## Original Article

# Testes size increases with sperm competition risk and intensity in bony fish and sharks

Amy G. Rowley,[a,b,ⓘ] Toby S. Daly-Engel,[c] and John L. Fitzpatrick[b,ⓘ]

[a]School of Medical Sciences, Faculty of Biology, Medicine and Health, University of Manchester, Oxford Road, Manchester M13 9PL, UK, [b]Department of Zoology, Stockholm University, Svante Arrhenius väg 18B, SE-10691 Stockholm, Sweden, and [c]Department of Biological Sciences, Florida Institute of Technology, W University Blvd, Melbourne, FL 32901, USA

Female multiple mating provides the opportunity for sexual selection to continue after gamete release, generating strong selection on male reproductive traits. In particular, in species where female multiple mating is common, males are expected to invest more in testicular tissue to afford them a numerical advantage during sperm competition. However, although relative testes size (correcting for body size) is a commonly used proxy of the strength of sperm competition, there is surprisingly scant direct evidence linking male investment in testes with genetic estimates of multiple paternity across species. Here, we test the hypothesis that testes size is associated with genetic estimates of sperm competition risk (multiple paternity percentage) and intensity (number of sires per brood) in fishes, the most diverse and specious vertebrate group. We provide conclusive evidence that relative testes size is larger in species experiencing a higher risk and intensity of sperm competition, a finding that remains consistent among sharks and bony fishes (including in separate analyses focused only on cichlids). These findings shed new light on evolutionary processes governing sperm competition in a basal vertebrate lineage and validate the now-widespread use of relative testes mass as a proxy for sperm competition risk and intensity in fishes.

*Key words:* **polyandry, sexual selection, sperm competition.**

## INTRODUCTION

Female genetic monogamy appears to be the exception rather than the rule in animals (Birkhead and Møller 1998; Birkhead et al. 2009; Taylor et al. 2014). Females frequently mate with multiple males during a single reproductive period (Birkhead and Møller 1998), leading to sperm competition, an extended form of male–male competition between sperm from rival males to fertilize ova (Parker 1970). Sperm competition generates strong selection on males to produce ejaculates that are better at fertilizing ova than those of their rivals (Simmons and Fitzpatrick 2012; Rowley and Fitzpatrick 2016). Critically, sperm number is a crucial factor influencing the outcome of sperm competition, as males that ejaculate a greater number of sperm enjoy a competitive advantage in fertilizations (Martin et al. 1974; Parker 1982; Stoltz and Neff 2006; Boschetto et al. 2011). At a functional level, males with larger testes are capable of producing more sperm (Marconato and Shapiro 1996; Schärer et al. 2004; Lüpold et al. 2009; Ramm and Stockley 2010; Rowe and Pruett-Jones 2011; Ramm and Schärer 2014). But

due to the costs associated with sperm production (Wedell et al. 2002), male investment in testicular tissue should be tailored to the level of sperm competition their ejaculates are likely to experience. Therefore, a long-standing prediction from sperm competition theory is that selection will act to increase male investment in testes size as the probability that females will mate multiply (i.e., sperm competition risk) and the number of partners a female has (i.e., sperm competition intensity) increase (Parker and Ball 2005; Parker and Pizzari 2010).

Indeed, increases in relative testes size (correcting for body size) represent a widespread response to sperm competition risk both within and across species (Simmons and Fitzpatrick 2012). Within species, comparisons between males with alternative reproductive tactics (Montgomerie and Fitzpatrick 2009), among males from populations where the level of sperm competition differs (Parker et al. 1997; Firman and Simmons 2008), and between populations experimentally exposed to different levels of sperm competition (Hosken and Ward 2001; Hosken et al. 2001; Simmons and García-González 2008) all demonstrate a positive association between sperm competition and male investment in testicular tissue. Similarly, comparative studies routinely demonstrate increased testicular investment in response to sperm competition

Address correspondence to A. Rowley. E-mail: amy.rowley@zoologi.su.se.

risk (reviewed by Simmons and Fitzpatrick 2012). Increases in testes size in response to sperm competition are thought to be so common that relative testes mass is used extensively as a proxy measure for sperm competition risk across species (Balshine et al. 2001; Gage and Freckleton 2003; Calhim et al. 2007; Immler and Birkhead 2007; Gómez Montoto et al. 2011; Simmons and Fitzpatrick 2012). However, direct evidence that testicular investment reflects interspecific variation in sperm competition risk remains limited. Most comparative studies examining how sperm competition influences male investment in testes rely on comparisons between species with different social mating systems, or use behavioral metrics (e.g., mating rate) as an indicator of sperm competition risk, 2 approaches that may not accurately reflect competitive dynamics at the genetic level (Birkhead and Møller 1998; DeWoody and Avise 2001; Griffith et al. 2002). Surprisingly, direct evidence of positive correlations between relative testes size and the frequency of genetic polyandry has been demonstrated in less than a handful of studies, all of which focused on either mammals (Ramm et al. 2005; Soulsbury 2010) or birds (Møller and Briskie 1995).

Here, we evaluate the relationship between testicular investment and multiple paternity in fishes, the largest and most diverse vertebrate group, which correspondingly display the most complex and varied range of mating strategies and behaviors of any vertebrate group (Desjardins and Fernald 2009). Fish reproduction encompasses an astonishing variety of behaviors from genetic monogamy to polygynandry (DeWoody and Avise 2001), and multiple paternity frequencies vary considerably across species (Coleman and Jones 2011; Fitzpatrick et al. 2012). Therefore, fishes represent an ideal model for investigating the relationship between multiple paternity rates and male investment in testes size. Moreover, as is the case with other taxa (e.g., Griffith et al. 2002), there are discrepancies between behavioral and genetic mating systems in fishes (e.g., Sefc et al. 2008; Chapman et al. 2013). Yet, the few available comparative studies of fishes to date have examined how investment in testicular tissue is influenced by behavioral estimates of mating systems (Stockley et al. 1997; Pyron 2000; Balshine et al. 2001; Molloy et al. 2007; Erisman et al. 2009; Fitzpatrick et al. 2009). Here, we take advantage of the wealth of information now available on the genetic mating systems of fish species to consider the effect of female polyandry on patterns of investment in testes across both major fish lineages, elasmobranchs (sharks and rays), and bony fishes.

