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Original Article

Diet affects ejaculate traits in a lizard with condition-dependent fertilization success

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Sexually selected traits are often driven to costly extremes by persistent directional selection. Energy acquisition and allocation can therefore influence variation in traits subject to both precopulatory and postcopulatory sexual selection, though the later have received much less attention. We tested the condition dependence of sperm morphology, sperm count, and fertilization success in a promiscuous lizard (*Anolis sagrei*) by 1) collecting sperm samples from wild males that varied naturally in body condition, 2) experimentally altering the body condition of captive males through dietary restriction, and 3) analyzing genetic paternity data from competitive mating trials between captive males that differed in body condition. In both wild and captive males, the length of the sperm midpiece decreased with body condition. Experimental food restriction decreased sperm production, decreased length of the sperm head, increased length of the sperm midpiece, and increased variance in sperm morphology within individuals. When restricted to a single copulation, males on high-intake diets exhibited a slight but nonsignificant fertilization advantage. Reanalysis of a previous experiment in which high- and low-condition males were sequentially allowed to copulate ad libitum for 1 week revealed a significant fertilization bias in favor of high-condition males. When controlling for mean treatment effects on the proportion of offspring sired and on sperm phenotypes, multiple regression revealed negative correlations between fertilization success and sperm head length, midpiece length, and sperm count. Collectively, our results suggest that condition-dependent fertilization success in *A. sagrei* may be partially mediated by underlying condition dependence of sperm morphology and sperm count.

Key words: ***Anolis sagrei*, paternity analysis, postcopulatory sexual selection, sperm competition, sperm morphology.**

INTRODUCTION

Sexually selected traits often exhibit condition dependence in their expression because chronic directional selection due to female choice or male competition can drive them to expensive and exaggerated states, the costs of which can only be borne by individuals in good condition (Andersson 1986; Cotton et al. 2004). Condition dependence is therefore predicted to maintain both phenotypic and genetic variation in sexually selected traits even in the face of strong selection and is consequently thought to be of general evolutionary significance (Falconer and Mackay 1981; Rowe and Houle 1996). Though most studies of condition dependence have focused on elaborate traits subject to precopulatory sexual selection (e.g., ornaments, weapons), traits experiencing postcopulatory sexual selection (e.g., ejaculate size and quality) can also be costly to produce and equally important for male fitness (Dewsbury 1982; Perry and Rowe 2010). Consequently, an understanding of the condition dependence of fertilization success and of features of the ejaculate that contribute to this component of fitness is necessary to fully understand how variation in male condition influences the dynamics of sexual selection (Cotton et al. 2004).

Whereas individual sperm cells are relatively inexpensive, ejaculates are often costly to produce (Dewsbury 1982; Van Voorhies 1992; Olsson et al. 1997). Hence, their quality and composition can change based on diet and nutritional state (Gage and Cook 1994; Merrells et al. 2009; Perry and Rowe 2010; Rahman et al. 2013; Tigreros 2013). Likewise, male fitness is often influenced by sperm count (Laskemoen et al. 2010; Boschetto et al. 2011), sperm morphology (LaMunyon and Ward 1998; Simmons and Kotiaho 2002; Fitzpatrick et al. 2012; Johnson et al. 2013; Bakker et al. 2014), and sperm quality (Gage and Cook 1994; Malo, Garde, et al. 2005; Malo, Roldan, et al. 2005; Devigili et al. 2012), particularly in promiscuous species that experience strong postcopulatory sexual selection. Though the condition dependence of both sperm morphology and fertilization success has been examined in several species, they have rarely been assessed simultaneously within a species (Amitin and Pitnick 2007). This comparison is critical to our understanding of the evolution of condition-dependent traits because it can help identify the causes and consequences of intraspecific variation in sexually selected traits.

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Fertilization success of males has been shown to change with body condition or diet in several species (Bonduriansky and Rowe 2005; Tigreros 2013; Zikovitz and Agrawal 2013). For species that experience high levels of sperm competition, increased sperm production can be critical for male fitness (Møller 1989; Møller and Briskie 1995; Boschetto et al. 2011; Kelly and Jennions 2011). However, to prevent functional and competitive sperm from being diluted by faulty or low-quality cells, increased sperm production must be matched by the maintenance of sperm quality. Aspects of sperm morphology that increase sperm performance (e.g., velocity, longevity), such as head size (Malo et al. 2006; Pitcher et al. 2009), midpiece length (Lüpold, Calhim, et al. 2009; Firman and Simmons 2010), and tail length (Malo et al. 2006; Lüpold, Calhim, et al. 2009; Mossman et al. 2009; Helfenstein et al. 2010) may be related to sperm quality. The quality of an ejaculate may also be reflected in morphological variance among cells, and increased selection on sperm can lead to reduced variation in sperm morphology (Calhim et al. 2007; Immler et al. 2008; Kleven et al. 2008; Fitzpatrick and Baer 2011). Though sperm morphology is linked to fertilization success in several species (Simmons and Kotiaho 2002; Laskemoen et al. 2010; Fitzpatrick et al. 2012; Johnson et al. 2013; Bakker et al. 2014), a link between condition dependence of sperm morphology and its effects on male fitness has only been demonstrated in a few species (LaMunyon and Ward 1998; Amitin and Pitnick 2007; García-González and Simmons 2007). Males that produce large and costly sperm are more successful at fertilization in some species (LaMunyon and Ward 1998), but in other species, effects of diet or condition on fitness occur without associated differences in sperm morphology (Schulte-Hostedde and Millar 2004; Amitin and Pitnick 2007).

