

Research



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Evolutionary biology

Fine-scale genital morphology affects male ejaculation success: an experimental test

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The evolution of male genital traits is usually ascribed to advantages that arise when there is sperm competition, cryptic female choice or sexual conflict. However, when male–female contact is brief and sperm production is costly, genital structures that ensure the appropriate timing of sperm release should also be under intense selection. Few studies have examined the role of individual structures in triggering ejaculation. We therefore conducted a series of anatomical manipulations of fine-scale features of the complex intromittent organ (gonopodium) of a freshwater fish with internal fertilization (*Gambusia holbrooki*) to determine their effects on sperm release. Mating in *G. holbrooki* is fleeting (less than 50 ms), so there should be strong selection for control over the timing of sperm release. We surgically removed three features at the tip of the gonopodium (claws, spines, awl-shape) to test for their potential role in triggering ejaculation. We show that the ‘awl-shape’ of the tip affects sperm release when a male makes contact with a female, but neither gonopodial claws nor spines had a detectable effect. We suggest that the claws and spines may instead function to increase the precision of sperm deposition (facilitating anchorage and contact time with the female’s gonopore), rather than the initiation of ejaculation.

1. Introduction

Male genitalia show higher divergent and more rapid evolution than other morphological traits [1]. While natural selection favours genitalia that ensure effective coupling and sperm delivery, sexual selection is known to be a key force in genital diversification. Male genitalia and allied appendages that contact the female reproductive tract often evolve owing to the advantage they confer under sperm competition, cryptic female choice and sexual conflict [2]. Many studies have described differences in genital morphology between species or across populations and attributed them to selection for greater insemination success (e.g. [3]). However, supporting experimental evidence comes primarily from invertebrates [4–7]. Little is known about the role of fine-scale genital features of vertebrates in the timing of ejaculation, which is a fundamental component of a successful copulation. Insemination failure can result from: (i) ineffective sperm deposition and/or retention or (ii) incomplete or incorrect insertion into the female so that males do not receive the necessary cues for ejaculation. Cryptic female choice and male–male competition appear to select for genital evolution in the former case [8], whereas natural selection for mechanical compatibility seems to be more important in the latter [9]. Understanding how genital traits affect ejaculation helps to distinguish between these two processes. Unfortunately, existing studies of vertebrates are mainly correlational because of the technical challenge of manipulating genitalia [10].