## METHODS

### Data collection

Estimates of multiple paternity were obtained from the literature from 152 studies, representing 107 fish species, including bony fishes ($n = 77$) and elasmobranchs (sharks and rays, $n = 30$). We define the multiple paternity percentage as the proportion of the total number of broods/nests/litters (henceforth collectively referred to as "broods") tested for parentage where 2 or more males sired progeny, which served as a proxy of *sperm competition risk*. Where available, we also recorded the mean number of sires per brood for each species, which was used as a proxy of *sperm competition intensity*. We only considered data derived from wild populations, where multiple paternity was assessed using polymorphic microsatellite loci, and where at least one parent was genotyped at the candidate loci. When multiple paternity estimates were available from multiple populations or from a number of studies for a

given species, we combined the data from the populations/studies by calculating the mean percentage of multiple paternity weighted by brood number.

The methods used to estimate multiple paternity typically differ between egg laying (oviparous) and live bearing (ovoviviparous or viviparous) species based on variation in their reproductive biology. In egg laying (oviparous) species, at least one parent typically remained associated with fertilized eggs. In these oviparous species, one parental genotype can be determined from the associated individual and the presence of offspring showing an alternative genotype indicates the genetic contribution of nonassociated individuals to the brood (as is the case in nest guarding species [e.g., *Micropterus salmoides*], mouth brooding cichlids [e.g., *Tropheus moorii*], and seahorses and pipefishes). In some bony fish species, males display fixed alternative reproductive tactics, with typically larger "guarding" males monopolizing a female or guarding a nest, while smaller "satellite" or "sneaker" males try to fertilize eggs by stealth (Montgomerie and Fitzpatrick 2009). Alternative reproductive tactics are common in fish (Montgomerie and Fitzpatrick 2009), and likewise are common in our dataset (e.g., *Porichthys notatus*, *Telmatochromis temporalis*, *Pomatoschistus minutus*). In species with alternative reproductive tactics, guarding males may occasionally take over the nest of another male, which already contains eggs that have been fertilized by the displaced male. In such nest takeover events, the presence of offspring unrelated to the guarding male would not indicate sperm competition. Therefore, in species with alternative reproductive tactics, we classified any litters where the dominant or guarding male was found to be unrelated to the entire brood as probable nest takeover events and excluded such cases when calculating the proportion of multiply-sired litters. For live bearing (ovoviviparous or viviparous) species, where eggs or embryos are retained in the female's body cavity, the maternal genotype was subtracted from the offspring genotypes and the number of contributing sires inferred from the number of remaining alleles represented in the brood.

We then searched the literature for data on testes and body mass of sexually mature males for each of these 107 species, using their common and Latin names in combination with search terms including "testes"/"testis mass," "body mass," and "gonadosomatic index." We also used testes and body mass data from our own field collections or obtained through personal communications with researchers in the field (Supplementary Table S1). As testes mass varies temporally in many species (Montgomerie and Fitzpatrick 2009), we used maximum measurements taken during the breeding period of each species whenever possible. Where raw data were not reported, testes and body mass values were obtained from personal communication with the authors of the original studies or extracted when possible using the program GraphClick v3.0.3 (Boyle et al. 2012). In some studies, gonadosomatic index (GSI, calculated as testes mass/body mass × 100) was reported in lieu of testes mass. Despite criticisms of its use as a metric of investment in testicular tissue (Tomkins and Simmons 2002), GSI continues to be widely used by researchers in fish biology. When only GSI data were available, we calculated testes mass from GSI values using male body mass data. In the case of species that display morphologically distinct alternative reproductive tactics, we only included testes mass of the dominant or guarding males, as satellite or sneaker males experience a higher risk of sperm competition than guarding males and thus their inclusion is likely to yield an artificially high measure of investment in testes. Testes and body mass data was available for 40 of the 107 species with multiple paternity estimates. However,

the number of broods sampled differs dramatically among the species where multiple paternity data were available (across all 107 species mean brood number = 43.05; range: 4–408). Because low brood numbers can provide unreliable estimates of multiple paternities, we excluded species from which <10 broods were sampled. This exclusion criteria removed 4 species from our dataset (in these 4 species ≤5 broods were examined). Thus, the final sample size for analyses was 36 species ($n = 24$ bony fishes, $n = 12$ sharks). When we included the 4 species where <10 broods were examined in our analyses, the results did not qualitatively change (data not shown). Mean brood size was reported for 16 species ($n = 4$ bony fishes, $n = 12$ sharks), and ranged from 3.2 to 22.8, though note that only 2 of these species are external fertilizers, which typically have higher fecundity.

## Phylogeny

Despite recent advances in phylogenies for both bony fishes (e.g., Rabosky et al. 2013), and elasmobranchs (e.g., Naylor et al. 2012), no detailed phylogeny that encompasses both lineages is currently available. We therefore built a phylogeny incorporating the 36 species for which both multiple paternity and testes mass data was available. We obtained sequences for 3 mitochondrial genes (NADH dehydrogenase subunit 2, 16S ribosomal RNA and cytochrome B) and one nuclear gene (recombination activating gene 1) from GenBank using Geneious v9.1.5 (Kearse et al. 2012). Sequences were aligned using the MUSCLE plugin for Mesquite v3.04 (Maddison and Maddison 2015) and used to estimate

