



Environmental variation promotes colour morph-specific behavioural differences in a cichlid fish

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Colour polymorphisms are among the most striking examples of intraspecific variation. If vulnerability in a given habitat depends on body colour, heterogeneity in the environment may drive behavioural differences between differently coloured individuals. We tested this prediction in a colour-polymorphic fish species, the red devil, *Amphilophus labiatus*. In *Amphilophus* cichlids, 'dark' and 'gold' morphs have previously been linked to differences in morphology, aggressive behaviour and mate choice, but it is unknown whether the morphs also differ in other key behaviours, or whether any such differences are sensitive to environmental factors. By testing activity, boldness and exploration, our laboratory experiment provided moderate evidence for an environmental variable, substrate type, having colour morph-specific effects on behaviour: dark morph red devils explored their environment more extensively when the environmental background (substrate) was dark brown than when it was light coloured, whereas gold morph individuals did not differ in their behaviour in relation to substrate colour. These results show that environmentally driven behavioural differences may be context dependent and have a role in maintaining colour morph-specific behavioural strategies. Hence, the evolution of morphological traits, such as colour morphs, cannot be fully understood without considering the behavioural phenotypes that have coevolved with them in interaction with the environment.

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A central goal in evolutionary ecology is to uncover the mechanisms that generate and maintain variation among individuals. Species with discrete colour polymorphisms are important model systems for understanding mechanisms that underlie this variation (Bond, 2007; Roulin, 2004; Svensson, 2017). Interestingly, an individual's body colour may be coupled with other key traits, such as metabolic rate, immune function and stress response (Friesen et al., 2017; McKinnon & Pierotti, 2010; Pryke et al., 2007). Selection on individuals with different colours is sometimes also influenced by potential mates (Eakley & Houde, 2004; Kokko et al., 2007), rivals (Dijkstra et al., 2005; Lehtonen, 2014; Tyers et al., 2021) and would-be predators (Abrams & Rowe, 1996; Bond, 2007; Reznick & Endler, 1982). Here, variation in the environment (e.g. in complexity, visibility or background colour) can also be important (Kekäläinen et al., 2010; Svanbäck & Eklöv, 2011). For example, individuals

with different colours may differ in how well they are able to blend into their surroundings, which may affect their risk of being detected by predators (Endler, 1978; Kjernsmo & Merilaita, 2012; Stevens & Ruxton, 2019). Here, the behaviour of an organism may also be crucial via, for instance, background choice and other behavioural strategies of camouflage (Kjernsmo & Merilaita, 2012; Stevens & Ruxton, 2019). In *Anolis sagrei* lizards, exploratory behaviour was found to be favoured in the absence, and avoidance of the ground in the presence, of predators (Lapiedra et al., 2018). Similarly, predation risk has been found to affect temperament in fish (Archard & Braithwaite, 2011; Dingemans et al., 2007; Harris et al., 2010).

The strategy by which individuals strive to increase their fitness can differ between colour morphs. For instance, in Gouldian finches, *Chloebia gouldiae*, red-headed males are dominant and aggressive, whereas the more passive strategy of black-headed males appears to buffer them against social stress responses (Pryke et al., 2007). Colour morphs may also differ in other behavioural contexts, such as mate choice (Hurtado-Gonzales et al.,

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2010) and antipredator responses (Thaker et al., 2009). Similarly, phenotype-dependent conspicuousness in a given environment may impact an individual's mating success (Endler, 1992; Heindl & Winkler, 2003) or predation risk (Cook et al., 2012; Endler, 1980; Godin & McDonough, 2003; Young et al., 2011). Such differences in conspicuousness have the potential to influence the spatial distribution of differently coloured individuals in fish (Endler, 1978, 1980; Young et al., 2011) and other animals (Cook et al., 2012; Edelaar et al., 2019; Galeotti et al., 2003). Behavioural responses that relate to conspicuousness may also carry significant costs (Creel & Christianson, 2008), such as delays in reproduction (Ruxton & Lima, 1997) and disruption of foraging or movement patterns (Creel & Christianson, 2008), sometimes promoting spatial segregation of colour morphs (Edelaar et al., 2008), assortative mating (Jiang et al., 2013) or even speciation (Gray & McKinnon, 2007). Hence, environmental variation has the potential to induce state-dependent behaviours (Niemelä & Dingemans, 2018), including behavioural differences between colour morphs.

