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# Sexually asymmetric colour-based species discrimination in orangethroat darters

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Female mate preferences, male mate preferences and male–male competition can all potentially play roles in promoting speciation by sexual selection. The orangethroat darter, *Etheostoma spectabile*, is a sexually dichromatic fish in which males compete for access to females. We quantified male and female responses to conspecific versus heterospecific fish under normal light, dim light and blue light, the last of which impaired colour perception. We found that behavioural isolation was likely mediated primarily by male behavioural discrimination against heterospecific fish, with females showing no evident mate preferences. Furthermore, male aggression towards conspecific rivals was reduced in blue light, suggesting that the evolution of male coloration may contribute to speciation through male–male interactions.

Sexual selection has long been hypothesized to drive speciation through coevolution of male ornaments and female preference, which results in behavioural isolation when two populations diverge in both traits to such a degree that females of one population no longer recognize males of the other population as potential mates (Fisher, 1930; Lande, 1981). Under traditional sexual selection theory, females are expected to show conspecific mate recognition for the same reason that they are expected to be choosy in selecting mates: female gametes are more costly to produce than male gametes, hence the cost of making a 'mistake' with a mate of inferior quality or incorrect species should be correspondingly higher for females than for males (Bateman, 1948; Wirtz, 1999).

However, male preferences for females have also been reported from multiple taxa, and may be selectively advantageous if males invest heavily in reproductive behaviours (e.g. parental care; Bonduriansky, 2001). Just as choosy females should be reticent to mate with heterospecific males due to negative fitness consequences, choosy males may similarly be predicted to discriminate against heterospecific females; as a result, the evolution of behavioural isolation may rely on both sexes (Kozak, Reisland, & Boughmann, 2009; O'Rourke & Mendelson, 2010; Svensson, Karlsson, Friberg, & Eroukhmanoff, 2007). Further complicating the picture is male–male competition, which is increasingly recognized as a selective force underlying male ornament diversification (West-Eberhard, 1983). By promoting male trait divergence and/or modulating the effect of female preferences, male–male competition may directly contribute to speciation (Dijkstra & Groothuis, 2011; Hunt, Breuker, Sadowski, & Moore, 2009; Seehausen & Schluter, 2004). These three forces (female mate preferences, male mate preferences and male–male competition) may all interact to shape the form of selection on sexually dimorphic traits and behavioural isolation, and ideally all three should be considered when examining how sexual selection can drive speciation.

Darters (Percidae: Etheostomatinae) are a group of North American freshwater fishes that show high diversity (approximately 250 species) and widespread sexual dichromatism (Near et al., 2011; Page, 1983). Behavioural isolation appears to be the most important reproductive barrier between darter species, evolving faster and farther towards completion than other isolating mechanisms such as postzygotic hybrid inviability (Mendelson, 2003). Previous research suggests that behavioural isolation may

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be based on preferences for species-specific male coloration; both male and female *Etheostoma zonale* and *Etheostoma barrenense* prefer to associate with fish models painted in conspecific colours (Williams & Mendelson, 2011, 2013). Darters thus represent potentially fruitful subjects for understanding how the diversification of male coloration may facilitate speciation.

The orangethroat darter, *Etheostoma spectabile*, and the rainbow darter, *Etheostoma caeruleum*, are members of the subgenus *Oligocephalus* and are similar in morphology, behaviour and ecology. Males of both species show blue-green and orange-red breeding coloration. The most obvious visual difference between male *E. spectabile* and *E. caeruleum* is the presence of an orange-red colour patch on the anal fin of the latter. They have widely overlapping ranges in the eastern United States and often co-occur in close proximity (Kuehne & Barbour, 1983; Page, 1983). Both species spawn during the spring and lack parental care; males are nonterritorial and follow gravid females while attempting to drive away conspecific rivals. Females that are ready to spawn bury themselves shallowly in the substrate; with the arrival of one or more males, the fish release eggs and sperm (Winn, 1958). There is little evidence for inviability in *E. spectabile*–*E. caeruleum* hybrids, at least in the F1 generation (Hubbs & Strawn, 1957; Linder, 1958). Nevertheless, reproductive isolation between these species is heightened to near-completion in sympatry relative to allopatry, suggesting that there is selective pressure against heterospecific mating (Zhou & Fuller, 2014). Female *E. spectabile* show no preference for more colourful males or for conspecific males in dichotomous choice trials (Pyron, 1995; Zhou & Fuller, 2012). Sexual selection on male coloration, and any resultant effects on behavioural isolation, may therefore occur via alternative processes to female preference, such as male–male competition.

## METHODS

We conducted a series of behavioural observation trials to assess (1) the relative contributions to sexual selection and behavioural isolation by male and female *E. spectabile*, and (2) the signalling role of male coloration in within- and across-species interactions. To determine whether male coloration encodes behaviourally relevant information, we used coloured lighting to disrupt colour perception by the fish. Similar behavioural experiments using lighting manipulation have been conducted in guppies, *Poecilia reticulata* (Long & Houde, 1989), threespine sticklebacks, *Gasterosteus aculeatus* (Brooks & Endler, 2001; Milinski & Bakker, 1990) and cichlids (*Haplochromis nyererei* complex; Seehausen & van Alphen, 1998), wherein female preferences for a particular colour expressed by the males could be eliminated using lighting conditions that impeded the perception of that colour. As a prerequisite step for informing our lighting treatments, we investigated the visual pigments of *E. spectabile* and *E. caeruleum* via microspectrophotometry.

### *Microspectrophotometry*

Adult *E. spectabile* ($N = 2$ females) and *E. caeruleum* ($N = 1$ female, 2 males) were collected by seine net from a tributary of the Embarras River (Douglas Co., IL, U.S.A.) in June 2009 and transported to Cornell University (Ithaca, NY, U.S.A.). Microspectrophotometry was performed following the methods described in Loew (1994). The fish were dark-adapted for at least 12 h and then euthanized by cervical transection under infrared light. The eyes were enucleated, hemisected and placed in Sorensen's phosphate buffer (pH 7.2) with 6% sucrose, whereupon the retinas were separated from the pigment epithelium with razor blades and tungsten needles. Pieces of retina were sandwiched between two coverslips sealed with grease and transferred to the microspectrophotometer.