phylogenetic relationships between species within a Bayesian framework. At this stage, 2 species (*Symphmodus ocellatus* and *Petrochromis fasciolatus*) were removed as their sequences failed to align, reducing the total sample size to 34. The best fitting substitution model for each aligned gene sequence was determined using jModelTest v2.1.1 (Darriba et al. 2012) by comparing Akaike Information Criterion (AIC) values. The most appropriate substitution model was found to be GTR + I + G for all 4 genes. However, these models were later simplified to HKY to aid convergence. Phylogenies were estimated from the aligned sequences using the programs BEAUTi and BEAST (Bayesian evolutionary analysis by sampling trees) version 1.8.3 (Drummond *et al.* 2012) under an uncorrelated lognormal relaxed molecular clock, a Yule speciation process and unlinked speciation model for all genes. We constrained a number of well-resolved monophyletic clades (bony fishes, elasmobranch fishes, carcharhiniformes, carcharhin005, cichlids, poecilids) and set the ucld.mean (the mean of the prior distribution for mutation rate) to 0.66 with upper and lower values of 1 and 0, respectively, with a uniform distribution based on sensitivity testing that resulted in chain convergence. We ran a Markov Chain Monte Carlo (MCMC) simulation using a chain length of 50 million iterations with parameters logged every 1000 generations. Effective sample size (ESS) values were assessed in Tracer to determine convergence of the Bayesian chain, with a minimum ESS score of 200 indicating adequate mixing of the MCMC. We then used TreeAnnotator v2.1.2 (Rambaut and Drummond 2014) to construct a maximum clade credibility tree using mean node heights and a burn-in of 10%. A consensus phylogeny was viewed in FigTree version 1.4.2
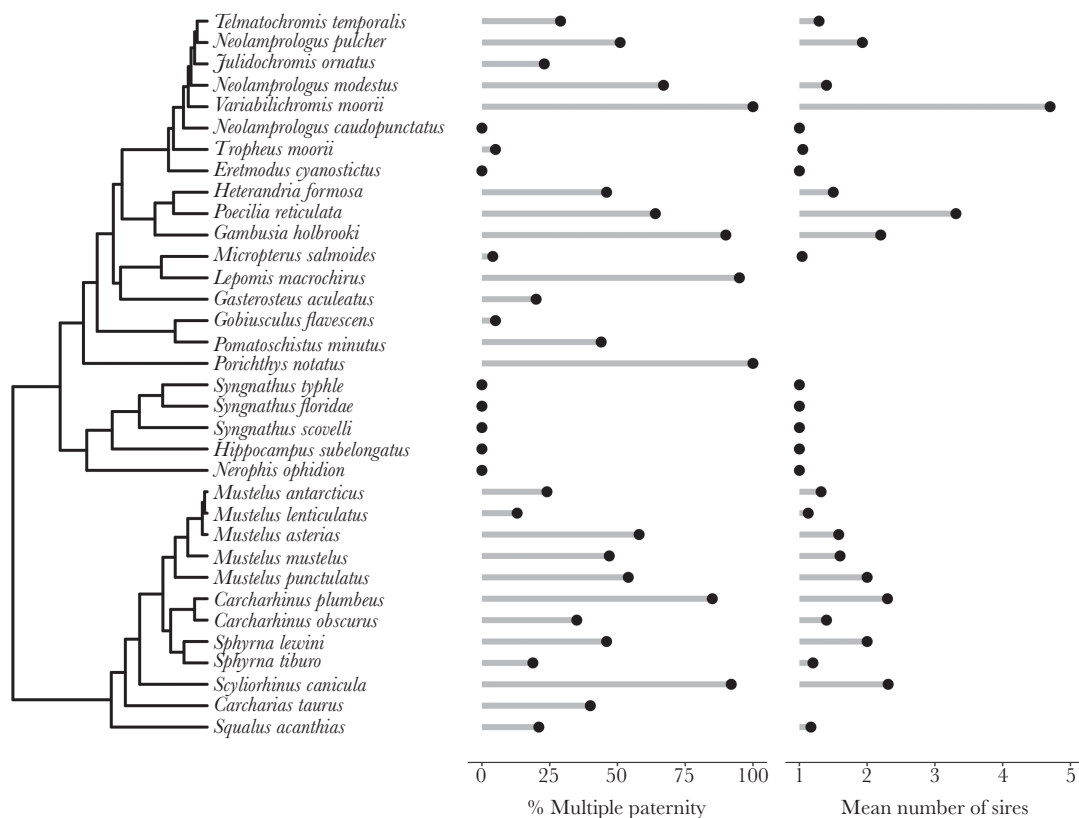
**Figure 1**
Variation in sperm competition risk and intensity across fishes. Phylogenetic relationships between fish species in our dataset, with points representing the percentage of litters sired by multiple males for each species (risk), and the average number of sires per brood within each species (intensity).

(Rambaut and Drummond 2015) and was generally consistent with existing phylogenies of bony and cartilaginous fishes (Naylor et al. 2010; Rabosky et al. 2013, Figure 1).

## Phylogenetic analyses

We tested the relationship between the percentage of multiple paternity and testes mass using phylogenetically controlled generalized least squares (PGLS) linear models (Freckleton et al. 2002) to account for statistical nonindependence as a result of shared ancestry. To assess phylogenetic dependence of the data, likelihood ratio tests were used to calculate the maximum-likelihood value of the phylogenetic scaling parameter $\lambda$, a measure of phylogenetic signal ranging from 0 (low phylogenetic signal) to 1 (high phylogenetic signal) (Pagel 1999; Freckleton et al. 2002). Importantly, estimates of $\lambda$ become less robust when the sample size in PGLS models drops below 20 species (Freckleton et al. 2002), which occurs in some of the models presented below. To determine if our results were sensitive to uncertainty in $\lambda$ estimates driven by low sample sizes, we examined the confidence intervals around $\lambda$ for each model. We then repeated all analyses with $\lambda$ estimates constrained to the upper and lower extremes (i.e., $\lambda = 0$ or $\lambda = 1$, respectively). Our results did not qualitatively change when the constrained $\lambda$ estimates were within the confidence intervals calculated in likelihood ratio tests (data not shown). However, analyses with low sample sizes should be interpreted cautiously, particularly in taxon-specific analyses. To control for the allometric relationship between testes mass and body size, body mass was included as a covariate in all models that included testes mass. All data were log10 transformed prior to analyses (this included multiple paternity percentage data, where we used log10 transformation rather than an arcsine square root transformation, as the latter has been criticized as generating uninterpretable model outputs; Warton and Hui 2011). Although our proxy for sperm competition intensity (i.e., the number of sires per brood) constitutes count data, we used Gaussian distributions in our models as sire number was never included as a response variable in any model. All analyses were performed in R version 2.15.1 (R Core Team 2012).

