



**The geographical variation in colouration-based behaviours of the
Arabian killifish (*Aphaniops dispar*)**

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Abstract

Animal colouration promotes essential biological functions, including sociosexual communication, thermoregulation, prey-predator interaction and protection from harmful radiation, which may be under conflicting pressures. One common technique to cope with colouration-based competing demands is colour change. However, colour change is susceptible to opposing selection pressures and varies intricately throughout taxonomic levels, and many areas remain unresolved, including how it evolves and interacts with other behaviours. In this thesis, I studied the geographical variation of colour change and its interaction with habitat preference in freshwater and marine populations of the Arabian killifish (*Aphaniops dispar*). In Chapter 2, I explored the taxonomic status of the species by examining morphometric, colour pattern and phylogenetic differences among populations. In Chapter 3, I examined the geographical variation of the achromatic and chromatic colour change and assessed their importance in conferring crypsis. In Chapter 4, I investigated the interaction between achromatic colour change, habitat preference and camouflage, and how these aspects varied between populations. The findings from Chapter 2 revealed two distinct phylogenetic groups that were difficult to identify using morphometric and colour pattern analyses, with one marine population potentially representing a cryptic species. The results from Chapter 3 indicated that all populations showed a greater ability to change colour achromatically, specifically becoming dark, which enhanced their camouflage. However, the chromatic colour change was more complex and varied within and between populations, but the marine populations generally showed greater chromatic responses than their freshwater counterparts. The outcomes of Chapter 4 showed that when given the choice between multiple achromatic substrates, the freshwater and marine populations did not change colour and displayed a similar habitat preference for darker substrates, despite being uncamouflaged against them. These findings highlight the importance of studying variation in colour change at the population level to understand its evolution at higher levels.

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Declaration

I, the author, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not been previously been presented for an award at this, or any other, university.

I acknowledge that the work presented in Appendix 4 is a review article published in the Journal of Experimental Biology (**Alfakih, A., Watt, P. J., & Nadeau, N. J.** (2022). The physiological cost of colour change: Evidence, implications and mitigations. *Journal of Experimental Biology*, 225(10), jeb210401. <https://doi.org/10.1242/jeb.210401>). This article was conceived and written by me with comments and contributions from Dr Penelope J. Watt and Dr Nicola J. Nadeau.

Chapter 1: General Introduction

Animal colouration

Animal colouration has been a subject of interest over centuries to many scientists who worked to understand its mechanisms, functions, diversity and evolution (e.g., Cott, 1940; Gamble & Keeble, 1900; Poulton, 1890; Stevens & Merilaita, 2011). Animal colouration can be a consequence of physical structures, chemical pigments, or a combination of the two (Bagnara & Hadley, 1973; Lloyd & Nadeau, 2021; Prum & Torres, 2003; Shawkey & D'Alba, 2017; Shawkey & Hill, 2005), though some animals can produce colour through bioluminescence (Widder, 2010). Colouration promotes essential biological functions (Cuthill et al., 2017; Endler, 1978, 1983) involving sociosexual signals that convey dominance, territorial and nuptial behaviours (Burmeister et al., 2005; John et al., 2021; Ligon & McGraw, 2016; Stuart-Fox & Moussalli, 2008), thermoregulation (Gómez et al., 2018; Smith et al., 2016a, 2016b), prey-predator interactions (Langridge et al., 2007; Ruxton et al., 2004; Siddiqi et al., 2004; Stevens & Merilaita, 2009), and protection from damaging radiation (Garcia et al., 2004; Lahti & Ardia, 2016). Animal colouration and its functions are influenced by a set of biotic and abiotic factors and may be under conflicting pressures (Cuthill et al., 2017; Endler, 1978, 1983; Kelley et al., 2016; Stuart-Fox & Moussalli, 2008, 2009).

Colour change: types and mechanisms

Animals exhibit fixed or changeable appearances, but optimising the functions that colour confers can be challenging for fixed-appearance species. For example, species with unchangeable appearances may incur opportunity costs when foraging or mating due to spatiotemporal variation of the visual properties of their habitats, leading them to stay motionless in restricted habitats (Ruxton et al., 2004). Other animals have evolved strategies to cope with colouration-based competing demands, such as possessing resilient body colouration (Cuthill et al., 2017) that change at different paces and directions (i.e., reversible and irreversible) (Caro et al., 2016; Umbers et al., 2014). For example, some animals change their colour ontogenetically in one direction during their development (Booth, 1990; Wilson et al., 2007). Other groups change colour reversibly at varying rates, which may occur rapidly (milliseconds to hours) to generally enable them to cope with unpredictable changes or slowly (days to months) so they can cope with more predictable changes (Caro et al., 2016; Duarte et al., 2017; Umbers et al., 2014). The reversible changes can be the

result of moults, as in some birds and mammals (Zimova et al., 2018), or due to changes in pigments and/or reflecting platelets within specialised cells termed chromatophores (Bagnara & Hadley, 1973), which are widespread in molluscs (Allen et al., 2010; Hanlon et al., 1999), arthropods (Umbers et al., 2014), amphibians (Garcia & Sih, 2003; Kang et al., 2016), reptiles (Ligon & McGraw, 2016; Stuart-Fox & Moussalli, 2008) and fish (Sköld et al., 2012, 2016). The pigment-based colour change is governed by two mechanisms (Bagnara & Hadley, 1973): (1) morphological change caused by pigment production and destruction, and (2) physiological change caused by the motility of preexisting pigments inside the chromatophores. However, these mechanisms are interlinked, and animals can display colour change using both types of mechanisms (Bagnara & Hadley, 1973; Sköld et al., 2016; Sugimoto, 1993). To illustrate, the chameleon prawn (*Hippolyte varians*) shows slow changes from red to green colours and vice versa over several weeks (Green et al., 2019) and also shows rapid changes in a daily rhythmic pattern that takes place over 90-120 minutes (Gamble & Keeble, 1900). Why morphological and physiological colour changes are under operation in the same species remains unknown.

The cues that trigger colour change vary and may be attributed to a number of factors, such as changes in the visual properties of habitats (Green et al., 2019; Smithers et al., 2017, 2018), the presence of conspecifics or heterospecifics (Kodric-Brown, 1998; Langridge et al., 2007; Ligon, 2014; Stuart-Fox et al., 2008), the lighting conditions (Vroonen et al., 2012), temperature (Gómez et al., 2018; Smith et al., 2016a, 2016b), odours (e.g., predator olfactory cues) (Tibblin et al., 2020), season (Pellitteri-Rosa et al., 2020; Zimova et al., 2018) and internal cues such as receptivity (Stuart-Fox, 2014) or stress (Detto et al., 2008). Animals subjected to multiple stimuli at once may vary their use of colour change. To illustrate, some fish, such as the males of Pecos pupfish (*Cyprinodon pecosensis*), display bright colouration for nuptial displays, but when faced with predators, they rapidly “*shut down*” these colours and become more cryptic (Kodric-Brown, 1998). Moreover, the magnitude of the changes may vary according to the need for the change. For example, the three-spined stickleback (*Gasterosteus aculeatus*) changes its brightness in accordance with the brightness of the given substrates, but the response is enhanced if an additional predator olfactory cue or simulated attack is added (Tibblin et al., 2020). These differences in the use of colour change lead to an open question, asking how and why the colour change varies.

Variation in colour change

Variation may occur in how colour change is expressed and embodied in terms of form (i.e., achromatic and chromatic) and its speed (slow and rapid) and magnitude due to the multiple functionalities of colouration. The changes in colour can occur in the body background colouration of animals and their patterns (Stuart-Fox, 2014; Tosetto et al., 2023). Animals may exhibit achromatic colour change (grey tone) by adjusting their brightness from dark to light and vice versa (Rodgers et al., 2003; Stuart-Fox, 2014) or chromatic colour change by varying their hue (the actual colours) and saturation (the intensity of colour) (e.g., Duarte et al., 2016; Green et al., 2019). There are animals capable of both of these forms of colour change, such as crabs (Stevens et al., 2014b), insects (Eacock et al., 2017, 2019), lizards (Stuart-Fox, 2014), frogs (Choi & Jang, 2014; Kang et al., 2016) and fish (Smithers et al., 2018; Stevens et al., 2014a). In animals that have the ability of both achromatic and chromatic colour change, their ability may be better in one subtype than another (i.e., better changes in brightness than in colour or vice versa) (e.g., Choi & Jang, 2014; Kang et al., 2016; Stevens et al., 2014a), but to what extent and why one type is more improved than the other is not fully known.

Nonetheless, like other plastic traits, the ability to change colour appears to vary intricately throughout taxonomic levels, which has not been thoroughly investigated (Cadena et al., 2017). The capacity to change colour is susceptible to opposing selection pressures (e.g., sociosexual, natural and thermoregulatory) (Kang et al., 2016; Stuart-Fox, 2014; Stuart-Fox & Moussalli, 2009). Although studying colour change variation among closely related species (e.g., John et al., 2023; Stuart-Fox & Moussalli, 2009) can provide insightful knowledge into the evolution of colour change (Clarke & Schluter, 2011), which is an obvious knowledge gap (Duarte et al., 2017; Hutton et al., 2015; Stuart-Fox & Moussali, 2009), it is still challenging to establish how this variation has arisen between species. Therefore, it is necessary to investigate the variation in colour change at a lower taxonomic level—among populations of a particular taxon—to shed more light on the evolution of colour change.

Colour change varies between disconnected populations in terrestrial (Batabyal & Thaker, 2017; Cadena et al., 2017; Choi & Jang, 2014; Munguia et al., 2013; Spadavecchia et al., 2023; Stuart-Fox et al., 2006; Stuart-Fox & Moussalli, 2009; Whiting et al., 2022) and aquatic environments (Pinto et al., 2020; Westley et al., 2013; Whiteley et al., 2009, 2011). These few examples of interpopulation differences in colour change give the impression that it is

not attributable to any one factor or combination of factors that apply to all colour-changing species. To illustrate, some species have evolved colour-changing abilities adapted to their local habitats (e.g., Cadena et al., 2017), while others maintain variation in colour change regardless of inhabiting similar or different habitats (e.g., Westley et al., 2013). This suggests that the processes that drive the evolution of the population variation in colour change vary between and among taxa, and are still poorly understood. In general, within a prey-predator context, when some populations experience stronger natural selection for camouflage than others, their capacity to change colour is predicted to differ (Stuart-Fox et al., 2006). It is also predicted that the divergence in habitat visual properties is paralleled by a divergence in the ability of a population to change colour (Cadena et al., 2017). Moreover, most animals live in environments with several predators (Stuart-Fox & Moussalli, 2009), each with specific visual abilities. Thus, selection for the increased colour change capacity may correspond to the diversity (Whiteley et al., 2011) and/or the abundance of visually guided predators (Stuart-Fox et al., 2006; Stuart-Fox & Moussalli, 2009).

Interaction between colour change and habitat choice behaviours

In many species, the phenotype-environment match is the outcome of a combination of habitat preference and colour plasticity (Boyle & Start, 2020), because each technique may help compensate for the limitations of the other (Smithers et al., 2018). The effects of the habitat components such as predator diversity and richness on colour-changing animals are not entirely known. In general, animals are expected to (1) select complex (Kjernsmo & Merilaita 2012; Merilaita, 2003) and/or matching habitats (Edelaar et al., 2008) to reduce their need for colour change (Green et al., 2019; Polo-Cavia & Gomez-Mestre, 2017; Rodgers et al., 2013), (2) select substrates that accommodate their ability to change colour if the substrates beyond their ability of changing colour (Polo-Cavia & Gomez-Mestre, 2017; Smithers et al., 2018), or (3) display compromised colour or patterns that approximately match multiple visual habitats (Hanlon et al., 1999). Only a few studies have attempted to study the interactions between colour change and habitat preferences, and these have focused on how the ability to change colour can affect the choice decision (e.g., Rodgers et al., 2013; Ryer et al., 2008; Smithers et al., 2018). This was done by acclimating the animals to contrasting backgrounds and then giving them a choice between multiple backgrounds. This approach may induce animals to expend energy on colour change, which then influences their choice. However, the free choice between multiple background colours and

its effects on colour change *per se* is more realistically relevant (see below), but seems unaddressed comprehensively so far.

In real scenarios, animals need to move from one point to another, and ironically, colour change in a broad sense may be thought to aid crypsis during locomotion via continuous alteration in colour. However, colour change is limited in many animals and entails physiological costs (Duarte et al., 2017; Stuart-Fox & Moussalli, 2009), and it may not always be performed effectively. Therefore, changing colour might be highly expensive, specifically when it is integrated with energetically depleting tasks such as movements. However, the relationship between colour plasticity and habitat selection is more intricate and is influenced by a variety of parameters such as body size (Green et al., 2019), diet (Gilby et al., 2015), and morphological constraints (Tyrie et al., 2015). In general, there is a negative relationship between colour change and habitat preference, with species that change colour slowly having higher habitat preferences than those that change colour rapidly (Stevens & Ruxton, 2019). However, a few studies have examined the colour change and habitat preference combined into a single taxon (e.g., Green et al., 2019; Magellan & Swartz, 2013; Smithers et al., 2018; Tyrie et al., 2015), and it is rare that studies have been conducted on colour change and habitat choice in different populations of a single species (e.g., Spadavecchia et al., 2023). As previously mentioned, colour change may vary spatially (i.e., among populations), but it is unknown if habitat choice in colour-changing species varies in the same way. Therefore, there is a need to study how multiple backgrounds may affect colour change and whether there are differences between populations in a single species in the use of colour change and background choice.

Euryhaline fish species are excellent models for studying the geographical variation in phenotypic traits among populations, owing to their tolerance of different environmental conditions and the potential to become landlocked (Schultz & McCormick, 2013). Freshwater and marine populations of euryhaline fish species exhibit intraspecific divergence in adaptive traits, including those that are physiological, behavioural, and morphological (Coscia et al., 2013; Freyhof et al., 2017; Schultz & McCormick, 2013). Fish are well known for their colour change capacity (Sköld et al., 2012, 2016), and there may be many phylogenetically diverse euryhaline fish species that display colour change, which will provide benefits to study the evolution of colour change. Yet comparative studies on colour plasticity remain unexplored among freshwater and marine populations in these groups of animals.

Thesis questions

In this thesis, the following questions will be explored :

1. Do achromatic and chromatic colour changes vary among freshwater and marine populations of a euryhaline fish species?
2. How does habitat choice mediate the ability to change colour in a euryhaline species? Does habitat choice vary among freshwater and marine populations?

To answer these questions, I studied freshwater and marine populations of the Arabian killifish (*Aphaniops dispar sensu lato*) from the Red Sea basin in Saudi Arabia, which covers around 1000 km. It was important to determine whether the studied populations belonged to the same *A. dispar sensu stricto* or different species due to the underestimation of their taxonomic placement in the chosen area compared to other areas in the Arabian Peninsula where the taxonomy is more resolved (e.g., Esmaeili et al., 2020; Freyhof et al., 2017). Therefore, the following additional question was addressed: (3) do the freshwater and marine populations of the Arabian killifish, *A. dispar sensu lato*, from the study locations represent the same species or different species?

The Arabian killifish as a model

The Arabian killifish (Figure 1.1) is an appropriate model to answer my thesis questions for a host of reasons. It is worth noting that much of the information presented in this chapter is specific to the genus *Aphaniops* but holds true to all of the populations used in my study, which are discussed in subsequent chapters. This is due to the fact that the majority of *Aphaniops* populations in and around the Arabian Peninsula have long been identified as one species, *A. dispar*, by which studies on ecology, morphology and physiology came from different locations. But, recent studies, including findings from this thesis (Chapter 2), have revealed that only populations from the Saudi Arabian Red Sea basin and the East Mediterranean Sea constitute *A. dispar sensu stricto*. Thus, the information here pertains to the genus *Aphaniops* unless otherwise specified for the Arabian killifish *A. dispar*.



Figure 1.1: A male (bottom) and females (top) of the Arabian killifish *A. dispar* from Wadi Al Arj, Saudi Arabia. Photo credit: Ateah Alfakih.

Aphaniops species are eurycious and can be found in a wide range of environments (Coad, 2010; Wildekamp, 1993). They are eurythermal species that can withstand temperatures ranging from more than 4.2 to approximately 40°C (Al-Daham et al., 1977; Al-Johany & Yousuf, 1993; Haas, 1982; Schunter et al., 2021). Furthermore, they are euryhaline and can survive in both freshwater and hypersaline seawater, including *A. dispar* from Saudi Arabia (Bonzi et al., 2021). Recent research has suggested that although hydrologically disconnected, the Arabian killifish may have moved between freshwater and saltwater habitats within a short timescale (Schunter et al., 2021), as was shown to tolerate direct transition between extreme salinity regimes (Bonzi et al., 2021).

Owing to their physiological plasticity, members of the genus *Aphaniops* may have gained an advantage that allowed them to colonise a variety of environments (Bonzi et al., 2021). They are distributed in the East Mediterranean Sea, Arabian Gulf, Arabian Sea, and the Red Sea and their adjacent inland waters (Coad, 2010; Hrbek & Meyer, 2003; Krupp, 1983; Wildekamp, 1993). Thus, *A. dispar* can be easily obtained from several habitats, including lagoons, brackish estuaries, freshwater wadis (ephemeral streams) and springs. *Aphaniops* species can be easily bred and maintained for experiments and longitudinal studies, and some populations of *Aphaniops* have been used in the aquarium trade (Coad, 2010). This ease of breeding and maintenance may apply to the *A. dispar* and the other congener

species within the genus *Aphaniops*, allowing studies of evolutionary and behavioural ecology to be broadened from population to species level.

Nevertheless, when Nardo described the genus *Aphanius* in 1827, which currently includes *Aphaniops* Hoedeman, 1951, and means “resembling *Aphanius*” (Esmaeili et al., 2020; Wildekamp, 1993), he did not explain the meaning of this generic name. However, the name *Aphanius* originated from Greek, which means inconspicuous (Esmaeili et al., 2020; Wildekamp, 1993), but I found no mention of the reason behind the choice of this name. The term *Aphanius* may truly refer to inconspicuousness. By living in freshwater streams and marine habitats, the *Aphaniops* representatives, including the Arabian killifish, are not only subjected to variable salinity and temperature but are susceptible to predation by a variety of visually guided predators (Egan, 2007; Freyhof et al., 2020) that may possess different visual capabilities (Figure 1.2). These predators include fish (Coad, 2010; Llewellyn-Smith, 2011), snakes (personal observations), birds (Freyhof et al., 2020) and invertebrates (personal observations). Thus, at least to the human eye in nature, *A. dispar* “frequently” exhibits levels of background matching (e.g., Figure 1.3), which may prevent observers from detecting it, which in turn might have led Nardo to choose the genus name *Aphanius*. My observations before the commencement of the PhD programme revealed that the Arabian killifish can change colour rapidly, which has not previously been observed in this species or its congeners. As a result, it is unknown to what degree *A. dispar* is capable of changing colour and employing it in nature and how it would vary between extreme habitats.



Figure 1.2: Some of the natural predators of *Aphaniops* in Saudi Arabia. (A) A white-throated kingfisher (*Halcyon smyrnensis*) feeding on a male of *A. stoliczkanus* from the Eastern province of Saudi Arabia, © Turki Alazwari. (B) A common kingfisher (*Alcedo atthis*) feeding on a female of *A. stoliczkanus* from the Eastern province of Saudi Arabia, © Ali Alhassan. (C) A sooty gull (*Ichthyaelus hemprichii*) from Dana Bay on the Gizan coast, and (D) a hamerkop (*Scopus umbretta*) from Wadi Qanuna searching in shallow water where mostly only *Aphaniops* fish live. (E) A crab and a school of *A. dispar* from Jeddah, Saudi Arabia. (F) An Arabian cobra (*Naja arabica*) feeding on the Arabian killifish's heterospecific (*Garra tibanica*) from Wadi Al Arj, Saudi Arabia, © Naif Almalki. (G) A water beetle feeding on a male of *A. dispar* from Wadi Nawan, Saudi Arabia. The photos C, D, E and G were taken by me, and the credited photos A, B and F were used with permission from the owner.



Figure 1.3: Example of background matching in the Arabian killifish in a shallow depth from Wadi Nawan, Saudi Arabia. The photo contains over 25 individuals of the Arabian killifish, 6 of which were magnified in the selected rectangle. Photo credit: Ateah Alfakih.

The differences in water salinity and predators between freshwater and marine ecosystems, along with other factors such as drought or tide fluctuations, may influence how Arabian killifish survive in both ecosystems. Findings from recent studies suggested that *A. dispar* shows geographical variation in diet (Schunter et al., 2021) and physiology (Bonzi et al., 2021) between freshwater and marine environments in Saudi Arabia. Additionally, freshwater populations of *Aphaniops* species show smaller bodies than their marine counterparts (Coad, 2010), and the colour patterns, especially of males, vary from one location to another (Krupp, 1983; Wildekamp, 1993). Moreover, embryos of *Aphaniops*, identified as the Arabian killifish (*A. dispar*), were shown to possess a novel type of pigment cell termed fluoroleucophores (Hamied et al., 2020). However, the function of this pigment is not known.

The species epithet “*dispar*” refers to the pronounced sexual dimorphism (Rüppell, 1829), where males possess elaborated colouration with various blue to whitish colour patterns and large yellowish to hyaline elongated fins (Figure 1.1). In contrast, females have silvery flanks, vertical dark stripes and smaller translucent fins (Figure 1.1). During mating, *Aphaniops* males exhibit highly intense blue, orange, and yellow colouration on their flanks (Coad, 2010). These colour changes are also seen in the Arabian killifish. Males of *A. dispar* may rapidly adjust their nuptial colouration by decreasing the saturation of their ornamental colouration (e.g., when disturbed) (personal observation), which is found in other fish species (Kodric-Brown, 1998). Furthermore, *Aphaniops* males perform aggressive displays associated with the establishment of temporal territories (Haas, 1982). These nuptial displays of *A. dispar* males are accompanied by very dark colouration on the dorsal part of their bodies that highly contrasts the substrates of the established territories (personal observations), which may mediate sociosexual interactions, but have adverse effects on survival. The observed sexual dimorphism and sex-specific behaviours add an additional advantage to exploring whether there is any variation between males and females in colour change for camouflage and associated behaviours such as habitat choice.

All of the aforementioned characteristics of *Aphaniops* species make the Arabian killifish an excellent candidate for investigating geographical variation in colour change and colouration-based behaviours such as habitat preference. Particularly interesting is the evolutionary history of *Aphaniops* in and around Arabia and its counterpart genera of the Aphaniidae family in Europe, Asia and Africa. These genera are highly diverse and neighbour each other geographically over relatively short distances (Wildekamp, 1993;

Freyhof & Yoğurtçuoğlu, 2020). Additionally, members of *Aphaniops* and *Aphanius* seem to display conserved plastic traits such as euryhalinity and eurythermality (Kottelat & Freyhof, 2007; Freyhof et al., 2020). The ability to change colour may also be a conserved trait as well among members of Aphaniidae in freshwater and marine ecosystems. The results from this thesis will serve as a foundation for future studies on the evolution of colour change in the euryhaline Aphaniidae fishes. Comparative studies on colour change among and within the taxonomically resolved Aphaniidae species will provide an opportunity to investigate the evolution of colour change.

Thesis content

In this thesis, the ultimate goal is to explore the geographical variation in colour change and related behaviours, specifically habitat choice, between freshwater and marine populations of a euryhaline species of fish. In order to do this, I investigated the taxonomy of the fish from the populations studied and conducted experiments to determine their colour change ability and substrate preference. The thesis chapters are presented as follows:

1. The taxonomic status of the Arabian killifish *Aphaniops dispar sensu lato* from the Saudi Arabian Red Sea basin (Chapter 2).
2. The geographical variation of colour change in the Arabian killifish (*Aphaniops dispar*) from freshwater and marine ecosystems (Chapter 3).
3. Multiple background types attenuate the need for colour change in the Arabian killifish (*Aphaniops dispar*) from freshwater and marine ecosystems (Chapter 4).

In the first empirical chapter (Chapter 2), I studied eight populations (three marine and five freshwater) to gain general insight into the taxonomic construction of the Arabian killifish in the Saudi Arabian Red Sea basin. By utilising molecular, morphological and colour pattern approaches, I was able to determine the taxonomic status of the Arabian killifish populations in the western part of Saudi Arabia. The findings from this chapter updated the previous knowledge (i.e., Esmaeili et al., 2020; Freyhof et al., 2017; Teimori et al., 2018) of the species in the study area. The results revealed two distinct phylogenetic clusters in the Saudi Arabian Red Sea, termed molecular operational taxonomic units (MOTUs): (1) the Gizan MOTU from a single marine location in Gizan on the southwesternmost coast of Saudi Arabia and (2) the widespread *dispar* MOTU from the southern to northern freshwater and marine locations of the west of Saudi Arabia, which groups with the East Mediterranean

populations. These MOTUs were difficult to identify by analysing the morphological and colour pattern characteristics, and it is most likely that the Gizan MOTU represents a distinct cryptic species and, therefore, is referred to in this thesis as *A. cf. dispar*.

In the second empirical chapter (Chapter 3), I examined the geographical variation in colour change among four populations (two marine and two freshwater) that belonged to the two MOTUs identified in Chapter 2. By using a digital camera and analysing calibrated photographs from a bird's visual system, I looked at the ability of each population to change colour (1) achromatically by analysing the luminance (the perceived brightness by the visual model) changes in response to white and black backgrounds, and (2) chromatically by analysing the hue and saturation changes on beige, brown and green backgrounds. Then, I analysed the luminance and colour contrasts between the fish and the given substrates to better understand how these changes contributed to the fish camouflage. The results showed that all populations showed a greater ability to change colour achromatically than chromatically. The fastest and greatest changes were observed on the black backgrounds compared to the white ones for all populations, and the most isolated population (Khaybar within the *dispar* MOTU) showed almost no response to a white background. The chromatic changes were complex, but the marine population belonging to the Gizan MOTU and Jeddah population within the *dispar* MOTU had a greater ability to change colour than the freshwater populations within the *dispar* MOTU.

In the third empirical chapter (Chapter 4), by using the photography and visual modelling approaches as in Chapter 3 in addition to video recording, I examined how the fish from one marine and one freshwater population within the *dispar* MOTU choose between multiple achromatic substrates, and how these substrates mediate the colour change ability. I looked at the luminance camouflage of the fish on all the given substrates to determine its relationship to both colour change and substrate choice. The results showed that fish from both populations exhibited increased fidelity to the black substrates by spending more time on them compared to the white and the neutral intermediate grey substrates. Moreover, the fish did not show significant changes in luminance and did not appear camouflaged to the avian visual model on all substrates, especially the white. To the best of my knowledge, except for a recent study that was published while writing this thesis (i.e., Spadavecchia et al., 2023), studying the interpopulation differences in colour change and its interaction with habitat choice among a single taxon seems unexplored. Thus, I further extended the

discussion of how colour change, habitat choice and other related factors such as refugia, shoaling, habitat complexity and conspecific presence may interact with each other.

In the last chapter (Chapter 5), I discussed the findings from the previous chapters in a wider context. I discussed how my thesis contributes to the present understanding of the geographical variation in colour change as well as the future perspectives and limitations of our knowledge of this subject.

