



## Spawning coloration, female choice and sperm competition in the redbside dace, *Clinostomus elongatus*

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Understanding pre- and postcopulatory mechanisms of sexual selection (male–male competition and female choice) can provide insight into the evolution of male ornamentation. In this study, we use an integrative approach to study sexual selection by incorporating measures of both mechanisms using the redbside dace, *Clinostomus elongatus*, a freshwater, externally fertilizing fish found in streams of eastern North America. We measured the success of individual male redbside dace in both pre- and postspawning sexual selection and related the success of males to a range of traits, including spectral properties and the size of their red spawning coloration, body size metrics and sperm quality. We found significant sexual dimorphism in the relative amount and spectral properties (saturation and brightness) of the red spawning coloration, with males being more ornamented than females. Using dichotomous mate choice trials, we found that females did not favour males with relatively larger red spawning coloration area and more saturated or brighter red spawning coloration. Males with relatively more red spawning area would be favoured in male–male postspawning sperm competition because they possess sperm with higher velocity, and sperm velocity was determined to be the key predictor of sperm competition success as assessed by paternity analyses related to in vitro sperm competition trials. These results suggest that prespawning selection via female choice is not driving the evolution of red spawning coloration and that postspawning selection via male–male competition is primarily determined by sperm velocity, which was correlated with the relative amount of red spawning coloration.

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A central aim of evolutionary biology is to understand how selection acts on traits in natural populations. For example, sexual selection acts on secondary sexual traits when there is variation in reproductive success associated with these traits, which arises from two mechanisms: intrasexual selection (male–male and female–female competition) and intersexual selection (e.g. female and male mate choice) (reviewed in Andersson 1994; Andersson & Simmons 2006). Although the importance of both of these mechanisms for the evolution of secondary sexual characters in males has been acknowledged, most researchers focus their attention on one or the other mechanism within the context of their study system. This can be problematic, however, because even though both mechanisms may operate at the same time, they do not necessarily act in the same direction (reviewed by Hunt et al. 2009). For example, the two mechanisms can reinforce each other, as in the western rainbow fish, *Melanotaenia australis*, and hissing

cockroaches, *Gromphadorhina portentosa*, where male–male competition and female choice both act to favour large body size in males (Clark & Moore 1995a, b, c; Young et al. 2010). Alternatively, if sexual conflict is pronounced, one mechanism may oppose the other (reviewed in Arnqvist & Rowe 2005). In water striders, *Aquarius remigis*, for example, male–male competition appears to select for larger body size, whereas female choice appears to select against larger body size (Sih et al. 2002). As such, when differences in the relative strengths of the two mechanisms exist, the outcome for sexual selection will depend on an interaction between the magnitude and direction of each selective force (see Hunt et al. 2009). Differentiating between the effects of female choice and male–male competition within a mating system is therefore critical in order to better understand the relative contribution of the two mechanisms in the evolution of sexually selected traits.

A recent review of 83 studies (of 51 species) that investigated both male–male competition and female mate choice acting on an apparent sexually selected trait, male body size, found that in most cases selection was linear and that both mechanisms were more likely to be reinforcing rather than opposing (Hunt et al. 2009). However, the generality of these results are questionable because

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although body size is commonly thought to be important in sexual selection, it is also a metric that is crucial in many other contexts (e.g. different life history stages) and is likely to be influenced by natural selection as well. As such, Hunt et al. (2009) suggest that there is a need for more empirical studies of both mechanisms that examine traits that are limited in expression to the adult stage and that have little relevance outside of courtship and mating, such as colourful male secondary sexual characters (e.g. Setchell 2005; Tarof et al. 2005). In addition to considering sexual traits other than body size, there is also a need to examine how both pre- and postcopulatory processes might affect the evolution of male secondary sexual characters. For example, if there is a relationship between male secondary sexual characters and sperm quality (Blount et al. 2001; Peters et al. 2004), postcopulatory sperm competition success by more ornate males might reinforce or oppose precopulatory female choice on male sexual traits. Predicting the directionality of the relationship between sperm quality metrics and sexual ornamentation is difficult because two different hypotheses make opposite predictions. The phenotype-linked fertility hypothesis (Sheldon 1994) suggests that male sperm quality and ornament elaboration will positively covary, whereas, the sperm competition hypothesis (Parker 1998) predicts a negative association between investment in sperm quality and ornamentation related to mate acquisition. For example, in guppies, *Poecilia reticulata*, males with relatively more orange body coloration (a trait subject to precopulatory female choice: Magurran 1995; Pitcher et al. 2003) have more and faster sperm (Pitcher & Evans 2001; Locatello et al. 2006; Pitcher et al. 2007) and a paternity advantage following artificial insemination of females with equal numbers of sperm from two competing males (Evans et al. 2003). Whereas, in Arctic charr, *Salvelinus alpinus*, the most brightly coloured males (i.e. males with more intense red sexual ornamentation) have the lowest fertilization probability following artificial fertilization of female's eggs using equal numbers of sperm from two competing males (Liljedal et al. 2008).

