The relative effect of parasites and social status on sperm traits in Arctic charr

L. Figenschou¹, I. Folstad*¹, G. Rudolfsen¹², S.A. Hanssen³, R. Kortet⁴, P.A. Skau⁵, J.E. Killie⁶, I.C. Oskam⁷, and H. Strand⁸

Addresses:
¹Faculty of Bioscience, Fishery and Economy, University of Tromsø, N-9037 Tromsø, Norway
²Norwegian Radiation Protection Authority (NRPA), Department of Environmental Radioactivity, The Fram Centre, N-9296 Tromsø, Norway
³Norwegian Institute for Nature Research, N-9296 Tromsø, Norway.
⁴Department of Biology, University of Eastern Finland, P.O. Box 111, FI-80101 Joensuu, Finland.
⁵Centre of Clinical Evaluation and Documentation. Northern Norway Regional Health Authority, N 9000 Tromsø, Norway.
⁶The Norwegian College of Fishery Science, University of Tromsø, N-9037 Tromsø, Norway.
⁷Norwegian School of Veterinary Science, Norwegian University for Life Sciences, N-0033 Oslo, Norway.
⁸The University Hospital of Northern Norway, N-9000 Tromsø, Norway.

Corresponding author:
* Ivar Folstad
E-mail: ivar.folstad@uit.no
Phone: +47 77644384 / +47 90088764

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Dominance and infections affect ejaculates
Abstract

Sperm production and sperm swimming speed, which most likely affect fertilization under sperm competition, are modified by proximate mechanisms. In a comprehensive observational study of free-living and reproductively active Arctic charr (Salvelinus alpinus) we examined the possible modulating effects of male social status (including ornamental development), parasite intensities and immunity on novel traits indicative of ejaculate quality (e.g., ATP in sperm cells, levels of immunoglobulines attached to sperm cells, and the degree of DNA-fragmentation in sperm cells). A multiple regression model showed no relationship between DNA-fragmentation in sperm cells and abdominal coloration of charr, suggesting that sperm of colourful males are not better protected against oxidative stress. We found, however, that males with traits indicative of high social status had low amounts of ATP in sperm cells and also sperm cells with low swimming speed. Sperm production, on the other hand, was strongly predicted by parasite intensities only, and these associations were mainly positive. Our results suggest that the presence of parasites seems to stimulate sperm production similar to what is observed among males entering subordinate reproductive roles. Thus, if resistance towards parasites is influencing parasite intensities, resistance may also indirectly be important for the “choice” of reproductive role and in turn investment in primary sex traits.

Key words:
Arctic charr, parasite intensity, social status, immunity, sperm quality, sexual selection
INTRODUCTION

Among males of many species there is notable variation in competitive ability. This variation is particularly pronounced during the reproductive season, and in many species it is convenient to categorize males in two distinct tactics, dominants and subordinates, based on their reproductive behaviour and appearance (e.g., Gross 1996). There are different costs associated with these male mating tactics, and in dynamic, role-flexible species males are assumed to quickly adopt the phenotype giving the highest genetic contribution to the next generation (Taborsky 2001).

Dominant males usually develop striking sexual ornamentation and a number of mechanisms, including the well-known handicap model (Zahavi 1975, 1977), have been proposed to explain the maintenance of variation in these sexually selected traits (reviewed in Andersson 1994; Birkhead and Møller, 1998). Two mechanisms derived from the handicap model emphasize the relationship between development of secondary sex traits and development of sperm traits (Folstad and Karter 1992; Sheldon 1994). According to the “phenotype-linked fertility hypothesis” male secondary sexual characters are honest indicators of sperm quality because intensely coloured males should be better protected towards oxidative stress (Sheldon 1994; Pike et al. 2009). For example, in species where trade-offs for limited amounts of carotenoids exist between ornamental development or development of antioxidant support for sperm functioning (Peters et al. 2004; Velano et al. 2008), colourful males are suggested to have higher body supplies of antioxidants and hence also a more efficient antioxidant protection of sperm DNA compared to drab males. Free radicals, which may be inactivated by antioxidants (Garratt and Brooks 2012), can cause reduced sperm motility (Delamirande and Gagnon 1992), inhibit sperm-oocyte fusion (Aitken et al. 1989) and reduce fertility (Wishart 1984). Moreover, excessive free radicals may lead to oxidative damage, resulting in damage to the DNA of the sperm (Blount et al. 2001), which in turn may
cause irreversible changes in the genetic composition of offspring. Thus, as a male’s antioxidant-based ornamentation may reveal not only direct benefits to females, i.e., fertility insurance, but also indirect benefits such as lowered probability for mutations in offspring (Blount et al. 2001; Velano et al. 2008), females may use antioxidant-based sexual signals to choose prospective mates (Peters et al. 2004; Helfenstein et al. 2010).