Chapter 2: The taxonomic status of the Arabian killifish *Aphaniops dispar* *sensu lato* from the Saudi Arabian Red Sea basin

Abstract

Although *Aphaniops* is the only genus among the Eurasian killifish family Aphaniidae found in freshwater and marine habitats in Arabia, its taxonomic status remains unresolved. Initially, many *Aphaniops* populations were hypothesised to represent only one widespread species, *Aphaniops dispar*, but ultimately, molecular-based studies rejected this hypothesis, validating nine species from Arabia, Africa and Iran. However, the taxonomic status of the Red Sea populations of *A. dispar* is unknown, especially in Saudi Arabia. Therefore, this study aims to determine whether the populations of *A. dispar* from Saudi Arabia represent a single or different species. I utilised morphometric, colour pattern and molecular methods on multiple freshwater and marine populations along the Red Sea basin in Saudi Arabia. The findings revealed some differences between populations in morphology and colour patterns, particularly in male morphs. Moreover, the molecular analysis grouped the freshwater populations with the closest marine populations, suggesting that these are not distinct species, and instead identified two distinct molecular operational taxonomic units (MOTUs), separating the southernmost population (Gizan) from all other Red Sea basin populations. The southern MOTU I found in Gizan may represent a new cryptic species that is genetically distinct but morphologically similar to other populations in the Red Sea.

Introduction

The Eurasian killifish family (Aphaniidae: Cyprinodontiformes) contains about 45 members of small fishes, native to the Old World and widely distributed from the Mediterranean Sea to the Indian Ocean, including the Arabian Gulf, the Arabian Sea, the Red Sea and the inland waters adjacent to these seas (Freyhof et al., 2017, 2020; Wildekamp, 1993). The inland waters of Anatolia and the Iranian Plateaus house the highest species diversity of the Aphaniidae members, making them global hotspots for killifish (Hrbek & Meyer, 2003). Prior to 2020, *Aphanius* Nardo, 1827 was considered a large genus with 44 species (Esmaeili et al., 2020), but currently, the term *Aphanius* is mostly a common synonym for many species belonging to eight new proposed genera (*Anatolichthys*, *Aphaniops*, *Aphanius*, *Apricaphanius*, *Esmaeilius*, *Kosswigichthys*, *Paraphanius*, *Tellia*) (Esmaeili et al., 2020; Freyhof & Yoğurtçuoğlu, 2020). However, only two Mediterranean species, *Aphanius almiriensis* Kottelat et al., 2007, and *A. fasciatus* Valenciennes, 1821, have been placed under the genus *Aphanius* in the newly proposed separation of the Aphaniidae genera (Freyhof & Yoğurtçuoğlu, 2020). Following this, the genus names *Aphanius* and *Aphaniops* are interchangeable, with the latter in this chapter referring to the current and historical discussions.

Pertaining to the Arabian peninsula and its surroundings, only one genus, *Aphaniops* Hoedeman, 1951, is currently present in coastal and adjacent inland waters, which represents nine valid species: *A. dispar* Rüppell, 1829, *A. furcatus* Teimori et al., 2014, *A. ginaonis* Holly, 1929, *A. hormuzensis* Teimori et al., 2018, *A. kruppi* Freyhof et al., 2017, *A. richardsoni* Boulenger, 1907, *A. sirhani* Villwock et al., 1983, *A. stiassnyae* Getahun & Lazara, 2001, and *A. stoliczkanus* Day, 1872 (Esmaeili et al., 2020; Freyhof et al., 2017; Freyhof & Yoğurtçuoğlu, 2020; Teimori et al., 2018). Regardless of the new generic name *Aphaniops*, historically, at least seven of these nine species—apart from *A. furcatus* and *A. sirhani*—were hypothesised to represent populations of only one single taxon, *A. dispar*, with two subspecies: one landlocked in the Dead Sea (*A. d. richardsoni*) and the widespread Arabian killifish (*A. d. dispar*) (Krupp, 1983; Krupp and Schneider, 1989; Villwock et al., 1983; Wildekamp, 1993).

The hypothesis of the one widespread taxon was largely influenced by the limitations in the methods used for species identification, as similarities in appearance occurred between the populations. Meristic analyses suggested overlap between the populations and even

between different species (see below). The morphological and meristic characteristics of allopatrically distributed species can often be remarkably similar, making identification challenging (Hrbek et al., 2006). *Aphaniops* species may possess high variability in morphometric and meristic traits that overlap between closely related species (e.g., Getahun & Lazara, 2001), making species identification problematic (Teimori et al., 2022); for an exception, see Coad (1980). Moreover, variations in the number of fish specimens examined may have led to different results (see Krupp, 1983; Villwock et al., 1983, for some instances).

However, the one-species hypothesis has persisted for an extended period of time, despite the clearly reported morphological interpopulation variations, especially in the colour patterns (Krupp, 1983; Wildekamp, 1993). Ultimately, geographic locations and colour patterns were proposed as the best approach for identifying members of the *Aphaniops* group due to inconsistency in meristic analysis within and among populations (Krupp, 1983), though there have been contrasting views regarding the usefulness of the morphological and meristic analyses in the separation of populations for resolving taxonomic designations, such as subspecies level (Wildekamp, 1993). As a result, lately, *A. dispar* has been treated as a species group rather than as a single taxon (Charmapila et al., 2020; Esmaeili et al., 2020). Despite the long history of ichthyologists contending with each other over the description of members of the *A. dispar* group (see Wildekamp, 1993 for detailed synonymy), Hrbek and Meyer (2003) appeared to be the first to suggest that *A. dispar* was not a single species, based on molecular analysis, thus indicating that the postulated populations of this taxon were not monophyletic. However, with the advances in molecular-based studies, the one-taxon hypothesis was consensually rejected, validating the nine species from Arabia, Africa and Iran (Freyhof et al., 2017; Freyhof & Yoğurtçuoğlu, 2020; Teimori et al., 2018, 2022).

Currently, the landlocked subspecies in the Dead Sea *A. d. richardsoni*, has been validated as *A. richardsoni* (Freyhof et al., 2017), and the widespread *A. d. dispar* has been represented by the remaining six species: *A. dispar*, *A. ginaonis*, *A. hormuzensis*, *A. kruppi*, *A. stiassnyae* and *A. stoliczkanus* (Freyhof & Yoğurtçuoğlu, 2020; Teimori et al., 2022). So far, based on molecular and colour pattern data (Charmapila et al., 2020), only three of these species are present in Arabia: *A. dispar* from the Red Sea, *A. kruppi* from Oman and *A. stoliczkanus* from the Arabian Gulf catchment, including the eastern coast of Saudi Arabia (Esmaeili & Hamidan, 2023; Freyhof et al., 2017). Although differences in colour patterns can be helpful to some extent for species identification (Charmapila et al., 2020), relying on

one methodology may lead to conflicting outcomes. For example, a high resemblance in colour patterns can be observed in killifish species that live in close proximity, such as *A. ginaonis*, *A. hormuzensis* and *A. kruppi*, despite being genetically distant (Freyhof et al., 2017; Teimori et al., 2018, 2022; Wildekamp, 1993), and different morphs have been identified across a wider range, as in *A. kruppi* and *A. stoliczkanus* populations, despite being genetically similar (Freyhof et al., 2017).

While research on killifish has been carried out extensively around Saudi Arabia, the Red Sea populations in Saudi Arabia have received minimal attention. Colour pattern and morphological characteristics have not been examined in the majority of Saudi Arabian Red Sea populations, and molecular materials have only been collected from a single location, Thulbah, in the north of the country (Freyhof et al., 2017). Therefore, the taxonomic status of *A. dispar* from the Saudi Arabian Red Sea remains unknown despite the repeated suggestion of additional unidentified species, especially in the northernmost part of the region (Freyhof et al., 2017; Teimori et al., 2018). It is assumed that the marine populations of *A. dispar* display continuous gene flow, whereas landlocked freshwater populations are prone to genetic isolation and may represent independent species (Freyhof et al., 2017). Several populations in Saudi Arabia have been identified as landlocked (Krupp, 1983), but recent genomic research from the central and near-south Saudi Arabian Red Sea has indicated that there are hydrological connections and, hence, genetic connectivity between freshwater and marine populations (Schunter et al., 2021). This raises the question of what groups the distant allopatric populations of *A. dispar* in Saudi Arabia might belong to.

Assuming the presence of only one species is challenging in light of the aforementioned contradictions and given the large geographical areas that *A. dispar* occupies in the Red Sea basin, which are extremely ecologically varied and may contribute to the evolutionary history of the fish. In this study, morphometric, colour pattern and molecular methods were used to determine whether populations of *A. dispar sensu lato* found in freshwater (Khaybar and Nawan) and marine (Gizan and Jeddah) ecosystems represent a single species that has multiple populations or different species. The results will serve as a baseline for future research on these interesting populations, and their apparently rapid switches between freshwater and marine environments, particularly for colour-based behaviours. Studies on the geographical variation in phenotypic traits have been extensively addressed in specific models, such as guppies (Endler, 1995; Houde, 1997) and sticklebacks (French et al., 2018). Using the Arabian killifish would expand the scope of evolutionary and behavioural

ecology studies, allowing for a more comprehensive understanding of how adaptive traits evolve among ecosystems across different levels, from populations to species and genera.

Methods

Sample collection

A total of 160 individuals of the Arabian killifish, *A. dispar sensu lato*, were collected from 2020 to 2022 from four main locations along the western coastline of Saudi Arabia (20 males and 20 females per location) (Figure 2.1; Table S2.1). These locations included two freshwater populations (Khaybar and Nawan) and two marine populations (Gizan and Jeddah). These populations were used for morphometric, colour pattern and molecular analyses. Additionally, four other populations, namely Arj, Deba, Hali and Qanuna, were used merely for molecular analyses (Figure 2.1; Table S2.1). The Arabian killifish is neither threatened nor protected. Permission for the collection and the study was obtained from the Saudi National Center for Wildlife (2020) prior to the start of the study. Except for springs in Khaybar, for which a separate permit was obtained from the Royal Commission for AlUla (2022), the collection sites were open to the public, and no additional licences or permits were required to access them. After completing the photography, all fish involved in morphometric and colour pattern analyses were returned to their original site. The collections of fish samples for molecular analysis were authorised by the Saudi National Center for Wildlife with exportation documents E-SA-21-000007 and E-SA-22-000097.

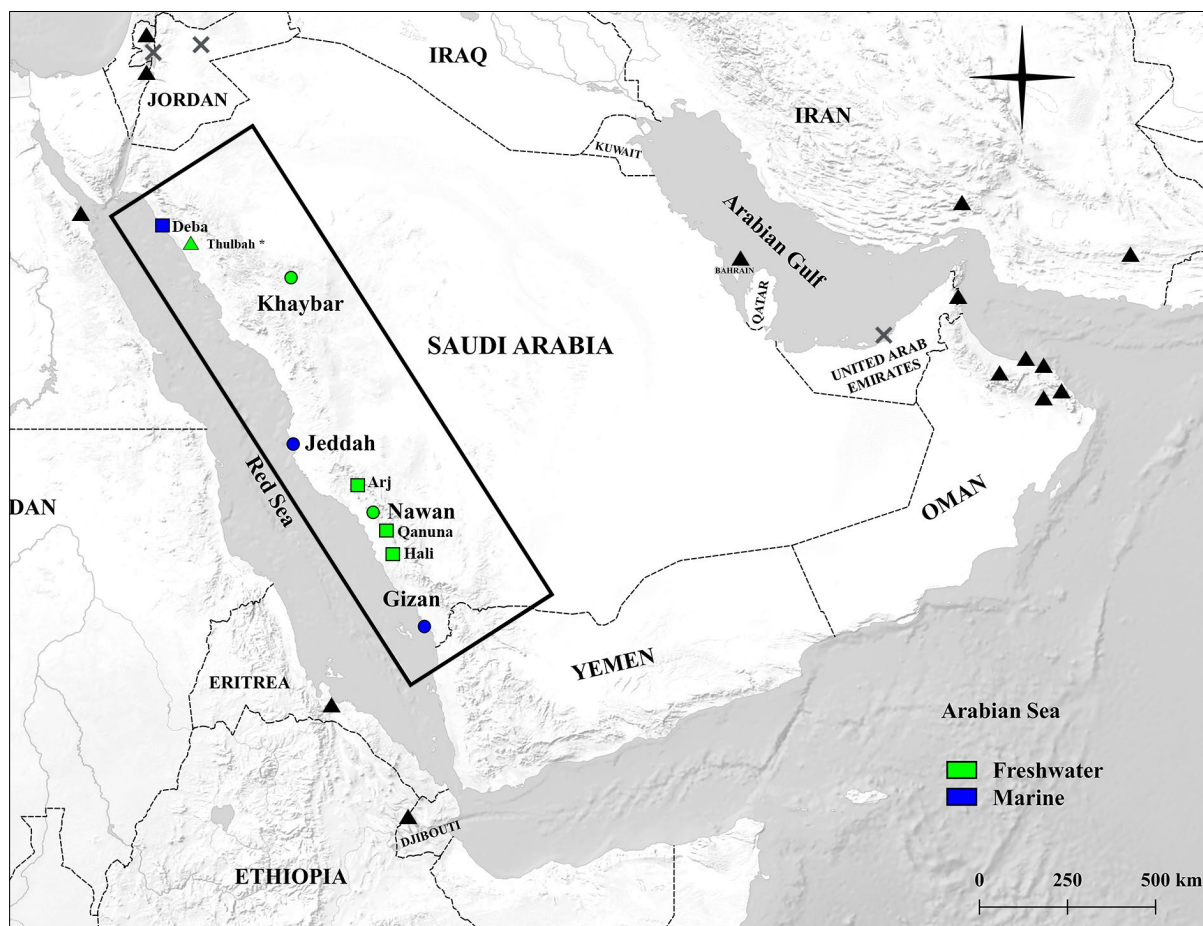


Figure 2.1: A map illustrating the locations of four main populations (circles) used for the morphological, colour pattern and molecular analyses along the western coastline of Saudi Arabia. The map includes two marine populations represented by blue circles (Gizan and Jeddah), two freshwater populations represented by green circles (Khaybar and Nawan), and four additional populations exclusively used for the molecular analyses represented by squares (freshwater: Arj, Hali and Qanuna; marine: Deba). The black crosses represent locations of molecular samples collected by Geiger et al. (2014). The black triangles indicate locations of molecular samples collected by Freyhof et al. (2017), with the exception of the freshwater pond in Thulbah, Saudi Arabia, which is represented by a green triangle.

Morphometric analysis

Images of 20 male and 20 female live fish from each of the four main populations were captured from the left side of each fish inside a photography box (Figure 2.2) in a photography room under an EyeColor Arc MT70D lamp (Iwasaki Electric Co., LTD) with a Nikon D3300 camera equipped with either 105 mm f/2.8G Micro-Nikkor lens or 18–55 mm Nikon lenses. Some individuals were photographed in the shade in the field. A known 4 cm

length in the photography box was used as a scale bar (Figure 2.2). I measured 22 morphological characteristics using ImageJ software (Schneider et al., 2012), following previous approaches used on killifish from Saudi Arabia (Freyhof et al., 2020; Krupp, 1983) (Figure 2.3; Table 2.1). I minimised potential differences in the length of fish body parameters due to variation in body size by standardising the morphometric parameters with Elliott et al. (1995) allometric formula:

$$M_{adj} = M \times \left(\frac{MSL}{SL}\right)^b ,$$

where *M_{adj}* is the size-adjusted measurement of the original characteristic of the focal fish, *M* is the original measurement of the characteristic of the focal fish, *SL* is the standard length (from the tip of the snout to the caudal fin base) of the focal fish, and *MSL* is the mean *SL* of all fish from all samples. I used the observed data to find the parameter *b* for each body characteristic. This was done by calculating the slope of the regression of log (*M*) for all fish from all samples on log (*SL*) for all fish from all samples.

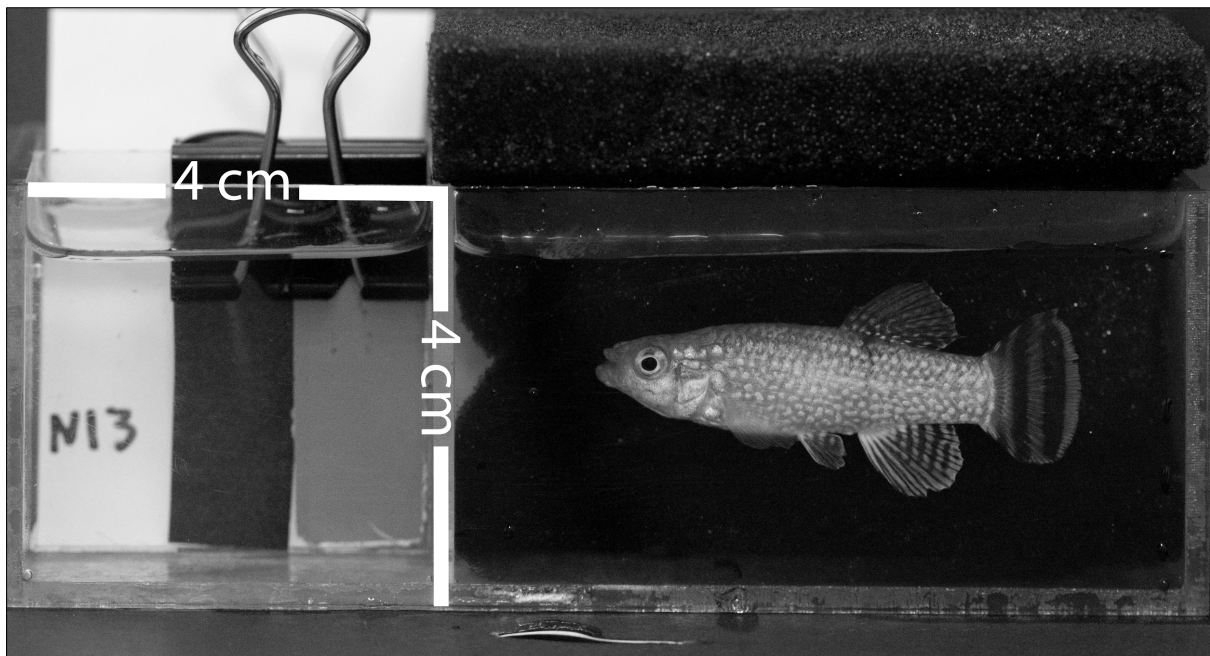


Figure 2.2: The photography box, along with a 4 cm known length as a scale bar.

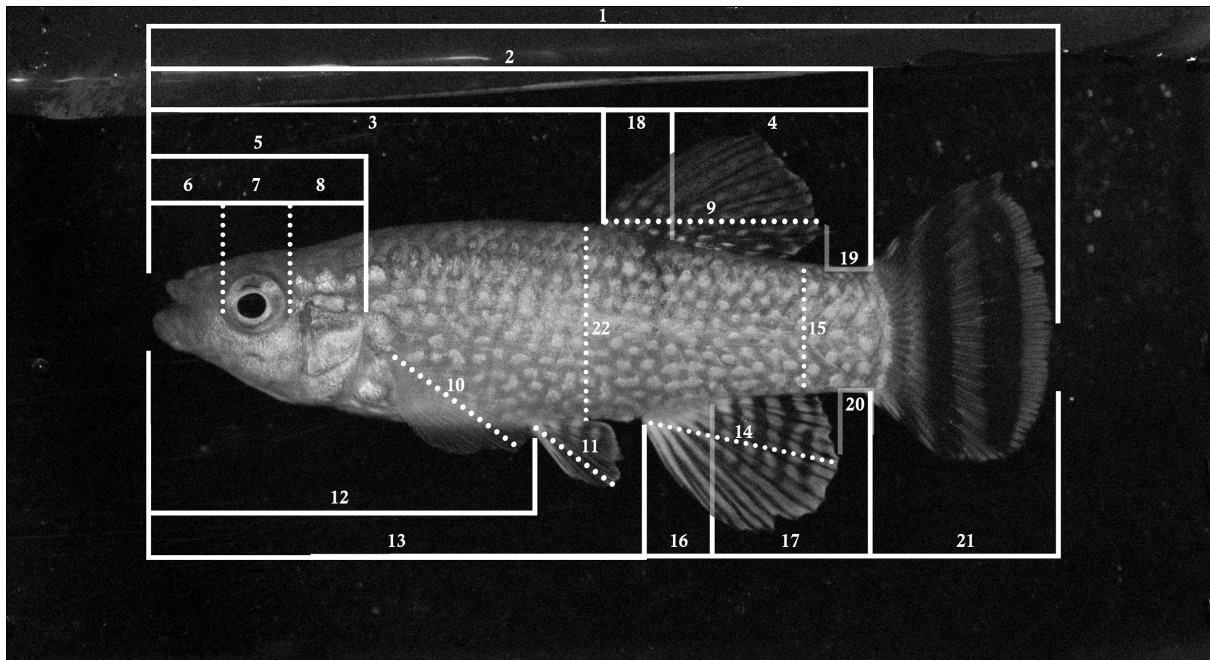


Figure 2.3: Illustration of the point-to-point method used for the 22 morphometric measurements taken for male and female individuals.

Table 2.1: The 22 morphological characteristics used for the point-to-point measurements in males and females from 4 main populations as depicted in Figure 2.3. The abbreviations inside the parentheses are used in Appendix 1A.

Number of measurements	Abbreviation	Measured area
1	TL	Total length
2	SL	Standard length
3	PRDL	Pre-dorsal length
4	PODL	Post-dorsal length
5	(HL)	Head length
6	(SNL)	Snout length
7	EYE	Eye diameter
8	PORBL	Postorbital length
9	DFL	Dorsal fin length
10	PFL	Pectoral fin length
11	PLVCFL	Pelvic fin length
12	PRPL	Pre-pelvic length
13	PRANL	Pre-anal length
14	AFL	Anal fin length
15	(DCP)	Depth of caudal peduncle

16	AFBL	Anal fin base length
17	(CPL)	Caudal peduncle length
18	DFBL	Dorsal fin base length
19	DFTCFB	Dorsal fin tip to caudal fin base
20	ANTCFB	Anal fin tip to caudal fin base
21	CFL	Caudal fin length
22	(BD)	Body depth

Colour pattern analysis

The image of each of the 20 male individuals from each population was scored for six colour pattern characteristics (Table 2.2); two males (one from Jeddah and one from Nawan) were excluded from the analysis due to a lack of clarity in the images. The characteristics of interest were (1) the number of bars, (2) the presence of arranged blotches at the caudal peduncle, (3) the presence of elongated vertical or horizontal patterns, (4) the presence of vermiculation and (5) the scatter of the blotches, and (6) the area size of the blotches on each fish flank, which was quantified using ImageJ software in proportion to the focal individual's standard length. The images were converted to 8-bit greyscale, and auto thresholding was applied with a manual adjustment, if required, to accurately capture the regions of interest. The blotches were selected using a rectangular or polygon selection tool with dimensions approximately set at 25% × 15% of the standard length. For the analysis, three regions were selected, excluding areas of bars that did not form spots to avoid the potential impact on the analysis. The area size of the blotches was measured using the "Analyze Particles" function, which extracted and measured the area of multiple spots within a selected region. A minimum size threshold of 0.0001 cm² was applied to eliminate small speckles that could introduce noise. Finally, the average area size of the blotches was calculated.

Table 2.2: A summary of the six male colour pattern characteristics and the data processing used for colour pattern analysis. See (Appendix 1B; Figure S2.7) for sample images illustrating the scoring approach of characteristics 1–5.

No.	Pattern characteristic	Definition	Data scoring	Data conversion
1	Number of bars	The bar is defined as the wide dark background colouration that is well separated by the vertical narrow light lines emerging from the ventral side up to the lateral line.	Numerical: count	None
2	Arranged blotches at the caudal peduncle	The arranged blotches are defined as the white blotches that are aligned vertically which do not constitute a white line.	Logical: Yes, No	Numerical: 0, 1
3	Elongated patterns	The light blotches that are not round or ovate and extend vertically or horizontally.	Logical: Yes, No	Numerical: 0, 1
4	Vermiculation	The irregular white blotches that are not round, ovate or elongated.	Logical: Yes, No	Numerical: 0, 1
5	Scatter of blotches	The distribution form of the white blotches that are randomly distributed on the flank which do not constitute arranged blotches.	Categorical: No scatter, scatter at the front, scatter at the mid-front and scatter across all body.	Numerical: 0, 1, 2 and 3 respectively
6	Average pattern size	The average area size of blotches as proportion to the standard length of the fish (i.e., the average area size (cm ²) ÷ SL) × 100).	Numerical: continuous	None

Statistical analysis

I ran principal component analysis (PCA) on the scaled data of the twenty-two morphometric characteristics for males and females separately; because the SL was constant across all samples after the standardisation, it was excluded from the PCA analyses. However, I removed two females (one from Gizan and one from Nawan) from the PCA analysis due to a lack of clarity in the images. To explore the differences between populations based on pattern characteristics, I converted the nonnumerical colour pattern scores described above to numerical variables (Table 2.2). I then performed a PCA on the scaled data of the six colour pattern characteristics. Females possessed only narrow vertical stripes; hence, only the differences in the number of vertical stripes were reported (Appendix 1B).

I conducted statistical tests (ANOVA or Kruskal-Wallis rank sum test) on the extracted PC scores of the first and second major principal components PC1 and PC2 from both PCAs of male and female morphometrics and from the PCA of male colour patterns to assess the significance of differences between the four populations. The choice of the statistical test depended on two factors: (i) the homogeneity of variance, determined by Levene's test for homogeneity of variance of the data, and (ii) the normality of the ANOVA residuals, determined by the Shapiro-Wilk normality test. If the data met the assumptions of homogeneity of variance and the residuals of ANOVA were normally distributed, I reported the results from the one-way ANOVA test. If the assumptions were not met, I used and reported a Kruskal-Wallis rank sum test (hereinafter Kruskal-Wallis test). After conducting the one-way ANOVA and Kruskal-Wallis test, a post-hoc analysis was carried out. For the one-way ANOVA, the Tukey honest significant differences test (Tukey test) was used, while for the Kruskal-Wallis test, the Wilcoxon rank sum test (Wilcoxon) with Bonferroni adjustment of P values was employed. All analyses were performed in R version 4.2.3 (R Core Team 2023).

After conducting the PCAs on scaled data from twenty-one morphometric traits, I observed that three variables exhibited significant loadings on PC2 for males, and similarly, three variables displayed substantial loadings on PC1 for females (see Results). Thus, I conducted additional statistical tests on the raw standardised data for both males and females to examine the differences between populations in each trait using the same approach for the statistical tests described above (Appendix 1A). As I analysed only six

colour pattern characteristics of the males, additional tests were also performed to look at the differences among populations based on each colour pattern characteristic (Appendix 1B; Figures S2.8–S2.14).

Molecular analysis

A total of 32 fish were collected from eight populations (four fish per population); the four main locations (Gizan, Jeddah, Khaybar and Nawan) in addition to the other three freshwater locations (Arj, Hali and Qanuna) and a marine location (Deba) (Figure 2.1; Table S2.1). The fish were euthanised with an overdose of clove oil and preserved immediately in 100% ethanol at room temperature. Fin clips were obtained from the preserved specimens, immediately air dried for 1 h, and placed inside tracing paper.

DNA Extraction

Genomic DNA was extracted using ammonium acetate methods (Nicholls et al., 2000; Richardson et al., 2001) from ~7 mm of each of the 32 fin clips placed in 1.5 ml eppendorf tubes with 250 µl of Digsol buffer (Table S2.2) and 10 µl of 10 mg/ml Proteinase K. The tubes were centrifuged at 14,000 rpm / 18 °C for 1 min and placed in a rotating oven at 55 °C overnight to obtain maximum digestion. After digestion, each eppendorf was filled with 300 µl of 4 M ammonium acetate, vortexed repeatedly over 15 min at room temperature, and centrifuged at 14,000 rpm / 18 °C for 10 min. The supernatant from each eppendorf was aspirated into a clean eppendorf, and then 1 ml of 100% ethanol was added, vortexed, and centrifuged at 14,000 rpm / 18 °C for 10 min. The supernatant ethanol was then discarded, and 500 µl of 70% ethanol was added to the eppendorfs and inverted several times to rinse the pellets, and then centrifuged at 14,000 rpm / 18 °C for 5 min. The supernatant was gently discarded, and eppendorfs were placed upside down on tissue for 20 h at room temperature to allow ethanol to evaporate fully. After thoroughly drying, 100 µl of low TE (TE₁₀E_{0.1}) (Table S2.2) was placed in each eppendorf to resuspend the pellets. The eppendorfs were briefly vortexed and flicked to dislodge pellets and then transferred to a water bath for 30 min at 65 °C with flicking every 10 min to dissolve pellets. Samples were stored at –20 °C.

