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Secondary sexual characters and sperm traits in coho salmon *Oncorhynchus kisutch*

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A study was undertaken to examine secondary sexual characters (spawning colouration and overall body size) in relation to sperm metrics in one alternative reproductive tactic of coho salmon *Oncorhynchus kisutch*: large hooknose males that spawn in dominance-based hierarchies. Males with less intense red spawning colouration had higher sperm velocities than males with darker red spawning colouration. There was no relationship between male body size and sperm metrics. These results suggest that within an alternative reproductive tactic, variation in sperm competition intensity may select for a trade-off between investment in sexual colouration and sperm quality.

Key words: longevity; motility; sexual selection; sperm competition; sperm quality.

INTRODUCTION

Sperm competition is common in fishes, especially among external fertilizing species with alternative life histories (Taborsky, 1998; Knapp & Neff, 2008). In these species, males with primary access to sexually active females *via* more ideal spawning positions, either by defending nests or by achieving high rank in a spawning hierarchy, are known as favoured males, whereas males that use a sneaking tactic that result in less than optimal spawning positions during spawning are known as disfavoured males (Parker, 1990, 1998; Kortet *et al.*, 2004). In accord with sperm competition theory (Parker, 1990; Ball & Parker, 1996), males that spawn in the disfavoured position have been found to invest more in spermatogenesis to compensate for their less than optimal proximity to females during egg release (Stoltz & Neff, 2006; Knapp & Neff, 2008), resulting in relatively larger testes and more sperm per unit volume of milt (Gage *et al.*, 1995; Neff *et al.*, 2003), faster sperm (Vladic & Jarvi, 2001;

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Burness *et al.*, 2004) or longer-lived sperm (de Fraipont *et al.*, 1993; Gage *et al.*, 1995; Uglem *et al.*, 2001) compared to males spawning in the favoured position (Knapp & Neff, 2008). Researchers often assume that all individuals within a given alternative reproductive tactic experience an equal intensity of sperm competition. It is unlikely, however, that all males within a particular reproductive tactic face the same sperm competition intensity since males will differ in their proximity to females during ejaculation, and, by extension, in their ability to co-ordinate their sperm release with female egg release (Stoltz & Neff, 2006). Despite these differences in sperm competition intensity, variation in spermatogenesis and sperm velocity has rarely been explored within a reproductive tactic. This study set out to test whether male coho salmon *Oncorhynchus kisutch* (Walbaum) within a single alternative reproductive tactic invest in sperm related traits as function of their likelihood of spawning in a favoured or disfavoured position.

Oncorhynchus kisutch typically breed in freshwater streams in North America. Their mating system resembles that of other semelparous Pacific salmon *Oncorhynchus* sp. with external fertilization: females compete for oviposition territories and males compete to fertilize eggs (Sandercock, 1991; Fleming & Reynolds, 2004; Quinn, 2005). Females spawn multiple times in a series of nests and defend those nests from superimposition by other females. Males do not help to build nests or defend them; rather, they invest in competing for access to ovipositing females. This competition results in dominance-based spawning hierarchies, with favoured positions closest to the female and disfavoured positions more distant from the female (Fleming & Gross, 1994). Secondary sexual characters, such as larger body size and more intense reddish-pink spawning colouration, are known to positively correlate with favourable spawning positions within the spawning hierarchy (Fleming & Gross, 1994). Moreover, proximity of males to the nest (which is presumably affected by a male's position within the dominance hierarchy) is correlated with order of entry into a female's nest at oviposition (Gross, 1985), and males in favoured positions often enjoy enhanced fertilization success (Schroder, 1981; Hutchings *et al.*, 1999; Jones & Hutchings, 2002; Blanchfield *et al.*, 2003). Sperm competition is intense within this hierarchy because several males may attempt to fertilize the eggs of a single ovipositing female during a spawning event (Gross, 1985).

