

# Intraspecific evidence from guppies for correlated patterns of male and female genital trait diversification

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The role of sexual selection in fuelling genital evolution is becoming increasingly apparent from comparative studies revealing interspecific divergence in male genitalia and evolutionary associations between male and female genital traits. Despite this, we know little about intraspecific variance in male genital morphology, or how male and female reproductive traits covary among divergent populations. Here we address both topics using natural populations of the guppy, *Poecilia reticulata*, a livebearing fish that exhibits divergent patterns of male sexual behaviour among populations. Initially, we performed a series of mating trials on a single population to examine the relationship between the morphology of the male's copulatory organ (the gonopodium) and the success of forced matings. Using a combination of linear measurements and geometric morphometrics, we found that variation in the length and shape of the gonopodium predicted the success of forced matings in terms of the rate of genital contacts and insemination success, respectively. We then looked for geographical divergence in these traits, since the relative frequency of forced matings tends to be greater in high-predation populations. We found consistent patterns of variation in male genital size and shape in relation to the level of predation, and corresponding patterns of (co)variation in female genital morphology. Together, these data enable us to draw tentative conclusions about the underlying selective pressures causing correlated patterns of divergence in male and female genital traits, which point to a role for sexually antagonistic selection.

**Keywords:** sexual selection; genitalia; coevolution; sexual conflict

## 1. INTRODUCTION

Male genitalia typically exhibit striking patterns of diversity and specificity and are among the most rapidly evolving morphological traits in animals with internal fertilization [1]. Indeed, genital structures exhibit far greater levels of complexity than is thought necessary for simply transferring sperm to females, and there is now widespread evidence that sexual selection is responsible for such complexity [1,2]. Much of this evidence comes from studies linking variation in male genital morphology to either mating success [3–6] or paternity success [7–11], although further evidence comes from comparative studies revealing positive associations between the rate of divergent evolution of male genitalia and the intensity of sexual selection acting on these traits (e.g. [12]).

An increasing body of work has focused on the potential for sexual selection to fuel coevolutionary patterns of selection on male and female reproductive traits. To date, the available studies revealing such patterns have focused

on interspecific variation in male genitalia and associated covariance in female reproductive traits [13–20]. Yet, there is also considerable intraspecific variation in male genital morphology in some species [21–24], although in many of these cases the functional basis for such variation remains enigmatic, and the evolutionary relationships with female reproductive traits have yet to be elucidated.

Poeciliids are a family of livebearing fishes with internal fertilization in which the males' anal fin is modified to form a copulatory organ, the gonopodium. The shape and length of the male gonopodium exhibit substantial inter- and intraspecific variability [25–28], and this morphological variation is thought to be associated with the frequency with which males employ gonopodial thrusting, a coercive mating tactic in which males forcibly inseminate females [25,29–31]. Among the poeciliid family, guppies (*Poecilia reticulata*) also exhibit substantial intraspecific divergence in male genital traits, which in turn is thought to reflect differences in the relative occurrence of forced matings among populations [32]. Guppies inhabit freshwater bodies throughout Venezuela and Trinidad (West Indies). Many Trinidadian streams exhibit strong discontinuities in the level of predation along their course, with lowland rivers typically characterized

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by high levels of predation in relation to upstream sections of the same river [33]. In all habitats, male guppies employ both courtship and forced matings to achieve copulations, but the extent to which either tactic is employed varies with the level of predation, with courtship being more prevalent in low-predation populations and forced matings more common in high-predation populations [34]. Forced matings, in turn, may be costly for females, both in terms of lost foraging opportunities and potential reductions in fecundity [35], but also because they may impose direct physical damage to the females' genital region caused by the complex bony structures at the distal tip of the male's gonopodium [25,36].

In this paper, we seek to understand the functional significance and evolutionary basis for variation in male genital morphology in natural guppy populations. We employ three complementary approaches to address these questions. First, we use geometric morphometric techniques to test whether variation in the shape of the gonopodium's distal tip is associated with insemination success during forced copulations. We focus only on the tip of the gonopodium for these analyses as this is the portion of the male's intromittent organ that enters the female's genital tract during copulation [30,37]. Second, we determine whether male genital morphology varies predictably among populations according to the level of predation, since predation intensity covaries with the relative prevalence of forced matings [34] and therefore potentially with the strength or direction of selection acting on male genital morphology. Third, because forced copulations are likely to be costly for females [38], we anticipated that counter-selection would result in the divergence of female genital traits among populations. This final analysis therefore also serves as a prospective test for intraspecific covariance in male and female genital morphologies, and lays the foundations for future studies designed to test among competing hypotheses to explain the evolutionary origins for such relationships.

## 2. METHODS

### (a) *Male genital morphology and mating success*

#### (i) *Mating trials*

We initially conducted a series of mating trials on  $n = 36$  male–female pairs taken from the Lower Quaré population, a high-predation site in the Oropouche drainage of Trinidad's Northern Mountain Range. Fish were transported to the University of the West Indies where females were isolated individually in 51 containers (containing river weed and an airstone) until they produced a brood. Offspring were removed immediately and each post-partum female was subsequently maintained individually within the same container for a further 14 days before taking part in the mating trials. This process of isolating females for 14 days prior to the trials was essential because it ensured that all females were at the same reproductive stage (approximately mid-way through their brood cycle) and sexually unreceptive (post-partum females are only sexually receptive to male courtship for 2–3 days after producing a brood and are never sexually receptive during the middle of their brood cycles; see [39]). The focal males were maintained in mixed-sex groups until 3 days before the mating trials, at which time they were isolated in 51 containers to ensure that they had replenished sperm stores when entering the mating trials [40]. On the

evening before each trial, females were placed individually in a  $45 \times 30 \times 30$  cm mating tank (filled to 20 cm) and allowed to settle overnight. The mating tanks contained a small amount of river weed and natural river stones collected from the Lower Quaré site. On the following morning, a male was placed in the tank and left for 2 h to interact with the female. We then observed the male's sexual behaviour for 15 min, recording the number of gonopodial thrust attempts (no prior courtship, male swings his gonopodium forward at least  $90^\circ$  and attempts a forced mating) that resulted in genital contact with the female ('contact success'). Such contacts were immediately recognizable from the females' 'startle' response and were used as a measure of 'success' because they potentially signal intromission. After each observation, the two fish were left in the mating arena until 5 h had elapsed since the trial commenced, at which point the male and female were separated so that we could recover sperm from the female's reproductive tract (see below). Immediately after the mating trials, males were anaesthetized (Benzocaine) and digitally photographed. Standard length for each male was estimated to within 0.5 mm by taking the distance (in mm) between the male's snout and the tip of his caudal peduncle. Gonopodium length was taken as the distance (in mm) between the base of the gonopodium and its distal tip. Each anaesthetized male was then killed through ice immersion and preserved in Dietrich's fixative (30% pure ethanol, 10% formalin, 2% glacial acetic acid, 58% DI  $H_2O$ ) for the subsequent geometric morphometric analysis of gonopodial shape (see below).

