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COMPLEX PATTERNS OF MULTIVARIATE SELECTION ON THE EJACULATE OF A BROADCAST SPAWNING MARINE INVERTEBRATE

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Assessing how selection operates on several, potentially interacting, components of the ejaculate is a challenging endeavor. Ejaculates can be subject to natural and/or sexual selection, which can impose both linear (directional) and nonlinear (stabilizing, disruptive, and correlational) selection on different ejaculate components. Most previous studies have examined linear selection of ejaculate components and, consequently, we know very little about patterns of nonlinear selection on the ejaculate. Even less is known about how selection acts on the ejaculate as a functionally integrated unit, despite evidence of covariance among ejaculate components. Here, we assess how selection acts on multiple ejaculate components simultaneously in the broadcast spawning sessile invertebrate *Mytilus galloprovincialis* using the statistical tools of multivariate selection analyses. Our analyses of relative fertilization rates revealed complex patterns of selection on sperm velocity, motility, and morphology. Interestingly, the most successful ejaculates were made up of slower swimming sperm with relatively low percentages of motile cells, and sperm with smaller head volumes that swam in highly pronounced curved swimming trajectories. These results are consistent with an emerging body of literature on fertilization kinetics in broadcast spawners, and shed light on the fundamental nature of selection acting on the ejaculate as a functionally integrated unit.

KEY WORDS: **Disruptive selection, free spawning, mussel, nonlinear selection, postcopulatory sexual selection, selection analysis, sperm competition.**

Ejaculates are composed of numerous components that play a fundamental role in determining male reproductive fitness. To fulfill their primary (naturally selected) function of fertilizing eggs, motile sperm often have to swim toward immobile eggs. In the course of this journey, sperm may encounter a hostile female reproductive tract (Birkhead et al. 1993; Eberhard 1996) or, in the case of externally fertilizing species, extrinsic environmental stressors that limit their ability to reach their target (Morisawa et al. 1983; Billard 1986). In most species, only a tiny fraction of the millions of sperm contained within a typical ejaculate actually reach an egg (Birkhead et al. 1993). Therefore, there is intense natural selection on ejaculates to ensure male fertility. Additionally, in most sexually reproducing species ejaculates fulfill a secondary (sexually selected) function of competing with sperm from rival males to fertilize a female's eggs (sperm competition, Parker 1970) or maximizing the likelihood that females use their sperm during this competition (cryptic female choice, Eberhard 1996). As a result, ejaculates are also under intense selection to ensure the male's paternity during episodes of postmating sexual selection. Surprisingly, despite the fundamental link between ejaculates and fitness, our understanding of how either of these selective processes operates on the ejaculate as a

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functionally integrated unit is extremely limited (Pizzari and Parker 2009).

Ejaculates are best considered as composite traits comprising multiple components that serve many, and sometimes opposing, functions (Pizzari and Parker 2009). An increasing number of studies have revealed complex phenotypic associations among ejaculate components that have individually been linked to reproductive fitness. For example, both sperm morphology and sperm swimming velocity, which are often assumed to be positively associated (Gomendio and Roldan 1991, 2008), have been shown to influence male fertilization success in noncompetitive and/or competitive contexts (e.g., Donnelly et al. 1998; Au et al. 2002; Gage et al. 2004; Malo et al. 2005; García-González and Simmons 2007). Yet the phenotypic association between these ejaculate components is inconsistent among species, with several studies reporting positive, negative, or no phenotypic correlations between sperm length and swimming velocity (e.g., Malo et al. 2006; Pitcher et al. 2007; Skinner and Watt 2007; Humphries et al. 2008; Mossman et al. 2009; Firman and Simmons 2010; Fitzpatrick et al. 2010; Helfenstein et al. 2010). Underlying many of these phenotypic associations are complex patterns of genetic covariance among ejaculate traits, which in many cases point to potential constraints on ejaculate evolution (reviewed by Simmons and Moore 2009). Indeed, a growing number of quantitative genetic studies have revealed negative genetic correlations between various ejaculate components (e.g., Simmons and Kotiaho 2002; Moore et al. 2004; Birkhead et al. 2005; Mossman et al. 2009; Simmons and Moore 2009; Evans 2011). For example, morphological components of sperm, including flagellum and midpiece size (Birkhead et al. 2005) and flagellum and head size (Evans 2011), are negatively genetically correlated in the zebra finch, *Taeniopygia guttata*, and guppy, *Poecilia reticulata*, respectively. These negative genetic correlations highlight the potential for trade-offs to constrain the evolutionary responses of ejaculates in the face of selection. However, out of necessity and/or tractability, when assessing how ejaculate traits influence reproductive fitness (usually relative fertilization success), previous studies have typically focused on ejaculate components individually rather than assessing how multiple ejaculate components simultaneously influence fitness.