The goal of the present study was to investigate the relationship between sperm quality metrics and secondary sexual characters within one alternative reproductive tactic of *O kisutch*. This study focused on large 'hooknose' males only (derived from the exaggerated snout, which develops at maturity), and not on small precocious sneaking males (*i.e.* 'jacks', Gross, 1985). Sperm competition theory predicts that hooknose males likely to occupy disfavoured positions in spawning hierarchies, as indicated by reduced expression of secondary sexual characters, would allocate more resources to sperm production and velocity to compensate for relatively poor access to spawning females. To test these predictions, body size metrics and red abdominal spawning colouration were examined in relation to sperm related metrics in *O. kisutch*.

MATERIALS AND METHODS

FISH COLLECTION AND BODY SIZE MEASUREMENTS

On 6 November 2007, 22 fish were collected from an autumn run in the Credit River (43°34′ N; 79°42′ W), which flows into Lake Ontario, using standard electroshocking methods. *Oncorhynchus kisutch* have been stocked in Lake Ontario for 40 years (Crawford, 2001). Hooknose males were selected to encompass a wide range of variation in secondary sexual characters (*i.e.* body size and spawning colouration). The mid-eye-to-hypural-flexure (MEH) length (L_{MEH}), caudal peduncle depth, hump height, snout length and body depth to the nearest mm were measured for each male (Table I). MEH is preferable to total length, fork length or mass for analyses of morphological variation in maturing salmonids because it is not confounded with snout or hump development or fin erosion (Hendry & Berg, 1999; Kinnison *et al.*, 2003).

SPAWNING COLOUR ASSESSMENT

Reflectance spectrometry was used to measure the intensity of male abdominal spawning colouration as it applied to integument pigmentation. The spawning colouration of each male was measured using an Ocean Optics reflectance spectrometer (USB 4000; detector range: 200–1100 nm; www.oceanoptics.com) and a xenon pulse lamp (PX-2; illumination range: 220–750 nm; Ocean Optics). A bifurcated fibre-optic probe delivered light from the light source to the measurement area and delivered reflected light from the measurement area to the spectrometer (R-400-7-UV-VIS; Ocean Optics). The probe was encased in a matt-black holder, which maintained the probe at a fixed distance to the measurement area and excluded external light. All spectrometry readings were made relative to a white standard, which reflected >97% of light at the wavelengths used in the analyses (Labsphere WS-1; Ocean Optics). Before collecting

TABLE I. Mean and estimates of intraspecific variation [(S.D.) and range] for sperm traits and secondary sexual characters of male hooknose *Oncorhynchus kisutch* ($n = 22$). Sperm velocity measurements included average path velocity (V_{AP}, average velocity on the smoothed cell path), straight line velocity (V_{SL}, average velocity on a straight line between the start and the end points of the track) and curvilinear velocity (V_{CL}, average velocity on the actual point-to-point track followed by the cell)

Trait	Mean ± S.D.	Range
Sperm metrics		
V_{AP} (μm s^{-1})	120·2 ± 16·1	95·7–158·6
V_{SL} (μm s^{-1})	97·0 ± 19·3	57·6–131·4
V_{CL} (μm s^{-1})	142·3 ± 17·4	116·4–176·2
Longevity (s)	27·3 ± 6·5	18–45
Sperm density per ml ($\times 10^6$)	106·3 ± 40·0	37·5–222·2
Total sperm length (μm)	31·0 ± 1·9	27·4–35·6
Sperm head length (μm)	3·2 ± 0·08	3·1–3·4
Flagellum length (μm)	27·8 ± 1·9	24·3–32·2
Secondary sexual characters		
Snout length (mm)	81·8 ± 7·0	71–97
Hump height (mm)	87·2 ± 11·1	66–115
Mid-eye-to-hypural-flexure length (mm)	593·1 ± 28·7	538–656
Caudal peduncle depth (mm)	62·8 ± 5·6	56–76
Body depth (mm)	166·6 ± 13·3	150–194

reflectance readings, the surface of the scales was wiped to minimize specular glare that might result from the reflection of water. Reflectance was measured at three consistent land-marked locations (at the lateral line) on the body of each male: (1) at the anterior end of the pectoral fin, (2) at the posterior insertion point of the dorsal fin and (3) at the posterior insertion point of the pelvic fin. Two readings were collected on each body region location, each of which comprised 20 consecutive measurements averaged by the spectrometer operating software (OOIBase 32; Ocean Optics). These two readings were then averaged within each body region, and because the body regions did not differ substantially in reflectance (see below), these readings were averaged to obtain a single mean reflectance spectrum for each male [Fig. 1(a)].