Quantification of DNA

To quantify the DNA, 2 µl of each sample was added to a separate well in a clean BMG plate, and 2 µl of seven DNA standards (Promega) deconcentrated with low TE (0, 3.125, 6.25, 12.5, 25, 50 and 100 ng/µl) were added to separate wells. Then 200 µl of Quantifluor mix (50 µl Quantifluor dsDNA dye (Promega), 1 ml 20X TBE and 19 ml ddH₂O) was added to the samples and the standards. Readings were obtained using FLUOstar Optima (BMG Labtech, Germany). The extraction method was efficient and yielded suitable concentrations of DNA (10.3–86.6 ng/µl), which were then diluted to ~10 ng/µl with low TE.

Polymerase chain reaction (PCR)

LCO1490 and HCO2198 primers (Folmer et al., 1994) were used to amplify the cytochrome c oxidase subunit I (COI) gene. To prevent cross-contamination, I used filter-tip pipetting to prepare a 10 µl PCR reaction mix (5 µl Qiagen PCR Master mix, 1 µl of 10 pmol/µl LCO1490 primer, 1 µl of 10 pmol/µl HCO2198 primer, 1 µl ddH₂O and 2 µl DNA) of each sample. On a Mastercycler X50s Thermocycler (Eppendorf), I used a thermocycle programme consisting of 95 °C for 15 min, 32 cycles of 94 °C for 30 s, 55 °C for 90 s and 72 °C for 60 s, with a final extension at 72 °C for 10 min. The products were stored for 20 h at –20 °C. PCR products were checked by running 4 µl PCR on a 1% Agarose gel (LE Agarose CSL-AG500, Cleaver Scientific Ltd, Warwickshire, UK) stained with Ethidium Bromide in 1x TBE under 96 volts for 45 min and against 5 µl of quick-load 100-bp DNA ladder (New England BioLabs). The gel was visualised under UV transmission on an E-Box Imaging System (Vilber).

Purification

Purification of PCR products was done by adding 2 µl of illustra ExoProStar 1-Step (GE Healthcare, UK) to 5 µl of each PCR product. Then, they were incubated at 37 °C for 15 min and 80 °C for 15 min on a Peltier thermal cycler (DNA Engine TETRAD, MJ Research) to inactivate enzymes. After that, the plate was cooled down using ice, and 33 µl of ddH₂O was added to each sample and centrifuged briefly in a minicentrifuge. The total 40 µl volume of each purified PCR product was split into two eppendorfs, each containing 20 µl. The purified samples were Sanger sequenced by GENEWIZ (Takeley, UK) using BigDye and 5 µM of 10 pmol/µl LCO1490 and 5 µM of 10 pmol/µl HCO2198 primers, where 29 out of the 32 samples qualified for molecular analyses.

Sequence preparation and alignment

Forward and reverse sequence traces from all successfully Sanger-sequenced PCR products were visually inspected in Geneious Prime 2023.0.4 (Kearse et al., 2012) to select the best materials to construct phylogenetic trees, as subsequently detailed. The forward and reverse sequences were aligned and combined to create consensus sequences. The obtained consensus sequences were then trimmed with an error probability limit of 0.01, producing 28 aligned consensus sequences and 1 single-read reverse sequence. Next, I searched for open reading frames (ORFs) where bases at the beginning of sequences were manually deleted if a 2nd or 3rd codon position was found. All the final sequences started at the 1st codon position, with translated bases indicating no stop codons, resulting in quality 89.3%–97.8% with post trim length 620–692 bp. Sequence data prepared in this study are all available in the GenBank Nucleotide Database with accession numbers OR841348 and OR863925–OR863952, as stated in (Appendix 1C; Table S2.1). I imported 2 *COI* barcode sequences of *Paraphanius mento* as an outgroup species and 58 sequences of *Aphaniops* spp. from NCBI GenBank that were used in the Freyhof et al. study on *Aphaniops* around Arabia (2017), which included the barcodes provided by Geiger et al. (2014) (Appendix 1C), and set their appropriate ORFs. Then, I aligned those barcodes with the 29 new sequences using MUSCLE by codon in MEGA 11 (Tamura et al., 2021) and trimmed the ends of the alignment to remove regions with less than 95% site coverage, resulting in 606 sites for the subsequent phylogenetic constructions. The nucleotide substitution saturation test (Xia et al., 2003; Xia & Lemey, 2009) was used to assess substitution saturation in the *COI* barcode region using DAMBE 7.3.32 (Xia, 2018). The saturation test revealed that the Index of Substitution Saturation (I_{ss}) was substantially less than the Critical Index of Substitution Saturation ($I_{ss.c}$) for symmetrical and asymmetrical topologies, indicating that the *COI* sequences were not saturated and thus suitable for phylogenetic analysis.

Phylogenetic construction

I employed the jModelTest2 (Darriba et al., 2012), accessed via CIPRES (<https://www.phylo.org/>), for the selection of the best nucleotide substitution models for constructing the phylogenetic trees: maximum likelihood (ML), Bayesian inference (BI) and neighbour joining (NJ) trees, which were all conducted in Geneious Prime. The choice of the model was based on the Bayesian Information Criterion (BIC) and Decision Theory. The construction of the ML tree was done using the PhyML (Guindon et al., 2010) plugin with 1000 bootstraps, utilising the HKY with a Gamma distribution with four classes and setting

the gamma parameter to 0.1490 and the transition/transversion bias value to 7.70693. The reconstructed consensus ML tree was then used for species delimitation using Poisson Tree Processes (PTP) (Zhang et al., 2013) via (<http://mptp.h-its.org/>). I summarised the estimations of Kimura 2-parameter genetic distances (K2P) (Kimura, 1980) between the groups delimited by PTP in MEGA 11 for all 606 sites in the dataset. The K2P genetic distances (Appendix 1C; Tables S2.3, S2.4) are expressed as percentages in the discussion, which offer information comparable to other related research (e.g., Freyhof et al., 2017; Teimori et al., 2020). I used the HKY+G model with four gamma rate categories and a gamma parameter of 0.1490 for Bayesian Inference (BI) analysis. The base frequencies for A, C, G and T were estimated as 0.242, 0.293, 0.1856 and 0.2794, respectively. The transition/transversion ratio was estimated to be 7.7069. The analysis was carried out via the MrBayes (Huelsenbeck & Ronquist, 2001) plugin. I executed two simultaneous runs of four Markov chains for 1000,000 generations, followed by a burn-in, which discarded 20% of the initial trees. The construction of the NJ was done utilising the HKY model with 10,000 replicates using the Geneious tree builder. The phylogenetic tree figures were constructed and visualised using the Interactive Tree of Life (iTOL, v 6.8.1: <https://itol.embl.de/>) (Letunic & Bork, 2007). I reported the genera names on the phylogenetic trees as reported by Esmaili et al. (2020) and Freyhof and Yoğurtçuoğlu (2020). The species names were reported based on the work of Freyhof et al. (2017) pertaining to *Aphaniops spp.* in and around Arabia.

Results

Morphometric analysis

The PCA analyses for both male and female datasets produced 21 components corresponding to the 21 morphometric measurements. The first two major principal components for males accounted for 40.39% of the total variance, with PC1 contributing 23.51% and PC2 contributing 16.89% (Figure 2.4A; Table 2.3). However, the third and fourth components contributed 11.67% and 7.9%, respectively, which were not further analysed. There were marginally significant differences among populations in PC1 (Kruskal-Wallis test: $\chi^2 = 7.992$, $df = 3$, $p = 0.0462$), where only Khaybar and Nawan populations exhibited a near, but not, significant difference (Wilcoxon: $p = 0.057$) (Figure 2.4B). The most substantial loadings on PC1 were DFL, PLVCFL, AFL, DFTCFB and ANTCFB (Table 2.3).

In addition, there were highly significant differences among populations in PC2 (ANOVA: $F(3,76) = 26.55$, $p < 0.0001$), whereby each population from the freshwater (Khaybar and Nawan) differed significantly from each of the marine (Gizan and Jeddah) (Tukey test: $p < 0.0001$), but no significant differences were found between Khaybar and Nawan (Tukey test: $p = 0.88$) or between Gizan and Jeddah (Tukey test: $p = 0.42$) (Figure 2.4C). The most substantial loadings on PC2 were SNL, CPL and BD (Table 2.3; Appendix 1A; Figures S2.1–S2.3).

The first two major principal components for females accounted for 40.03% of the total variance, with PC1 contributing 23.81% and PC2 contributing 16.23% (Figure 2.5A; Table 2.3). However, the third and fourth components contributed 8.61% and 7.43%, respectively, which were not further analysed. There were highly significant differences among populations in PC1 (Kruskal-Wallis test: $\chi^2 = 40.359$, $df = 3$, $p < 0.0001$), where the Jeddah population differed from all the other populations (Wilcoxon: $p < 0.0001$) and no significant differences occurred between the rest (Figure 2.5B). The most substantial loadings on PC1 were HL, DCP and CPL (Table 2.3; Appendix 1A; Figures S2.4–S2.6). There were no significant differences among populations on PC2 (ANOVA: $F(3,74) = 1.083$, $p = 0.362$) (Figure 2.5C), where the most substantial loadings were DFL, PFL and PRANL (Table 2.3).

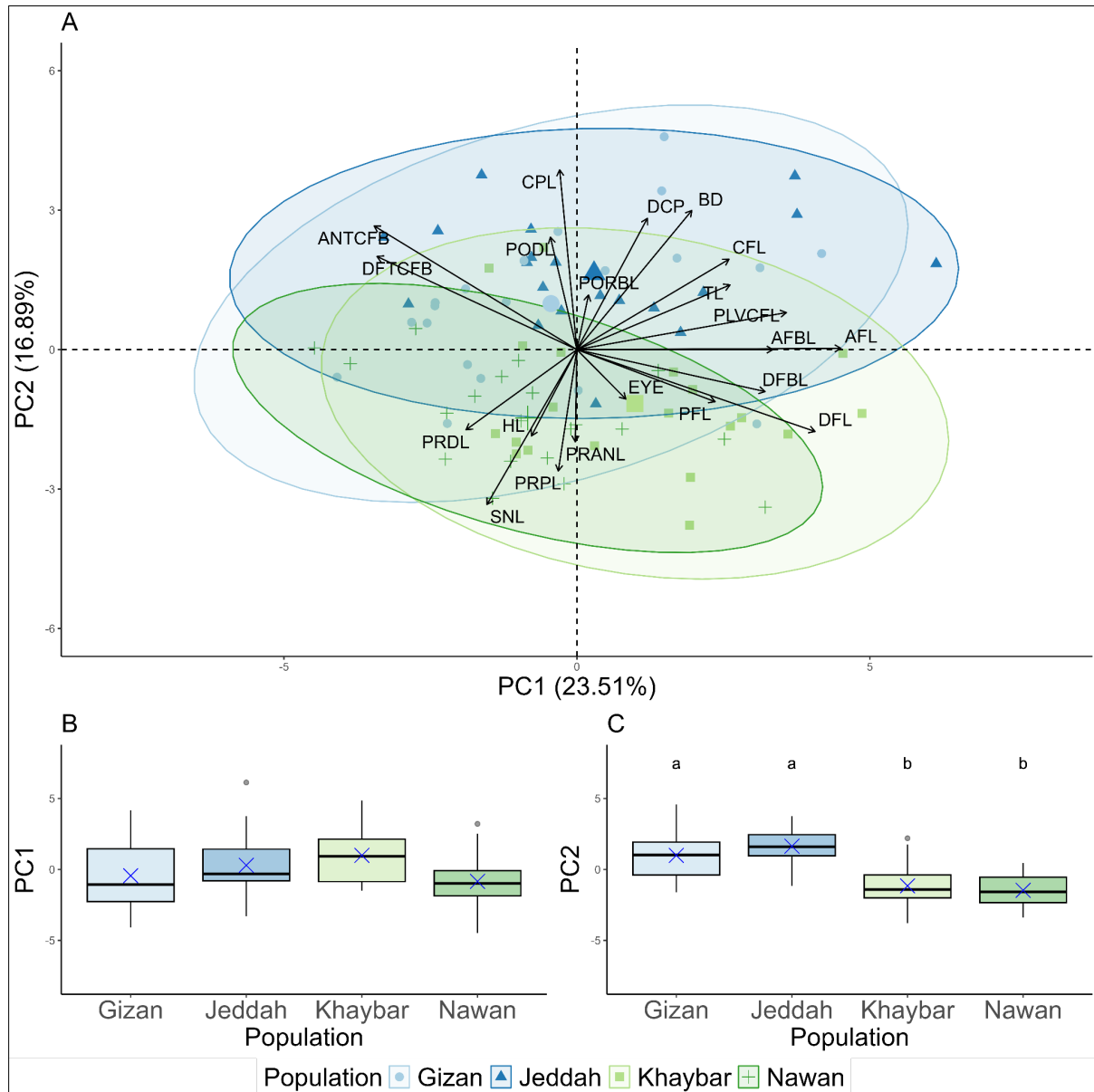


Figure 2.4: Outputs of the principal component analysis (PCA) on the 21 morphometric characteristics of male individuals of *A. dispar* from two freshwater (■ Khaybar and + Nawan) and two marine habitats (● Gizan and ▲ Jeddah) showing (A) a biplot of the two major principal components PC1 and PC2, and box plots of extracted values from PC1 (B) and PC2 (C) showing the differences among populations based on all the morphometrics. The black lines represent the medians, and the blue crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The grey dots represent the outliers. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations.

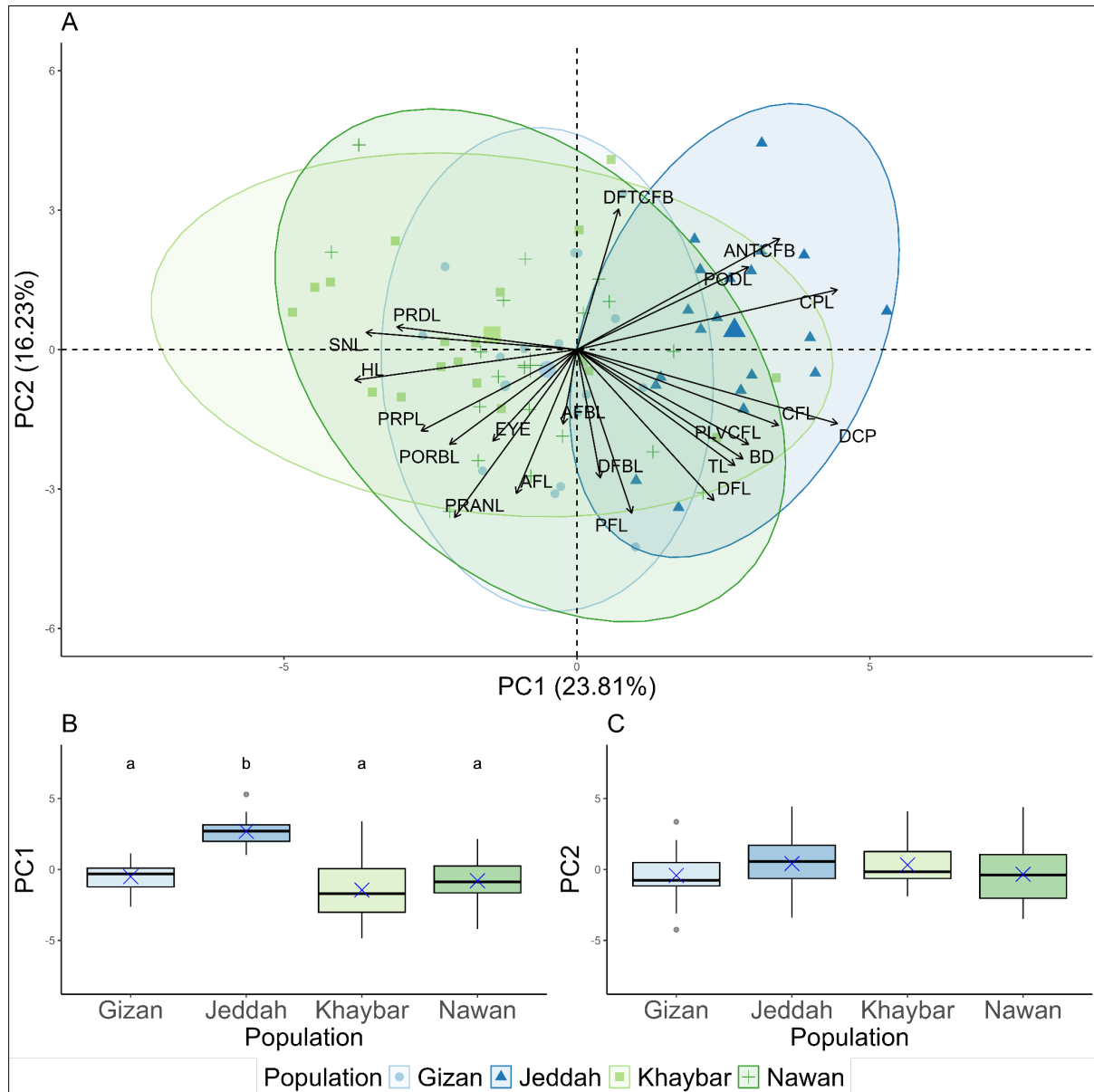


Figure 2.5: Outputs of the principal component analysis (PCA) on the 21 morphometric characteristics of female individuals of *A. dispar* from two freshwater (■ Khaybar and + Nawan) and two marine habitats (● Gizan and ▲ Jeddah) showing (A) a biplot of the two major principal components PC1 and PC2, and box plots of extracted values from PC1 (B) and PC2 (C) showing the differences among populations based on all the morphometrics. The black lines represent the medians, and the blue crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The grey dots represent the outliers. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations.

Table 2.3: Contribution percentage, Eigenvalues, and loading values of 21 morphometric characteristics on the first two principal components of male and female individuals of *A. dispar* from two freshwater (Khaybar and Nawan) and two marine habitats (Gizan and Jeddah). Variables in boldface indicate the most substantial contributions to the principal components (load value > 0.3). The acronyms of variable loadings are described in Table 2.1.

	Males		Females	
	PC1	PC2	PC1	PC2
% Variance	23.50	16.89	23.81	16.23
Eigenvalue	4.93597053	3.54768671	4.99917895	3.40844819
Variable Loadings				
PRDL	-0.16807	-0.18066	-0.24376	0.04693
PODL	-0.04016	0.25334	0.23232	0.17092
HL	-0.06889	-0.19443	-0.30071	-0.06296
SNL	-0.13622	-0.34890	-0.28547	0.03548
EYE	0.07416	-0.11140	-0.11374	-0.18890
PORBL	0.01761	0.12317	-0.17226	-0.19588
DFL	0.36094	-0.18447	0.18547	-0.31164
PFL	0.20942	-0.11728	0.07429	-0.33794
PLVCFL	0.31750	0.08349	0.23249	-0.19587
PRPL	-0.02824	-0.27343	-0.21080	-0.16801
PRANL	-0.00244	-0.20782	-0.16572	-0.34690
AFL	0.40010	0.00285	-0.08192	-0.29748
DCP	0.10677	0.29582	0.35283	-0.15291
AFBL	0.29699	0.00004	-0.01871	-0.15380
CPL	-0.02614	0.40515	0.35260	0.12360
DFBL	0.28519	-0.09451	0.03166	-0.26515
DFTCFB	-0.30294	0.20988	0.05639	0.29056
ANTCFB	-0.30731	0.27869	0.27454	0.22963
CFL	0.23062	0.20374	0.27293	-0.15641
BD	0.17413	0.31351	0.22474	-0.22572
TL	0.23209	0.14579	0.21394	-0.23955

The PCA analysis for male colour patterns produced 6 principal components corresponding to the 6 colour pattern measurements. The first two principal components accounted for 78.95% of the total variance, with PC1 contributing 50.84% and PC2 contributing 28.11% (Figure 2.6A; Table 2.4). However, the third to sixth components contributed 8.39%, 5.6%, 5.1% and 1.93%, respectively, which were not further analysed.

There were significant differences among the populations in PC1 (Kruskal-Wallis test: $\chi^2 = 58.457$, $df = 3$, $p < 0.0001$), where all populations differed significantly from each other (Wilcoxon: $p < 0.01$) except Gizan and Jeddah (Figure 2.6B). The most substantial loadings on PC1 were the number of bars, arranged blotches at the caudal peduncle, elongated patterns, vermiculation and scatter of blotches (Table 2.4). The PCA of male colour patterns revealed that the marine populations of Gizan and Jeddah had a high degree of similarity, clustering together with a great overlap in PC1, where they had a higher number of bars and more arranged blotches at the caudal peduncle compared to the freshwater population (Figure 2.6A), with the Gizan population having a slightly higher number of bars than Jeddah (Appendix 1B). The number of bars ranged from 2–8 in Gizan individuals (median = 3; mean = 3) and 1–5 in Jeddah individuals (median = 1; mean = 1.3). Khaybar exhibited the highest score for the presence of elongated patterns and vermiculation among all populations (Figure 2.6A), with no significant differences among the other populations in these two traits (Appendix 1B). The freshwater Khaybar and Nawan populations had significantly more scattered blotches across their bodies compared to the marine populations of Gizan and Jeddah (Figures 2.6A, S2.13; Appendix 1B).

Also, there were significant differences among populations in PC2 (Kruskal-Wallis test: $\chi^2 = 48.927$, $df = 3$, $p < 0.0001$), where all populations differed significantly from each other (Wilcoxon: $p < 0.05$) except Gizan and Khaybar (Figure 2.6C). The most substantial loadings on PC2 were the average pattern size, elongated patterns, vermiculation and scatter of blotches (Table 2.4). In PC2, Khaybar populations exhibited the largest blotch size among all populations, followed by Gizan individuals, but no significant difference was observed between them, whereas Jeddah and Nawan had the smallest blotch size, and no significant difference was observed between them (Figure 2.6A; Appendix 1B). However, females in all populations had varying vertical stripes, with Gizan having 15–20 (median = 18; mean =

17.95), Jeddah 13–20 (median = 16; mean = 16.15), Khaybar 9–13 (median = 11; mean = 11.05) and Nawan 11–17 (median = 16; mean = 14.46) (Appendix 1B; Figures S2.14).

Table 2.4: Contribution percentage, Eigenvalues, and loading values of 6 colour pattern characteristics on the first two principal components of male individuals of *A. dispar* from two freshwater (Khaybar and Nawan) and two marine habitats (Gizan and Jeddah). Variables in boldface indicate the most substantial contributions to the principal components (load value > 0.3). The abbreviations inside the parentheses are used to explain Figure 2.6 and in Appendix 1B.

	PC1	PC2
% Variance	50.84	28.11
Eigenvalue	3.0507012	1.6868633
Variable loadings		
Average pattern size (APS)	0.16756	-0.61096
Number of bars (NB)	-0.46293	-0.29697
Arranged blotches at the caudal peduncle (ABP)	-0.47904	-0.24067
Elongated patterns (EP)	0.34980	-0.48059
Vermiculation (V)	0.41268	-0.36201
Scatter of blotches (SB)	0.48526	0.34438

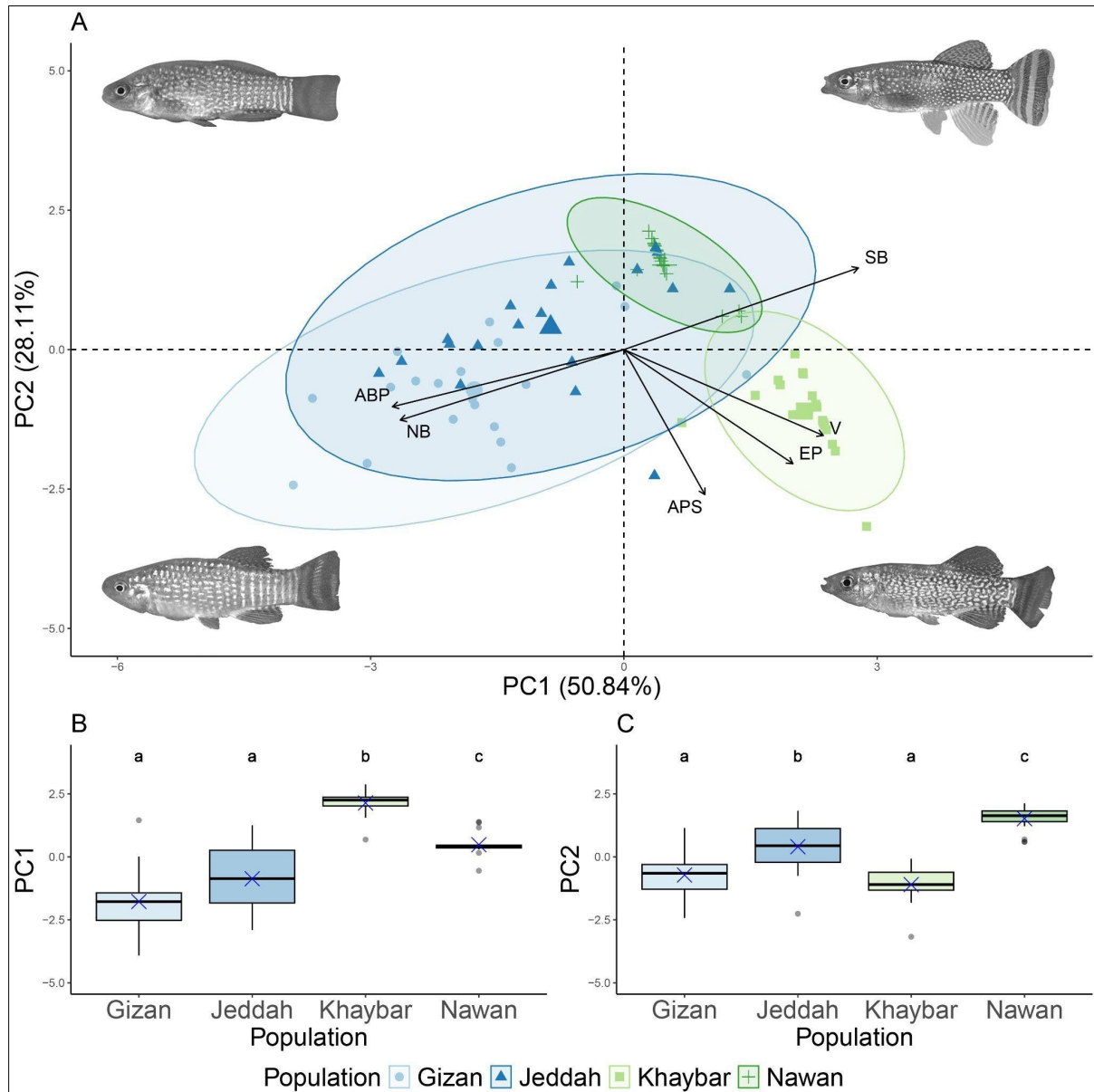


Figure 2.6: Outputs of the principal component analysis (PCA) on the six colour pattern characteristics of male individuals of *A. dispar* from two freshwater (■ Khaybar and + Nawan) and two marine habitats (● Gizan and ▲ Jeddah) showing (A) a biplot of the two major principal components PC1 and PC2, and box plots of extracted values from PC1 (B) and PC2 (C) showing the differences among populations based on all the colour pattern metrics. The black lines represent the medians, and the blue crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The grey dots represent the outliers. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations. The colour patterns are depicted in the figure by random representative males from Jeddah (top left), Gizan (bottom left), Nawan (top right) and Khaybar (bottom right). The abbreviations in the figure are provided in Table 2.4.

