The vertical distributions and spawning site choices of red and yellow bluefin killifish *Lucania goodei* colour morphs

Chia-Hao Chang[1] | Jessica E. Schult[2] | Jonathan Sanders[3] | Shih-Hui Liu[4] |
Rebecca C. Fuller[1]

[1]Department of Animal Biology, School of Integrative Biology, University of Illinois, Champaign, Illinois

[2]College of Veterinary Medicine, University of Illinois, Champaign, Illinois

[3]School of Integrative Biology, University of Illinois, Champaign, Illinois

[4]Biodiversity Research Center, Academia Sinica, Taipei City, Taiwan

Correspondence
Chia-Hao Chang, Department of Animal Biology, School of Integrative Biology, University of Illinois, Champaign, Illinois 61820.
Email: chiahao0928@gmail.com

A genetic colour polymorphism is present in bluefin killifish *Lucania goodei*, where red and yellow anal-fin morphs coexist in clear springs, but the source of balancing selection is unknown. In a field study, vertical distributions did not differ between the morphs and there was little evidence that light environments differed qualitatively over the 200 cm at which fish were collected. A greenhouse study showed that both morphs preferred to spawn at shallow depths and hence vertical distribution and spawning site choice are unlikely to explain the maintenance of the colour polymorphism.

KEYWORDS

colour polymorphism, Cyprinodontiformes, depth, Fundulidae, light environment, microhabitat

Genetic colour polymorphisms in fishes are ideal for studying how genetic variation is maintained in a population. First, many colour morphs are determined by one or a few genes, so they are highly heritable. Second, a large number of individuals can be quickly categorized as distinct colour morphs, allowing evolution to be easily quantified in wild populations (Rosenblum *et al*., 2012; Wellenreuther *et al*., 2014). Third, the appearance of colour patterns can easily vary in aquatic habitats due to the highly heterogeneous nature of light in water.

The conspicuousness of a colour signal is dependent on lighting environment, including ambient light, transmission properties of the water, and visual background. Thus, a colour morph that dwells in a heterogeneous habitat could be either cryptic or conspicuous depending on its location (Endler, 1990; Endler & Mielke, 2005). Colour polymorphisms can be maintained via spatial variation in light when colour morphs differ in reproductive success and survival in different microhabitats within a population. Aquatic lighting habitats vary tremendously due to materials in the water column (*e.g.* tannins, algae, and

particulates), tree cover, and depth. Empirical studies on many fish species support the notion that spatially variable lighting environments affect the evolution of colour patterns (Culumber & Tobler, 2016; Endler, 1983; Fuller & Noa, 2010; Gray *et al*., 2008; Hurtado-Gonzales *et al*., 2014; Magellan & Swartz, 2013; Morrongiello *et al*., 2010; Whitney *et al*., 2018).

The bluefin killifish *Lucania goodei* Jordan 1880 lives in various lighting habitats throughout Florida, ranging from springs to swamps. The colour polymorphism is sex-limited, with highly variable colour morphs (red, yellow or blue) observed in the anal fins of males. Differences in lighting environments across populations affect the abundance of blue males. Blue morphs are common in tannin-stained swamps, while red and yellow morphs are more common in clear springs (Fuller, 2002). Although spatially variable selection across populations probably explains why blue males are common in swamps and rare in springs (R. C. Fuller, unpubl. data), it is still unclear how multiple colour morphs are maintained within populations. Here, we

focus on the maintenance of red and yellow morphs in clear-water environments. The red and yellow colour morphs are largely controlled by a locus of large effect where the yellow allele (Y) is dominant to the red allele (y). Males with yellow and red colouration are particularly common in clear-water habitats. Fuller and Johnson (2009) and Johnson *et al.* (2018) ruled out the hypothesis that negative frequency-dependent selection (NFDS) in male mating success plays a role in maintaining *L. goodei* colour polymorphism. Nevertheless, data from Johnson *et al.* (2018) implied that the vertical distribution of fish in a water column may have some influence.

