Body condition influences sperm energetics in lake whitefish (*Coregonus clupeaformis*)

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Abstract: Theoretical models predict that individual males will increase their investment in ejaculates when there is a risk of sperm competition. Because the production of ejaculates is assumed to be energetically costly, only those males in good physical condition should be capable of producing ejaculates of high quality. We studied ejaculate investment (relative testis size, controlling for body size) as well as the size, behaviour, and energetics of spermatozoa in lake whitefish (*Coregonus clupeaformis*), a species in which males provide nothing but sperm to females during reproduction. Ejaculate investment was condition dependent, with males in better body condition having relatively larger testes. Sperm adenosine triphosphate (ATP) levels were unaffected by male condition alone, but increased with testis size when controlling statistically for both male size or body condition. Thus, males investing in relatively larger testes produced spermatozoa with higher energetic capacity. We also discovered testis asymmetry in this species, a phenomenon not previously reported in fishes, though widespread in other taxa. The mass of the (larger) left testis was a better predictor of sperm ATP stores than the mass of the (smaller) right testis (controlling for body size), suggesting that testis size asymmetry might be related to the production of high-quality sperm.

Résumé : Les modèles théoriques prédisent que les mâles individuels soumis à un risque de compétition spermatique vont augmenter leur investissement dans leur éjaculat. Comme on présume que la production d'éjaculat a un coût énergétique élevé, seuls les mâles en bonne condition physique devraient être capables de produire des éjaculats de grande qualité. Nous étudions l'investissement dans l'éjaculat (taille relative des testicules, compte tenu de la taille corporelle), ainsi que la taille, le comportement et les relations énergétiques des spermatozoïdes chez le grand corégone *(Coregonus clupeaformis)*, une espèce chez laquelle le mâle ne fournit que le sperme aux femelles durant la reproduction. L'investissement dans l'éjaculat est fonction de la condition, car les mâles en meilleure condition corporelle possèdent des testicules relativement plus gros. Les concentrations d'adénosine triphosphate (ATP) dans les spermatozoïdes ne sont pas affectées par la condition seule, mais elles augmentent en fonction de la taille des testicules, une fois qu'on a tenu compte statistiquement de la taille du corps et aussi de la condition corporelle des mâles. Ainsi, les mâles qui investissent dans des testicules relativement plus gros produisent des spermatozoïdes de capacité énergétique supérieure. Nous avons aussi découvert une asymétrie des testicules chez cette espèce, un phénomène non encore signalé chez les poissons, bien que courant dans d'autres taxons. La masse du testicule gauche (plus gros) s'avère être une meilleure variable explicative des réserves d'ATP des spermatozoïdes que la masse du testicule droit (plus petit), une fois qu'on a fait les corrections pour tenir compte de la taille corporelle; cela laisse croire que l'asymétrie de la taille des testicules peut être reliée à la production de spermatozoïdes de qualité supérieure.

[Traduit par la Rédaction]

Introduction

Sperm competition is widespread in nature and occurs when the sperm of two or more males compete to fertilize a female's ova (Parker 1970). It is a powerful influence on selection, responsible for variation in male behaviour, anatomy, and physiology (Birkhead and Møller 1998). Sperm are produced in the testes, and because the size of the testes reflects ejaculate investment (Scharer et al. 2004), the testes are predicted to be larger in species with more intense sperm competition. Comparative studies across numerous vertebrate taxa have detected general support for this prediction (e.g., Mammals: Harcourt et al. 1981; Fish: Stockley et al. 1997; Amphibians: Byrne et al. 2002).

Within species, ejaculate quality (e.g., sperm density, motility, swimming speed, and adenosine triphosphate (ATP) stores) also varies among individuals (reviewed in Snook 2005) and can result in differential fertilization success (e.g., Casselman et al. 2006). Numerous factors have been shown to influence ejaculate quality, including age (e.g., Evans and

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Geffen 1998), body size (Skinner and Watt 2007), mating tactic (e.g., Uglem et al. 2001; Vladic and Järvi 2001), and social status (e.g., Rudolfsen et al. 2006).