The brown anole (*Anolis sagrei*) is a small, promiscuous lizard that likely experiences strong postcopulatory selection due to the high rates of multiple mating and multiple paternity that occur in wild populations (Tokarz 1998; Calsbeek and Bonneaud 2008). Female anoles store sperm for several months after mating, and 80% of females from wild populations produce offspring by more than 1 sire when using stored sperm (Calsbeek and Bonneaud 2008). We used several complementary approaches to explore the condition dependence of sperm morphology, sperm count, and fertilization success in *A. sagrei*. First, we examined ejaculates of wild males that varied naturally in body condition to test for correlations between body condition and sperm morphology in a natural context. Second, we experimentally altered body condition by manipulating the diets of captive males to directly test for condition dependence of sperm count and sperm morphology. Next, we sequentially mated 2 males from different diet treatments to the same female, then genotyped the progeny of each female to test for condition-dependent fertilization success in a situation where each male was allowed a single copulation. Finally, we reanalyzed data from a previous mating experiment to test for condition-dependent fertilization success in pairs of captive males that varied naturally in body condition and were allowed to mate ad libitum with the same female. We predicted that 1) sperm morphology (head, midpiece, and tail length) would be correlated with natural variation in body condition and influenced by diet treatment, 2) sperm count (cells per ejaculate) would be influenced by diet and increase with body condition, 3) within-male variance in sperm morphology (head, midpiece, and tail length) would be influenced by diet and decrease with body condition, indicating a more stringent maintenance of sperm quality when sufficient resources are available, and 4) fertilization success would be influenced by diet and biased in favor of high-condition males.

METHODS

Natural variation in body condition

To assess variation in sperm morphology as a function of natural variation in body condition, we collected 21 adult *A. sagrei* males from February Point, near Georgetown on Great Exuma, the Bahamas (23°30′N, 75°45′W). We collected males in early June of 2013, during the middle of their prolonged breeding season, which extends from approximately February through September (Tokarz et al. 1998). For each male, we measured snout–vent length (SVL, nearest 1 mm), body mass (nearest 0.1 g), and collected a sperm sample into a glass microcapillary tube by applying pressure to the abdomen, anterior to the cloaca. We fixed sperm cells in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 5 min, at which point the fixative was removed and the cells were dried on slides. We stained cells with Sperm Blue™ (Microptic SL, Barcelona, Spain) and then imaged them with an Olympus Magnafire camera (Olympus America, Melville, NY) at ×100 magnification using differential interference contrast microscopy. We measured the length of the head, midpiece, and tail for 25 sperm cells per male using ImageJ (NIH, Bethesda, MD), then calculated the mean and coefficient of variation (CV) in each measure for each male. We used a resampling procedure to confirm that sampling 25 cells per individual was sufficient to reach asymptotically low levels of variance in our measures of individual means and CVs for each measure of sperm morphology (Supplemental Figure 1). We did this through simulated resampling (with replacement) of a variable number of cells (range 2–25 cells), with 1000 simulations at each sample size for 34 individual males.

For each individual, we estimated body condition in 2 ways: 1) as residuals from the regression of $\log_{10}$ mass on $\log_{10}$ SVL (residual index, R_i) and 2) as a scaled mass index (M_i), which was recently proposed as a superior alternative to the residual index (Peig and Green 2009, 2010). We then tested for correlations between R_i or M_i and individual means and CVs for sperm head, midpiece, and tail length using ordinary least-squares regression. These condition indices have the advantage of generating a body condition phenotype for each individual, which facilitates visualization of correlations, though some authors advocate for the use of multiple regression with mass and length as separate independent variables (García-Berthou 2001; Freckleton 2002). Therefore, we also performed multiple regressions with $\log_{10}$ mass and $\log_{10}$ SVL as independent variables in models with individual means or CVs of sperm head, midpiece, and tail as response variables. In brown anoles, these 3 approaches (R_i, M_i, multiple regression) tend to generate similar results in subsequent analyses (Cox and Calsbeek 2014). All statistical analyses were performed using JMP (Version 9, SAS Institute Inc., Cary, NC).

Diet manipulation

We collected 34 adult *A. sagrei* males and 34 females from the same population on Great Exuma in January 2012 and acclimated them to captivity for 1 year before initiating diet treatments. Adults were housed individually in small plastic cages at 82 °F and 60% relative humidity on a 12L:12D photoperiod. We separated the 34 male *A. sagrei* into 2 treatment groups placed on different diets: a high-intake diet consisting of 5 crickets per feeding or a low-intake diet consisting of 1 cricket per feeding (n = 17 males per treatment). We fed each group 3 times per week (i.e., 15 or 3 crickets per male, per week). All crickets were approximately the same size (3/8 inch) and were dusted weekly with vitamin and mineral supplements

(Repta-Vitamin, Fluker Farms, Port Allen, LA). We maintained males on these diet treatments for 5 months to induce a change in body condition (Figure 1) and to ensure that this change in condition had sufficient opportunity to affect spermatogenesis. Reptiles with long or continuous reproductive seasons, such as *A. sagrei*, produce sperm continually throughout the reproductive season, with spermatogenic cycles typically lasting 2 months (Gribbins 2011). We monitored body condition every 2 weeks during the diet treatment to ensure that sperm used during competitive matings (see below) were produced when body condition differed between treatment groups (Figure 1). To statistically confirm that our diet treatment affected condition, we used repeated-measures Anova to test for a time-by-treatment interaction on body condition, using time as a within-subjects effect and diet treatment as an among-subjects effect.

After 16 weeks of dietary manipulation, we collected a sperm sample from each male to assess treatment effects on sperm morphology using the methods described above. To assess sperm count, we applied pressure to the lower abdomen of each male until he stopped releasing sperm. Ejaculates were collected into a microcapillary tube and transferred to 500 µL of PBS with 4% PFA to fix the cells. We mixed this suspension by tapping and pipetted 10 µL onto a hemocytometer to determine the total cell count. This measure of sperm count is correlated with estimates obtained by collecting whole ejaculates from the female reproductive tract immediately following mating (n = 18 males, r^2 = 0.49, P = 0.001). Measures of sperm morphology were obtained immediately after competitive mating trials, and measures of sperm count were obtained 1 week after competitive mating trials (see below).