In this article, we estimate the direction, form, and strength of selection on ejaculate components. Specifically, we determine how ejaculate traits are shaped by linear (i.e., directional) and nonlinear (i.e., stabilizing, disruptive, or correlational) selection within a multidimensional framework using the statistical tools of multivariate selection analyses. This approach recognizes that ejaculates are composed of multiple components on which selection is unlikely to act in isolation (Lande and Arnold 1983; Phillips and Arnold 1989; Schluter and Nychka 1994; Blows 2007). Here, we focus on the broadcast spawning mussel *Mytilus galloprovincialis*, which offers a remarkable opportunity to assess how selection acts on ejaculates. In this species, spawning can be readily mimicked in the laboratory under highly controlled experimental conditions in which confounding factors that are known to influence reproductive fitness (e.g., gamete density and age; Hoysak and Liley 2001; Pizzari et al. 2008) can be taken into account. Furthermore, like other broadcast spawning marine invertebrates, *M. galloprovincialis* offers an excellent opportunity to estimate relative fertilization rates while accounting for male-by-female interaction effects at fertilization (due to gametic incompatibilities; Evans and Marshall 2005) and pairwise interactions between rival males (García-González 2008; García-González and Evans 2011). In broadcast spawning marine invertebrates, variation in adult density, synchrony of gamete release, and environmental conditions can result in fertilizations taking place under ecological conditions ranging from extreme sperm limitation, where sperm competition is unlikely and fertilization is limited by the availability of sperm, to polyspermic conditions, where sperm competition is likely to be intense (Levitan and Petersen 1995; Yund 2000; Levitan 2004, 2010). Here, we mimic the ecological conditions of sperm limitation and focus on how selection operates in noncompetitive fertilization trials when ejaculates are fulfilling their primary, naturally selected function of fertilizing eggs.

Methods

GAMETE COLLECTION AND FERTILIZATION PROTOCOL

The mussel *M. galloprovincialis* is a cosmopolitan marine invertebrate that dominates intertidal communities in many temperate and subpolar regions of the northern and southern hemispheres (Daguin and Borsa 2000). Live adults were collected from Claremont Jetty, Western Australia (31°59′20.1″S, 115°46′53.5″E) in July 2010 and placed in recirculating seawater tanks maintained at 16°C until they were required (within a week of collection). Mussels were induced to spawn by raising their temperature to 30°C, which induces gamete release in *Mytilus* species. After the onset of gamete release (where males and females were identified), mussels were removed from the warm water bath, washed in clean seawater to prevent contamination of gametes from other individuals, and placed individually in a cup containing 250 mL seawater where they continued to release gametes. Using these methods, we collected gametes from 21 females and 114 males.

To provide a '"common" fertilization environment in which fertilization rates could be estimated, we pooled 100 mL of an egg/seawater solution from each of the 21 females (with a standardized density of ∼15,000 eggs/mL for each female). By pooling eggs in this way, we minimized stochastic variation in the fertilizing ability of eggs from different females and potential male-by-female interaction effects at fertilization, which are common

in broadcast spawning invertebrates (e.g., Evans et al. 2007) and known to characterize fertilization in *M. galloprovincialis* (Evans et al. 2012). The use of a common fertilization environment facilitated the comparison of ejaculate traits among males without having to address female effects (e.g., egg size/quality/ripeness) or male-by-female interactions in our analyses. This precaution was necessary because our aim was to derive an estimate of "relative fitness" for each male (see below), which in nature would depend on a male's ability to successfully fertilize eggs from numerous females during successive spawning events. The pooling of eggs from multiple female genotypes was therefore necessary to simulate a broad genetic background and thereby generate standardized measures of relative fitness for each focal male.

We subdivided the mixed pool of eggs by placing 10 mL of the egg/seawater solution into 114 petri dishes, which were gently mixed with an aerator. We then collected sperm/seawater samples from each of the 114 males for the sperm velocity and morphology measures (see Sperm Analyses) and fertilization trials. In broadcast spawning marine invertebrates, sperm begin to age once diluted (Levitan et al. 1991). Therefore, sperm were collected from the dense aggregations that accumulated at the bottom of the 250-mL cup and were only diluted when assessing sperm swimming speed just prior to fertilizations. Fertilization assays were performed by adding 100 μl of the sperm sample to the egg solution in the petri dishes, which were left under aeration for 1 h. We then added 1 mL of 10% buffered formalin to the sperm–egg mixture to halt the fertilization process. These fixed samples were used to determine fertilization rates for each male by counting the number of eggs undergoing cleavage and/or with polar body formation among 200 randomly selected eggs per sample. The remainder of the sperm sample was preserved with 10% buffered formalin and used later to measure sperm morphology and determine sperm density in each sample (see Sperm Analyses and Gamete Density and Age). Thus, in our experiment, all $n = 114$ males experienced a standardized fertilization environment (i.e., the same combination of females), enabling us to generate comparable estimates of relative "fitness" (fertilization rates) in our multivariate selection analyses (see Multivariate Selection Analyses).