Another indicator mechanism of sexual selection (Andersson and Simmons 2006), the “immunocompetence handicap hypothesis” (Folstad and Karter 1992), suggests that the immune system competes for resources with sexually selected ornaments, and that variation in ornamental display reflects variation in immunocompetence. Yet, immune activity may also be related to ejaculate quality because sperm cells are perceived as “non-self” by the immune system and are exposed to immunological attacks in the testes and epididymis (Friberg 1982; Hogarth 1982; Roitt et al. 1993). This attack, which may reduce male fertility (Skau and Folstad 2005), is often manifested as high levels of immunoglobulins (Ig) on sperm cell surfaces (Chamley and Clarke 2007). Males that are forced to fight infection by up-regulating immune function pay a cost of reduced sperm quality. Thus, males with genetic resistance against parasites may have an advantage as they may be better able to lower their immune activity during spermatogenesis and in turn produce ejaculates of higher quality (Folstad and Skarstein 1997; Hillgarth et al. 1997). Thereby, parasite intensity and immune responses may not only be related to the development of secondary sexually selected traits, and to male social dominance, but also to primary sex traits under sexual selection, i.e., sperm quantity and quality. Associations between variation in primary sex traits and traits indicative of immunity have been documented (Liljedal et al. 1999; Hosken and O'Shea 2001; Liljedal and Folstad 2003; Kortet et al. 2004; Måsvær et al. 2004) and in insect models negative trade-offs between immune activity and sperm production have repeatedly been documented (e.g., Hosken 2001; Kerr et al. 2010; Simmons 2011).
The arctic charr (*Salvelinus alpinus*), an externally fertilizing fish with a lek-like mating system (Figenschou et al. 2004) and high levels of sperm competition (Sørum et al. 2011), is excellent for studies of sexual selection (Skarstein et al. 2001; Liljedal and Folstad 2003; Rudolfsen et al. 2006). Free-living charr reproduce in shallow waters annually and both sexes develop a red carotenoid-based abdominal spawning coloration with males more intensely ornamented than females (Skarstein and Folstad 1996). Males interact vigorously before and during arrival of sexually mature females and arriving females are guarded closely by one of the larger, more aggressive and dominant males (Sørum et al. 2011; pers. obs.). Size differences between males may be large within a spawning population (Figenschou et al. 2004) and during female egg release the nearby, often smaller and more subordinate males regularly dart into the spawning site that offers no protection against sneakers, and release their milt (Sørum et al. 2011; [http://naturweb.uit.no/amb/evolution/](http://naturweb.uit.no/amb/evolution/)). Sperm velocity plays a key role in fertilization (Liljedal et al. 2008), and charr males have the capacity to rapidly adjust velocity and density of own sperm in response to changes in hierarchical position, i.e., social status (Rudolfsen et al. 2006). The different male mating tactics in charr seem to be conditional and plastic, with relative body size as the most important determinant of tactic choice (Sigurjonsdottir and Gunnarsson 1989).

Sperm production and sperm quality (e.g., swimming speed) are modified by proximate mechanisms, and several studies of sexually selected variables and their associations with male ejaculate investment have been conducted in charr (Skarstein and Folstad 1996; Liljedal et al. 1999; Skarstein et al. 2001; Liljedal and Folstad 2003; Masvaer et al. 2004; Rudolfsen et al. 2006; Serrano et al. 2006; Haugland et al. 2009). However, the actual pathways for the interactions between ejaculate characteristics, parasite pathogenicity, immunological activity, social status and secondary sex traits are not clear. We conducted a comprehensive observational study in which we, in unprecedented detail, examined traits
indicative of ejaculate quality (i.e., testes mass, sperm production, percentage of motile sperm cells, sperm velocity, ATP in sperm cells, levels of Ig attached to sperm cells, and the degree of DNA-fragmentation in sperm cells) and the possible modulating effects of male social status (including ornamental development), parasite intensities (eight species) and immunity (four parameters) on these sexually selected traits (Figure 1). To our knowledge, the present study is the first comprehensive analysis on this topic, not only in fishes but in any taxa.
MATERIALS AND METHODS

Fish sampling and handling

In the early part of the lekking period, 15-17 September 2005, we caught 123 sexually mature males from Lake Fjellfrøsvatn, Northern Norway (69°4’N, 19°20’E). All males were collected from one spawning ground and had spawning coloration and gonads holding mature sperm. During sampling, which occurred at daytime, the fish never stayed in the gill nets for more than 15 minutes and individuals with external signs of injuries were excluded from the study. Following capture, 95 males were transported to the field laboratory where each was put to death by a distinct blow to the head before blood and milt samples were collected. An additional sample of males was used to assess male social status in the experiment described below.