In nature, *L. goodei* typically inhabit depths of 5–100 cm (R. C. Fuller, pers. obs.). Males and females spawn eggs on both floating vegetation as well as on vegetation deeper in the water column (<150 cm) (Fuller, 2001). A previous experiment (Sandkam & Fuller 2011) demonstrated that *L. goodei* showed no vertical preference for siring sites, but Johnson *et al.* (2018) recently found that red males proportionally sired more offspring compared with yellow males on deeper spawning substrata, especially when red males were rare. The authors then proposed two non-mutually exclusive explanations for this phenomenon. First, because clear water selectively absorbs longer wavelengths of light, red males might create greater contrast with the background at greater depths, providing a stronger signal that may draw the attention of females. Second, yellow males might expel red males from floating substrata, particularly when they are common. This would cause red males to frequently stay at the bottom. These two potential explanations imply that red and yellow morphs might diverge in their preference to live and spawn at distinct depths. One drawback of the previous studies (Johnson *et al.*, 2018; Sandkam & Fuller, 2011) is that they used depths < 50 cm, which might be insufficient to clearly distinguish the spawning site preference between shallow and deep substrata.

In this study, we first hypothesized that red male *L. goodei* occur at greater depths than yellow males. This hypothesis was based on two observations. First, previous studies suggested that females prefer male colour patterns that contrast with the available light spectrum (Fuller & Noa, 2010; Fuller *et al.*, 2010; Johnson *et al.*, 2013). Second, as discussed above, red males have greater mating success than yellow males in deeper places in clear water. To test this hypothesis, we conducted a field study to determine whether red males inhabit deeper regions than yellow males. *Lucania goodei* were collected with minnow traps placed at different depths along a 300 m stretch of the Wakulla River beginning at the CR 365 Bridge (30° 12′ 49″ N; 84° 15′ 41″ W; Upper Bridge, Wakulla County, FL) from June 2 to June 6, 2017 during the breeding season (Lee *et al.*, 1980). We used three types of minnow traps (Frabill 42 × 23 cm crawfish trap, 34 × 18 cm glass minnow trap, and 34 × 25 cm flexible fish trap), which were baited with dog food and fish bait mixed with bread and placed at different depths. Traps were set up for about 2 hr and then subsequently checked. For each trap, we recorded depth, counted the number of *L. goodei* and other fish species (mainly cyprinids). A two-sample *t*-test was used to determine whether red male *L. goodei* occur at greater depth than yellow males. All the statistical analyses in this study were conducted using R version 3.2.3 (www.r-project.org). For *L. goodei*, we recorded the standard length (L_S), sex, and male anal-fin colour.

Overall, 2,237 individual fish were sampled in this study, of which 433 were *L. goodei*. A sample of *c.* 60 adult *L. goodei* was transported to the University of Illinois at Urbana-Champaign and housed in a communal oval stock tank (185 cm in length × 86 cm in width × 65 cm in height) located in a climate-controlled greenhouse for the behavioural assay on depth preference.

We next asked whether the lighting environments varied greatly as a function of depth. Downwelling irradiance spectra were measured at 0, 50, 100, and 145 cm depths with a USB-2000 spectrometer with a cosine corrector and 200 cm length optical fibre (QP1000-2-UV-VIS) (Ocean Optics, Dunedin, FL, U.S.A.; www.oceanoptics.com). The spectrometer was calibrated with a deuterium-tungsten lamp (Ocean Optics) with SpectraSuite software. We recorded the downwelling irradiance (photons nm^{-1} cm^{-2} sec^{-1}) from 300 to 700 nm between 1,000 and 1,200 hrs on June 2, 2017.