SPERM ANALYSES

For each male, sperm motility was measured with computer-assisted sperm analysis (CASA) using an aliquot of the sperm sample (diluted ×10) that was subsequently used for the fertilization trials described above. Two separate 2 μl aliquots from each male were placed individually into separate wells of a 12-cell multitest slide (MP Biomedicals, Aurora, OH), previously coated with 1% polyvinyl alcohol to prevent sperm from sticking to the glass slide (Wilson-Leedy and Ingermann 2007). These samples were analyzed using the CEROS Sperm Tracker (Hamilton Thorne Research, Beverly, MA, USA). The threshold values for defining static cells were predetermined at 19.9 μm/sec for VAP (average path velocity) and VCL (curvilinear velocity), and 4 μm/sec for VSL (straight-line velocity). Sperm motility measures were based on an average of 240 ± 6.4 SE sperm tracks per sample. We performed replicate sperm motility measures for each male; within-sample repeatability (Lessells and Boag 1987) for all sperm velocity measures was high (intraclass correlation coefficients ± SE: VAP: $r = 0.91 \pm 0.02$; VCL: $r = 0.89 \pm 0.02$; VSL: $r = 0.81 \pm 0.03$). Given this consistency in our sperm velocity measures, we used the mean of the two values for each male in the subsequent analysis. CASA yields several highly correlated measures of sperm motility (Table 1), which in turn can generate statistical issues due to multicolinearity of the data. Thus, rather than including all sperm motility measures in our analyses, we condensed CASA measures using a principal component analysis (PCA). The PCA generated two principal components with eigenvalues >1 (Table 1) that were used in subsequent analyses. Three measures of sperm velocity (VCL, VSL, and VAP) and the percentage of motile sperm contributed primarily to the first composite sperm motility score (PC1), whereas sperm path straightness and linearity contributed to the second composite motility score (PC2). Measures of sperm motility were taken within 7.4 ± 0.4 min (mean ± SE, range: −5 to 22 min) of the fertilization trials.

Table 1. Principal component analysis of sperm velocity variables generated by computer-assisted sperm analysis in mussels.

	Principle component	
Sperm trait	PC1	PC2
Percent of motile sperm	0.35	0.05
VAP: Smooth path velocity	0.42	−0.17
VSL: Straight-line velocity	0.42	0.16
VCL: Curvilinear path sperm	0.41	−0.23
ALH: Amplitude of lateral head displacement	0.29	−0.42
BCF: Beat cross frequency	−0.37	0.18
STR: Straightness (ratio of VSL/VAP)	0.17	0.65
LIN: Linearity (ratio of VSL/VCL)	0.31	0.51
Eigenvalue	5.1	1.9
Percent of variance explained	63.3	86.6

Measures of sperm length and sperm density (see Gamete Density and Age) were estimated from preserved sperm samples. We measured sperm flagellum length, sperm head width (W), and sperm head length (L) from 30 individual spermatozoa per male from digital photographs taken with a Leica DFC320 digital camera mounted to a Leica DM1000 microscope at 400× magnification. The midpiece in mussel sperm is not clearly visible under

light microscopy and was therefore not considered in our analyses. Sperm morphology was measured using the software ImageJ 1.43 (http://rsb.info.nih.gov/ij/download.html). Sperm head volume was calculated using the formula for a prolate spheroid (volume = $4/3\pi W^2L$), where W = sperm head width and L = sperm head length.

GAMETE DENSITY AND AGE

In externally fertilizing species, fertilization success can be influenced by gamete density and gamete age (Hoysak and Liley 2001; Pizzari et al. 2008; Havenhand and Schlegel 2009). We therefore accounted for both of these variables using two approaches. First, prior to performing fertilization trials we broadly standardized sperm densities among samples using a spectrophotometric method (see Supporting Information). This method ensured that there was sufficient experimental variation in sperm density among ejaculates (counts estimated using an improved Neubauer haemocytometer; mean ± SE sperm density: 8501 ± 439 sperm/μl; range: 2350–27,250 sperm/μl), while also ensuring that ceiling effects (i.e., fertilization rates of 100%) did not confound our analyses (see Supporting Information). This is consistent with natural spawning events in broadcast spawning marine invertebrates, which are generally characterized by conditions of sperm limitation resulting in highly variable fertilization rates that rarely reach 100% (Levitan and Petersen 1995). Furthermore, the final density of sperm used in fertilization trials (8.5×10^{-4} sperm/mL) is at the lower end of the range of concentrations where sperm becomes limited during fertilizations in broadcast spawning marine invertebrates (Pemberton et al. 2003). As we were interested in assessing ejaculate traits in this study, we ensured that egg density did not vary among fertilization trials.