A fundamental assumption underlying much of sexual selection theory is that traits associated with male–male competition and female mate choice should be costly to produce (Andersson 1994). Production of ejaculates can be energetically demanding (Olsson and Madsen 1998), and thus only males in good body condition are predicted to produce high-quality ejaculates. In fishes, males in better body condition have been shown to have higher fertilization success (e.g., Rakitin et al. 1999), and the eggs they fertilize have higher survival rates (Wedekind and Müller 2004), but relations between ejaculate investment (testis size and sperm parameters) and body condition factors have been rarely considered (e.g., Casselman and Montgomerie 2004; Jonsson and Jonsson 2006).

In the current study, we examined intraspecific variation in ejaculate investment in male lake whitefish, *Coregonus clupeaformis*. The mating system of lake whitefish is not well understood, but they are considered to be group spawners (Wedekind et al. 2001), suggesting that sperm competition is likely to be an important, selective force in this species. We examined relations among ejaculate investment (testis size); ejaculate quality (swimming speed, percent motility, density, and ATP stores of sperm); and the age, size, and body condition of individual male lake whitefish to test the predictions that (*i*) ejaculate investment is dependent upon body condition — males in good condition should have larger testes and higher-quality ejaculates than males in poor condition; and (*ii*) younger and (or) smaller males have greater relative investment in testes and ejaculate quality than larger, older males to compensate for their presumed disfavoured role during spawning as a result of dominance by larger males.

Like males of many other vertebrates (Birkhead et al. 1998), male lake whitefish have asymmetrical testes (see below), with the left testis being larger than the right. Reasons for testis size asymmetry are generally unknown, though one suggestion has been that the smaller testis provides insurance in case of damage or disease impairing sperm production in the larger testis. If the larger testis becomes impaired, then the smaller testis may increase in size to compensate, thereby reducing the degree of testis asymmetry (Møller 1994; Birkhead et al. 1998). Thus, this directional asymmetry may be positively related to measures of male quality, with high-quality males having more asymmetrical testis (Møller 1994). To test this hypothesis, we examined the relative importance of right and left testis size as predictors of sperm energetics.

Materials and methods

Sampling and handling

Adult male lake whitefish were captured in 2002 during fall spawning in the Bay of Quinte, Lake Ontario, Canada. Twenty-five males were captured in traps, transported to shore, and held in aerated holding tanks filled with lake water at 7 °C until they were stripped of milt (within 8 h of being brought to shore). Twenty-four additional males were stripped of milt immediately following removal from the trap so that we could preserve additional sperm samples for morphological analysis; no measures of sperm motility or ATP content were performed on these 24 males. Milt was collected by applying pressure to the male's abdomen and taking up the extruded milt into 1 mL syringes. Samples for motility and ATP content were used immediately; all other samples were stored on ice for up to 4 h before processing. Milt contaminated with either urine or feces was discarded.

Following milt collection, all fish were killed and their fork length (FL ± 1 mm) and body mass (±1 g) recorded. Left and right testes were removed and weighed (±1 g). Male age for 47 individuals was determined from sagittal otoliths, following Casselman and Montgomerie (2004).

Ejaculate quality

See Burness et al. (2004) for details of our methods for measuring sperm motility. We first used 750 µL of Cortland's solution (250 mmol·L^{-1} NaCl, 10 mmol·L^{-1} KCl, 3 mmol·L^{-1} CaCl$_2$, 24 mmol·L^{-1} NaHCO$_3$, at pH 7.91, ~500 mosmol) to dilute 5 µL of the milt without activating the sperm. We then placed a drop of this solution into a hemocytometer chamber and initiated motility by flooding the sample with lake water at 7 °C. We recorded sperm motility on videotape using a high-resolution CCD camera mounted on a negative phase contrast microscope with the microscope stage maintained at ambient lake temperature (7–10 °C).