The Midas cichlid species complex (*Amphilophus* spp.) is an important model system in evolutionary ecology (Elmer et al., 2010; Kautt et al., 2020). Multiple lake habitats (such as rocky, sandy, vegetation-covered and limnetic), differing in the type and colour of the substrate, are available for, and used by, these Neotropical fish (Elmer et al., 2010; McCrary et al., 2008; Recknagel et al., 2014). Interestingly, many *Amphilophus* species and populations exhibit a well-established, genetically determined 'dark' and 'gold' colour polymorphism, with the colour morphs being linked to differences in morphology (gold morph being slightly more robust: Kusche et al., 2015), aggression (among wild adult fish, more aggression is directed towards the same morph individuals: Lehtonen, 2014), mate choice (assortative mating by colour: Elmer et al., 2009; Lehtonen, 2017), ability to change colour (dark morph individuals are better at matching their colour within minutes to that of the background: Dickman et al., 1990; Sowersby et al., 2015), parental care (more coordinated care when with a partner of the same morph: Lehtonen, 2017) and social dominance (gold morph is more dominant: Barlow & Ballin, 1976). However, whether the colour morphs differ with regard to other key behaviours, such as activity, boldness or exploration, or whether any such differences are sensitive to environmental factors, is not known. Here, we addressed this knowledge gap using an introduced population of the colour-polymorphic red devil, *Amphilophus labiatus*, to investigate the boldness, activity and exploration tendencies of dark and gold individuals against two different environmental backgrounds. We expected that fish would adjust their behaviours to minimize their conspicuousness in their current environmental setting. In particular, we predicted that the fish would perceive themselves less conspicuous on a dark background (here, substrate), allowing them to be bolder and more active and explorative than when on a light background. We also expected that this response may differ between colour morphs, with dark individuals predicted to be bolder and more explorative than the gold morph, particularly on a dark background, because gold morph individuals are expected to be more conspicuous to predators (Torres-Dowdall et al., 2014), and less capable of changing their coloration to match that of the background (Dickman et al., 1990; Sowersby et al., 2015).

METHODS

The red devil is native to Nicaragua but has been introduced to many parts of the world, including the artificial Hazelwood Pondage in southeastern Australia, where a population of red devils established in the past few decades (Sowersby et al., 2015, 2020). More than half of the individuals in this population are of the gold

colour morph (Sowersby et al., 2015; Wong & Lehtonen, 2015), whereas in the native range in Nicaragua the proportion of gold morph individuals is typically around 10% (Elmer et al., 2010). Similar to the natural lake environment, the Hazelwood Pondage contains different substrate types with respect to coarseness and colour (Sowersby et al., 2020; T.K. Lehtonen & B.B.M. Wong, personal observations). The fish used in this study were collected from Hazelwood in October 2015 and transported to Monash University Clayton campus, where they were maintained in multiple holding tanks of ca. 200 litres at 24–25 °C on a diet of thawed brine shrimp and commercial fish food pellets. The trials were run in December 2015–January 2016.

To investigate colour morph- and environment-dependent behavioural differences, we used maze arenas, which have been widely employed for assessments of behaviour in a range of fish species, including cichlids (e.g. Bertram et al., 2018; Brand et al., 2021; Hope et al., 2020; Kotschal et al., 2014; Salena et al., 2022; Wallace et al., 2022). Each arena contained a 3 cm layer of either a 'dark brown' or 'light' sand substrate (Fig. 1), and we tested the behaviour of dark ($N = 24$, total length \pm SE: 117 ± 3 mm) and gold ($N = 35$, 117 ± 2 mm) individuals (Figs. 2 and 3). In arenas of both types, we had placed green markers ca. 4 cm apart on the substrate (Fig. 1). At the beginning of a trial, the focal fish was placed in the corner of the arena inside a cage with mesh walls to allow acclimation for 15 min. The cage was then removed, and the arena was video recorded (with an Olympus TG-4 camera) from above for 25 min (which was close to the maximum nonstop recording time supported by the camera). Altogether, each focal individual was tested, in a randomized order (established by a random number generator), in four different arenas, two of which had light substrate and the other two had dark brown substrate. We changed the positions of the arenas, and the water in them, multiple times, and their substrate once, during the study. Overall, we had 236 trials, of which one (a gold fish on a light substrate) was not available for analyses (see below) due to a technical mishap with the camera. From the recordings of the four trials per fish, we quantified multiple measures of behaviour. First, the time taken from the start of