Finally, we conducted a behavioural experiment to test whether red and yellow males preferred to spawn at different depths. Male preference for spawning location was measured by allowing a single male to mate with several females in a tall cylindrical vat containing spawning substrata at several depths. Two white PVC cylindrical water vats (75 cm diameter × 106 cm in height and 76 cm diameter × 112 cm in height) were placed in a climate-controlled greenhouse from June 5 to July 21, 2017. Vats were surrounded by black plastic bags, so the light source was limited to the downwelling light. Each vat was filled with water to a depth of 100 cm and was equipped with an ultraviolet sterilizer for algae control and an air-powered filter for water circulation and nitrogen removal. Mops, each containing 100 16 cm strands of green yarn, were used as spawning substrata. Two mops were secured at each depth of 0 cm (floating position), 45 cm (middle position) and 90 cm (bottom position). One male, with either a red or yellow anal fin, and five females were released in each vat. The fish were fed three times per day (morning, noon and evening) with frozen brine shrimp *Artemia* sp. and bloodworms *Chironomus* sp. The fish were placed in the vats on either Saturday or Sunday, and mops were added on Sunday evening. The mops were checked twice per day for 5 days. Eggs were removed and maintained in a dilute solution of methylene blue (*c.* 1–2 mg l^{-1}) for a few days and the undeveloped eggs were excluded from the analysis. Males were only used once, but females were sometimes used multiple times with at least 1 week of rest between replicates. We assumed that the location of eggs with respect to depth reflects, at least in part, male preference for depth. This is a reasonable assumption given that, in nature, females visit males on patches of vegetation that they guard from other males (Fuller, 2001). Hence, male territoriality limits the locations at which spawning can occur. Twelve replicates were conducted (six red, and six yellow morphs). We asked whether male colour morph (red *v.* yellow), mop position (floating, middle, or bottom), and the interaction between the male colour morph and mop position affected where males sired their eggs. To do this, we used a binominal model with a logit link function using the glmer function from the lme4 package in R. The experimental vat was treated as a random effect. Since the initial model suffered from over dispersion, we also included observation level as a random effect (Harrison, 2014). The final model was as follows: (number of eggs at a given mop position,

number of eggs at alternate positions) ~ mop position + colour morph + mop position: colour morph + (1|vat) + (1|observation). We used a type 3 analysis in the car package to determine the effects of each term.

In the field study, 433 *L. goodei* were collected; 125 individuals were adult males. Forty-nine males had a red anal fin, while 54 males had a yellow anal fin. The remaining males had either blue, blue–red or blue–yellow anal fins. We collected males at depths ranging from 44 to 207 cm. A two sample *t*-test showed that there was no significant difference between the sampling depths of red and yellow morphs (t_{101} = −0·474, $p > 0.05$) (Figure 1(a)). Although the overall intensity of spectral irradiance decreased with the increasing depth, the shape of the irradiance curves at 0, 50, 100 and 145 cm depth were very similar (Figure 1(b)).

The laboratory preference experiment indicated no differences in spawning depths between red and yellow morphs. A total of 2,402 eggs were collected and 351 of them were undeveloped. Each vat had at least 40 developed eggs. Neither colour morph (χ^2 = 0·369, $p > 0.05$) nor the interaction between colour morph and mop position (χ^2 = 0·741, $p > 0.05$) had significant effects on where males sired their eggs, but the effect of mop position was statistically significant (χ^2 = 71·344, $p < 0.001$). Floating mops were preferred over middle and bottom mops as spawning substrata. There was no notable preference between middle and bottom mops ($p > 0.05$) (Figure 1(c)).

The main hypothesis in this study was that red morphs in *L. goodei* prefer greater depths than yellow morphs, and that this behavioural difference contributes to the maintenance of the red–yellow polymorphism, but neither the field data nor the behavioural data confirmed this prediction. Red and yellow males did not differ in the depths at which they were found in nature nor did they differ in where they preferred to spawn with females. Furthermore, the underlying assumption of our study, *i.e.* that lighting environments differ dramatically with depth, was not upheld. In retrospect, this is perhaps not surprising as the Wakulla River is very clear and clear water has low attenuation over the depths we measured, even in the red wavelengths. Clearly, this does not preclude effects due to differences in lighting environment over greater depths or in different types of water (Dalton *et al.*, 2010; Seehausen *et al.*, 2008). Also, we cannot rule out the potential that red males migrate from greater depths than those measured in this study. In addition, there are potentially effects due to horizontal spatial variation such as shade, canopy cover, or differences in visual background (*e.g.* water column *v.* vegetation) that could potentially affect the maintenance of the colour polymorphism (Chunco *et al.*, 2007).