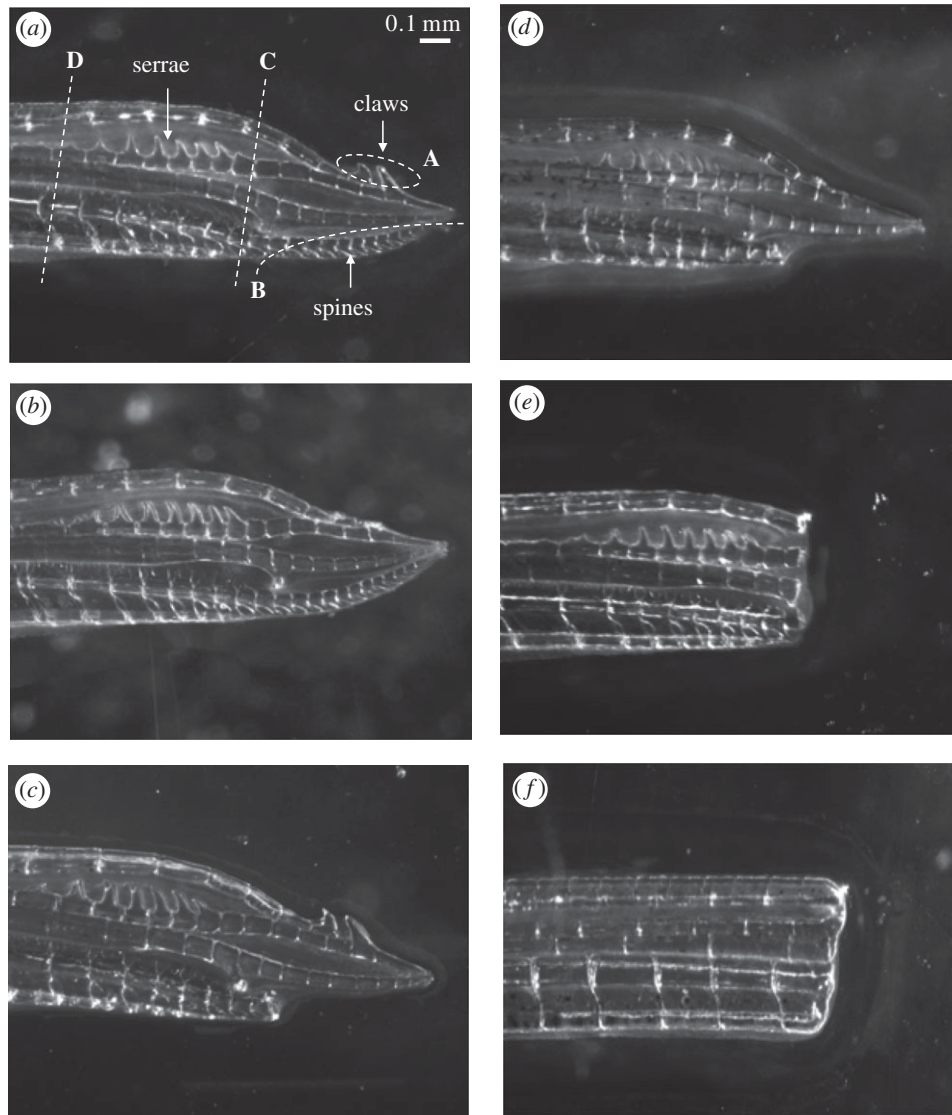


Figure 1. Surgery on the gonopodium tip: (a) control (no ablation); (b) claws removed (cut A in a); (c) spines removed (cut B); (d) claws and spines removed (cuts A and B); (e) distal part of shape altered (cut C); (f) whole tip removed (cut D). White dashed lines represent ablated locations.

Poeciliid fishes exhibit internal fertilization involving one of the briefest copulations known (20–50 ms; electronic supplementary material, Video S1). Males employ a coercive torque-thrust motion [11], whereby a male twists his body, swings his gonopodium (a modified anal fin) forward and expels spermatophores into the female's genital tract along a groove formed by the folded gonopodium [12–14]. Females are usually unreceptive, so there is strong selection on males to mate coercively [15]. This selects for the ability to control the timing of ejaculation during the brief 20–50 ms that a pair's genitals are in contact. Various fine-scale features such as claws, spines and serrae near the gonopodial tip (figure 1a) have presumably evolved in response to selection to ensure ejaculation at the appropriate time [14,16]. Specifically, it has been claimed that a male's *spines* provide sensory stimuli inducing sperm release upon penetration of the female's urogenital aperture [12]; that the *claws* grasp the female's genitalia and prolong the mating duration; and that the *serrae* anchor the male to stabilize the twisting direction of the folded gonopodium [12,13]. These structures should therefore determine the efficiency of sperm release, but except for a key study demonstrating the importance of claws in sperm transfer in guppies [17], experiments to test these hypotheses are lacking.

We conducted anatomical manipulations to test which gonopodium traits affect sperm release by male *Gambusia holbrooki*. Males frequently attempt to mate (sometimes >1 attempt/min; [18]). Costs of sperm production [19] should favour males that only ejaculate after successfully inserting their gonopodium into a female. Owing to the gonopodium length (erected tip position is behind the eye; [20]) and the sheer speed of the mating process, it is unlikely that males use visual cues, but rather rely on mechano-sensory stimuli from the tip to confirm insertion. Here, we demonstrated no effect of gonopodium tip ablation on male sexual motivation or sperm production. We then tested whether ablation led to a decline in sperm reserves (indicative of ejaculation) when males interacted with females. Finally, we surgically removed fine-scale gonopodial features to investigate their individual effects on ejaculation success.

2. Material and methods

Fish were collected from the wild in Canberra, Australia and maintained in single-sex stock tanks under a 14 L:10 D cycle at $28 \pm 1^\circ\text{C}$. Mature females were kept in single-sex tanks for more than four weeks to ensure that they were not gravid at the start of the experiment.

