within one sperm head provides evidence of diploid gene expression from both alleles. Our results provide concrete evidence that the protamines present in sperm heads are transcribed during the diploid phase of sperm development from both alleles in the genome. This increases the likelihood that the allelic, and thus possibly overall timing, of protamine expression may vary widely across different species.

2. Materials and Methods

All flies and crosses were maintained on standard Bloomington recipe media (Bloomington Drosophila Stock Center, Bloomington, IN, USA) and flies were housed at 22°C on a 14 h : 10 h light:dark cycle. Transgenic *D. simulans* and *D. mauritiana* flies with GFP- and RFP-tagged protamines were kindly provided by Dr. John Belote. Transgenic *D. simulans* lines possessed either a GFP-tagged protB (genotype: w^+; pBac{3xP3-EGFP, ProtB-EGFP}11B) or a RFP-tagged protB transgene (genotype: w; P{w8, ProtB-DsRed-monomer, w^+}3A). Likewise, *D. mauritiana* transgenic lines also possessed either a GFP-tagged protB transgene (genotype: w; P{w8, ProtB-EGFP, w^+}8A) or a RFP-tagged protB transgene (genotype: w; P{w8, ProtB-DsRed-monomer, w^+}13A).

Five-day-old virgin *D. simulans* males carrying the protB-GFP transgene were mated with five-day-old virgin *D. simulans* females carrying the protB-RFP transgene. The reciprocal cross was also made. The same set of crosses was performed with equivalent *D. mauritiana* GFP and RFP transgenic flies. Testes of newly eclosed transgenic hybrid males (1-2 days old) were dissected in Testes Buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl) and squashed using a cover slip. Images of fluorescent sperm were captured using fluorescent imaging on a Leica DMI6000 B inverted microscope and were analyzed using MetaMorph. Some samples were captured using Z-stacking and deconvolved with AutoQuant deconvolution software.

We did note that transgenic flies possessing RFP-tagged protamines exhibited a lower fluorescent intensity than those expressing GFP-tagged protamines. Therefore, contrast and brightness levels were adjusted for some images to allow for clear visualization of the presence or absence of fluorescence. Images of sperm with only GFP- or RFP-tagged protamines were not adjusted; however, contrast and brightness levels of sperm from transgenic hybrids required minor changes to offer better simultaneous visualization of both fluorescent protamines.

3. Results and Discussion

Previous work on mammals found that protamines, used for packaging DNA into sperm heads, are expressed from the haploid genome after meiosis. Although it has been shown that protamines are also expressed in the insect *D. melanogaster*, and are expressed in diploid cells prior to meiosis [11], it has not been shown whether this expression occurs from a single allele, as in mammals, or if both alleles are expressed. Additionally, diploid expression has yet to be confirmed in other species of *Drosophila*. Here, we created transgenic hybrid flies that can produce protamines tagged by two fluorophores (GFP and RFP) from the diploid genome, but only one fluorophore (GFP or RFP) from the haploid genome. This allows us to determine if protamines are expressed during the haploid or diploid phase of the developing sperm, and if they are expressed in diploid cells, whether their expression derives from a single allele or both alleles.

To ensure the dual fluorescence from RFP and GFP in the transgenic hybrids is not a product of autofluorescence, male flies with only one transgene were dissected and sperm were scored for both red and green fluorescence (Figures 1(a)–1(l)). Transgenic flies possessing either RFP- or GFP-tagged protamines in *D. simulans* (Figures 1(a)–1(f)), as well as *D. mauritiana* (Figures 1(g)–1(l)), exhibited only one signal (Figures 1(c), 1(f), 1(i), 1(l)). Male transgenic hybrids possessing both the GFP and RFP transgenes had sperm that fluoresced both green and red in *D. simulans* (Figures 2(a)–2(f)) and *D. mauritiana* (Figures 2(g)–2(l)). Signal from RFP- and GFP-tagged protamines could be seen without adjustments; however, contrast and brightness levels were adjusted to enhance visualization of the weaker RFP fluorophore. Although it was not possible to determine at which cellular stage protamines are expressed, since transcription of the fluorophore labelled protein may occur at an earlier stage than translation, we can definitively say that two fluorophores are present in each sperm head, and thus expression must occur within a diploid cell. Therefore, this provides concrete evidence that protamine expression, at least in the *melanogaster* subgroup of *Drosophila*, occurs at the diploid phase from both alleles, rather than in the haploid phase from a single allele, as observed in mammals [15–17].