Social status

Due to practical limitations, we could not directly assess social status from observations of behavioural interactions on the spawning ground. Instead, we relied on indirect estimates. In general, body size is a very important factor predicting dominance among spawning male charr (Sigurjonsdottir and Gunnarsson 1989, see also Noakes 1980), and in order to estimate the effect of size on social dominance we pair-wise caged 28 size-matched males (14 pairs) captured simultaneously at the same spawning ground as the focal individuals of our primary study. Pair-wise comparisons showed that the longest and heaviest male in each pair most often became dominant ($F_{(1, 26)} = 17.09, p < 0.001$; mean within-pair length difference was 1.7 cm, range 0.1 to 5.9 cm). Moreover, the individual’s length (and mass) was positively correlated with the individual’s total number of aggressive acts (length, $r = 0.44$, $P = 0.019$) and the individual’s number of aggressive acts per minute (length, $r = 0.40$, $P = 0.034$; see Liljedal and Folstad (2003) for a more detailed description and evaluation of the methods.
The intensity of abdominal coloration tended to be positively correlated with fish length \((r = 0.40, P = 0.098, n = 18)\), but was not significantly associated with the total number of aggressive acts \((r = 0.19, P = 0.457, n = 18)\) or the number of aggressive acts per minute \((r = 0.12, P = 0.64, n = 18)\). It should be noted that the size-differences between the dominants and the subordinates in a natural group of spawning charr is usually larger than in our experimental setup (pers. obs.). A principal component analysis resulting from variation in length, mass and age was consequently used as an indicator of social status in our primary study (see below).

**Ejaculate analysis and primary sex traits**

All males were caught in the early part of the lekking period (c.f., Figenschou 2004) and before spawning activity was observed at the spawning ground. Therefore, sperm depletion is unlikely confounding our results. All sperm sampling was executed by one person, who carefully dried the area around the genital pore of each male to avoid water contamination and activation of sperm. Milt stripping was carried out by applying repeated, bilateral pressure from the anterior part of the abdomen towards the genital pore. To reduce handling time, all ejaculate measurements were conducted in the random sequence in which the fish were handled. Milt volume was estimated to the nearest 0.1 ml using 1 ml syringes and thereafter stored at 4°C. Spermatocrit, which is the percentage of a given volume of milt that is occupied by cells (i.e., sperm density), was measured by centrifuging about 10 µl homogenized milt in a capillary tube for 195 s at 11500 rpm with a Compur mini-centrifuge (Compur-electronic Gmbh, Munich, Germany). Testes mass was measured to the nearest 0.001 g. Video recording of activated sperm was done within 2 h after the milt was collected using a CCD black and white video camera (XCST50CE PAL, Sony, Tokyo, Japan) mounted on a negative phase-
contrast microscope (Olympus CH30, Olympus, Tokyo, Japan) with a 10x objective. Motility was initiated by adding 4.5 µl water after placing less than 0.12 µl of sperm on a cooled (5–7 °C) standard counting chamber (Leja products BV, Nieuw-Vennep, Netherlands). Sperm movement was recorded from activation until movement ceased (between 60 and 90 s). Each male is represented with two recordings of moving sperm cells that each has evenly distributed cells (mean = 182, sd = 97.8, both 10 s post activation). The recordings were later analysed using computer assisted sperm analysis (HTM-CEROS sperm tracker, CEROS v.12, Hamilton Thorne Research, Beverly, MA, USA), which has been shown to be an objective tool for studying sperm motility in fish (Kime et al. 1996; Kime et al. 2001). The image analyser was set at; frame rate 50 Hz, number of frames 25, minimum contrast 10 and minimum cell size 5 pixels. For each male we quantified sperm motility 10 s after activation. Each motility measurement lasted 0.5 s. The parameters assessed were mean average path velocity (VAP), mean straight-line velocity (VSL), mean curvilinear velocity (VCL), and percentage of motile cells. Relative static cells having a VAP <10 mm and a VSL <20 mm were excluded from the motility analysis.

As the relationship between testes mass and social status may be allometric, we constructed a gonadosomatic index (gonad mass/body mass). Hereafter, the testes mass refers to this constructed variable. A similar approach was used to measure relative milt volume (milt volume/body mass). Moreover, to measure sperm production we multiplied spermatocrit with milt volume.

**Colour analysis of ornamentation**

We captured digital images of each male’s abdomen with a Nikon Coolpix 995. The camera was fixed on an adjusted photo-rack that had a constant illumination to which the white balance of the cameras was calibrated. From a standardized area of the belly of the fish (see
Skarstein and Folstad 1996) we produced numeric estimates of red, green, and blue intensities using Adobe Photoshop 7.0. Thereafter, red intensity was calculated as \( \text{red} / (\text{red} + \text{green} + \text{blue}) \) and termed raw data (Villafuerte and Negro 1998).

Morphological traits and parasites

In the laboratory we measured fish mass (mean 254 g, range 122 – 630 g) and fish length from nose to caudal cleft (mean 26.6 cm, range 21.3 – 35.7 cm). Age was estimated by counting hyaline zones on otoliths immersed in glycerol (mean 6.8 years, range 5 – 10 years).

