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Evolutionary biology

Insulin-insensitivity of male genitalia maintains reproductive success in *Drosophila*

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For most arthropod species, male genital size is relatively implastic in response to variation in developmental nutrition, such that the genitals in large well-fed males are similar in size to those in small poorly-fed males. In *Drosophila melanogaster*, reduced nutritional plasticity of the male genitalia is a consequence of low insulin sensitivity through a tissue-specific reduction in the expression of *FOXO*, a negative growth regulator. Despite an understanding of the proximate developmental mechanisms regulating organ size, the ultimate evolutionary mechanisms that may have led to reduced *FOXO* expression in the genitalia have not been fully elucidated. Here we show that restoring FOXO activity in the developing genitalia reduces the male genital size and decreases various aspects of male reproductive success. These data support the hypothesis that sexual selection has acted on the male genitalia to limit their nutritional plasticity through a reduction in *FOXO* expression, linking proximate with ultimate mechanisms of genital evolution.

1. Introduction

In most animals, body proportion stays relatively consistent across a range of body sizes in a population, such that both large and small individuals have the same shape. However, some morphological traits do not follow this pattern and are disproportionally larger or smaller in individuals of different sizes. Perhaps the most obvious examples are of exaggerated secondary sexual characteristics used by males to compete for females, e.g. the horns of rhinoceros beetles and stalked eyes of diopsid flies [1,2]. These traits are disproportionally large in larger individuals and referred to as hyperallometric traits. Less charismatic but probably more prevalent are traits that show the opposite pattern and are disproportionally small in larger individuals, referred to as hypoallometric traits, e.g. brain size in mammals and genital size in male arthropods [3,4]. While the selective pressures that generate hyperallometric traits have been well studied, those generating hypoallometric traits are less well understood. Perhaps the best studied hypoallometric traits are arthropod genitalia, and there are a number of alternative hypotheses to account for their low covariance with body size, most proposing reduced reproductive success in males with atypically sized genitalia [4–6]. Because hypoallometric traits show relatively little variation in a population, however, testing the effects of their size on fitness is challenging.

In contrast to the lack of clarity over the ultimate evolutionary causes of genital hypoallometry, the proximate developmental mechanisms that have been the target of selection are increasingly well understood. In *Drosophila*, the posterior lobe of the genital arch is thought to be involved in reproductive isolation [7]. The lobe scales hypoallometrically with body size and is less sensitive than other traits to genetic and environmental size regulators [8,9]. Perhaps the most important environmental regulator of size is developmental nutrition, which works

through the insulin/insulin-like growth factor signalling (IIS) pathway to control the rate of cell growth and division. A key component of the IIS pathway is the negative growth regulator FOXO, a forkhead transcription factor, which slows growth when nutrition and IIS activity are low. In *D. melanogaster*, the male genitalia express low levels of *FOXO*. This renders the genitalia insulin-insensitive and allows them to maintain growth and lobe size when IIS activity in the rest of the body is low and the size of other traits is reduced [10].

While increasing *FOXO* expression in the posterior lobe restores nutritional sensitivity [10], it is unclear what impact this has on male mating success. We addressed this question by upregulating both FOXO and insulin-receptor (InR) activity in the posterior lobe of the genital arch alone, imposing changes in IIS to produce males with an expanded range of genital sizes beyond that found in wild-type populations, including very small genitalia. We then tested these males' mating ability, both when placed singly with females, and when made to compete directly or indirectly with other males.

2. Material and methods

A complete description of the methods is provided in the electronic supplementary material, and the data and R scripts used to analyse them are provided on Dryad.