We began by comparing broad differences in multiple paternity percentage and the number of sires per brood between bony fish and elasmobranchs (hereafter referred to as "sharks," as no rays were present in our final dataset). To investigate evolutionary responses in testes to sperm competition risk/intensity, we first examined the relationship between multiple paternity percentage/number of sires per brood and relative testes mass across all fish species in the dataset. We then subdivided the dataset into 2 major taxonomic groups, bony fish and sharks, to examine taxa-specific responses in testes size to sperm competition. Among bony fish, there was sufficient data among one family of bony fish, the Cichlidae ($n = 8$), to evaluate the relationship between relative testes mass and sperm competition risk and intensity at a lower taxonomic level. We refrained from directly comparing the relationship between testes size and sperm competition risk/intensity between bony fish and sharks, as such an analysis confounds inherent differences in fertilization and reproductive mode between these taxonomic groups (i.e., most bony fish are external fertilizers while all sharks are internal fertilizers, and most bony fish are oviparous while all sharks in this study are ovoviviparous or viviparous). Mating systems and multiple paternity frequency can vary among populations in fishes (e.g., Daly-Engel et al. 2007; Portnoy et al. 2007). To account for possible population-specific effects, we performed a secondary set of analyses restricted to species for which testes measurements came from individuals from the same

geographical location as those used to determine multiple paternity frequency. Finally, to examine the possibility that our results are confounded by inclusion of the seahorses and pipefishes ($n = 5$) in our sample, which exhibit monogamous or sequentially polygynous mating systems due to the uncommon strategy of male pregnancy (Wilson et al. 2003; Stölting and Wilson 2007), we also performed all analyses on a dataset that excluded seahorses and pipefishes.

## RESULTS

Sperm competition risk and intensity varied widely across fishes (Figure 1). Multiple paternity percentage among bony fishes ranged from 0% in the cichlids *Eretmodus cyanostictus* and *Neolamprologus caudopunctatus* and all seahorses and pipefishes, to 100% in the plainfin midshipman (*P. notatus*) and *Variabilichromis moorii*. Among sharks, multiple paternity ranged from 13% in the rig (*Mustleus lenticulatus*) to 92% in the small-spotted catshark (*Scyliorhinus canicula*) (Figure 1). The mean number of sires per brood among bony fishes ranged from 1 in *E. cyanostictus*, *N. caudopunctatus* and all seahorses and pipefishes to 4.7 in *V. moorii*. Among sharks, mean number of sires ranged from 1.1 in the rig to 2.3 in the small-spotted catshark (*S. canicula*) and sandbar shark (*Carcharhinus plumbeus*) (Figure 1).

Mean multiple paternity percentage did not differ between bony fishes ($33.8 \pm 7.9\%$, $n = 22$) and sharks ($44.5 \pm 7.2\%$ [SE], $n = 12$; PGLS, $\lambda = 0.68$, df $= 32$, intercept: estimate $= 0.84 \pm 0.42$, $t = 1.98$, $P = 0.06$; taxonomic group: estimate $= 0.75 \pm 0.69$ [SE], $t = 1.08$, $P = 0.29$, $r = 0.33$, lower CI $= -0.05$, upper CI $= 3.98$). When seahorses and pipefishes were removed from the analysis, the mean multiple paternity percentage in bony fishes increased to $43.7 \pm 8.9\%$ ($n = 17$), which again did not differ from sharks ($\lambda < 0.001$, df $= 27$, intercept: estimate $= 1.36 \pm 0.13$, $t = 10.37$, $P = 0.25$; taxonomic group: estimate $= 0.23 \pm 0.20$, $t = 1.16$, $P = 0.26$, $r = 0.22$, lower CI $= -0.83$, upper CI $= 3.13$). The mean number of sires did not differ significantly between bony fish ($1.59 \pm 0.22$, range: $1-3.3$, $n = 17$) and sharks ($1.64 \pm 0.13$, range: $1.2-2.3$, $n = 12$; $\lambda < 0.001$, intercept: estimate $= 0.14 \pm 0.04$, $t = 3.21$, $P = 0.004$; taxonomic group: estimate $= 0.06 \pm 0.07$, $t = 0.81$, df $= 25$, $P = 0.43$, $r = 0.17$, lower CI $= -1.13$, upper CI $= 2.82$), and this result was unchanged when seahorses and pipefishes were removed from the analysis ($\lambda < 0.001$, df $= 20$, intercept: estimate $= 0.21 \pm 0.05$, $t = 3.85$, $P = 0.001$; taxonomic group: estimate $= -0.01 \pm 0.08$, $t = -0.11$, $P = 0.91$, $r = -0.02$, lower CI $= -2.07$, upper CI $= 1.85$). Multiple paternity percentage was tightly correlated with mean number of sires per brood ($\lambda = 0.60$, df $= 25$, intercept: estimate $= 0.64 \pm 0.23$, $t = 2.78$, $P = 0.01$; number of sires: estimate $= 2.57 \pm 0.46$, $t = 5.61$, $P < 0.001$, $r = 0.75$, lower CI $= 3.08$, upper CI $= 8.07$).

## Sperm competition risk, intensity, and testicular investment

We found a significant positive relationship between relative testes mass and both multiple paternity percentage, our proxy measure of sperm competition risk, and the mean number of sires per brood, our proxy measure of sperm competition intensity, across all fishes in our dataset (Table 1, Figure 2). The positive association between relative testes mass and sperm competition risk or intensity remained significant when seahorses and pipefishes were excluded from the analyses (Table 1). Likewise, the results remained qualitatively unchanged when only geographically matched data were included in the analyses (sperm competition risk: $\lambda = 0.38$, df $= 23$, intercept: estimate $= -2.60 \pm 0.21$, $t = -12.22$, $P < 0.001$; multiple paternity percentage: estimate $= 0.48 \pm 0.11$, $t = 4.47$,

**Table 1**

<mark>PGLS regressions between testes size and sperm competition risk</mark>/intensity