The measurement of absorbance has been described in detail elsewhere (Loew, 1994; Provencio, Loew, & Foster, 1992). Briefly: absorbance was recorded from single cone and rod cells in 1 nm increments from 750 to 300 nm and then back to 750 nm. The data were smoothed using the ‘smooft’ digital filtering routine (Press, Flannery, Teukolsky, & Vetterling, 1987) and the smoothed spectrum was then overlaid with the unsmoothed spectrum and inspected by eye to ensure no shift in the apparent maximum. The absorbance maximum ($\lambda_{max}$) was determined by fitting vitamin $A_1$ and $A_2$ template curves from Lipitz and Cronin (1988), using the Mansfield's method as described in MacNichol (1986). Data that did not meet the selection criteria given in Loew (1994) were disregarded. We averaged the $\lambda_{max}$ values of the three cell types from each individual.

### *Experimental Observations*

Adult *E. spectabile* and *E. caeruleum* were collected by seine net (dimensions 106 × 122 cm) from three adjacent tributaries of the Salt Fork River, Illinois in April and May of 2013, during the breeding season of these species. Both species were encountered at all three sites during the collection period. The fish were maintained in group tanks segregated by species and sex, at a temperature of 20 °C and a 13:11 h light:dark cycle. Fish were fed frozen bloodworms (chironomid larvae) daily; behavioural observations were performed prior to feeding on that day.

Behavioural trials took place in a 38-litre aquarium (bottom area 25 × 50 cm) with naturalistically coloured gravel substrate. Illumination was provided by six 15 W ‘daylight’ fluorescent tubes (Damar F15t/8d) positioned directly above the tank. Three light quality treatments were used: control (no filter), grey and blue. The grey treatment was achieved by passing the overhead light through four 0.6 ‘Neutral Density’ filter sheets (LEE Filters). Similarly, the blue lighting treatment was achieved via a single ‘Winter Blue’ colour filter sheet (LEE Filters). The blue lighting treatment was designed to suppress stimulation of the long-wavelength-sensitive cones in the retina (see Results), and thus interfere with the fish's ability to discern the orange and red components of male breeding coloration.

To assess the effectiveness of the lighting filters, we measured the downwelling irradiance using an Ocean Optics USB 2000 spectrophotometer connected to a 400 μm diameter patch cord connected to a cosine corrector. A calibrated deuterium-halogen 2000 lamp (Ocean Optics) was used to calibrate the spectrophotometer, and data were taken using SpectraSuite software. The grey filter yielded light with a similar profile to the control at much lower intensity, whereas the blue filter virtually eliminated wavelengths above 530 nm while allowing through blue light of 400–530 nm (Fig. 1). We multiplied the irradiance and the absorbance of the two cone types from 400 to 600 nm, and summed the area beneath the two curves to obtain a rough estimate of the total light available to the fish's eye in the different light quality treatments. The amount of available light under the grey filter was approximately 0.3% that of the control, whereas the available light under the blue filter was approximately 4% that of the control. Hence, the grey filter allowed through much less light overall than the blue filter, but with similar spectral characteristics to the control.

To further verify that the blue filter selectively obscured the orange-red components of male mating coloration, we used the Ocean Optics spectrophotometer to measure the coloration of a single male *E. spectabile*. The fish was first anaesthetized in a 0.03% tricaine methanesulfonate (MS-222) solution, which has been used

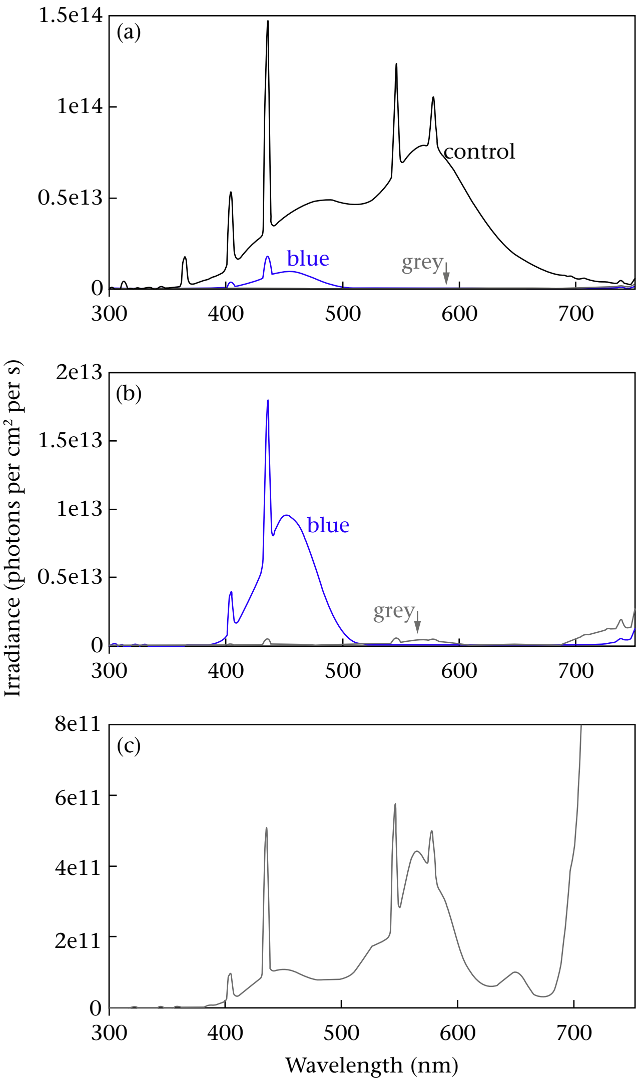

**Figure 1.** Irradiance profiles for (a) all three light quality treatments, (b) blue and grey lighting and (c) grey lighting only.

in previous studies of darter coloration (Gumm & Mendelson, 2011). Reflectance data were taken using a reflectance probe held at a 45° angle to the sampled surface, and coupled with a deuterium-tungsten light source. We took four measurements each from the blue-green centre of the anal fin and the orange/red bars in the second dorsal fin (Fig. 2). Given that blue-green reflects predominantly below 530 nm whereas orange-red reflects predominantly above it, the blue filter should have been effective in preventing the fish from perceiving the latter.