Phylogenetic analysis of 89 *COI* sequences using the ML method revealed two major clades, namely, the Red Sea clade and the Arabian Gulf clade, supported by a bootstrap value of 67.4% in the ML tree (Figures 2.7, S2.17). There was a disagreement between the ML, BI and NJ trees in the split of the Red Sea (including East Mediterranean and Dead Sea populations) and Arabian Gulf clades, with the Arabian Gulf clade nested within the Red Sea clade in the BI tree and vice versa in the NJ tree (Figures S2.15, S2.16). However, the same major subclades were supported in all analyses, albeit the phylogenetic relationships between subclades differed between methods. In the Red Sea, three distinct hyperpopulations were identified in all the phylogenetic trees. These included (i) the northern populations, which consisted of the Khaybar and Thulbah freshwater populations, along with their marine counterpart population in Deba (support values: ML = 83.7, BI = 0.92 and NJ = 89.47). (ii) The central-southern populations, which included the marine population in Jeddah, formed a cluster with all the central-southern freshwater populations (Arj, Hali, Nawan and Qanuna) (support values: ML = 49.8, BI = 0.5 and NJ = 64.09). (iii) The southernmost marine population from Gizan (support values: ML = 100, BI = 1 and NJ = 99.95).

The central-southern populations from Saudi Arabia were found to be monophyletic, with the northern populations in the ML and NJ trees grouping together with the East Mediterranean populations. Likewise, the Gizan population in both trees formed a monophyletic group to all the Saudi Arabian Red Sea and East Mediterranean populations (Figures 2.7, S2.15, S2.17). In contrast, in the BI tree, the Red Sea populations were paraphyletic: central-southern populations had the deepest split, followed by the northern populations, the East Mediterranean populations and then the Gizan population (Figure S2.16). Overall, although the exact phylogenetic relationships of the clades were not well resolved, the separation of the Gizan population from the others in the Red Sea was consistently well supported, with all other Red Sea populations consistently being more similar to the East Mediterranean populations than they were to the Gizan population (Figures 2.7, S2.15–S2.17).

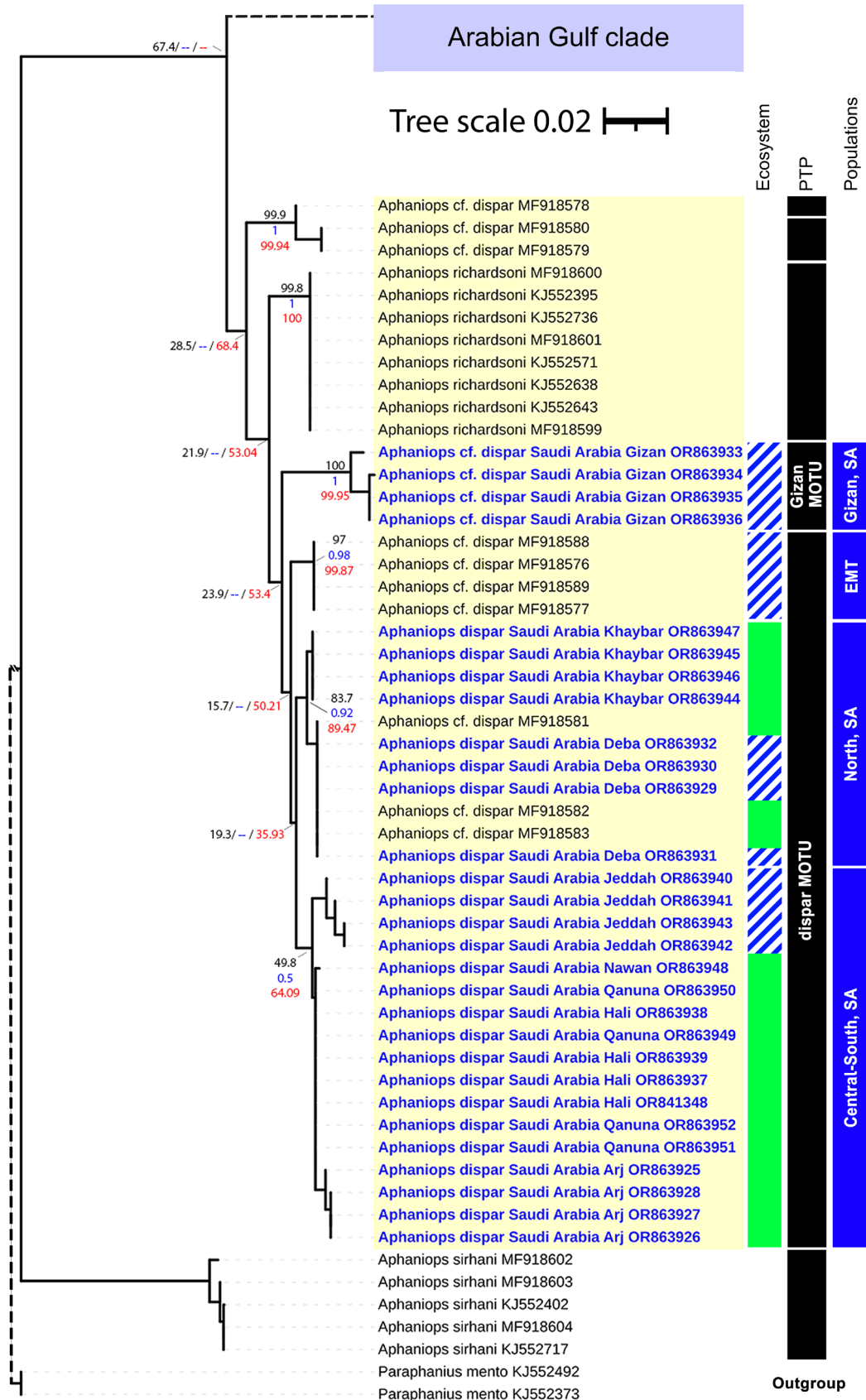


Figure 2.7: Phylogenetic relationships inferred from the mitochondrial *COI* barcode region using the consensus maximum likelihood tree. The tree was constructed using the HKY model with a Gamma distribution (4 classes), a G parameter of 0.1490 and a transition/transversion value of 7.70693. Numbers on nodes as ordered from left to right on

top to bottom represent support values obtained from 1000 bootstrap replicates using the ML method (black), posterior probability values from BI (blue), and bootstrap values obtained from 10000 bootstrap replicates using the NJ method (red). The Arabian Gulf clade is collapsed and highlighted in blue, while the Red Sea clade is highlighted in yellow. The solid black bars represent 6 out of the 11 delimited MOTUs using the PTP approach (5 out of 11 MOTUs are not shown, which belong to the collapsed Arabian Gulf clade (see Appendix 1C, Figure S2.17)). The solid blue bars represent the Saudi Arabian (SA) and East Mediterranean (EMT) populations within the *dispar* MOTU, as well as the Gizan MOTUs from Saudi Arabia. The solid green bars represent the freshwater populations within the *dispar* MOTU. The blue diagonal striped bars represent the marine populations within the *dispar* and Gizan MOTUs. Labels in blue boldface represent the samples collected in this study from Saudi Arabia. The scale bar indicates 0.02 substitutions per site, but does not apply for the shortened branches to the Arabian Gulf clade and the outgroup.

The species delimitation method using ML tree outputs identified 11 putative molecular operational taxonomic units (MOTUs) (Figures 2.7, S2.17). These MOTUs comprised five from the Red Sea clade, five from the Arabian Gulf clade and one MOTU designated for *A. sirhani*. Regarding the populations in Saudi Arabia, two distinct MOTUs were identified. The first lineage, referred to as the "Gizan MOTU", was found in the Gizan population. The second lineage was observed in the Saudi central-southern and northern populations and East Mediterranean populations, referred to as the "*dispar* MOTU".

The estimates of evolutionary divergence between all groups, based on the K2P model over *COI* sequence pairs, ranged from 0% to ~70% for the 89 sequences. The mean distance among Saudi Arabian populations within the *dispar* MOTU was ~1%. A minimum genetic distance of 0% was observed between proximate locations (measured by hydrological distance) in the central-southern freshwater populations Hali and Qanuna (160 km apart), as well as in the northern populations and between the freshwater Thulbah and marine Deba populations (120 km apart). The maximum genetic distance was ~2.1% between the central Jeddah and northern populations of Deba and Thulbah. Furthermore, I observed that the average genetic divergence between the East Mediterranean populations and the Saudi populations within the *dispar* MOTU was ~1.8%. Remarkably, the average distance from Gizan to all *dispar* MOTU members was ~4.6%, greater than that of *dispar* MOTU to other distal valid species like *A. richardsoni* from the Dead Sea (~3.2%).

Discussion

Species delimitation can be challenging, particularly when there is limited availability of studies, as is the case with the Arabian killifish. This challenge may be further compounded by relying on only one method or a few materials. Here, I utilised morphological, colour pattern and molecular approaches aiming to evaluate the taxonomic status of Arabian killifish *A. dispar sensu lato* populations from the Saudi Arabian Red Sea basin.

Morphometric analyses have been shown to be effective in distinguishing some geographically distant populations of the *A. dispar* group (Teimori et al., 2012) as well as morphologically semi-identical species like *Apricaphanius spp.* (Doadrio et al., 2002). However, the PCA conducted on 21 morphometric characteristics of both male and female individuals indicated limited (but significant) differences between populations. The presence of extensive overlap among populations in the PCA plots suggests that the morphometric characteristics do not differ substantially across the Saudi populations. This finding aligns with the majority of previous conclusions on the morphometry of the *Aphaniops* species (Teimori et al., 2018). This study found that the differences in SNL, CPL and BD on the PC2 of males and HL, CPL, and DCP on the PC1 of females were significant (Appendix 1A) and contributed to some extent to the discrimination between populations. Nevertheless, in practice, these characteristics might be difficult to use in taxonomy within the genus *Aphaniops* compared to other characteristics, such as fins (Charmapila et al., 2020; Freyhof et al., 2017; Wildekamp, 1993), which have been commonly used in the identification key for *Aphaniops* species.

However, the analysis of males' colour patterns effectively discriminated between the populations and revealed the presence of distinct male morphs. Males from marine populations in Gizan and Jeddah exhibited the greatest similarities among the analysed populations. The only differences between them were that Gizan males displayed a larger blotch size and had a slightly higher number of bars. Superficially, both Gizan and Jeddah populations resemble *A. stoliczkanus* in having vertical dark bars on the posterior flank and vertically arranged blotches. When comparing Gizan and Jeddah's male colour patterns to freshwater populations, they overlapped with Nawan, which is situated midway (350–390 km) between the two marine populations. However, Nawan had unique characteristics, namely the small scattered blotches, the lack of vertical dark bars and the vertical arrangement of blotches on the posterior flank. Khaybar was found to be the most

morphologically distinct population in Saudi Arabia, mainly displaying irregular vermiculation, scattered blotches, and more elongated and larger blotches than the other populations. Khaybar males look like males of *A. richardsoni* from the Dead Sea; still, they can be easily differentiated by comparing the length of dorsal and anal fins, which were noted to be large in Khaybar, similar to the other Saudi populations examined in morphological analyses, but are significantly smaller in *A. richardsoni* (Krupp, 1983; Wildekamp, 1993). The bars on males have been used as a general guideline to distinguish between the Arabian Gulf and Red Sea populations of the *A. dispar* group. It has been observed that the Arabian Gulf populations have a higher number of bars, which gradually decrease as one moves towards the northern Red Sea and are absent in the East Mediterranean populations (Krupp, 1983). Interestingly, the spatial distribution of the males' patterns follows the hypothesised trajectory of the *Aphaniops* invasion around Arabia (Krupp, 1983). It is, therefore, intriguing to ask why the bars are displaced by blotches and vermiculation northward.

In contrast, female colour patterns are not an efficient predictor to distinguish between populations of the Arabian killifish, as most females of *Aphaniops* species around Arabia exhibit vertical stripes. In this study, females from all populations displayed varying numbers of vertical stripes, with those from Khaybar having the fewest stripes. Indeed, the number of stripes can be influenced by pigment concentration, resulting in variations within and between populations (see Freyhof et al., 2017; Hamidan & Shobrak, 2019 for some instances). The persistence of vertical stripes in females among *Aphaniops* species might facilitate predator avoidance by grouping with matching-shoal females, as in the Mediterranean killifish *A. fasciatus* (Cattelan & Griggio, 2020), or by blending in visually matching or visually complex habitats, as in the least killifish (*Heterandria formosa*) (Kjernsmo & Merilaita, 2012). The variation in males' colour patterns among populations of *Aphaniops* suggests that they are sexually selected traits. However, to date, it appears that there have been no advances in the studies on the ecological and evolutionary significance of the colour patterns in Aphaniidae.

Phylogenetic analyses contrasted the morphometrics and colour pattern analyses, but proved efficient in elucidating population differences among *Aphaniops* species. My study aligns with previous studies conducted on *Aphaniops* species, especially within the Arabian Gulf clade, which has a high level of resolution (Charmvila et al., 2020; Esmaeili et al., 2020; Freyhof et al., 2017; Hrbek & Meyer, 2003; Teimori et al., 2018, 2022). Thus, in this study,

the focus is specifically on phylogenetic relationships within groups closely related to the Saudi Arabian *Aphaniops* populations from the Red Sea.

Two distinct hyperpopulations were discovered in Saudi Arabia within the *dispar* MOTU. First, the central-southern population that included the marine population in Jeddah and the freshwater populations in Arj, Hali, Nawan and Qanuna. Second, the northern population that included the freshwater populations of Thulbah and Khaybar in the northernmost part of the Saudi Arabian Red Sea, together with the marine population of Deba. The average distance between the northern and central-southern Saudi Arabian populations within the *dispar* MOTU was around 1.4%. However, there was a clear difference in the magnitude of genetic distance based on latitude, meaning that populations from either the same or different ecosystems at closer proximity appeared more related than those that were farther apart.

Inland freshwater populations have been considered landlocked inside Arabia since the Pleistocene (Krupp, 1983), with little attention given to the potential connection between the freshwater populations and their marine counterparts. In the northern population, I found that the freshwater population Thulbah and the marine population Deba, despite being 120 km apart, exhibited a genetic distance of 0%. These findings strongly lend support to recent research indicating that gene flow between the *dispar* MOTU ecosystems is continuous and may occur through flash flooding (Schunter et al., 2021). Avian dispersal may also play a significant role (Silva et al., 2019) in explaining the low genetic distances observed in the southern freshwater populations (Hali, Nawan and Qanuna). Ultimately, these findings reject the suggestion of landlocked populations in the Red Sea drainage systems of Saudi Arabia. The colour pattern differences between freshwater and marine populations, despite the lack of large genetic differentiation, may indicate rapid local adaptation. Furthermore, the high similarity among marine populations may indicate convergent evolution, and it is intriguing to study the driving forces underlying these similarities and dissimilarities.

Previous research suggested that *A. dispar* populations from the East Mediterranean Sea were separate from those in the northernmost Saudi Arabian Red Sea (Freyhof et al., 2017; Temori et al., 2020). This suggestion was based on molecular data from limited samples collected from the North Saudi Arabian, Egyptian Red Sea and the East Mediterranean Sea. However, the findings in this chapter significantly challenge this suggestion, demonstrating that these populations clustered with most populations from Saudi Arabia and, hence,

cannot be regarded as distinct species based on the similarities in mitochondrial DNA genetic distance, phylogenetic position and PTP outputs.

Furthermore, the results uphold the view that the East Mediterranean Sea populations are not likely to be Lessepsian migrants (i.e., populations migrating from the Red Sea to the East Mediterranean Sea through the Suez Canal) (Krupp, 1983; Villwock et al., 1983). This view is based on the relatively large genetic distance of 1.8% compared to the spatial distance of approximately 230 km between Deba and the nearest East Mediterranean Sea population—i.e., el-Guna, which was collected by Freyhof et al. (2017)—given the genetic distance of the Saudi populations that are spatially distant (e.g., Deba vs. Jeddah, which are approximately 760 km apart and showed a genetic distance of 2.06%). Similar findings were observed in populations of the Comber (*Serranus cabrilla*) from the Red Sea and the East Mediterranean Sea, which showed a great genetic distance compared to the spatial distance (Bos et al., 2020). This may suggest that the Red Sea and the East Mediterranean Sea populations of *A. dispar* were isolated by vicariant events long before the opening of the Suez Canal, and then the East Mediterranean Sea population became anti-Lessepsian migrants to the Red Sea afterwards. Nevertheless, more research is needed to investigate the population structures and genetic diversity within the *dispar* MOTU populations from the Red Sea and East Mediterranean Sea, which could potentially clarify the causes of genetic divergence of *A. dispar*.

A key finding in my study is the evolutionary placement of Gizan MOTU in the southernmost part of the Saudi Arabian Red Sea. Based on the morphological characteristics discussed above, this MOTU likely represents a new cryptic species, referred to here as *A. cf. dispar*, in order to emphasise the uncertainty about the species designation. Indeed, Gizan MOTU did not group with *dispar* MOTU members of the Red Sea clade in Saudi Arabia and the East Mediterranean Sea. In fact, Gizan MOTU had a greater genetic distance from *dispar* MOTU members than *dispar* MOTU had from other distal species, such as *A. richardsoni* from the Dead Sea. This suggests that Gizan MOTU has had a long period of divergence from the *dispar* MOTU lineage. Gizan MOTU may be a subpopulation, i.e., an extension of a larger population similar to *A. dispar* and *A. stoliczkanus*. It is worth noting that Gianferrari (1932, 1933a, 1933b) described two new species from Somalia, namely *Cyprinodon darrorensis* and *C. zaccarinii*, which generally matched the observed morphological characteristics of Gizan MOTU. However, both are currently synonyms for *A. dispar*. The inclusion of new materials from Saudi Arabia in this study supports the distinction of all the

African inland populations in Djibouti and Eritrea presented by Chiozzi et al. (2017) and Freyhof et al. (2017) from all the populations in the Red Sea clade. The findings in this chapter suggest that these populations are eligible for the description and designation of new species. *Aphaniops* populations in Saudi Arabia show interesting geographical differences in colour-based behaviours in freshwater and marine populations from distant locations, so it is important to know if these are composed of the same species. It is also important to determine the taxonomic status of other closely related populations in the Red Sea near Saudi Arabia. Therefore, acquiring additional molecular materials from Yemen and northern Somalia could potentially result in classifying the Gizan MOTU as a new taxon and could also help resolve the taxonomic status of *Aphaniops* in the Red Sea. Additional molecular research using nuclear markers is necessary to ascertain whether the Gizan population is distinct in terms of mitochondrial DNA only and to provide insights into the gene flow between the Red Sea basin populations.

Conclusion

This study examined the taxonomic status of Arabian killifish populations in the Saudi Arabian Red Sea basin using morphological, colour pattern and molecular methods. Morphometric analyses of the four main populations (Gizan, Jeddah, Khaybar and Nawan) indicated limited significant interpopulation variations. In contrast, colour patterns revealed distinct morphs, but the differences in colour patterns were not aligned with the geographical distribution or the phylogenetic relationship of the studied populations. Phylogenetic analyses indicated notable population variations within the Arabian killifish; Jeddah, Khaybar and Nawan were placed in one MOTU, with the central-southern population of Jeddah and Nawan being more closely related than the northern population of Khaybar. The study also found another distinct MOTU present in the southernmost region of the Saudi Arabian Red Sea, Gizan, which had a morphological affinity for the population of Jeddah but was genetically distant from all the populations of Saudi Arabia and may be a new cryptic species.

Chapter 3: The geographical variation of colour change in the Arabian killifish (*Aphaniops dispar*) from freshwater and marine ecosystems

Abstract

Widely distributed animals may exhibit substantial plastic physiological, morphological and behavioural responses to environmental changes. One such extraordinary form of plasticity is colour change, which varies substantially among most taxonomic groups. Most studies on colour change have focused on a focal population, which has left a gap in our knowledge pertaining to the evolution of colour change in animals. Thus, examining colour change variation across populations of a taxon can shed light on how it evolves. Colour plasticity is influenced by spatial and temporal contextual variables, including visual habitat heterogeneity, predator diversity, and their interaction. Using the Arabian killifish (*Aphaniops dispar*) as a model, I examined the geographical variation of colour change in two freshwater and two marine populations from the west of Saudi Arabia, separated by vicariant and distance barriers, and that differed in visual characteristics and predator diversity. I used digital photography and visual modelling to investigate the ability of individuals in each of the populations to change colour when presented with achromatic (black and white) and chromatic (beige, brown and green) backgrounds. When presented with a black background, I found that individuals in all populations were capable of becoming darker at varying rates, but in a similar manner. Becoming lighter was more challenging, with individuals from all populations changing less than on black backgrounds, with one freshwater population exhibiting almost no response. However, there were complex interpopulation differences in chromatic responses, with marine populations exhibiting generally greater responses to the given backgrounds than freshwater populations. Here, I discuss the evolutionary and ecological factors possibly underlying these variations and their repercussions.

Introduction

Animals that are widely distributed and inhabit diverse habitats with different biotic and abiotic compositions may vary in their physiological (Cooper et al., 2018; Staurnes et al., 1992), morphological (Endler, 1978; Macedonia et al., 2003) and behavioural characteristics (Foster, 1999; Foster & Endler, 1999). Animals' behaviours are often associated with their colouration, and colouration has been shown to play a crucial part in providing vital biological functions (Endler, 1978; Stevens & Merilaita, 2011), including signalling (Dollion et al., 2020; Endler, 1978; Hutton et al., 2015; Poulton, 1890), thermoregulation (Smith et al., 2016a, 2016b), ultraviolet screening (Garcia et al., 2004) and crypsis (Cott, 1940; Duarte et al., 2017; Stuart-Fox & Moussalli, 2011). Colouration is an evolutionarily labile trait subject to many spatiotemporal influential factors (Caro et al., 2016), and it can vary within and between populations of the same species (Cullen et al., 2022; Greenwood et al., 2011, 2012). Furthermore, the colouration of animals in non-contiguous populations may differ in response to environmental factors (Endler, 1983; Magurran, 2005; McPhail, 1969). The most impressive example of this can be found in multiple pigmented epigean (surface-dwelling) cavefish species and their depigmented hypogean (subterranean) counterparts, which have lost their pigment owing to the lack of functionality (Dowling et al., 2002; Romero & Green, 2005).

In addition to evolutionary change, many animals can maximise the benefit of their colouration by plastically changing it in response to their habitat, such as its visual properties (Barbosa et al., 2008; Green et al., 2019; Ramachandran et al., 1996; Smithers et al., 2017), temperature (Smith et al., 2016a, 2016b), the presence of heterospecifics (Kodric-Brown, 1998) or conspecifics (Ligon, 2014), and ambient light (Vroonen et al., 2012). Colour change is categorised broadly into two subtypes (Bagnara & Hadeley, 1973): morphological colour change, which is slow (days to months) and involves an increase or decrease in pigment, and physiological colour change, which is rapid (seconds to minutes) and involves a movement of pigment. The capacity to change colour can vary substantially among most groups of colour-changing animals (Stuart-Fox & Moussalli, 2009), although this has been rarely examined (Cadena et al., 2017), in comparison to geographical variations in other behaviours. It is evident that colour change varies among closely related species. For example, investigations into 21 groups of dwarf chameleons (*Bradypodion spp.*) revealed differences in the facultative response (i.e., the ability to fine-tune the response to multiple stimuli) of colour change to bird and snake predators, with some lineages responding

differently and others similarly (Stuart-Fox & Moussalli, 2009). In addition, recent research on two sympatric species of scorpionfishes, *Scorpaena* spp., showed that black scorpionfish (*S. porcus*) aggregated 50% of their dispersed pigments faster than the Madeira rockfish (*S. maderensis*) when placed on white substrates after being acclimated on black backgrounds (John et al., 2023).

Although studying differences in colour change between closely related species can provide valuable insight into the evolution of colour change (Clarke & Schluter, 2011), it is a clear knowledge gap (Duarte et al., 2017; Hutton et al., 2015; Stuart-Fox & Moussalli, 2009), and it remains difficult to infer how these variations have arisen among species. Despite this, much is still unknown about the evolution of colour plasticity within a particular species. Examining the variation in colour change among populations of a specific taxon can, therefore, cast considerable light on the evolution of colour change. While most studies focused on a focal population, a search of the literature revealed several lines of evidence that colour change varies between disconnected populations in terrestrial (Batabyal & Thaker, 2017; Cadena et al., 2017; Choi & Jang, 2014; Munguia et al., 2013; Spadavecchia et al., 2023; Stuart-Fox et al., 2006; Stuart-Fox & Moussalli, 2009; Whiting et al., 2022) and in aquatic environments (Pinto et al., 2020; Westley et al., 2013; Whiteley et al., 2009, 2011). Differences in colour change do not appear to be attributed to a single factor or combination of factors applicable to all species, indicating that the processes that explain the evolution of population variation in colour change vary between and within species and remain inadequately understood. Due to a lack of phylogenetic and longitudinal studies and limited access to ecological data for each geographical region, such as habitat parameters and biodiversity, it is difficult to determine how colour change has diverged among populations; thus, additional research is required.

Environment-specific requirements, such as physiological (Alfakih et al., 2022), sociosexual, thermoregulatory and camouflage, as well as their interactions, may drive variations in colour change (Stuart-Fox & Moussalli, 2009). Generally, if some populations are subject to stronger natural selection for camouflage than others, it is anticipated that there will be differences in their capacity to change colour (Stuart-Fox et al., 2006). Thus, spatial and temporal environmental differences play a significant role in shaping the evolution of plasticity, including colour change (Doughty & Reznick, 2004). It is broadly predicted that, as variation in habitat visual properties increase, so does the capacity to change colour (Cadena et al., 2017), though this is not always the case. For instance, four populations of

brown trout (*Salmo trutta*) were found to differ in the magnitude and endpoint of their pigmentation change despite the similarity of their environments (Westley et al., 2013), and comparable findings were observed in dwarf chameleons (*Bradypodion transvaalense*) (Stuart-Fox et al., 2006). The majority of animals, however, reside in ecosystems that have several predators in their microhabitats (Stuart-Fox & Moussalli, 2009), which possess different visual capacities. Thus, prey species may evolve increased colour-based defensive tactics (Caro et al., 2016), and selection for increased colour change capacity may accord with the increase in visually guided predator diversity (Whietely et al., 2011) or abundance (Stuart-Fox et al., 2006; Stuart-Fox & Moussalli, 2009).

Nonetheless, the effects of predator diversity on colour change *per se* have not been adequately addressed and are only marginally acknowledged. Stuart-Fox and Moussalli (2009) provided evidence that suggests the loss of facultative colour change in *Bradypodion* chameleons may be an adaptation to geographically variable local predation regimes. A recent study strengthens this suggestion by showing that relaxed predation pressure influenced the extent of colour change in the invasive Jackson's chameleon (*Trioceros jacksonii xantholophus*) in Hawaii, by which they changed colour during social context and when presented with predator models in a way that contrasted the non-native Hawaiian habitats more than the original populations did on their local environments (Whiting et al., 2022). Predation and visual habitats are inseparable factors that affect colour change. Antipredator responses vary spatially depending on predator exposure, leading to population-specific strategies and colouration-based behaviours (e.g., fish: Magurran et al., 1993).