Second, although sperm and eggs remain viable for several hours after gamete release in many marine invertebrates (e.g., Sprung and Bayne 1984), gamete quality (i.e., swimming speed and percent motile), and fertilization efficiency decrease with gamete age (e.g., Levitan 2000). Therefore, we recorded and controlled for gamete age for each sample by noting the time that elapsed between the onset of spawning and the time that sperm were added to the egg mixture. Egg age was included in the analyses as this can influence fertilization rates (Babcock and Keesing 1999; Hoysak and Liley 2001). The mean (± SE) age of gametes used in our experiment was 135 ± 4.7 min and 274 ± 7.9 min for sperm and eggs, respectively. These gamete age values are well within the 6–11 h range (after spawning) where successful fertilization occurs in the closely related *M. edulis* (Sprung and Bayne 1984).

CORRELATION ANALYSIS

To explore potential phenotypic relationships among ejaculate morphology, motility, and density, we calculated correlation and partial correlation coefficients from a multivariate correlation model including all ejaculate traits examined in this study, including sperm age. Partial correlation coefficients of ejaculate traits were generated with respect to all other ejaculate traits in the model. Coefficients were generated in JMP version 9.0.0 (SAS Institute Inc. 2010).

MULTIVARIATE SELECTION ANALYSES

We used selection analyses and response surface methodology (Lande and Arnold 1983; Phillips and Arnold 1989; Blows and Brooks 2003; Reynolds et al. 2010) to characterize the form, intensity, and direction of selection acting on the ejaculate. Prior to analyses, all gamete traits were standardized to have a mean of zero and standard deviation of one, whereas fertilization rates were converted to measures of relative fitness by dividing each male's fertilization score by the mean across all samples (Lande and Arnold 1983). We used JMP version 9.0.0 (SAS Institute Inc. 2010) to estimate the univariate linear selection gradients (β) and the matrix of quadratic and correlational selection gradients (γ) (Lande and Arnold 1983) using multiple regressions on the following seven gamete traits: sperm head volume, sperm flagellum length, composite sperm motilities PC1 and PC2, sperm density, sperm age, and egg age. To obtain accurate estimates, all quadratic selection gradients ($\boldsymbol{\gamma}_{ii}$) were doubled following multiple regressions (Stinchcombe et al. 2008).

When testing for nonlinear selection, we used a canonical rotation of the matrix of nonlinear selection gradients (gamma matrix) to find the major axes of the response surface. This process generated new composite trait scores (eigenvectors, **m1**, **m2**... **m7**), each describing a major axis of the response surface. This method provides improved estimates of nonlinear (e.g., disruptive, stabilizing) selection over traditional second-order polynomial regression models by accounting for correlational selection among the measured traits (Phillips and Arnold 1989; Blows and Brooks 2003). The strength of selection along each eigenvector is given by its eigenvalue, the significance of which is traditionally assessed using the double regression method (Bisgaard and Ankenman 1996). However, the double regression method can inflate type I error rates due to the false assumption that nonsignificant nonlinear terms have zero eigenvalues (Reynolds et al. 2010). We therefore used the recently prescribed permutation-based approach (Reynolds et al. 2010), which avoids inflating type I error rates when testing for nonlinear selection. Permutation tests were performed in R version 2.10.1 (R Foundation for Statistical Computing 2009) using the R code generously supplied in the supplementary material of Reynolds et al. (2010).

To visualize significant nonlinear axes of selection, we generated nonparametric fitness surfaces (Schluter 1988; Schluter and Nychka 1994). Although nonparametric surfaces do not necessarily reflect the parametric parameter estimates generated from

selection analyses, they estimate fitness surfaces without a priori assumptions and therefore offer less constrained visualizations of quadratic approximations (Schluter 1988; Schluter and Nychka 1994). The predicted values and Bayesian standard errors for the cubic splines were generated using Schluter's GLMS 4.0 (http://www.zoology.ubc.ca/~schluter/software.html) in R version 2.10.1. The smoothing parameters were chosen from values that minimize the generalized cross-validation (GCV) scores. Fitness surfaces of the significant major canonical axes were visualized by fitting thin-plate splines using the Tps function of the fields package of R.