We analyzed videotapes using a CEROS (v. 12) sperm analysis system (Hamilton Thorne Research, Beverly, Massachusetts, USA). For each male, we calculated sperm swimming speed (VAP; average smoothed path velocity) for up to 35 spermatozoa per field of view, for each of seven time intervals from 0.5 to 90 s after activation, using criteria for selection of individual sperm reported in Burness et al. (2004). Here, we report only VAP (Casselman and Montgomerie 2004) at 10 s after activation, because the majority of fertilizations have been reported to occur within the first few seconds following ejaculation in externally fertilizing fishes (Vladic and Järvi 1997; Hoysak and Liley 2001). We use median VAP in all analyses to minimize the influence of both outliers and skewed distributions of swimming speeds (Casselman et al. 2006). At 10 s after activation, we measured VAP for a mean of 23 sperm per field of view (±1.3 standard error (SE), range 9–33 sperm, *n* = 24 fish).

To calculate the percentage of sperm that were motile within an ejaculate, we placed a piece of acetate over the computer monitor and marked each spermatozoon as either swimming or immotile at both 10 s and 60 s after activation. We performed this analysis twice for each of 10 males at 10 s after activation and found that the estimates were highly repeatable (repeatability = 0.95, *P* < 0.001).

To preserve sperm for morphological analysis, we diluted milt 151-fold in fixative (2.5% glutaraldehyde in 0.1 mol·L^{-1} sodium cacodylate buffer, pH 7.0), then spread the solution evenly across a microscope slide and allowed it to air-dry. Sperm tail length was measured from digitized images of sperm from these slides (see Burness et al. 2004). Sperm concentration was measured by counting the number of sperm in 10 haphazardly chosen hemocytometer cells (see Leach and Montgomerie 2000).

To measure sperm ATP content, 20 µL of fresh milt, from each of the males for which we measured sperm motility,

Table 1. Descriptive statistics for morphological and ejaculate traits of male lake whitefish (*Coregonus clupeaformis*).

Trait	Mean±SE	95% CL	Range	n
Morphological				
Age (years)	8.8±0.3	8.2–9.3	5–12	45
Fork length (mm)	452.9±5.0	442.9–463.0	360–538	47
Body mass (g)	1001.9±38.1	925.1–1078.6	472–1712	47
Fulton's K	1.05±0.01	1.03–1.08	0.89–1.25	47
Left testis mass (g)	8.38±0.56	7.26–9.51	3–19	47
Right testis mass (g)	6.74±0.46	5.81–7.68	3–19	47
Ejaculate				
Median sperm length (μm)	38.4±0.4	37.6–39.2	30.4–44.7	47
Sperm density (no. $\times 10^6 \cdot (\mu$L milt$)^{-1}$)	6.25±0.21	5.83–6.67	3.26–9.85	47
Median VAP (μm·s^{-1}) at 10 s after activation	112.5±2.0	108.5–116.8	93.2–129.1	23
Percent motility at 10 s after activation	60.6±2.8	54.8–66.4	34–79	23
Percent motility at 60 s after activation	32.1±3.6	24.7–439.5	5–68	24
ATP levels (pmol·(million sperm)$^{-1}$)	139.0±9.1	120.1–157.9	51.5–236.5	23

Note: Two outliers were removed from all analyses and one additional outlier was removed from the ATP analysis. SE, standard error; CL, confidence limit; VAP, average smoothed path velocity (sperm swimming speed).

was frozen on dry ice in the field and stored at –80 °C until ATP content was measured (see Burness et al. 2004). ATP was extracted from frozen samples in ice-cold 3% perchloric acid, followed by sonication and centrifugation for 2 min at 15 000g. Samples were then neutralized, and ATP content was determined (per 10^6 spermatozoa) by quantifying bioluminescence following addition of luciferin–luciferase.

Body condition

We calculated two measures of male body condition: (*i*) Fulton's condition factor (K) as $K = M \cdot FL^{-3} \times 10^5$ (where M is mass) and (*ii*) residuals from the regression of log(soma mass) on log(FL). Soma mass was calculated by subtracting the total mass of the testes from body mass so that body and testes masses for each individual would be independent. In all cases, both measures of condition yielded the same results, so we report only the latter. In some cases, we included soma mass and FL as predictor variables in a multiple regression, such that the partial coefficient of soma mass represents body condition (Schulte-Hostedde et al. 2005*b*).