In this study we used mate choice trials, spectral and sperm analysis, competitive in vitro fertilization and paternity assignment to examine factors affecting the evolution of a male secondary sexual character. We did this by quantifying prespawning female mate choice and postspawning male–male competition in the redbreasted dace, *Clinostomus elongatus*. The redbreasted dace is a small, externally fertilizing cyprinid native to headwater streams in the basins of the Mississippi River and Lakes Michigan, Huron, Erie and Ontario (Parker et al. 1988). Sexual selection has not been explored in this species, but the sexual dichromatism exhibited in sexually mature adults during the spawning season suggests that it may be present in this species' mating system (Andersson 1994). Throughout the year, adult redbreasted dace have pink coloration on their body just behind their head, but during the breeding season both sexes develop intense red spawning coloration. Based on a chemical test that identifies the presence of carotenoid pigments in animal tissues (McGraw et al. 2005) and preliminary high performance liquid chromatography analyses, the red spawning coloration is carotenoid based (T. E. Pitcher & K. McGraw, unpublished data). The red spawning coloration manifests itself as an intense red stripe extending from the opercula to below the dorsal fin or beyond (Fig. 1). Males appear to possess a larger area of more intensely red spawning coloration than females, although this has yet to be quantified. Throughout their range, redbreasted dace, which become sexually mature at the age of 3 years (they can live to be 4 years old), spawn between mid-May and early June (Scott & Crossman 1998; Werner 2004). Like some other small minnows, redbreasted dace typically parasitize gravel nests built by larger minnows or suckers such as creek chub, *Semotilus atromaculatus*. These nests are found just upstream or downstream of fast riffles in



**Figure 1.** Male redbreasted dace showing typical sex-specific spawning coloration. The circle indicates the location where reflectance spectrometry measurements were collected. Note that the bluish colour on the dorsal surface is not structural coloration but a reflection from excess water, which causes specular glare by reflecting incident light. Prior to taking spectral readings, we removed excess water by wiping the body surface with a lint-free delicate task wiper.

coarse sand or fine gravel. Redbreasted dace leave the eggs to be guarded by the larger species and provide no parental care to their offspring. During spawning, males typically congregate in dense schools downstream from creek chub nests, with the females positioned on the outer regions of the school. Spawning typically begins when a female moves upstream towards a creek chub nest and is followed by up to six males (Koster 1939; T. E. Pitcher, unpublished data). During spawning, multiple males crowd around the female and release their sperm as she deposits her eggs into a gravel nest. Females carry between 400 and 1500 eggs, depending on their body size (Scott & Crossman 1998; Werner 2004). Spawning of eggs by females and sperm from multiple males can be repeated every few seconds for about 1 min, with several minutes often elapsing between each spawning bout. Unlike other minnows that have a lek-like mating system (e.g. European minnow, *Phoxinus phoxinus*, Jacob et al. 2009), behavioural observations of wild redbreasted dace prior to spawning suggest that prespawning male–male competition plays a limited role in this mating system because males do not appear to defend territories or have consistent spawning hierarchies (T. E. Pitcher, personal observation). However, it is likely that postspawning intrasexual competition in the form of sperm competition, the competition between sperm of two or more males for the fertilization of ova (Parker 1970), is an important selective factor in this mating system. Recently, Pitcher et al. (2009a) documented significant intermale variance in sperm size and shape metrics (total sperm length, sperm head length, flagellum length and sperm head length to width ratio) for redbreasted dace and also found positive relationships between these morphometric indices and sperm velocity. Because sperm velocity correlates with sperm competition success in many fish species (e.g. Gage et al. 2004), these findings suggest that male redbreasted dace vary in their sperm competitive ability.

We examined the success of individual male redbreasted dace in both pre- and postspawning sexual selection and related the success of males to a range of traits, including body size, spectral properties and size of the red spawning coloration, and sperm quality. Our first objective was to quantify sexual dimorphism in this species and to test whether females show preferences for sexually dimorphic male traits. Using mate choice trials in which females were allowed to choose between two males, we predicted that the degree of female preference for a particular male would increase as a function of the difference in phenotype between the two males; that is, females should be more responsive to more colourful (i.e. more ornamented) males. Our second objective was to examine postspawning male–male competition by using in vitro sperm competition trials to determine which sperm traits are important for competitive fertilization success and whether males that are more colourful possess these sperm traits. To accomplish this objective, we investigated the importance of sperm velocity, density and longevity with respect to competitive fertilization success by conducting in vitro fertilization trials using sperm from pairs of males and eggs from individual females. We then used

microsatellite markers to assign paternity to the offspring, estimate relative fertilization success (i.e. paternity) for each male and relate relative fertilization success to differences in sperm traits and ornamentation between competing males. Finally, we determined whether female choice and male–male competition favour the same or different male sexual traits, and thus whether pre- and postspawning sexual selection to reinforce or oppose each other, respectively.

## METHODS

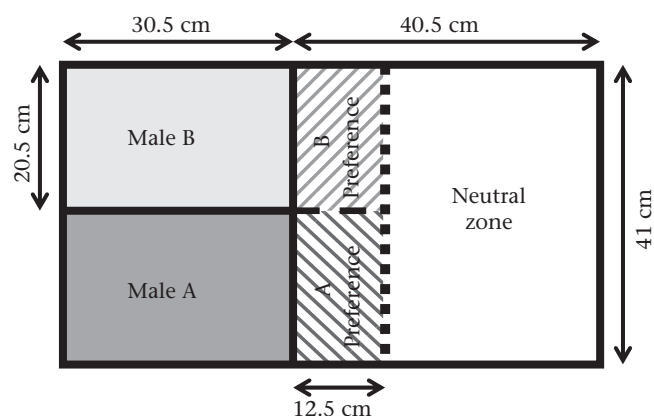
### *Study Species and General Field Methods*