| Predictors | λ | df | Estimate | SE | t | P | r | Lower CI | Upper CI |
|---|---|---|---|---|---|---|---|---|---|
| *All fishes* | | | | | | | | | |
| (a) Sperm competition risk | | | | | | | | | |
| Intercept | 0.24 | 31 | −2.69 | 0.14 | −19.39 | **<0.001** | | | |
| Multiple paternity percentage | | | 0.49 | 0.08 | 6.10 | **<0.001** | 0.74 | 3.59 | 8.54 |
| Body mass | | | 0.90 | 0.04 | 23.53 | **<0.001** | 0.97 | 17.36 | 29.65 |
| (b) Sperm competition intensity | | | | | | | | | |
| Intercept | 0.81 | 24 | −2.31 | 0.23 | −10.01 | **<0.001** | | | |
| Number of sires per brood | | | 1.61 | 0.31 | 5.16 | **<0.001** | 0.73 | 2.69 | 7.56 |
| Body mass | | | 0.87 | 0.06 | 14.58 | **<0.001** | 0.95 | 10.01 | 19.09 |
| *Bony fishes* | | | | | | | | | |
| (c) Sperm competition risk | | | | | | | | | |
| Intercept | <0.001 | 19 | −2.78 | 0.11 | −26.19 | **<0.001** | | | |
| Multiple paternity percentage | | | 0.45 | 0.08 | 5.79 | **<0.001** | 0.80 | 3.07 | 8.43 |
| Body mass | | | 0.90 | 0.06 | 15.64 | **<0.001** | 0.96 | 10.30 | 20.92 |
| (d) Sperm competition intensity | | | | | | | | | |
| Intercept | <0.001 | 13 | −2.65 | 0.10 | −26.33 | **<0.001** | | | |
| Number of sires per brood | | | 1.83 | 0.39 | 4.76 | **<0.001** | 0.80 | 2.04 | 7.38 |
| Body mass | | | 0.88 | 0.07 | 12.13 | **<0.001** | 0.96 | 7.09 | 17.10 |
| *Sharks* | | | | | | | | | |
| (e) Sperm competition risk | | | | | | | | | |
| Intercept | <0.001 | 9 | −1.73 | 0.41 | −4.25 | **0.002** | | | |
| Multiple paternity percentage | | | 0.58 | 0.21 | 2.72 | **0.02** | 0.67 | 0.35 | 4.99 |
| Body mass | | | 0.65 | 0.06 | 10.68 | **<0.001** | 0.96 | 5.42 | 15.87 |
| (f) Sperm competition intensity | | | | | | | | | |
| Intercept | <0.001 | 8 | −0.94 | 0.23 | −4.14 | **0.003** | | | |
| Number of sires per brood | | | 1.44 | 0.42 | 3.40 | **<0.01** | 0.77 | 0.79 | 5.89 |
| Body mass | | | 0.61 | 0.06 | 10.62 | **<0.001** | 0.97 | 5.13 | 16.05 |
| *Cichlids* | | | | | | | | | |
| (g) Sperm competition risk | | | | | | | | | |
| Intercept | <0.001 | 5 | −3.22 | 0.40 | −7.99 | **<0.001** | | | |
| Multiple paternity percentage | | | 0.42 | 0.15 | 2.77 | **0.04** | 0.78 | 0.12 | 5.27 |
| Body mass | | | 1.29 | 0.46 | 2.83 | **0.04** | 0.78 | 0.16 | 5.35 |
| (h) Sperm competition intensity | | | | | | | | | |
| Intercept | <0.001 | 4 | −3.33 | 0.39 | −8.51 | **0.001** | | | |
| Number of sires per brood | | | 1.32 | 0.48 | 2.76 | **0.05** | 0.81 | -0.01 | 5.37 |
| Body mass | | | 1.63 | 0.44 | 3.70 | **0.02** | 0.88 | 0.48 | 6.78 |
| *All fishes (excluding pipefishes)* | | | | | | | | | |
| (i) Sperm competition risk | | | | | | | | | |
| Intercept | 0.30 | 26 | −2.67 | 0.21 | −12.25 | **<0.001** | | | |
| Multiple paternity percentage | | | 0.48 | 0.11 | 4.48 | **<0.001** | 0.66 | 2.14 | 6.75 |
| Body mass | | | 0.90 | 0.04 | 20.29 | **<0.001** | 0.97 | 14.44 | 26.08 |
| (j) Sperm competition intensity | | | | | | | | | |
| Intercept | 0.82 | 19 | −2.11 | 0.29 | −7.28 | **<0.001** | | | |
| Number of sires per brood | | | 1.53 | 0.33 | 4.57 | **<0.001** | 0.72 | 2.10 | 6.96 |
| Body mass | | | 0.85 | 0.07 | 12.65 | **<0.001** | 0.95 | 8.17 | 17.06 |

Sperm competition risk assesses the relationship between testes mass and the percentage of litters sired by multiple males, while sperm competition intensity assesses the relationship between testes mass and the mean number of sires per brood. Body mass was included as a covariate in all models to control for the allometric relationship between body and testes size. The phylogenetic scaling parameter λ indicates the level of phylogenetic dependence of the data, ranging from 0 (low phylogenetic signal) to 1 (high phylogenetic signal). The degrees of freedom (df), estimate and t-statistic (t) are presented for each model, with significant results ($P < 0.05$) highlighted in bold.
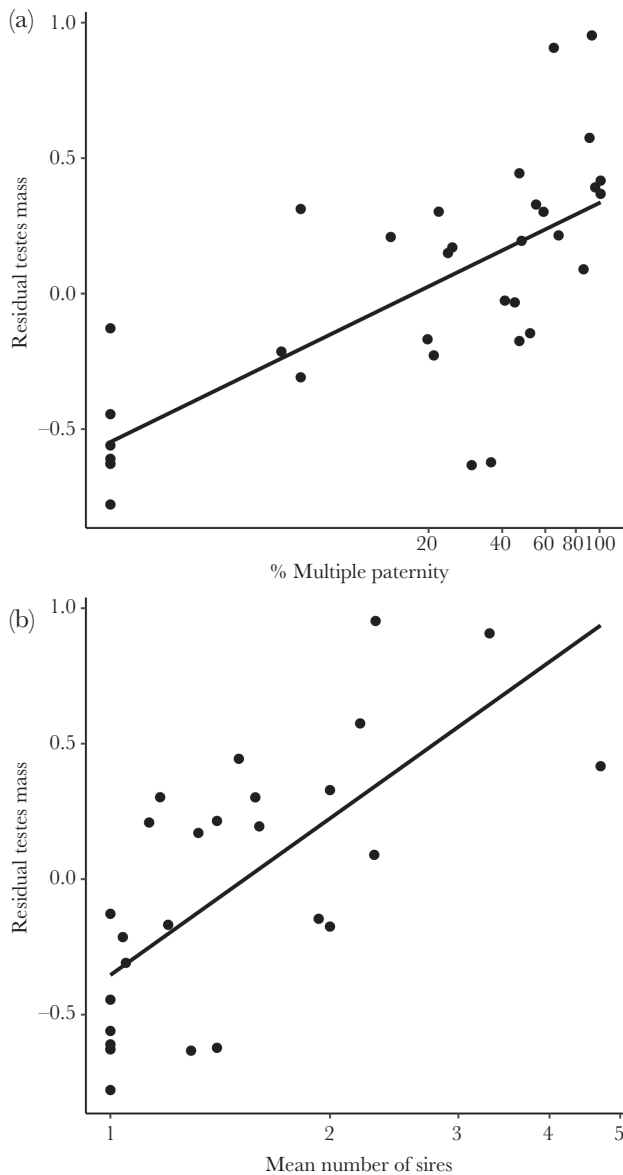
$P < 0.001$, $r = 0.68$, lower CI = 2.09, upper CI = 6.78; body mass: estimate = $0.88 \pm 0.05$, $t = 17.19$, $P < 0.001$, $r = 0.96$, lower CI = 11.85, upper CI = 22.47; sperm competition intensity: $\lambda = 0.81$, df = 18, intercept: estimate = $-2.18 \pm 0.28$, $t = -7.92$, $P < 0.001$; number of sires: estimate = $1.60 \pm 0.34$, $t = 4.69$, $P < 0.001$, $r = 0.74$, lower CI = 2.17, upper CI = 7.13; body mass: estimate = $0.83 \pm 0.07$, $t = 11.12$, $P < 0.001$, $r = 0.93$, lower CI = 6.98, upper CI = 15.18).