The results from this study, in addition to previous studies [11, 15–17], raise some interesting questions: are there benefits between haploid versus diploid expression of protamines? Why is there a temporal difference in protamine expression between *Drosophila* and other organisms, where protamine expression has been characterized? Perhaps the answer lies in the sharing of haploid-expressed transcripts

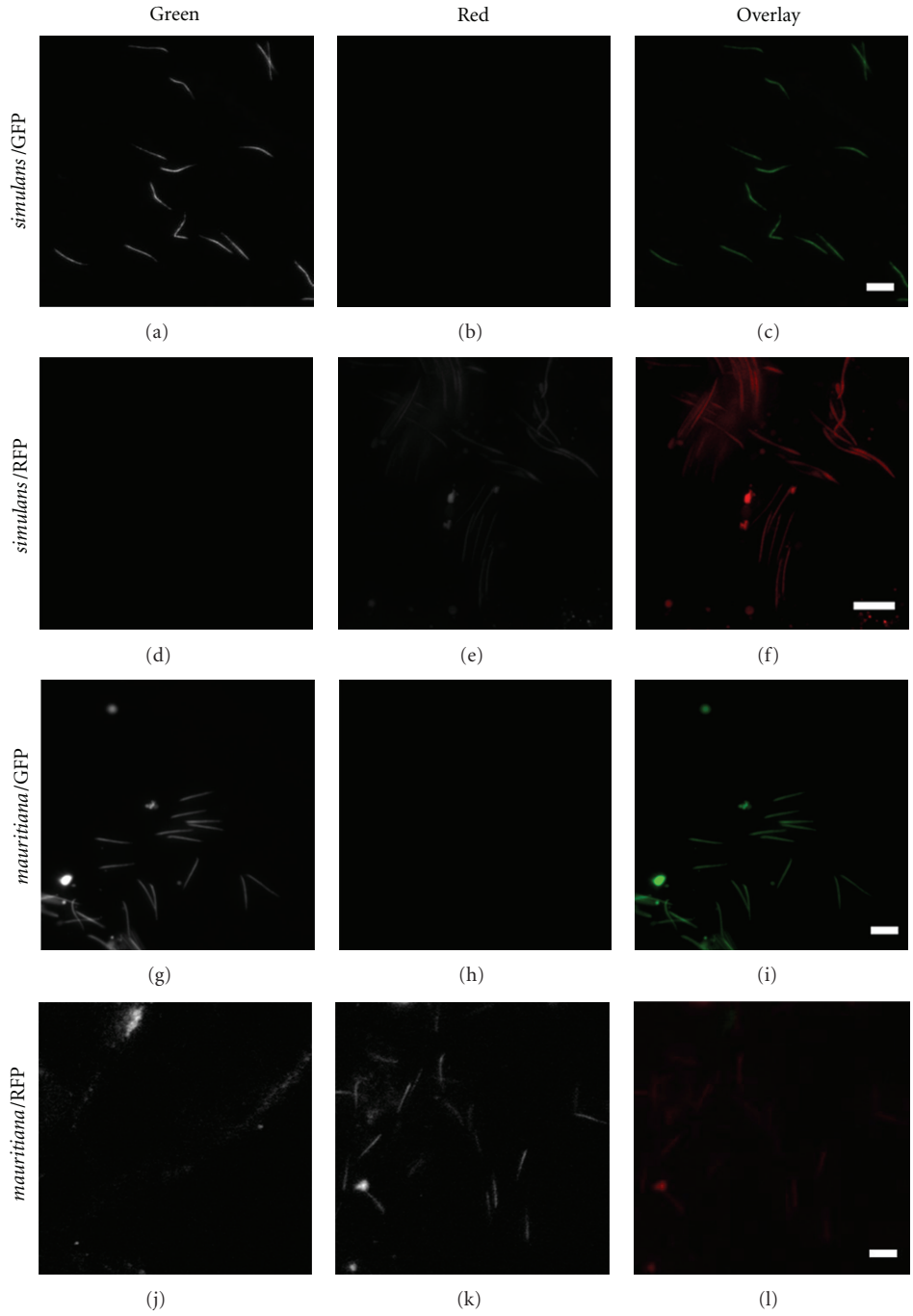

Figure 1: *D. simulans* (a–f) and *D. mauritiana* (g–l) sperm heads possessing GFP-tagged (a–c, g–i) or RFP-tagged (d–f, j–l) protamine. Sperm containing the protB-GFP transgene only fluoresce green (a, c, g, i) and do not reveal any red light autofluorescence (b, c, h, i). Similarly, sperm containing only the protB-RFP transgene only fluoresce red (e, f, k, l), with no green autofluorescence (d, f, j, l). Images (a–c) and (g–l) were taken at 40x magnification, while images (d–f) were taken at 63x magnification. Bars represent 10 μm.

between connected sperm heads. In mammals, protamine transcripts are shared though cytoplasmic bridges connecting the nonindividualized sperm after meiosis are complete [18]. Even though each protamine is only transcribed from the haploid genome, the individual sperm has access to the transcripts from the diploid genome due to these cytoplasmic bridges. It is possible that nonindividualized sperm heads are not equally sharing postmeiotic transcripts, so it is unclear what the degree of access to both protamines truly is within each sperm head [19]. If sharing is indeed unequal, subtle differences in sperm head packaging may exist between individualized sperm heads due to differences in the protamine