Spleen size, which is related to filtering capacity and immune function in fish (Hadidi et al. 2008), was estimated by drying the spleen for 6 days at 80°C and then weighed to the nearest mg (mean 16 mg, range 3 – 55 mg) and relative spleen weight (spleen mass/body mass) was calculated. Blood sugar was estimated with blood glucose electrodes (Precision Plus, MediSence, UK). In addition, blood smears were fixed in methanol and later stained using the May-Grünwald-Giemsa method. Smears were scanned at 1000 x magnification and erythrocytes, lymphocytes and heterophils were counted in three independent areas. We calculated the lymphocyte/erythrocyte- and heterophil/lymphocyte-ratios by averaging the ratios from the three counts of each blood smear. The heterophil/lymphocyte ratio (H/L-ratio) is known to increase in response to various stressors, including infectious diseases (e.g., Dein et al. 1986; Gross and Siegel 1983), whereas decreased lymphocyte levels are indicative of immunosuppression with a concomitant increase in susceptibility to infections (Siegel 1985; Fitzgerald 1988). Moreover, after dissection of the gills, eyes, buccal cavity, stomach, intestine, liver, kidney, swimbladder, pylorus and gonads, eight different macroparasite species were counted (i.e., the digeneans *Phyllidostomum umblae, Diplostomum* spp., and *Crepidostomum* spp.; the cestodes *Diphyllobothrium* spp., *Eubothrium salvelini*, and...
Cyathocephalus truncatus; the nematode Cystidicola farionis; and the crustacean Salmincola sp.).

ELISA measurement of total serum IgM

In fish, the major plasma Ig found is a tetramer called IgM (hereafter termed Ig) and in order to measure the serum concentration of Ig, a quantitative capture sandwich enzyme linked immunosorbent assay (Elisa) was developed based on immunoreagents. Briefly, 96-well immunoassay plates (Costar) were coated with 5 µg/mL of R@C-IgM in 0.05 M Carbonate buffer, pH 9.6 for 2 hours at room temperature. After washing the plates (20 mM PBS, pH 7.4, 0.05 % Tween 20) the plates where blocked by incubating for 2 hours at room temperature with 1% dried milk (fat free) in PBS. Serum from the sampled fish along with purified charr Ig (as quantitative reference) were 2-fold diluted in 20 mM PBS, pH 7.4, including 0.05 % Tween 20, and incubated over night at 4°C. To visualize the binding activities, the plates where incubated for 2 hours overlaid with Biotin-R@C-Ig, followed by horse radish peroxidase conjugated streptavidin (HRPO-streptavidin, ICN Biomedicals Inc., USA) for another 2 hours. Finally, 100 µL of the TMB-substrate (1 mg/mL tetra-methyl-benzidine, Sigma) in 50 mM phosphate-citrate buffer (pH 5.0, Sigma) supplied with 2 µL 30% H₂O₂ per 10 mL was added to visualize photometrically the amount of char-Ig bound to the wells (Spectramax 190, Molecular Devices, CA, USA). The Elisa assay was fine tuned to give a substrate conversion yielding OD₄₅₀nm ~ 1.0 – 1.5 at 50 % of maximum binding capacity within 30 minutes. The substrate conversion was stopped by adding H₂SO₄ to a final concentration of 0.5 M in the wells. Between incubation steps in the procedure, the plate was washed in 20 mM PBS, pH 7.4, 0.05 % Tween 20 on a Skan Washer 400 (Skatron, Molecular Devices, CA, USA).
**ATP measurements**

From each fish, 20 µL of sperm was transferred in duplicate to cryotubes and immediately frozen in liquid nitrogen. The samples were kept at -196°C until the ATP analysis. In the laboratory we added 1 mL of cold 3% perchloric acid to the frozen cryotubes, before the content was exposed for two sonication bursts, each lasting for 5-6 sec at 40 watts. Subsequently, the homogenized content was transferred to 2 mL centrifuge-tubes and centrifuged at 15,000 r/min (5764 g) for two minutes. Finally, 180 µL of the supernatant was transferred to a new centrifuge-tube, and 75 µL neutralization buffer was added. The neutralization buffer consisted of a mixture of 6 mL saturated TRIS buffer, 6 mL 2 M potassium chloride and 33 mL 1 M potassium hydroxide. ATP concentration in the neutralized samples was measured on a BioOrbit 1251 Luminometer with ATP Reagent SL 11-501-M (BioThema AB, Sweden). The assay was calibrated by a series of ATP standards (BioThema AB, Sweden) diluted in a mixture of perchloric acid and neutralization buffer equal to the sample matrix.

**Flow cytometry for Ig attached to sperm**

In the field, 50 µL fresh milt was transferred to 50 mL Nunc bottles for cell culture, containing 5 mL of growth medium (M-199, medium 199 containing hanks salts without bicarbonate, with streptomycin 100 µg/mL and penicillin 60 µg/mL, Sigma Chemicals, no. M5017). The bottles were kept in Ziploc bags and filled with pure oxygen and refrigerated at 4°C. Once a day the bottles were gently rotated and oxygen was replaced every second day. To quantify immunoglobulins attached to sperm cells, 26.01 – 418.06 µL (mean 88.19 µL, based on individual variation in spermatocrit, i.e., sperm cell density in ejaculate) of cell suspension were added to 200 µL with PBSA, shaken and centrifuged for 4 min at 10,000 rpm. The supernatant was then removed and 20 µL of rabbit-anti-charr-immunoglobulin was
added and incubated for 45 min at room temperature (21°C). A total of 200 µL of PBSA was then added and the sample was shaken and centrifuged for 4 min at 10,000 rpm.