#### (ii) *Sperm recovery from females*

As a measure of insemination success, we recovered and counted sperm extracted from the female's reproductive tract at the completion of each behavioural trial. Sperm recovered from females in this way must have arisen from forced copulations that took place during the mating trials because sperm are not recoverable from the female's reproductive tract more than 7–8 days post-parturition [41,42]. Females in our trials had been isolated from males for 14 days, meaning that they were both sexually unresponsive and without freshly inseminated sperm. The technique for extracting sperm from recently mated females is described in detail elsewhere [43], but briefly this process involves flushing the female's genital pore five times with saline solution (0.9% NaCl). The number of sperm recovered in these flushes was then estimated using an Improved Neubauer haemocytometer [44].

### (b) *Population comparisons*

#### (i) *Populations surveyed*

We sampled guppy populations from upstream and downstream sites within each of five river systems in Trinidad's Northern Mountain Range. Four of these rivers (Aripo, Tacarigua, Quaré and Turure) exhibited the typical upstream–downstream predation gradient (upstream sites = low predation, downstream sites = high predation) seen in many Trinidadian rivers [39], while the fifth (Oropouche) was characterized by high levels of predation both in the upstream and downstream sites [45]. By incorporating the Oropouche River in the analyses, we attempted to account for variation in physical and ecological factors (other than predation intensity) that may drive interpopulation diversification of phenotypic traits. Thus, differences in male or female traits between the two Oropouche sites

would not be owing to the effects of predation, but some other (unmeasured) variable. All of the rivers sampled here have been extensively surveyed for predator abundances in previous studies [38,45], and we have monitored these populations to confirm their predation status since these original surveys (e.g. [46]).

(ii) *Characterizing variation in male genital traits*

We examined variation in the shape of the gonopodium's distal tip from  $n = 225$  males taken from the 10 populations (at least 20 males per population) and the 36 males used in the mating trials (above). For both analyses, we estimated the length (in mm) of each male's gonopodium from photographs of fresh specimens before preserving the males in Dietrich's fixative (see above). All males were preserved within one week of each other and were maintained under identical conditions before being photographed again for the analysis of gonopodium shape. Fixation times and holding conditions were therefore standardized among populations and these conditions did not differ between the mating trials and population comparisons. In all cases, a digital image of the lateral (left) side of each male's gonopodium was captured with a Leica DFC320 fitted to a Leica MZ75 stereomicroscope under transmitted light- and dark-field illumination. Images were captured at  $\times 50$  magnification to include the distal tip of the gonopodium (electronic supplementary material, figure S1a).

We examined variation in the shape of the gonopodium's distal tip using geometric morphometric analyses [47]. We digitized eight fixed landmarks on each image (electronic supplementary material, figure S1a) using TPSDIG2 software [48]. Partial warps and uniform components were subject to relative warp analysis using the program TPSRELW [49]. Relative warps analyses correspond to a principal components analysis and serve to reduce multivariate shape data to relative warps that describe most of the variation in shape. The relative warp analyses (including the uniform component) returned five relative warp scores (RWS-1–5), explaining 90 per cent of variance in gonopodial shape in the mating trials and 86 per cent in the population comparison trials. In our subsequent analyses of male genital traits (see below), variation in gonopodial shape could be visualized as deformations of a thin-plate spline (tps), where each tps plot displays deformations of the consensus configuration corresponding to a point in the space spanned by a particular pair of relative warps (§3).

(iii) *Female genital morphology and offspring size measurements*

Approximately 20 females from each of the 10 populations were included in our analysis of female genital tract morphology (total  $n = 195$ ). Immediately after collection, gravid females were isolated individually in a 41 container until they produced their first brood. At this stage, we counted the number of offspring produced by each female and measured the maximal width of each offspring's head (using the eye as a landmark) to within 0.05 mm for five offspring per brood. After giving birth, females were isolated individually for a further 3–4 days before being killed with an overdose of anaesthetic. In this way, all females were preserved at approximately the same reproductive stage (3–4 days postpartum) to minimize variation among females in the anatomy of the reproductive tract, which can occur during gestation [25]. Females were then preserved individually in Dietrich's fixative until required for the morphological

analyses. As with males, fixation times for females did not vary systematically among populations.

Dissections were subsequently carried out under a stereomicroscope. Each female was placed with her ventral side exposed and a scalpel was used to make an incision from the gills to the anus. The ventral abdomen muscles and skin were then removed to expose the peritoneal cavity. We carefully removed visceral organs and fat within the cavity, leaving the ovary and the oviduct intact. At this stage, an image of each female's oviduct was captured using a digital camera fitted to the stereomicroscope. Images were stored and analysed using Leica IM500 image manager software. Oviduct length was taken from the point closest to the egg sac to the urogenital sinus (i.e. the oviduct opening, in which males insert their gonopodial tips). Oviduct width was estimated mid-way along this tract (electronic supplementary material, figure S2. NB: the oviduct is roughly cylindrical, and its width did not change substantially along the entire length of the tract.). We specifically focused on this portion of the female's reproductive tract as this is the region that is physically contacted by the gonopodium during copulation (mean  $\pm$  s.d. oviduct length =  $2.37 \text{ mm} \pm 0.48$ ; gonopodium =  $4.24 \pm 0.29$ ). In a subsample of 45 females, we took two independent measurements of each trait (i.e. oviduct length and width) and tested for consistency in these measures by calculating the intraclass coefficient for repeated measures [50]. The two measures were performed by the same operator working blind of the identity of each sample. Intraclass correlation coefficients ( $R$ ) for both traits were high (oviduct length: ANOVA  $F_{42,85} = 38.2$ ,  $p < 0.001$ ,  $R = 0.95$ ; oviduct width:  $F_{43,87} = 107.8$ ,  $p < 0.001$ ,  $R = 0.98$ ).