Both red and yellow males preferred to spawn on floating mops. Sandkam and Fuller (2011) previously suggested that *L. goodei* do not have a preference for siring depth. The authors suggested that previously reported differences between the number of eggs found in floating versus bottom mops was mainly due to egg cannibalism, since *L. goodei* primarily forage on the bottom for invertebrates (Loftus & Kushlan, 1987). In the current study, three safeguards were utilized to limit egg cannibalism. First, the bottom mops were not placed on the vat bottom. Instead, the mops were 10 cm above the vat bottom. Second, *L. goodei* were fed three times per day to minimize hunger. Third, the mops were checked twice per day to restrict the time when eggs
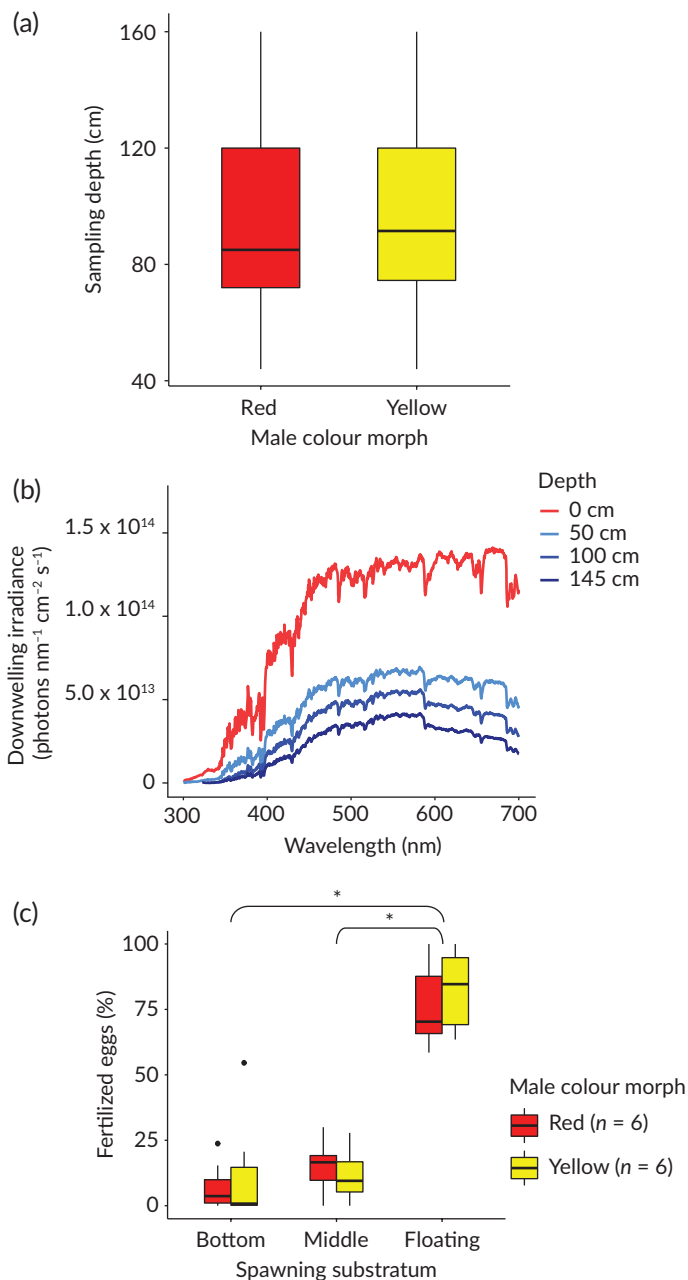
FIGURE 1 (a) Field study: Box plot showing the median (—), 25th and 75th percentiles (box) and range (◊) of the sampling depth of male *Lucania goodie* red (*n* = 49) and yellow (*n* = 54) tail morphs of. (b) Downwelling irradiance at different depths in clear-water habitat of *L. goodie*.; (c) depth preference: Box plot showing the median (—), 25th and 75th percentiles (box), 95% range (◊) and outliers (●) of the fertilized eggs found on bottom, middle and floating spawning substratum. ✳ significant differences between *L. goodie* spawning mop positions at *p* < 0.01

were in the water. These combined efforts are expected to have greatly reduced egg cannibalism in our experiment. In addition, the sample sizes of this study are larger than those of Sandkam and Fuller (2011). We confidently assert that *L. goodei* did indeed prefer to spawn at floating mops.