(a) Confirmation that an intact gonopodium tip is required for sperm release

Males were anaesthetized for 10 s in ice water and photographed on a glass slide to measure their standard length (snout tip to base of caudal fin) using *ImageJ* [21]. We randomly assigned size-matched males (21.30 ± 0.14 mm) into an ablated ($n=44$) and control ($n=46$) group (two-sample *t*-test, $t_{88} = -0.069$, $p=0.945$). While under anaesthetic, ablated group males were placed under a dissecting microscope, their gonopodium swung forward and we removed the gonopodial tip with a blade (Diplomat Blades, Victoria, Australia). Control males underwent the same manipulation without ablation. An ablated and a control male were then placed on either side of a 7 l tank divided by a mesh barrier and given 3 days to recover. When the experiment started, we stripped males' sperm reserves (Day 0). On Day 8 (allowing for full sperm replenishment; [19]), we again stripped males to record their baseline sperm count. Following 8 days, replenishment, we presented each male with a female (Day 16). We rotated females between tanks daily for 4 days to maintain male mating interest. On Day 20, we measured each male's post-mating sperm count.

(b) Testing which feature(s) of the gonopodium tip control the initiation of ejaculation

The presence of the gonopodial tip was necessary for triggering ejaculation (see §3). We therefore focused on the role of four structures (figure 1a): (i) *claws*; (ii) *spines*; (iii) the terminal segment and (iv) the mid-section (including *serrae*) of the tip's awl-like shape. We conducted two sets of experiments involving fine-scale anatomical manipulations of anaesthetized males performed under a stereo microscope (Leica M165C). The first set contained four treatments: control ($n=50$; no traits removed; figure 1a), claws removed ($n=43$; figure 1b), awl-shape altered (only distal part ($n=45$; figure 1e), or entire tip removed ($n=48$; figure 1f)). Given the significant effect of removing the awl-like shape (see *Results*), we conducted a second set of experiments with three treatments: control ($n=25$), spines removed ($n=23$; figure 1c), and claws and spines removed ($n=32$; figure 1d) to test whether the absence of spines and/or claws on a gonopodium with an intact awl-shape affected ejaculation. Size-matched males (first: 21.24 ± 0.13 ; second: 20.65 ± 0.15 mm) were haphazardly assigned to treatments (ANOVA, first: $F_{3,182} = 0.938$, $p=0.423$; second: $F_{2,77} = 0.908$, $p=0.408$). Males were then placed in individual 2 l tanks for 3 days to recover. We repeated the procedure in §2a to examine changes in sperm number.

(c) Ablation effect on male mating behaviours

We tested whether the lack of any decline in sperm count of ablated males (see §3) reflected a failure to ejaculate upon contacting a female, rather than a behavioural artefact of reduced mating motivation. We randomly assigned virgin males (22.49 ± 0.10 mm) to control and entire tip removed groups ($n=70$ per group; two-sample *t*-test, $t_{138} = -0.556$, $p=0.579$). Following a 3-day recovery, each male was individually introduced to a 4 l aquarium with a female behind a mesh screen. After 10 min acclimation, we raised the barrier and, for 20 min, recorded the number of copulation attempts (male positioned below female gonopore and thrusting his gonopodium). Trials were also videoed (PowerShot G7X Mark II video camera, Canon, Japan) to obtain the time spent associating with the female and total distance swum using Ethovision XT software (Noldus Information Technology, The Netherlands).