We captured redbreasted dace in the wild during the spawning season from Rathburn Run in Wayne County, OH, U.S.A. (40°48.658'N, 082°01.400'W) between 28 May and 1 June 2008. Overall, 50 males and 22 females were captured using standard seining techniques. All males ( $N = 50$ ) and a subset of females ( $N = 7$ ) were used to test for sexual dimorphism among a suite of candidate phenotypic traits, including standard length and metrics of the red spawning coloration (hue, saturation, brightness and relative area (corrected for body area)). Forty-four of the males and all 22 females were used to examine which traits females preferred using dichotomous mate choice trials. Next, 45 of the males were used in comparisons of sexual ornamentation to sperm quality traits (sperm data were not available for five of the males). Finally, we captured an additional 27 redbreasted dace ( $N = 18$  males, 9 females) to determine which sperm traits are related to competitive fertilization success. We could not use the same males used to examine sexual ornamentation and sperm quality traits because of the need to expedite the eggs back from the field to the laboratory for immediate fertilization. We investigated the importance of sperm velocity, number and longevity with respect to fertilization success by conducting *in vitro* fertilization competitions using sperm from pairs of males and eggs from a single female. Next, we used microsatellite markers to assign paternity to the offspring, estimate relative fertilization success for each male, and then relate relative fertilization success to difference in sperm traits between competing males.

### *Female Choice Trials*

Mate choice trials were conducted in a dichotomous choice Plexiglas tank in which the partitions were watertight and all sides of the tank were transparent with the exception of the wall separating the males, which was opaque to prevent male–male interactions (see Fig. 2). The tank was filled with stream water and placed on the bed of the stream from which individuals were collected, and was positioned so that there would be approximately even levels of light entering both male sections. Females were chosen haphazardly from those collected, and pairs of males were chosen by eye to have similar body size. A removable opaque partition was used to separate the female from the males for a 5 min acclimation period. The partition was then removed and 23 min behavioural trials were recorded using a digital camcorder (Sony Handycam DCR-SR200) with a polarized lens filter (Sony MC Circular PL) to reduce glare from the water surface. After individuals were used in the behavioural trials they were measured for a variety of potential secondary sexual characters and a milt sample was collected from each male (see *Sperm Quality Measurements* below).

Footage of the mate choice trials was reviewed and the total amount of time the female spent in the neutral and preference zones (see Fig. 2) was determined to the nearest second. Preference time with a male was determined as time the female spent in the preference zone (dotted line in Fig. 2) on that male's half of the tank



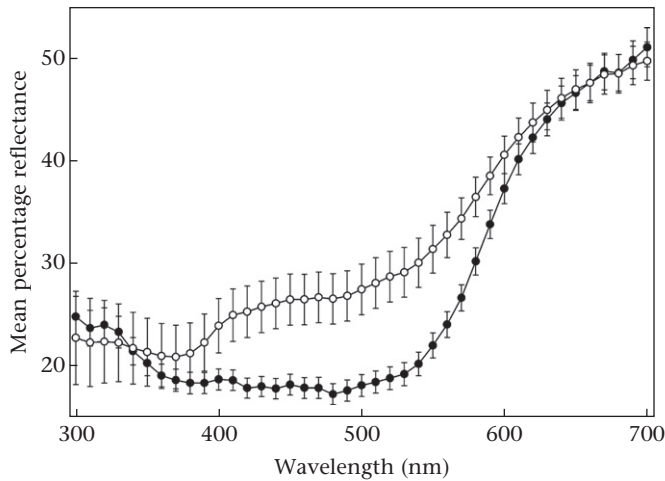
**Figure 2.** Schematic top view of the dichotomous choice tank used for assessing mate choice in redbreasted dace. The diagram shows the sections containing the two males (left side) and the larger section comprising the neutral zone and preference zones, which contained the female (see text for details).

when she was interacting with that male (approach and visual inspection of males by females was considered to be interaction). Time spent in the neutral zone was counted as not showing preference for either male. We examined female behavioural data in two ways: by comparing the number of times that a female spent more time with the more ornamented male (using a binomial test), and by using a preference function asking whether a female spent significantly more than 50% of the time with the more ornamented male (see *Statistical Analyses* below for details). Three behavioural trials were discarded because one male escaped from the tank and two females did not inspect either male during the duration of the trial.

### *Male and Female Phenotypic Measurements*

At the completion of a behavioural trial, individuals were anaesthetized in a water bath containing 0.15 g/litre of MS-222. Next, each fish was immediately photographed with a ruler for scale using a digital camera (Canon PowerShot A570IS) and weighed to document the standard length ( $\pm 0.1$  cm), body mass ( $\pm 0.001$  g), condition ( $= (\text{body mass (g)})(100)/(\text{standard length (cm)}^3)$ ), the body area ( $\text{mm}^2$ ) and the area of red spawning coloration area ( $\text{mm}^2$ ; i.e. area of red integument pigmentation; see Fig. 1). The area of red spawning coloration and total body area were quantified for the right side of each individual using ImageJ analysis software (available at [www.rsbi.info.nih.gov/ij/](http://www.rsbi.info.nih.gov/ij/)) to calculate the relative area covered by red integument pigmentation (see Pitcher et al. 2003), hereafter referred to as residual red spawning coloration area.