Results

Multivariate linear correlation analyses did not reveal any significant associations among sperm morphology (head volume and flagellum length), sperm density, and sperm motility measures (Table 2).

We examined whether gamete traits influenced fitness by assessing the standardized univariate linear, and quadratic and correlational nonlinear selection gradients using relative fertilization rates as a fitness measure. Multiple regression analyses revealed significant, albeit weak ($\beta = -0.044$), negative linear selection on sperm age, indicating that relatively older sperm fertilized fewer eggs than relatively younger sperm (Table 2). We found no other evidence for linear selection on any other trait (Table 2). However, composite sperm motility PC1 exhibited significant nonlinear positive quadratic selection, and we detected negative correlational selection on sperm flagellum length and composite sperm motility PC2 (Table 2). These results reveal nonlinear selection on ejaculate traits in *M. galloprovincialis*.

To further characterize nonlinear selection, we performed a canonical rotation of the $\boldsymbol{\gamma}$ matrix of nonlinear selection gradients. The overall pattern of selection on ejaculate traits revealed a fitness peak at one end of the phenotypic distribution, albeit with nonlinear concave curvature in the **m2** and **m4** axes (Fig. 1a). In particular, we detected two significant axes of nonlinear selection (**m2** and **m4**), with **m2** displaying concave curvature (positive quadratic) in the fitness surface and **m4** displaying largely directional nonlinear selection (Fig. 1a; Table 3). The **m2** axis was primarily loaded negatively by the composite sperm motility PC1 (Table 3), which itself was primarily loaded positively by three measures of sperm velocity, VCL, VSL, and VAP, and by the percentage of motile sperm (Table 2). Males with larger values on the **m2** axis had the highest relative fertilization rates, indicating that males who produced ejaculates with slower swimming sperm and fewer motile sperm (i.e., low composite sperm motility PC1 values) were selectively advantaged. The other significant axis of nonlinear selection, **m4**, which was considerably weaker

Table 2. The vector of standardized linear selection gradients (β), the matrix of standardized quadratic and correlational selection gradients (γ), and the phenotypic correlation matrix for the ejaculate traits analyzed. For β and γ matrices, the response variable is the relative fertilization rates. The standardized quadratic selection gradients are on the diagonal axis (highlighted in gray) and the correlational selection gradients are listed above the diagonal. All selection gradients are presented ± standard error. The phenotypic correlations of sperm traits are listed below the diagonal. Partial phenotypic correlations, which are partialed with respect to all other ejaculate variables examined (including sperm age), are shown in parentheses. Egg age was not included in the phenotypic correlation analyses. Estimates and partial correlations in bold are significant. $^{*}P < 0.05$; $^{**}P < 0.01$.

	β	γ					
		Sperm density	Sperm head volume	Sperm flagellum length	Sperm motility PC1	Sperm motility PC2	Sperm age	Egg age
Sperm density	−0.016	0.008±0.020	−0.007±0.017	−0.015±0.020	0.018±0.016	−0.006±0.015	0.005±0.026	0.012±0.026
Sperm head volume	−0.015	0.07(−0.04)	0.022±0.025	0.019±0.016	0.020±0.020	0.008±0.017	0.032±0.025	0.029±0.029
Sperm flagellum length	0.002	−0.08(−0.05)	−0.02(0.04)	−0.002±0.018	0.021±0.012	**−0.039±0.016***	0.029±0.025	0.008±0.026
Sperm motility PC1	−0.001	0.02(0.08)	−0.16(−0.09)	0.11(0.08)	**0.046±0.022***	0.012±0.016	−0.014±0.020	−0.010±0.022
Sperm motility PC2	0.001	0.13(0.03)	0.08(−0.04)	0.08(0.13)	0.0(0.05)	0.009±0.016	−0.022±0.021	−0.015±0.022
Sperm age	−0.044**	–	–	–	–	–	−0.093±0.054	0.000±0.041
Egg age	−0.017	–	–	–	–	–	–	0.080±0.063