To test for treatment effects on sperm morphology, we used nested Anova with head length, midpiece length, or tail length as the dependent variable, 25 individual cells per each of 34 individual males as observations, and male identity as a random effect nested within diet treatment. To compare effects of natural and experimentally induced variation in body condition on sperm morphology, we tested for correlations between body condition (R_i or M_i) and individual means for head length, midpiece length, and tail length of sperm. As described above, we also used multiple regressions with $\log_{10}$ mass and $\log_{10}$ SVL as covariates and individual means of sperm head, midpiece, and tail length as response variables. To test whether dietary restriction resulted in increased variance in sperm morphology within individuals, we calculated within-individual CV for each sperm morphological component (head length, midpiece length, tail length). We used t-tests to determine whether variance differed between treatment groups (n = 17 males in each group). We also used a t-test to determine whether diet impacted sperm count.

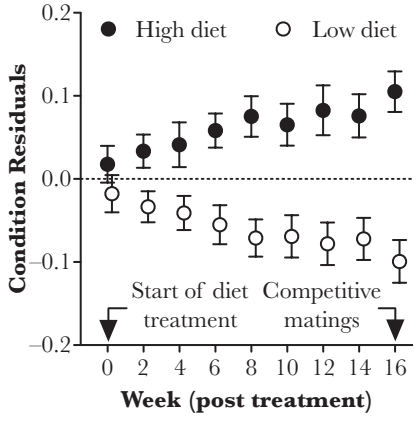

Figure 1
Experimental timeline plotting mean (± standard error) body condition for high-intake and low-intake groups from the start of the diet treatment (week 0) to the onset of competitive matings (week 16). Condition is expressed as residuals from separate regressions of $\log_{10}$ body mass on $\log_{10}$ SVL at each time point.

Competitive fertilization trials

To test for condition dependence of fertilization success, we mated males from each group competitively to females so that ejaculates from high- and low-condition males would be in direct competition for fertilization. Pairs of males (n = 17), one from each treatment group, were matched for body size (SVL) and for approximately the same relative difference in body condition across pairs. First, 1 male from a pair was allowed to copulate once with a female. After 1–2 days, the female was allowed to copulate once with the second male in the pair. Both males were then allowed to recover for 1 week (to prevent sperm depletion), and the procedure was repeated with a second female. The order of the males was reversed with the second female, to account for observed first-male paternity advantage in this species (Duryea et al. 2013). The order of each mating pair was balanced with respect to treatment, so that half of the males in each treatment mated first in the first round of mating, and half mated first in the second round of mating.

We monitored all trials from behind a blind so that we could remove males immediately after mating and prevent multiple copulations. After mating, we housed females in individual cages with potted plants in which they oviposited (mean ± standard error of the mean = 5.55 ± 0.90 eggs per female, range 0–16) at approximately 10-day intervals over an ensuing 3-month period. Female brown anoles can store sperm and produce viable eggs for upwards of 4 months after mating (Calsbeek and Bonneaud 2008). Eggs were removed from potted plants, placed individually in small containers with a mixture of vermiculite and deionized H_2O (1:1 by weight), and incubated at 28 °C and 80% relative humidity for 2 weeks, until embryo sex could be determined, at which point all embryos were sacrificed for genetic material. We stored embryonic and parental tissue in 95% ethanol at 4 °C until DNA extraction.

Paternity analysis

We extracted genomic DNA from parental and embryonic tissue (tail clip, 1 mm) by incubating samples for 180 min at 55 °C and denaturing for 10 min at 99 °C in 150 µL of 5% Chelex® resin (Bio-Rad, Inc.) in purified H_2O plus 1-µL Proteinase K (20 mg/mL, Qiagen, Chatsworth, CA) per sample. After centrifugation, we collected 30 µL of supernatant from these extractions to genotype individuals at 10 microsatellite loci: AAGG-38, AAAG-61, AAAG-68, AAAG-70, AAAG-76, AAAG-77, AAAG-91, AAAG-94 (Bardeleben et al. 2004), and ACAR11, ACAR23 (Wordley et al. 2010). We performed polymerase chain reaction (PCR) using Qiagen Multiplex PCR Kits with 1 µL of template DNA in a total volume of 10 µL. PCR cycles consisted of 1 denaturation step at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 90 s, and extension at 72 °C for 90 s, with a final extension step at 60 °C for 30 s. We ran all PCR reactions on an Eppendorf Mastercycler (Applied Biosystems, Carlsbad, CA) and performed fragment analysis using a GeneScan LIZ500 size standard on an ABI 3130 Genetic Analyzer (Applied

Biosystems). We scored genotypes using GeneMarker v2.2.0 software (SoftGenetics, State College, PA).

We analyzed paternity using a likelihood-based method implemented in the program CERVUS 3.0 (Kalinowski et al. 2007). Initial allele-frequency analysis revealed that 1 locus was not in Hardy–Weinberg equilibrium (AAAG-68) and that 2 loci (AAAG-38, AAAG-94) had 8 null alleles. These 3 loci were removed from the analysis, leaving a total of 7 informative loci (Supplementary Table S1). To assess the power of our markers, we ran a simulation of parentage analysis for 200 offspring and 34 potential sires using the allele frequencies generated above, while assuming 100% of potential sires sampled, a 5% genotyping error rate, and a minimum number of typed loci set to 4. These simulated data were used to determine the likelihood-odds ratio (LOD) scores in the paternity analysis. We analyzed paternity using the "one parent known" option in CERVUS, which allowed us to specify the known mother and the 2 candidate sires for each offspring.