The red spawning coloration was measured on the right side of the body for each of the males and a subset of females using reflectance spectrometry (see Figs 1, 3). The coloration of the red integument pigmentation was measured using an Ocean Optics reflectance spectrometer (USB 4000, detector range 200–1100 nm) and a xenon pulse lamp (PX-2, illumination range 220–750 nm) (see Pitcher et al. 2009b). Light was delivered to the sample area and the reflected light was transmitted to the spectrometer via a bifurcated fibre-optic probe (R-400-7-UV-VIS). The probe tip was encased in a matte-black rubber holder that excluded external light and maintained a fixed distance between the probe and the measurement surface. The system was calibrated using a white standard that reflects more than 97% of the wavelengths used in our analyses (Labsphere WS-1). Prior to collecting reflectance measurements, the body surface was wiped with a lint-free delicate



**Figure 3.** Mean  $\pm$  SE reflectance spectra (see text for details) for male (closed circles,  $N = 50$ ) and female (open circles,  $N = 7$ ) reddsides.

task wiper to remove excess water that could cause specular glare by reflecting incident light.

Reflectance was measured at one landmark position on the red spawning coloration (see Fig. 1), immediately behind the operculum (with the probe tip positioned so that the entire opening was on the red integument pigmentation and no light could enter through the operculum opening). One reading consisting of 20 consecutive measurements (averaged by the spectrometer operating software, OOIBase 32) was taken at the one landmark point on the red spawning coloration (see Fig. 3, Table 1). We used the program CLR (see Montgomerie 2008) to calculate three colorimetric variables to approximate the three dimensions of colour: hue, saturation and brightness. We calculated hue as the wavelength at which each reflectance spectrum reached 50% of its maximum value (Montgomerie 2006). We calculated saturation as the difference between maximum and minimum reflectance, divided by brightness (Montgomerie 2006). We calculated brightness as the average percentage reflectance across the entire spectrum; lower brightness values are indicative of increased pigmentation (Montgomerie 2006).

#### Sperm Quality Measurements

After reflectance measurements were completed, milt was collected from males by holding each one upside down and applying gentle pressure to the abdomen. The milt was then collected as it emerged using a pipette and then placed in a 1.6 ml Eppendorf tube. Care was taken to prevent exposure of the milt to activation by water. Tubes containing the milt were stored in a cooler and transported back to the laboratory for further analysis. Sperm were activated with 10  $\mu$ l of water from the stream where the fish were collected at 12 °C, the mean water temperature during spawning. Five seconds after activation, sperm velocity was videorecorded through a microscope and analysed with sperm-tracking software (see Pitcher et al. 2009a, b for details). Briefly, videorecording was performed using a CCD B/W video camera (at 50 Hz vertical frequency) mounted to an external negative phase-contrast microscope (CX41 Olympus) with a 10 $\times$  magnification objective. Recordings were then analysed using HTM-CEROS sperm-tracking package (CEROS version 12, Hamilton Thorne Research, Beverly, MA, U.S.A.), an objective tool for the study of sperm motility in fish (see Kime et al. 2001; Rurangwa et al. 2004). The system was set at the following parameters: number of

frames = 60, minimum contrast = 11, minimum cell size = 8 pixels. Three aspects of sperm velocity are measured by this software: the average path velocity (the average velocity along a smoothed cell path), the straight line velocity (the average velocity along a straight line from the starting point to the ending point of the path) and the curvilinear velocity (the average velocity along the actual path followed by the cell) at 5 s postactivation. These velocity estimates corresponded to the mean velocity of all motile cells analysed (mean  $\pm$  SD number of sperm examined per male =  $47 \pm 27$ ); 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. We used principal components (PC) analysis to summarize variation in sperm velocity (see Results, Table 2). One informative PC axis was extracted (hereafter referred to as sperm velocity) that explained 73.9% of the variation in overall sperm velocity and received strong positive loadings from all three velocity estimates.

Sperm longevity was also estimated as the time from activation until about 95% of the spermatozoa within the field of view were no longer motile (i.e. showed no forward movement; see Gage et al. 2004). Estimates of sperm density came from counts taken on an 'improved Neubauer chamber' haemocytometer under 400 $\times$  magnification (see Pitcher et al. 2007, 2009a, b for details). Briefly, the numbers of sperm in five of the 25 larger squares were counted (each was further subdivided to simplify counting). The mean number of sperm in five squares was then multiplied by 25 (to estimate the number of sperm in all squares), again by 10 (the depth of the chamber in  $\mu$ m) and then again by 10 (the original volume of the sample examined in  $\mu$ l). The estimated densities were expressed as the number of sperm per millilitre of stripped ejaculate.

#### In Vitro Sperm Competition Trials

Five of the nine triads (each consisting of two males and a female) used in the in vitro sperm competition trials were wild-caught using standard seining techniques from the same stream as the fishes used above (i.e. Rathburn Run) between 29 May and 31 May 2008. Four additional triads were wild-caught from the North Fork Licking River (Morrow County, OH, U.S.A., 40°11.673'N, 082°42.488'W) on 2 June 2008. All individuals used in the sperm competition trials were from the same population.

Holding each individual upside down, we collected milt or egg samples after drying the body surface with a lint-free delicate task wiper. The milt was collected and processed in the same manner as described above. A subsample of the female's eggs were collected by applying gentle pressure to her abdomen, while holding her over a small circular petri dish (55 mm diameter, 13 mm depth). Milt and eggs were kept in a cooler for transport back to the laboratory for sperm quality assessment (see above for details) and sperm competition trials. Fin clips were collected from all adults and preserved in 95% ethanol for paternity assignment genotyping.

Sperm competition trials were conducted in the same small petri dishes that eggs were initially collected in. Males were paired so that the difference in storage time of their respective milt samples was minimized. We simultaneously applied 2  $\mu$ l of milt from each of the two males to the same place in the egg mass (from one female) using two micropipettes. Stream water from the site of collection was then poured to the same level in all dishes ( $\sim$  18 ml). Petri dishes were then covered and left for 10 min to allow fertilization to occur; sperm longevity is known to have a mean of 34 s in this species (Pitcher et al. 2009a). Next, the eggs were transferred to rearing tanks (see below) modified from the design of Barber & Arnott (2000).