SPERM TRAIT ASSESSMENT

Milt was collected from each individual by applying gentle pressure to the fish's abdomen. The milt was then placed in a plastic bag. Care was taken to ensure that the milt was not exposed to any water to prevent activation of the sperm. The bags were stored at *c*. 10° C (the temperature of the river water) and transported back to

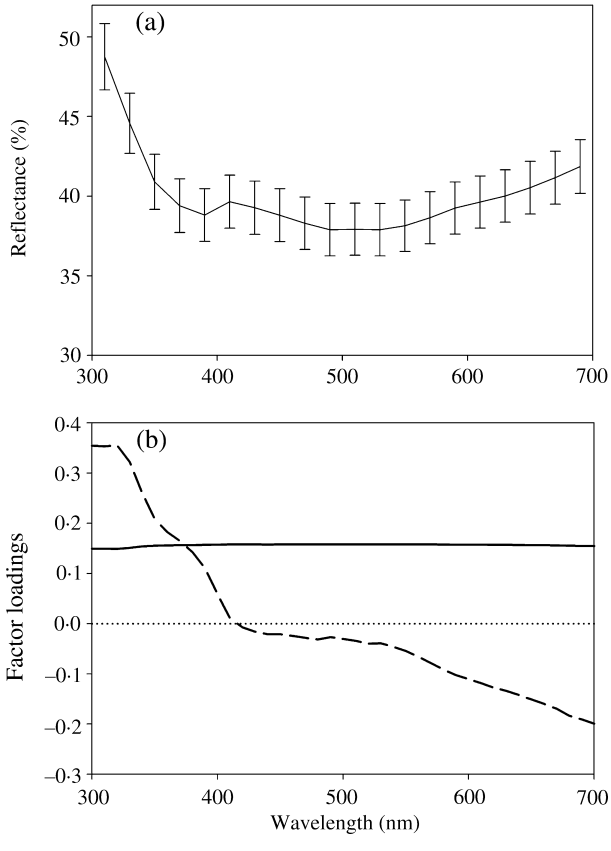

FIG. 1. (a) Mean ± S.E. reflectance spectra for male *Oncorhynchus kisutch* ($n = 22$). (b) Factor loadings relative to wavelength for a principal components analysis (PCA) derived from reflectance spectra of male *O. kisutch* abdomens (integument pigmentation – reddish spawning colouration): ——, loadings on the first principal component axis (PC1); ----, loadings on PC2.

a laboratory for further analysis. Sperm velocity (after activation with 10 μl of water from the river where fish were collected) was video recorded through a microscope and analysed with sperm-tracking software. Video recording was performed using a CCD B/W video camera module (Hamilton Thorne; www.hamiltonthorne.com) at 50 Hz vertical frequency, mounted on an external negative phase-contrast microscope (CX41 Olympus; www.olympusamerica.com) with a ×10 magnification objective. Video recordings were analysed using the HTM-CEROS sperm tracking packing (CEROS version 12; Hamilton Thorne), an objective tool for studying sperm motility in fishes (Kime *et al.*, 2001, Rurangwa *et al.*, 2004), set at the following recording variables: number of frames = 60; minimum contrast = 11; minimum cell size = 3 pixels. The following variables were used to assess for each male's sperm: average path velocity (V_{AP}, average velocity on the smoothed cell path), straight line velocity (V_{SL}, average velocity on a straight line between the start and the end points of the track) and curvilinear velocity (V_{CL}, average velocity on the actual point-to-point track followed by the cell) at 5 s post-activation (Table I). Post-activation of 5 s was used as the standard because fertilization of eggs decreased after 10 s but did not differ significantly between 0, 5 and 10 s (Liley *et al.*, 2002). These velocity estimates corresponded to the mean velocity of all motile cells analysed; that is, for each male, the velocity of each individual sperm cell was measured but the estimate used in the final analyses corresponded to a mean over all individual sperm cells. Sperm longevity was also estimated as the time from activation until *c.* 95% of the spermatozoa within the field of view were no longer motile, *i.e.* showing no forward movement (Table I).