We tested the effects of light quality (control, grey or blue) and rival male identity (conspecific or heterospecific) on darter reproductive behaviour in a 3 × 2 factorial design (Fig. 3). Thus, behavioural trials were conducted in sets of six. A single behavioural trial involved three fish: a focal male *E. spectabile*, a rival male (either an *E. spectabile* or an *E. caeruleum*) and a gravid female *E. spectabile*. Thus, the same focal male and female were used in all six trials, whereas the two rival males were each used for three trials. The rival male *E. spectabile* were selected to be subdominant to the focal male *E. spectabile*. Relative dominance was determined prior to the experiment via observations of male interactions in the group tanks; when two given males met each other, the male that gave way over multiple encounters was assessed as subordinate to the other. The results of our behavioural trials upheld these dominance assessments, in that rival male *E. spectabile* never performed more reproductive or aggressive behaviours than focal male *E. spectabile*.

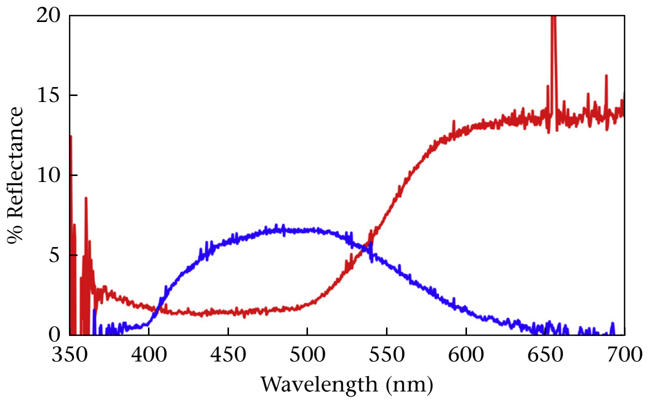

**Figure 2.** Average reflectance spectra for the blue-green in the anal fin (blue line) and the orange-red in the second dorsal fin (red line), from a male *Etheostoma spectabile*.

Before the trials began, the focal male was placed in the observation tank and allowed to acclimate for 1 h. Each behavioural trial began with the introduction of a rival male and female and lasted 30 min. The six light quality/rival male identity treatments were presented in random order, with 5 min between trials under normal light. We were able to use the same female for all six trials because a female *E. spectabile* will engage in many successive spawning bouts over a period of up to several days, releasing only a few eggs per bout until her clutch is expended (Winn, 1958; M. Zhou, personal observation). Thus, females could and did spawn throughout the 3 h time span of a behavioural trial set. We tested 12 focal males, for a total of 72 behavioural trials. The female did not spawn in 3 of the 12 sets of trials; excluding these trials from our analyses did not qualitatively change the results.

### *Behavioural Assays*

We scored three measures of male–female interaction (female pursuit, nosedigs and spawning) and two measures of male–male interaction (fin flares and attacks) for each behavioural trial.

#### *Male–female interactions*

(1) Female pursuit was assessed as follows: the trial was divided into 30 s blocks, and a male was recorded as having followed the female during a block if he remained within one body length of the

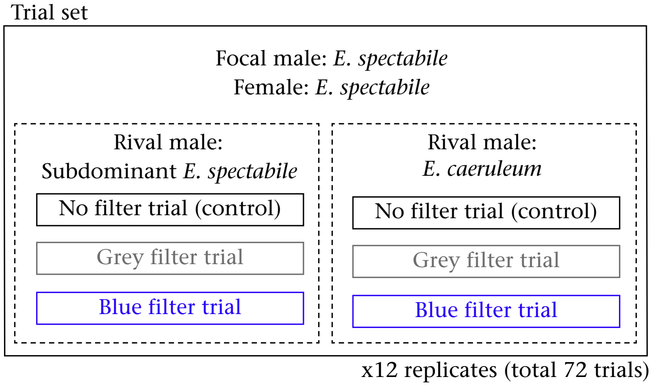

**Figure 3.** Diagram of experimental set-up. Behavioural trials were conducted in sets of six, and 12 sets of trials were performed in total. Within a set, the focal male *Etheostoma spectabile* and female *E. spectabile* were reused for all six trials, while the rival male *E. spectabile* and *Etheostoma caeruleum* were reused for three trials each.

female for at least five consecutive seconds. Female pursuit was then scored as the proportion of blocks (out of 60 total) in which the male followed the female.

(2) Nosedigs are a behaviour in female *E. spectabile* and *E. caeruleum*, wherein the female digs head-first into the substrate. As nosedigs are typically performed only in the presence of a male and closely precede spawning, they represent a direct measure of female preference. We recorded the identity of male(s) present within one body length for all nosedigs.

(3) Spawning involves the female burying herself shallowly in the substrate, whereupon she is joined by one or more males and they release eggs/sperm. For each spawning bout, the identity of the male(s) involved was recorded. If only one male participated in the spawning, then he was given a score of ‘1’; if both males participated, the male that initiated spawning was given a score of ‘0.75’ and the male that secondarily joined the spawning (the sneaker) was given a score of ‘0.25’. These scores were then summed to produce a per-trial spawning score for each male. Instances in which the female adopted a spawning position but was not subsequently joined by a male (due to interference from the rival male) were recorded as ‘incomplete’ and excluded from analyses.

