Secondary sex traits, parasites, immunity and ejaculate quality in the Arctic charr

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Ejaculate quality may limit male reproductive success. Sperm cells are immunologically perceived as non-self in the male reproductive tract and may therefore be attacked by the immune system. Males may consequently have to suppress their immune system in order to produce high-quality ejaculates. This suppression may be influenced by the current level of parasite infections, suggesting that only parasite-resistant males are able to produce high-quality ejaculates. In a study of naturally infected male Arctic charr sampled during their spawning period, we found that the density of circulating granulocytes, spleen mass and the intensity of infection by one nematode species located outside the testes were negatively associated with ejaculate quality. This suggests that a male's extra-testicular immune environment may affect the production of high-quality ejaculates and that parasite infections located in the extra-testicular soma may influence ejaculate quality, a trait most likely under directional selection. Moreover, male fertilization potential was negatively correlated with their red spawning coloration. In conclusion, these results emphasize the importance of parasites and immunity as factors generating variability in sperm quality, suggesting that parasite resistance may be of importance for maintaining variance in reproductive success even after copulation.

Keywords: sperm; granulocytes; parasite resistance; immunosuppression; secondary sex traits; Arctic charr

1. INTRODUCTION

If females are at risk of not having their eggs fertilized because some males have low sperm quality, selection should favour certain reproductive strategies among females. First, females should avoid mating with males that have ejaculates of poor quality (Nakatsuru & Kramer 1982). Second, females should allow several males to fertilize their eggs by initiating sperm competition. Sperm competition exists in a variety of species and it seems to increase the males' investment in sperm production (Smith 1984; Ginsberg & Huck 1989; Birkhead & Møller 1989; Stockley *et al.* 1997). That is, males from species with sperm competition have larger testes and release more sperm per ejaculate relative to males in species with low risk of sperm competition (Møller 1989; Møller & Briskie 1995; Stockley *et al.* 1997). Although the presence of sperm competition may be independent of the need of females to ensure fertilization, it is observed that females getting their eggs fertilized under sperm competition achieve a higher fertilization success and higher offspring survival relative to conspecific females with eggs fertilized by a single male (Shapiro *et al.* 1994; Keil & Sachser 1998). Consequently, female choice, sperm competition or a combination of these two mechanisms should favour males with high-quality ejaculates.

Females are known to select the most ornamented males (Andersson 1994), and it has recently been suggested

that when mating with the most ornamented male, a female may acquire both a high probability of fertilization and heritable parasite resistance for her offspring (Folstad & Skarstein 1995, 1997; Hillgarth *et al.* 1997). According to this scenario, males must undergo immune suppression during sperm production because sperm cells are non-self to the diploid organism, and thus they are targets of immunological attack in the male reproductive tract (Friberg 1982; Hogarth 1982; Roitt *et al.* 1993). Ejaculate quality will therefore be affected by a male's ability to downregulate his immune responses, a process potentially regulated by immunosuppressive androgens (Wyle & Kent 1977; Grossman 1984; Grossman & Roselle 1986; Folstad & Karter 1992). Androgens are known to stimulate sperm production and the development of secondary sex traits, but simultaneously suppress the immune system. This interaction between the immune system and ejaculate quality suggests an important role for infectious organisms, since a male's potential for immunosuppression may be restricted by his current level of infections (Folstad & Skarstein 1995, 1997; Hillgarth *et al.* 1997). According to this model, testes and the remaining soma have different optima for immunological activity, which are balanced according to individual time-specific needs. Consequently, a heavily infected male may be unable to bear the costs of elevated androgen level during sperm production because concomitant immunosuppression could seriously lower his future reproductive success. Males which are unable to increase their androgen level during sperm production will therefore have low ejaculate quality.