Sperm density was estimated by counting sperm cells in an 'improved Neubauer chamber' haemocytometer (www.hausserscientific.com) under ×400 magnification (Pitcher & Neff, 2007; Pitcher *et al.*, 2007). The numbers of sperm in each of five larger squares on the haemocytometer were counted. There were 25 of these large squares on the haemocytometer and each of these large squares had 16 smaller squares within it. Sperm were counted in the four large corner squares and the large centre one (80 smaller grids). The mean number of sperm per large square count (*i.e.* mean of the five counts) was multiplied by 25 (to obtain the mean per 5 × 5 large-square grid) and again by 10 (the depth of the chamber in μm). This number was then multiplied by the initial volume of the sample to estimate the sperm density. Sperm densities are expressed as the total number of sperm per ml of a male's stripped ejaculate (Table I).

A sub-sample of milt (20 μl) was used to assess sperm morphometry using techniques similar to those described by Leach & Montgomerie (2000). Sperm in saline solution (with 2·5% glutaraldehyde) were dispensed onto a glass slide to present two-dimensional images for measurement. Sperm were observed at ×1000 magnification under oil immersion and digital images were taken. The head and flagellum were measured separately (the midpiece was too small to discern using light microscopy and was indistinguishable from the head component) using ImageJ software (http://www.rsb.info.nih.gov/ij/) and care was taken to only measure intact sperm without flagellar damage. Head length (which included the midpiece) was the measurement from the insertion of the flagellum across the midline of the sperm head to its forward apex; flagellum length was measured from its insertion to the end of the terminal filament. Total sperm length was determined by combining the head length and the flagellum length. Ten sperm per male were measured and the mean of the measurements taken on total, head and flagellum length were used in all analyses (Table I).

STATISTICAL ANALYSES

Principal components analysis (PCA) was used to summarize variation in overall body size (*i.e.* L_{MEH}, caudal peduncle depth, hump height, snout length and body depth). One informative PC axis was extracted (hereafter referred to as body size) that explained 70% of the variation in overall body size. PCA was also used to summarize variation in reflectance (Endler, 1990; Montgomerie, 2006). PCA is a useful way to summarize variation in colour because it reduces complex variation in reflectance spectra into a few simple variables that are not intercorrelated and it makes no assumptions

about the visual system of the signal receiver (Endler, 1990; Montgomerie, 2006). PCA was conducted by calculating average reflectance values in 10 nm bins between 300 and 700 nm. These binned data were then used as variables in the analysis; each male served as an independent observation. Using the broken-stick criterion (Jackson, 1993), two informative PC axes were extracted that together explained 99·5% of the variation in colour. The first PC axis (colour PC1) explained 97% of the variation in reflectance, whereas the second axis (colour PC2) explained 1·92% of the variation in reflectance. As is typical of PCA conducted on reflectance data (Endler, 1990), the first axis received moderate, positive loadings across all wavelengths and can thereby be considered a measure of brightness; males with more negative PC1 colour scores had a more pigmented, darker colouration [Fig. 1(b)]. By contrast, the second axis received high positive loadings from ultraviolet (UV) wavelengths (300–400 nm), moderate negative loadings from blue–yellow wavelengths (400–600 nm) and high negative loadings from orange–red wavelengths (600–700 nm). This second axis can therefore be considered an axis of variation between colours with a relatively high UV component *v.* colours with a relatively high red component and is hereafter referred to as colour identity; males with more negative PC2 colour scores were redder in colour [Fig. 1(b)]. The spectral measurements were highly repeatable (Lessels & Boag, 1987) within body regions for both PC1 (repeatabilities: 0·82–0·92, $P < 0·001$) and PC2 (repeatabilities: 0·84–0·95, $P < 0·001$). Although there were some differences between regions for PC1, measurements across all three regions were repeatable within individuals for both PC1 (repeatability: 0·63, $P < 0·05$) and PC2 (repeatability: 0·87, $P < 0·001$). Finally, PCA was used to summarize variation in the three sperm velocity metrics (V_{AP}, V_{SL} and V_{CL}). One informative PC axis (hereafter referred to as sperm velocity) was extracted that explained 88·6% of the variation in velocity.