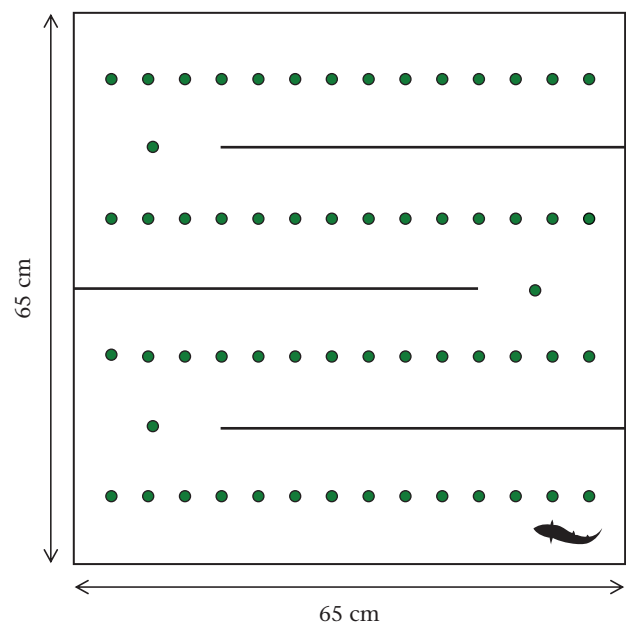


Figure 1. The experimental arena with dimensions at the substrate level (water depth: ca. 22 cm). The top view schematic also shows the position of the focal fish at the beginning of the replicate and green markers placed on the substrate (either light or dark brown).

the trial until the fish started to move was used as a proxy for boldness (Wilson & McLaughlin, 2007). In some of these trials, the focal fish did not move at all. For the remainder ($N = 125$ trials, performed by 50 individuals, see Figs 2 and 3 for sample sizes per treatment), we also quantified the number of markers the focal fish passed within the first 5 min after it had started to move, i.e. the new area it explored, as a proxy for exploration (Conrad et al., 2011) and the total number of markers passed (i.e. distance moved) within those 5 min as a proxy for activity (Wilson & Godin, 2009). Finally, we assessed the extent to which boldness, exploration and activity were correlated. After the trials, each individual was quickly digitally photographed against 1 mm grid lines, which were later used as a scale when assessing the individual's total length using ImageJ 1.51k software (NIH, U.S.A., <https://imagej.net/software/imagej/>).

Statistical Analyses

We used R 4.2.1 software (R Core Team, 2022) for all analyses. We were interested in the effects of the morph and substrate on boldness, activity and exploration, while accounting for the possible effects of fish size and the number of times the fish had already been tested. Accordingly, our full models (see below for additional details) included colour morph (dark versus gold), substrate background (dark brown versus light) and their interaction, as well as the individual's total length and the trial order (from 1 to 4). To account for each fish being tested in four trials, fish ID was used as a random effect. Using this general framework, to assess boldness (the time it took for an individual to move), we applied a mixed-effects Cox model (MECM; 'coxme' package). This type of analysis uses information about all individuals, including those that did not start to move within the first 20 min. For activity and exploration, we applied a generalized linear mixed model (GLMM), with a negative binomial distribution, as appropriate for over-dispersed count data (Zuur et al., 2013; 'glmer.nb' in the 'lme4' package). When more information about pairwise contrasts was needed, we used the 'emmeans' function/package.

