Spawning coloration and sperm quality in a large lake population of Arctic charr (Salmonidae: *Salvelinus alpinus* L.)

Running headline: Coloration and sperm quality in Arctic charr

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Abstract

The modern theories of sexual selection predict that male sexual ornaments may have evolved as reliable signals of male fertilization efficiency. However, among the studies of fishes with external fertilisation, the results have yielded ambiguous evidence. Here, we present data on the phenotypic relationships between red spawning coloration and ejaculate quality (spermatocrit, sperm motility) from Arctic charr, *Salvelinus alpinus*. We studied two generations (F_1 and F_2) of males from a large lake population, reared in a standardized hatchery environment, to see whether differential hatchery history, or duration of hatchery selection, affected the variation in ejaculate characteristics or abdominal coloration. After controlling for body length, there was no difference between the hatchery generations in these traits. However, the degree of redness increased with fish size. We found a positive correlation between sperm velocity and sperm longevity, indicating a functional integration between these sperm features across generations. Sperm velocity was also positively correlated with male redness. Therefore, our finding suggests that the carotenoid-based ornamentation in Arctic charr may provide information about differences between males in their fertilization potential.

ADDITIONAL KEYWORDS: ornamentation – ejaculate quality – sexual selection – sperm competition – teleost

INTRODUCTION

Conspicuous male ornaments during reproduction are commonly found in various animal species. Especially in species where males offer females gametes but neither resources nor parental care, indirect genetic benefits to the offspring (i.e., good genes or compatible genes; Mayrs & Hill, 2004; Neff & Pitcher, 2005) can form a major selective force in the evolution of female mate choice (Andersson, 1994; Møller & Alatalo, 1999; Kokko *et al.*, 2003). However, females could also directly benefit from choosing more elaborately ornamented males if they thereby improve the changes of mating with more fertile males. This assumption is the background for the phenotype-linked fertility hypothesis, which states that the male display of sexual ornaments reliably advertises male fertility via condition dependency of the ornaments and the sperm (Sheldon, 1994).

Increasing attention has been recently devoted to the idea of a phenotypic relationship between sexually selected characters and ejaculate quality (e.g., Pizzari, Jensen & Cornwallis, 2004; Malo *et al.*, 2005; Parker *et al.*, 2006; Rogers *et al.*, 2008), but the studies have yielded contradictory results. Among fishes, there is ambiguous evidence for a linkage between the expression of sexual ornaments and various sperm quality indices. Positive correlations have been found in some of the studies (Måsvær, Liljedal & Folstad, 2004; Kortet *et al.*, 2004; Locatello *et al.*, 2006; Pitcher, Rodd & Rowe, 2007), whereas others have found no or even negative associations (Liljedal *et al.*, 1999; Skinner & Watt, 2007; Liljedal, Rudolfsen & Folstad, 2008). Moreover, there can be a significant intra-specific variation with respect to which components of the male's phenotype potentially predict insemination success (Pitcher & Evans, 2001). These findings may partly result from the fact that such phenotypic relationships have often been tested in populations where varying environmental effects may obscure the underlying genetic associations between male ornaments and fertilization efficiency.

The prevalence of carotenoid-based ornaments, particularly in the versatile signaling of fishes and birds, has made them an ideal study subject in the context of sexual selection. Carotenoids usually appear as red-yellows, and these integumentary colours in feathers and skin are generally assumed to serve as reliable signals of the bearer's health, vigour and genetic quality (Olson & Owens, 1998; Møller *et al.*, 2000). Since primary and secondary sex traits are promoted by the same sex hormones, androgens (Folstad & Skarstein, 1997; Hillgarth, Ramenofsky & Wingfield, 1997), the expression of carotenoid-derived colour signals may be directly associated with the males' capacity for sperm production. Furthermore, the allocation of carotenoids for ornamental purposes might reflect low immune activity within the body and further the reduced exposure of 'non-self' sperm cells to an autoimmune attack (see Folstad & Skarstein, 1997; Liljedal, Folstad & Skarstein, 1999). Another explanation rests on the role of carotenoids as antioxidants, i.e., functioning to inactivate free radicals, which have a deleterious effect on both sperm quality and the substrates responsible for male ornamentation (von Schantz *et al.*, 1999; Blount, Møller & Houston, 2001). However, it has been recently suggested that carotenoid-based sexual traits may rather signal the availability of non-pigmentary antioxidants (Bertrand, Faivre & Sorci, 2006; Pike *et al.*, 2007; Pérez, Lores & Velando, 2008).Either way, intense carotenoid-dependent ornamental traits may imply high body supplies of antioxidants, which have a potential to reduce the susceptibility of sperm to oxidative stress and thus increase fertility (Greco *et al.*, 2005 and references therein).