Euryhaline fish species are highly adaptable to various ecological systems and may potentially become landlocked (Schultz & McCormick, 2013). This adaptability makes them valuable models for studying geographical resilience in phenotypic traits, such as colour change. Here, I examine the geographical differences in the achromatic and chromatic colour change between populations of the Arabian killifish (*Aphaniops dispar sensu lato*) from the Red Sea basin in Saudi Arabia. Natural populations of the Arabian killifish have the capacity to change colour (personal observations), but the colour changeability in the genus *Aphaniops* has not been documented. This species is euryhaline and eurythermal, meaning that it can tolerate a broad spectrum of water salinity and temperature (Haas, 1982; Krupp, 1983). Hence, it has a wide geographical range and thrives across multiple ecosystems, including hypersaline marine and freshwater ecosystems, with some populations believed

to be landlocked (Krupp, 1983). Here, I present the first study on colour plasticity in members of the *Aphaniops dispar* complex, and the first study pertaining to the variations in colour change among different ecosystems, comprising two freshwater and two marine populations that are geographically separated by distance and land barriers.

The Arabian killifish generally lives in shallow water (Bonzi et al., 2021), whereas in marine systems, it occupies shallow lagoons, mangrove forests (Walton et al., 2021), and coral reefs (Freyhof et al., 2020). The lagoon locations on a large scale are consistently homogenous across many sites on the Saudi Arabian and Yemeni Red Sea coasts (Figure S3.1; Krupp et al., 2006; Rasul, 2015; Scott et al., 2022). Lagoons mostly consist of sand and mud, light yellowish to dark brown in colour, with the latter likely derived from freshwater wadis (ephemeral streams) during floodings (Rasul, 2015; Scott et al., 2022). In comparison, wadis are more visually heterogeneous, consisting of aquatic plants, eutrophicated patches, rocks, and gravel pools (Figure S3.2; García et al., 2015). The Arabian killifish is susceptible to predation from birds in both ecosystems and from snakes in freshwater environments (Egan, 2007; Freyhof et al., 2020). In freshwater environments, the most diverse fauna is generally found in the southernmost regions of Saudi Arabia (Boland & Alsuhaibany, 2020; Egan, 2007; Gasperetti, 1988). Moreover, fish are key predators of the killifish (Coad, 2010), where they occur in the killifish marine microhabitats (Lieske & Myers, 2004; Zajonz et al., 2002), such as the Crescent grunter (*Terapon jarbua*), which is known to hunt *Aphaniops* species (Llewellyn-Smith, 2011), but no piscivorous fish is present in freshwater habitats. These profound differences in the patterns of predator diversity might cast a better understanding of the interaction between habitat and predator heterogeneity.

This chapter aimed to quantitatively measure the ability of colour change in the Arabian killifish, and examine the geographical variation among two freshwater and two marine populations of the Arabian killifish in the ability to change colour achromatically (luminance: the perceived brightness by the visual model as detailed below) in response to black and white backgrounds, and chromatically (hue: the actual colours, and saturation: the intensity of colour) in response to beige, brown and green backgrounds. Here, I hypothesise that colour change will vary in accordance with variations of the general visual characteristics of the environments and predators between populations, predicting that (i) populations occupying more visually diverse environments will exhibit greater achromatic and chromatic colour-changing ability in terms of both speed and magnitude of colour change, and (ii) biotic

components, including the abundance and diversity of visual predators, will amplify this ability.

Methods

Study species and collection sites

I collected mixed-sex wild-caught individuals of the Arabian killifish opportunistically (Figure 1.1) from four locations in the west of Saudi Arabia, including two landlocked freshwater populations from internal wadis: Khaybar (25°44'38.6"N 39°15'19.3"E) and Nawan (19°39'25.1"N 41°16'57.7"E), and two marine populations: Gizan (16°50'07.9"N 42°34'34.4"E) and Jeddah (21°29'24.9"N 39°10'58.9"E) (Figure 2.1). The sampling locations were chosen based on the ecosystem types (freshwater and marine) and the latitudinal distance between the populations to represent the extreme allopatry of this species, and to ensure differences in faunal diversity. In marine environments, the salinity was typically around 35 parts per thousand (ppt), whereas in freshwater streams, the salinity was nearly 0 ppt. The marine locations were predominantly homogeneous, with Gizan consisting of light brown sand, grey gravel with some seagrass and algae (Figure 3.1A) and Jeddah primarily composed of large dark rocks with crevices and some greyish gravelled and light patches and some algae (Figure 3.1B). The substrates at the northern freshwater location Khaybar were highly dark brown with some green biofilms (Figure 3.1C), where the population is located in small springs surrounded by farms and basaltic volcanic rocks. The southern freshwater location Nawan, which was more visually heterogeneous than the other study locations, consisted of brown to dark grey sand, brown and green algae, reed plants, and gravel with varying brightness (Figure 3.1D).



Figure 3.1: Some representative images of the visual habitats in the study areas showing the marine locations (A) Gizan and (B) Jeddah, and the freshwater locations (C) Khaybar and (D) Nawan. Photo credits: Ateah Alfakih.

Fish collection

Four hundred fish in total ($n = 100$ fish per site) were collected using cuboid-shaped nylon traps (L 54 × W 25 × H 25 cm) (Figure S3.3A) to avoid any harm or damage to them. This was done by placing bread bait inside the traps and submerging them in shallow water at random places for a few minutes. Fish were gently removed from the traps and placed in 12 L plastic containers containing water from their collection site to reduce stress, and portable air pumps were used to provide oxygen. Fish from each population were housed indoors near the collection area in two tanks for each experiment (L 50 × W 30 × H 40 cm: ~20 fish/tank) (Appendix 2; Figure S3.3B). The salinity was maintained for each population by housing individuals from the freshwater population directly in freshwater, while the marine fish were placed in saline water at ~35 ppt by mixing fresh water with Red Sea salt mix (Red Sea). Since the Arabian killifish is eurythermal (Coad, 2010; Haas, 1982), the temperature was maintained at ~28 °C using tank heaters, which is within the recorded water temperature of both ecosystem types (Bonzi et al., 2021; Schunter et al., 2021). Each tank was under a 12 h:12 h light:dark cycle and supplied with a tank pump with a filter to circulate and oxygenate the water, and fish were fed twice daily with commercial flake food (Siso Bio-tech CO., LTD). Fish were acclimated to the husbandry tanks under the above conditions for at least 24 hours before being used in the experiments.

Ethical note

The Arabian killifish is neither threatened nor protected (Freyhof, 2014). The collection and the study was carried out with permission from the Saudi National Center for Wildlife (2020) prior to the start of the study. Except for springs in Khaybar, by which a separate permit was acquired from the Royal Commission for AlUla (2022), the collection sites were open to the public, and no additional licences or permits were required to access them. The fish were returned to their original site after completing the experiments.

Achromatic backgrounds

The purpose of the first experiment was to determine whether individuals from the freshwater and marine populations were capable of altering their luminance when exposed to achromatic (black and white) backgrounds. Each background was made of waterproof paper (Xerox Premium NeverTear Paper; Antalis, London, UK). I used the Xerox white paper for the white backgrounds and measured the mean reflectance of the paper (see Image analysis for reflectance measurements), which was 85.5%. Black backgrounds were generated in Inkscape version 0.92.3 (<https://inkscape.org>) by specifying the RGB values to 0:0:0 (Smithers et al., 2017). The black backgrounds were printed with an HP Color LaserJet M254dw printer 600 dpi (Hewlett-Packard, USA), and the mean reflectance of the paper was measured which was 5.9%. To acclimate the fish prior to the experiments and to ensure that they all had the same starting point, midpoint grey backgrounds with an intermediate reflectance relative to the average reflectance of the black and white backgrounds were created using a method similar to that of Kang et al. (2016) and Smithers et al. (2017). I created 100 greyscale gradients in Adobe Photoshop 2019 (Adobe Systems), resulting in a grid of different greyscale blocks with RGB values ranging from (black) 0 to (white) 255 (Figure S3.4). The grid was printed and photographed (see Photography methods below), and the reflectance of each grid block was measured. The closest midpoint grey colour between the black and white papers was selected to generate another grid. This process was repeated until I obtained an intermediate grey substrate which reflected 45.7% with an RGB value of 151 (Figure S3.4).

Chromatic backgrounds

The chromatic backgrounds were designed primarily using the previously described approach. Rather than indiscriminately selecting colours, the objective was to produce broad representations of natural environments rather than perfectly replicating them, as the study focused on colour change rather than habitat matching. Therefore, prior observations were made during this study to assess the broad visual characteristics of different marine and freshwater locations in the west of Saudi Arabia to cover as many potential natural colours as possible (Figures S3.1, S3.2). I subjectively divided the colours of the observed habitats into one common background and two opposing backgrounds. The colour green was

selected for the common substrate to be similar to the algal substrates seen usually in the freshwater and marine ecosystems. To reduce complexity, I utilised sand colours for the opposing backgrounds, which broadly varied slightly in both habitats, being primarily brown in freshwater streams and beige in coastal areas (Figures S3.1, S3.2); the light sand colour seems to be a consistent feature of the Saudi Arabian Red Sea coast (Scott et al., 2022).

I photographed wet algal and sand substrates from the marine and freshwater locations from a height (~1.5 m) in the visible human spectrum because water absorbs a substantial portion of light, and light penetration varies with water depth, affecting the colours and reflectance of backgrounds. The images were captured in the shade to eliminate overexposure, using a Nikon D3300 equipped with an 18–55 mm Nikon lens in raw format and had a Spectralon 99% reflectance standard. The white balance of these photographs was then corrected in Camera Raw (Adobe Systems) using the reflectance standard. The average values of the RGB channels of the substrates were then measured in Photoshop and input into Inkscape to print the final colours (Figure S3.5). In this experiment, I intended to examine the changes in the fish's actual colours (hue and saturation) by placing individuals from the four locations on chromatic backgrounds. The perceived differences in brightness between the created starting backgrounds (i.e., the grey acclimating backgrounds) and the printed coloured backgrounds were eliminated. This was done by measuring the average reflectance of the printed coloured backgrounds and then selecting the corresponding grey starting points with a similar reflectance to the printed coloured backgrounds, in the same way as described for the achromatic backgrounds above (Figure S3.5; Table S3.1).

Experimental procedure

For each population, I used 20 different and randomly selected mixed-sex fish for each type of the achromatic background: black and white, and the chromatic background: beige, brown, and green. This means that 20 fish were used for each background type per population. Based on prior research (Smithers et al., 2017, 2018; Stevens et al., 2014a), 20 is a sufficient sample size for a statistically robust analysis. In each experiment (Figures 3.2, 3.3), each fish was placed and kept for 10 minutes in an oxygen-supplied tray (L 12 × W 12 × H 10 cm) containing the starting grey backgrounds under constant photography conditions (see below). This was to ensure that all the individuals had the same starting point, allowing them to acclimatise and diminish any colour changes induced by stress (Smithers et al.,

2017; Stevens et al., 2014a). After being acclimated for 10 minutes, the individuals were swiftly transferred to the photography containers with the test backgrounds (L 7 × W 7 × H 7 cm) and immediately photographed (minute 0), and at minutes 1, 8 and 15 (Figure S3.6). The photography containers were filled with oxygenated water that was salinity-matched to their original locations (0 ppt for freshwater and 35 ppt for marine) and had a temperature of ~28 °C that matched the husbandry conditions. Acclimating and photography containers were washed with water after each experiment to avoid olfactory cues being transferred to subsequent test individuals, and tested fish were returned to the acclimating containers for 10 minutes and then to another husbandry tank and provided with food. Transferring fish to and from the photography containers was performed using a small, transparent plastic tray immersed in water; this method proved efficient in reducing stress because fish did not exhibit flight (escape) response to this handling.

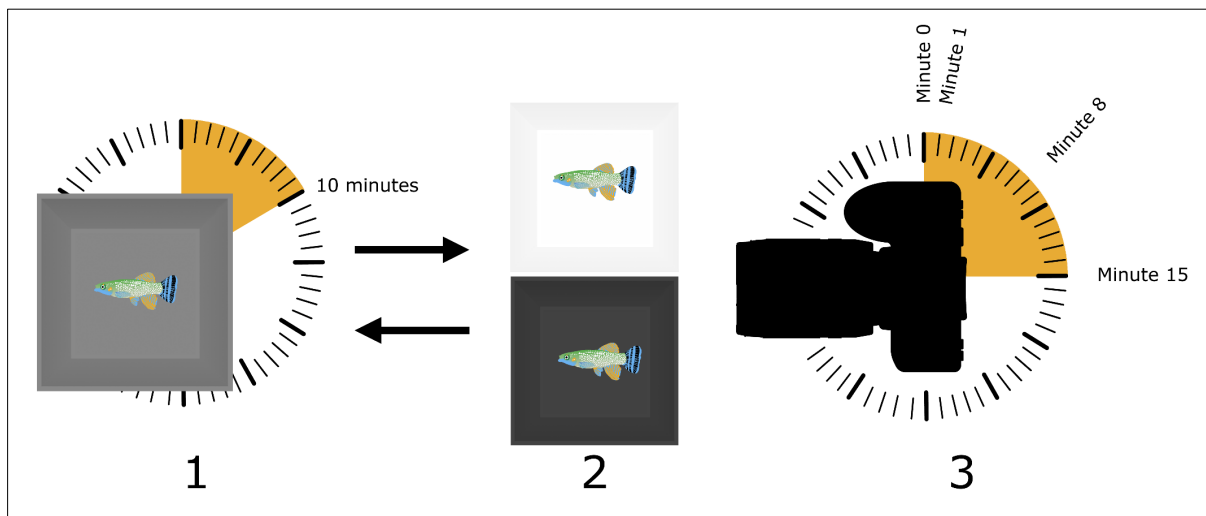


Figure 3.2: Schematic diagram showing the experimental procedure for the achromatic backgrounds for each of the studied populations. (1) Placing each fish (20 different fish/ black background experiment and 20 different fish/ white background experiment) in the intermediate acclimating background for 10 minutes; the upper arrow indicates transferring each of the 40 individuals to the photography containers (20 fish/ black background and 20 fish/ white background). (2) Placing the fish in the photography container. (3) Photographing each fish at minutes 0, 1, 8, and 15. The lower arrow indicates returning the individuals to their intermediate acclimating background for 10 minutes.

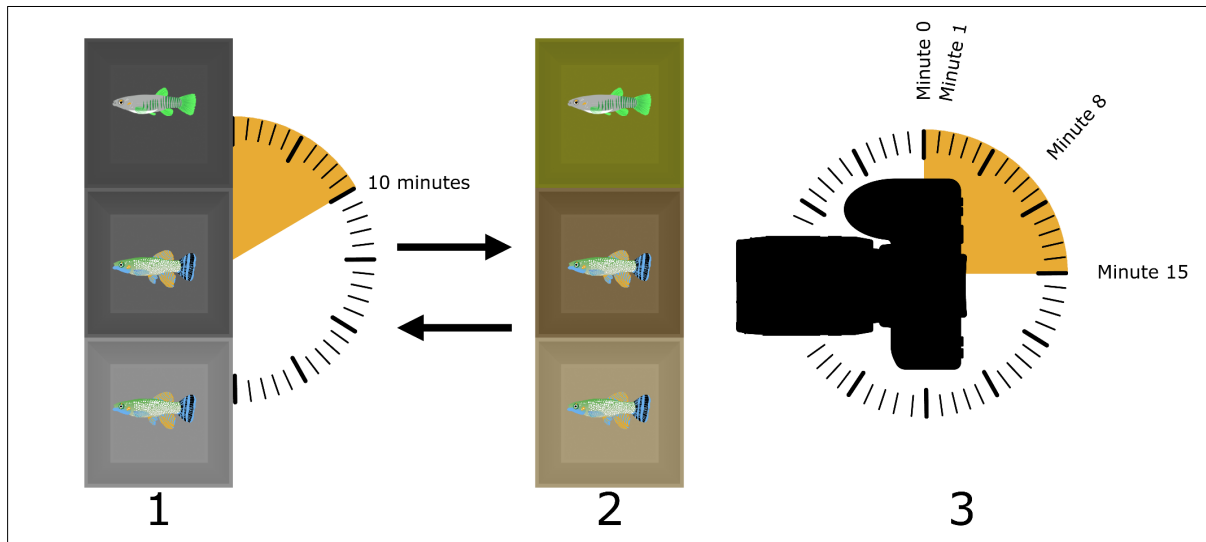


Figure 3.3: Schematic diagram showing the experimental procedure for the chromatic backgrounds for each of the studied populations. (1) Placing each fish (20 different fish/ beige background experiment, 20 different fish/ brown background experiment and 20 different fish/ green background experiment) in the reflectance-corresponding acclimating grey backgrounds for 10 minutes: the upper arrow indicates transferring the individuals to the photography containers. (2) Placing the fish in the photography containers (from top to bottom: green, brown, and beige backgrounds). (3) Photographing each fish at minutes 0, 1, 8, and 15. The lower arrow indicates returning the individuals to their acclimating grey backgrounds.

Photography

Photographs of the fish were captured in a photography room with a Nikon D7000 digital camera that had been converted to capture images in the visible and ultraviolet (UV) spectra of electromagnetic wavelength (Advanced Camera Services, Norfolk, U.K.). The camera was equipped with a Nikon 105mm f/2.8G Micro-Nikkor lens, and placed approximately 95 cm above the photography containers utilising a tripod (Figure S3.6). Due to the high mobility of the fish, it was challenging to take photographs in the UV range, so only images in the visible spectrum 420-680 nm were taken using a Baader UV/IR-Cut/L filter (Baader Planetarium, Mammendorf, Germany). All photographs were captured in raw format (.NEF) with fixed ISO (400) and aperture (F3) under EyeColor Arc MT70D lamp (Iwasaki Electric Co., LTD). The shutter speed was manually adjusted depending on the background colour, but maintained across the study for all the populations (i.e., each background colour had a fixed shutter speed for all the 4 studied populations). The light was diffused using a custom-

built tent made with PVC pipes 110 × 80 × 125 cm and covered with white PEVA covers from the sides as well as with a black-matte foam board over the tent to prevent specular reflection (Figure S3.6). Each image had a known scale bar of 3 cm along with 4.88% and 41.73% reflectance standards (ColorChecker Passport MSCCPP-B: X-Rite) for all background types except for the white background, which had 41.73% and 98.24% reflectance standards. These standards were chosen to fit within the expected reflectance values of the fish and backgrounds to prevent any inconsistencies in lighting conditions, which would lead to deficient colour quantification. The photography settings were maintained throughout the study, and photographs were captured from a uniform distance and angle across the study.

Image analysis

The aim of this study was to examine the geographical variations in *A. dispar*'s ability for achromatic change (luminance) and chromatic change (hue and saturation), in addition to see if the changes affected luminance and colour camouflage. Images were analysed using non-invasive approaches on ImageJ software (Schneider et al., 2012) using a Multispectral Image Calibration and Analysis Toolbox (MICA) (Troscianko & Stevens, 2015). This tool linearised and standardised the raw nonlinear images of the fish and their backgrounds to calibrated linear multispectral images with regard to the 4.88%, 41.73% and 98.24% grey standards, which encompassed reflectance information of the visible RGB channels. The linearisation of the photographs was used to control for any fluctuations in light conditions to obtain objective measurements (Stevens et al., 2007; Troscianko & Stevens, 2015). The dorsal view of each individual from the snout tip to the base of the caudal fin (excluding the pectoral fin and the dorsal fin when it was extended over the body) was chosen as regions of interest (ROI) for luminance, colour and camouflage quantification. I randomly selected ~1 cm² of the photographed test backgrounds for each image as ROIs at minute 0. The collected fish had an average length of 3.5 cm ± 0.67 (standard deviation), measured from the tip of the snout to the base of the caudal fin using ImageJ.

To discuss the biological significance of the changes in the colouration of the Arabian killifish—luminance, luminance camouflage and colour camouflage were analysed from a predator visual model. Despite the lack of data concerning the key predators of *A. dispar* (but see examples in Coad, 2010 for fish and Freyhof et al., 2020 birds) and their visual

systems, this fish and cyprinodontiformes in general, are likely to be important prey to birds in all ecosystems (Figure 1.2; Silva et al., 2019). Thus, the calibrated photos were mapped from the camera spectral sensitivity into the trichromatic version of the peafowl's (*Pavo cristatus*) spectral sensitivity (Hart, 2002) in MICA (Troschianko & Stevens, 2015), which has been frequently used as a model of avian vision (e.g., Barnett et al., 2018; Kang et al., 2015; Nokelainen et al., 2020). The mapping technique resulted in images with channels' values corresponding to long-wave (LW), medium-wave (MW), short-wave (SW) sensitive cones, and double cone type (dbl).

The changes in luminance were directly analysed from the dbl catch values of the peafowl model. Camouflage due to changes in luminance and colour was analysed against the test backgrounds as perceived by the avian predator model and expressed as Just Noticeable Differences (JNDs). The achromatic camouflage was calculated via the modified version (Siddiqi et al., 2004) of Vorobyev and Osorio's (1998) receptor noise limited model (RNL). I used a Weber fraction of 0.2, as reported by Olsson et al. (2018). I used a Weber fraction of 0.06 (Olsson et al., 2015) as a noise for the most abundant cone cell of the peafowl visual model, which is MW based on the cone ratios of the peafowl (SW 1.9: MW 2.2: LW 2.1) (Hart, 2002). Then I estimated the noise in the SW and LW cones using $e_i = \sqrt{\frac{\eta^{MW}}{\eta^i}} e^{MW}$ (Olsson et al., 2018), where i is the cone type (SW or LW), η^i is the ratio of each of these cones to the MW cone, and e^{MW} is the Weber fraction 0.06 of MW. The final noise in each of these photoreceptors was (SW= 0.0645; MW= 0.06; LW= 0.0614). Based on the discrimination model described above, the obtained digits of the luminance and colour JNDs specified whether the luminance and colour of two objects were perceptible to the receiver model. Generally, a JND value of < 1 indicates that the two stimuli are indistinguishable by the receiver vision, and values between 1 to 3 might mean the two stimuli can be detectable under good viewing conditions. Increment in JNDs over 3 shows that the two objects' colours become more distinguishable (Siddiqi et al., 2004).

In order to measure the saturation and hue of fish and backgrounds, I first standardised each of the LW, MW, and SW catch values to remove the brightness differences (Stevens, 2011). This was done by dividing each catch value by the sum of all catch values (e.g., $sLW = \frac{LW}{LW + MW + SW}$) (Kelber et al., 2003; Stevens, 2011). Then, these values were mapped into a trichromatic colour space using Cartesian coordinates where $x = \frac{1}{\sqrt{2}} (sLW - sMW)$

and $y = \frac{\sqrt{2}}{\sqrt{3}} \left(sSW - \frac{sLW + sMW}{2} \right)$ (Kelber et al., 2003). Saturation was then calculated as the distance from the achromatic centre (Stevens, 2011) in the trichromatic colour space, $\sqrt{x^2 + y^2}$ in which higher values indicate that colours are more saturated. Hue is difficult to generate from colour space (Stevens, 2011), thus, the hue was simply calculated as the difference between standardised middle to the longer-shorter wavelengths, $sMW - \frac{sLW + sSW}{2}$ similar to previous studies (e.g., Komdeur et al., 2005; Smithers et al., 2018; Stevens et al., 2014). A value of 0 from this calculation means that the subject measured is achromatic and has equal cone catch values across all three channels. According to the printed backgrounds, the beige, brown and green hues had average values of (0.005, 0.015 and 0.075, respectively). Therefore, I could determine whether changes in hue were directed to the backgrounds' hues (see Results).

Statistical analyses

All statistical analyses were performed in R version 4.2.3 (R Core Team, 2023). I performed linear mixed models via the “lme4” package (Bates et al., 2015), and significant differences were obtained from the package “lmerTest” utilising Satterthwaite’s method (Kuznetsova et al., 2017). Each background treatment was analysed separately with the following colour metrics: luminance, hue, saturation, luminance JND and Colour JND as response variables; Colour JND was only analysed for the chromatic backgrounds (beige, brown and green). The test time (minutes 0, 1, 8 and 15), fish population and fish sex were treated as fixed categorical variables, and fish length as a fixed numerical variable with the fish identities as random effects to account for the repeated measures.

Fish length and sex were initially added as interaction terms in the models. However, they were treated as fixed covariates after visually comparing the fit of the models and by using the Akaike Information Criterion (AIC) and Bayesian Information Criterion (BIC). The final model syntax was as follows: Colour metric ~ TestTime * FishPopulation + FishLength + FishSex + (1|FishID). Some variables were transformed to log (*log*) or square root (*sqrt*) scales to obtain normally distributed residuals. Each linear mixed model was followed by post hoc analyses via the Tukey’s honest significant difference test (Tukey test) to obtain the significant differences in the means of the colour metrics values between the time points

within each population using the package “emmeans” (Lenth, 2023) with the “regrid” function to back transform the transformed models.

Results

Colour change on achromatic backgrounds

Black background

There were significant differences between populations in luminance change indicated by the interaction between population and time ($\log(\text{LMM})$: $F(9,228) = 11.4243$, $p < 0.0001$) (Figure 3.4A). The Tukey post hoc test between minutes 00–15 revealed highly significant changes for all the populations ($p < 0.0001$) (Figures 3.4A, 3.5). Luminance changes in the black background experiment occurred faster—within the first minute for all the populations (Tukey test: $p < 0.0001$) apart from Khaybar population (Tukey test: $p = 0.0003$) (Figure S3.7), where luminance changed steadily. Changes between minutes 01–08 continued, with decreased significance observed in all the populations (i.e., not as highly significant as between minutes 00–01). However, no significant differences were observed between minutes 08–15, except for Khaybar population, which retained a considerable change in luminance (Tukey test: $p = 0.0019$). There were significant differences between populations in the changes of luminance JND revealed by the interaction between population and time ($\sqrt{\text{LMM}}$: $F(9,228) = 11.721$, $p < 0.001$). Luminance JND reduced greatly within the first minute of the experiments for all the populations (Tukey test: $p < 0.0001$) except for Khaybar population (Tukey test: $p = 0.027$). However, apart from the population of Jeddah, which showed a significant and directional trend towards the detection threshold (i.e., $\text{JND} = 1$) (Tukey test: $p < 0.0001$) (Figure 3.4B), there was a non-significant improvement in camouflage for all the other populations by the end of the experiments (minutes 00–15).

There were significant differences between populations in hue change indicated by the interaction between population and time ($\log(\text{LMM})$: $F(9,228) = 11.1286$, $p < 0.0001$). As in luminance changes, the Tukey post hoc test between minutes 00–15 revealed highly significant differences in hue for all the populations in the black background experiment ($p < 0.0001$), which changed faster within the first minute of the experiment for all the populations ($p < 0.0001$) except for Khaybar population ($p = 0.0023$). The significant

changes continued between minutes 01–08 but disappeared for all the populations between minutes 08–15, except for Khaybar population, which retained the significant change (Tukey test: $p = 0.0041$). There were significant differences between populations in saturation change indicated by the interaction between population and time ($\log(\text{LMM})$: $F(9,228) = 12.4184$, $p < 0.0001$). As in luminance and hue changes, the Tukey post hoc test between minutes 00–15 revealed highly significant differences in saturation for all the populations ($p < 0.0001$), which were faster within the first minute of the experiment for all the populations ($p < 0.0001$) except for Khaybar population ($p = 0.0181$). There was no significant difference for all the populations between minutes 08–15, except for Khaybar population, which retained the significant change (Tukey test: $p = 0.0014$). There were no significant effects of fish length and sex on all colour metrics in the black background experiment.