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The Arctic charr (*Salvelinus alpinus*) is well suited for studies of both pre- and post-copulatory mechanisms. Before the spawning period both sexes develop a carotenoid-based spawning coloration (Scalia *et al.* 1989), which is sexually dichromatic, males being redder than females (Skarstein & Folstad 1996). The ornament seems to incur a cost to immunity, as a negative association between lymphocyte density and spawning coloration has been documented (Skarstein & Folstad 1996). Moreover, development of red spawning coloration may lead to increased parasite exposure, since carotenoids needed for ornamental development are acquired by consumption of small crustaceans, which in turn are intermediate hosts for common parasites (Folstad *et al.* 1994; Skarstein & Folstad 1996). Both males and females spawn with several different mates during the spawning period, but generally males have a larger variance in the number of mates (Fabricius & Gustafson 1954). Sperm competition in Arctic charr is documented, where large males guard their mates against smaller, sneaky breeding, males (Sigurjónsdóttir & Gunnarsson 1989). As females receive nothing but sperm during spawning (Fabricius 1953), a choosy female may thus be selecting high fertilization probability, 'good genes' for her offspring or a combination of these two benefits. Additionally, according to the models outlined by Folstad & Skarstein (1995, 1997) and Hillgarth *et al.* (1997), female charr may also obtain 'good genes' and high fertilization probability by allowing sperm competition.

Given the above scenario we predict ejaculate quality in male Arctic charr to be negatively associated with intensities of parasite infections and immune activity. Spawning coloration should, on the other hand, be positively associated with ejaculate quality. These predictions were examined in an observational study of reproductively active male charr.

2. MATERIAL AND METHODS

(a) *Fish sampling*

During 21–29 September 1995 we caught 46 sexually mature male Arctic charr in lake Fjellfrøsvatn, northern Norway. The sampling occurred at night-time on four different spawning grounds which were identified by the presence of sexually mature and mating Arctic charr. The spawning grounds were no more than 500 m apart. The point of time of each catch was recorded, and time intervals, in hours, between catches were calculated (hereafter referred to as sampling time), in order to detect changes in physiological variables during the spawning season. During sampling the fish never remained in the gill nets for more than three hours, a capture period previously shown not to cause any changes in ornamental coloration or blood parameters (Skarstein & Folstad 1996). After sampling, we transported the fish in water-filled buckets to the field laboratory where we collected samples of semen and blood. Thereafter each fish was killed, photographed and frozen.

(b) *Sperm*

To prevent spilling semen during fish sampling we took special care not to apply pressure to the fish's abdomen. Additionally, before the collection of semen we dried the area around the genital pore to prevent water from diluting the semen and activating sperm cells. We then squeezed the semen from the

seminal tract by gently pressing the abdomen. All sperm sampling was done by one person.

To evaluate ejaculate quality we measured spermatocrit and sperm motility for each fish. By centrifuging a homogenized proportion of the semen in a capillary tube for three minutes at 11 500 rpm, with a Compur mini centrifuge, we could estimate spermatocrit, which is the percentage of a given volume of semen that is occupied by cells. We obtained motility measures by video recording activated sperm cells of 25 males through a microscope at $\times 100$ magnification. The percentage of motile sperm cells were later estimated from the videotapes. Both spermatocrit and sperm motility are repeatable measures ($r^2 = 0.83$, $n = 16$, $p < 0.0001$, and $r^2 = 0.42$, $n = 10$, $p < 0.05$, respectively). Spermatocrit is known to be a reliable measure of sperm density in the seminal fluid in salmonids (Bouck & Jacobson 1976; Aas *et al.* 1991), and both sperm density and sperm motility are widely used as measures of ejaculate quality (Gibson & Jewell 1982; Aas *et al.* 1991; Møller 1991; Billard 1992; Warner *et al.* 1995; Marconato & Shapiro 1996). Additionally, spermatocrit has been shown to correlate positively with fertilizing success in another salmonid, the *Salmo salar* (Aas *et al.* 1991).

(c) *Blood*

In order to estimate the density of circulating lymphocytes and granulocytes we made blood smears from blood samples collected from the caudal vessel of each fish. Granulocytes, which are an important part of the non-specific immune system, attack foreign cells by phagocytic activity or enzymes released during lysis (Iwama & Nakanishi 1996). Lymphocytes, on the other hand, are a part of the specific immune system involved in antibody production (Iwama & Nakanishi 1996). From the blood smears we calculated the lymphocyte:erythrocyte and granulocyte:erythrocyte ratios with a repeatability of $r^2 = 0.73$, $p < 0.0001$, and $r^2 = 0.82$, $p < 0.0001$, respectively ($n = 125$ in both cases). Furthermore, we estimated haematocrit, which is the percentage of red blood cells in a given volume of blood, by centrifuging a proportion of the blood sample in a capillary tube with a compur mini centrifuge for three minutes at 11 500 rpm. Densities of lymphocytes and granulocytes in the blood sample were estimated by multiplying haematocrit by the lymphocyte:erythrocyte and granulocyte:erythrocyte ratios, respectively (Skarstein & Folstad 1996).