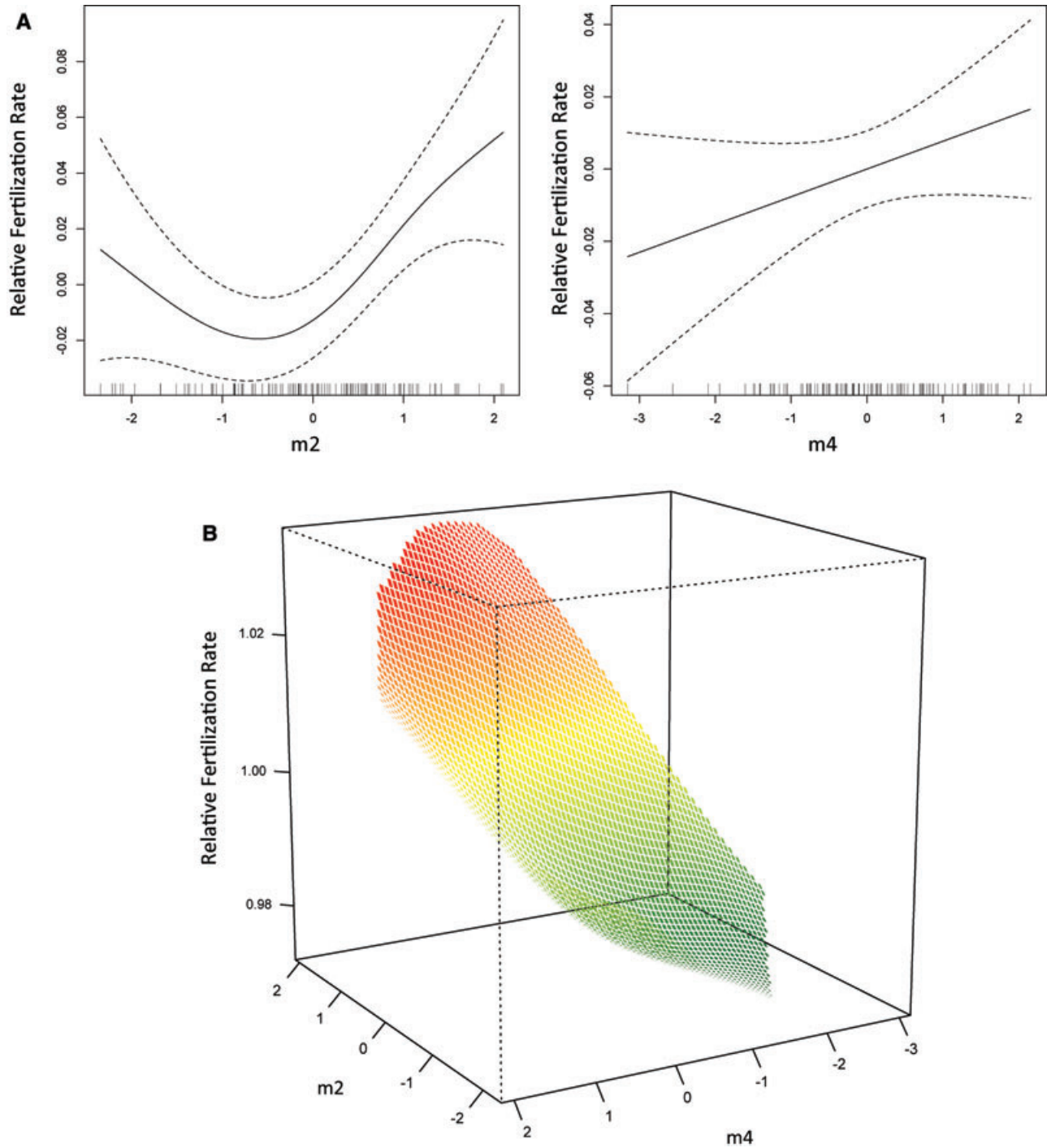

Figure 1. **Visualizations of selection on the m2 and m4 major canonical axes. (a) A univariate cubic spline visualization of selection on m2 and m4 axes, which were both characterized by positive nonlinear selection coefficients. Cubic spline plots are generated from predicted values. The solid lines represent the fitted spline and the dotted lines represent ± 1 Bayesian standard error. (b) The fitness surface of the two significant axes of nonlinear selection, m2 and m4, demonstrating a fitness peak at one end of the phenotypic distribution.**

than **m2**, was heavily loaded by positive sperm density values and by negative sperm head volume and composite sperm motility PC2 values (primarily positively loaded by sperm path straightness and linearity). Males with larger values on the **m4** axis had the highest relative fertilization rates, indicating that dense ejaculates with sperm that had small head volumes and sperm that swim in curved paths were selectively advantaged.