Condition dependence of fertilization success

We tested for condition-dependent fertilization success using data from the fertilization trials described above, in which condition was experimentally altered via dietary manipulation, and by analyzing data from a previous study in which captive-bred males varied naturally in condition (Cox et al. 2011). The design of this previous study was similar to that described above, with each female mated sequentially to 2 males, and each pair of males mated to 2 females, once as the first and once as the second male to mate. In contrast to the present study, pairs of males in the previous study were matched for age (rather than size), each male was allowed to mate ad libitum with each female for 1 week (rather than being limited to 1 copulation), and males were classified into "high" and "low" condition groups on the basis of positive or negative residuals from the regression of $\log_{10}$ body mass on $\log_{10}$ SVL (rather than being assigned to diet treatments). We filtered this dataset to include only those pairs that comprised 1 high-condition male and 1 low-condition male ($n = 20$ pairs of sires). Only 30 of the 40 females to which these males were mated produced offspring, similar to the present study, in which 22 of 34 females produced offspring ($n = 14$ pairs of sires). Therefore, we tested for condition-dependent fertilization success in 2 ways for each experiment. First, we considered each female as a unit of observation and compared the proportion of her offspring sired by high- versus low-condition males using paired *t*-tests (statistically equivalent to testing whether the proportion of offspring sired by either group differs from the null expectation of 0.5). Second, to avoid double-counting male pairs, we conducted analogous *t*-tests but considered each male pair as the unit of observation, pooling the progeny they sired across both females to calculate their proportional paternity. With each approach, we conducted weighted (by the total number of offspring from which proportional paternity was calculated) and unweighted tests.

To test whether sperm morphology and sperm count were correlated with fertilization success independent of any overall treatment effects on these variables, we standardized the proportion of offspring sired, sperm morphology, and sperm count within each treatment by subtracting the treatment mean from each male's individual value and dividing by the treatment standard deviation [SD]. To avoid any potential biases due to first- or second-male mating advantage (Duryea et al. 2013), we conducted 3 separate analyses with different response variables: 1) the proportion of offspring sired when the male was the first male to mate with a female (P_1), 2) the proportion of offspring sired when the male was the second male to mate with a female (P_2), and 3) the average proportion of paternity that each male achieved across both of his trials, once as the first and once as the second male to mate (P_{avg}). We used these estimates of proportional paternity as response variables in separate multiple regressions that included treatment-standardized measures of each male's average sperm head length, midpiece length, tail length, and sperm count (square-root transformed) as independent variables. We confirmed that these analyses were not strongly influenced by multicollinearity by examining variance inflation factors that are (VIF < 1.6 for all models, Marquardt 1970).

RESULTS

Condition dependence of sperm morphology and sperm count

Diet treatment significantly altered male body condition (R_i), such that males in the high-intake treatment were significantly more massive for a given length than males in the low-intake treatment (treatment: $F_{1,33} = 19.39$, $P < 0.0001$; Figure 1). This effect of diet on condition increased over time (time × treatment: $F_{1,33} = 7.44$, $P < 0.0001$; Figure 1). Diet treatment also affected sperm morphology. Males on a high-intake diet had marginally longer sperm heads and significantly smaller sperm midpieces, relative to low-intake males, while sperm tail length did not differ between treatment groups (Table 1, Figure 2). Treatment also significantly affected within-male variance in sperm morphology, such that males on the high-intake diet had marginally lower variance in head and tail length and significantly lower variance in midpiece length (Table 1, Figure 2). Males on the high-intake diet also had higher sperm counts ($6.92 \pm 3.42 \times 10^6$ cells per ejaculate) than males in the low-intake group ($3.82 \pm 3.22 \times 10^6$ cells per ejaculate; $t = 2.526$, $P = 0.017$).

Natural patterns of covariance in body condition and sperm morphology generally corroborate this experimental evidence for condition dependence. In the wild, we detected a weak negative correlation between natural variation in body condition and mean midpiece length, but found no relationship between body condition and mean head length or tail length (Figure 3). These results were robust to the choice of R_i, M_i, or multiple regression (i.e., correlations with body mass while controlling for SVL) to assess body condition (Supplementary Table S2). Correlations were similar across

Table 1

(a) Results of 3 nested Anovas testing for difference in mean head length, midpiece length, and tail length of sperm between diet treatments, with male identity nested within treatment as a random effect in each model. (b) Results of paired *t*-tests for differences in individual CV for sperm morphology between diet treatment groups

(a) Nested Anovas (means)	df	*F*	*P*
Head length	1, 32.87	3.13	0.086
Midpiece length	1, 32.87	8.33	0.007*
Tail length	1, 32.87	0.44	0.513
(b) *t*-tests (CV)	df	*t* ratio	*P*
Head length	16	1.91	0.065
Midpiece length	16	2.67	0.011*
Tail length	16	1.92	0.068

*Significant ($P < 0.05$) effects.