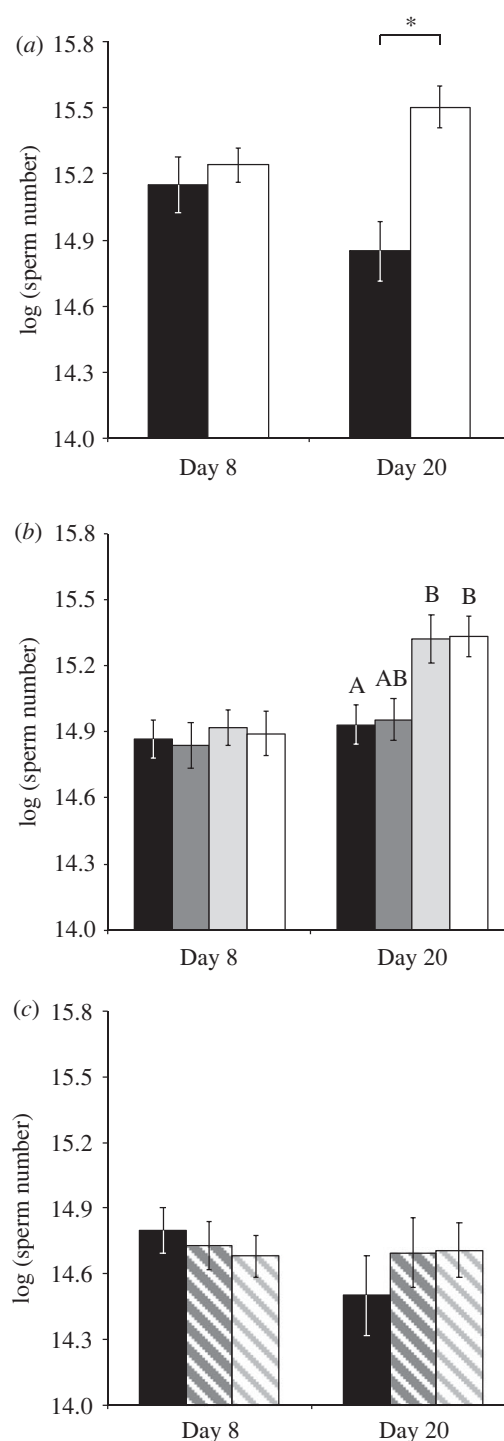


Figure 2. (a) Log-transformed sperm number of males with/without a gonopodial tip on Day 8 (baseline sperm reserves) and Day 20 (after interacting with a female). (b,c) Log-transformed sperm number of different tip treatments. Black = intact tip, white = entire tip removed, dark grey = claws removed, light grey = distal part of tip removed, dark grey stripe = spines removed, light grey stripe = claws and spines removed. Letters indicate significant differences using Tukey's tests. Means \pm s.e.

(d) Sperm count

After being anaesthetized, a male was placed on a slide covered in 1% polyvinyl alcohol solution. We swung his gonopodium forward and pressed his abdomen to eject all sperm bundles. We hydrated the ejaculate with 100 μ l of extender medium (207 mM NaCl, 5.4 mM KCl, 1.3 mM CaCl₂, 0.49 mM MgCl₂, 0.41 mM MgSO₄, 10 mM Tris (Cl); pH 7.5), collected the solution with a pipette and transferred it to an Eppendorf. We diluted the sperm with extender medium based on the stripped amount. We vortexed the solution for 30 s to break up sperm bundles and mixed the sperm

Table 1. Ablation effects on mating behaviours.

behaviour	estimate	s.e.	test statistic		
				value	<i>p</i>
mating attempts	0.157	0.298	$\chi^2_{1,136}$	0.279	0.598
association time	18.900	28.097	$F_{1,136}$	0.453	0.502
total moving distance	-121.831	92.316	$F_{1,136}$	1.742	0.189

with a 10 μ l pipette. We then placed 3 μ l of the solution into a 20-micron capillary slide (Leja) and used CEROS Sperm Tracker (Hamilton Thorne Research) under 100 \times magnification to record the sperm count for five subsamples per sample (repeatability: $r \pm$ s.e. = 0.876 ± 0.007 , $p < 0.001$, $n = 712$ male-days). Sperm counts were therefore performed blind to male treatment.

(e) Statistical analyses

We ran a generalized linear model with negative binomial error for mating attempts and separate general linear models for other traits, with treatment as a fixed factor and standardized body size as a covariate. We used sperm count on Day 8 as the dependent variable to test whether surgery affected sperm replenishment, and on Day 20 for the ablation effect on sperm release (controlling for Day 8 sperm count). We included the treatment \times body size interaction in full models to test whether gonopodial structures more strongly influence ejaculation by smaller, less attractive males [17,22,23]. We ran Tukey's *post hoc* tests to examine pairwise differences if the models in §2b indicated a significant treatment effect. Sperm data were log-transformed to meet model assumptions. Summary statistics are presented as mean \pm s.e. and significance as $p \leq 0.05$ (two-tailed). No outliers were found (Cook's Distance < 1). Our planned analyses were pre-registered online (<https://osf.io/ejt5p>).

3. Results and discussion

Controlling for body size (see electronic supplementary material), there was no surgery effect on sperm replenishment (Day 8 sperm count), either when males had their entire gonopodium tip removed (§2a: $F_{1,86} = 1.137$, $p = 0.289$) or when specific features were removed (§2b: set 1: $F_{3,178} = 0.033$, $p = 0.992$; set 2: $F_{2,74} = 0.221$, $p = 0.802$). Ablated and control males did not differ in their mating behaviour (table 1). We therefore attributed any differences in Day 20 sperm count to the rate of sperm release.