Finally, we estimated female body size by taking the distance between the oviduct opening and the snout, rather than the more usual measurement of standard length (as in males—see above). We used this measurement because caudal fin tissue had been taken from each female for genetic analyses in a different study [51]. This method of estimating female size was strongly correlated with standard length, as confirmed by correlating both measurements using a subsample of females (Pearson correlation:  $r = 0.95$ ,  $n = 33$ ,  $p < 0.0001$ ).

(c) *Statistical analysis*

(i) *Mating trials*

We initially tested whether gonopodium length and shape influenced the likelihood of successful genital contact during the mating trials (i.e. our measure of 'contact success'). For this analysis, we used a generalized linear model (with binomial error distribution and a logit link function), in which the number of thrusts that resulted in contact between the males' and females' genitalia was entered as the dependent variable (with the total number of mating attempts as the binomial denominator). The predictor variables in this analysis included male body size (standard length), female body size (snout-to-oviduct opening), gonopodium length and the shape variables RWS-1–5. Three replicates could not be included in these regressions because photographs were unavailable for geometric morphometric analyses. Hence, our final analyses were based on  $n = 33$  replicates.

Next, we tested whether variation in gonopodium length and shape was associated with the number of sperm recovered from females (hereafter 'sperm recovered'). To address this question, we used linear multiple regression using the

same predictor variables described above. In the analysis of sperm recovered, a log transformation was used to improve the distribution of the data.

To estimate the effect of the predictors in both analyses (contact success and sperm recovered), we used an information theoretic approach [52,53], which recognizes the uncertainty in model selection inherent in other ‘best model’ approaches by estimating the average effect of each predictor over the total possible combinations of predictors. We first calculated the Akaike information criterion, with second-order correction (AICc) for small samples, for models including the intercept only to those containing all predictors ( $n$  models = 2048). For the generalized linear model, we accounted for overdispersion by using the quasi-AICc (QAICc). The regression coefficient ( $b$ ) of each predictor was obtained for all possible models using the RSEARCH function in GENSTAT. Models were then ranked according to their AICc or QAICc values (smallest = best), which were used to weight each model on the basis of its likelihood [53]. The likelihood of being the best model in each case was calculated as a function of the difference from its AICc value and the AICc value of the best model (also called ‘Akaike weight’, see formula (3) in [52]). The Akaike weight is an estimate of the probability that a given model is the best of all  $n$  models (total Akaike weights sum to 1). For each predictor, an average parameter (in this case the regression coefficient  $b$ ) was calculated by summing the parameter values in each model (models in which the predictor was not selected had a value of zero), weighted for the likelihood of each model. This is a type of shrinkage estimator that gives less weight to the predictors with a weak relationship to the response [52]. For the regression coefficients, 95% confidence intervals (CIs) were calculated from the variance in the predictor coefficients [52]. Regression coefficients whose 95% CI did not include zero were considered statistically significant. Statistical analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA) and GENSTAT 12 (VSN International Ltd, Hemel Hempstead, UK). All probabilities are two-tailed.

#### (ii) Population comparisons of male and female traits

We used linear mixed-effects models with restricted maximum likelihood (REML) estimation to compare male and female genital traits between predation regimes among populations (rivers). The initial multivariate model included RWS-1–5 as response variables, with predation as a fixed factor (two levels, high and low predation), and both river (five levels) and sampling locality (10 levels) entered as random effects. In these analyses, we also included gonopodium length as a covariate. Univariate linear mixed-effects models (including the same fixed and random terms as the multivariate model) were then used to identify the specific traits contributing to the divergence in male genital size and shape among populations.

In the analysis of female traits, estimates of oviduct width and length were entered as response variables, with predation (fixed) and both river and sampling locality (random) entered as factors. In these analyses, we also included female body length (which was correlated with oviduct measures—§3) and mean offspring size (which varied among populations—§3) as covariates. In both analyses (males and females), we ensured homogeneity of slopes by testing the significance of the interaction between the fixed factor (predation) and the covariates. These interaction

Table 1. Regression coefficients for the relationship between male success in coercive copulations, expressed as proportion of gonopodium contacts (contact success) and number of sperm transferred during the mating trials (sperm recovered), and male and female traits (body size and genital size/morphology). (Average regression coefficients ( $b$ ) and their 95% CI were obtained using a model-averaging procedure (§2). Significant effects are indicated in bold font.)

	$b$	95% CI
<i>(a) contact success</i>		
constant	-3.294	-25.037 to 18.449
male body size	-0.053	-0.172 to 0.066
female body size	0.003	-0.028 to 0.034
gonopodium length	<b>0.465</b>	<b>0.137–0.793</b>
RWS-1	-0.391	-4.067 to 3.285
RWS-2	1.400	-3.735 to 6.534
RWS-3	-2.676	-8.379 to 3.028
RWS-4	-4.508	-14.116 to 5.099
RWS-5	1.336	-12.11 to 14.782
<i>(b) sperm recovered</i>		
constant	-0.747	-19.966 to 18.473
male body size	0.095	-0.013 to 0.202
female body size	<b>0.048</b>	<b>0.008–0.088</b>
gonopodium length	-0.042	-0.422 to 0.338
RWS-1	<b>11.992</b>	<b>10.419–13.565</b>
RWS-2	0.035	-2.364 to 2.434
RWS-3	<b>4.086</b>	<b>1.512–6.661</b>
RWS-4	-0.272	-2.604 to 2.059
RWS-5	-2.053	-4.348 to 0.242

terms were all non-significant ( $p > 0.20$ ), satisfying the assumptions of these models.