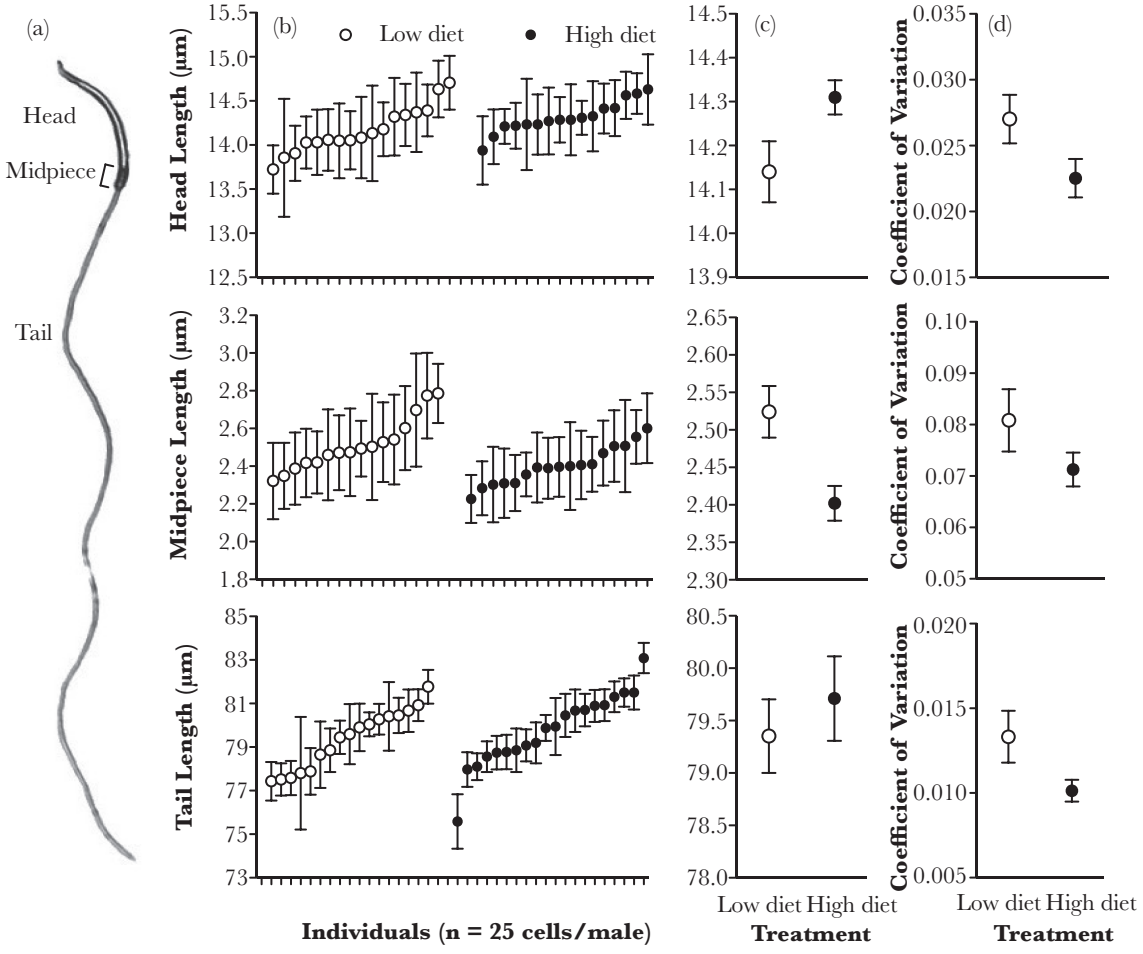

Figure 2

(a) *Anolis sagrei* sperm cell. (b) Individual means (±SD) for head length, midpiece length, and tail length of 25 sperm cells per individual for each of 17 males from the low-intake diet treatment and 17 males from the high-intake diet treatment, grouped by treatment and ranked by mean within each group. (c) Treatment means (± standard error [SE]) of individual means in head length, midpiece length, and tail length. (d) Treatment means (±SE) of individual CV in head length, midpiece length, and tail length.

experimentally induced variation in body condition. We found a significant negative relationship between body condition and mean midpiece length, but no correlations with mean head length or tail length, and these results were again robust to the method used to assess condition (Figure 3, Supplementary Table S2). Though we found significant differences in the variance in sperm morphology between treatment groups, we found no relationship between natural variation in body condition and variation in the length of the sperm head ($P > 0.49$ for all 3 methods), midpiece ($P > 0.31$ for all 3 methods), or tail ($P > 0.41$ for all 3 methods).

Condition dependence of fertilization success

Loci used in paternity analysis had an average polymorphism information content of 0.72, with a nonexclusion probability of 0.0009 (Supplementary Table S1). In total, we genotyped 188 offspring, and only 3 of these could not be reliably assigned to a sire. Of the remaining offspring, 82% ($n = 152$) were assigned with >95% confidence and 18% ($n = 33$) were assigned with >80% confidence, assuming a 0.05 error rate.

In our reanalysis of data from a previous study in which captive-bred males varied naturally in body condition (Cox et al. 2011), we found a significant fertilization advantage associated with high condition (Figure 4a), irrespective of whether we used individual females ($n = 30$; weighted: $t = 2.47$, $P = 0.010$; unweighted: $t = 1.74$, $P = 0.046$) or pairs of males as units of observation ($n = 20$; weighted: $t = 2.73$, $P = 0.007$; unweighted: $t = 1.94$, $P = 0.034$). In the present experiment, we did not detect any overall fertilization advantage associated with the high-intake diet (Figure 4b), irrespective of whether we used individual females ($n = 22$; weighted: $t = 0.19$, $P = 0.57$; unweighted: $t = 0.13$, $P = 0.45$) or pairs of males as units of observation ($n = 14$; weighted: $t = 0.05$, $P = 0.52$; unweighted: $t = 0.32$, $P = 0.38$). When we conservatively limited male pairs to the subset that produced offspring by each of 2 females, we observed similar overall patterns of condition dependence in both studies (Figure 4a,b, $n = 9$ pairs of sires), but neither trend was significant due to low statistical power (previous study: $t = 1.34$, $P = 0.11$; this study: $t = 0.74$, $P = 0.24$; df = 8 for each test).

After standardizing fertilization success and sperm phenotypes to a mean of zero and units of SD within each diet treatment, we found no significant relationships between P_1 or P_2 and any aspect of sperm morphology or sperm count. However, when we used the average proportion of paternity for each male across both mating