The Arctic charr (*Salvelinus alpinus* L.) is a highly suitable model species for studying the associations between secondary sexual ornaments and sperm traits (e.g., Måsvær *et al.*, 2004). Charr males fertilize the eggs externally, and neither males nor females provide parental care to offspring after spawning (Sigurjónsdóttir & Gunnarson, 1989). Before and during the

breeding season, mature males form hierarchical groups and develop a red abdominal ornamentation that is correlated with parasite intensities and immune activity (Skarstein & Folstad, 1996; Liljedal *et al.*, 1999; Skarstein, Folstad & Liljedal, 2001). Previous studies from wild-caught charr have revealed that the degree of carotenoid coloration may be related to male fertility traits as well. Måsvær *et al.* (2004) found that redder males had higher sperm production (i.e., testes mass, milt mass and sperm cell numbers produced) than paler males (but see Liljedal *et al.*, 1999). Yet a recent insemination experiment demonstrated that more colourful male charr have lower parentage success than their less conspicuous rivals when ejaculates with equal sperm numbers compete (Liljedal *et al.*, 2008). This suggests that the lower competitive ability of the ejaculates from more intensively coloured males was due to sperm traits other than sperm numbers.

In this study, we examined whether the two important phenotypic traits, red spawning coloration and body size, are correlated with appropriate measures of male reproductive quality (or primary sexual traits) in a large lake Arctic charr (Lake Inari, Finland) population, reared in a standard environment (fish hatchery). Both the first and the second hatchery generations of wild-collected fish were used to examine the alternative hypothesis that differential hatchery history had an effect on these traits.

MATERIALS AND METHODS

Study fish and their sampling

The experiment was carried out in mid-October 2007 at the facilities of Sarmijärvi Aquaculture station (Finnish Game and Fisheries Research Institute), in north-eastern Finland. The charr were descended from the nearby Lake Inari population and represented the first (F_1) and second (F_2) hatchery generations (year classes 2002 and 2001, respectively), both of which had been produced by pairwise fertilizations (F_1: n = 300 pairs; F_2: n = 76 pairs).

Further, the F_2 group had been produced by specifically avoiding matings between close relatives. The fish were reared similarly in oblong outdoor tanks (area 200 m^2, water volume 200 m^3) and fed continuously (*ad libitum*) with carotenoid-rich salmonid food (Rehuraisio Emo-Vital®; astaxanthin content 80 mg kg^{-1}). The temperature of the inflow water as well as the lightning conditions followed natural rhythm.

Prior to measurements, the fish were fasted for more than three weeks. Twenty randomly chosen mature males from both generations were stripped for all available milt and placed into discrete group-specific tanks for five days to prohibit spawning activity during the replenishment of their sperm reserves. The anaesthetized fish (MS-222) were measured for total length (L_T) to the nearest mm and photographed on a grey background under standardized light conditions for later analysis. Thereafter, each fish was carefully dried around the genital pore to avoid sample contamination and the produced milt was collected in individual Petri dishes by pressing the abdomen towards the vent.