We used the *Poxn-GAL4* driver to drive the expression of *UAS-InR.CA* (constitutively active insulin-receptor), *UAS-FOXO.3X* (constitutively active FOXO) and *UAS-GFP* (control) in the posterior lobe of the genital arch of male flies, referred to as LG (large genital), SG (small genital) and CG (control genital) males, respectively. Unwanted neuronal *GAL4* expression was eliminated with *elav-GLA80* [11]. Where necessary, paternity was assigned using the presence/absence of *ubi-GFP*. Flies were isolated at pupation and adults were exposed to potential mates and video-recorded in one of three assays: single-male, direct male–male competition, or indirect male–male competition. Females, which were all *Poxn-GAL4, elav-GAL80; UAS-GFP*, were allowed to oviposit for 48 h, and the number of eclosing adults was counted. Three morphological traits were recorded for each male fly: pupal case area (a proxy for body size), wing area and area of the posterior lobe of the genital arch. We tested for the influence of male morphology on several aspects of male mating success: courtship latency, courtship duration, copulation latency, copulation duration, the probability of copulation, the probability of siring offspring and the number of offspring.

3. Results

Changes in the activity of the IIS pathway expanded the range of posterior lobe sizes among males (figure 1*a*; electronic supplementary material, table S1). Specifically, activating FOXO in the posterior lobe of the genital arch of SG males significantly decreased lobe size by 29% compared with control CG males. By contrast, driving the expression of a constitutively active form of InR in the lobe of LG males did not significantly increase lobe size compared with CG males. LG males had slightly (2.8%) but significantly larger wings than CG males but not SG males. Body size (pupal size) did not vary with genotype.

Males with smaller genitalia had lower mating success when singly mated with females. Across all genotypes, there was a significantly positive relationship between genital size and the probability of a male copulating (electronic supplementary material, figure S1 and table S2) and producing offspring (figure 1*b*; electronic supplementary material, table S3). These trends reflected differences in the mating success of the males of different genotypes. SG males were significantly less likely to both copulate and sire offspring than CG or LG males (electronic supplementary material, figure S2), and produced fewer offspring if they did (electronic supplementary material, figure S3). While LG males enjoyed the same copulation success as CG males, they were slightly less likely to sire offspring (electronic supplementary material, figure S2), although they produced the same number of offspring if they did (electronic supplementary material, figure S3).

Males with larger genitalia had a competitive advantage in both the direct and indirect competition assays. For pairs of directly competing males, the male with the larger posterior lobes was more likely to sire offspring (figure 1*c*; electronic supplementary material, table S4). Correspondingly, the male that mated had, on average, larger posterior lobes than the unsuccessful male (electronic supplementary material, figure S4 and table S5). The male with the larger wing was also more likely to sire offspring (figure 1*c*; electronic supplementary material, table S4), although this did not translate into a significant difference in wing size between successful versus unsuccessful males (electronic supplementary material, table S5). Body size had no influence on the probability of mating (electronic supplementary material, table S4) and did not differ between successful versus unsuccessful males (electronic supplementary material, table S5). For two males mated to the same female in sequence, the male with larger genitalia was also more successful: females that produced offspring from either or both males had more offspring from the male with the larger posterior lobes, when controlling for mating order (figure 1*d*; electronic supplementary material, table S6). Males with larger bodies also sired more offspring in each pair of indirectly competing males (figure 1*d*; electronic supplementary material, table S6). While there was a trend among indirectly competing males for the males that sired offspring to have larger genitalia and wings than males that did not, this trend was not significant (electronic supplementary material, table S7).

We found no significant effect of male genital size class or morphology on courtship latency or duration, nor on copulation duration (electronic supplementary material, tables S8 and S9). Copulation latency was, however, marginally effected by genotype, and was longest for SG and shortest for LG males (although no pairwise comparison was significant) (electronic supplementary material, figure S6).