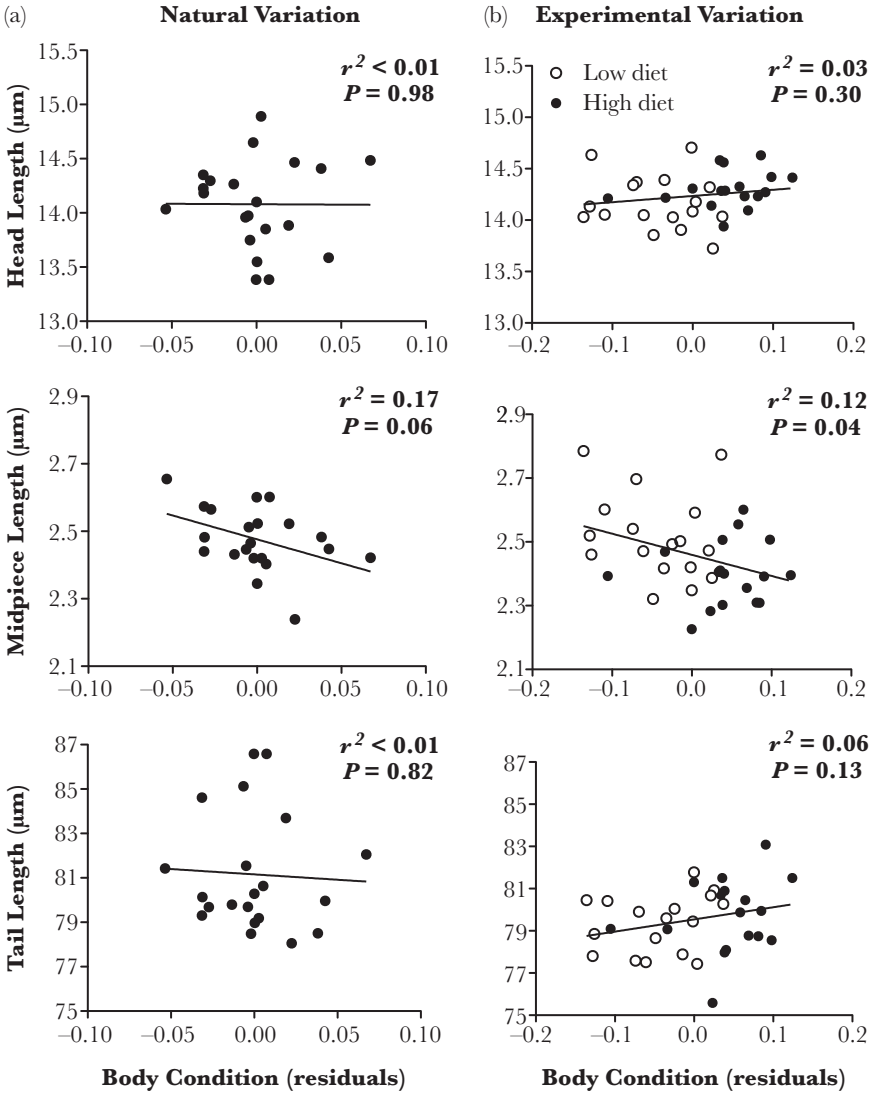

Figure 3
(a) Correlations between natural variation in body condition and individual means for head length, midpiece length, and tail length of sperm across 21 wild males from Great Exuma, the Bahamas. (b) Correlations between experimental variation in body condition and individual means for head length, midpiece length, and tail length of sperm across 17 high-intake and 17 low-intake males in captivity. Individual means are derived from 25 cells per male. Body condition is derived from residuals of regressions of $\log_{10}$ body mass on $\log_{10}$ SVL.

trials (P_{avg}) as our response variable, multiple regressions revealed significantly negative partial correlations between P_{avg} and sperm head length, sperm midpiece length, and sperm count (Table 2).

DISCUSSION

Over the past decade, there has been mounting empirical evidence that ejaculate traits are important determinants of male fitness (LaMunyon and Ward 1998; Gage et al. 2004; García-González and Simmons 2005, 2007; Boschetto et al. 2011). Sexual selection for ejaculate quantity and quality has presumably driven some aspects of sperm morphology and production to be costly and therefore condition dependent (Gage and Cook 1994; Simmons and Kotiaho 2002; Malo, Roldan, et al. 2005; Perry and Rowe 2010; Gasparini et al. 2013; Rahman et al. 2013). Though traits associated with sperm quantity (e.g., sperm count) have obvious potential costs, traits that are indicative of sperm quality (e.g., sperm morphology and performance) may have more subtle energetic costs that are attributable to the maintenance of spermatogenesis (Hill 2011). Our results demonstrate that sperm count and some aspects of sperm morphology are condition dependent in *A. sagrei*. Males in lower body condition due to dietary restriction produced fewer and more variable sperm, as well as sperm with larger midpieces (Figure 2). We observed a similar negative relationship between body condition and midpiece length in wild males that varied naturally in body condition (Figure 3). Males in higher body condition also tended to sire more offspring in competitive mating trials with size- or age-matched males in lower condition (Figure 4). Though we cannot directly link this apparent condition dependence in fertilization success to underling condition dependence in sperm morphology or sperm count, the proportion of offspring sired by each male was negatively correlated with sperm head length, midpiece length, and sperm count, even after removing the overall effects of diet treatment on each measure. Below, we

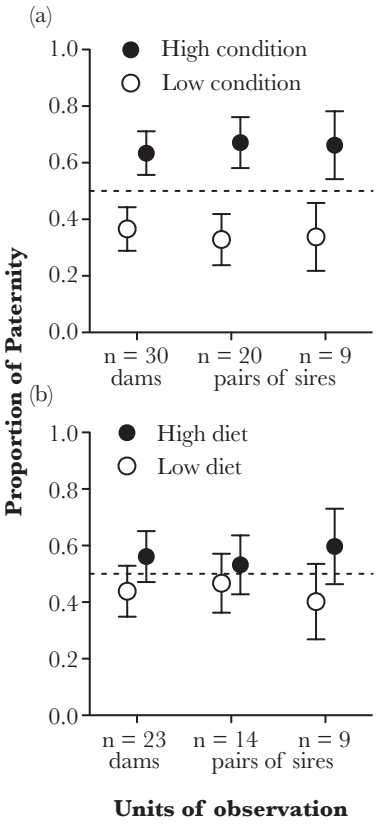

Figure 4

Mean (± standard error) proportion of progeny sired by males that were (a) categorized into high- and low-condition pairs on the basis of natural variation in body condition (data reanalyzed from Cox et al. 2011) and (b) assigned to high-intake and low-intake diet treatments (this study). For each experiment, condition dependence was assessed in 3 ways: 1) using each dam as a unit of observation and estimating the proportion of paternity for each of her 2 mates (n = 30 and 23 dams), 2) using each pair of potential sires as a unit of observation and estimating the proportion of paternity for each male across 1 dam (if the other dam did not produce offspring) or across both dams (n = 20 and 14 pairs of sires), and 3) using each pair of potential sires as a unit of observation but restricting the comparison to the subset of pairs for which both dams produced offspring (n = 9 and 9 pairs or sires). See text for details.

discuss these findings as part of the emerging body of literature on the condition dependence of traits subject to postcopulatory sexual selection and their potential fitness consequences.