Lastly, due to the widespread interest in behavioural correlations, particularly in the context of behavioural syndromes (Sih et al., 2004; Wolf & Weissing, 2012), we assessed correlations between the three behaviours by running pairwise repeated measures correlation analyses ('rmcorr' in R) on log-transformed values of the behavioural scores (after which they passed the Shapiro–Wilk test of normality). Note that one potential source of any such correlation is that the behaviours of interest were measured using the same replicates.

Ethical Note

Before and after the experiment, the fish used in this study were kept in holding aquaria of ca. 200 litres (with a maximum of 20 individuals in each tank). They were fed daily with thawed brine shrimp and commercial fish food granules. The holding tanks and experimental arenas were kept at 24–25 °C with a 12:12 h day/night cycle. To ensure high water quality, we changed 40% of the water in the tanks weekly.

This study complies with all relevant federal and state laws, as well as national, institutional and ASAB/ABS guidelines for the care and use of animals in research. Fish were collected under a scientific licence from the Victorian Department of Environment and Primary Industries (Permit number RP1241) and, after the completion of trials, they were retained for future, unrelated studies. Fish were handled with care. During the size measurements and behavioural assessments in novel environments, fish

may have experienced temporary stress but not any physical harm. The work was approved by the Animal Ethics Committee of Monash University, Australia (BSCI/2012/23 and BSCI/2016/10).

RESULTS

Boldness: Latency to Move

The interaction between morph and substrate did not have a significant effect on boldness, i.e. the time it took for an individual to move (MECM: $\beta \pm SE = 0.1399 \pm 0.3927$, $z = 0.36$, $P = 0.72$). In addition, neither morph (MECM: $\beta \pm SE = 0.2354 \pm 0.4115$, $z = 0.57$, $P = 0.57$) nor substrate (MECM: $\beta \pm SE = 0.03792 \pm 0.3036$, $z = 0.12$, $P = 0.90$) had a significant effect (Fig. 2). Individuals with a smaller total length tended to be bolder (MECM: $\beta \pm SE = 0.01993 \pm 0.01226$, $z = 1.63$, $P = 0.10$) and presentation order also had a significant effect (MECM: $\beta \pm SE = 0.4968 \pm 0.09186$, $z = 5.41$, $P < 0.001$), with fish being bolder during the earlier presentations.

Activity: Total Distance Moved within 5 min

The morph*substrate interaction did not have a significant effect on activity, i.e. the number of markers the individual passed within the first 5 min after they started to move (GLMM: $\beta \pm SE = 0.2214 \pm 0.2608$, $z = 0.849$, $P = 0.40$). Similarly, morph (GLMM: $\beta \pm SE = 0.2564 \pm 0.1989$, $z = 1.289$, $P = 0.20$; Fig. 3a), substrate (GLMM: $\beta \pm SE = 0.2431 \pm 0.2015$, $z = 1.207$, $P = 0.23$; Fig. 3a) and total length (GLMM: $\beta \pm SE = 0.002797 \pm 0.005394$, $z = 0.519$, $P = 0.60$) did not have a significant effect. Activity decreased over successive trials (GLMM: $\beta \pm SE = 0.3429 \pm 0.05507$, $z = 6.228$, $P < 0.001$).

Exploration: New Area Covered within 5 min

The effect of the morph*substrate interaction on exploration, i.e. the new area covered during the first 5 min, approached significance (GLMM: $\beta \pm SE = 0.3646 \pm 0.2111$, $z = 1.727$, $P = 0.084$), with the fish being bolder on dark substrate (GLMM: $\beta \pm SD = 0.3436 \pm 0.1626$, $z = 2.113$, $P = 0.035$) and the morph effect being nonsignificant (GLMM: $\beta \pm SD = 0.2407 \pm 0.1550$, $z = 1.553$, $P = 0.12$). Regarding the other two effects we accounted for, total length did not have a significant effect (GLMM: $\beta \pm SD = 0.0023 \pm 0.0040$, $z = 0.560$, $P = 0.58$), whereas presentation order did (GLMM: $\beta \pm SD = 0.1539 \pm 0.0461$, $z = 3.339$, $P = 0.0008$), with exploration scores being higher in earlier than later trials. To better understand the tentative interaction between morph and substrate, we assessed pairwise contrasts for the two morphs and found that dark morph individuals explored more on the dark brown than light substrate ('emmeans' contrast assessment: $\beta \pm SE = 0.344 \pm 0.163$, $z = 2.11$, $P = 0.035$; Fig. 3b), whereas gold individuals did not adjust their behaviour ('emmeans' contrast assessment: $\beta \pm SE = 0.021 \pm 0.136$, $z = 0.135$, $P = 0.88$; Fig. 3b).