Subsequently, the supernatant was removed and 20 µL of FITC conjugated swine-anti-canine-immunoglobulin (F0261 from DAKO, Glostrup, Denmark) was added. The fluorescence intensities obtained were then analyzed by FacsCalibur flow cytometer with CellQuest 3.3 software system (Becton-Dickinson, Mountain View, CA). A gate was set in a forward scatter/side scatter dotplot, containing the majority of platelets, and events from 5000 to 25,000 gated cells were acquired and analyzed.

DNA fragmentation

The sperm chromatin structure assay (SCSA) monitors the susceptibility of sperm chromatin DNA to acid-induced denaturation, as reflected in the % DFI (DNA fragmentation index), and has proven to be a sensitive tool when studying fertility in mammals (Evenson et al. 2000). However, the SCSA has not previously been used for analysis of sperm quality in charr. Thawed milt samples stored in 500 µL cryotubes were analyzed by a Coulter EPICS XL flow cytometer (Beckman Coulter Ltd, Luton, England), equipped with a 15 mW argon laser with excitation at 488 nm. Both light-scatter and fluorescence data were collected in linear mode. Green fluorescence was detected using a 505 – 545 nm BP filter (FL1) while red fluorescence was detected using a 660-900 nm BP filter (FL4). Discriminator as well as photo multiplier settings were found to be different from that of mammalian species both with regard to sperm morphology and chromatin structure. Thus, where necessary these settings were modified. For samples that showed low sperm concentration, the flow rate was increased from “low” to “medium” in order to analyse these samples within comparable time as samples with appropriate sperm concentrations. A cytogram of green versus red DNA fluorescence was used to identify sperm with denaturated DNA. A gate was set around the sperm with
increased red DNA fluorescence (denatured DNA) compared to the main population to
determine the percentages of sperm with single stranded DNA. This percentage was
calculated as percentage of the total sperm population, and is reported as the DFI.

**Statistical analyses**

As not all measurements were obtained for all individuals, sample sizes differ among
analyses. Variables that deviate from normal distribution were log-transformed. In order to
reduce the number of variables, we used three principal component (PC) analyses for the
variables indicative of (i) social status, (ii) sperm swimming speed, and (iii) parasite
intensities.

**(i) Social status**

Fish length, mass and age were highly correlated ($r = 0.7$ to $0.96$, $P < 0.0001$, $n = 92$ to 95),
whereas the associations between the three former variables and red intensity were significant,
but not as strong ($r = 0.3$ to $0.37$, $P < 0.003$, $n = 92$ to 95). Using the varimax approach to the
correlation matrix, we retained two PC’s (eigenvalues = 2.79 and 0.81). PC1, hereafter termed
“social status”, explained 71 % of the variation and was positively correlated with fish length,
body mass and age. PC2 explained 20 % of the variation and was positively correlated with
abdominal coloration (correlation = 0.95). Therefore, we termed PC2 “abdominal coloration”.

**(ii) Sperm swimming speed**

The three measured variables associated with sperm swimming performance (average path
velocity, straight-line velocity and curvilinear velocity; all measured 10 s after activation)
where combined in a separate PC (eigenvalue = 2.91). The resulting sperm swimming speed
PC1 accounted for 97% of the variance in the original data and was hereafter termed “sperm
swimming speed”.

**(iii) Parasite intensities**
The third PC analysis reduced the number of parasite variables from eight (see above) to four statistically independent axes that reflected different aspects of overall parasite intensity (eigenvalues = 2.71, 1.23, 1.03 and 0.88, cumulative percentages of variance explained = 0.34, 0.49, 0.62 and 0.73, respectively). Many of charr parasites either use amphipod or copepod intermediate hosts and the most correlated variables in parasite PC1 was the amphipod transmitted parasites *C. farionis, Crepidostomum* spp., *C. truncatus* (r = 0.7 to 0.84) and *P. umblae* (r = -0.62). The strongest associated parasite species to PC2 was that of the copepod transmitted parasites *Diphyllobothrium* spp. (r = 0.64), *E. salvelini* (r = 0.37) and *Salmincola* sp (r = -0.60), and that of the direct transmitted parasite *Diplostomum* spp. (r = 0.51). No clear pattern between transmission vehicle was apparent for PC3 and PC4.

Regression analysis

To evaluate the association between different ejaculate characteristics and social status, parasites and immunity, we used backwards stepwise multiple regressions (type III sum of squares). All eleven predictors (see Results) were included in the initial models (P to enter or leave = 0.1) and in order to check the validity, and to test the explanatory power of our models, we compared the final backward stepwise regression models with Akaike’s Information Criterion (AIC) (see Helu et al. 2000; Garvin et al. 2008). AIC is a statistical model selection criterion that accounts for model complexity. The two different methods produced concordant results (not shown). As stepwise procedures are known to suffer from a multiple-testing problem with greatly inflated Type I error rates (Mundry and Nunn 2009), we also conducted separate regression and correlation analysis (results not shown). Finally, all associations within the group of dependent variables and within the modulator variables (Fig. 1) were tested by simple regressions (in StatView for Windows 5.0.1.) and, if not otherwise
stated, results from these bivariate analyses are reported below. The main results are appropriately reported for later meta-analysis (Nakagawa and Cuthill 2007).