Data analyses

We used both JMP (v. 6.0.3) and R (v. 2.4.1) to analyze mean (or median) ejaculate traits per male. We $\log_{10}$-transformed data for age, FL, Fulton's K, and the masses of body, soma, and testes in all analyses to normalize residuals (see Table 1 for descriptive statistics on all measured variables).

For multiple regression analyses, we first performed hierarchical partitioning analysis (Mac Nally 2000) to choose the most important predictor variables (from testis mass, soma mass, body length, and sperm length) influencing different indices of ejaculate quality (e.g., ATP levels, swimming speed, sperm density). This is an especially useful approach in a study like this where independent variables may be significantly correlated and the sample sizes are relatively small. These selected variables were then entered into a multiple regression analysis of all possible models, and Schwarz's Bayesian information criterion was used to determine the best minimal m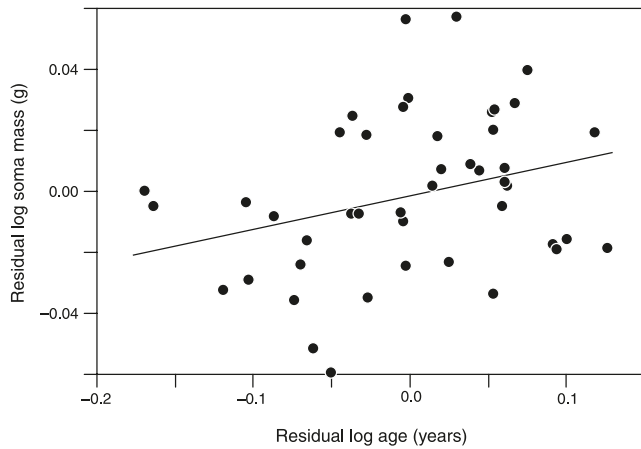odel in each case. When building models, we considered not only the statistical results but also the biological relevance of each variable. Thus, in the analysis of the different ejaculate traits, we screened for the independent effects of both (*i*) body condition (measured as either residual soma mass or Fulton's K) and (*ii*) ejaculate investment (measured as absolute testes mass). In the analysis of sperm ATP, we also included sperm length as a potential independent variable, as longer sperm are expected to require more energy for forward propulsion (Cardullo and Baltz 1991).

Our initial screening of the data indicated three outliers that we removed from subsequent analyses, as follows. First, we removed one male that was by far the oldest fish in our sample (16 years vs. 5–12 years for the rest of the sample) and an exceptionally old fish for this population (J. Casselman, Queen's University, Department of Biology, Kingston, ON K7L 3N6, personal communication). This male was in particularly poor condition for its size, suggesting that it might have been senescent. Second, we excluded one male with unusually small testes for his body size and condition (>3 standard deviations (SD), below the predicted value for this sample), suggesting he may not have been in full breeding condition. Finally, we excluded a male from all analyses involving sperm ATP because his sperm ATP level was particularly low (15.5 pmol·(million sperm)$^{-1}$ vs. 51.5–236.5 for the rest of the sample), indicating that this milt sample had likely thawed before the assay. The removal of these males from analyses resulted in normally distributed residuals and an increase in statistical power (and thus smaller P values), but analyses with and without these outliers showed the same patterns.

Results

There was a significant positive relation between log(male age) and log(FL) ($r = 0.69$, $n = 44$, $P < 0.001$). In a multiple regression with log(soma mass) as the dependent variable, there was a significant effect of log(age) (partial $r = 0.29$, $F_{[1,42]} = 3.9$, $P = 0.05$, $n = 45$), with log(FL) held constant.

Fig. 1. Partial regression plot showing relation between body condition (residual soma mass) and age in male lake whitefish (*Coregonus clupeaformis*), with both variables log-transformed and controlling for log(fork length).