4. Discussion

In *Drosophila melanogaster*, low levels of *FOXO* expression in the male genitalia limit the negative growth effects of FOXO activity when IIS and nutrition are low and render genital size hypoallometric to body size [10]. Our data show that increased FOXO activity in the genitalia reduces multiple aspects of male mating success. Females are less likely to copulate a male where FOXO is activated in the posterior lobes, less likely to produce offspring from him, and produce fewer offspring with him if she does mate. Selection against males with smaller posterior lobes is also observed when females are exposed to two competing males, either at the same time

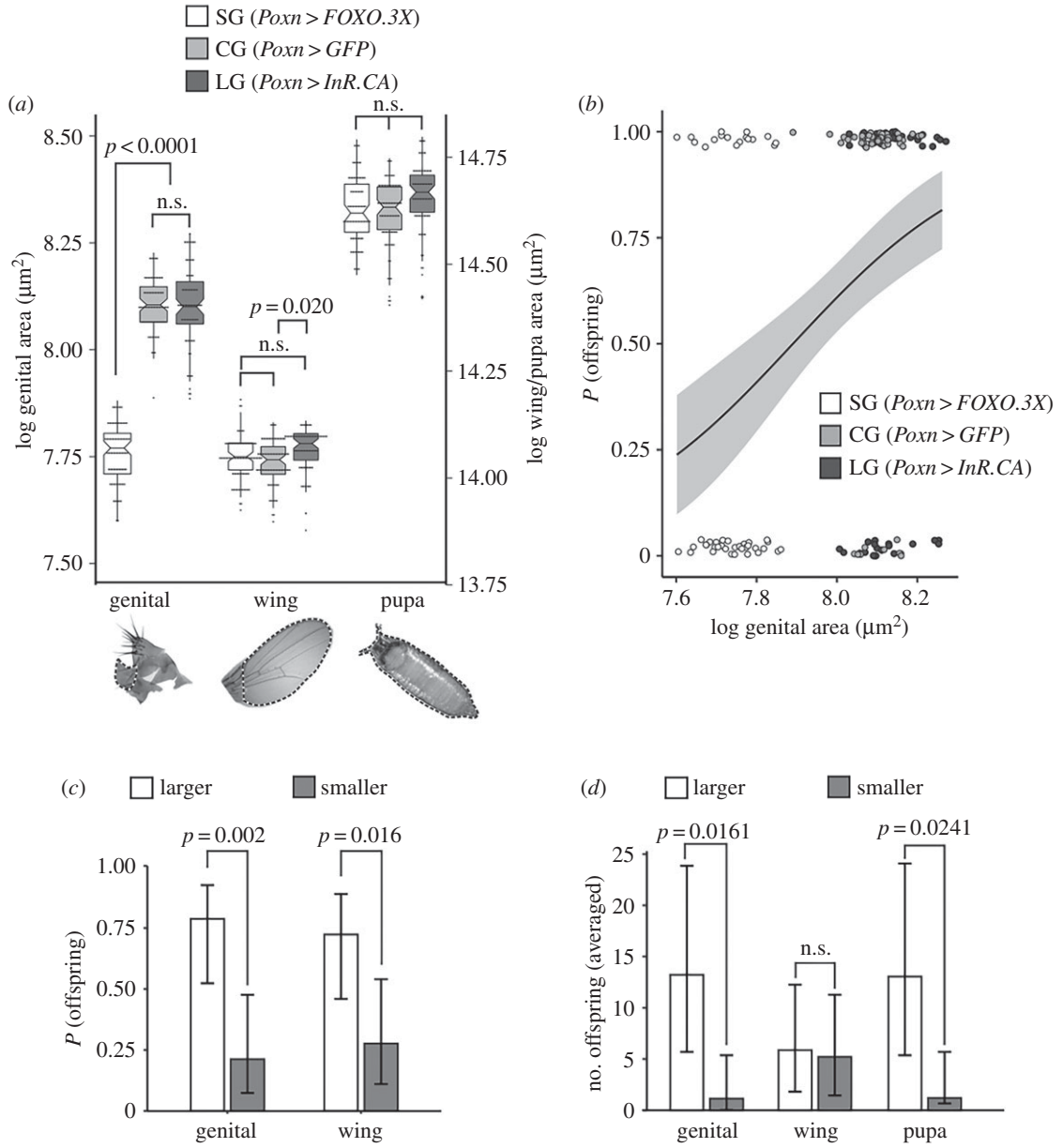

Figure 1. The influence of insulin signalling in the genitalia on male mating success. (*a*) Effect on trait size of driving expression of a constitutively active form of FOXO (*FOXO.3X*) and InR (*InR.CA*) using *Poxn-GAL4*. Images below axis show traits (not to scale) with measurement area outlined by a broken line. (*b*) Effect of genital size on the probability of siring offspring. (*c*) Effect of a male's genital and wing size relative to a competing male on the probability of him siring offspring, for direct competition. (*d*) Effect of a male's genital/wing/pupal size relative to a competing male on his number of offspring, for indirect competition. Error bars/shading show 95% confidence intervals. See electronic supplementary material for statistical details. Significance is taken at $p < 0.05$.

or sequentially. Collectively, these data support the hypothesis that, in *Drosophila*, there is selection against activation of FOXO in the genitalia—and a corresponding selection against reduced genital size—when nutrition and insulin signalling are low. Previous studies have explored the effect of posterior-lobe morphology on male mating success [11,12]. Our study links the proximate with the ultimate mechanisms that generate genital hypoallometry.

While our data reveal the fitness advantages for males to reduce FOXO activity in their genitalia, why they enjoy this advantage is less clear. One possibility is that males with small genitalia are less able to transfer sperm to females, possibly owing to physical incompatibility between male and female genitalia. While we did not measure sperm transfer, a previous study that examined the effect of posterior-lobe size and shape on male mating success found that males with smaller lobes transferred fewer sperm [11]. A second non-exclusive explanation is that females are engaging in cryptic female choice and choosing not to use the sperm from males with reduced genitalia. If male genital size and shape were an indicator of conspecificity then there would be strong selection on females to avoid mating with, or using sperm from, males with small genitalia. However, a study that damaged the lobes of *D. simulans* found no evidence for post-copulatory sexual selection on lobe morphology, although lobe damage did reduce male reproductive success [12]