(d) *Ornamentation*

To estimate the intensity of red coloration in the individual spawning ornamentation, we photographed each fish's abdomen under standardized light conditions. These pictures were later analysed using Adobe Photoshop (Macintosh 2.5) to produce numerical estimates for three colour properties: hue, saturation and brightness, with repeatabilities of $r^2 = 0.95$, $p < 0.0001$, $r^2 = 0.89$, $p < 0.0001$, and $r^2 = 0.90$, $p < 0.0001$, respectively ($n = 71$ in each case). Both photography and picture analyses were done according to Skarstein & Folstad (1996). We examined the three colour properties separately, because they may be of independent importance in signalling individual quality (Wedekind 1992; Skarstein & Folstad 1996). An individual was considered more ornamented if its colour had a redder hue, was brighter and more saturated (Skarstein & Folstad 1996).

(e) *Dissection and parasites*

In the laboratory we measured body length and whole (non-gutted) mass for each fish. Thereafter, we dissected the spleen and testes and measured the dried mass of both organs. The

Table 1. Prevalence, mean intensity (mean) and standard deviation (s.d.) of the different parasite groups from reproductively active male Arctic charr

variable	prevalence (%)	mean	s.d.
<i>Salmincola</i> spp.	33	0.71	1.56
<i>Cystidicola farionis</i>	96	102.00	128.38
<i>Phyllidostomum umblae</i>	93	83.79	63.79
<i>Diphyllbothrium</i> spp.	100	24.92	35.16
<i>Cyathocephalus truncatus</i>	29	2.18	5.41
<i>Eubothrium salvelini</i>	96	5.10	4.39
<i>Diplostomum</i> spp.	100	117.90	57.13
<i>Crepidostomum</i> spp.	98	117.67	206.79

spleen is believed to be the major antibody-producing organ in teleost fish (Manning 1994) and has a homogenous morphology, with no clear distinction between splenic red pulp and white pulp as in mammals. Lymphocyte proliferation occurs in the ellipsoids scattered about in the entire splenic volume (Manning 1994), and a spleen with high mass can thus be interpreted as providing high immunological activity. Fish age was estimated by counting the number of growth zones on the otoliths under a stereomicroscope (repeatability: $r^2 = 0.89$, $n = 10$, $p < 0.001$). We recorded macroparasites by investigating the eyes, the gills, the outside and inside of the gastrointestinal tract, the swim bladder and the kidney tubules. Eight parasite groups were identified: the digeneans *Phyllidostomum umblae*; *Diplostomum* spp.; *Crepidostomum* spp.; the cestodes *Diphyllbothrium* spp.; *Eubothrium salvelini*; *Cyathocephalus truncatus*; the nematode *Cystidicola farionis*; and the crustacean *Salmincola* sp. Prevalence and mean intensity are given in table 1.

(f) Statistical analyses

To investigate the potential confounding effects of spawning ground and age on our dependant variables (i.e. sperm and immunological variables), we first used mixed models ANOVA, entering spawning ground as the randomized factor and age as the fixed factor, together with fish mass, testes mass and sampling time as covariates. Neither spawning ground nor age significantly improved any of the models. We consequently removed both variables from the analyses and used multiple regression models. The independent variables, and the above mentioned covariates, were entered in accordance with the forward selection procedure (Kleinbaum *et al.* 1988). As fish mass and length were highly correlated ($r^2 = 0.87$, $n = 46$, $p < 0.0001$), we only used fish mass in the analyses. Distributions were tested using Kolmogorov–Smirnov one-sample tests. To meet the assumption of normality we log-transformed saturation and testes size, and square-root-transformed spleen mass. Additionally, following Conover & Iman (1981), all parasite intensities were rank-transformed. Outliers were removed following Schlotzhauer & Littell (1991). We carried out all statistical analyses using SAS software (PROC MIXED, Littell *et al.* 1996) and Statsoft Statistica for Macintosh (version 3.0). As we did not obtain a measure of all parameters from all individuals, sample size varied between analyses.