### 3. RESULTS

#### (a) Male genital morphology and the success of forced matings

We found that males with relatively longer gonopodia achieved significantly higher contact success than those with shorter gonopodia, whereas gonopodium shape (as described by RWS-1–5) was not significantly associated with contact success (table 1). However, we did find significant effects of gonopodium shape (RWS-1 and RWS-3) on the number of sperm recovered after the mating trials (table 1). Both of these relative warp scores (RWS-1 and RWS-3) were associated with variation in the length of the male’s gonopodial tip (electronic supplementary material, figure S1*b*). Overall, males with relatively short gonopodial tips (i.e. positive values of RWS-1 and RWS-3) were more successful at transferring sperm to females than males with relatively long gonopodial tips. To visualize overall differences in gonopodial shape between minimally and maximally successful males (i.e. those that transferred the least and most sperm, respectively), we used the program TPSREGR [54], which enabled us to characterize total shape variation across the samples analysed and relate this variation to insemination success (electronic supplementary material, methods). Overall, these plots confirmed our conclusions from the analysis of the relative warp scores (table 1) by revealing that relatively short gonopodial tips were associated with

Table 2. Population comparisons of male traits. ((a) Mean values (marginal means estimated from the linear mixed model) with their standard errors (s.e.), and sample sizes for body size and gonopodium length. (b) Results of the univariate mixed-model analyses (REML) on relative warp scores (RWS) 1–5 describing shape variation in the distal tip of the male copulatory organ (gonopodium). The percentage of variance explained by each relative warp (RW) is also provided.)

		body size (mm)	gonopodium length (mm)	RWS-1	RWS-2	RWS-3	RWS-4	RWS-5
<i>(a) population</i>								
high predation ( $n = 136$ )	mean	15.84	4.26 <sup>a</sup>					
	s.e.	0.15	0.055					
low predation ( $n = 85$ )	mean	18.02	4.20					
	s.e.	0.21	0.071					
<i>(b) univariate analysis<sup>b</sup></i>								
RW variance explained (%)	—	—	—	39.95	20.58	14.05	6.62	5.11
predation	<i>F</i>	<b>44.76</b>	0.56	<b>19.08</b>	0.37	<b>10.81</b>	0.03	0.52
	<i>p</i>	0.002	0.48	0.012	0.57	0.009	0.88	0.50
	d.f.	1, 4.5	1, 6.9	1, 4.1	1, 5.3	1, 9.0	1, 5.6	1, 6.4
body size (covariate)	<i>F</i>	—	<b>26.22</b>	—	—	—	—	—
	<i>p</i>	—	<0.001	—	—	—	—	—
	d.f.	—	1, 211.3	—	—	—	—	—

<sup>a</sup>One missing value.

<sup>b</sup>Univariate mixed models (REML) in which predation (two levels) was entered as a fixed factor, river (five levels) and sampling locality (10 levels) as a random factors and body size, where appropriate, as a covariate. Linear measurements were log-transformed. Significant effects are highlighted in bold.

higher insemination success (electronic supplementary material, figure S1c). Finally, we detected a weak but significant relationship between female body size and the number of sperm recovered (table 1).

### (b) Population comparisons of male and female genital morphology

Our analysis revealed overall significant divergence among populations in male genital traits, which in turn was attributable to differences in the level of predation at each site. Although high-predation males had relatively longer gonopodia than their low-predation counterparts (mean fitted values reported in table 2a), this difference was not significant even when we controlled for differences in body size (table 2b). By contrast, the shape of the distal portion of the gonopodium differed significantly between predation regimes (linear mixed-effects MANOVA: predation,  $F_{5,16.7} = 6.36$ ,  $p = 0.002$ ; gonopodium length (covariate),  $F_{5,412.2} = 1.55$ ,  $p = 0.17$ ; results were similar if the covariate was removed from the model). Subsequent univariate analyses revealed that two of the relative warps (RWS-1 and RWS-3, accounting for 40 and 14% of the overall variance in male genital morphology, respectively) differed significantly between and high- and low-predation populations (table 2b). Briefly, males from high-predation populations had gonopodia with less elongated distal tips (evident from variance in RWS-1; figure 1a) and reduced hook angles (RWS-3; figure 1b) than their low-predation counterparts.

Our analysis of female genital morphology focused on the width and length of the oviduct. These two measurements were correlated with female body length, which in turn was positively associated with the mean size of offspring at parturition ( $r = 0.31$ ,  $p < 0.0001$ ,  $n = 195$ ). In our analysis of female oviduct length and width, we therefore included both female body length and the

mean size of offspring as covariates in the model. This analysis revealed that oviduct width (but not length) differed consistently with predation intensity, when controlling for variation in female body length and offspring size (table 3).

In summary, females from high-predation populations had relatively wider oviducts than their low-predation counterparts. This difference is unlikely to be owing to the production of larger offspring in these populations, because females from high-predation streams tend to produce smaller, not larger offspring [55]. Indeed, our measurements of mean offspring size among populations confirmed that females from high-predation sites produced significantly smaller offspring than their low-predation counterparts (table 3).

### (c) Covariance between male and female genital morphology

Given the significant divergence among populations in both male and female genital morphology, we looked for evidence of coevolutionary patterns of selection on these traits using multiple regression in which oviduct width was entered as a response variable and RWS-1 and RWS-3 were included as predictors. We specifically focus on these components of male and female genital shapes as they varied among populations in relation to predation intensity, and in the case of male genital traits, both RWS-1 and RWS-3 were associated with insemination success in the behavioural trials. Because oviduct width was positively correlated with both female body length and the mean size of offspring at birth (see above), we also included female body length and mean offspring size as covariates in this analysis. This enabled us to test how oviduct width covaried with male genital shape among populations when controlling for differences in female and offspring body size. We then used the model-averaging procedure described above to identify