At the completion of the sperm competition trial, each batch of eggs was gently poured into an individual submerged egg tray. The trays consisted of short cylinders of transparent Plexiglas (7.5 cm

interior diameter, 2.5 cm tall sides) with bottoms covered with 400  $\mu\text{m}$  mesh to allow water circulation around the eggs. These trays were then placed in a stand designed to hold them in the centre of the tank, above an airstone to ensure that highly oxygenated water would circulate around the eggs; water tension prevented the air bubbles from passing directly through the mesh, so the bubbles were moved to the side, leaving the eggs undisturbed. There were two such set-ups per rectangular tank, with an extremely fine mesh (150  $\times$  150  $\mu\text{m}$ ) secured between them to prevent larvae from crossing between sides. Half-tank water changes were conducted at approximately 8 h intervals throughout rearing. Eyespots were visible to the naked eye approximately 24 h postfertilization, and larvae began to hatch approximately 48 h postfertilization. Fertilized eggs (that had visible eyespots) were collected from each family prior to hatching and prior to any mortality, which could affect the paternity estimates for each male in competition if the embryo viability of either male's offspring differed (see Garcia-Gonzalez 2008). Offspring samples were preserved in 95% ethanol for subsequent DNA extraction.

DNA was extracted from adult fin clips (two males and one female per competition trial) and all of the larvae dissected from the available eggs. Fin clips or larvae were dried of ethanol and placed in 96 well plates with digestion buffer and proteinase K solution before being incubated at 37 °C and agitated gently overnight. Extraction was then performed using a Janus Automated Liquid Handling System (Perkin Elmer Life and Analytical Sciences, Dowers Grove, IL, U.S.A.) following the protocol of Elphinstone et al. (2003). Paternity of offspring was determined using four polymorphic microsatellite markers recently developed for reddsides (Pitcher et al. 2009c); RSD 42A (dinucleotide repeat motif), RSD-86 (dinucleotide repeat motif), RSD2-91 (tetranucleotide repeat motif) and RSD-142 (tetranucleotide repeat motif). The loci were amplified using polymerase chain reaction with the following protocol: denature for 2 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 30 s annealing at 58 °C (53 °C for RSD-142), 30 s extension at 72 °C, then a final extension step of 2 min at 72 °C. Fluorescently labelled primers were used and the fragment sizes determined by polyacrylamide gel electrophoresis using a Licor 4300 DNA Analyzer system. Allele size was estimated using Gene ImagR (version 4.05) software. In all cases we were able to identify alleles in the offspring that were diagnostic for paternity identification; thus, paternity was determined via both exclusion of one of the two possible males and positive inclusion of the other males. Paternity was assigned to an average of 27 offspring (range 10–51) for the nine sperm competition trials. Paternity was measured for each male by dividing the number of offspring he sired by the total number of offspring genotyped.

### Statistical Analyses

Because the area of red spawning coloration on males was a function of their body area (Spearman correlation:  $r_s = 0.81$ ,  $N = 50$ ,  $P < 0.001$ ), the residual area of red spawning coloration was used as an index of how much red spawning coloration males possessed controlling for body size. We estimated the residual area of red spawning coloration using the least squares residuals of the regression of the log of red coloration area on the log of the total area of the body. We used a MANOVA to examine which of the candidate phenotypic traits (standard length, body mass, condition, residual area of red spawning coloration, and the hue, saturation, brightness of the red spawning coloration) showed significant sexual dimorphism or dichromatism. Next, we assayed whether females showed a preference for any of the significant sexually dimorphic traits using standard tests associated with dichotomous choice trials, binomials for the number of females that spent more

time with the more ornamented male, and one-sample *t* tests (with the null hypothesis set to 0.50 or 50%) for preference strength (quantified as the proportion of time that the female spent in the non-neutral zone with the more ornamented male). Finally, Spearman correlations were used to assess the relationship between male ornamentation traits and each of the males' sperm quality metrics (i.e. sperm velocity, longevity and density).

For the sperm competition trials, we examined the relationships between sperm quality metrics (velocity, longevity and density) and each male's paternity share. We did not examine the relationship between paternity and male redness because spectral properties were not available for the males used in the in vitro sperm competition trials. We arbitrarily subtracted the percentage paternity and values of sperm quality (sperm velocity, longevity and density) of the male with the higher ID number (ID numbers were arbitrarily assigned based on the order in which males were collected) from that of the male with the lower ID number, resulting in relative measures of traits (e.g. differences in sperm velocity and differences in paternity). Because paternity estimates are more accurate when more offspring are examined (e.g. Neff & Pitcher 2002) and because the number of offspring to which we could assign paternity varied between pairs of males, the relationships between the differences in sperm traits and the differences in paternity were examined collectively using a weighted multiple linear regression (see Pitcher et al. 2003). Similar results were also found when the nonweighted regressions were used (data not shown).