We also do not know in what direction selection is acting. Some theories of male genital hypoallometry argue that it is a consequence of stabilizing selection on male genital size, either because a female uses genital size as an indication of conspecificity, or because she is physically unable to mate with males with inappropriately sized genitalia [4]. Alternatively, females could impose directional selection on the male genital size that is strong in males with small genitalia

but weak or absent in males with large genitalia [5,6]. However, since we could not generate males with larger genitalia than wild-type, we are unable to assay their impact on reproductive success.

It is intriguing that, unlike for other traits [13,14], activating InR in the genitalia of LG males does not increase their size. Thus, whatever mode of selection has been applied to the genitalia, it appears to have pushed to reduce *FOXO* expression and insulin sensitivity to a minimum. Nevertheless, LG males did show slightly reduced reproductive success when mated singly with females. This is unlikely to be due to their slightly larger wings: wing size was positively correlated with male mating success in our competitive assays of mating success. Nor is it likely to be due to genetic background effects—all transgenes were backcrossed into a common background for five generations—or *InR.CA* expression outside of the genitalia—*Poxn-GAL4, elav-GAL80* detectably drives expression only in the developing genitalia [11]. One possibility is that, while hyperactivation of the IIS pathway does not increase posterior lobe size, it does impact other aspects of lobe morphology, not measured in this experiment.

We did not explore the effect of female genital size on male mating success, but future studies should address this important (and understudied) aspect of genital evolution.

Ethics. Research was conducted according to Michigan State University guidelines.

Data accessibility. Data and R scripts for analysis are available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.44bm68r [15].

Authors' contributions. A.P.D. and A.W.S. conceived of the study. A.P.D. designed and conducted the experiments. A.P.D. and A.W.S. analysed the data and drafted the manuscript. All authors approved the final version of the manuscript and agree to be held accountable for the content therein.

Competing interests. We declare we have no competing interests.

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