Fig. 2. Relative total testes mass (controlling for body length) in male lake whitefish (*Coregonus clupeaformis*) increases with body condition (soma mass controlling for body length). This partial regression plot illustrates the trend found using multiple regression analysis. In this plot, $y = 3.3 \times 10^{-15} + 3.5x$ ($r = 0.60$, $P < 0.0001$, $n = 47$).


Thus the body condition of males improved as they aged from 5 to 12 years (Fig. 1).

As predicted, males in good condition had heavier testes for their body size than males in poor condition, with condition measured as soma mass controlling for body length (partial $r = 0.61$, $n = 47$, $P < 0.0001$; Fig. 2). In a separate multiple regression analysis that included age, body length (FL), and residual soma mass (as an index of condition) as predictor variables (all log-transformed), the effect of age on testes size was not significant ($F_{[1,41]} = 1.4$, $P = 0.25$), but the effects of both residual soma mass ($F_{[1,41]} = 25.8$, $P < 0.0001$) and FL ($F_{[1,41]} = 24.4$, $P < 0.0001$) were both positive and significant. Thus, testis size was influenced by both body size and condition but not by male age.

The best minimal multiple regression model to explain sperm ATP levels included testes mass, body length (FL), and soma mass as predictor variables (Table 2) and explained almost half of the variation in the ATP content of spermatozoa. In this model, sperm ATP levels increased significantly with relative investment in testes (controlling for body length and soma mass) but decreased with body condition (soma mass controlling for body length and testes mass). Thus, larger testes (relative to body size) produced sperm with higher ATP content (Fig. 3a), but the production of this ATP, in testes of a particular relative size, appears to have resulted in poorer overall body condition (Fig. 3b). Sperm ATP levels were not significantly related to male condition alone (i.e., without controlling for testes mass), with condition measured as soma mass controlling for body length (partial $r = -0.19$, $F_{[1,20]} = 0.30$, $P = 0.59$), though the relation is negative, as in the multiple regression model shown in Table 2.

None of the models predicting sperm density, sperm swimming speed (VAP at 10 s after activation), or sperm motility (percent motile at 10 s after activation) were significant. Thus, these variables were not significantly correlated with male soma mass, FL, sperm length, or testes mass, nor were multiple regressions containing any combination of these variables significant ($P > 0.10$ in each case).

The left testis was larger than the right testis (Table 1) in 38 of 47 males, and this difference (mean = 1.64 g, 95%
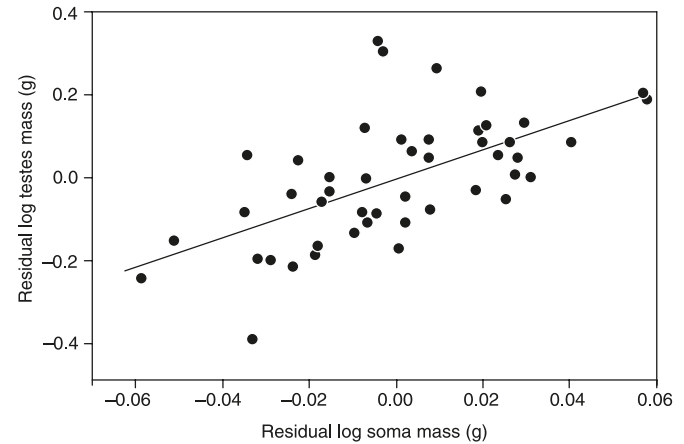
Table 2. Multiple regression model to predict ATP per sperm in lake whitefish (*Coregonus clupeaformis*).

| Predictor variable	Standardized Beta	F	P
log(soma mass) (g)	−2.86	10.2	0.005
log(fork length) (mm)	2.2	7.2	0.020
log(combined testes mass) (g)	0.9	12.6	0.002

Note: Whole model: $F_{[3,19]} = 5.11$, $P = 0.009$, $R^2 = 0.45$. The partial regression coefficients (Standardized Beta) indicate both the sign and relative magnitude of each relation when controlling for all other variables in the model.