**Results**

**Red intensity of ornament**

In simple regression analyses, abdominal red intensity (from the Principal Component Analysis) did not correlate significantly with any of the seven sperm characteristics variables ($r = -0.12$ to $0.09$, $P = 0.27$ to $0.96$ and $n = 76$ to $95$), whereas the original red intensity measurements (the raw data) of skin coloration correlated negatively with sperm swimming speed ($r = -0.22$, $P = 0.036$, $n = 94$) and positively with sperm production ($r = 0.23$, $P = 0.027$, $n = 95$). None of the other sperm traits were significantly related to male skin coloration (the raw data, $r = -0.11$ to $-0.03$, $P = 0.277$ to $0.763$ and $n = 79$ to $95$).

**Parasites and sperm**

In addition to the results presented in Table 1, parasite PC1 (amphipods transmitted species) was, in simple regression analysis, positively related with milt volume ($r = 0.467$, $P < 0.0001$, $n = 93$) and testes mass ($r = 0.25$, $P = 0.015$, $n = 94$), but negatively with spermatocrit ($r = -0.24$, $P = 0.02$, $n = 94$). Parasite PC3 was, on the other hand, negatively related to milt volume ($r = -0.27$, $P = 0.008$, $n = 93$).

**Parasites and immunity**

Parasite PC1 was negatively related to Ig in blood ($r = -0.37$, $P < 0.001$, $n = 94$) and to Ig in seminal fluid ($r = -0.27$, $P < 0.046$, $n = 56$), but positively related to spleen mass ($r = 0.36$, $P$
Parasite PC4 was positively related to Ig in seminal fluid \( (r = 0.26, P < 0.048, n = 56) \). No other associations between the four parasite PCs and the immune variables were significant \( (r = -0.17 \text{ to } 0.2, P = 0.06 \text{ to } 0.9 \text{ and } n = 56 \text{ to } 94) \).

**Immune variables and sperm**

Ig in blood and Ig in seminal fluid were positively correlated \( (r = 0.39, P = 0.002, n = 57) \). Ig in seminal fluid was weakly, negatively associated with sperm production \( (P = 0.044) \) and testes mass \( (P = 0.054) \). Ig in blood was negatively related to both spleen mass \( (r = -0.40, P < 0.0001, n = 84) \) and sperm production \( (r = -0.20, P = 0.042, n = 95) \), whereas none of the immune variables was associated with the percentage of motile sperm cells \( (P = 0.08 \text{ to } 0.90) \). The amount of lymphocytes in blood, spleen mass and blood sugar did not correlate significantly with other variables than those shown in Table 1.

**DNA-fragmentation, social status, immunity and parasites**

The degree of DNA fragmentation in sperm cells was not significantly related to social status, parasite intensities, or to any of the immune variables \( (P = 0.168 \text{ to } 0.88; \text{ Table 1}) \). Moreover, there was no significant association between the amount of Ig attached to sperm cell surface and sperm swimming speed \( (r = 0.06, P = 0.58, n = 84) \), nor was the amount of Ig attached to sperm cells significantly related to any of the predictor variables \( (P = 0.097 \text{ to } 0.981) \).

**Correlations among primary sex traits**

Sperm swimming speed was positively related to the percentage of motile sperm cells in the ejaculate \( (r = 0.28, P = 0.007, n = 94) \) and as expected to the amount of ATP in sperm cells \( (r = 0.33, P < 0.001, n = 94) \). Moreover, sperm swimming speed was negatively related to testes mass \( (r = -0.33, P = 0.001, n = 94) \). Testes mass was in turn positively associated with sperm
production \((r = 0.42, P < 0.0001, n = 95)\), but negatively related to both the percentage of
motile cells \((r = -0.27, P = 0.007, n = 94)\) and the amount of ATP in sperm \((r = -0.21, P = 0.041, n = 95)\). Moreover, the relationship between testes mass and male social status, spleen
mass and abdominal coloration (see Table 1) are most likely allometric. Thus large males are
dominant, red and have large testes and spleens. The degree of DNA fragmentation was
negatively related to the percentage of motile sperm cells \((r = -0.22, P < 0.05, n = 78)\). Yet,
contradictory to our predictions, DNA fragmentation was not correlated with the amount of Ig
attached to sperm cells or to any of the other primary sex traits \((r = -0.16 \text{ to } +0.16, P = 0.112
\text{ to } 0.946 \text{ and } n = 71 \text{ to } 94)\).
Parasite intensities and male social status were the most significant correlates of ejaculate quality in charr. That is, in the multivariate model, male social status predicted sperm swimming speed and the amount of ATP in sperm cells, whereas sperm production was predicted by parasite intensities only. Moreover, individual levels of lymphocytes in the bloodstream predicted the amount of Ig attached to sperm cells, suggesting an immunological interaction of the male germ line.