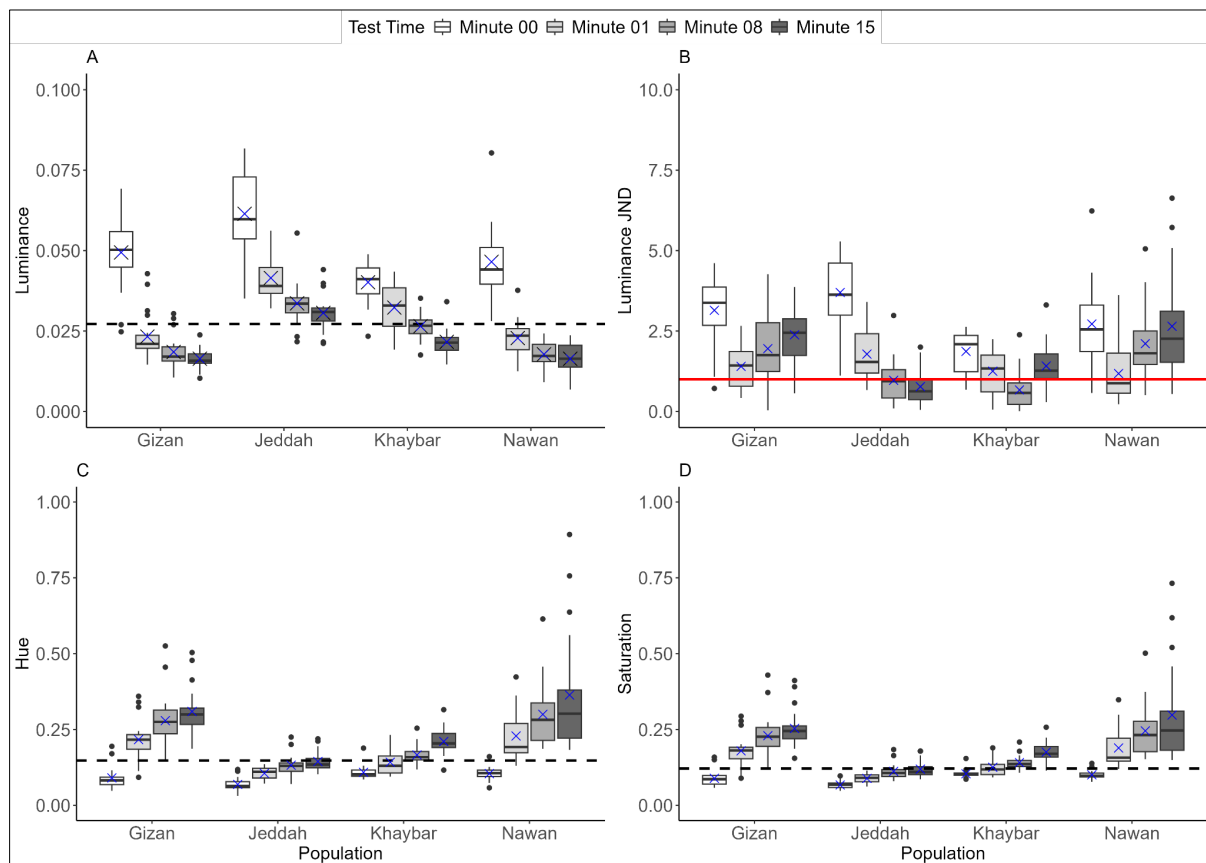


Figure 3.4: Changes in (A) the luminance, (B) luminance JND, (C) hue and (D) saturation within 15 minutes for all the populations ($n = 20$ per population) in the black background experiment. The boxes show the means (blue crosses), medians (black lines) and inter-quartile ranges. The whiskers represent the lowest and highest values within $1.5 \times$ the inter-quartile ranges, and the dots represent the outliers. The horizontal black dashed lines represent the average values of the backgrounds' luminance (A), hue (C) and saturation (D)

for all the populations, and the horizontal solid red line represents the detection threshold of luminance contrast at 1 JND by the avian model (B).

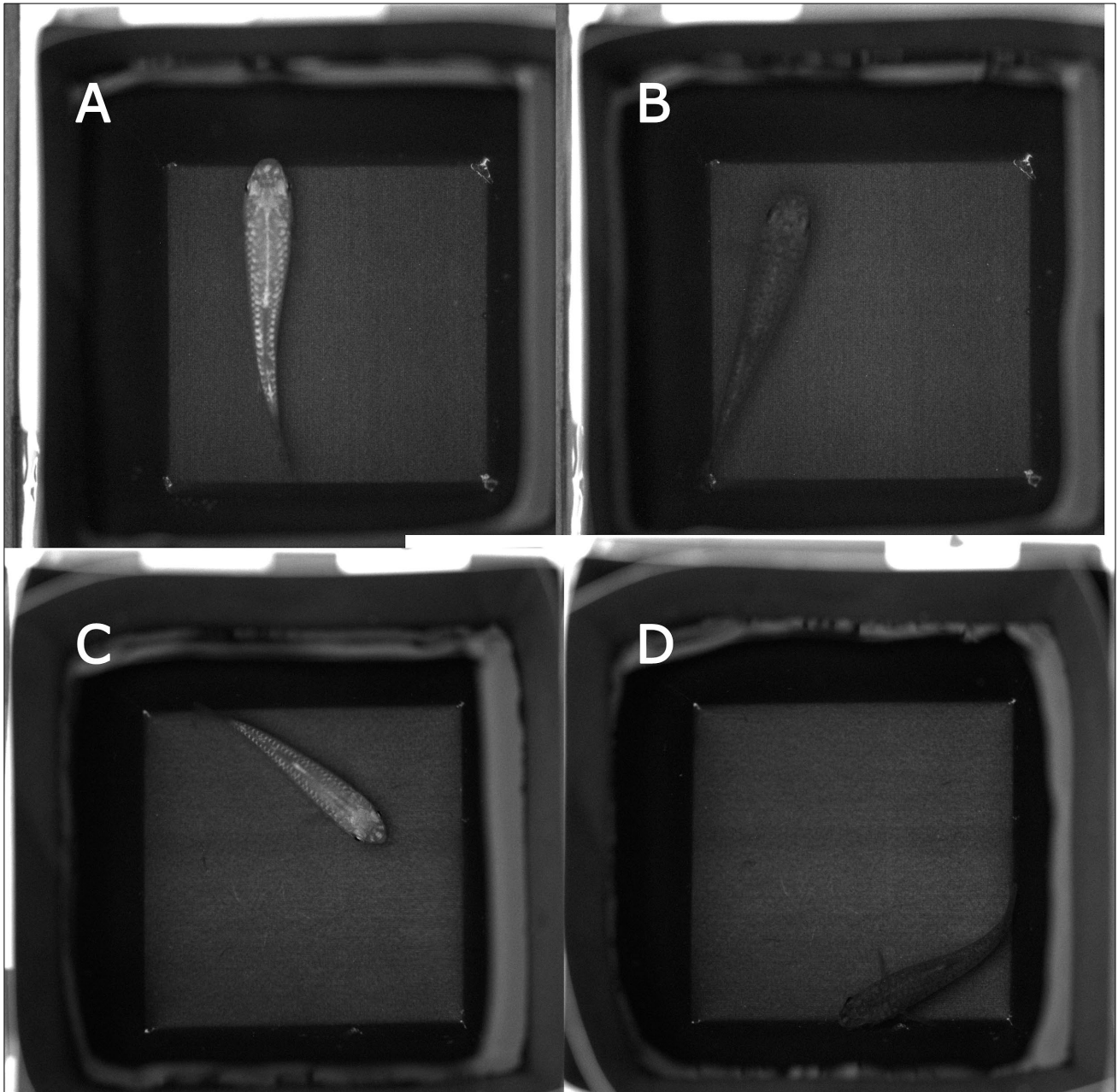


Figure 3.5: Unmodelled images (RGB images in grey mode) illustrating the changes in the brightness of an Arabian killifish from the marine population of Jeddah in the black experiment at minutes 0 (A) and 15 (B), as well as an individual from the freshwater population of Nawan at minutes 0 (C) and 15 (D).

White background

There were significant differences between populations in luminance change revealed by the interaction between population and time (LMM: $F(9,228) = 2.251$, $p = 0.0198$) (Figure

3.6A). In contrast to the black background experiment, luminance changes in white background experiment occurred mainly between minutes 00 and 15 for all the populations except Khaybar population, by which luminance did not change significantly (Tukey test between minutes 00–15 in the populations of [Gizan: $p = 0.0079$], [Jeddah: $p = 0.0001$], [Nawan: $p = 0.0343$], and [Khaybar: $p = 0.9998$]; Figures 3.7, S3.8). Only Jeddah population showed a significant change between minutes 01–08 (Tukey test: $p = 0.0166$) due to the decreased luminance within the first minute which increased afterwards (Figure 3.6A). There was a significant effect of fish length on luminance (LMM: $F(1,74) = 15.018$, $p = 0.0002$). Additionally, the interaction between population and time revealed significant differences between populations in the changes of luminance JND ($\log(\text{LMM})$: $F(9,228) = 2.366$, $p = 0.0142$). Luminance JND changes in the white background experiment mainly occurred within the last minute of the experiment for all the populations except for Khaybar population (Tukey test between minutes 00–15 for the populations of [Gizan: $p = 0.0088$], [Jeddah: $p < 0.0001$], [Nawan: $p = 0.0466$], and [Khaybar: $p = 0.9998$]). There was a significant effect of fish length on luminance JND ($\log(\text{LMM})$: $F(1,74) = 8.040$, $p = 0.006$).

There were slightly significant differences between populations in hue change indicated by the interaction between population and time (LMM: $F(9,228) = 2.293$, $p = 0.0176$). The Tukey post hoc test between time points indicated that the significance observed in hue changes in the white background experiment was due to changes in Jeddah population between minutes 00–01 ($p = 0.0117$) and 00–08 ($p = 0.003$) and in Gizan population between minutes 01–15 ($p = 0.0155$), but no significant differences were observed by the end of the experiments (Figure 3.6C). There were significant differences between populations in saturation change indicated by the interaction between population and time ($\sqrt{\text{LMM}}$: $F(9,228) = 2.238$, $p = 0.0206$). In the white background experiment, the Tukey post hoc test between time points indicated that the significance observed in saturation changes was due to changes in Jeddah population between minutes 01–15 and 08–15 ($p = 0.0161$ and $p = 0.0537$, respectively) and in Nawan population between minutes 00–15 ($p = 0.0108$). However, no significant differences were observed by the end of the experiments for the other populations (Figure 3.6D). There was a significant effect of fish length on saturation ($\sqrt{\text{LMM}}$: $F(1,74) = 10.709$, $p = 0.0016$). There were no significant effects of fish sex on all colour metrics in the white background experiment.

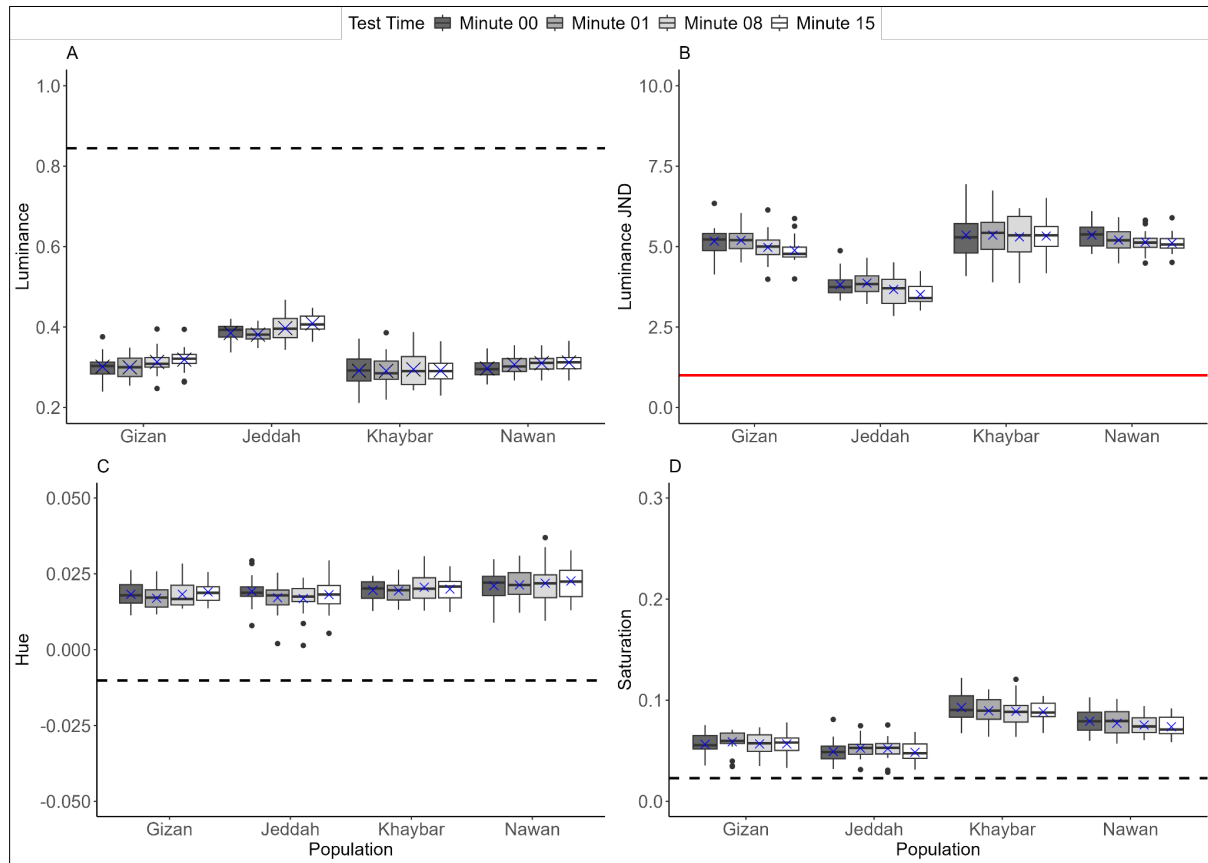


Figure 3.6: Changes in (A) the luminance, (B) luminance JND, (C) hue and (D) saturation within 15 minutes for all the populations ($n = 20$ per population) in the white background experiment. The boxes show the means (blue crosses), medians (black lines) and inter-quartile ranges. The whiskers represent the lowest and highest values within $1.5 \times$ the inter-quartile ranges, and the dots represent the outliers. The horizontal black dashed lines represent the average values of the backgrounds' luminance (A), hue (C) and saturation (D) for all the populations, and the horizontal solid red line represents the detection threshold of luminance contrast at 1 JND by the avian model (B).

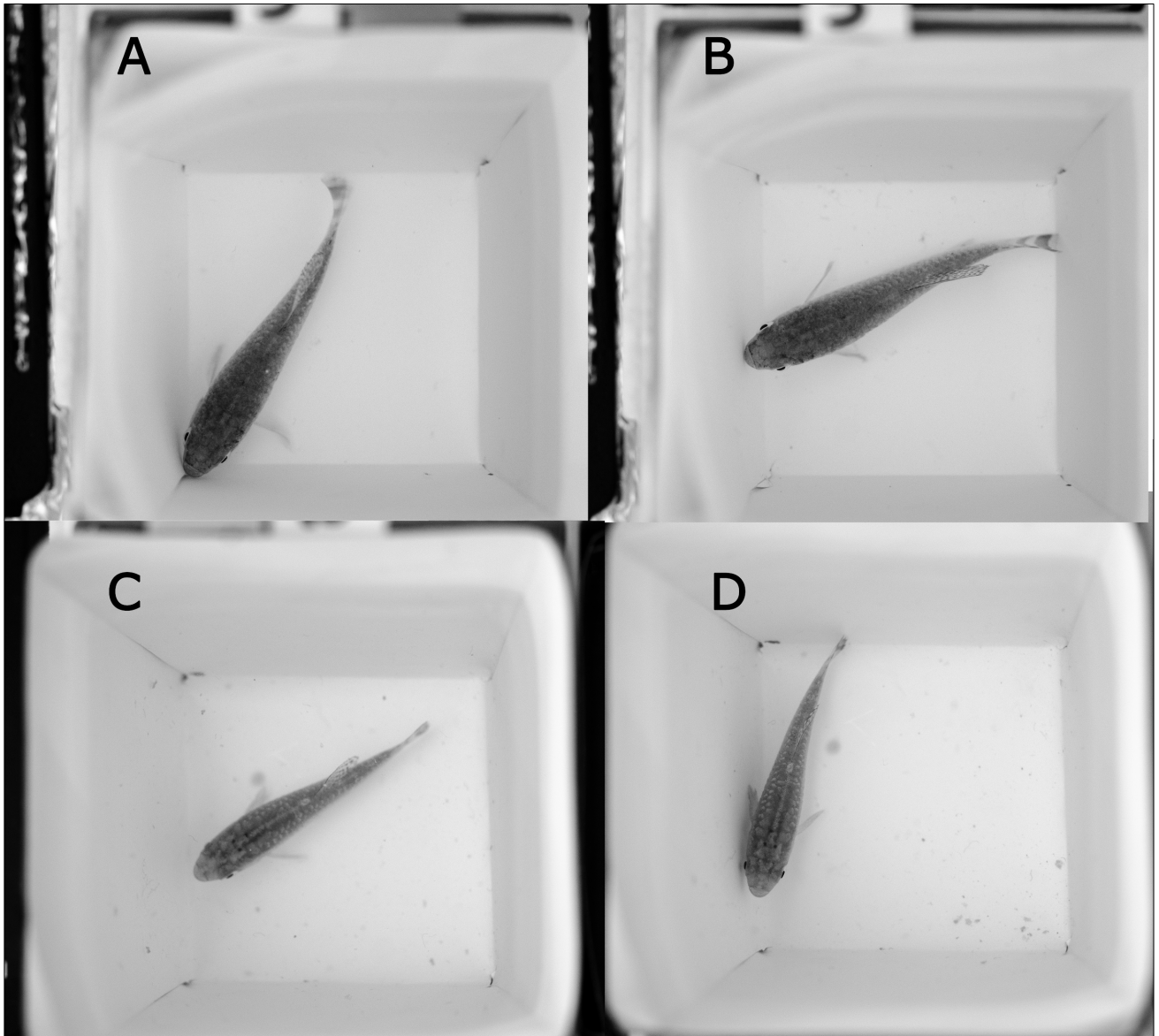


Figure 3.7: Unmodelled images (RGB images in grey mode) illustrating the changes in the brightness of an Arabian killifish from the marine population of Gizan in the white experiment at minutes 0 (A) and 15 (B), as well as an individual from the freshwater population of Khaybar at minutes 0 (C) and 15 (D).

Colour change on chromatic backgrounds

The findings consist of responses to three background colours, each of which has analyses of five colour metrics. The results of luminance, hue, saturation, colour JND and Luminance JND are summarised in Table 3.1.

Table 3.1: Summary of the statistical analyses of the luminance, hue, saturation, colour JND and Luminance JND changes in the chromatic background experiments (beige, brown, and green) for individuals from the four populations. Ticks (✓) indicate a significant difference (Tukey test: $p < 0.05$) in colour metric values between minutes 00 and 15, and crosses (X) indicate that no significant changes were observed during the experiments. Circles (○) indicate that at least a significant difference was observed between two time points at the beginning or in the middle of the experiments. While (M) means that the statistical significance was marginal (Tukey test: $p \approx 0.05$). Some significant changes in hue were not directed towards the hue values of the backgrounds (Figures 3.8B, 3.9B, 3.10B), which might be likely maladaptive (mala). Each shade of the orange colour represents one of the results categories mentioned above.

Background colour	Population				Colour metric
	Nawan	Jeddah	Gizan	Khaybar	
Beige	X	✓	X	X	Luminance
	X	X	✓ mala	X	Hue
	✓	✓	X	X	Saturation
	✓	✓	X	X	Colour JND
	X	✓	X	X	Luminance JND
Brown	X	X	✓	X	Luminance
	X	✓ mala	X	X	Hue
	X	✓	✓	○	Saturation
	X	X	✓	○	Colour JND
	X	X	✓	X	Luminance JND

Green	X	○	✓	X	Luminance
	X	✓	✓ mala	X	Hue
	○ M	X	✓	X	Saturation
	X	✓ mala	✓	○	Colour JND
	X	○	✓	X	Luminance JND

Beige background

There were significant differences between populations in luminance change indicated by the interaction between population and time ($\sqrt{\text{LMM}}$: $F(9,228) = 3.779$, $p = 0.0002$). The pairwise Tukey post hoc test indicated that Gizan, Khaybar and Nawan populations did not change luminance (Figure 3.8A). Jeddah population showed significant changes by the end of the experiment ($p = 0.0005$). There were significant differences between populations in the changes of luminance JND demonstrated by the interaction between population and time (LMM: $F(9,228) = 2.971$, $p = 0.0023$). However, when further investigating this using the pairwise Tukey post hoc test between minutes, Gizan, Khaybar and Nawan populations showed no significant changes in luminance JND, and only Jeddah population showed significant changes (Tukey test between minutes 00–15: $p = 0.0034$) (Figure 3.11A). There was a significant effect of fish length on luminance ($\log(\text{LMM})$: $F(1,74) = 9.771$, $p = 0.0025$) and on luminance JND (LMM: $F(1,74) = 8.914$, $p = 0.0038$) (see Appendix 2 for detailed differences).

There were no significant differences between populations in hue change as shown by the interaction between population and time (LMM: $F(9,228) = 1.096$, $p = 0.3667$). However, there was a significant effect of fish length on hue (LMM: $F(1,74) = 8.100$, $p = 0.0057$) (see Appendix 2 for detailed differences). There were significant differences between populations in saturation change indicated by the interaction between population and time ($\log(\text{LMM})$: $F(9,228) = 4.766$, $p < 0.0001$). The pairwise Tukey post hoc test indicated that only Jeddah and Nawan populations changed saturation significantly by the end of the experiments ($p < 0.0001$ for each population).

There were significant differences between populations in the changes of colour JND shown by the interaction between population and time ($\sqrt{\text{LMM}}$: $F(9,228) = 3.862$, $p = 0.0001$). In this experiment, only Jeddah and Nawan populations showed highly significant changes (Tukey test: $p = 0.0002$ and $p < 0.0001$, respectively), whereas Gizan and Khaybar populations showed no significant differences in colour JND values. Nevertheless, all individuals from all the populations in the beige background experiment were chromatically detectable, and only Jeddah population closely matched the beige background (Figure 3.8D). There were no significant effects of fish sex on all colour metrics in the beige background experiment.

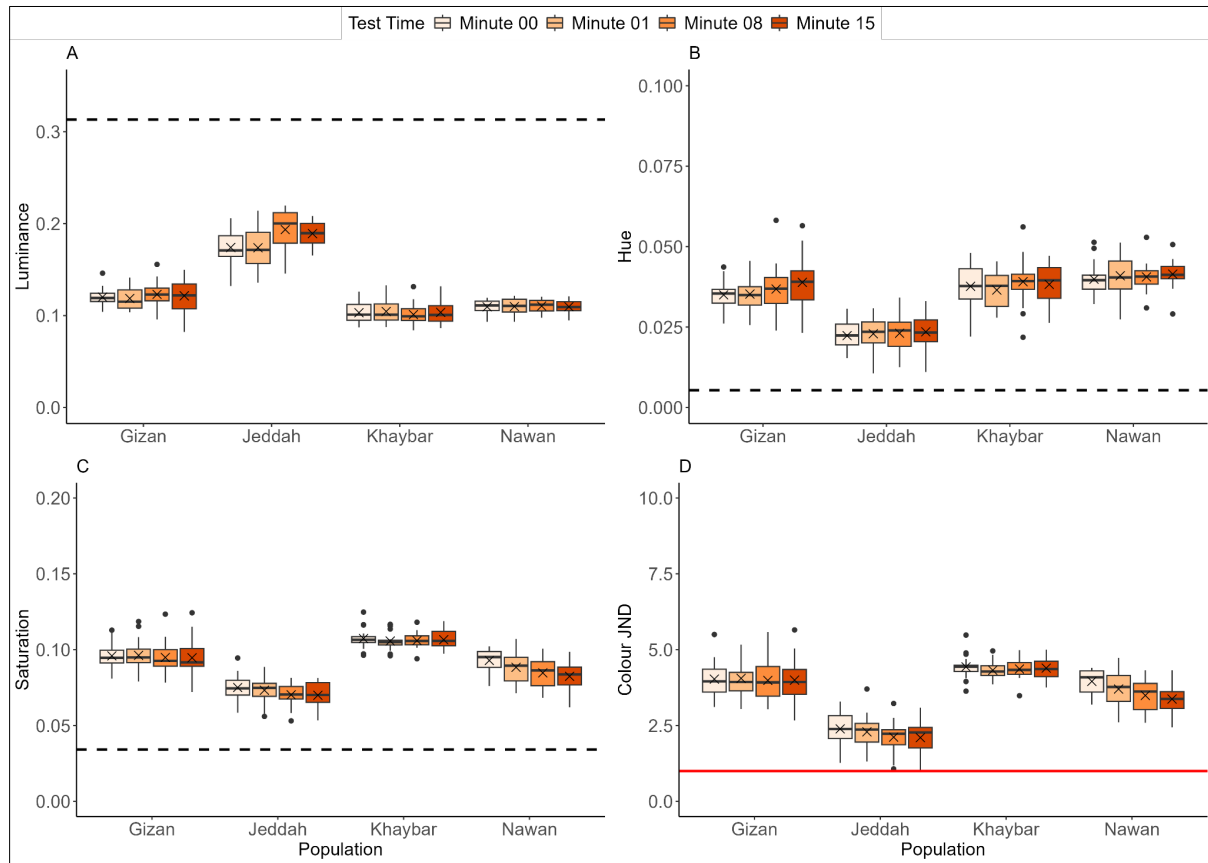


Figure 3.8: Changes in (A) the luminance, (B) hue, (C) saturation and (D) colour JND within 15 minutes for all the populations ($n=20$ per population) in the beige background experiment. The boxes show the means (black crosses), medians (black lines) and inter-quartile ranges. The whiskers represent the lowest and highest values within $1.5 \times$ the inter-quartile ranges, and the dots represent the outliers. The horizontal black dashed lines represent the average values of the backgrounds' luminance (A), hue (B) and saturation (C) for all the populations, and the horizontal solid red line represents the detection threshold of colour contrast at 1 JND by the avian model (D).

Brown background

There was a non-significant trend for differences between populations in luminance change indicated by the interaction between population and time ($\text{sqrt(LMM)}: F(9,228) = 1.870, p = 0.0574$). The pairwise Tukey post hoc test indicated that Jeddah, Khaybar and Nawan populations did not change luminance (Figure 3.9A). However, the marine population of Gizan showed significant changes in luminance by the end of the experiments (Tukey test: $p = 0.0006$). There were marginally significant differences between populations in the changes of luminance JND displayed by the interaction between population and time (LMM: $F(9,228) = 1.936, p = 0.0480$). The Tukey post hoc test between minutes showed that

Jeddah, Khaybar and Nawan populations exhibited no significant changes in luminance JND except for the Gizan population (Tukey test between minutes 00–15: $p = 0.0004$) (Figure 3.11B).

There were significant differences between populations in hue change demonstrated by the interaction between population and time ($\log(\text{LMM})$: $F(9,228) = 4.715$, $p < 0.0001$) where Gizan, Khaybar and Nawan populations did not change hue (Figure 3.9B) compared to Jeddah population, which showed significant changes by the end of the experiment (Tukey test: $p < 0.0001$). There was a significant effect of fish length on hue ($\log(\text{LMM})$: $F(1,74) = 6.696$, $p = 0.0116$). There were significant differences between populations in saturation change indicated by the interaction between population and time (LMM: $F(9,228) = 3.896$, $p = 0.0001$). The pairwise Tukey post hoc test indicated that in the brown background experiment, only the marine Gizan and Jeddah populations changed saturation significantly by the end of the experiments ($p = 0.0005$ and $p = 0.0277$), and the freshwater population of Khaybar showed only a significant change at the beginning of the experiments (minutes 00–01: $p = 0.0001$).