The positive association between testicular investment and sperm competition risk and intensity remained consistent across a range of taxonomic levels of analysis. When we examined bony fish and sharks separately, relative testes size was significantly related to multiple paternity percentage in both taxonomic groups (Table 1).

Similarly, both bony fishes and sharks exhibited significantly positive associations between relative testes mass and the mean number of sires per brood (Table 1). Moreover, consistent with the results from our wider analyses, male cichlids also invested more in testes mass (correcting for body size) as sperm competition risk and intensity increased (Table 1), although it should be noted that only 8 species were included in this analysis.

## DISCUSSION

Testes mass (corrected for body size) is a commonly used metric of the strength of postcopulatory sexual selection (Møller 1991; Briskie and Montgomerie 1992; Dunn et al. 2001; Calhim and Birkhead

**Figure 2**
Relationships between testes mass relative to body size and (a) the percentage of litters sired by more than one male (sperm competition risk) and (b) the mean number of sires per brood (sperm competition intensity) across species. Data presented in this figure are not controlled for phylogeny.

robust response in testes size to sperm competition and validate the use of relative testes mass as a proxy for sperm competition risk and intensity in fishes.

Sperm competition risk is highly variable across fishes, from strict genetic monogamy in some bony fish species (though no sharks), to universal polyandry in others (see Figure 1). While this variation is evident across the various taxonomic scales we tested, the positive relationship we observed between testicular investment and sperm competition remained remarkably consistent between all fishes in our dataset and when we examined fishes at increasingly reduced taxonomic scales (i.e., sharks, bony fish and cichlids). In addition, we also detected a positive relationship between testicular investment and sperm competition when we included or excluded seahorses and pipefishes from our analyses and when we constrained our analyses to only consider species where testicular and genetic data were collected from the same geographical location. Thus, overall, our results suggest that female multiple mating imposes selection on sperm number, driving males to increase sperm-production capacity by investing in relatively larger testes in fishes.

The similar evolutionary responses in testicular investment in response to sperm competition risk and intensity between sharks and bony fish was surprising at first glance, particularly given the dramatic differences in reproductive biology between these taxonomic groups. For example, all sharks in our dataset are internal fertilizers, while most of the bony fish examined are external fertilizers. Recent theoretical arguments suggest that evolutionary shifts from external to internal fertilization result in relaxed selection on testes size, as sperm dilution effects are less extreme in internal fertilizers (Parker 2014). However, the shark species examined in our dataset were significantly larger than the bony fish we examined (PGLS: $\lambda = 0.00$, df = 32, taxonomic class: estimate = $3.41 \pm 0.38$, $t = 9.08$, $P < 0.001$, $r = 0.85$, lower CI = 6.09, upper CI =12.01), and large female body size can generate strong dilution effects, even in the absence of external fertilization (Immler et al. 2011; Lüpold and Fitzpatrick 2015). However, our dataset did not allow us to adequately disentangle the role of fertilization in shaping the strength of postcopulatory sexual selection, as the difference between sharks and bony fish was largely synonymous with differences in fertilization mode. Moreover, our sample of 34 fish species represents a small fraction (~0.1%) of this large and diverse taxonomic group. Therefore, a crucial next step is to clarify the role of fertilization mode in shaping testicular investment across a wider range of fish species.

Our findings demonstrate that the extent of female polyandry strongly determines the strength of sexual selection on males, shaping the evolution of male reproductive physiology in fishes. These results are consistent with patterns found in other taxonomic groups (Møller and Briskie 1995; Ramm et al. 2005; Soulsbury 2010) and validate the use of testes mass corrected for body size as a metric of sperm competition in fishes. Our proxy measures of sperm competition risk and intensity are conservative estimates of the degree of female polyandry, as these proxies are unable to incorporate the potential effects of postmating processes such as cryptic female choice or intrauterine cannibalism (Sefc et al. 2008; Chapman et al. 2013) that may eliminate certain males either before fertilization occurs or before the brood is analyzed for parentage. Moreover, future studies should also consider the potential for interactions between male investment in sperm producing tissue, male investment in other sexually selected traits (e.g., sperm and genital morphology) and the size and number of eggs available to fertilize during a reproductive event (sensu Stockley et al. 1996). Nevertheless, the broad

2007). Yet surprisingly few comparative studies have linked testicular investment with genetic estimates of polyandry (Møller and Briskie 1995; Ramm et al. 2005; Soulsbury 2010). Moreover, despite the widespread use of fish as a model for studying sexual selection (Coleman et al. 2009; Montgomerie and Fitzpatrick 2009; Evans et al. 2010), how female multiple mating shapes male investment in testicular tissue remained previously unclear in fishes. In this study, we found that males' investment in testes increased commensurately with sperm competition risk (i.e., multiple paternity percentage) and intensity (i.e., the number of sires per brood) in fishes. This primary finding of our study is consistent with the theoretical prediction that an increase in either risk or intensity of sperm competition should select for greater investment in sperm production (Parker and Ball 2005). Our results therefore demonstrate a

patterns we observed between testicular investment and sperm competition risk and intensity at various levels of taxonomic scales points to a strong evolutionary response among fishes.

## SUPPLEMENTARY MATERIAL

Supplementary data are available at *Behavioral Ecology* online.

## REFERENCES

Balshine S, Leach BJ, Neat F, Werner NY, Montgomerie R. 2001. Sperm size of African cichlids in relation to sperm competition. Behav Ecol. 12:726–731.