## RESULTS

There was no significant sexual dimorphism with respect to standard body length ( $F_{1,55} = 0.20$ ,  $P = 0.66$ ), body mass ( $F_{1,55} = 0.13$ ,  $P = 0.72$ ), condition factor ( $F_{1,55} = 1.70$ ,  $P = 0.19$ ) or the hue of the red spawning coloration ( $F_{1,55} = 2.94$ ,  $P = 0.09$ ) (see Table 1). By contrast, there was significant sexual dimorphism in the saturation ( $F_{1,55} = 8.35$ ,  $P = 0.006$ ), brightness ( $F_{1,55} = 18.19$ ,  $P < 0.001$ ) and the residual area ( $F_{1,55} = 18.55$ ,  $P < 0.001$ ) of red spawning coloration, with males having larger values than females for saturation and residual area and lower values for brightness (i.e. males are 'darker' than females) (see Table 1).

During the dichotomous mate choice trials, females spent on average 73.7% of the time (on average 14.8 min of the 20 min behavioural trial, range 4.8–19.9 min) associating with one of the two males (i.e. time spent in the preference zones). The preferences of focal females varied widely, ranging from some females preferring the more ornamented male to other females preferring the less ornate males. The number of females that spent more time with the

**Table 1**

Mean, standard deviation and range (minimum–maximum) of trait values in male ( $N = 50$ ) and female ( $N = 7$ ) reddsides

Trait	Mean $\pm$ SD (range)	
	Male	Female
Standard length (cm)	7.6 $\pm$ 0.3 (6.8–8.2)	7.6 $\pm$ 0.3 (7.3–8.2)
Mass (g)	5.58 $\pm$ 0.64 (4.36–7.18)	5.49 $\pm$ 0.48 (5.00–6.24)
Condition	1.28 $\pm$ 0.09 (1.15–1.53)	1.22 $\pm$ 0.09 (1.10–1.33)
Brightness (%)	19.1 $\pm$ 9.4 (6.7–56.9)	35.0 $\pm$ 7.9 (28.0–51.2)
Saturation (%)	1.94 $\pm$ 0.67 (0.8–3.9)	1.16 $\pm$ 0.61 (0.3–2.2)
Hue (nm)	598.1 $\pm$ 6.5 (585–616)	593 $\pm$ 12.6 (574–608)
Residual red spawning coloration area (%)	20.0 $\pm$ 2.0 (15–23)	17.0 $\pm$ 1.0 (15–18)

Residual red spawning coloration area is shown here as a percentage of the body covered in red; however, in all analyses, we used residuals derived from the regression of area of red spawning coloration on overall body surface area (see text for details).

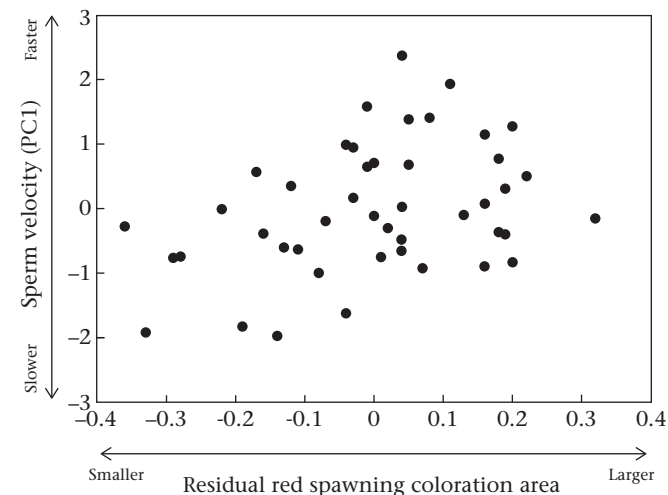
**Table 2**  
Mean, standard deviation and range (minimum–maximum) for sperm traits of reddsided dace ( $N = 45$ )

Trait	Mean	SD	Range
Path velocity ( $\mu\text{m/s}$ )	143.6	21.7	100–188
Straight line velocity ( $\mu\text{m/s}$ )	95.4	29.7	12.8–172.9
Point-to-point velocity ( $\mu\text{m/s}$ )	165.3	17.7	129.7–196.2
Longevity (s)	34.0	4.9	27–51
Density per ml ( $\times 10^6$ )	11.1	4.0	4.4–21.4

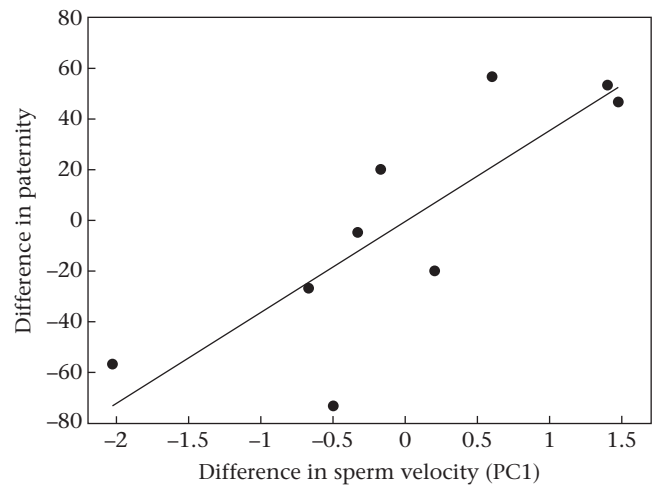
more ornamented male did not differ from chance for any of the dimorphic traits examined (binomial tests: residual red spawning coloration: 8/19,  $P = 0.14$ ; saturation: 13/19,  $P = 0.052$ ; brightness: 7/19,  $P = 0.10$ ). In addition, females showed no significant preference based on the proportion of association time for either the more colourful male in the dichotomous choice test for any of the dimorphic traits examined (one-sample  $t$  tests (null hypothesis = 50%): residual red coloration: mean =  $0.51 \pm 0.04$ ,  $t_{18} = 0.23$ ,  $P = 0.82$ ; saturation: mean =  $0.56 \pm 0.04$ ,  $t_{18} = 1.49$ ,  $P = 0.15$ ; brightness: mean =  $0.51 \pm 0.04$ ,  $t_{18} = 0.23$ ,  $P = 0.82$ ). No significant preferences (using binomial and one-sample  $t$  tests) were found for traits that were not dimorphic (i.e. length, condition and hue), and the preference-related results did not differ when we controlled for body size (length) or condition (data not shown).