confidence limit (CL) = 0.71–2.57 g, $n = 47$) is significant within males (paired t test on untransformed data, $t = 3.54$, $P = 0.0009$, $n = 47$). Though left and right testes masses of individual males were correlated (intraclass correlation coefficient = 0.62, $n = 47$), the left testis explained only 38% of variation in the right testis. Interestingly, the size of the left (partial $r = 0.63$, $F_{[1,19]} = 12.3$, $P = 0.002$; Fig. 3b) rather than the right testis (partial $r = 0.48$, $F_{[1,19]} = 5.8$, $P = 0.03$) was a better predictor of sperm ATP levels (in both cases controlling for body length and soma mass) in the sample of 23 males for which we measured sperm ATP. Moreover, right testis mass did not contribute significantly (partial $r = 0.28$, $F_{[1,18]} = 1.5$, $P = 0.24$) to a multiple regression model predicting sperm ATP levels from body length, soma mass, and left and right testis sizes, whereas left testis mass did (partial $r = 0.52$, $F_{[1,18]} = 6.6$, $P = 0.02$), suggesting again that the left testis mass was a better predictor of sperm ATP. No other ejaculate traits made a significant contribution to these multiple regression models.

Discussion

Our results are the first to suggest that individual males investing heavily in testicular tissue also invest differentially in the energetic content of individual spermatozoa. We also found that testis size is asymmetrical in this species, with the left testis larger than the right. Moreover, close to 50% of

Fig. 3. Partial regression plots showing relations between residual adenosine triphosphate (ATP) content of lake whitefish (*Coregonus clupeaformis*) sperm and (*a*) residual mass of the left testis (partial $r = 0.63$, $y = 6.0 \times 10^{-13} + 182.8x$, $P = 0.001$), with both variables controlling for body length and soma mass; and (*b*) residual soma mass as an index of body condition ($r = -0.41$, $y = -2.0 \times 10^{-13} + 700.2x$, $P = 0.05$), with both variables controlling for body length and mass of left testis. Soma mass was calculated as body mass minus combined testes mass in each case. All variables except ATP were log-transformed for these analyses.
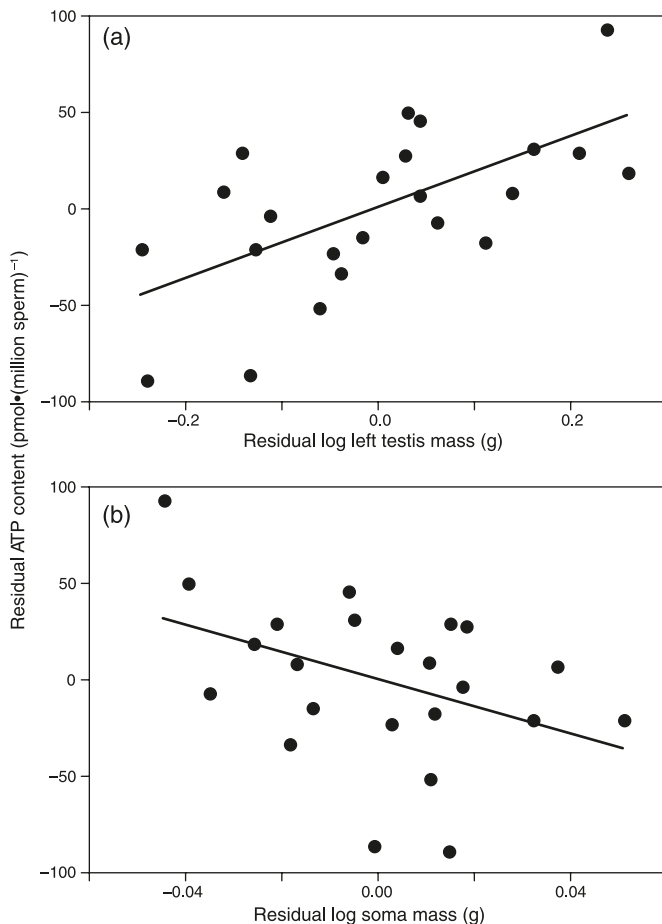
the variation in energetic content (ATP levels) of individual spermatozoa was explained by a model that included the size of the left testis, soma mass, and body length. In fact, inclusion of the right testis in that analysis contributed little to the model predicting spermatozoan energy stores. The larger size of the left testis suggests that it produces sperm at a higher rate than the right testis (sensu Scharer et al. 2004). Although such testicular size asymmetry has been reported in birds and frogs (e.g., Møller 1994; Hettyey et al. 2005), we are unaware of any other study that has reported this in fishes. The functional importance of such asymmetry is unclear (Birkhead et al. 1998), but our results suggest that the increased size of the left testis reflects increased investment in the energetic content of spermatozoa, something that has not previously been reported. Møller (1994) argued that directional asymmetry was positively correlated with male quality, but subsequent tests of this hypothesis have yielded equivocal results (Birkhead et al. 1998; Merilä and Sheldon 1999; Hettyey et al. 2005). The discovery of directional