It is still unclear how the colour polymorphism is maintained in *L. goodei*. One possibility is a trade-off between male aggression and

female mate choice. Fuller and Noa (2010) focused on the same population and found that females, which were reared and tested in a clear water environment, preferred red over yellow males. Johnson *et al.* (2018), however, found, that red males did not have an advantage on the preferred floating substrata. One possibility is that yellow males were competitively dominant to red males. In another system, the persistence of colour polymorphism in the pygmy swordtail *Xiphophorus pygmaeus* Hubbs & Gordon 1943 (Kingston *et al.*, 2003), seems to be maintained by this opposition between male–male competition and female choice.

Disassortative mating has been suggested as a major force in the maintenance of morphological polymorphisms in many animals (Follett *et al.*, 2007; Houtman & Falls, 1994; Rolán-Alvarez *et al.*, 2012; Takahashi & Hori, 2008). Although female *L. goodei* do not express red or yellow colouration, they still carry the colour-determining alleles. Disassortative mating could potentially maintain the colour polymorphism. Alternatively, the persistence of the colour polymorphism could also be explained by over-dominance if heterozygotes (Yy) have higher fitness than either homozygote (YY or yy). Monitoring genetic markers for the red and yellow alleles would help to test these hypotheses.

In conclusion, our study found that male *L. goodei* with red anal fins did not inhabit deeper waters than males with yellow anal fins and that both red and yellow males preferred to spawn at the same depth. Therefore, we ruled out the possibility that differentiation in preferred depth could maintain the colour polymorphism of the *L. goodei* in clear water environments. In light of these data, we speculate that either a trade-off between male-male competition and female choice, disassortative mating, or overdominance accounts for the coexistence of red and yellow morphs in a *L. goodei* population.

The treatment of animals was approved by the Institutional Animal Care and Use Committee under protocol #15246.

REFERENCES

Calsbeek, R., Bonvini, L., & Cox, R. M. (2010). Geographic variation, frequency-dependent selection, and the maintenance of a female-limited polymorphism. *Evolution*, 64, 116–125.

Chunco, A. J., McKinnon, J. S., & Servedio, M. R. (2007). Microhabitat variation and sexual selection can maintain male color polymorphisms. *Evolution*, 61, 2504–2515.

Culumber, Z. W., & Tobler, M. (2016). Spatiotemporal environmental heterogeneity and the maintenance of the tailspot polymorphism in the variable platyfish (*Xiphophorus variatus*). *Evolution*, 70, 408–419.

Dalton, B. E., Cronin, T. W., Marshall, N. J., & Carleton, K. L. (2010). The fish eye view: Are cichlids conspicuous? *Journal of Experimental Biology*, 213, 2243–2255.

Endler, J. A. (1983). Natural and sexual selection on color patterns in poeciliid fishes. *Environmental Biology of Fishes*, 9, 173–190.

Endler, J. A. (1990). On the measurement and classification of colour in studies of animal colour patterns. *Biological Journal of the Linnean Society*, 41, 315–352.

Endler, J. A., & Mielke, P. W. J. (2005). Comparing entire colour patterns as birds see them. *Biological Journal of the Linnean Society*, 86, 405–431.

Follett, P. A., Calvert, F., & Golden, M. (2007). Genetic studies using the orange body color type of *Nezara viridula* (Hemiptera: Pentatomidae): Inheritance, sperm precedence, and disassortative mating. *Annals of the Entomological Society of America*, 100, 433–438.

Fuller, R. C. (2001). Patterns in male breeding behaviors in the bluefin killifish, *Lucania goodei*: A field study (Cyprinodontiformes: Fundulidae). *Copeia*, 2001, 823–828.

Fuller, R. C. (2002). Lighting environment predicts the relative abundance of male colour morphs in bluefin killifish (*Lucania goodei*) populations. *Proceedings of the Royal Society B*, 269, 1457–1465.

Fuller, R. C., & Johnson, A. M. (2009). A test for negative frequency-dependent mating success as a function of male colour pattern in the bluefin killifish. *Biological Journal of the Linnean Society*, 98, 489–500.

Fuller, R. C., & Noa, L. A. (2010). Female mating preferences, lighting environment, and a test of the sensory bias hypothesis in the bluefin killifish. *Animal Behaviour*, 80, 23–35.

Fuller, R. C., Noa, L. A., & Strellner, R. S. (2010). Teasing apart the many effects of lighting environment on opsin expression and foraging preference in bluefin killifish. *The American Naturalist*, 176, 1–13.