Birkhead T, Hosken DJ, Pitnick S. 2009. Sperm biology: an evolutionary perspective. Oxford, UK: Elsevier/Academic Press.

Birkhead TR, Møller AP. 1998. Sperm competition and sexual selection. 1st ed. San Diego (CA): Academic Press.

Boschetto C, Gasparini C, Pilastro A. 2011. Sperm number and velocity affect sperm competition success in the guppy (*Poecilia reticulata*). Behav Ecol Sociobiol. 65:813–821.

Boyle MA, Samaha AL, Rodewald AM, Hoffmann AN. 2012. Evaluation of the reliability and validity of GraphClick as a data extraction program. Comput Human Behav. 29:1023–1027.

Briskie JV, Montgomerie R. 1992. Sperm size and sperm competition in birds. Proc Biol Sci. 247:89–95.

Calhim S, Birkhead TR. 2007. Testes size in birds: quality versus quantity—assumptions, errors, and estimates. Behav Ecol. 18:271–275.

Calhim S, Immler S, Birkhead TR. 2007. Postcopulatory sexual selection is associated with reduced variation in sperm morphology. PLoS One. 2:e413.

Chapman DD, Wintner SP, Abercrombie DL, Ashe J, Bernard AM, Shivji MS, Feldheim KA. 2013. The behavioural and genetic mating system of the sand tiger shark, *Carcharias taurus*, an intrauterine cannibal. Biol Lett. 9:20130003.

Coleman SW, Harlin-Cognato A, Jones AG. 2009. Reproductive isolation, reproductive mode, and sexual selection: empirical tests of the viviparity-driven conflict hypothesis. Am Nat. 173:291–303.

Coleman SW, Jones AG. 2011. Patterns of multiple paternity and maternity in fishes. Biol J Linn Soc 103:735–760.

Daly-Engel TS, Grubbs RD, Bowen BW, Toonen RJ. 2007. Frequency of multiple paternity in an unexploited tropical population of sandbar sharks (*Carcharhinus plumbeus*). Can J Fish Aquat Sci. 64:198–204.

Darriba D, Taboada GL, Doallo R, Posada D. 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat Methods. 9:772.

Desjardins JK, Fernald RD. 2009. Fish sex: why so diverse? Curr Opin Neurobiol. 19:648–653.

DeWoody JA, Avise JC. 2001. Genetic perspectives on the natural history of fish mating systems. J Hered. 92:167–172.

Drummond AJ, Suchard MA, Xie D, Rambaut A. 2012. Bayesian phylogenetics with BEAUti and the BEAST 1.7. Mol Biol Evol. 29:1969–1973.

Dunn PO, Whittingham LA, Pitcher TE. 2001. Mating systems, sperm competition, and the evolution of sexual dimorphism in birds. Evolution. 55:161–175.

Erisman BE, Craig MT, Hastings PA. 2009. A phylogenetic test of the size-advantage model: evolutionary changes in mating behavior influence the loss of sex change in a fish lineage. Am Nat. 174:E83–E99.

Evans JP, Pilastro A, Schlupp I. 2010. Ecology and evolution of Poeciliid fishes. Chicago: The University of Chicago Press.

Firman RC, Simmons LW. 2008. The frequency of multiple paternity predicts variation in testes size among island populations of house mice. J Evol Biol. 21:1524–1533.

Fitzpatrick JL, Kempster RM, Daly-Engel TS, Collin SP, Evans JP. 2012. Assessing the potential for post-copulatory sexual selection in elasmobranchs. J Fish Biol. 80:1141–1158.

Fitzpatrick JL, Montgomerie R, Desjardins JK, Stiver KA, Kolm N, Balshine S. 2009. Female promiscuity promotes the evolution of faster sperm in cichlid fishes. Proc Natl Acad Sci USA. 106:1128–1132.

Freckleton RP, Harvey PH, Pagel M. 2002. Phylogenetic analysis and comparative data: a test and review of evidence. Am Nat. 160:712–726.

Gage MJG, Freckleton RP. 2003. Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length. Proc Biol Sci. 270:625–632.

Gómez Montoto L, Varea Sánchez M, Tourmente M, Martín-Coello J, Luque-Larena JJ, Gomendio M, Roldan ER. 2011. Sperm competition differentially affects swimming velocity and size of spermatozoa from closely related muroid rodents: head first. Reproduction. 142:819–830.

Griffith SC, Owens IP, Thuman KA. 2002. Extra pair paternity in birds: a review of interspecific variation and adaptive function. Mol Ecol. 11:2195–2212.

Hosken DJ, Garner TW, Ward PI. 2001. Sexual conflict selects for male and female reproductive characters. Curr Biol. 11:489–493.

Hosken DJ, Ward PI. 2001. Experimental evidence for testis size evolution via sperm competition. Ecol Lett. 4:10–13.

Immler S, Birkhead TR. 2007. Sperm competition and sperm midpiece size: no consistent pattern in passerine birds. Proc Biol Sci. 274: 561–568.

Immler S, Pitnick S, Parker GA, Durrant KL, Lüpold S, Calhim S, Birkhead TR. 2011. Resolving variation in the reproductive trade-off between sperm size and number. Proc Natl Acad Sci USA. 108:5325–5330.

Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, Buxton S, Cooper A, Markowitz S, Duran C, et al. 2012. Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 28:1647–1649.

Lüpold S, Fitzpatrick JL. 2015. Sperm number trumps sperm size in mammalian ejaculate evolution. Proc R Soc B. 282:20152122.

Lüpold S, Linz GM, Rivers JW, Westneat DF, Birkhead TR. 2009. Sperm competition selects beyond relative testes size in birds. Evolution. 63:391–402.

Maddison WP, Maddison DR. 2015. Mesquite: a modular system for evolutionary analysis. Version 3.04. Available from: http://mesquiteproject. org.

Marconato A, Shapiro DY. 1996. Sperm allocation, sperm production and fertilization rates in the bucktooth parrotfish. Anim Behav. 52:971–980.

Martin PA, Reimers TJ, Lodge JR, Dziuk PJ. 1974. The effect of ratios and numbers of spermatozoa mixed from two males on proportions of offspring. J Reprod Fertil. 39:251–258.

Møller AP. 1991. Sperm competition, sperm depletion, paternal care, and relative testis size in birds. Am Nat. 137:882–906.