3. RESULTS

(a) Sperm versus colour

In order to test whether hue, saturation and brightness significantly explained variance in spermatocrit we

Figure 1. Relationship between spermatocrit and granulocyte density in somatic blood in reproductively active male Arctic charr. The line is the linear regression line with the equation $y = -2.790x + 13.683$.

entered these three variables, as well as sampling time, testes mass and fish mass, in the multiple regression model. Sampling time (partial $r = -0.49$, $n = 46$, $p < 0.001$), saturation (partial $r = -0.35$, $n = 46$, $p < 0.01$), testes mass (partial $r = 0.37$, $n = 46$, $p < 0.01$), and fish mass (partial $r = -0.30$, $n = 46$, $p < 0.01$) explained a significant proportion of the variance in spermatocrit. The same procedure was used to test if any of the three colour properties significantly explained any variance in sperm motility. Only sampling time entered this model (partial $r = -0.47$, $n = 25$, $p < 0.05$). Thus, spawning coloration could explain some of the variance in spermatocrit but not in sperm motility. However, the sample size in the latter analysis is small.

(b) Colour versus immunity

To test if spawning coloration could explain the variance in immune variables, we first entered spleen mass as the dependent variable. Spleen mass was only related to saturation (partial $r = 0.41$, $n = 46$, $p < 0.01$). On the other hand, lymphocyte density was related to fish mass (partial $r = -0.32$, $n = 46$, $p < 0.05$) and hue (partial $r = 0.33$, $n = 46$, $p < 0.05$, note that a smaller value for hue means redder fish). Thus, immune activity correlates both positively (spleen mass) and negatively (lymphocyte density) with spawning coloration.

(c) Sperm versus immunity

Granulocyte density (partial $r = -0.55$, $n = 46$, $p < 0.001$, figure 1), spleen mass (partial $r = -0.24$, $n = 46$, $p < 0.05$), testes mass (partial $r = 0.28$, $n = 46$, $p < 0.05$), fish mass (partial $r = 0.37$, $n = 46$, $p < 0.001$) and sampling time (partial $r = -0.20$, $n = 46$, $p < 0.05$) all explained a significant proportion of the variance in spermatocrit. On the other hand, sperm motility was only related to granulocyte density (partial $r = -0.52$, $n = 25$, $p < 0.01$, figure 2).

Figure 2. Relationship between percentage of motile sperm cells and granulocyte density in somatic blood in reproductively male Arctic charr. The line is the linear regression line with the equation $y = -8.777x + 88.586$.

(d) *Sperm versus parasites*

The infection intensities of eight parasites were tested for their associations with spermatocrit. Sampling time (partial $r = -0.55$, $n = 45$, $p < 0.001$) and the intensity of the nematode *Cystidicola farionis* (partial $r = -0.28$, $n = 45$, $p < 0.05$) explained a significant proportion of the variance in spermatocrit. Additionally, intensities of the cestodes *Diphyllobothrium* spp. correlated negatively with spermatocrit, although not significantly so (partial $r = -0.22$, $n = 45$, $p = 0.06$).

(e) *Parasites versus immunity*

The suggested model (Folstad & Skarstein 1995, 1997; Hillgarth *et al.* 1997) predicts that parasite intensity should negatively affect ejaculate quality by activating the immune system. We therefore examined whether parasite intensities were related to immunity variables. Variation in granulocyte density was positively related to sampling time (partial $r = 0.51$, $n = 43$, $p < 0.001$) and the intensity of *Cystidicola farionis* (partial $r = 0.42$, $n = 43$, $p < 0.001$). Variation in spleen mass was positively related to fish mass (partial $r = 0.52$, $n = 43$, $p = 0.001$) and the density of *Cystidicola farionis* (partial $r = 0.24$, $n = 43$, $p = 0.06$).

4. DISCUSSION

Spermatocrit was negatively associated with granulocyte density, spleen mass, and the intensity of *C. farionis* in reproductively active male charr. Additionally, sperm motility was negatively associated with granulocyte density. These observations are in accordance with the prediction that immune activity and infectious organisms may negatively affect ejaculate quality. However, con-

trary to our prediction, the saturation of the spawning coloration was negatively associated with spermatocrit.