Remarkably, all four principal components derived from the parasite intensities were related to sperm production, and parasites were also the only variables predicting sperm production. Parasites may reduce ejaculate quality (Skau and Folstad 2003) and reproductively active male charr isolated from natural spawning activity show a negative relationship between certain parasite intensities and sperm production (Måsvær et al. 2004). Although negative relationships were also found in the present study, the predominant relationships between parasite intensities and sperm production were positive. Such associations may result from parasite intensities tailoring host’s reproductive investment. The accumulated parasite level, which we measured to be higher among dominant males, is probably temporary – at least for some parasite species. A previous cross-sectional study from the same population of Arctic charr showed that the monthly establishment rate of the amphipod transmitted cestode *C. truncatus* to Arctic charr increased throughout summer and exhibited a peak in late autumn, which corresponded with the spawning period of the fish (Amundsen and Forsgren 2003). The establishment rate then decreased and was at a minimum in early summer. In our study, the parasite *C. truncatus* was strongly positive correlated with male social status (*r* = 0.70) and also negatively related to Ig levels (*r* = -0.32), suggesting that dominant males reduce their immune activity and tolerate the parasite during the spawning period, but remove them afterwards. Although this capacity for co-existence may
explain the positive relationship between sperm production and intensities of some parasite species, an alternative explanation seems at least as attractive. A recent experimental study of charr showed that antigen treated males prevented from spawning activity produced ejaculates with higher sperm density than sham injected males (Figenschou et al. 2012). This association seems not to result from terminal investment by hosts, as antigen treated males did not allocate more carotenoids to ornamental development (see below). Rather, it is likely that parasites may reduce a male’s ability to attain and maintain high social status and, in order to reproductively compensate for low status, they consequently prepare for sperm competition. Thus, parasites may shift host reproductive investment from social dominance towards investment in ejaculate quality, shown as high spermatocrit levels and high sperm velocity, typically characterising individuals of low social status. Additionally, high parasite intensities may reduce spawning opportunities and consequently cause a build-up in sperm density in our sample of reproductively active males. In sum, parasites seem to have a large impact on reproductive decisions in Arctic charr males.

Indices of social status were the strongest predictors of sperm velocity in the present study. Both social status and abdominal spawning coloration were negatively related to swimming speed of sperm, and the latter correspond to our previous finding that the most brightly coloured male in pair-wise sperm competitions also had the lowest fertilization probability (Liljedal et al. 2008). For external fertilizers both theoretical models and empirical observations suggest that swimming speed of sperm is of large importance for fertilization success (Ball and Parker 1996; Levitan 2000; Kime et al. 2001; Kupriyanova and Havenhand 2002; Burness et al. 2004; Burness et al. 2005; Schulte-Hostedde and Burness 2005; Figenschou et al. 2007), and the velocity of a charr’s sperm relative to the velocity of the sperm of competing males is found to be a good predictor of male fertilization success (Liljedal et al. 2008, Egeland 2012). Sperm velocity is also a plastic trait in charr and
experimental attainment of dominance can result in a rapid decrease of sperm swimming speed in previously subordinate males (Rudolfsen et al. 2006). Ejaculates of subordinates also show high sperm speed (and numbers) compared to ejaculates of dominants (Rudolfsen et al. 2005) and may fully compensate for the average 0.68 s delay in spawning synchrony experienced by subordinates in our studied population (Sørum et al. 2011, Egeland et al. 2012). Additionally, the difference in sperm velocity between dominant and subordinate charr is most predominant among the fastest sperm cells (Serrano et al. 2006; Haugland et al. 2009), which are those cells most likely to fertilize eggs. The proximate explanation for the negative association between social status and sperm swimming speed in the present study may be related to differences in ATP levels as suggested by (i) the positive relationship between sperm speed and ATP levels, and (ii) the negative relationship between social status and ATP levels. Thus, ATP may be traded-off differently between dominant and subordinate males during the one-month long spawning season. This explanation, which relies heavily on energy being a limited resource for reproductively active males, fit the recent suggestion that the seemingly sexually selected adipose fin in salmonids (Järvi 1990; Fleming and Gross 1994) may have evolved as a signal of energy stores (Haugland et al. 2012) and be indicative of sperm swimming speed (Egeland 2012). Immunological responses do not seem to influence swimming speed of sperm as there was no significant relationship between sperm velocity and the amount of immunoglobulins on sperm surfaces. Yet, the effect of immunoglobulins on sperm surfaces may, as reported in internal fertilizers, be more prominent during interactions with female fluids (Ayvaliotis et al. 1985; Bronson 2000) and strong male-female interaction effects on swimming speed of sperm in ovarian fluid have been also documented in charr (Urbach et al. 2005).