Residual red spawning coloration area among males was significantly related to sperm velocity ( $r_s = 0.36$ ,  $N = 45$ ,  $P = 0.014$ ; see Fig. 4) and marginally related to sperm density ( $r_s = 0.28$ ,  $N = 45$ ,  $P = 0.06$ ), but was not significantly related to sperm longevity ( $r_s = 0.23$ ,  $N = 45$ ,  $P = 0.13$ ). The hue, saturation and brightness of male red spawning coloration was not significantly related to sperm velocity (all  $P > 0.10$ ), density (all  $P > 0.59$ ) or longevity (all  $P > 0.38$ ). Results of correlations of male traits (ornamentation and body size metrics) versus individual sperm traits (path velocity, straight line velocity, point-to-point velocity, longevity, density) are given in the Appendix (Table A1).

Multiple regression (all  $\beta$  values are standardized; overall model:  $r^2 = 0.85$ ,  $F_{2,8} = 9.49$ ,  $P = 0.017$ ) showed that the relative sperm longevity ( $\beta = -0.18$ ,  $P = 0.46$ ) and relative sperm number ( $\beta = 0.30$ ,  $P = 0.19$ ) were not significantly associated with sperm competition success. However, relative sperm velocity was positively and significantly related to sperm competition success, as measured by each male's relative share of paternity in the in vitro competitive fertilization trials ( $\beta = 0.69$ ,  $P = 0.02$ ; Fig. 5).



**Figure 4.** Relationship between residual red spawning coloration area and sperm velocity in reddsided dace (see text for details).



**Figure 5.** Partial regression plot (based on multiple regression analysis, see text for details) showing the relationship between fitted partial residual differences in sperm velocity and differences in paternity in reddsided dace.

## DISCUSSION

Using mate choice trials, spectral and sperm analyses and paternity assignment, we found that red spawning coloration in male reddsided dace is not subject to sexual selection arising from prespawning female choice but is indirectly correlated with male–male postspawning competition. First, we documented sexual dimorphism in the relative amount of red spawning coloration as well as the spectral properties (saturation and brightness) of the red spawning coloration, with males being more ornamented than females. Second, we found that females did not favour males that had relatively larger red spawning coloration areas or spawning coloration with longer wavelength hues. Third, we found that males with relatively more red spawning area would likely be favoured in male–male postspawning sperm competition because they possessed sperm with higher velocity, and sperm velocity was determined to be the key predictor of sperm competition success as assessed by paternity in our in vitro sperm competition trials.

We documented sexual dimorphism in the saturation, brightness and relative area of red spawning coloration, as well as a nearly significant difference in hue, with males showing a greater degree of trait elaboration. Our findings suggest that these traits are most likely secondary sexual characters. However, we found no sexual dimorphism in body size in reddsided dace, which is consistent with Pyron's (1996) phylogenetic examination of 58 species of North American minnows. Pyron (1996) found that the main ecologically relevant variable related to sexual size dimorphism was territory guarding behaviour, a behaviour that does not appear to be present in the reddsided dace mating system (T. E. Pitcher, personal observation).

Females showed no behavioural preference for males with relatively more red spawning coloration or spawning coloration that was brighter, more saturated or had higher hue values. This finding is in contrast to other mate choice studies on other minnows such as *Phoxinus phoxinus*, where females prefer more colourful males (Kekalainen et al. 2010). The lack of female preference for red spawning coloration in reddsided dace may arise because of differences in its mating system compared to other minnow species. For example, reddsided dace males do not defend a territory and do not display in leks, as do most other minnows (see Pyron 1996). There is also the possibility that our female choice data may describe association preference only, and not actual

mating intent, but this seems unlikely for a variety of reasons. First, we performed the behavioural trials when males and females were spawning (individuals all possessed bright red spawning coloration and breeding tubercles), suggesting that mating was most likely a priority for both sexes at the time. Second, it has been shown in several fish species that a female's visual response in dichotomous trials can be a good predictor of a male's subsequent reproductive success (e.g. Kodric-Brown 1993; Walling et al. 2010). It is also possible that the statistical tests of the behavioural data did not have enough power to detect a relationship between male coloration metrics and female preference measures. However, our sample size ( $N = 19$ ) was larger than that of another study of minnows ( $N = 16$ , Kekalainen et al. 2010) that also used dichotomous choice behavioural data and found that females spent significantly more time on the side of the more colourful (redder) males using both a binomial test and  $t$  test.