Sperm quality measurements

Sperm quality parameters were quantified immediately following stripping of males. Spermatocrit, which is defined as the percentage of a given volume of semen that is occupied by cells, was measured by centrifuging a homogenized proportion of the milt in a capillary tube for three minutes at 11 500 rpm with a mini-centrifuge (Compur-electronic Gmbh, Munich, Germany). Computer-assisted sperm analysis was employed to estimate variation in sperm velocity (see Rudolfsen *et al.*, 2006 for a more detailed description about the method). Briefly, sperm activity was initially video-recorded for 40 seconds after activation, that is, the precise moment that the subsample of pure milt was exposed to 4,5 µl of water on a cooled (c. 5 ºC) microscope slide (Leja Products BV, Nieuw-Vennep, The Netherlands). Recordings were made using a CCD B/W video camera (Sony XC-ST50CE PAL, Tokyo, Japan) attached

to a negative phase-contrast microscope (Olympus CH30, Tokyo, Japan) with a 10× magnification objective. Video recordings were later analysed using the HTM-CEROS sperm tracker software (CEROS v.12, Hamilton Thorne Research, Beverly, MA, USA). The parameters measured were: average path velocity (VAP), straight line velocity (VSL) and curvilinear velocity (VCL) (Rurangwa *et al.*, 2004). The velocity estimates were based on the mean velocity of all motile cells (i.e., those exceeding the pre-determined threshold values VAP > 10 μm s^{-1} and VSL > 20 μm s^{-1}) recorded at 10, 20, 30 and 40 s following activation. The percentage of motile cells 40 s after activation was used as an estimate of sperm longevity. For statistical analyses, the average over two replicates within each male was used for each motility measure. For four individuals, sperm swimming speed could not be reliably quantified and consequently they were excluded from final analyses.

Colour assessment

To estimate the red spawning coloration on the abdominal region, digital images were measured using a graphical user interface designed for the MATLAB environment (InFotonics Center©, University of Joensuu, Finland) to produce numerical estimates for the red, green and blue intensities in the RGB mode (Stevens *et al.*, 2007). The means of these colour parameters were calculated for each fish within two specified areas (Fig. 1), and these values were further averaged. Red intensity was calculated according to the formula: I_R = red/(red+green+blue) (e.g., Liljedal *et al.*, 2008), and this measure was highly correlated with two alternative colour measurements, hue and saturation, defined in the converted HSV colour model (r = -0.682, n = 40, P < 0.001 and r = 0.973, n = 40, P < 0.001, respectively). Unlike saturation, I_R showed normal sample frequencies and was therefore selected along with hue for a colour variable. Hue is expressed as an angle on a continuous circular scale (0-

360°) so that the hue scores closer to zero represent a higher degree of red coloration (e.g., Skarstein & Folstad, 1996). It can be thus said to be a more qualitative measure of redness.

Statistical analyses

We used SPSS for Windows v. 15.0 (SPSS, Inc., IL, Chicago, USA) for the statistical analysis. Variable distributions were checked for normality and variance homogeneity to verify the assumptions of parametric statistics. The arc-sine square-root transformation was applied to sperm longevity and I_R variables to fulfil their normality assumption. Since there were no ovarian fluid gradients that could have directed the course of sperm cells (Urbach, Folstad & Rudolfsen, 2005), cell trajectories were not expected to be linear and thus the measures of the actual point-to-point track followed by the cells (VCL) was expected to be the most relevant indicator of sperm swimming speed. In addition, the two other velocity parameters were highly correlated with VCL (both $r > 0.98$, both $P < 0.001$, $n = 36$) and so we omitted them in the analyses as redundant variables.