Given the complexity of the immune system, measuring only three variables, i.e. spleen mass, lymphocyte density and granulocyte density, makes this study a limited test of the prediction that immune activity should negatively affect sperm quality. However, two of the three immunological variables measured, granulocyte density and spleen mass, were negatively related to ejaculate quality. Granulocytes may be of particular importance, as they are the most commonly observed white blood cells in the seminal fluid. High granulocyte counts in semen have been associated with low ejaculate quality in other vertebrates (Berger *et al.* 1982; Wolff & Anderson 1988; Wolff *et al.* 1990; Eggert-Kruse *et al.* 1992; Chan *et al.* 1994; Wolff 1995; Yanushpolsky *et al.* 1996). Our measurements of granulocyte density are, however, not from seminal fluid, but from somatic blood, and the negative association between granulocyte density outside the testes and spermatocrit suggests that granulocytes in somatic blood have access to sperm storage areas. This is likely since the blood–testes barrier is penetrable for white blood cells (Maddocks & Setchell 1990; Turek & Lipshultz 1994) and sperm may, additionally, not remain behind the blood–testes barrier during their whole life-span within the male (Wolff 1995). Thus, at some point, sperm may be exposed to the extra-testicular immune environment. In conclusion, our results suggest that high immunological activity outside the testes may have negative effects on the production of high-quality ejaculates. Males which can suppress their extra-testicular immune activity during the spawning period, may thus gain fitness benefits.

It is noteworthy that only one out of eight parasites were associated with spermatocrit. However, this particular parasite was also the only parasite associated with our evaluated immune variables. That is, males with a high intensity of *C. farionis* had large spleens and a high granulocyte density, and the high immune activity in these males may explain the negative association between *C. farionis* and ejaculate quality. Parasite infections may alternatively reduce ejaculate quality by reducing the amount of resources available for sperm production, and both these trade-off mechanisms may be mediated by hormones such as androgens. As parasite infections are known to negatively influence androgen levels (Isserhoff *et al.* 1986; Folstad & Karter 1992), they may interfere with the allocation of resources to sperm production. Independently of which of these two proximate mechanisms might explain our results, both indicate that parasites may be important for maintaining variation in ejaculate quality, a trait most likely under directional selection. As rapidly co-evolving infectious organisms may be particularly good candidates for maintaining heritable variance in the ability of individuals to develop sexually selected traits (Hamilton & Zuk 1982), they may also be important in maintaining heritable variation in a male's ability to produce high-quality ejaculates.

The relationship between sexual ornamentation and sperm quality may be influenced by immunity (Folstad & Skarstein 1995, 1997; Hillgarth *et al.* 1997). Individual charr with saturated spawning coloration had low spermatocrit. This is contrary to our prediction that both

spawning coloration and ejaculate quality should be positively correlated with extra-testicular immune suppression. Immune variables were related to both spawning coloration and ejaculate quality. For example, a male with a large spleen has a saturated spawning coloration and also a low spermatozoa value. Although not all of these relationships are in the predicted directions, we cannot exclude the importance of sperm-immune system interactions on ornamental development. Ejaculate quality in fish is indeed also influenced by individual reproductive behaviour, for example, time since last ejaculation and frequency of previous ejaculations (Nakatsuru & Kramer 1982; Büyükhatipoglu & Holtz 1984). Consequently, the negative relationship between spawning saturation and spermatozoa may result from saturated males being more attractive and thus ejaculating more often than the less saturated males. However, these first observations on how sexual ornamentation and sperm quality may be influenced by immunity clearly warrant more exact studies.

In conclusion, granulocyte density, spleen mass and intensity of *C. farionis* were negatively correlated with ejaculate quality. Consequently, parasite-resistant males may experience lower costs in the production of high-quality ejaculates relative to less resistant males, and parasite infections may thus be an important source for maintaining variance in ejaculate quality among males. Contrary to our prediction, spermatozoa was negatively related to the expression of secondary sex traits, suggesting that female Arctic charr mating with redder males have a lower fertilization probability. In summary, our results emphasize the importance of parasites and immunity as factors generating variability in sperm quality, and suggest that parasite resistance may even be of importance for maintaining variance in reproductive success taking place after copulation.

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