There were significant differences between populations in the changes of colour JND indicated by the interaction between population and time ($\log(\text{LMM})$: $F(9,228) = 2.381$, $p = 0.0136$). In the brown background experiment, only Gizan population showed a significant shift in colour JND by the end of the experiments (Tukey test: $p = 0.0029$). Khaybar population changed only at the beginning of the experiment (Tukey test: $p = 0.0272$) (Figure 3.9D). As in the beige background experiment, all individuals from all the populations in the brown background experiment were chromatically detectable, and only Jeddah population had more similarities to the brown backgrounds (Figure 3.9D). There were no significant effects of fish sex on all colour metrics in the brown background experiment.

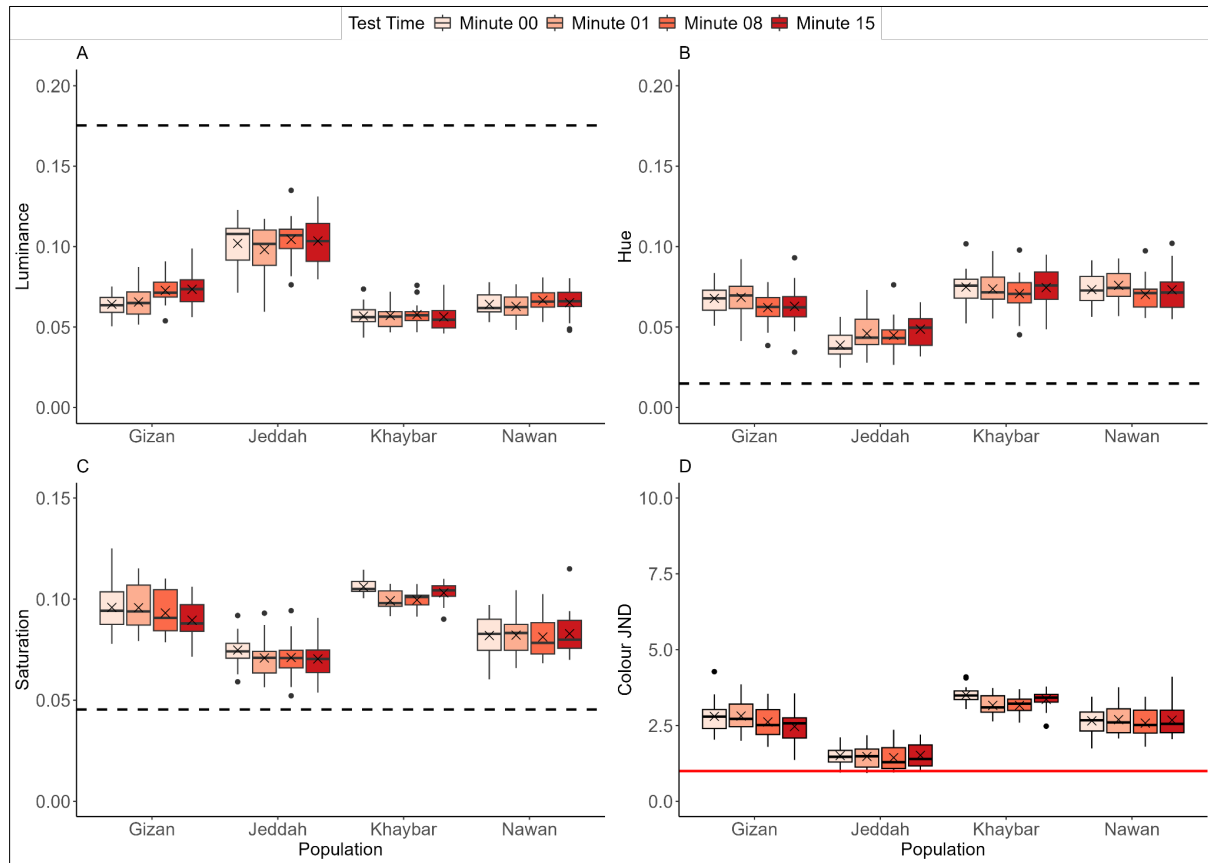


Figure 3.9: Changes in (A) the luminance, (B) hue, (C) saturation and (D) colour JND within 15 minutes for all the populations ($n = 20$ per population) in the brown background experiments. The boxes show the means (black crosses), medians (black lines) and inter-quartile ranges. The whiskers represent the lowest and highest values within $1.5 \times$ the inter-quartile ranges, and the dots represent the outliers. The horizontal black dashed lines represent the average values of the backgrounds' luminance (A), hue (B) and saturation (C) for all the populations, and the horizontal solid red line represents the detection threshold of colour contrast at 1 JND by the avian model (D).

Green background

There were significant differences between populations in luminance change revealed by the interaction between population and time (LMM: $F(9,226.552) = 3.209$, $p = 0.0011$). The pairwise Tukey post hoc test indicated that Khaybar and Nawan populations did not change luminance (Figure 3.10A). However, the marine populations of Gizan and Jeddah showed significant changes in luminance, with Gizan population showing changes by the end of the experiment (Tukey test: $p = 0.0006$). Jeddah population showed significant changes in the middle of the experiment (Tukey test between minutes 01–08: $p = 0.0057$) (Figure 3.10A). There was a significant effect of fish length on luminance (LMM: $F(1,73.351) = 4.172$, $p =$

0.0447) (see Appendix 2 for detailed differences). There were significant differences between populations in the changes of luminance JND indicated by the interaction between population and time ($\sqrt{\text{LMM}}$: $F(9,226.207) = 3.063$, $p = 0.0018$). When further investigating this using the pairwise Tukey post hoc test between minutes, Khaybar and Nawan populations showed no significant changes in luminance JND (Figure 3.11C). However, the marine populations of Gizan and Jeddah showed significant differences in luminance JND by the end of the experiments, parallel to changes in luminance, whereas Gizan population showed changes by the end of the experiments (Tukey test: $p = 0.0008$). Jeddah population displayed significant changes in the middle of the experiment (Tukey test between minutes 01–08: $p = 0.0238$). There was a significant effect of fish length on luminance JND (LMM: $F(1,72.998) = 4.861$, $p = 0.0306$) (see Appendix 2 for detailed differences).

There were significant differences between populations in hue change demonstrated by the interaction between population and time ($\log(\text{LMM})$: $F(9,226.588) = 3.243$, $p = 0.0010$). The pairwise Tukey post hoc test indicated that Khaybar and Nawan populations did not change the hue. However, the marine populations of Gizan and Jeddah showed significant changes by the end of the experiments ($p = 0.0337$ and $p < 0.0001$, respectively). There were significant differences between populations in saturation change indicated by the interaction between population and time (LMM: $F(9,226.597) = 2.733$, $p = 0.0048$). The pairwise Tukey post hoc test indicated that only the marine Gizan population changed saturation significantly by the end of the experiments ($p = 0.0001$), and only the freshwater Nawan population showed a marginally significant change in the middle of the experiments (minutes 01–08: $p = 0.0457$).

There were significant differences between populations in the changes of colour JND exhibited by the interaction between population and time (LMM: $F(9,227.034) = 3.314$, $p = 0.0008$). The pairwise Tukey post hoc test between minutes revealed that, in the green background experiment, the marine populations of Gizan and Jeddah changed colour JND significantly by the end of the experiments ($p = 0.0267$ and $p = 0.0095$, respectively), but the freshwater Khaybar population showed a change only between minutes 01–15 ($p = 0.0060$), and no differences were observed in Nawan population. In the beige background experiment, the avian model found some individuals from all the populations to be undetectable from the background (Figures 3.8D, 3.9D, 3.10D), possibly due to their natural

green hues. There were no significant effects of fish sex on all colour metrics in the green background experiment.

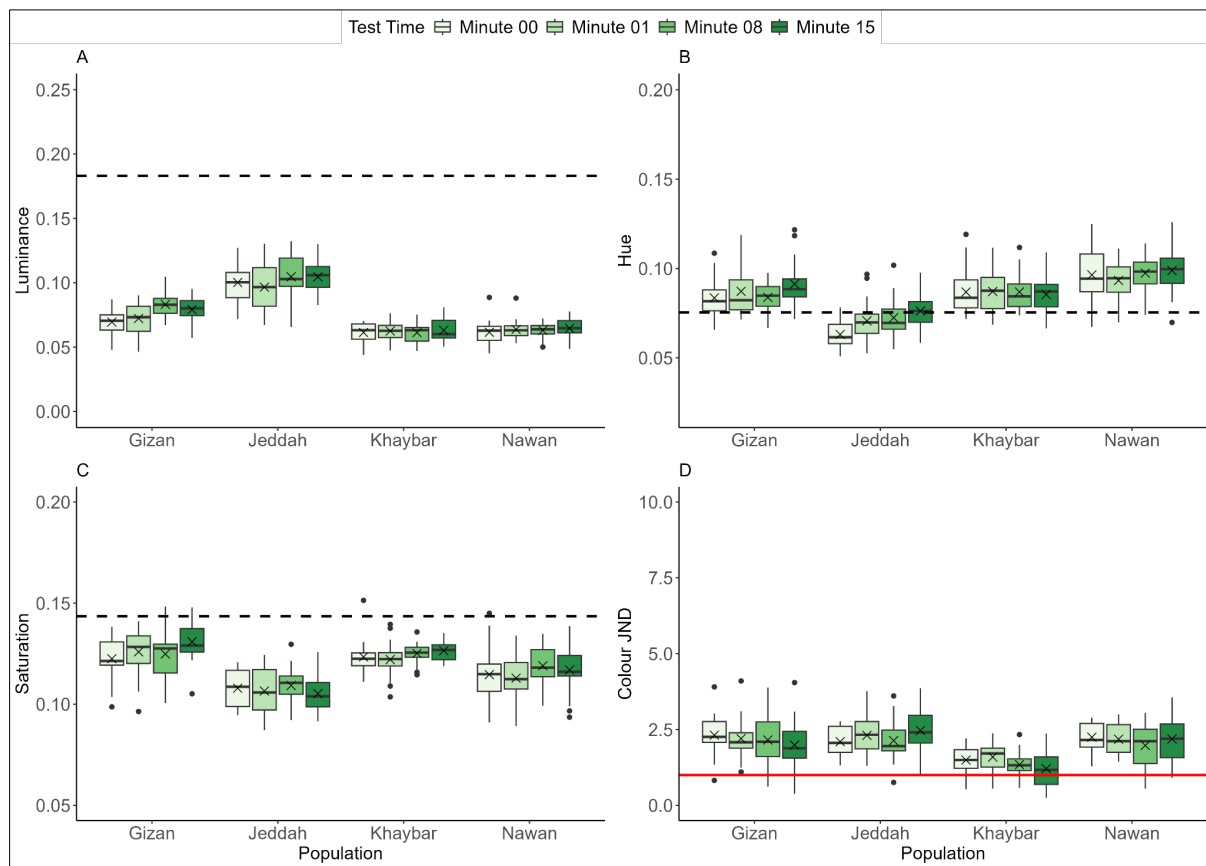


Figure 3.10: Changes in (A) the luminance, (B) hue, (C) saturation and (D) colour JND within 15 minutes for all the populations ($n = 20$ per population; only Nawan population had 19 individuals at minute 08) in the green background experiments. The boxes show the means (black crosses), medians (black lines) and inter-quartile ranges. The whiskers represent the lowest and highest values within $1.5 \times$ the inter-quartile ranges, and the dots represent the outliers. The horizontal black dashed lines represent the average values of the backgrounds' luminance (A), hue (B) and saturation (C) for all the populations, and the horizontal solid red line represents the detection threshold of colour contrast at 1 JND by the avian model (D).

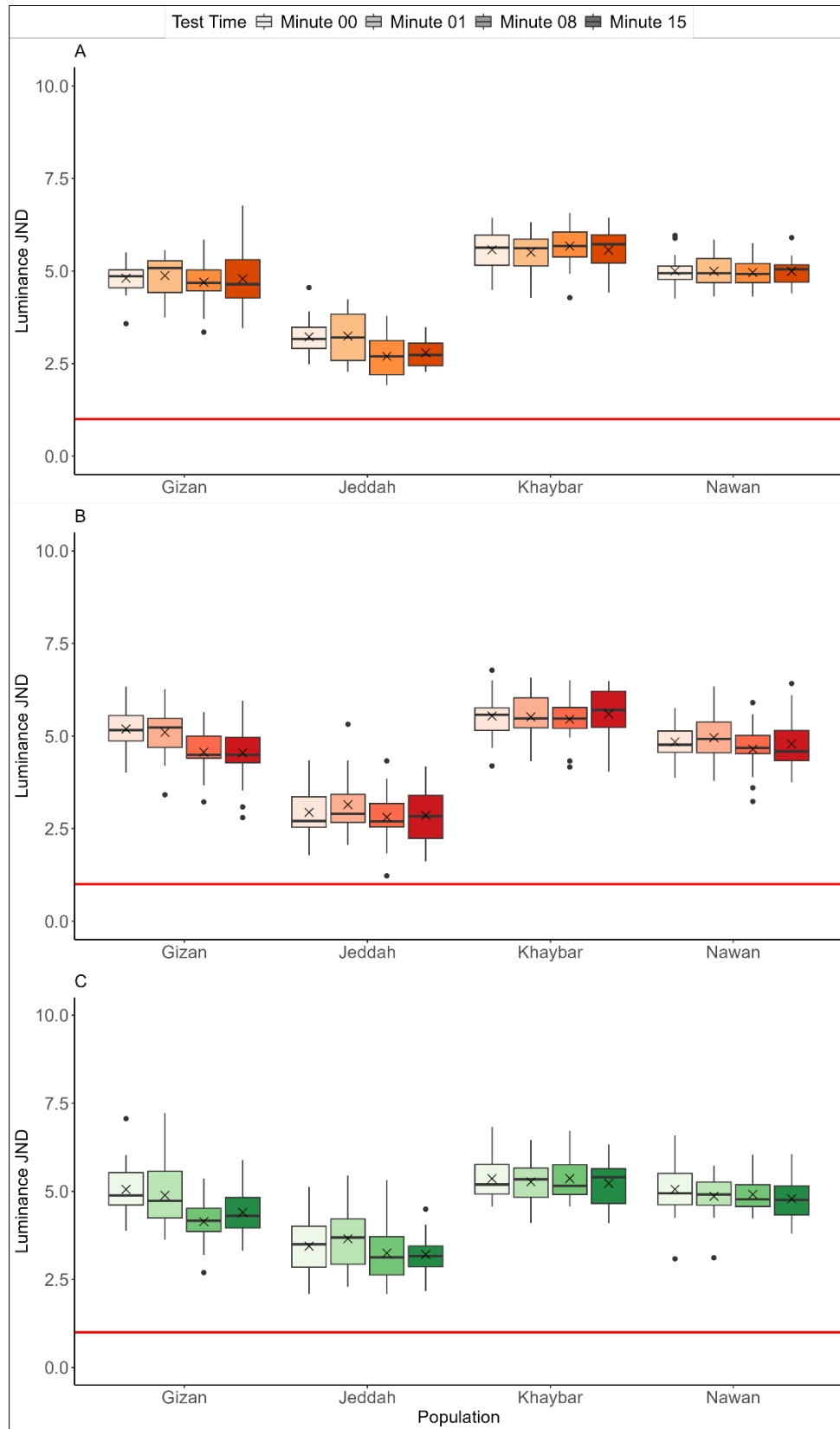


Figure 3.11: Changes in the luminance JND within 15 minutes in (A) the beige, (B) brown, and (C) green experiments ($n = 20$ per population; only Nawan population had 19 individuals in the green background experiment at minute 08)). The boxes show the means (black crosses), medians (black lines) and inter-quartile ranges. The whiskers represent the lowest and highest values within $1.5 \times$ the inter-quartile ranges, and the dots represent the outliers.

The horizontal solid red line represents the detection threshold of luminance contrast at 1 JND by the avian model. The light-to-dark coloured guides correspond to the test time.

Discussion

In this study, I investigated the geographical variation in the capacity for achromatic and chromatic colour change among marine and freshwater *A. dispar* populations. Since this is the first study on colour change in a member of this promising family, Aphaniidae, I thoroughly extended the discussion to serve as a baseline for future work on *A. dispar* and other Aphaniidae members. The findings indicated that the freshwater and marine populations of the Arabian killifish can change colour rapidly within a few minutes and showed increased ability for achromatic colour change (luminance) in response to the black and white substrates, compared to chromatic colour change in response to the beige, brown and green substrates. Additionally, there was an inter-population variation in colour change capabilities. Although all the populations exhibited the same directional responses to black backgrounds (i.e., became darker); the relative initial (after the minute 1) and final (after the minute 15) change rates varied among them (Appendix 2; Figures S3.7, S3.8). There are some possibilities for the apparent inequalities in changing luminance when on the black backgrounds. First, fish showed varying magnitudes of shifting luminance because they differed in their luminance at the start of the experiment, and hence the quantity of pigments constrained the dispersion (i.e., the more pigments the fish had, the greater they displayed darkening response). To some extent, this might be valid; the population of Jeddah had the greatest starting luminance and a 30.69% relative shift to dark during the first minute. Furthermore, the populations of Gizan and Nawan had nearly similar average luminance at the beginning and also had similar relative change rates; after one minute, both showed more than 48% changes.

On the contrary, despite having the lowest luminance (perhaps due to high quantity of melanosomes) at the start of the experiment that could have demonstrated advanced ability in dispersing pigments, the population of Khaybar exhibited slower and weaker changes, decreasing luminance by 18.5% during the first minute and 44.9% by the end of the experiment (Figures S3.7, S3.8). Alternatively, the variation in response to changing luminance could be predicted due to geographical variation in selective pressures, such as habitat brightness. In the white background experiment, all the populations showed a similar final increase in luminance (i.e., luminance at minute 15 relative to minute 00) ranging from

5.3–6.6%, except Khaybar population, which showed only a 0.95% increase (Figure S3.8). A combination of the two explanations appears reasonable; it could be that the populations of Gizan, Jeddah and Nawan might have convergently evolved greater changing abilities but differed in rates due to morphological and/or physiological constraints. In contrast, the population of Khaybar might have diverged into a decreased capacity to alter luminance to black and white backgrounds. These findings raise the question of why the changes in the two directions (i.e., black vs. white) differ substantially in all the populations.

Although extremely black and white surfaces are uncommon in nature, the utilisation of extreme backgrounds' brightness (i.e., very dark and very light) may disclose information about the limits of colour plasticity (Choi & Jang, 2014). I observed significant within-population differences in response to black and white backgrounds; all the populations changed luminance stronger and faster in the black background experiment than in the white one, with most changes occurring within the first minute in the black substrates and taking 8 to 15 minutes on the white backgrounds. The changes in brightness to white has also proven challenging in different freshwater species than on the brown and green backgrounds (Cox et al., 2009).

There are two non-exclusive possibilities for the varying response among processes in changing luminance (darkening vs. lightening) within a population. First, aggregating pigments in fish can be challenging or constrained by their pigment quantity, and the high concentration of pigments within chromatophores can make the animals appear dark despite the occurrence of the pigment aggregation. Therefore, significant changes to white may become pronounced through the apoptosis of pigments (Sugimoto et al., 2005) during the process of shifting to morphological colour change (Alfakih et al., 2022). Second, the artificial substrates impeded the pigment aggregation responses to white backgrounds, which are infrequent in typical environments (Choi & Jang, 2014; Smithers et al., 2018; Stevens et al., 2014a), or increased the stress on the fish. I found that Jeddah and Gizan populations slightly darkened their bodies upon being placed on the white backgrounds during the first minute. This darkening response was also found in other colour-changing fish species (da Silva et al., 2020), suggesting that, while it can be used to some extent for instantaneous background matching, pigment aggregation in the killifish is more likely a response to more predictable changes, such as seasonal changes (Caro et al., 2016).

Contrary to the white backgrounds, substrates with low brightness might be more common, and being on a dark background and shifting to a dark colouration may impair predator vision, resulting in reduced predation potential on fish (Sumner, 1934, 1935a, 1935b). Several lines of evidence demonstrated that different fish species prefer dark substrates more frequently (Bradner & McRobert, 2001; Brown & Thompson, 1937; Kjærsmo & Merilaita, 2012; Smithers et al., 2018).

Despite being acclimatised to the same intermediate grey backgrounds, fish from the same population exhibited differing luminance, hue, and saturation values at the beginning of both black and white background experiments. This variation was consistently observed across all chromatic treatments as well and was not due to changes in pigmentation. Instead, the initial differences were caused by the reflective properties of the fish's scales, which mirrored some of the background colour. Consequently, fish on lighter backgrounds appeared lighter, while those on darker backgrounds appeared darker.

The marine populations of Gizan and Jeddah appeared to be more capable of changing colour on chromatic backgrounds than the freshwater populations of Khaybar and Nawan (Table 3.1). I found no evidence for consistent differences between and within populations in chromatic changes (hue, saturation or even luminance), with some populations displaying changes in one colour metric and not in the other metrics. These results confirmed the previous observations made on four different populations of dwarf chameleons *B. transvaalense* where responses were not consistent in luminance and colour contrast against two different substrates (Stuart-Fox et al., 2006). There are possibly two explanations for the differences in achromatic and chromatic colour change among and within populations. First, antipredator responses may have varied depending on predator exposure leading to population-specific antipredator strategies (Magurran et al., 1993), thus the variation seen could be attributed to differential predator selective pressures, which resulted in the marine populations convergently evolving with a higher capacity for chromatic change due to higher diversity of predators in the Red Sea shores (Boland & Alsuhaibany, 2020; Lieske & Myers, 2004) compared to the freshwater populations, which may face fewer predators. To illustrate, an introduced population of Jackson's chameleon *T. j. xantholophus* in Hawaii displayed different colour contrasts than the original population in Kenya when changing colour and luminance, which has been attributed to relaxed selection by visual predators (Whiting et al., 2022). Second, chromatic changes may differ due to carryover effects emerging from prolonged exposure to multiple avian and aquatic predators in the

marine environments. Females of *Aphaniops* members reach a size of approximately 2.5 cm within 3–4 months, while males are generally smaller in standard length at this age (Coad, 2010; Freyhof et al., 2020). The mean standard length of the collected individuals in this study was $3.5 \text{ cm} \pm 0.67$ (standard deviation), meaning that the fish collected were mature and likely had been exposed to their local predators for several months. The exposure to predators might have increased their chromatic response through learning, presumably from continuous prey-predator interactions (Kelley & Magurran, 2003). Despite the fact that it is rarely discussed, at least one study on the zebrafish (*Danio rerio*), demonstrated some evidence of the role of learning in modulating colour change (Hatamoto & Shingyoji, 2008). The first explanation that pertains to the marine populations evolving a higher capacity for chromatic changes may be unlikely given that molecular data suggested connectivity and probably continuity of gene flow between the marine and freshwater populations (Schunter et al., 2021), as in Nawan and Jeddah (Chapter 2).

In both the achromatic and chromatic experiments, all adjustments in luminance and saturation were directed towards the average values of the corresponding backgrounds. This might mean that the increased directional plasticity could be an adaptation to the higher visual heterogeneity of the natural habitats (Clark & Schluter, 2011). Some of the changes in hue for the marine populations of Gizan and Jeddah, however, deviated from the average hue values of the backgrounds (Table 3.1; Figures 3.8B, 3.9B, 3.10B). I also noted some significant fluctuations in luminance and saturation on the brown and green substrates that occurred at the beginning or middle of the experiments and then reversed (Table 3.1; Figures 3.9D, 3.10A, C). These oscillatory-style changes are consistent with previous findings on a variety of colour-changing species, including fish (Whiteley et al., 2010; Clarke & Schluter, 2011; John et al., 2023) and amphibians (Kang et al., 2016) but their occurrence remains unresolved though energetic expenditure on pigment maintenance during changing colour may play a partial role (Alfakih et al., 2022).

Our findings indicated that significant changes in luminance on the white and black backgrounds increased the fish's visual similarities to their mismatched substrates. However, while the improvement in camouflage in the white experiments was statistically significant, all individuals were highly detectable (Figure 3.6B). By the end of the experiments, however, all the populations in the black background experiment reached, on average, better matches to the black backgrounds, and some individuals were undetectable ($< 1 \text{ JND}$). With the exception of Jeddah population, which had the best match at the end of

the experiments, all the other populations had the greatest concealment around the first or eighth minute (Figure 3.4B). Despite all the populations changing considerably in luminance, this occurred because fish were darker than the backgrounds (Figure 3.4A), leading the RNL model to determine them as less camouflaged than previous time points (Figure 3.4B). Luminance contrast is context-dependent (van den Berg et al., 2020); therefore, it cannot be corroborated whether fish darker than the black substrates were detectable or well camouflaged because this would necessitate behavioural validation (van den Berg et al., 2020). Furthermore, the visual capability of the killifish is not known as they might perceive luminance and colour differently than the used avian visual model. Generally, darkening body colour and being on dark substrates may reduce predation likelihood (Sumner, 1934, 1935a, 1935b).

The potential significance of hue and saturation changes in modifying achromatic camouflage cannot be ruled out, but changes in these colour attributes may be by-products of changes in luminance (John et al., 2023; Stevens et al., 2014b) and might even be perceptually small, contributing little to overall achromatic concealment (Stevens et al., 2014b). It has been found that transitioning from dark to white colour was accompanied by an increase in saturation (Kang et al., 2016). However, how different types of chromatophores operate concurrently is not well known (Bagnara & Hadley, 1973), and histological studies are required to describe chromatophore configuration during colour change.

In all chromatic experiments, there were statistically significant changes in chromatic contrasts (i.e., colour JNDs) for some populations. By closely looking at the data, those modifications were mainly accompanied by significant changes in saturation. In other animals, such as the Japanese tree frog (*Hyla japonica*), saturation was shown to be more effective than hue for achieving background matching (Choi & Jang, 2014; Kang et al., 2016). In contrast, hue changes were not influential in improving chromatic camouflage. For example, in the green experiment, Jeddah population exhibited significant directional hue shifts (Figure 3.10B), but these shifts had a negative effect and reduced camouflage (Figure 3.10D). However, determining why hue and saturation remain operational is challenging, given their negligible significant contribution to concealment. They may have persisted as vestiges when selection pressures were alleviated or absent (Rayner et al., 2022), or equally valid, they are novel responses under positive selection. Alternatively, altering colour simultaneously to match multiple colour attributes in the visual environment (e.g., brightness,

hue, and saturation) is physiologically constrained (Choi & Jang, 2014). Although changes in chromatic experiments were complicated, and some may appear to be malfunctioning, they may be adaptive in another context (Norris & Lowe, 1964; Waring, 1963), for example, inducing shifting to morphological colour change. Nonetheless, all individuals had high luminance JNDs which were achromatically visible in the chromatic experiments (Figures 3.11A–C), despite some achromatic modifications exhibited by the marine populations. The ineffective colour change could probably have been caused as a result of the fish gathering more visual information from the walls of the photography containers rather than the basal substrates, which might have been perceived as darker. As a result, their colour responsiveness might have been altered, which may also explain why the fish became darker than the black backgrounds in the achromatic experiments (Figure 3.4A). However, the sensory limitations and the use of photoreceptors to estimate colour and luminance differences are not known in the Arabian killifish. Studies on these aspects are lacking in many animals, which need more investigation using behavioural and neurophysiological approaches (Bullough et al., 2023).