Møller AP, Briskie JV. 1995. Extra-pair paternity, sperm competition and the evolution of testis size in birds. Behav Ecol Sociobiol. 36:357–365.

Molloy PP, Goodwin NB, Côté IM, Reynolds JD, Gage MJ. 2007. Sperm competition and sex change: a comparative analysis across fishes. Evolution. 61:640–652.

Montgomerie R, Fitzpatrick JL. 2009. Reproductive biology and phylogeny of fishes. 1st ed. Jamieson BGM, editor. Enfield (New Hampshire): Science Publishers.

Naylor GJP, Caira JN, Jensen K, Rosana KAM, Straube N, Lakner C. 2012. Elasmobranch phylogeny: a mitochondrial estimate based on 595 species. In: Carrier JC, Musick JA, Heithaus MR, editors. Biology of Sharks and Their Relatives. Boca Raton, FL: CRC Press. p. 31–56.

Pagel M. 1999. Inferring the historical patterns of biological evolution. Nature. 401:877–884.

Parker GA. 1970. Sperm competition and it's evolutionary consequences in the insects. Biol Rev. 45:525–567.

Parker GA. 1982. Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. J Theor Biol. 96:281–294.

Parker GA. 2014. The sexual cascade and the rise of pre-ejaculatory (Darwinian) sexual selection, sex roles, and sexual conflict. Cold Spring Harb Perspect Biol. 6:a017509.

Parker GA, Ball MA. 2005. Sperm competition, mating rate and the evolution of testis and ejaculate sizes: a population model. Biol Lett. 1:235–238.

Parker GA, Ball MA, Stockley P, Gage MJ. 1997. Sperm competition games: a prospective analysis of risk assessment. Proc Biol Sci. 264:1793–1802.

Parker GA, Pizzari T. 2010. Sperm competition and ejaculate economics. Biol Rev Camb Philos Soc. 85:897–934.

Portnoy DS, Piercy AN, Musick JA, Burgess GH, Graves JE. 2007. Genetic polyandry and sexual conflict in the sandbar shark, *Carcharhinus plumbeus*, in the western North Atlantic and Gulf of Mexico. Mol Ecol. 16:187–197.

Pyron M. 2000. Testes mass and reproductive mode of minnows. Behav Ecol Sociobiol. 48:132–136.

Rabosky DL, Santini F, Eastman J, Smith SA, Sidlauskas B, Chang J, Alfaro ME. 2013. Rates of speciation and morphological evolution are correlated across the largest vertebrate radiation. Nat Commun. 4: 1958.

Rambaut A, Drummond AJ. 2014. TreeAnnotator v2. 1.2. Available at: http://beast.community/treeannotator

Rambaut A, Drummond AJ. 2015. FigTree, ver. 1.4. 2. Available from: http://beast.community/figtree.

Ramm SA, Parker GA, Stockley P. 2005. Sperm competition and the evolution of male reproductive anatomy in rodents. Proc Biol Sci. 272:949–955.

Ramm SA, Schärer L. 2014. The evolutionary ecology of testicular function: size isn't everything. Biol Rev Camb Philos Soc. 89: 874–888.

Ramm SA, Stockley P. 2010. Sperm competition and sperm length influence the rate of mammalian spermatogenesis. Biol Lett. 6:219–221.

R Core Team. 2012. R: a language and environment for statistical computing. Vienna (Austria): R Foundation for Statistical Computing.

Rowe M, Pruett-Jones S. 2011. Sperm competition selects for sperm quantity and quality in the Australian Maluridae. PLoS One. 6:e15720.

Rowley AG, Daly-Engel TS, Fitzpatrick JL. 2018. Data from: testes size increases with sperm competition risk and intensity in bony fish and sharks. Dryad Digital Repository. doi:10.5061/dryad.fd3nq57

Rowley AG, Fitzpatrick JL. 2016. Sperm competition. In: Kilman R, editor. Encyclopaedia of evolutionary biology. Oxford, UK: Academic Press. p. 245–248.

Schärer L, Ladurner P, Rieger R. 2004. Bigger testes do work more: experimental evidence that testis size reflects testicular cell proliferation activity in the marine invertebrate, the free-living flatworm *Macrostomum* sp. Behav Ecol Sociobiol. 56:420–425.

Sefc KM, Mattersdorfer K, Sturmbauer C, Koblmüller S. 2008. High frequency of multiple paternity in broods of a socially monogamous cichlid fish with biparental nest defence. Mol Ecol. 17:2531–2543.

Simmons LW, Fitzpatrick JL. 2012. Sperm wars and the evolution of male fertility. Reproduction. 144:519–534.

Simmons LW, García-González F. 2008. Evolutionary reduction in testes size and competitive fertilization success in response to the experimental removal of sexual selection in dung beetles. Evolution. 62:2580–2591.

Soulsbury CD. 2010. Genetic patterns of paternity and testes size in mammals. PLoS One. 5:e9581.

Stockley P, Gage MJG, Parker GA, Moller AP. 1996. Female reproductive biology and the coevolution of ejaculate characteristics in fish. Proc R Soc B Biol Sci. 263:451–458.

Stockley P, Gage MJ, Parker GA, Møller AP. 1997. Sperm competition in fishes: the evolution of testis size and ejaculate characteristics. Am Nat. 149:933–954.

Stölting KN, Wilson AB. 2007. Male pregnancy in seahorses and pipefish: beyond the mammalian model. Bioessays. 29:884–896.

Stoltz JA, Neff BD. 2006. Sperm competition in a fish with external fertilization: the contribution of sperm number, speed and length. J Evol Biol. 19:1873–1881.

Taylor ML, Price TA, Wedell N. 2014. Polyandry in nature: a global analysis. Trends Ecol Evol. 29:376–383.

Tomkins JL, Simmons LW. 2002. Measuring relative investment: a case study of testes investment in species with alternative male reproductive tactics. Anim Behav. 63:1009–1016.

Warton DI, Hui FK. 2011. The arcsine is asinine: the analysis of proportions in ecology. Ecology. 92:3–10.

Wedell N, Gage MJG, Parker GA. 2002. Sperm competition, male prudence and sperm-limited females. Trends Ecol Evol. 17:313–320.

Wilson AB, Ahnesjö I, Vincent AC, Meyer A. 2003. The dynamics of male brooding, mating patterns, and sex roles in pipefishes and seahorses (family Syngnathidae). Evolution. 57:1374–1386.