Associations Between Behaviours

An individual's activity was positively associated with the area it explored (repeated measures correlation: $r_{74} = 0.8662$, 95% confidence interval, CI = 0.7951–0.9139, $P < 0.0001$) and negatively associated with the latency to move (i.e. more active individuals were bolder; repeated measures correlation: $r_{74} = -0.2550$, 95% CI = -0.4569 to 0.0282, $P = 0.026$). There was no significant correlation between exploration and the latency to move (repeated measures correlation: $r_{74} = -0.1226$, 95% CI = -0.3414 to 0.1090, $P = 0.29$).

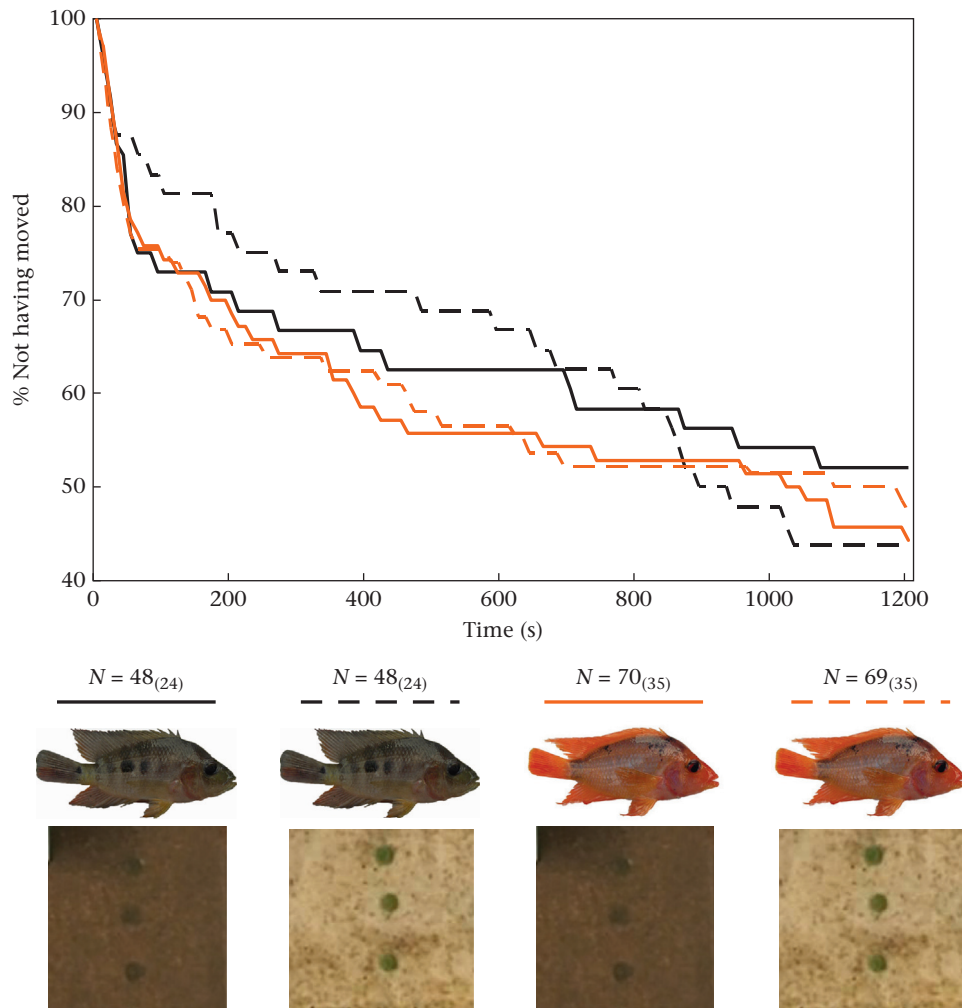


Figure 2. Percentage of trials, over time, in which the focal fish had not started to move, for individuals of dark and gold morphs, tested on light and dark brown substrates. Sample sizes are indicated both as numbers of trials and as individuals (in parentheses). The different treatments are visualized in the bottom part of the figure.