Condition dependence of ejaculate traits

We found that sperm morphology, particularly the size of the midpiece, was condition dependent in both a wild population and in response to experimental diet treatments. We also found that sperm count was condition dependent in our experimental diet treatments. In promiscuous species, such as *A. sagrei*, males who are able to produce more competitive ejaculates (e.g., higher sperm count, morphologically sound cells, greater velocity, increased cellular longevity), are expected to be more successful in sperm competition. Our findings are generally consistent with other studies that have measured sperm count (Gage and Cook 1994; Malo, Roldan, et al. 2005; Gasparini et al. 2013; Rahman et al. 2013; but see Perry and Rowe 2010), and sperm morphology (Simmons and Kotiaho 2002; Bonanno and Schulte-Hostedde 2009; Rahman et al. 2013) in relation to body condition. In general across these studies, males in high body condition produce more sperm than males in low body condition, though relationships between body condition and sperm morphology are more variable across these studies. The differences we observed between treatment groups in sperm morphology and sperm quantity suggest that sperm may be energetically costly to produce in large quantities, in high quality, or with particular morphologies (Perry and Rowe 2010).

In some cases, traits that are condition dependent are predicted to experience positive selection for size or symmetry and therefore require greater energy expenditure for their production and maintenance (Rowe and Houle 1996). Consistent with this idea, when food quantity or quality is low, total sperm length often decreases (Alavi et al. 2009; Merrells et al. 2009; Rahman et al. 2013) whereas within-individual variation in sperm morphology increases (Hellriegel and Blanckenhorn 2002). However, other studies found no relationship between condition and sperm length (Gage and Cook 1994; Amitin and Pitnick 2007; Gasparini et al. 2013). Unfortunately, the majority of these studies did not separately quantify the length of each sperm component, but looked only at total sperm length. Those studies that have quantified individual morphological components of sperm size have found both

Table 2

Regression analyses testing for fitness consequences of variation in sperm head length, midpiece length, tail length, and sperm count (square-root transformed) using 3 measures of fertilization success as dependent variables in separate analyses: P_1 is the proportion of paternity when males were the first to mate with a female, P_2 is the proportion of paternity when males were the second to mate with a female, and P_{avg} is the average proportion of paternity across P_1 and P_2

Model	P_1 (df = 21)		P_2 (df = 20)		P_{avg} (df = 16)	
Effect	F	P	F	P	F	P
Multiple regression						
Head length	0.106	0.748	3.030	0.099	6.958	0.021*
Midpiece length	0.469	0.502	0.594	0.449	8.267	0.014*
Tail length	1.158	0.296	0.081	0.777	0.892	0.363
Sperm count	0.190	0.667	2.424	0.136	5.437	0.026*
Univariate regressions						
Head length	0.291	0.594	1.115	0.302	4.580	0.048*
Midpiece length	0.293	0.593	0.083	0.774	1.521	0.235
Tail length	0.880	0.358	0.121	0.730	0.414	0.529
Sperm count	0.102	0.752	0.289	0.597	0.538	0.474

Males are only included in the analysis of P_{avg} if both of their mates produced offspring. All dependent and independent variables were first standardized to a mean of zero, and units of SD within each diet treatment to eliminate any collinearity due to overall treatment effects. Results are shown separately for a single multiple regression with all 4 ejaculate phenotypes as separate independent variables and as separate univariate regressions for each ejaculate phenotype.

context- and nutrient-dependent variation in cellular morphology (Alavi et al. 2009; Merrells et al. 2009; Immler et al. 2010). In the present study, we found no association between body condition and total sperm length, but we did find a weak effect of diet treatment on the length of the sperm head, and a strong effect of diet treatment on the length of the sperm midpiece, suggesting that males in better condition produce sperm with slightly larger heads, but smaller midpieces.

High-condition males produced sperm with slightly smaller midpieces than those of low-condition males in the wild and in captivity, a result that encompasses both natural and experimentally induced variation in body condition. Although correlations between body condition and midpiece length were generally weak ($0.12 < r^2 < 0.17$) and effects of diet on midpiece size were fairly modest and subject to considerable variation among individuals (Figure 2), midpiece size has been linked with cellular performance and may play a critical role in male fitness (Bakker et al. 2014). The midpiece of the sperm cell contains its mitochondria, which are potentially costly to produce in high numbers. In comparative studies of birds, mammals, rodents, fish, and snakes, polygamous species and/or species with larger testes tend to produce sperm with larger midpieces, suggesting that sperm competition may select for large midpiece size (Breed and Taylor 2000; Anderson et al. 2005; Lüpold, Calhim, et al. 2009; Lüpold, Linz, et al. 2009; Tourmente et al. 2009). Within species, Gouldian finches placed in environments with greater male–male competition produced sperm with larger midpieces, which demonstrates that individual variation in sperm morphology may respond to environmental cues of intrasexual competition (Immler et al. 2010). This association between competitive environment and midpiece size within and among species may occur because midpiece size is often positively related to measures of performance, such as velocity (Firman and Simmons 2010) and longevity (Helfenstein et al. 2010), suggesting that larger midpieces could improve cellular performance and potentially increase fertilization success.