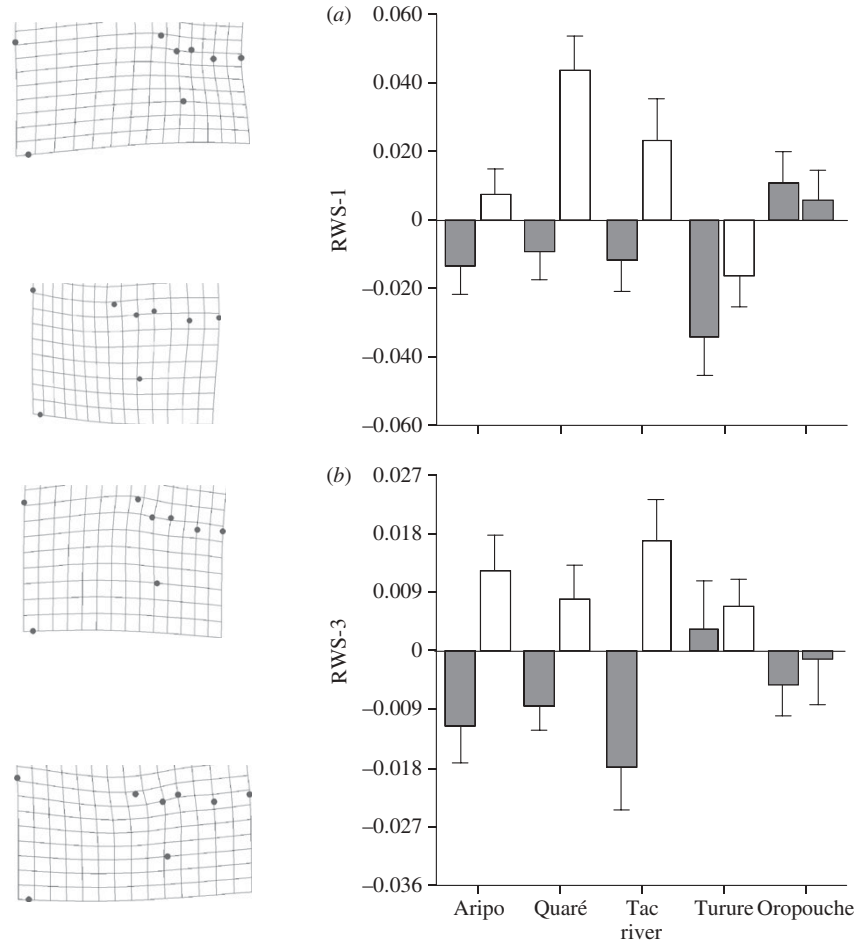


Figure 1. Variation in gonopodial shape among natural guppy populations. Mean ( $\pm$  s.e.) values for (a) relative warp score 1 (RWS-1) and (b) RWS-3 in relation to predation regime in 10 guppy populations. The thin-plate splines (panels on the left-hand side of graphs) were generated by the program TPSRELW [49] and depict the observed variation in the shape of the gonopodium's distal tip (i.e. positive and negative values) captured by both relative warp scores. White bars, low predation; black bars, high predation.

Table 3. Population comparisons of female traits. ((a) Mean values (estimated from the linear mixed-effects model) with their standard errors (s.e.) and sample sizes for female traits according to predation regime. (b) Results from the univariate mixed-model analyses (REML) on these traits.)

		body size (mm) <sup>a</sup>	oviduct length (mm)	oviduct width (mm)	mean offspring size (mm)
(a) population					
high predation ( $n = 112$ )	mean	13.993	2.374	0.789	1.265
	s.e.	1.707	0.487	0.218	0.128
low predation ( $n = 76$ )	mean	14.039	2.382	0.743	1.428
	s.e.	1.812	0.463	0.189	0.153
(b) univariate analysis <sup>b</sup>					
predation	<i>F</i>	0.02	0	6.60	<b>8.00</b>
	<i>p</i>	0.89	0.98	0.046	0.030
	d.f.	1, 5.3	1, 6.5	1, 5.5	1, 6.0
body size (covariate) <sup>a</sup>	<i>F</i>		<b>65.19</b>	<b>27.36</b>	<b>16.91</b>
	<i>p</i>		<0.001	<0.001	<0.001
	d.f.		1, 177.5	1, 97.7	1, 187.4
mean offspring size (covariate)	<i>F</i>		0.01	3.06	
	<i>p</i>		0.94	0.09	
	d.f.		1, 145.5	1, 31.8	

<sup>a</sup>Distance between the genital opening and the snout (§2).

<sup>b</sup>Univariate mixed-effects models (REML) in which predation (two levels) was entered as fixed factor, river (five levels) and sampling locality (10 levels) as random factors and body size and mean offspring size, where appropriate, as covariate. Significant effects are highlighted in bold.

Table 4. Covariation between female oviduct width and gonopodium shape, after statistically controlling for the variation of female and offspring body size. (Average regression coefficients (*b*) and their 95% CI were obtained using a model-averaging procedure (see §2 for more details). Significant effects are indicated in bold font.)

	<i>b</i>	95% CI
constant	1.389	−4.463 to 7.241
female body size <sup>a</sup>	0.422	−0.039 to 0.884
offspring size <sup>a</sup>	−0.019	−0.242 to 0.204
RW1	<b>−1.195</b>	<b>−0.253 to −2.136</b>
RW3	0.184	−0.796 to 1.164

<sup>a</sup>Log transformed.

predictors that were more strongly associated with variation in oviduct width. This analysis revealed a significant negative association between relative oviduct width and RWS-1 (table 4), revealing that increasingly wide oviducts were associated with lower RWS-1 scores (i.e. relatively shorter gonopodium tips; figure 2).

#### 4. DISCUSSION

The results from our mating trials revealed that variation in both the size and shape of the male's intromittent organ was associated with the success of forced matings. Males with relatively longer gonopodia were more successful at achieving genital contacts with females than those with relatively short gonopodia, although surprisingly we found no difference in the relative length of the gonopodium among populations (c.f. [32]), despite the higher prevalence of forced matings in high-predation sites. We also found a significant relationship between male genital shape and insemination success (number of sperm recovered from the female's genital tract). This latter finding accords with early work on this species and other poeciliid fishes, showing that the distal tip of the gonopodium plays an important role in sperm transfer. For example, Rosen & Gordon [30] observed that in the genera *Poecilia* and *Xiphophorus*, only the tips of the gonopodium were inserted into the female's gonopore. They further suggested that contact between the male and female during copulation was maintained by 'holdfast' devices that project from rays 3 and 4 of the gonopodium (e.g. hooks). These findings were subsequently corroborated in xiphophorin fishes by Clark *et al.* [37], who demonstrated experimentally that these structures were necessary for successful insemination. More recent work has revealed that the experimental removal of the paired hooks from the male guppy's gonopodial tip impairs subsequent sperm transfer [56]. Importantly, the effects of this manipulation on insemination success were only apparent during forced (unsolicited) matings [56], again underscoring our suspicion that variation in this region of the gonopodium is shaped by divergent male-mating tactics among populations.