Data were all normally distributed (Shapiro–Wilks tests) except for sperm density, which was $\log_{10}$ transformed to normalize its distribution. Relationships between secondary sexual characters (abdominal spawning colouration and overall body size) and sperm metrics were examined using Pearson correlations. Variation among males in sperm morphometry metrics (total sperm length, head length and flagellum length) was analysed using one-way ANOVAs.

RESULTS

Oncorhynchus kisutch spawning colouration brightness was not significantly related to sperm density ($r = 0·07$, $n = 22$, $P > 0·05$) or sperm longevity ($r = 0·25$, $n = 22$, $P > 0·05$) but was positively related to sperm velocity [($r = 0·46$, $n = 22$, $P < 0·05$; Fig. 2(a)], such that lighter, less pigmented males had higher sperm velocity. Spawning colouration identity was not significantly related to sperm density ($r = -0·07$, $n = 22$, $P > 0·05$) or sperm longevity ($r = 0·02$, $n = 22$, $P > 0·05$) but was positively related to sperm velocity [$r = 0·53$, $n = 22$, $P < 0·05$; Fig. 2(b)], such that males with decreased red reflectance and increased UV reflectance had higher sperm velocity. Although there was variation in sperm morphology among the males (total length; $F_{21,198}$, $P < 0·001$; head length; $F_{21,198}$, $P > 0·05$; flagellum length $F_{21,198}$, $P < 0·001$; Fig. 3), there was no significant relationship between sperm morphology metrics and sperm velocity ($P > 0·05$) spawning colouration brightness ($P > 0·05$) or identity ($P > 0·05$). Body size was not significantly correlated with any of the sperm metrics (all $P > 0·05$). Sperm longevity and sperm velocity were positively correlated ($r = 0·46$, $n = 22$, $P < 0·05$; Fig. 4).