asymmetry in lake whitefish and its relationship with sperm energetics indicates that fish may be an appropriate model to test Møller's (1994) hypothesis further.

Why whitefish preferentially invest in one testis is not clear, but the negative relation between sperm energetic content and body condition, holding testis size constant, certainly hints at energetic constraints. Consistent with the presumed costly nature of sperm production (Nakatsuru and Kramer 1982; Olsson and Madsen 1998), we also found a negative relation between sperm ATP content and soma mass, independent of testis mass and body size. This supports the hypothesis that males in good condition before spawning allocate more energy to ejaculates and as a result are in relatively poor condition at spawning (Casselman and Montgomerie 2004). Given the links between sperm energy content and fertilization success detected for other species (e. g., Atlantic salmon, *Salmo salar*; Vladic and Järvi 2001) and the heritability of condition in fish (Kause et al. 2003), females may use ejaculate traits to select for the sperm of males in good condition, thereby exhibiting postcopulatory female choice (e.g., Rakitin et al. 1999). It would therefore be informative to manipulate the condition of individual males to explore the impact on sperm ATP levels, motility, and fertilization success.

A positive relation between testis size and body condition has been reported previously in other taxa (e. g., Simmons and Kotiaho 2002; Schulte-Hostedde et al. 2005*a*), but not, as far as we are aware of, in fishes, even though there are presumably fitness benefits for male fish with relatively large testes. For example, in yellow-pine chipmunks (*Tamias amoenus*), males with relatively large testes had higher annual reproductive success than males with smaller testes (Schulte-Hostedde and Millar 2004). Any positive covariation between ejaculate investment (as indexed by testis size) and body condition would indicate that there may be reproductive benefits accrued by individuals in good condition. Links between condition and reproductive success have been shown previously in whitefish (*Coregonus* sp., Wedekind and Müller 2004) and other fish species (Atlantic cod, *Gadus morhua*; Rakitin et al. 1999), but the physiological mechanisms relating condition and reproductive success are not clear.

ATP stored in spermatozoa prior to ejaculation is probably the primary source of energy for sperm motility in fishes (e.g., Burness et al. 2004). We provide here the first evidence that this sperm energy content is influenced by variation in both body condition and testis size within a population. We suggest two exciting avenues for future work: (*i*) male body condition could be manipulated experimentally to identify the role that body condition plays on sperm energetics, testis asymmetry, and male secondary sexual traits, and (*ii*) the importance of sperm energetics to male fertilization success could be explored. For example, sperm within an ejaculate could be separated based on mitochondrial content using a fluorescence activated cell sorter so that the fertilization success of sperm differing in mitochondrial content could be tested directly. Because our conclusions are necessarily based on correlation analysis, it is impossible to assign cause and effect with complete confidence. This is true of virtually all studies conducted to date on the effects of body condition on ejaculate traits. The results presented here, however, suggest that controlled experiments involving the manipulation of body condition by altering the diet and

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exercise of male fishes would be a fruitful avenue for further research on ejaculate traits.

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