Gray, S. M., Dill, L. M., Tantu, F. Y., Loew, E. R., Herder, F., & McKinnon, J. S. (2008). Environment-contingent sexual selection in a colour polymorphic fish. *Proceedings of the Royal Society B*, 275, 1785–1791.

Harrison, X. A. (2014). Using observation-level random effects to model overdispersion in count data in ecology and evolution. *PeerJ*, 2, e616.

Houtman, A. M., & Falls, J. B. (1994). Negative assortative mating in the white-throated sparrow, *Zonotrichia albicollis*: The role of mate choice and intra-sexual competition. *Animal Behaviour*, 48, 377–383.

Hurtado-Gonzales, J. L., Loew, E. R., & Uy, J. A. C. (2014). Variation in the visual habitat may mediate the maintenance of color polymorphism in a poeciliid fish. *PLoS One*, 9, e101497.

Johnson, A. M., Chang, C.-H., & Fuller, R. C. (2018). Testing the potential mechanisms for the maintenance of a genetic color polymorphism in bluefin killifish populations. *Current Zoology*.

Johnson, A. M., Stanis, S., & Fuller, R. C. (2013). Diurnal lighting patterns and habitat alter opsin expression and colour preferences in a killifish. *Proceedings of the Royal Society B*, 280, 20130796.

Kingston, J. J., Rosenthal, G. G., & Ryan, M. J. (2003). The role of sexual selection in maintaining a colour polymorphism in the pygmy swordtail, *Xiphophorus pygmaeus*. *Animal Behaviour*, 65, 735–743.

Lee, D. S., Platania, S. P., & Burgess, G. H. (1980). *Atlas of North American freshwater fishes*. Raleigh, NC: North Carolina State Museum of Natural History.

Loftus, W. F., & Kushlan, J. A. (1987). Freshwater fishes of Southern Florida. *Bulletin of the Florida Satate Museum Biological Sciences*, 31, 147–344.

Magellan, K., & Swartz, E. R. (2013). Crypsis in a heterogeneous environment: Relationships between changeable polymorphic colour patterns and behaviour in a galaxiid fish. *Freshwater Biology*, 58, 793–799.

Morrongiello, J. R., Bond, N. R., Crook, D. A., & Wong, B. B. M. (2010). Nuptial coloration varies with ambient light environment in a freshwater fish. *Journal of Evolutionary Biology*, 23, 2718–2725.

Rolán-Alvarez, E., Saura, M., Diz, A. P., Rivas, M. J., Alvarez, M., Cortés, B., … Iglesias, L. (2012). Can sexual selection and disassortative mating contribute to the maintenance of a shell color polymorphism in an intertidal marine snail? *Current Zoology*, 58, 463–474.

Rosenblum, E. B., Sarver, B. A. J., Brown, J. W., Roches, S. D., Hardwick, K. M., Hether, T. D., … Harmon, L. J. (2012). Goldilocks meets Santa Rosalia: An ephemeral speciation model explains patterns of diversification across time scales. *Evolutionary Biology*, 39, 255–261.

Sandkam, B. A., & Fuller, R. C. (2011). The effects of water depth and light on oviposition and egg cannibalism in the bluefin killifish *Lucania goodei*. *Journal of Fish Biology*, 78, 967–972.

Seehausen, O., Terai, Y., Magalhaes, I. S., Carleton, K. L., Mrosso, H. D. J., Miyagi, R., … Okada, N. (2008). Speciation through sensory drive in cichlid fish. *Nature*, 455, 620–626.

Takahashi, T., & Hori, M. (2008). Evidence of disassortative mating in a Tanganyikan cichlid fish and its role in the maintenance of intrapopulation dimorphism. *Biology Letters*, 4, 497–499.

Wellenreuther, M., Svensson, E. I., & Hansson, B. (2014). Sexual selection and genetic colour polymorphisms in animals. *Molecular Ecology*, *23*, 5398–5414.

Whitney, J. L., Donahue, M. J., & Karl, S. A. (2018). Niche divergence along a fine-scale ecological gradient in sympatric color morphs of a coral reef fish. *Ecosphere*, *9*, e02015.