To test if hatchery generations differed in their sperm or colour properties, we first used multivariate analysis of covariance in which the three sperm measures or two colour measures were the dependent variable(s), fish length was a covariate, and generation was a fixed factor. To examine if the decrease in VCL differed between generations, we used a repeated measurements ANOVA, using measurements of sperm velocity from 10 to 40 s after activation as the within-subject variable. Otherwise, only the VCL 10 s post-activation was included in the analyses, as this measurement is the one that is most likely to be under selection (Levitan, 2000). Controlling for the effect of male length (L_T), partial correlation coefficients between sperm and colour variables were formed. Following Nakagawa (2004), we did not use Bonferroni or similar corrections for the multiple comparisons ($n = 10$

192 pairwise tests), but present all test probabilities as two-tailed. As we did not obtain the
193 measures of sperm velocity parameters from all males, sample sizes varied.
194
195 RESULTS
196 There was a significant difference in body size between the hatchery generations due to their
197 different ages (mean length ± SE = 48.7 ± 0.74 cm and 55.2 ± 1.19 cm for F_1 and F_2
198 generations, respectively; *t*-test, $t_{33} = -4.551$, $P < 0.001$). The sperm traits (spermatocrit,
199 VCL, longevity) did not differ between the hatchery generations (MANCOVA, $F_{3,31} = 1.153$,
200 $P = 0.343$) nor were they related to male length (covariate: $F_{3,31} = 1.185$, $P = 0.332$).
201 However, the spermatocrit showed a negative relationship with fish length among the males
202 from the first hatchery generation ($r = -0.540$, $P = 0.014$, $n = 20$), but not the second ($r =$
203 0.189, $P = 0.426$, $n = 20$). Sperm swimming speed declined significantly from 10 to 40
204 seconds after sperm activation (repeated measures ANOVA, time: $F_{3,32} = 165.01$, $P < 0.001$),
205 but there was no generation-specific effect of the rate of decline (generation: $F_{1,34} = 0.008$, P
206 $= 0.931$; time × generation: $F_{3,32} = 1.275$, $P = 0.300$) (Fig. 2).
207 There was no difference in the variation of red coloration (I_R, hue) between generations
208 (MANCOVA, $F_{2,36} = 1.056$, $P = 0.220$), when the effect of L_T was taken into account ($F_{2,36} =$
209 6.114, $P = 0.005$). Redness increased with male body length (Fig. 3A).
210 As the two hatchery generations did not differ from each other with respect to coloration
211 or sperm features, we combined the data for further partial correlation analyses (see Table 1
212 for the respective correlation coefficients). After controlling for male body length, we found a
213 strong positive correlation between sperm longevity and VCL ($P < 0.001$, $n = 36$).There were
214 also moderate, though non-significant, interrelationships between spermatocrit and both
215 sperm motility traits (Table 1). VCL showed a significant correlation with both red intensity
216 ($P = 0.004$, $n = 36$; Fig. 3B) and red hue ($P = 0.007$, $n = 36$). The significant influence of

redness on VCL was also revealed by the univariate analyses of covariance (I_R = covariate: $F_{1,32} = 7.667$, $P = 0.009$; $I_R \times$ generation: $F_{1,32} = 2.670$, $P = 0.112$, or alternatively, hue = covariate: $F_{1,32} = 5.688$, $P = 0.023$; hue × generation: $F_{1,32} = 1.409$, $P = 0.244$).

DISCUSSION

We found some evidence for the phenotype-linked fertility hypothesis that sperm velocity was positively associated with the degree of male red spawning coloration among a large lake population of Arctic charr, reared in a controlled environment. Sperm motility has been found to predict male fertilization success (Froman *et al.*, 1999; Rurangwa *et al.*, 2004), and under sperm competition, sperm velocity is probably the prime determinant of paternity (Birkhead *et al.*, 1999; Levitan, 2000; Gage *et al.*, 2004; Liljedal *et al.*, 2008; Rudolfsen *et al.*, 2008; Fitzpatrick *et al.*, 2009). Our observations are thus consistent with the phenotype-linked fertility hypothesis (Sheldon, 1994): the expression of a sexual ornament may signal a male reproductive trait that potentially determines male fertility.