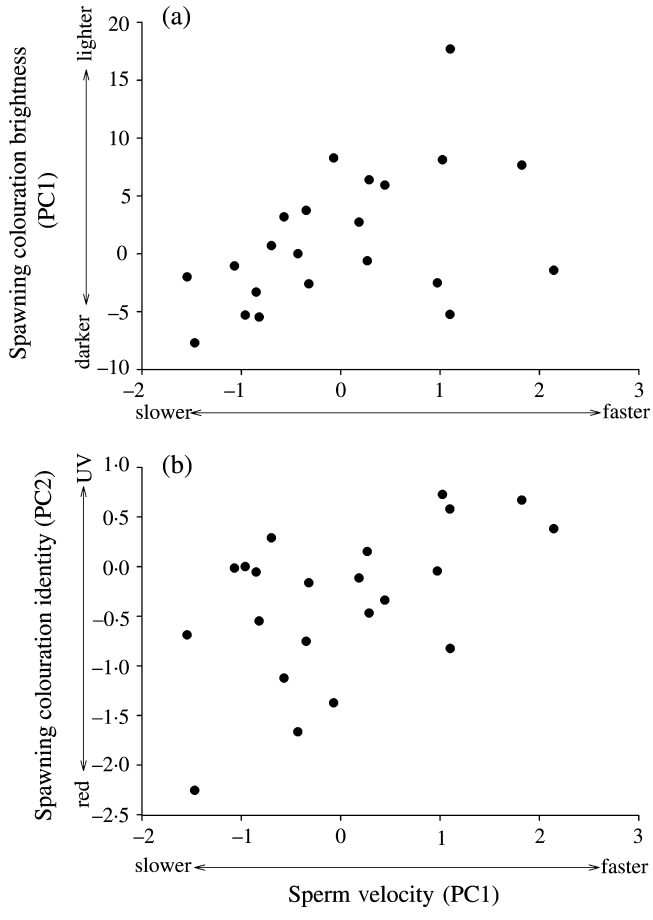

FIG. 2. Relationship between sperm velocity and abdominal spawning colouration (a) brightness (colour PC1) and (b) identity (colour PC2) in *Oncorhynchus kisutch*. Positive colour brightness and colour identity scores indicate lighter and less red abdominal spawning colouration, respectively. Positive sperm velocity scores indicate faster sperm velocity.

DISCUSSION

The data did not support the prediction that disfavoured males should invest relatively more in spermatogenesis, as sperm density was not inversely related to body size or spawning colouration. Furthermore, body size did not relate to sperm velocity or longevity. Males with less intense red spawning colouration, however, were found to possess sperm with higher velocity compared to males with more intense red spawning colouration. These results suggest that within an alternative reproductive tactic, males may trade-off their investment in ornamental colouration and sperm quality.

The observed relationship between spawning colouration and sperm velocity is potentially attributable to the carotenoids responsible for the reddish–pink abdominal spawning colouration in salmonids (Rajasingh *et al.*, 2007). Since carotenoids can only be obtained through the diet, the intensity of spawning

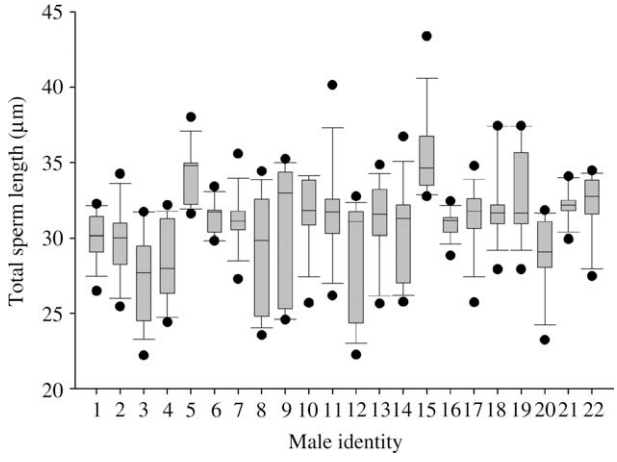

FIG. 3. Total sperm length variation within and among male *Oncorhynchus kisutch*. Box plots show medians, 1 interquartile range (IQR) as boxes, whiskers extending to the extreme values within the median, 1·5 IQR, and outliers as circles (>1·5 IQR).

colouration may indicate dietary carotenoid access and nutritional condition (Bjerkeng *et al.*, 2000; Grether *et al.*, 2001). In addition, these pigments act as antioxidants by intercepting and neutralizing free radicals (Goodwin, 1984; Aitken, 1995; Halliwell & Gutteridge, 1999), and as such, carotenoid-based ornaments are negatively affected by parasitism and can serve as honest indicators of immune health (von Schantz *et al.*, 1999).

Although a negative relationship between carotenoid ornamentation and sperm velocity may at first seem paradoxical, it is important to consider that carotenoids also play a direct role in male reproductive function in ways that can influence sperm quality and quantity in salmonids (Ahmadi *et al.*, 2006).