Despite this general pattern across species, other studies have found negative intraspecific relationships between body condition and midpiece length (Bonanno and Schulte-Hostedde 2009), and between body condition and adenosine triphosphate content of sperm cells (Burness et al. 2008), comparable with our results for *A. sagrei*. Because the midpiece may be one of the most energetically expensive portions of the cell to produce, this pattern could represent a trade-off in investment between midpiece size and sperm number (Parker and Begon 1993). One explanation for the opposing pattern across species and the pattern we observed in our study may be that midpiece size is more important for long-term storage and viability in the female reproductive tract, but may not affect paternity when competition for fertilization occurs over shorter intervals. Species that store sperm for months or years may rely on the mitochondria to increase cellular longevity (Tourmente et al. 2009), while glycolysis in the tail can provide the energy needed for short-term competition. In our study, females produced offspring from stored sperm over a 3-month span following mating, whereas in the wild, females mate repeatedly over a 6-month reproductive season, potentially minimizing the importance of sperm longevity. Producing more sperm with shorter midpieces could therefore be more beneficial in competition for fertilizations using stored sperm. Though our data cannot directly link sperm morphology with fertilization success, males producing smaller midpieces (as well as smaller sperm heads) sired a larger proportion of offspring even when controlling for overall treatment effects on proportional paternity and sperm morphology (Table 2).

Condition dependence of fertilization success

When we reanalyzed data from Cox et al. (2011) (Figure 4a), we found that males in high condition produced a greater proportion of offspring (63–68%) when competitively mated against males in low condition in a design that eliminated the potential for precopulatory male–male competition. We found a similar trend in the present study (Figure 4b), where males on a high-intake diet produced a slightly higher proportion of offspring (53–60%) than males on a low-intake diet, though this trend was not statistically significant. In the previous experiment (Cox et al. 2011), males were allowed unlimited access to females for 1 week, rather than being limited to a single copulation, as in the present study. This difference could influence relative fertilization success in several ways. First, males in high condition could have mated more frequently than males in low condition, therefore increasing the relative abundance of their sperm in the female reproductive tract beyond what would result from a single mating by each male. Second, even if low-condition males were able to mate as often as high-condition males, their ability to continuously produce ejaculates with high sperm counts could have declined more steeply with successive mating, relative to high-condition males. Lastly, though all males mounted and appeared to copulate with females in the present study, we could not confirm the actual transfer of ejaculates to the female for all trials. Collectively, these factors could explain why we only detected a slight condition dependence of paternity in the present study, whereas this effect was large and significant in a previous experiment (Cox et al. 2011).

Irrespective of these potential differences between studies, our results collectively indicate that male anoles in high condition tend to sire a higher proportion of offspring than males in low condition, even when direct, precopulatory male–male competition is eliminated. We cannot exclude the possibility that this apparent condition dependence of fertilization success actually reflects cryptic female choice (e.g., based on precopulatory assessment of male phenotypes) or some aspect of male behavior or performance unrelated to properties of the ejaculate per se (e.g., deeper intromission with more efficient sperm transfer). Nonetheless, our simultaneous documentation of condition dependence in sperm morphology and sperm count indirectly supports the hypothesis that properties of the ejaculate may contribute to these observed fitness differences. Moreover, our multiple regression analyses revealed significant correlations between the proportion of offspring sired and head length, midpiece length, and sperm count when proportional paternity was averaged over 2 matings (P_{avg}), though these same ejaculate phenotypes were unrelated to variance in P_1 or P_2 (Table 2). This difference may arise in part from the small number of offspring from which estimates of proportional paternity were derived (median 12.5, range 1–26 per male pair), such that averaging across multiple mates may provide a better representation of a male's competitive ability.

We emphasize that our evidence for a relationship between sperm phenotypes and fertilization success is subject to many caveats, including the correlative nature of the data, our relatively low sample sizes in terms of both parents and offspring, and the fact that significant effects are only detectable under certain analytical conditions (e.g., using P_{avg} rather than P_1 or P_2; using multiple regression rather than univariate comparisons). Under these conditions, we found that proportional paternity decreased as the average size of the sperm head and midpiece increased, even when controlling for overall treatment effects on proportional paternity and sperm phenotypes. In other species, midpiece size is often correlated with sperm velocity (Anderson and Dixson 2002; Malo

et al. 2006; Firman and Simmons 2010), and large head size may contribute to drag on the cell, preventing the cell from moving efficiently (Humphries et al. 2008). However, without a better understanding of the functional significance of sperm morphology in *A. sagrei*, and of the aspects of female reproductive anatomy and physiology that influence sperm performance, any adaptive interpretations are merely speculative. We also note that after removing overall treatment effects (i.e., when considering only variance within treatments), we observed a significant negative relationship between proportional paternity and sperm count using multiple regressions including all sperm phenotypes, a result that seems difficult to reconcile with any adaptive interpretation.

Quantifying the condition dependence of male phenotypes, as well as the fitness consequences of this condition dependence, may aid in our understanding of how phenotypic and genetic variance is maintained in the face of chronic sexual selection. Sperm morphology is highly variable among species, and often within species, but few studies have identified the processes that generate and maintain this intraspecific variation (Hellriegel and Blanckenhorn 2002; Simmons and Kotiaho 2002; Rahman et al. 2013). We have demonstrated that some aspects of sperm morphology and sperm count exhibit signatures of condition dependence in wild *A. sagrei* populations, as well as in direct response to dietary restriction in captivity. Moreover, we have shown that high-condition males tend to sire a greater proportion of offspring in competitive mating trials with low-condition males. These data suggest that condition-dependent reproduction may be mediated in part by the condition dependence of sperm quantity and quantity, and our results also provide limited correlative evidence that variation in sperm phenotypes may be associated with variation in fertilization success. The nature of this association, and its relevance for the evolution of sperm and ejaculate traits via sexual selection in *A. sagrei* and other organisms, will require a deeper understanding of the factors that influence sperm performance, along with more comprehensive tests for selection on sperm morphology and sperm count.

SUPPLEMENTARY MATERIAL

Supplementary material can be found at http://www.beheco.oxfordjournals.org/

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