DISCUSSION

We found that dark morph individuals swimming on a dark brown substrate explored their environment more extensively than on a light-coloured substrate, while gold morph individuals did not adjust their exploratory tendencies to substrate colour. Boldness (i.e. time until starting to move around) and activity (the distance moved within 5 min after having started) were not significantly affected by colour morph or substrate type. Hence, the results show that, in these cichlid fish, environment-driven behavioural differences are context dependent: behaviour did not vary according to morph or environment initially (regarding boldness to move) but only later, after the individuals had started to gather more information about their environment (i.e. when they had the opportunity to explore their surroundings).

The long-term continued coexistence of separate (colour) morphs in a population has fascinated ecologists and evolutionary biologists for decades, and a range of factors have been postulated to contribute to their persistence. We were particularly interested in the possibility that an individual's phenotype might influence the environmental setting in which it performs well or behaves in a specific manner. Such responses may be linked to assortative mating, niche separation or predation risk (Bond, 2007; Galeotti et al., 2003; Gray & McKinnon, 2007), and hence have a potential

role in speciation (Forsman et al., 2008; Gray & McKinnon, 2007; Puebla et al., 2007). Currently, we do not know to what extent the environment-dependent differences in behaviour between the different morphs, shown in the current study, contribute to foraging strategies, niche separation or assortative mating in the wild. In *Amphilophus* cichlids, breeding pairs are usually formed between individuals of the same morph (Elmer et al., 2009; Lehtonen, 2017), while morphological differences suggest that the gold morph may occupy a more benthic ecological niche (Kusche et al., 2015). During the breeding season, the only (and relatively minor) morph-related spatial segregation was observed between colour morph-assorted and nonassorted breeding pairs (Lehtonen, 2017). If the behavioural adjustment shown in our study allows the dark morph to forage against a wider range of backgrounds more efficiently than their gold counterparts, this benefit could help to explain why it is more numerous than the gold morph in its native range, despite the gold coloration having dominant genetic inheritance (see also Lehtonen, 2017; Sowersby et al., 2015; Torres-Dowdall et al., 2014). Environmental variation and frequency-dependent selection could then stabilize the frequencies at the observed levels (Roulin, 2004).

We propose that the main reason for exploration being highest for dark morph individuals swimming above a dark brown substrate is that this morph and substrate combination provides the