### *Male–male interactions*

(1) Fin flares are an intrasexual behaviour in male *E. spectabile* and *E. caeruleum* in which the first dorsal fin, and sometimes also the second dorsal fin and anal fin, are fully extended in response to the arrival of a rival male. We recorded the number of fin flares performed by each male during the trial.

(2) Attacks involve one male chasing and attempting to bite the other male. We recorded the number of attacks performed by each male during the trial.

### *Statistical Analyses*

We analysed the five behaviours assayed (female pursuit, fin flares, attacks, nosedigs received and spawning success) using general linear mixed models. First, we compared the behaviour of the focal male *E. spectabile* in trials with a conspecific rival male versus a heterospecific rival male. The factors included in the model were trial type (conspecific/heterospecific), light quality (control/grey/blue), and their interaction. For the analyses of nosedigs received and spawning success, we included female pursuit in the model as a covariate to control for the differing amount of time that each male spent close to the female. For the analysis of spawning success, we also repeated the analysis with nosedigs received as the covariate instead of female pursuit, so as to assess male reproductive success relative to female preference. The individual identity of the focal male was included as a repeated measure.

Second, we compared the behaviour of the focal male in the conspecific trials to that of the rival *E. spectabile*. The models used were the same as above, except with male identity (focal/rival) in place of trial type. Third, we compared the behaviour of the focal male in the heterospecific trials to that of the rival *E. caeruleum*. Again, we used the same model as above but with male species (*E. spectabile*/*E. caeruleum*) replacing trial type. The number of repeated measures per focal male was six for the first comparison, and three for the second and third comparisons. All analyses were performed in SAS (version 9.3, SAS Institute, Cary, NC, U.S.A.).

## RESULTS

### *Microspectrophotometry*

Both *E. spectabile* and *E. caeruleum* possessed single cones, double cones and rods (Fig. 4a). The single cones contained a

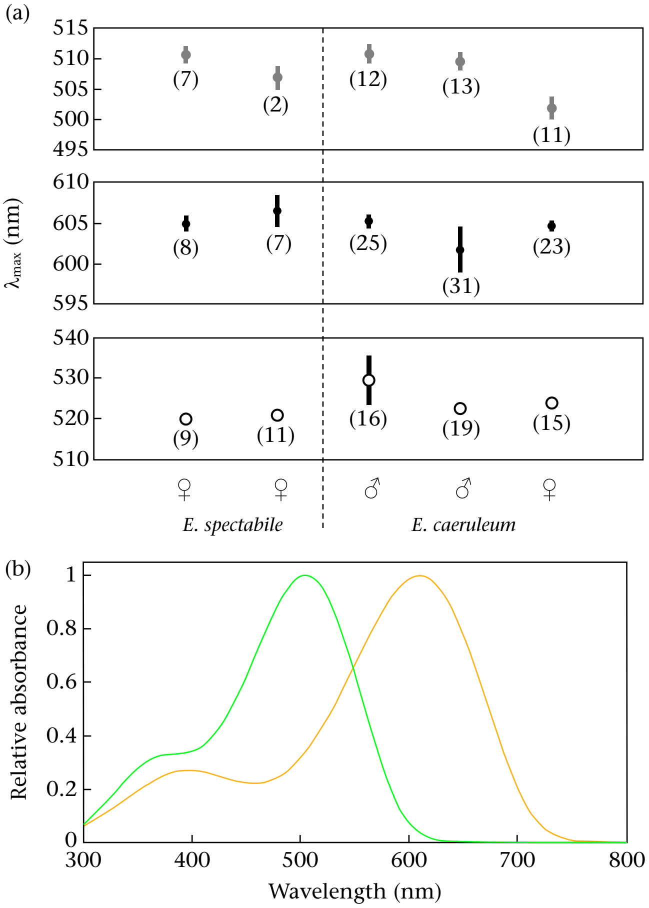

**Figure 4.** (a) Mean maximum absorbance (±SE) of medium-wavelength-sensitive (MWS) cones (grey circles, top), long-wavelength-sensitive (LWS) cones (black circles, middle) and rods (open circles, bottom) for each individual. The number of cells sampled is given in parentheses. (b) Relative absorbance spectra from an example of a MWS cone (green line) and an LWS cone (orange line).

middle-wavelength-sensitive pigment with a mean $\lambda_{max} \pm$ SE of 508.0 ± 2.8 nm (range 501.9–510.8 nm), whereas the double cones contained a long-wavelength-sensitive pigment with a mean $\lambda_{max} \pm$ SE of 604.7 ± 0.8 nm (range 601.8–606.6 nm) (Fig. 4b). The rods had a mean $\lambda_{max} \pm$ SE of 523.5 ± 1.7 nm (range 520.0–529.6 nm). In all samples, the $A_2$ template provided the best fit to the data. A two-cone visual system with middle- and long-wavelength-sensitive pigments is also found in other *Etheostoma* species (Gumm, Loew, & Mendelson, 2012).

### *Focal Male Behaviour*

Across all trials, focal male *E. spectabile* pursued and attempted to spawn with the female. The amount of time that focal males spent in proximity to the female did not differ between conspecific trials and heterospecific trials. Focal males also performed comparable numbers of fin flare displays to both subdominant male *E. spectabile* and male *E. caeruleum*. On the other hand, focal males vigorously attacked subdominant conspecific males to drive them away from the female, but seldom attacked heterospecific males (Table 1, Fig. 5a–c).