There are alternative mechanistic causes for the general association between male fertility and ornamental traits. In wild Arctic charr populations, both the males' carotenoid-based coloration and fertilizing potential can be mediated by the individual differences in resistance to parasitic infections (Skarstein & Folstad, 1996; Liljedal *et al.*, 1999; Måsvær *et al.*, 2004). Increased immune activity may canalize resources away from ornamentation and sperm production as well as interfere with developing sperm cells, which are regarded as non-self to the male (Hillgarth *et al.*, 1997; Folstad & Skarstein, 1997). Hence, the males that are resistant or capable of maintaining a low immunological defence against pathogens and parasites before and during reproduction will have more intense carotenoid-based coloration and higher sperm quality (Skarstein & Folstad, 1996; Liljedal *et al.*, 1999). Since our study fish had been housed in a controlled environment and fed with commercial food (i.e., there

was no parasite transmission through the diet), the differences of intrinsic resistance may have been less important in generating the between-individual variability in ornamental coloration and sperm quality. Alternatively, males exhibiting carotenoid-rich coloration may also be those with high body supplies of sperm-protective antioxidants (von Schantz *et al.*, 1999; Blount *et al.*, 2001). Carotenoids themselves may not play an active role in mitigating oxidative stress (Isaksson & Andersson, 2008), but instead, carotenoid-based ornaments could signal an individual's availability of a more general antioxidant defence (e.g., the dietary intake of vitamins A, C and E) (Hartley & Kennedy, 2004; Bertrand *et al.*, 2006; Pike *et al.*, 2007; Pérez *et al.*, 2008).

Previous studies of guppies (*Poecilia reticulata* Peters) suggest that independent of body size, carotenoid pigmentation can be positively linked with sperm movement (Locatello *et al.*, 2006; Pitcher *et al.*, 2007) as well as with the number of sperm deposited (Pilastro *et al.*, 2002; Pitcher *et al.*, 2007; see however Pitcher & Evans, 2001; Skinner & Watt, 2007). Interestingly, our results revealed that male body length (L_T) was not a significant predictor of ejaculate characteristics, though it did have a positive correlation with male redness. Although there did not seem to be a general trade-off between the attained body size (or age) and ejaculate quality, we found a negative correlation between spermatocrit and male length in the first hatchery generation. This is likely related to the differential social status of individuals (Rudolfsen *et al.*, 2006), which is determined by the relative body size within the group of males present, larger males being more dominant (Gross, 1996; in charr, see Blanchfield & Ridgway, 1999; Sigurjónsdóttir & Gunnarson, 1989). Subordinate charr males are known to rapidly invest more both in sperm quantity and quality, presumably to compensate for their disfavoured breeding position in relation to the dominant males (Liljedal & Folstad, 2003; Rudolfsen *et al.*, 2006; Haugland *et al.*, 2008, in press). Additionally, Liljedal *et al.* (2008) recently found that colourful males gain lower parentage than their less ornamented

counterparts when equal numbers of sperm from rival males compete for fertilization. The authors suspected that the more efficient sperm of the paler males could result, at least in part, from their lower social status. Also in the present study, the formation of size-related social hierarchies during the few days before the measurements is a potential confounding factor affecting the observed within-group relationships between coloration and sperm traits. The smaller individuals, especially among the F_1 fish, may have relatively increased their ejaculate quality (both sperm density and motility) as compared with the larger, more colourful fish.

Consistent with the observations in roach (*Rutilus rutilus* L.) (Kortet *et al.*, 2004) and African cichlid fishes (Cichlidae) (Fitzpatrick *et al.*, 2009), we found a positive correlation between spermatozoal velocity and sperm longevity. A functional inter-dependence between these features reflects the metabolic performance of the sperm cells well, and suggests that sperm energetics have been the target of particularly intense selection due to sperm competition (Fitzpatrick *et al.*, 2009). In a typical spawning situation of Arctic charr where several males simultaneously release their milt in close proximity to eggs (Sigurjónsdóttir & Gunnarson, 1989), initial sperm velocity may have precedence over endurance in terms of fertilization success (Levitan, 2000; Liljedal *et al.*, 2008; Fitzpatrick *et al.*, 2009). Also spermatocrit showed signs of covariance with both sperm motility variables, implying that these traits are phenotypically integrated to some extent rather than represent totally independent components of fertilizing efficiency. Unlike sperm number and milt volume, sperm motility may be less dependent on the intra-testicular steroid content, but is mostly determined by the intra-cellular ATP stores (Christen, Gatti & Billard, 1987; Cummins, 1998; Froman *et al.*, 1999, 2002). Since sperm motility is susceptible to the activity of maternally derived mitochondrial genes (Cummins, 1998; Pizzari & Birkhead, 2002), directional selection on sperm density and sperm motility may not occur contemporaneously (Froman *et al.*, 2002; Pizzari & Birkhead, 2002; but see Fitzpatrick *et al.*, 2009). It is also possible that