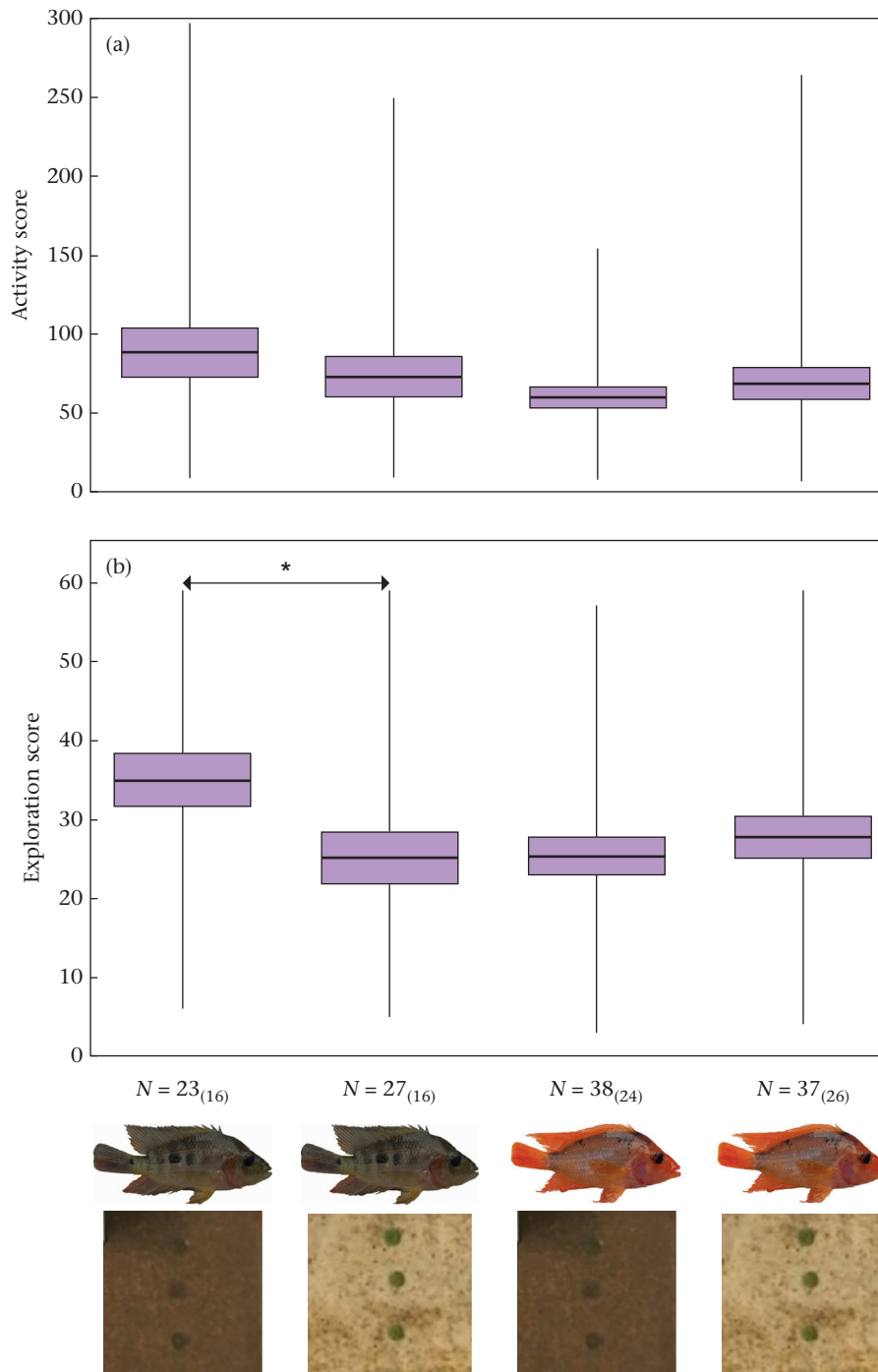


Figure 3. (a) Activity score, that is, the distance moved within 5 min, estimated by the overall number of markers passed within that time. (b) Exploration score, that is, the number of different markers passed within 5 min. The centre line shows the mean, the boxes indicate standard errors and whiskers the range of all observations. Sample sizes are indicated both as numbers of trials and as individuals (in parentheses). The different treatments are visualized in the bottom part of the figure. * $P < 0.05$ (pairwise contrast assessment; 'emmeans').

best background matching and lowest conspicuousness to visually hunting predators (see Sowersby et al., 2015). In contrast, the behaviour of the gold morph individuals might be less plastic or their conspicuousness less dependent on the background. However, assessments of the visual properties of the predators are needed to formally test these hypotheses. Avian predators that are easily capable of preying on red devils (and other similar sized fish) are common around both their natural and introduced lake environments (T.K. Lehtonen & B.B.M. Wong, personal observations

2005–2016). While our study was conducted in the laboratory in the absence of predators, each individual was netted immediately prior to the trial's acclimatization period, which may have resulted in them perceiving the environment as not only novel but also risky, given that some individuals did not move for the entire duration of the trial. The environment-driven differences imply that, in a heterogeneous environment, a single phenotype or strategy is unlikely to be optimal against all potential backgrounds (Stevens & Ruxton, 2019). For instance, when motionless, Pacific

tree frogs, *Pseudacris regilla*, with two colour forms (brown and green) were more likely to be attacked by garter snakes, *Thamnophis elegans*, when their body colour was mismatched with the shade of their background (Morey, 1990). We suggest that similar mechanisms could explain the higher exploration scores of dark morph individuals on a dark brown substrate. Therefore, the findings indicate that variation in habitat types can maintain intra-specific variation in behaviour.