Overall, we can gain a better understanding of how nonlinear selection influences mussel ejaculate traits by examining the fitness surface of the two significant axes of nonlinear selection

Table 3. **The M matrix of eigenvectors and estimates of nonlinear selection (λ) on the new latent axes described by the eigenvectors from the canonical analysis of γ and the associated *P*-values from permutation tests for the gamete traits measured in our analysis. The response variable is relative fertilization rate.**

M	Sperm density	Sperm head volume	Sperm flagellum length	Composite sperm motility PC1	Composite sperm motility PC2	Sperm age	Egg age	λ	*P*
M1	−0.06	−0.39	−0.26	−0.03	0.24	−0.13	−0.84	0.10	0.53
M2	−0.09	−0.35	−0.28	−0.84	−0.08	−0.04	0.27	0.07	0.02
M3	0.32	−0.01	−0.56	0.23	0.60	−0.22	0.35	0.05	0.08
M4	0.63	−0.58	0.05	0.23	−0.46	−0.06	0.08	0.03	0.02
M5	0.64	0.54	−0.15	−0.34	−0.07	0.26	−0.30	−0.01	0.77
M6	−0.26	0.22	−0.69	0.21	−0.60	−0.10	−0.03	−0.05	0.13
M7	−0.10	−0.24	−0.22	0.15	0.10	0.92	0.06	−0.11	0.36

(Fig. 1b). The fitness surface demonstrates a strong peak of relative fertilization success at the end of the phenotypic distribution with positive values of the **m2** and **m4** axes of nonlinear selection (Fig. 1b). Thus, ejaculates experience nonlinear selection on the combination of traits that load on positive values of the **m2** and **m4** nonlinear axes. Specifically, in this study selection favors ejaculates with a combination of slower swimming sperm, fewer motile sperm, small sperm head volume, curved sperm swimming paths, and high sperm density.

Discussion

Our study shows that the ejaculate of the mussel *M. galloprovincialis* is subject to multiple forms of selection and reveals that complex interactions among ejaculate traits can have an important bearing on individual fitness. Surprisingly, the strength of linear (i.e., directional) selection is relatively weak in *M. galloprovincialis*, as sperm age was the only ejaculate trait that exhibited a significant linear selection gradient, and there was no evidence of linear relationships among sperm morphology and motility. In contrast, mussel ejaculates experience multivariate selection on two major axes of nonlinear selection, indicating that nonlinear selection acts on combinations of ejaculate traits. Specifically, selection favors ejaculates with slower swimming sperm and a lower percentage of motile sperm (which loaded on the significant axis of nonlinear selection, **m2**) and dense ejaculates made up of sperm with smaller head volumes that swam in curved paths (**m4**) being selectively advantaged. Yet overall, the fitness surface of the two axes of nonlinear selection revealed a clear fitness peak at positive values of the **m2** and **m4** axes (Fig. 1b). Despite the limited role of linear selection on the ejaculate in mussels, our results demonstrate that fertilization success generates a pattern of selection that is largely directional in favoring one end of the phenotypic distribution of ejaculate traits. Collectively, our results highlight the importance of considering the multiple components of the ejaculate as a functionally integrated unit and assessing both linear and nonlinear forms of multivariate selection on ejaculates.

Our finding that relatively slow-swimming sperm conferred the greatest relative fitness in *M. galloprovincialis* seems initially counterintuitive and contrasts with the findings from several studies reporting the fitness benefits associated with producing relatively fast-swimming sperm, both in noncompetitive (Donnelly et al. 1998; Froman et al. 1999; Au et al. 2002; Kupriyanova and Havenhand 2002; Malo et al. 2005) and competitive contexts (Gage et al. 2004; Denk et al. 2005; Casselman et al. 2006; Liljedal et al. 2008; Gasparini et al. 2010; Boschetto et al. 2011). However, in marine invertebrates there may be substantial adaptive benefits favoring the evolution of slower swimming sperm, particularly under conditions of sperm limitation when slower swimming sperm may be able to search for eggs for longer periods of time (Levitan 1998a, b, 2002). For example, in a comparison of gamete traits among three congeneric sea urchin species Levitan (1998a) reported that *Strongylocentrotus droebachiensis*, the species most likely to experience sperm limitation due to low adult densities, produced slower swimming sperm compared with two other closely related urchin species where sperm limitation was less likely. As the fertilization conditions in the present study closely approximated sperm limitation conditions in broadcast spawning marine invertebrates (Pemberton et al. 2003), our results offer insights into how selection operates when the probability of sperm–egg encounters is reduced due to environmental factors (e.g., water depth, turbulence, and velocity) and the local density of reproductive adults. Additionally, a common characteristic of reproductive dynamics in broadcast spawning marine invertebrates is that gamete release by males and females is asynchronous, with males releasing gametes prior to females (Lotterhos and Levitan 2010). This asynchrony in gamete release between the sexes is likely to generate intense selective pressure against sperm senescence (Pizzari et al. 2008). Because sperm swimming speed and longevity are negatively correlated

in some externally fertilizing species (Burness et al. 2004), including broadcast spawning marine invertebrates (Levitan 2000), males that produce slower swimming sperm are likely to also produce sperm that can continue swimming in search of eggs for longer periods of time. Under such conditions, selection may favor slower sperm, as we have observed in the present study. Indeed, in a recent study of an externally fertilizing myobatrachid frog, *Crinia georgiana*, where sperm are long lived and fertilization does not occur instantaneously after contact between sperm and eggs, Dziminski et al. (2009) reported that males with slower swimming sperm had a fertilization advantage in the presence of competing sperm from a rival male.