Entire tip-ablated males had a lower rate of sperm release than control males (§2a: $F_{1,85} = 17.110$, $p < 0.001$). The sperm count of ablated males increased from Day 8 to 20 while that of control males decreased (figure 2a), demonstrating the role of the gonopodium tip in triggering ejaculation. The treatments in §2b set 1 then showed significant variation in the effects of removing different structures on sperm release ($F_{3,177} = 4.810$, $p = 0.003$, figure 2b). Although we hypothesized that fine-scale traits might be more important for smaller males [3,17,23], there was no treatment \times body size interaction affecting sperm release (see electronic supplementary material). This might reflect the fact that there is no consistent body size effect on male mating success in *G. holbrooki* [10,24]: smaller size increases the rate of success per mating attempt [15], but females prefer larger males [22], who tend to win male-male fights [25].

Control and claw-removed males had similar Day 20 sperm counts (Tukey's test, $p = 0.967$), suggesting that claws are not required for sperm release. This is consistent with

studies in other species showing that removal of putative holdfast traits lowers insemination success because of sperm being transferred to an inappropriate location, rather than the likelihood of ejaculation [16,17,26]. It has been hypothesized that gonopodial claws in poeciliids enhance the likelihood of sperm release by prolonging genital contact [27], but this seems unlikely to be true for *G. holbrooki*. We did not detect a difference in the sperm count of males with and without claws. However, claws might help males deposit sperm more precisely inside the female. Anecdotal observations of sperm bundles being released into the water are evidence that sperm release does not always equate to successful sperm transfer in *Gambusia* [28,29].

Males with the awl-like shape removed had lower sperm release than control males (Tukey's test, $p = 0.028$), irrespective of whether this modification included or excluded the less distal part containing the serrae (Tukey's test, $p = 0.998$; figure 2b). We therefore focused on two key features on the distal tip: claws and spines. Spines' removal, irrespective of the presence or absence of the claws, did not affect the sperm count (§2b, set 2: $F_{2,73} = 1.472$, $p = 0.236$; figure 2c). Although innervation of gonopodial spines has been reported in another poeciliid [12], no innervation has been detected in *G. holbrooki* spines [13]. The lack of an effect of claw removal corroborates the findings of the §2b, set 1 manipulations.

Given no detectable role of spines or claws on sperm release, we emphasize the functional significance of the gonopodium's awl-like shape to elicit ejaculation (figure 1). An indirect line of evidence that tip shape affects sperm release comes from comparative analyses, which indicate that species or populations where males adopt more coercive mating tend to have a narrower, more elongated tip [3,30].

At present, we cannot determine whether fewer, or possibly zero, ejaculations following tip ablation is owing to: (i) ablated males being unable to insert their gonopodium into the female when the tip is no longer pointed; (ii) males sensing the malformity owing to altered hydro-dynamics, although this seems improbable (table 1), or (iii) removal of structures on the distal tip, other than the claws or spines, which trigger ejaculation. Although females control copulation duration in some taxa [31], thereby determining whether males ejaculate, this seems unlikely to be the case in *G. holbrooki*, given their high-speed copulation. We can also reject the hypothesis that swinging the gonopodium forward transfers spermatophores to the tip in anticipation of sperm transfer [14]: tip-ablated males still performed this movement (table 1) but there was no decline in sperm reserves. This finding emphasizes the role of genital contact in triggering ejaculation [3,12]. Indeed, our clearest finding is a *post hoc* analysis that tip removal has a large effect on sperm release (control or claws removed versus distal or entire tip removed: $F_{1,181} = 14.568$, $p < 0.001$).

Aside from a study in guppies [17], ours is the only one to experimentally test for the functional role of gonopodial traits in ejaculation in poeciliids. Our results highlight that the distal shape of the gonopodium tip can be selected to minimize wastage of ejaculate resources, likely to be crucial for males exhibiting coercive mating (with its associated low copulation success rate; [32]). Importantly, we also show that holdfast devices (*claws*, *spines* and possibly *serrae*) have no, or an insignificant, effect on triggering ejaculation. Given that such structures show strong variation among poeciliid species [12], they may instead evolve because they affect the success, or precision, of sperm transfer to females [17], e.g. through prolonging contact with the gonopore.

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