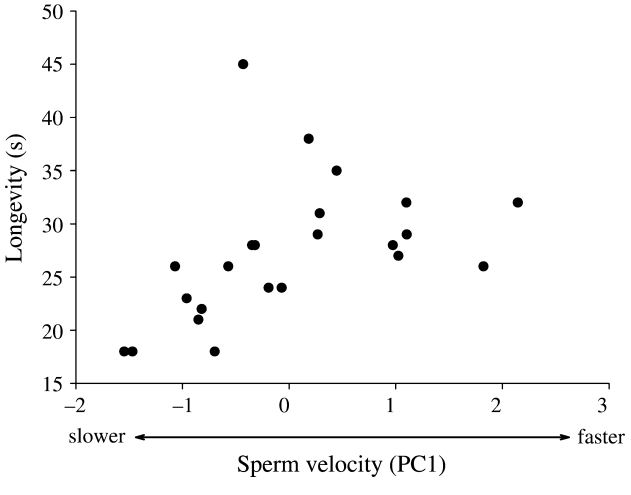

FIG. 4. Relationship between sperm longevity and sperm velocity in *Oncorhynchus kisutch*. Positive sperm velocity scores indicate faster sperm velocity.

Carotenoids are present in gonads, reproductive accessory glands and seminal plasma (Goodwin, 1984; Aitken, 1995; Bjerkeng *et al.*, 2000). In addition, the antioxidant capacity of carotenoids may directly affect sperm quality by neutralizing free radicals, which have a deleterious effect on sperm quality (Halliwell & Gutteridge, 1999; von Schantz *et al.*, 1999; Blount *et al.*, 2001). Antioxidants present in semen appear to reduce the susceptibility of sperm to oxidative stress and increase fertility (Blount *et al.*, 2001). In *O. kisutch*, males may trade-off carotenoid allocation between sexual ornamentation and sperm related metrics. Males with more intense red spawning colouration may use carotenoid reserves for the purposes of pre-spawning displays that establish favourable spawning hierarchy positions, whereas males with less intense red spawning colouration may use carotenoids to protect the integrity of their sperm to compensate for a less than optimal spawning position. Indeed, one recent study showed that males whose diets were supplemented with astaxanthin, the carotenoid pigment responsible for pinkish–red coloration in salmonids (Rajasingh *et al.*, 2007), had higher fertilization success than males with carotenoid-limited diets (Ahmadi *et al.*, 2006). Thus, carotenoid pigments may provide a mechanistic link between sexual ornamentation and sperm quality metrics in *O. kisutch*. In addition, sperm velocity did not negatively correlate with longevity but instead was positively correlated with this trait (Kortet *et al.*, 2004), which might be expected if carotenoids protect sperm quality in general. Furthermore, sperm morphology was not related to sperm velocity (Gage *et al.*, 2002) or spawning colouration, which rules out an explanation based on the hypothesis that longer sperm are more motile (Gomendio & Roldan, 1991; Gage & Freckleton, 2003). In light of these observations, it is perhaps not surprising that there was no relationship between body size and sperm quality metrics since a direct mechanistic link between these traits might be more difficult to establish.

It is also likely that some of the association between intensity of red spawning colouration and sperm velocity in *O. kisutch* is developmental in nature. Differences in sperm quality have been found in relation to ontogeny in many taxa (Calvo *et al.*, 1999; Evans *et al.*, 2002; Ceballos-Vazquez *et al.*, 2003; Green, 2003). For example, Evans *et al.* (2002) found that the area of ornamentation (*i.e.* orange caretenoid colouration) increases with age in male guppies *Poecilia reticulata* Peters, and this ornamentation correlates with several metrics of sperm quality (Pitcher & Evans, 2001; Pitcher *et al.*, 2007). As such, in the present study, the relationship between the intensity of red spawning colouration and the sperm velocity may well result, at least in part, from differences in the stage of development of the males examined; other than knowing that the males were sexually mature and spawning, the age of the males or when they arrived on the spawning grounds was unknown and therefore this hypothesis could not be tested directly.

In conclusion, sperm velocity was negatively related to the intensity of red spawning colouration. If the relationship between sperm velocity and a secondary sexual character observed in this study is widespread in nature, it may not only help explain dynamics associated with sperm competition among males with different levels of sexual ornamentation but also help provide an explanation for the evolution and maintenance of extravagant male ornaments in

non-resource-based mating systems; elaborate male sexual ornamentation may allow males to secure favoured position in spawning hierarchies, and disfavoured males may compensate by reducing investment in ornamental colouration and increasing investment in sperm quality.

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