We also found that female body size was a significant predictor of insemination success, although the strength of this relationship was relatively weak compared with the effects of gonopodium shape on sperm recovered (table 1). This finding accords with previous work on

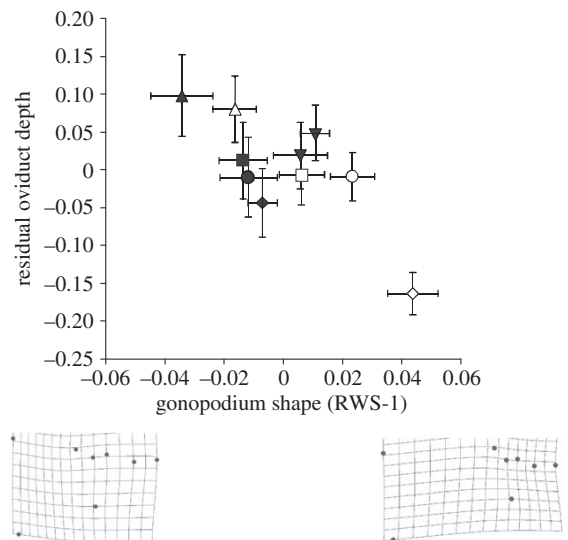


Figure 2. The relative width of the females' oviduct in relation to the shape of male gonopodium tip. For graphical purposes, relative oviduct width is estimated from the residuals of the regression of oviduct width on female body size. Plotted values are population means with standard errors for male (*x* error bars) and female (*y* error bars) traits. Key: filled symbols depict high-predation (HP) sites and open symbols low-predation (LP) sites. The thin-plate splines along the *x*-axis were generated by TPSRELW [49] and depict the observed variation in the shape of the gonopodium's distal tip (i.e. positive and negative values) captured by RWS-1. Squares, Aripo; circles, Tacarigua; diamonds, Quaré; upright triangles, Turure; inverted triangles, Oropouche.

the mosquitofish *Gambusia holbrooki*, where the probability of a female being inseminated through forced matings increased with female length [57]. Whether this relationship between female body size and insemination success is owing to males preferentially allocating mating effort (and/or sperm) towards larger (more fecund) females [58], or the increased ability of males to undermine the choice of relatively large females (owing to increased 'target' size, enhanced manoeuvrability, etc. [57]) remains to be tested.

Our population comparisons revealed that the shape of the distal tip of the gonopodium varied with predation intensity. Males from high-predation populations had gonopodia with less elongated distal tips and reduced hook angles than their low-predation counterparts. These patterns are therefore consistent with our findings from the mating trials, by indicating that the size and shape of the gonopodium vary predictably among populations that differ in the relative intensity of forced matings. The pattern of trait divergence for the genital hooks is also consistent with the general pattern for the poeciliid family, where the degree of gonopodial elaboration varies with mating system. Specifically, species that rely predominantly (or exclusively) on forced matings (i.e. without courtship) tend to have relatively 'unarmed' gonopodia, while those that rely more on courtship have far more elaborated gonopodia adorned with various 'holdfast' structures such as hooks, claws and serrae [25]. Although male guppies use both mating tactics

interchangeably, our data suggest that the extent to which either tactic is used in the population selects for variation in the relative shape of these traits. Interestingly, males from high-predation populations had more 'streamlined' hooks, potentially making them more adapted to the higher prevalence of forced matings by these males. However, hook shape was not a significant predictor of mating success in the single population used in our behavioural trials. This is probably because *among*-population variance for this trait greatly exceeds within-population variance. Indeed, visualizations of the five relative warp scores (using the software *TPSRELW*) describing male genital morphology in the mating trials revealed that none of these was associated with changes in the angle of the genital hooks (figures not provided). This suggests that there is very limited variability in this trait within a single population. Thus, population crosses or gonopodium experimental manipulation would be required to assess the role that genital hooks play in mediating the success of forced matings (but see [59]).

Our analysis also revealed corresponding patterns of trait diversification in female genital morphology (relative oviduct width). Although our current investigation, in conjunction with earlier work [30,37,56], enables us to draw tentative conclusions about the role that male genital shape plays in mediating the success of forced matings (above), we can only speculate about the significance of variability in female genital morphology. One possibility that we are currently pursuing is that variation in female genital tract morphology influences the success of forced matings, which in turn would potentially reduce the costs associated with the higher prevalence of forced matings in high-predation populations [35].

In summary, we have provided evidence for intraspecific (co)variance in male and female genital traits, and combined these comparative analyses with behavioural data that reveal an association between male genital morphology and the outcome of an antagonistic mating encounter. In order to provide evidence that these patterns of correlated trait divergence reflect a history of sexually antagonistic selection, a topic that is still generating considerable controversy [60], it is necessary to demonstrate: (i) that variation in female genital morphology reflects adaptations that limit the costs associated with male sexual harassment; (ii) that these patterns of morphological covariance are driven by differences in the level of sexual harassment endured by females among populations; and (iii) that patterns of genetic (co)variation underlying the expression of these traits do not impede their evolutionary potential. Our ongoing work addresses all of these questions.

All animal work was conducted according to the University of Western Australia's Animal Ethics Committee (Research Integrity Office, permit number 05/100/513).