Simply reducing overall light intensity without changing spectral characteristics had little effect on male behaviour. Specifically, the behaviour of focal males did not differ between the control and grey light quality treatments. Under blue lighting, however, focal

**Table 1**
Comparison of focal male *Etheostoma spectabile* behaviour in the presence of a conspecific versus heterospecific rival male

| Effect | *df* | *F* | *P* |
|---|---|---|---|
| **Female pursuit** | | | |
| Trial type | 1, 11 | 0.26 | 0.622 |
| Light quality | 2, 22 | 5.93 | 0.009 |
| Trial type∗light quality | 2, 22 | 0.01 | 0.086 |
| **Fin flares** | | | |
| Trial type | 1, 11 | 1.03 | 0.331 |
| Light quality | 2, 22 | 2.81 | 0.082 |
| Trial type∗light quality | 2, 22 | 0.91 | 0.417 |
| **Attacks** | | | |
| Trial type | 1, 11 | 13.36 | 0.004 |
| Light quality | 2, 22 | 3.49 | 0.048 |
| Trial type∗light quality | 2, 22 | 2.11 | 0.146 |
| **Nosedigs received** | | | |
| Trial type | 1, 11 | 0.45 | 0.517 |
| Light quality | 2, 22 | 2.80 | 0.083 |
| Trial type∗light quality | 2, 22 | 0.09 | 0.913 |
| Female pursuit | 1, 54 | 44.03 | <0.0001 |
| **Spawning success** | | | |
| Trial type | 1, 11 | 0.09 | 0.768 |
| Light quality | 2, 22 | 1.35 | 0.279 |
| Trial type∗light quality | 2, 22 | 0.02 | 0.979 |
| Female pursuit | 1, 54 | 37.19 | <0.0001 |

males spent less time pursuing the female and directed far fewer attacks towards the subdominant male (Table 1, Fig. 5a, c). There was also a nonsignificant tendency towards fewer fin flares under blue lighting, particularly in trials involving a heterospecific male (Table 1, Fig. 5b).

### *Subdominant Male Behaviour*

Invariably, subdominant male *E. spectabile* also attempted to pursue and spawn with the female. Under control and grey lighting, subdominant males were attacked by the dominant male and consequently spent less time in proximity to the female. Under blue lighting, subdominant and dominant males spent similar amounts of time close to the female, although the male identity by colour filter interaction term for female pursuit was not significant. Not surprisingly, the subdominant male performed fewer fin flares and virtually no attacks towards the dominant male, a pattern that was consistent regardless of light quality (Table 2, Fig. 5a–c).

### *Heterospecific Male Behaviour*

Male *E. caeruleum* showed behavioural discrimination against *E. spectabile* of both sexes, irrespective of light quality. Male *E. caeruleum* spent much less time in proximity to the female than the male *E. spectabile*. Male *E. caeruleum* also performed fewer fin flares towards, and almost never attacked, male *E. spectabile* (Table 3, Fig. 5a–c). Heterospecific spawning was uncommon (7.7% of all spawning bouts during behavioural trials with a heterospecific rival male, including bouts in which the male *E. caeruleum* acted as a sneaker) but occurred between multiple individual fish.

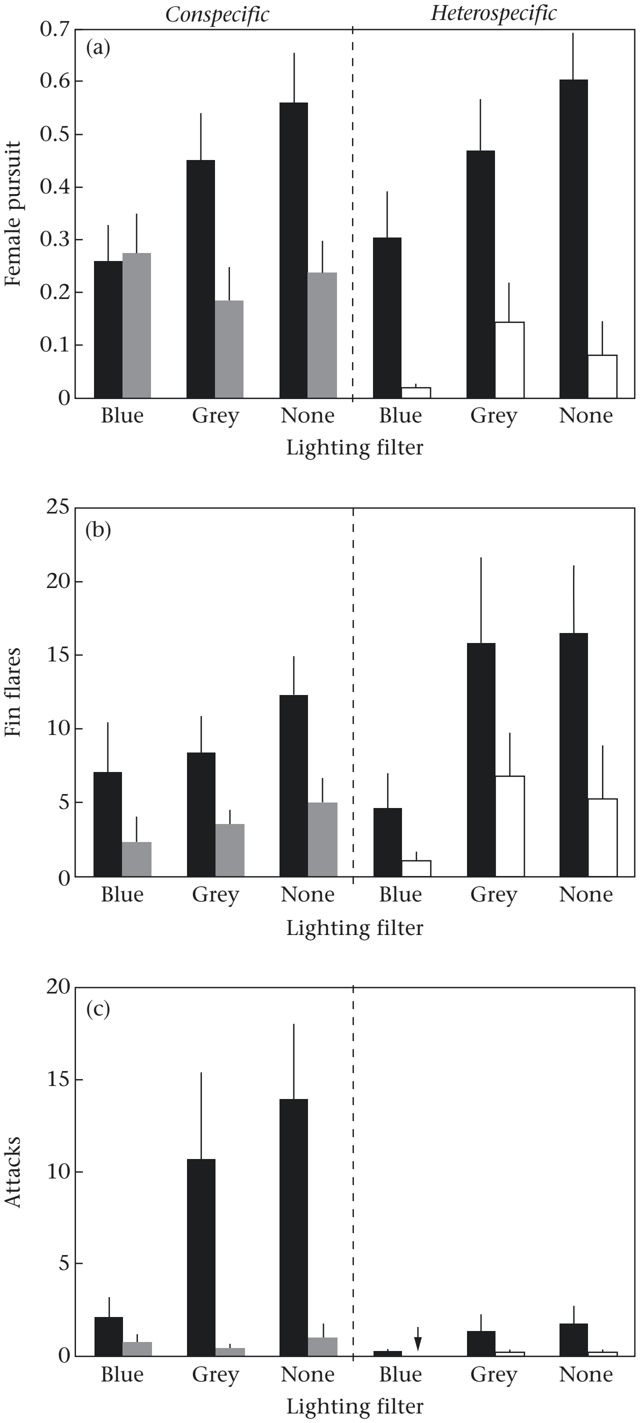

**Figure 5.** (a–c) Reproductive behaviours performed by focal male *Etheostoma spectabile* (black bars), subdominant male *E. spectabile* (grey bars) and male *Etheostoma caeruleum* (white bars) under the three light quality treatments.