While the environment-dependent link between colour and behaviour might be a response to selection on differently coloured individuals in different environments, our results do not exclude nonadaptive morph differences or proximate mechanisms such as pleiotropic effects or physical linkage between the 'colour gene' (Kratochwil et al., 2022) and genes responsible for behavioural differences between the morphs. Indeed, colour morphs in many animals have been found to be genetically linked to other traits (McKinnon & Pierotti, 2010). Interestingly, in *Amphilophus* cichlids, gold morph individuals gain their striking coloration by losing their melanophores, that is, pigment cells that produce and store melanin (Dickman et al., 1988). Melanin-based coloration has been found to have pleiotropic effects on other phenotypic traits (Ducrest, Keller, & Roulin, 2008). For instance, in the barn owl, *Tyto alba*, the size of melanin-based spots is associated with suites of antipredator responses, which are genetically influenced (van den Brink, Dolivo, Falourd, Dreiss, & Roulin, 2012). In close relatives of the red devil, different species and populations exhibit parallel colour morph differences in ecologically relevant morphological traits, which could result from a genetic link between an individual's colour morph and morphology (Kusche et al., 2015). While the flexible exploration behaviour of dark morph individuals is consistent with their better background matching ability (Sowersby et al., 2015), it does not appear to be linked to previously reported morph-specific patterns of social dominance (juvenile gold morph individuals are more dominant: Barlow & Ballin, 1976) or aggression (breeding individuals in the wild direct more aggression towards intruders of the same colour morph as themselves: Lehtonen, 2014). In this respect, note that the study population has reduced standing genetic variation compared to populations within the species' native range (Sowersby et al., 2020).

We also found that activity was positively associated with boldness and exploration. The result supports the idea that bold fish are likely to be quicker and more active in approaching novel objects or discovering food (Fraser et al., 2001). Note, however, that the behavioural traits were measured using the same trials, which may have induced the correlations. Even under these conditions, boldness and exploration were not significantly linked. Hence, our study does not indicate an important role of a boldness–exploration syndrome in red devils, in contrast to some other species of fish (Mazué et al., 2015; Wilson & Godin, 2009; Wisenden et al., 2011) and birds (van Oers et al., 2004; Verbeek et al., 1994).

Size-dependent predation risk, metabolism and energy reserves may facilitate behavioural differences (Blanckenhorn, 2000; Krause et al., 1998; Skalski & Gilliam, 2002; Sogard, 1997). In the introduced habitat, red devils had been exposed not to large piscivorous fish but, instead, to cormorants and other piscivorous birds (T.K. Lehtonen & B.B.M. Wong, personal observations). While there is no consensus about the prey size selectivity of these birds (Čech et al., 2008; Ovegård et al., 2021), size-dependent fitness effects of being bold (see Ballew et al., 2017; Brown & Braithwaite, 2004; Dowling & Godin, 2002), such as larger fish being targeted more often by avian predators, would be in line with the observed weak tendency of smaller red devils to be bolder.

To conclude, we found that dark morph individuals of red devils explored their environment more extensively on a dark brown than

light substrate. Interestingly, gold morph individuals did not similarly respond to their background. These results suggest that the type of environment (here: substrate colour) can play a role in maintaining colour morph-specific behavioural strategies in these fish. Our study also demonstrates that the evolution of morphological traits (such as colour morphs) cannot be fully understood without considering both the environment with which they interact and the behavioural phenotypes that coevolve with them.

Author Contributions

Topi K. Lehtonen: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization; Writing—original draft; Writing—review & editing. **Ulrika Candolin:** Funding acquisition, Supervision, Writing—review & editing. **Bob B.M. Wong:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing—review & editing.

Data Availability

Data are available at the Harvard Dataverse: <https://doi.org/10.7910/DVN/KZ7VDC>.

Declaration of Interest

None.

Acknowledgments

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