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## REFERENCES

- Eberhard, W. G. 1985 *Sexual selection and animal genitalia*. Cambridge, MA: Harvard University Press.
- Hosken, D. J. & Stockley, P. 2004 Sexual selection and genital evolution. *Trends Ecol. Evol.* **19**, 87–93. (doi:10.1016/j.tree.2003.11.012)
- Bertin, A. & Fairbairn, D. J. 2005 One tool, many uses: precopulatory sexual selection on genital morphology in *Aquarius remigis*. *J. Evol. Biol.* **18**, 949–961. (doi:10.1111/j.1420-9101.2005.00913.x)
- Langerhans, R. B., Layman, C. A. & DeWitt, T. J. 2005 Male genital size reflects a tradeoff between attracting mates and avoiding predators in two live-bearing fish species. *Proc. Natl Acad. Sci. USA* **102**, 7618–7623. (doi:10.1073/pnas.0500935102)
- Brooks, R. & Caithness, N. 1995 Female choice in a feral guppy population: are there multiple cues. *Anim. Behav.* **50**, 301–307. (doi:10.1006/anbe.1995.0246)
- Preziosi, R. F. & Fairbairn, D. J. 1996 Sexual size dimorphism and selection in the wild in the waterstrider *Aquarius remigis*: body size, components of body size and male mating success. *J. Evol. Biol.* **9**, 317–336. (doi:10.1046/j.1420-9101.1996.9030317.x)
- Arnqvist, G. & Danielsson, I. 1999 Copulatory behavior, genital morphology, and male fertilization success in water striders. *Evolution* **53**, 147–156. (doi:10.2307/2640927)
- Danielsson, I. & Askenmo, C. 1999 Male genital traits and mating interval affect male fertilization success in the water strider *Gerris lacustris*. *Behav. Ecol. Sociobiol.* **46**, 149–156. (doi:10.1007/s002650050604)
- House, C. M. & Simmons, L. W. 2005 Relative influence of male and female genital morphology on paternity in the dung beetle *Onthophagus taurus*. *Behav. Ecol.* **16**, 889–897. (doi:10.1093/beheco/ari066)
- Weninger, E. J. & Averill, A. L. 2006 Influence of body and genital morphology on relative male fertilization success in oriental beetles. *Behav. Ecol.* **17**, 656–663. (doi:10.1093/beheco/ark013)
- Hotzy, C. & Arnqvist, G. 2009 Sperm competition favors harmful males in seed beetles. *Curr. Biol.* **19**, 404–407. (doi:10.1016/j.cub.2009.01.045)
- Arnqvist, G. 1998 Comparative evidence for the evolution of genitalia by sexual selection. *Nature* **393**, 784–786. (doi:10.1038/31689)
- Brennan, P., Prum, R., McCracken, K., Sorenson, M., Wilson, R. & Birkhead, T. 2007 Coevolution of male and female genital morphology in waterfowl. *PLoS ONE*, e418. (doi:10.1371/journal.pone.0000418)
- Córdoba-Aguilar, A. 2005 Possible coevolution of male and female genital form and function in a calopterygid damselfly. *J. Evol. Biol.* **18**, 132–137. (doi:10.1111/j.1420-9101.2004.00796.x)
- Huber, B. A. 2003 Southern African pholcid spiders: revision and cladistic analysis of *Quamtana* gen. and Spermophora Hentz (Araneae: Pholcidae), male–female covariation. *Zool. J. Linn. Soc.* **139**, 477–527. (doi:10.1046/j.0024-4082.2003.00082.x)
- Arnqvist, G. & Rowe, L. 2002 Correlated evolution of male and female morphologies in water striders. *Evolution* **56**, 936–947. (doi:10.1111/j.0014-3820.2002.tb01406.x)