### *Female Preference*

No evidence for female preference was found either within or between species. Across all trials, males received nosedigs in proportion to the amount of time they spent in proximity to the female; that is, dominant male *E. spectabile* received more nosedigs than subdominant conspecific males, and male *E. spectabile* received more nosedigs than male *E. caeruleum*. When time spent in proximity to the female was accounted for, there was no difference in the number of nosedigs received by dominant versus subdominant conspecific males, or by conspecific versus heterospecific males. Nosedig performance was not affected by light quality (Tables 2, 3, Fig. 6a).

Male reproductive success was proportional to the amount of female pursuit performed by each male. Once time spent in proximity to the female was taken into account, there was no difference in spawning score between dominant and subdominant conspecific males, or between conspecific and heterospecific males. Again,

**Table 2**
Comparison of focal male *Etheostoma spectabile* versus rival male *E. spectabile* behaviour

| Effect | *df* | *F* | *P* |
|---|---|---|---|
| **Female pursuit** | | | |
| Male identity | 1, 11 | 9.36 | 0.012 |
| Light quality | 2, 22 | 1.50 | 0.244 |
| Male identity∗light quality | 2, 22 | 2.80 | 0.083 |
| **Fin flares** | | | |
| Male identity | 1, 11 | 9.15 | 0.012 |
| Light quality | 2, 22 | 1.54 | 0.236 |
| Male identity∗light quality | 2, 22 | 0.21 | 0.814 |
| **Attacks** | | | |
| Male identity | 1, 11 | 15.13 | 0.003 |
| Light quality | 2, 22 | 2.89 | 0.077 |
| Male identity∗light quality | 2, 22 | 2.79 | 0.083 |
| **Nosedigs** | | | |
| Male identity | 1, 11 | 1.16 | 0.305 |
| Light quality | 2, 22 | 3.94 | 0.035 |
| Male identity∗light quality | 2, 22 | 0.39 | 0.681 |
| Female pursuit | 1, 54 | 67.24 | <0.0001 |
| **Spawning success** | | | |
| Male identity | 1, 11 | 4.08 | 0.068 |
| Light quality | 2, 22 | 2.62 | 0.095 |
| Male identity∗light quality | 2, 22 | 0.06 | 0.944 |
| Female pursuit | 1, 54 | 57.7 | <0.0001 |

there was no effect of light quality (Tables 2, 3, Fig. 6b). Repeating these analyses using nosedigs received as the covariate yielded qualitatively identical results (not shown), indicating that the number of nosedigs received by a male predicted his spawning success. Females, once in spawning position (buried in the gravel), always spawned with the first male to arrive regardless of whether that male had been actively pursuing her to that point.

## DISCUSSION

### *Visual System of Darters*

The retinas of adult *E. caeruleum* and *E. spectabile* contain rods, middle-wavelength-sensitive single cones and long-wavelength-sensitive double cones. As these species appear to be insensitive to short and ultraviolet (UV) wavelengths of light, the lack of UV irradiance during our behavioural observations should not have affected the results. A dichromatic visual system based solely on rods and middle- and long-wavelength-sensitive cones has also been reported from darters in the subgenus *Ulocentra* (Gumm et al., 2012), as well as from species of *Perca* and *Sander* (Ali, Ryder, & Anctil, 1977; Loew & Lythgoe, 1978), suggesting that all adult percids may share this system. Short-wavelength-sensitive cones are present in juvenile yellow perch, *Perca flavescens* (Loew & Wahl, 1991), and the same may be true in darters. The peak sensitivity of rods and long-wavelength-sensitive cones in *E. caeruleum* and *E. spectabile* are within the range of *Ulocentra* species, whereas the peak sensitivity of middle-wavelength-sensitive cones are slightly lower (Gumm et al., 2012). Variation in photoreceptor peak sensitivity among darter species may reflect variation in the spectral characteristics of species-specific male breeding coloration (Gumm & Mendelson, 2011).

**Table 3**
Comparison of male *Etheostoma spectabile* and male *E. caeruleum* behaviour

| Effect | *df* | *F* | *P* |
|---|---|---|---|
| **Female pursuit** | | | |
| Male species | 1, 11 | 37.44 | <0.0001 |
| Light quality | 2, 22 | 3.25 | 0.058 |
| Male species∗light quality | 2, 22 | 1.41 | 0.265 |
| **Fin flares** | | | |
| Male species | 1, 11 | 7.06 | 0.022 |
| Light quality | 2, 22 | 3.32 | 0.055 |
| Male species∗light quality | 2, 22 | 0.58 | 0.566 |
| **Attacks** | | | |
| Male species | 1, 11 | 4.82 | 0.051 |
| Light quality | 2, 22 | 1.21 | 0.318 |
| Male species∗light quality | 2, 22 | 0.75 | 0.485 |
| **Nosedigs received** | | | |
| Male species | 1, 11 | 0.19 | 0.673 |
| Light quality | 2, 22 | 0.89 | 0.424 |
| Male species∗light quality | 2, 22 | 0.87 | 0.432 |
| Female pursuit | 1, 54 | 36.85 | <0.0001 |
| **Spawning success** | | | |
| Male species | 1, 11 | 0.03 | 0.857 |
| Light quality | 2, 22 | 0.48 | 0.627 |
| Male species∗light quality | 2, 22 | 0.26 | 0.775 |
| Female pursuit | 1, 54 | 25.44 | <0.0001 |