292 the mitochondrial genes themselves, or their interaction with nuclear genes, mediate a
293 relationship between some male ornaments and sperm quality (Pizzari *et al.*, 2004).
294 Predicting the phenotypic linkage of primary and secondary sex traits from captive-bred
295 brood stocks involves both advantages and drawbacks in comparison with wild fish. Most
296 importantly, the culture environment essentially reduces, though not totally remove, the
297 among-individual variation arising from confounding environmental effects. Nevertheless,
298 captive conditions are radically different from natural habitats, which can easily alter and/or
299 relax the selective pressures operating on various phenotypic traits and thus ultimately reduce
300 the frequency of genotypes adapted to reproduce in the wild (Lynch & O'Hely, 2001;
301 Wedekind, 2002; Frankham, 2008). Hatchery selection and inbreeding have been mentioned
302 among the major causes for the rapid genetic divergence of hatchery fish from their wild
303 ancestors (Verspoor, 1988; Wang & Ryman, 2001; Wedekind *et al.*, 2007). Both hatchery
304 generations in our study had been produced by a large number of pairwise fertilizations,
305 which is presumably an effective means to prevent the loss of genetic diversity as well as to
306 prevent the selection for efficient sperm owing to sperm competition. Furthermore, the
307 possibility of inbreeding among the F_2 fish had been minimized with controlled matings, and
308 thus also the inbreeding depression (genetic load) for sexual coloration (van Oosterhout *et al.*,
309 2003) or fertility traits (e.g., Gomendio, Cassinello & Roldan, 2000; Margulish & Walsh,
310 2002; Gage *et al.*, 2006) is unlikely in our sample. It remains to be investigated, however,
311 whether an exposure to artificial selection over multiple generations would result in less clear
312 phenotypic correlations between sperm traits and sexually selected ornaments.
313 In conclusion, our results give some support to the idea that male ornamental traits reveal
314 information about primary sexual traits. After controlling for environmental conditions and
315 body size, male abdominal redness was positively correlated with sperm motility. Thus, the

spawning coloration of Arctic charr males may provide information about differences between males' fertilization potential.

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FIGURE LEGENDS

Figure 1. A Fish illustration showing the abdominal areas from which the coloration was measured.

Figure 2. Mean sperm velocity (VCL) among males of the first (n = 20) and second (n = 16) hatchery generations measured at different times after activation. Vertical bars denote 95 % confidence intervals.

Figure 3. Relationship between the relative red intensity (non-transformed) and (A) fish total length and (B) sperm velocity (VCL) 10 seconds after activation among individuals of the first (dark circles) and second (open circles) hatchery generations. The curves were fitted by (A) $y = 32.7 + 24.7x$ and (B) $y = 88.8 + 41.6x$.

503 TABLES

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505 **Table 1.** Partial phenotypic correlations between red intensity (I_R), red hue, spermatocrit,
506 sperm velocity (VCL, 10 seconds after activation) and sperm longevity in sexually mature
507 male Arctic charr while controlling for the effect of fish body length. Sample sizes vary
508 between 36 and 40.

Parameter	Hue	Spermatocrit	VCL	Sperm longevity
I_R	-0.682***	0.168	0.475**	0.213
Hue		-0.108	-0.449**	-0.186
Spermatocrit			0.302	0.275
VCL				0.838***

*$P < 0.05$
**$P < 0.01$
***$P < 0.001$

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