- 17 Arnqvist, G. & Rowe, L. 2002 Antagonistic coevolution between the sexes in a group of insects. *Nature* **415**, 787–789. (doi:10.1038/415787a)
- 18 Peden, A. E. 1972 The function of gonopodial parts and behavioural pattern during copulation by *Gambusia* (Poeciliidae). *Can. J. Zool.* **50**, 955–968. (doi:10.1139/z72-128)
- 19 Tataric, N. J. & Cassis, G. 2010 Sexual coevolution in the traumatically inseminating plant bug genus *Coridromius* [transl. from English]. *J. Evol. Biol.* **23**, 1321–1326 (doi:10.1111/j.1420-9101.2010.01991.x)
- 20 Rönn, J., Katvala, A. & Arnqvist, G. 2007 Coevolution between harmful male genitalia and female resistance in seed beetles. *Proc. Natl Acad. Sci. USA* **104**, 10 921–10 925. (doi:10.1073/pnas.0701170104)
- 21 Andrade, C. A. C., Vieira, R. D., Ananina, G. & Klaczko, L. B. 2009 Evolution of the male genitalia: morphological variation of the aedeagi in a natural population of *Drosophila mediopunctata*. *Genetica* **135**, 13–23. (doi:10.1007/s10709-008-9247-9)
- 22 Mutanen, M. & Kaitala, A. 2006 Genital variation in a dimorphic moth *Selenia tetralumaria* (Lepidoptera, Geometridae). *Biol. J. Linn. Soc.* **87**, 297–307. (doi:10.1111/j.1095-8312.2006.00578.x)
- 23 Mutanen, M., Kaitala, A. & Monkkonen, M. 2006 Genital variation within and between three closely related *Euxoa* moth species: testing the lock-and-key hypothesis. *J. Zool.* **268**, 109–119. (doi:10.1111/j.1469-7998.2005.00029.x)
- 24 Mutanen, M., Rytönen, S., Linden, J. & Sinkkonen, J. 2007 Male genital variation in a moth *Pammene luedersiana* (Lepidoptera: Tortricidae). *Eur. J. Entomol.* **104**, 259–265.
- 25 Greven, H. 2005 Structural and behavioral traits associated with sperm transfer in Poeciliinae. In *Viviparous fishes* (eds M. C. Uribe & H. J. Grier), pp. 145–163. Homestead, FL: New Life Publications.
- 26 Rosen, D. E. & Bailey, R. M. 1963 The poeciliid fishes (cyprinodontiformes). Their structure, zoogeography, and systematics. *Bull. Am. Mus. Nat. Hist.* **126**, 1–176.
- 27 Ptaček, M. B. & Travis, J. 1998 Hierarchical patterns of covariance between morphological and behavioural traits. *Anim. Behav.* **56**, 1044. (doi:10.1006/anbe.1998.0874)
- 28 Evans, J. P. & Meisner, A. D. 2009 Copulatory structures: taxonomic overview and the potential for sexual selection. In *Reproductive biology and phylogeny of fishes* (ed. B. G. M. Jamieson), pp. 138–180. Enfield, NH: Science Publishers.
- 29 Reynolds, J. D., Gross, M. R. & Coombs, M. J. 1993 Environmental conditions and male morphology determine alternative mating behaviour in Trinidadian guppies. *Anim. Behav.* **45**, 145–152. (doi:10.1006/anbe.1993.1013)
- 30 Rosen, D. E. & Gordon, M. 1953 Functional anatomy and evolution of male genitalia in poeciliid fishes. *Zoologica* **38**, 1–47.
- 31 Rosen, D. E. & Tucker, A. 1961 Evolution of secondary sexual characters and sexual behavior patterns in a family of viviparous fishes (Cyprinodontiformes: Poeciliidae). *Copeia* **1961**, 201–212. (doi:10.2307/1439999)
- 32 Kelly, C. D., Godin, J. G. & Abdallah, G. 2000 Geographic variation in the male intromittent organ of the Trinidadian guppy (*Poecilia reticulata*). *Can. J. Zool.* **78**, 1674–1680. (doi:10.1139/cjz-78-9-1674)
- 33 Magurran, A. E. 2005 *Evolutionary ecology: the Trinidadian guppy*. Oxford, UK: Oxford University Press.
- 34 Endler, J. A. 1995 Multiple-trait coevolution and environmental gradients in guppies. *Trends Ecol. Evol.* **10**, 22–29. (doi:10.1016/S0169-5347(00)88956-9)
- 35 Magurran, A. E. & Seghers, B. H. 1994 A cost of sexual harassment in the guppy, *Poecilia reticulata*. *Proc. R. Soc. Lond. B* **258**, 89–92. (doi:10.1098/rspb.1994.0147)
- 36 Constantz, G. D. 1984 Sperm competition in poeciliid fishes. In *Sperm competition and the evolution of animal mating systems* (ed. R. L. Smith), pp. 465–485. Orlando, FL: Academic Press.
- 37 Clark, E., Aronson, L. R. & Gordon, M. 1954 Mating behavior pattern in two sympatric species of xiphophorin fishes: their inheritance and significance in sexual isolation. *Bull. Am. Mus. Nat. Hist.* **103**, 135–226.
- 38 Magurran, A. E. & Seghers, B. H. 1994 Sexual conflict as a consequence of ecology: evidence from guppy, *Poecilia reticulata*, populations in Trinidad. *Proc. R. Soc. Lond. B* **255**, 31–36. (doi:10.1098/rspb.1994.0005)
- 39 Houde, A. E. 1997 *Sex, color, and mate choice in guppies*, p. 210. Princeton, NJ: Princeton University Press.
- 40 Kuckuck, C. & Greven, H. 1997 Notes on the mechanically stimulated discharge of spermiozeugmata in the guppy, *Poecilia reticulata*: a quantitative approach. *Zeitschrift für Fischkunde* **4**, 73–88.
- 41 Matthews, I. M. & Magurran, A. E. 2000 Evidence for sperm transfer during sneaky mating in wild Trinidadian guppies. *J. Fish Biol.* **56**, 1381–1386. (doi:10.1111/j.1095-8649.2000.tb02150.x)
- 42 Clark, E. & Aronson, L. R. 1951 Sexual behaviour in the guppy, *Lebistes reticulatus*. *Zoologica* **36**, 49–66.
- 43 Pilastro, A. & Bisazza, A. 1999 Insemination efficiency of two alternative male mating tactics in the guppy (*Poecilia reticulata*). *Proc. R. Soc. Lond. B* **266**, 1887–1891. (doi:10.1098/rspb.1999.0862)
- 44 Matthews, I. M., Evans, J. P. & Magurran, A. E. 1997 Male display rate reveals ejaculate characteristics in the Trinidadian guppy *Poecilia reticulata*. *Proc. R. Soc. Lond. B* **264**, 695–700. (doi:10.1098/rspb.1997.0099)
- 45 Endler, J. A. & Houde, A. E. 1995 Geographical variation in female preferences for male traits in *Poecilia reticulata*. *Evolution* **49**, 456–468. (doi:10.2307/2410270)
- 46 Evans, J. P., Pilastro, A. & Ramnarine, I. W. 2003 Sperm transfer through forced matings and its evolutionary implications in natural guppy (*Poecilia reticulata*) populations. *Biol. J. Linn. Soc.* **78**, 605–612. (doi:10.1046/j.0024-4066.2002.00193.x)
- 47 Zelditch, M. L., Swiderski, D. L., Sheets, H. D. & Fink, W. L. 2004 *Geometric morphometrics for biologists*. p. 443. London, UK: Elsevier Academic Press.
- 48 Rohlf, F. J. 2005 *TPSDIG2, digitize landmarks and outlines*, v. 2.10. Stony Brook, NY: Department of Ecology and Evolution, State University of New York.
- 49 Rohlf, F. J. 2005 *TPSRELW relative warps analysis*, v. 1.45. Stony Brook, NY: Department of Ecology and Evolution, State University of New York.
- 50 Lessells, C. M. & Boag, P. T. 1987 Unrepeatable repeatabilities: a common mistake. *Auk* **104**, 116–121.
- 51 Neff, B. D., Pitcher, T. E. & Ramnarine, I. W. 2008 Inter-population variation in multiple paternity and reproductive skew in the guppy. *Mol. Ecol.* **17**, 2975–2984. (doi:10.1111/j.1365-294X.2008.03816.x)
- 52 Lukacs, P. M., Burnham, K. P. & Anderson, D. R. 2010 Model selection bias and Freedman's paradox. *Ann. Inst. Stat. Math.* **62**, 117–125. (doi:10.1007/s10463-009-0234-4)
- 53 Burnham, K. P. & Anderson, D. R. 2002 *Model selection and multimodel inference: a practical information-theoretic approach*, 2nd edn. New York, NY: Springer-Verlag.
- 54 Rohlf, F. J. 2007 *TpsREGR, thin plate spline regression*, v. 1.35. Stony Brook, NY: Department of Ecology and Evolution, State University of New York.

- 55 Reznick, D. N. & Endler, J. A. 1982 The impact of predation on life history evolution in Trinidadian guppies (*Poecilia reticulata*). *Evolution* **36**, 125–148.
- 56 Cheng, Y. Y. 2004 Sexual selection and the evolution of genitalia in the guppy (*Poecilia reticulata*). MSc thesis, University of Toronto, Toronto.
- 57 Pilastro, A., Giacomello, E. & Bisazza, A. 1997 Sexual selection for small size in the male mosquitofish (*Gambusia holbrooki*). *Proc. R. Soc. Lond. B* **264**, 1125–1129. (doi:10.1098/rspb.1997.0155)
- 58 Herdman, E. J. E., Kelly, C. D. & Godin, J. G. J. 2004 Male mate choice in the guppy (*Poecilia reticulata*): do males prefer larger females as mates? *Ethology* **110**, 97–111. (doi:10.1111/j.1439-0310.2003.00960.x)
- 59 Rowe, L., Cameron, E. & Day, T. 2003 Detecting sexually antagonistic coevolution with population crosses. *Proc. R. Soc. Lond. B* **270**, 2009–2016. (doi:10.1098/rspb.2003.2453)
- 60 Eberhard, W. 2010 Evolution of genitalia: theories, evidence, and new directions. *Genetica* **138**, 5–18. (doi:10.1007/s10709-009-9358-y)