### *Species Discrimination by Males and Females*

Behavioural isolation between *E. spectabile* and *E. caeruleum* was strong, with male *E. caeruleum* rarely spawning with female *E. spectabile*. Three observations from our data suggest that male behaviour may be the main factor underlying this isolation. First, male *E. spectabile* demonstrated the capacity for recognizing conspecific versus heterospecific males by behaving more aggressively towards male *E. spectabile* than towards male *E. caeruleum*. Second, male *E. spectabile* pursued female *E. spectabile* whereas male *E. caeruleum* did not, potentially indicating that males of both species could recognize conspecific versus heterospecific females. Third, female *E. spectabile* did not appear to discriminate against either subdominant conspecific males or heterospecific males. Relative to the amount of time that each type of male spent pursuing the female, female receptiveness (i.e. nosedigs and spawning) was similar across all types of males (Fig. 6a, b). Female *E. spectabile* showed no evident mate choice once in spawning position and invariably spawned with the first attending male. Thus, although male *E. caeruleum* spent little time near the female *E. spectabile*, once this was taken into account neither the number of nosedigs received nor spawning success differed between male *E. spectabile* and *E. caeruleum* (Fig. 6a, b). A lack of mate preferences in female *E. spectabile* would be consistent with their lack of intraspecific choosiness, as female *E. spectabile* do not show preferences for male size or coloration (Pyron, 1995), but it would be at odds with traditional sexual selection theory, which predicts that females should be choosier than males due to differential resource costs arising from anisogamy (Andersson, 1994; Saetre, Kral, & Bures, 1997; Wirtz, 1999). While we cannot definitively state that male *E. spectabile* chose conspecific females, given that only one female was available, the absence of overt female choice and the discrepancy in female pursuit by male *E. spectabile* versus *E. caeruleum* are consistent with male mate preferences being responsible for maintaining behavioural isolation.

If males and not females mediate behavioural isolation between *E. caeruleum* and *E. spectabile*, one possible explanation for this sexual asymmetry could be that a high cost of reproductive behaviours for male *E. spectabile* and *E. caeruleum* has selected for male species discrimination while alleviating the need for female choosiness. Pursuing and guarding females is highly time intensive, with our control trials showing dominant male *E. spectabile* spending on average over half their time close to the female; presumably, males thusly engaged in nature are exposed to predators and are not foraging. In addition, densities of male *E. spectabile* can reach 20–40 fish/m$^2$ in the wild, resulting in intense intrasexual competition (Pyron, 1995). The act of chasing off rival males in itself often preoccupies the dominant male, preventing him from

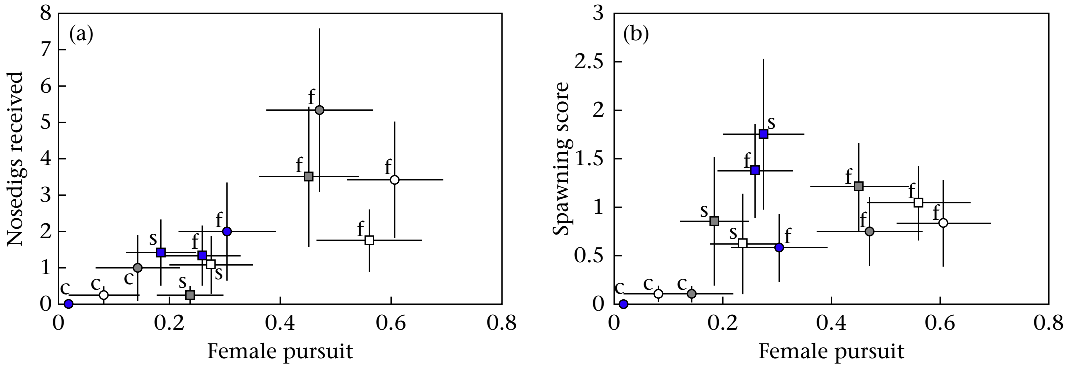

**Figure 6.** Plots showing the relationships between female pursuit and (a) nosedigs received and (b) spawning score. Square symbols indicate behavioural trials with a conspecific rival; circles indicate trials with a heterospecific rival. The colour of the each symbol indicates the light filter used in the trial (none, grey, or blue). Lowercase letters beside the data points denote the type of male ('f': focal male *Etheostoma spectabile*; 's': subdominant male *E. spectabile*; 'c': male *Etheostoma caeruleum*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

actually spawning with the female and presenting opportunities for other rival males to approach (Fuller, 1999; M. Zhou, personal observation). Male species discrimination in darters with mate guarding would thus be favourable so as to avoid the cost of pursuing an inappropriate heterospecific female or attacking a heterospecific male that poses no competition. Several studies have examined how high costs of male mating effort (e.g. from parental investment or male–male competition) can drive the evolution of male choosiness (Bonduriansky, 2001; Edward & Chapman, 2011; Wong & Jennions, 2003). In the damselfly *Calopteryx virgo*, male preference for conspecific females is stronger than the reciprocal female preference for conspecific males, possibly due to elevated predation risk for males performing courtship (Svensson et al., 2007).

Male behaviour seemed to ensure that female *E. spectabile* encountered dominant conspecific males far more often than subdominant conspecific males or heterospecific males. Curiously, we found that subdominant male *E. spectabile* had similar spawning success to dominant male *E. spectabile* across all light quality treatments, suggesting that reduced competitive ability is not detrimental to male fitness. Therefore, there may be little cost to the females' lack of choosiness as spawning with a less competitive male would not adversely affect the fitness of her offspring. Alternately, it may be that the confined area of the aquarium rendered dominant males unable to permanently drive away the subdominant rival and monopolize the female, resulting in the subdominant males obtaining inflated reproductive success relative to what would occur in nature. Anecdotal field observations of *E. spectabile* and *E. caeruleum* indicate that smaller, less dominant males are minimally successful in spawning with females (Reeves, 1907; Winn, 1958). A more detailed quantification of male fitness, under more naturalistic conditions, would better resolve whether intrasexual competition is an agent for sexual selection in these species.