We determined that sperm velocity was the primary determinant of sperm competition success in our competitive in vitro fertilization trials, such that males with faster sperm achieved higher levels of paternity than their competitors. Consistent with other studies (e.g. Gage et al. 2004; Linhart et al. 2005), this finding suggests that sperm competition in the reddsides mating system operates as a loaded raffle (Parker 1990), where the quality, rather than simply the number, of each male's sperm matters. Males with high-velocity sperm can have relatively higher paternity because their sperm will travel longer distances per unit time, and as such, will make more egg contacts per unit time (see Butts et al. 2009). Another reason sperm velocity is believed to be an important factor in teleost sperm competition success is the presence of a micropyle for fertilization. Teleost sperm have no mechanism for penetrating the cell membrane directly and are therefore required to enter the egg by using a canal, the micropyle, through the plasma membrane (Iwamatsu et al. 1991). In chum salmon, *Oncorhynchus keta*, it has been shown that the order of entry of sperm into the micropyle is an important determinant of fertilization success (Kobayashi & Yamamoto 1981). Higher-velocity sperm most likely have a higher probability of reaching the micropyle first, which is consistent with our findings. However, our paternity results need to be interpreted with some caution because the sperm from both males were applied in a controlled manner to the eggs (i.e. the position and timing were equalized), which is rarely the case in nature. During spawning, certain males are often able to position themselves closer to the female than others, and some may release sperm more quickly or in better synchrony with the timing of egg release (e.g. Stoltz & Neff 2006). For example, Yeates et al. (2007) showed that a delay of 2 s in releasing sperm reduces second-male fertilization success to 30% from an expected 50% in Atlantic salmon, *Salmo salar*. Therefore, future research should aim to observe spawning behaviour of reddsides dace, and replicate the position and timing effects observed in the wild in the laboratory to determine the relative importance of position, timing and sperm quality.

We found an indirect link between sperm competition success and male ornamentation: males in the experimental in vitro fertilization trials with faster sperm velocity were more likely to have a higher share of paternity and males from the field with more residual red spawning coloration area had sperm with higher velocity on average. We also found a marginally significant relationship between sperm density, which was not related to sperm competition success, and residual red spawning coloration area. However, the reason behind these associations between red spawning coloration and sperm-related traits remains unclear. There are several plausible explanations for this finding. First, the relative area of red spawning coloration in reddsides dace may be directly linked to carotenoid-based antioxidant resources that are presumed to affect sperm quality (reviewed in Blount et al. 2001).

The red spawning coloration of the reddsides dace is composed of carotenoid pigments (T. E. Pitcher, unpublished data), which several studies have linked to sperm quality. For example, darker carotenoid coloration is related to higher sperm velocity in coho salmon, *Oncorhynchus kisutch* (Pitcher et al. 2009b), as well as mallards, *Anas platyrhynchos* (Peters et al. 2004). The most prevalent hypothesis linking carotenoid coloration to sperm quality involves the role of carotenoids as antioxidants in the body. Both sperm and secondary sexual characters may be vulnerable to oxidative damage from free radicals, while the organism can only draw from a limited pool of antioxidants to mitigate this damage. Blount et al. (2001) therefore suggested that males who have a carotenoid pool that allows them to present more colourful ornaments can also better protect their sperm from oxidative damage or mutation. Second, it is possible that there is a genetic correlation between the area of red spawning coloration and sperm quality traits. This relationship could arise if red spawning coloration and sperm velocity are both pleiotropic consequences of some shared underlying genes. The area of carotenoid-based sexual coloration in other fishes, for example guppies, is Y-linked (Houde 1992), and may therefore be associated with sperm-related genes (see Roldan & Gomendio 1999). Third, a link between sperm velocity and red spawning coloration area may also be mediated by genetic load arising from inbreeding depression; ornament size is sometimes negatively correlated with inbreeding depression (e.g. van Oosterhout et al. 2003). Likewise, sperm traits have a genetic basis in many taxa (e.g. Woolley & Beatty 1967; Morrow & Gage 2001), and sperm abnormalities have often been associated with inbreeding depression (O'Brien et al. 1985; Wildt et al. 1987; Roldan et al. 1998; Gomendio et al. 2000; Margulis & Walsh 2002). Finally, it is also possible that some of the association between area of red spawning coloration and sperm velocity in reddsides dace is developmental. Differences in sperm number and quality have been found in relation to ontogeny in many taxa (e.g. Calvo et al. 1999; Ceballos-Vazquez et al. 2003; Green 2003), including fishes (e.g. Evans et al. 2002).

In conclusion, we combined several methods of measuring sexual selection in one mating system rather than the typical measure of either female choice or male–male competition. All of these methods consistently demonstrated that females do not appear to show preference for spectral properties or the amount of male ornamentation, and that male reddsides dace with relatively more ornamentation possess higher-velocity sperm on average, which in experimental competitive trials, was shown to be a primary determinant of sperm competition success. These results suggest that, in this mating system, selection arising from pre-spawning female choice does not appear to maintain male ornamentation and that post-spawning selection via male–male competition is primarily determined by sperm velocity, which is correlated with the relative amount of red spawning coloration.

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## Appendix

**Table A1**

Correlation matrix for individual sperm-related metrics (including the three sperm velocity metrics used to create the principal component for sperm velocity, PC1) in relation to ornamentation and body size metrics in the redbreast dace ( $N = 45$  in all cases)

Sperm metrics	Ornamentation/body size metrics						
	Brightness	Saturation	Hue	Residual red area	Standard length	Mass	Condition
Path velocity							
$r_s$	0.08	0.30	0.05	0.31	0.12	0.18	0.05
$P$	0.60	0.04	0.72	0.03	0.42	0.23	0.76
Straight line velocity							
$r_s$	0.11	0.11	0.11	0.46	-0.02	0.09	0.15
$P$	0.49	0.46	0.45	0.002	0.89	0.57	0.32
Point-to-point velocity							
$r_s$	0.01	0.18	0.03	0.15	0.17	0.16	-0.06
$P$	0.94	0.22	0.86	0.34	0.28	0.30	0.69

See text for details regarding each variable.