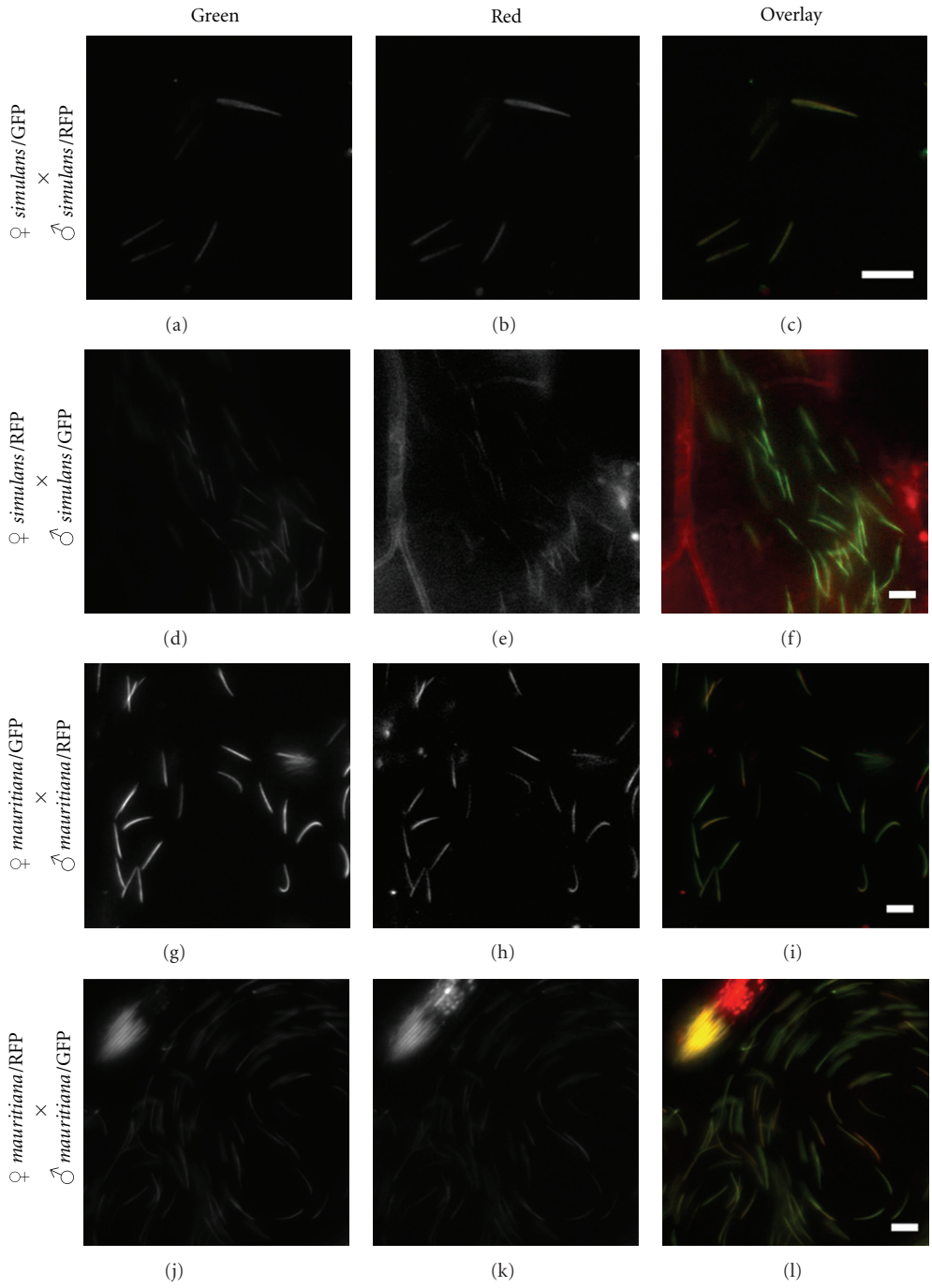

Figure 2: Transgenic hybrids in *D. simulans* (a–f) and *D. mauritiana* (g–l). *D. simulans* females with the transgene possessing the GFP-tagged protamine mated to *D. simulans* males with RFP-tagged protamine transgene (a–c), and the reciprocal cross (d–f) fluoresces both red and green (c, f). Similarly, *D. mauritiana* females with the transgene possessing the GFP-tagged protamine mated to *D. mauritiana* males with RFP-tagged protamine transgene (g–i), and the reciprocal cross (j–l) also fluoresces both red and green (i, l), thus suggesting that protamine expression occurs during the sperm cell's diploid phase. Images (a–c) were taken at 63x magnification, while images (d–l) were taken at 40x magnification. Bars represent 10 μm.

allele that is present in each sperm's haploid genome. This could have a profound effect on the sperm's fertilization success and the individual's overall fitness [20], resulting in strong purifying selection on protamine alleles. In contrast, organisms with protamine expression prior to meiosis from the diploid genome will ensure equal protamine transcripts across all sperm heads, and thus individual protamine alleles may have a lesser impact on sperm function. This

would prove to be especially important for species that are polygamous and undergo sperm competition within the reproductive tract [21, 22].

The expression of protamines during either the haploid or diploid phase in different species may indicate that there are benefits or costs to expression during one phase compared to the other. There may be ramifications of haploid gene expression which are alleviated by diploid expression. For example, protamine expression during the haploid phase may cause sperm from a single male to be more phenotypically different from each other, as well as from the diploid male [20]. As such, sperm derived from one male may potentially compete with each other, setting up a conflict of interest between the sperm and the male, as each sperm competes to successfully fertilize the egg, potentially affecting the male's ability to maximize his own fitness [23, 24]. Further studies may identify an advantage of protamine expression in the haploid versus diploid phase, and how species benefit uniquely to one expression pattern over the other.