Where they occur in sympatry, *E. spectabile* and *E. caeruleum* can often be found in close proximity. Given that they share the same breeding season and spawning habits, males and females of these species likely encounter each other in the wild as in our behavioural trials. Despite the apparent lack of demonstrable species discrimination in females, we found that positive and negative discrimination by males was sufficient to ensure that hybridization remained rare. During our behavioural trials, heterospecific spawning occurred when a male *E. caeruleum* happened upon a female *E. spectabile* already in spawning position or in the process of spawning. The likelihood of such an event is probably lower in nature than in the enclosed space of an aquarium, and thus hybridization between *E. spectabile* and *E. caeruleum* may be less frequent still than our data suggest. Behavioural isolation based mostly or entirely on male species recognition has seldom been documented in other taxa; one example occurs in *Heliconius* butterflies, in which females cannot resist male copulation attempts and thus male preference for conspecific colour patterns is responsible for preventing heterospecific mating (Bates, 1862; Jiggins, Naisbit, Coe, & Mallet, 2001).

### *Role of Male Breeding Coloration in Interspecies Interactions*

The behaviour of male, but not female, *E. spectabile* was affected by the disruption of colour perception under lighting filters. Dominant male *E. spectabile* spent less time pursuing the female, and launched fewer attacks on the subdominant male *E. spectabile*, when in blue light as compared to control or grey light. Fish behaviour in grey light was similar to the control, suggesting that behavioural differences under blue light were not simply due to reduced light intensity as the amount of available light was approximately 10 times greater under the blue filter than under the grey (Fig. 1b).

As the blue lighting filter blocked most light at wavelengths above ~530 nm, the fish's ability to detect and discriminate orange and red colour was likely impaired. The male breeding coloration of *E. spectabile* and *E. caeruleum* consists of blue-green and orange-red components; the latter is more predictive of species identity, and the presence/absence of an orange-red colour patch on the anal fin is the most obvious visual difference between males of these two species (Page, 1983; Zhou, Johnson, & Fuller, 2014). Thus, the blue filter may have disrupted the perception of species-specific cues in particular.

The lighting filters may have had an overall suppressive effect on male reproductive behaviour, as the amount of female pursuit, fin flares and attacks performed by focal male *E. spectabile* all trended downward from control/grey to blue light, although the effect was not always significant (Fig. 3a–c). One possible explanation for this pattern is that the blue filter mimicked lighting conditions at dawn and dusk, when sunlight becomes blue-shifted due to a disproportionate diminution of the orange part of the spectrum (Endler, 1991). Time of day and other factors that alter environmental light spectrum have been shown to modulate male courtship behaviour in the guppy (Endler, 1987). Since darters are diurnal (Page, 1983), their overall reproductive activity level may be reduced at dawn and dusk, and the blue filter could have caused a similar effect. However, since spawning did not decrease in blue light (Fig. 6b), it seems unlikely that our behavioural results could be solely attributed to such diurnal variation in activity.

That the blue lighting substantially reduced the frequency of male–male attacks, but not fin flare displays, suggests that these agonistic behaviours may differ in function. Male *E. spectabile* performed fin flares towards both conspecific and heterospecific males, while attacks were largely restricted to conspecific males. This pattern is consistent with fin flaring being a generalized signal relevant to multiple species, perhaps common to *Oligocephalus* or sexually dichromatic darters as a whole. Outside *Oligocephalus*, dorsal fin flaring has also been observed in male *E. zonale* and *E. barranense* (Williams & Mendelson, 2010). Flaring of the dorsal and anal fins may serve to advertise species-specific colour cues to approaching male darters, resulting in either escalation or de-escalation depending on the species of the receiver. In our trials, blue light may have impeded the ability of male *E. spectabile* to identify conspecific rivals, thus inhibiting further aggression. Such a signalling system would be comparable to dewlap displays in *Anolis* lizards. *Anolis* dewlaps exhibit high interspecific diversity in coloration (Fitch & Hillis, 1984; Nicholson, Harmon, & Losos, 2007), and male *Anolis* perform initial dewlap displays towards any novel object. Experiments with *Anolis marcanoi* have shown that subsequent escalation to overt aggression is dependent on the identification of the receiver as a conspecific male, with the species-characteristic colour of the dewlap being a definite factor (Losos, 1985).

### *Overall Conclusions*

Behavioural isolation between *E. spectabile* and *E. caeruleum* may be based mainly on male species discrimination, with nonevident female discrimination. Although behavioural isolation is widespread among darters and appears to be the primary mechanism for reproductive isolation (Mendelson, 2003), this pattern has not been reported in other species. In *E. zonale*, *E. barrenense* and *Etheostoma duryi*, both males and females preferentially associate with conspecific fish over heterospecific fish (Martin & Mendelson, 2013; Williams & Mendelson, 2010). On the other hand, in *Etheostoma flavum*, neither males nor females show preferences for conspecific fish (Martin & Mendelson, 2013). A sex difference in species discrimination is present in *Etheostoma nigripinne* but in the opposite direction to our results: females strongly prefer conspecific males while males do not consistently prefer conspecific females (O'Rourke & Mendelson, 2010). While these data sets are not directly comparable due to differences in experimental methodology, this apparent diversity in choosiness and discrimination behaviour hints that darter speciation may be complex, shaped by different selective forces in different clades. One potential contributing factor may be variation in spawning habits: *E. spectabile* and *E. caeruleum* are egg-buriers, *E. zonale*, *E. barrenense*, *E. duryi* and *E. flavum* are egg-attachers, and *E. nigripinne* shows territoriality and parental care (Simon & Wallus, 2005).

The breeding coloration of male *E. spectabile* and *E. caeruleum* appears to function primarily in interactions with other males rather than in attracting females, raising the possibility that male–male aggression within and across species may be an important driver of male colour diversification in darters. Relatively little is known about how interspecific competition may promote signal divergence and speciation (Grether, Losin, Anderson, & Okamoto, 2009). Male–male competition has been suggested to contribute to colour diversification among haplochromine cichlids in Lake Victoria, as males of novel colour morphs elicit less aggression from other males (Dijkstra & Groothuis, 2011; Pauers, Kapfer, Fendos, & Berg, 2008; Seehausen & Schluter, 2004). Our results suggest that male–male competition may be an underappreciated factor underlying male ornament diversification, particularly in taxa with weak or absent female preference.

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