Defects in chromatin and DNA structure are important parameters for assessing sperm quality and could be indicative of germ-line mutation rates and male fertility (Hendrich and
Bickmore 2001). Although sperm of colourful male birds have been shown to be better protected against oxidative stress (Helfenstein et al. 2010), we found no relationship between abdominal coloration in charr and DNA-fragmentation in sperm cells. Thus, we have no support for the hypothesis that ornamental expression reflects sperm mutation rate. Likewise, we found little support for an immunological influence on sperm quality as few of our predictors (i.e., parasites and immunological variables) were related to the amount of Ig attached to sperm cells. Still, the positive relationship between Ig attached to sperm cells and lymphocytes known to be involved in antibody production correspond with the contention that increased immunological activity may increase Ig-labelling of sperm (Folstad and Skarstein 1997; Skau and Folstad 2005). Yet, the increased Ig-labelling does not translate into reduced sperm swimming speed of sperm in water.

**Conclusion**

Although we found no support for carotenoid-based signalling of sperm quality, the observed associations between parasite intensities and sperm production in charr are remarkable. The mechanisms underlying these associations are still unclear, and we find little support for an adaptive immunological regulation of sperm traits. Rather the presence of parasites seems to stimulate sperm production similar to what is observed in males entering subordinate reproductive roles (Figenschou et al. 2012). Thus, if genetic resistance towards parasites is influencing parasite intensities, genetic resistance may also be important for the “choice” of reproductive role and, in turn, allocation of resources to primary sex trait development in charr.
Acknowledgements

We acknowledge Shinichi Nakagawa, Anders Pape Møller, Iain Barber, Bård Gunnar Stokke and two anonymous referees for their valuable comments on the manuscript, Sissel Kaino for accurate work and good humour and Goran Kauric for his very pedagogical “Flowcytometry-course” for dummies.
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Table 1

Results from a backward stepwise multiple regression model showing how social status, parasites and immunity predict seven primary sex traits of Arctic charr. All eleven predictors were included in the initial models. Variables included in the final model are indicated by their t and P values. Significant P values are in bold.

Figure 1

The flow-chart describing assumed pathway interactions and measured variables in our model organism, the Arctic charr. Although parasites and immunity are illustrated as effect modulators (solid lines), we cannot rule out that they alternatively may function as confounding variables (dotted lines). In sum, our findings suggest that parasites affect social status, which, in turn, affect sperm traits.
<table>
<thead>
<tr>
<th>Predictors</th>
<th>Testes mass</th>
<th>Sperm production</th>
<th>Sperm swimming speed</th>
<th>ATP in sperm</th>
<th>mtDNA translocation</th>
<th>Ig on sperm</th>
<th>DNA fragmentation</th>
<th>ATP in sperm</th>
<th>Ig on sperm</th>
<th>DNA fragmentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjusted R²</td>
<td>0.78</td>
<td>0.78</td>
<td>0.78</td>
<td>0.78</td>
<td>0.78</td>
<td>0.78</td>
<td>0.78</td>
<td>0.78</td>
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<td>0.78</td>
</tr>
<tr>
<td>r</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Social status</td>
<td>10.27</td>
<td>&lt; 0.0001</td>
<td>-4.50</td>
<td>&lt; 0.0001</td>
<td>-0.96</td>
<td>0.3406</td>
<td>-2.06</td>
<td>0.00</td>
<td>0.00</td>
<td>-2.06</td>
</tr>
<tr>
<td>Abdominal coloration</td>
<td>4.37</td>
<td>&lt; 0.0001</td>
<td>-1.85</td>
<td>0.0694</td>
<td>0.96</td>
<td>0.30</td>
<td>-4.33</td>
<td>0.0888</td>
<td>0.00</td>
<td>-4.33</td>
</tr>
<tr>
<td>Parasite PC1</td>
<td>-1.72</td>
<td>0.0888</td>
<td>-2.06</td>
<td>0.03</td>
<td>0.96</td>
<td>0.30</td>
<td>-4.33</td>
<td>0.0888</td>
<td>0.00</td>
<td>-4.33</td>
</tr>
<tr>
<td>Parasite PC2</td>
<td>6.01</td>
<td>&lt; 0.0001</td>
<td>4.21</td>
<td>&lt; 0.0001</td>
<td>-2.06</td>
<td>0.00</td>
<td>-4.33</td>
<td>0.0888</td>
<td>0.00</td>
<td>-4.33</td>
</tr>
<tr>
<td>Parasite PC3</td>
<td>-2.78</td>
<td>0.0067</td>
<td>-4.33</td>
<td>&lt; 0.0001</td>
<td>-2.06</td>
<td>0.00</td>
<td>-4.33</td>
<td>0.0888</td>
<td>0.00</td>
<td>-4.33</td>
</tr>
<tr>
<td>Parasite PC4</td>
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<td>&lt; 0.0001</td>
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<td>0.03</td>
<td>0.96</td>
<td>0.30</td>
<td>-4.33</td>
<td>0.0888</td>
<td>0.00</td>
<td>-4.33</td>
</tr>
</tbody>
</table>

Note: All p-values are significant at the 0.05 level.