Although many stages within spermiogenesis are conserved between *Drosophila* and mammals, there are major differences, including the findings from this paper, on the timing and genomic contribution towards protamine expression. Mice and humans have two protamines that likely arose due to a gene duplication event [25, 26]. These genes are haploinsufficient and require two fully functional copies in order to prevent male sterility [27]. *Drosophila* also possesses two protamine genes, again likely due to a gene duplication event, but each copy is not haploinsufficient [28]. In determining the functional significance of the protA and protB genes, it was surprising to discover that male flies with homozygous deletions for both protamine genes at the same time did not have a reduction in sperm motility or fertility, although approximately 20% had abnormally-shaped nuclei, suggesting some level of protamine functional redundancy [28]. Although fertility was not greatly impacted in these mutant flies, sperm that lacked both protA and protB were more sensitive to X-ray mutagenesis, indicating that the protamines may serve to protect DNA from damage in *Drosophila* [28].

Aside from the implications that sperm packaging has for male fertility, understanding DNA condensation and proper sperm head packaging also has applications from an evolutionary perspective, since there will be different selective pressures on a gene that is expressed only in a haploid state from those that are expressed in a diploid state [29, 30]. To understand the extent of differential protein expression in sperm heads, additional work in characterizing protamines across different taxa will need to be completed to further understand the evolutionary implications of diploid versus haploid gene expression.

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