The results from the present study reveal that selection favors variation in the type of swimming paths that sperm use when tracking eggs, and certain aspects of sperm morphology. Selection on sperm swimming paths is likely to influence fertilization success by improving the ability of sperm to efficiently locate unfertilized eggs. In broadcast spawning marine invertebrates, sperm are attracted to chemical signals (chemoattractants) released from unfertilized eggs (Eisenbach 1999; Eisenbach and Giojalas 2006). Physical models of sperm swimming trajectories indicate that sperm swimming in circular and helical paths sample chemoattractant gradients from unfertilized eggs more effectively (Friedrich and Julicher 2008). This may explain why sperm with curved swimming paths were selectively advantaged in our study and why circular sperm swimming paths are commonly observed in broadcast spawning species, where chemoattractants are spread diffusely in the external environment (Liu et al. 2011). In contrast, it is more difficult to interpret the observed pattern of selection on sperm morphology. For example, sperm head volume was subject to weak nonlinear selection that was clearly directional (see the cubic spline plot of the **m4** axes of nonlinear selection in Fig. 1a), where sperm with smaller head volumes had greater relative fertilization rates. In externally fertilizing species, sperm head morphology may be targeted by selection because of correlated responses with sperm swimming speed (via interactions with the sperm flagellum) or path type (sensu Humphries et al. 2008). Yet, we found no obvious evidence of selection on flagellum length, despite recent evidence of an association between flagellum length and sperm swimming velocity across a diverse set of taxa (Gomendio and Roldan 1991, 2008; Fitzpatrick et al. 2009; Lüpold et al. 2009), including broadcast spawning marine invertebrates (Fitzpatrick et al. 2010). However, we did detect a nonsignificant ($P = 0.08$) trend of nonlinear selection on the **m3** axis of selection, which was loaded heavily by negative values of sperm flagellum length and positive values of composite sperm velocity PC2. Although the effect size for the nonsignificant **m3** axis was larger than that of the significant **m4** axis, we refrain from overinterpreting this result and accept the null hypothesis based on our current analyses.

In this study, we sought to gain a basic understanding of how selection acts on multiple ejaculate traits simultaneously. To this end, we have focused on the primary (naturally selected) function of ejaculates by exploring patterns of selection within a noncompetitive framework that is biologically representative of the sperm-limited conditions that often face broadcast spawning invertebrates (Levitan and Petersen 1995; Levitan 1998b, 2002; Yund 2000). Under such conditions, natural selection will clearly favor adaptations that maximize the likelihood that sperm will encounter eggs (e.g., curved swimming trajectories that facilitate sperm–egg contact rates; see above). Nevertheless, although our study represents an important first step in exploring selection on the ejaculate, we acknowledge that sperm limitation is at one end of a continuum of fertilization conditions commonly experienced by broadcast spawning marine invertebrates and that an important future challenge is to determine how selection acts while ejaculates fulfill their second major function of competing to fertilize eggs with ejaculates from rival males (i.e., postmating sexual selection). However, establishing patterns of selection on ejaculates under conditions of sperm competition is not a trivial undertaking, not least because of the logistical challenges associated with deriving biologically relevant estimates of relative fitness. The main challenge in this regard is to derive estimates of relative fertilization success that avoid stochastic variation attributable to nonfocal sperm competitors, a problem that typically characterizes traditional sperm competition studies in which just two males compete to fertilize a female's eggs (García-González 2008). Nevertheless, the development of such approaches will facilitate a critical assessment of whether postmating sexual selection also generates stabilizing, directional, or disruptive selection on the ejaculate—each of which has been envisaged (Pizzari and Parker 2009). Moreover, contrasting the patterns of selection observed with and without postmating sexual selection promises exciting further insights into how selection operates on ejaculates. Finally, incorporating additional ejaculate traits that are known to influence male fitness (e.g., sperm viability: García-González and Simmons 2005; seminal fluid components: Poiani 2006; and gamete recognition proteins: Levitan and Ferrell 2006) into a multidimensional framework will help to further our understanding of how selection influences the primary and secondary functions of ejaculates.

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