While changes in hue and saturation of chromatophores may impact the receiver's perception of colours, their physical mechanisms are more challenging to comprehend than changes in luminance, which the latter are primarily attributable to melanosome dispersion and aggregation. The findings suggest that the luminance response to backgrounds is the primary change within visual environments mediated by physiological colour change, whereas chromatic responses involving independent hue and saturation changes may require an extended period and adjustments mediated by morphological colour change. In many animals, including fish species, brightness changes were reported to be easier than the actual colour changes (e.g., da Silva et al., 2020; Whiteley et al., 2011), which appeared to be a primary response for camouflage that may impede predator detection (Kang et al., 2016). Indeed, many animals rely on achromatic information to recognise small and moving elements (Osorio et al., 1999), and hue change may be less critical than brightness change for successful camouflage (Stuart-Fox et al., 2006). The Arabian killifish is highly mobile; hence, given its high mobility and small size, rapid achromatic responses may be more advantageous for impeding visual detection.

I found no effects of size on colour change in most treatments. In some treatments, when the fish length was added as a covariate in the luminance and luminance JND models in the white, beige and green experiments, and in the hue models in the beige and the brown

experiments in addition to the saturation model in the white experiments, I found significant correlations between those metrics and body length, indicating that, generally, larger individuals had darker appearances, higher hue and higher saturation values than the smaller ones, and hence were more detectable. I further included fish length as an interaction term, but no evidence for the effects of length on colour change was observed (see Appendix 2), although intrapopulation differences in effects of length on some colour changes were significant (Table S3.2). Colour change strengthens the defensive abilities of generally small fish species that are vulnerable to predators (Cox et al., 2009), and juvenile fish may also benefit from this plasticity (Skold et al., 2006). Because the Arabian killifish individuals occupy shallow water, I expect colour plasticity to persist throughout all life stages, unlike species, for example, that use colour change for mimicry, where only small individuals display colour change (Cheney et al., 2008). Changing colour is advantageous for increasing fitness, especially in ephemeral freshwater streams, so that they can reach maturity and mate before the drought season. It is unclear why the observed effects of size on colour change are inconsistent within and between populations (Table S3.2), but such differences may be partially attributable to physiological conditions associated with growth (Park et al., 2023).

For animals that rely on colour adjustments for concealment, selection should reduce within-population variation in colour change. Therefore, it is less likely to observe intrapopulation differences in colour change between sexes in animals with uniform body colouration unless trade-offs from other alternative behavioural strategies and differential physiological costs (Alfakih et al., 2022) or stress are involved (Gibson et al., 2009). For example, male colouration generally employed in sociosexual behaviours can be rapidly unexpressed when predators are present (Kodric-Brown, 1983, 1998). In animals that use colour shifts preliminary for temperature control, one study on the fiddler crab (*Uca pugilator*) found differences between males in the magnitude of change response among different populations (Munguia et al., 2013). The reported differences might be partly due to the male pattern differences, which may be sexually signalled to females (Dollion et al., 2020) and hence may differ between populations, affecting how each population changes colour. Such differences in colour change are apparent among species that use colour change primarily for signalling (Horr et al., 2023). However, it has been argued that males have an increased capacity for colour change in many fish families than females, which is used for inter- and intra-sexual signalling (Kodrci-Brown, 1998). In this study, I found no such differences between males and females in the ability to change luminance, hue and saturation within or

among populations when sex was included in the initial full models in all experiments, and also, sex did not affect the initial colour in the covariate model meaning that all sexes had similar initial colouration as reported earlier. This might imply that colour change has evolved for camouflage, since both sexes use it similarly for crypsis and has been co-opted for communication (Stuart-Fox & Moussalli, 2008). Preliminary observations on the study species indicated that males darken their bodies for territorial behaviours and undergo ephemeral chromatic colour change during nuptial displays; therefore, the differences between sexes may be context-dependent and transient.

Conclusion

In this study, I provided the first evidence of the geographical variation in colour plasticity among populations of a non-migratory euryhaline species of fish, *A. dispar*, residing in two extreme ecosystems that differ in the visual backgrounds and predator diversity. Despite genetic similarity between the investigated populations (Chapter 2), especially between the marine Jeddah and freshwater Nawan, a substantial variation in colour plasticity, particularly in chromatic responses, was observed. Counter to my first prediction, the freshwater population of Nawan, which inhabited more varied substrates, showed less ability to change colour compared to the marine populations in Gizan and Jeddah, which inhabited more homogenous environments. The second prediction, pertaining to the effects of abundance and diversity of visual predators on colour change, may be more relevant to the observed results in this chapter, but this needs to be confirmed in future. This variation in response suggests that the diversity of predators, as earlier stated, may exert greater selective pressure on colour plasticity than the heterogeneity of visual habitats, as the marine populations with more homogeneous habitats exhibited greater colour plasticity. Visual characteristics may also be crucial in shaping the capacity for colour change. As mentioned earlier, Saudi Arabia's freshwater streams are generally more visually diverse than coastal areas, exhibiting numerous achromatic and chromatic patches at fine-scale levels. However, the habitats of the northernmost freshwater population Khaybar were springs among basaltic volcanic rocks and uniformly dark brown due to irrigation and arboreal activities, which may have contributed to the lower white response compared to the southern freshwater counterpart. Due to a shortage of ecological data, it is challenging to disentangle the interaction effects of predator diversity and habitat heterogeneity on interpopulation variation in physiological colour change.

I recommend that future research on physiological colour change consider (1) longitudinal studies on colour change in comparison with temporal habitat changes, which are almost nonexistent in the literature; (2) removing the differences in colouration between populations by enabling animals to undergo morphological colour change for extended periods in uniform settings (e.g., intermediate visual habitat); or (3) using laboratory-bred individuals under uniform conditions.

Chapter 4: Multiple background types attenuate the need for colour change in the Arabian killifish (*Aphaniops dispar*) from freshwater and marine ecosystems

Abstract

Animals use their environments through movement and habitat selection for a variety of reasons, including avoiding unwanted observers in order to maximise survival and, thus, fitness. Animals can avoid being seen by simply choosing appropriate patches in their habitats that match or approximate their appearances; however, this strategy has drawbacks in that it may restrict animals to specific types of backgrounds and limit their opportunity to exploit their environments. To overcome this, animals may have evolved the ability to change colour, but this has limitations and costs. However, in many species, the phenotype-environment match is most likely a result of a combination of selecting a suitable habitat and effectively changing colour. Nevertheless, the interaction between colour plasticity and habitat selection, on the other hand, has received little attention and appears more intricate than expected, and is affected by multiple factors. Here, I investigate the interaction between colour change and behavioural choice of multiple achromatic substrates (black, neutral intermediate grey and white) in the Arabian killifish from freshwater and marine populations and examine how killifish from different ecosystems integrate these two strategies. I found that fish from both populations use colour change and background preference similarly, where they mostly chose black backgrounds over the other background types, which might have attenuated the killifish need to change colour.

Introduction

Animals utilise their environments through movement and selection of habitats that suit their needs to maximise fitness at various scales (Crist et al., 1992; Mayor et al., 2009), including for reproduction (Hendry et al., 2001), feeding (Werner & Hall, 1988), and hiding from unwanted observers (Kang et al., 2012). Camouflage is a common strategy in the animal kingdom, and it is critical to animal survival and directly affects fitness (Stevens & Merilaita, 2011). Among a host of different strategies, one way that camouflage can be achieved is by background matching, where the general appearance, including brightness, colour and patterns of animals, resembles the background against which they are viewed (Merilaita & Lind, 2005; Ruxton et al., 2004). Background matching has been well documented in many phyla, particularly in animals with fixed morphological appearances that can achieve background matching by selecting appropriate patches in their habitats (e.g., Kang et al., 2012; Lovell et al., 2013). This strategy, however, is not without its flaws, as it may restrict animals to particular types of backgrounds and thus affect the overall exploitation of their habitats (opportunity cost) (Ruxton et al., 2004). Additionally, it may render animals conspicuous during motility between backgrounds as the visual properties of habitats vary spatially or temporarily, affecting the putative optimal phenotype–environment match of animals and potentially affecting fitness.

In addition to habitat selection, animals have evolved different ways to increase phenotype–environment match (Cott, 1940; Stevens & Merilaita, 2011), including possession of compromised appearances (Briolat et al., 2021; Merilaita et al., 2001), polymorphism (Duarte & Flores, 2017) and colour plasticity (Caro et al., 2016; Duarte et al., 2017; Umbers et al., 2014). However, the ability of changing colour is subject to conflicting selection pressures (Kang et al., 2016; Stuart-Fox & Moussalli, 2009), and therefore, it is not unlimited and poses several costs. In many species, the phenotype–environment match is likely an interplay between habitat choice and colour plasticity (Boyle & Start, 2020), as employing the two strategies in tandem may act to overcome the shortfalls of both (Smithers et al., 2018). While it may be obvious how colour change acts to enhance phenotype–environment similarity, the effects of habitats on colour-changing animals, in turn, is not yet clear. Generally, animals are expected to select (1) complex (Kjernsmo & Merilaita, 2012; Merilaita, 2003) and/or matching habitats (Edelaar et al., 2008) to lower their need for colour change (Green et al., 2019; Polo-Cavia & Gomez-Mestre, 2017; Rodgers et al., 2013), (2) substrates that accommodate their ability to change colour if they cannot keep up with the

rate of the environmental changes (Polo-Cavia & Gomez-Mestre, 2017; Smithers et al., 2018) and (3) display colour or patterns that generally approximate multiple substrates (Hanlon et al., 1999). Nevertheless, the interaction between colour plasticity and habitat selection is more intricate than expected and is affected by multiple factors, for instance, body size (Green et al., 2019), nutrition (Gilby et al., 2015), and morphological constraints (Tyrie et al., 2015).

There is a generally negative correlation between colour change and habitat preference, with animals that have slow colour-change ability showing stronger microhabitat preferences than animals that can rapidly change colour (Stevens & Ruxton, 2019), but this is not always the case (see Alfakih et al., 2022). However, while studying habitat preference is a classic topic in behavioural ecology, research on colour-changing animals has received minimal attention, and there are limited studies investigating the two strategies combined in one taxon (e.g., Green et al., 2019). Only a few studies have attempted to experimentally relate colour change and habitat preference in rapidly colour-changing animals (Magellan & Swartz, 2013; Smithers et al., 2018; Tyrie et al., 2015), and it has proven difficult to find research quantifying the colour during substrate preference experiments (with the exception of Allen et al., 2010; Nakajima et al., 2022). Moreover, as colour change may vary geographically (Cadena et al., 2017), habitat choice may vary accordingly, but rarely has work been done on different populations of a single species in relation to colour change and habitat preference (e.g., Spadavecchia et al., 2023).

By inhabiting mostly shallow water in hypersaline lagoons or freshwater streams (Bonzi et al., 2021), the Arabian killifish (*Aphaniops dispar*) from different populations may face challenges from the continual changes of abiotic factors, as well as multiple threats from aquatic and terrestrial visually guided predators (Coad 2010, Freyhof et al., 2020). The Arabian killifish displays rapid colour plasticity (Chapter 3), which may facilitate camouflage in changing environments. Here, I ask, how does colour change interact with substrate choice in the Arabian killifish? and does the interaction between colour change and substrate choice differ among freshwater and marine populations?

Methods

Study species and collection sites

For this experiment, I used mixed-sexed wild-caught individuals of the Arabian killifish *A. dispar* collected in June 2022 from two locations. The marine individuals were collected from Jeddah (21°29'24.9"N 39°10'58.9"E) in the west of Saudi Arabia, and the freshwater individuals were collected from Nawan (19°39'25.1"N 41°16'57.7"E) in the south of Saudi Arabia; these habitats are ~370 km from each other with no connection between them (Figure 2.1). The sampling locations were selected based on the ecosystem types (freshwater and marine) and the latitudinal distance between populations to represent the extreme allopatry of this species. The salinity differs greatly between the two locations; in Jeddah, it was around 35 parts per thousand (ppt), and in Nawan, it was almost 0 ppt. The visual backgrounds also differ between these ecosystems, where Jeddah was primarily composed of large dark rocks with crevices and greyish gravelled and light patches with some algae (Figure S3.1). Nawan was more visually heterogeneous and consisted of brown to dark grey sand, brown and green algae, reed plants, and gravel with varying brightness (Figure S3.2). The marine site is likely to have a greater predator diversity than the freshwater site, including crustaceans, fish, and birds (Chapter 1; Figure 1.2). In the freshwater habitat, some birds and snakes, such as the Arabian cobra (*Naja arabicus*) (Figure 1.2) and the Wadi racer (*Platyceps rhodorachis*), which frequently observed feeding on fishes and amphibians (personal observations; Egan, 2007). Also, invertebrates such as water beetles were seen hunting *A. dispar* (personal observations) (Chapter 1; Figure 1.2).

Fish collection

A total of 80 fish samples ($n = 40$ fish per site) were collected using cuboid-shaped nylon traps (L 54 × W 25 × H 25 cm) to avoid any harm or damage to them (Figure S3.3A). This was done by placing bread bait inside the traps and submerging them in shallow water at random places for a few minutes. Once caught, the fish were placed in 12 L plastic buckets containing water from their collection site to minimise stress and supplied with oxygen using portable air pumps. Fish from each population were housed indoors in two tanks (L 50 × W 30 × H 40 cm: 20 fish/tank) (Figure S3.3B). The salinity was maintained for each population by housing individuals from the freshwater population directly in freshwater, while the marine fish were placed in saline water at ~35 ppt by mixing fresh water with Red Sea salt mix (Red Sea). Since the Arabian killifish is eurythermal (Coad, 2010; Haas, 1982), the temperature

was maintained at ~28 °C using tank heaters, within the recorded water temperature from both ecosystem types the killifish inhabits in Saudi Arabia (Bonzi et al., 2021; Schunter et al., 2021). Each tank was under a 12 h:12 h light:dark cycle and supplied with a tank pump with a filter to circulate and oxygenate the water, and fish were fed twice daily with commercial flake food (Siso Bio-tech CO., LTD). Fish were acclimated to the husbandry tanks under the above conditions for at least 24 hours before being used in the experiments.

Ethical note

The collection and the study were carried out with permission from the Saudi National Center for Wildlife (2020) before the study's commencement. The collection locations are public; therefore, no licences or further permits were required to enter those locations. After completing the experiments, all fish were returned to their original sites. Notably, the Arabian killifish is neither endangered nor protected (Freyhof, 2014).

Background creation and experimental design

The aim of this experiment was to determine whether individuals from the marine and the freshwater populations chose between different achromatic substrates (black, neutral intermediate grey and white) and how behavioural choices interacted with their ability of luminance change (i.e., perceived brightness). I first created two identical custom-made V-shaped chambers made of glass and sealed with silicone. The V-shaped chambers were designed to contain an intermediate grey starting substrate at the base, and a choice arena on each side of the chambers that was either counteractive white or black. The dimensions of each of the choice arenas were L 11 × W 9 × H 10 cm each (Figure 4.1). The creation of the achromatic backgrounds was the same as in Chapter 3 (Figure S3.4); the backgrounds were made of waterproof paper (Xerox Premium NeverTear Paper; Antalis, London, UK). I used the Xerox white paper for the white backgrounds and measured the mean reflectance of the paper, which was 85.5%. Black backgrounds were generated in Inkscape version 0.92.3 (<https://inkscape.org>) by specifying the RGB values to 0:0:0 (Smithers et al., 2017). The black backgrounds were printed with an HP Color LaserJet M254dw printer 600 dpi (Hewlett-Packard, USA), and the mean reflectance of the paper was measured which was 5.9%.

Therefore, the final intermediate grey with an intermediate reflectance relative to the average reflectance of the black and white backgrounds was created using the same method in Chapter 3. A grid of 100 greyscale gradients was created using Adobe Photoshop 2019,

resulting in RGB values ranging from 0 to 255 (Figure S3.4). The reflectance of each block was measured, and the intermediate grey substrate was obtained, which reflected 45.7% with an RGB value of 151 (Figure S3.4). The intermediate grey backgrounds were necessary to acclimate the fish before conducting the experiments and to ensure they all started from approximately the same point. To eliminate the mirror effect, which might influence fish decision behaviours (Cattelan et al., 2017), the final printed sheets were attached to the internal walls of the V-shaped compartments.

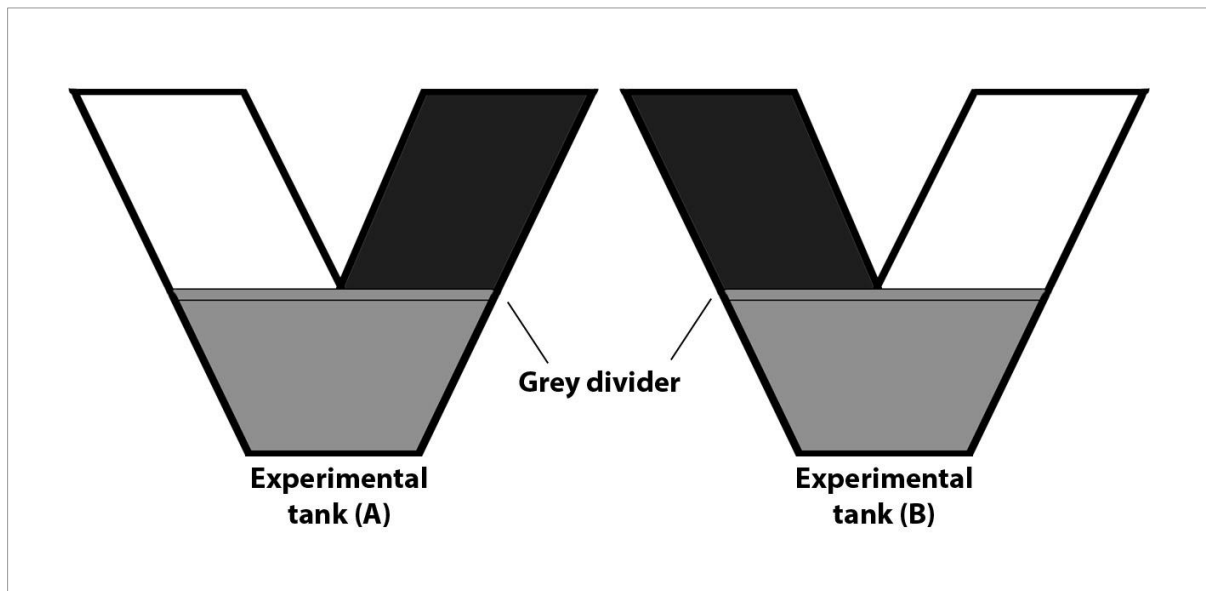


Figure 4.1: Schematic representation of the design of the experimental tanks showing the intermediate grey starting arenas, black arms, white arms and grey dividers.

Experimental procedure

In this experiment, I randomly used 26 mixed-sex fish from each population. Each fish was randomly assigned to one of the two V-shaped choice chambers in an alternate configuration (Figure 4.1), resulting in two groups of 13 fish assigned to each chamber. Fish were placed and kept for 10 minutes in an oxygen-supplied container (L 12 × W 12 × H 10 cm), which had a background with the same intermediate grey colour as the subsequent starting intermediate grey background. I did not supply the V-shaped compartments directly with oxygen pumps to eliminate any bias in choice due to aerobic differences; instead, the compartments contained oxygenated water to a depth of 50 mm that was salinity-matched to their original locations (0 ppt for the freshwater and 35 ppt for marine populations), and had a temperature of 28C° that matched the husbandry conditions. After being acclimated for 10 minutes, individuals were transferred to a photography container (7 × 7 × 7 cm) and

photographed on an intermediate grey background and then transferred to the starting intermediate grey backgrounds in the V-shaped tanks in the presence of grey dividers for 1 min (Figure 4.1).

I then started the video recording and immediately removed the grey divider, giving access to the choice arms. The video recording lasted for ~10 to ~11 minutes. The first 10 minutes were used for the video analysis. This duration is sufficient as the fish are able to change luminance within 1-8 minutes (Chapter 3). At the end of the experiments, individuals were quickly and gently transferred to the photography container and photographed again on the intermediate background to see whether they changed their luminance in response to any of the achromatic arenas. Transferring fish to and from the experimental compartments was performed using a small, transparent plastic tray immersed in water; this method proved efficient in reducing stress because fish did not exhibit flight (escape) response to this handling. Experimental tanks were washed after recording each focal fish to avoid olfactory cues being transferred to subsequent test individuals, and the tested fish were returned to the acclimating containers and then another husbandry tank and provided with food.

Photography

Photographs of the fish were captured in a photography room with a Nikon D7000 digital camera that had been converted to capture images in the visible and ultraviolet (UV) spectra (Advanced Camera Services, Norfolk, U.K.), and was equipped with a Nikon 105 mm f/2.8G Micro-Nikkor lens and approximately placed ~95 cm above the photography containers utilising a tripod (Figure S3.6). Due to the high mobility of the fish, it was challenging to take photographs in the UV range, so only images in the visible spectrum 420–680 nm were taken using a Baader UV/IR-Cut/L filter (Baader Planetarium, Mammendorf, Germany). All photographs were captured in raw format (.NEF) with fixed exposure time (1/20 sec), ISO (400) and aperture (F3) under EyeColor Arc MT70D lamp (Iwasaki Electric Co., LTD). The light was diffused using a custom-built tent made with PVC pipes L 110 × W 80 × H 125 cm and covered with white PEVA covers from the sides as well as with a black-matte foam board over the tent to prevent specular reflection (Figure S3.6). Each image had a known scale bar of 3 cm along with 4.88% and 41.73% reflectance standards (ColorChecker Passport MSSCPP-B: X-Rite) to prevent any inconsistencies in lighting conditions, which would lead to deficient colour quantification.

Video recordings and analysis

The videos were recorded under the previously described lighting conditions in AVCHD format using a Sony HDR CX405 camcorder using the default settings with the highest resolution. Since the videos are not used for colour quantification, I placed a 180 μm clear plastic sheet above each experimental compartment to prevent fish from jumping out of the compartment while unmonitored. Analyses of the video recordings were conducted using a custom-written stopwatch to time the duration spent in each background in seconds and allow exporting the outputs in CSV format (<https://github.com/AteahAlfakih/Stopwatch>). From the 10 min of each video file, I analysed the total duration spent on each background. The fish was considered to have situated on a specific substrate if half of its body (anterior part) was found on that substrate (Smithers et al., 2018).

Image analysis

The main aim of this study was to examine the interaction between the selection of the achromatic backgrounds with achromatic colour change. Analyses of luminance change (i.e., perceived brightness) and luminance camouflage were conducted using non-invasive approaches on ImageJ software (Schneider et al., 2012) using a Multispectral Image Calibration and Analysis Toolbox (MICA) (Troscianko & Stevens, 2015). This tool linearised and standardised the raw non-linear images of the fish and their backgrounds with regard to the 4.88% and 41.73% grey standards, which encompassed reflectance information of the visible RGB channels. The linearisation of the photographs is to control for any fluctuations in light conditions resulting from the camera responses to obtain objective measurements (Stevens et al., 2007; Troscianko & Stevens, 2015). The dorsal views of individuals, from the snout tip to the base of the caudal fin (excluding the pectoral fin and the dorsal fin when it was visible), were chosen as regions of interest (ROIs). For each image, I randomly selected $\sim 1\text{ cm}^2$ of the photographed grey backgrounds as an ROI, and I imported ROIs from separate black and white backgrounds photographed for the experiments in Chapter 3 under the same photography conditions (see Chapter 3). The collected individuals had an average length of the fish was $4.3\text{ cm} \pm 0.7$ (standard deviation), measured from the tip of the snout to the base of the caudal fin using ImageJ.

Luminance and luminance camouflage were analysed from a predator visual model. Despite the lack of data concerning the key predators of *A. dispar* (but see for fish: Coad, 2010; birds: Freyhof et al., 2020) and their visual systems, this fish and cyprinodontiformes in general are likely to be important prey to birds in both ecosystems (Silva et al., 2019). Thus,

the calibrated photos of fish were mapped from the camera spectral sensitivity onto the trichromatic version of the peafowl's (*Pavo cristatus*) spectral sensitivity (Hart, 2002) in MICA (Troscianko & Stevens, 2015), which has been frequently used as a model of avian vision (e.g., Barnett et al., 2018; Kang et al., 2015; Nokelainen et al., 2020).

The changes in luminance were directly analysed from the double cone catch values of the peafowl model. Luminance camouflage was analysed against the intermediate grey backgrounds in addition to the white and black backgrounds imported from the experiments in Chapter 3. As perceived by the avian predator model, the achromatic camouflage is expressed as Just Noticeable Differences (JNDs), via the modified version (Siddiqi et al., 2004) of Vorobyev and Osorio's (1998) receptor noise model. I used a Weber fraction of 0.2 from data from Olsson et al. (2018). Based on the discrimination model described above, the obtained digits of luminance JNDs specified whether the luminance of two objects was perceptible to the receiver model. Generally, a JND value of < 1 indicates that the two stimuli are undetected by the receiver vision and can be discriminated if JND values are between 1 to 3 in optimal viewing circumstances. Increment in JNDs over 3 shows that the two objects' luminance become more distinguishable (Siddiqi et al., 2004).

Statistical analyses

In this experiment, I used 26 fish from each population, but I excluded all the fish that tried to escape and showed aerial jumping behaviour from the final analyses, resulting in ($n = 17$; 8 males and 9 females) and ($n = 14$; 4 males and 10 females) from freshwater and marine populations, respectively. For background choice, I performed a linear mixed model (LMM) of the duration percentage for both populations with the background type and fish population as interacting factor fixed effects, while fish identification was treated as a factor random effect. The final model syntax was as follows: `lmer(DurationPercentage ~ FishPopulation * BackgroundColour + (1 | FishID), data = NawanJeddahHC)`. To test for sex differences in habitat choice, I performed separate LMMs for each population with background type and fish sex as interacting factor-fixed effects while fish identification was treated as a factor-random effect.

For luminance change, I used a paired T-test for analysing luminance before and after the choice trials for each population. For luminance camouflage analyses against each background before and after choice trials, I performed an LMM for each population separately, with background type and test time (before and after choice) as interacting factor-fixed effects and fish identification as a factor-random effect. All statistical analyses

were performed in R version 4.2.3 (R Core Team, 2023). Linear mixed models (LMMs) were utilised via an “lme4” package (Bates et al., 2015), and significant differences were obtained from the package “lmerTest” (Kuznetsova et al., 2017). Following each LMM model, Tukey post hoc pairwise comparisons (Tukey tests) were performed in case significant effects were observed from the models to compare differences in the mean between factor levels utilising the package “lsmeans” (Lenth, 2016).

Results

Background preference

There were significant differences in the amount of the time spent between the coloured backgrounds as indicated by the interaction between the time and the coloured backgrounds, where individuals from both populations significantly exhibited distinct preferences for particular background colours (LMM: $F(2, 87) = 3.520$, $p = 0.0339$). Both freshwater and marine fish showed a pronounced preference for the black over white substrates (Tukey tests: $p < 0.0001$ and $p = 0.0077$, respectively) (Figure 4.2). Moreover, the freshwater population also showed a significant preference for the black over the grey backgrounds (Tukey test: $p < 0.0001$) and no significant preference for the grey over the white backgrounds (Tukey test: $p = 0.4457$). Likewise, the marine population significantly preferred the black background over the grey one (Tukey test: $p = 0.04$) but not for the grey over the white one (Tukey test: $p = 0.9915$). No differences were observed between populations in the time spent on each of the three coloured backgrounds (Tukey test: $p > 0.1$). Preference did not significantly differ among sexes in the freshwater population (LMM: $F(2, 45) = 0.646$, $p = 0.53$) and the marine population (LMM: $F(2, 36) = 0.770$, $p = 0.47$).

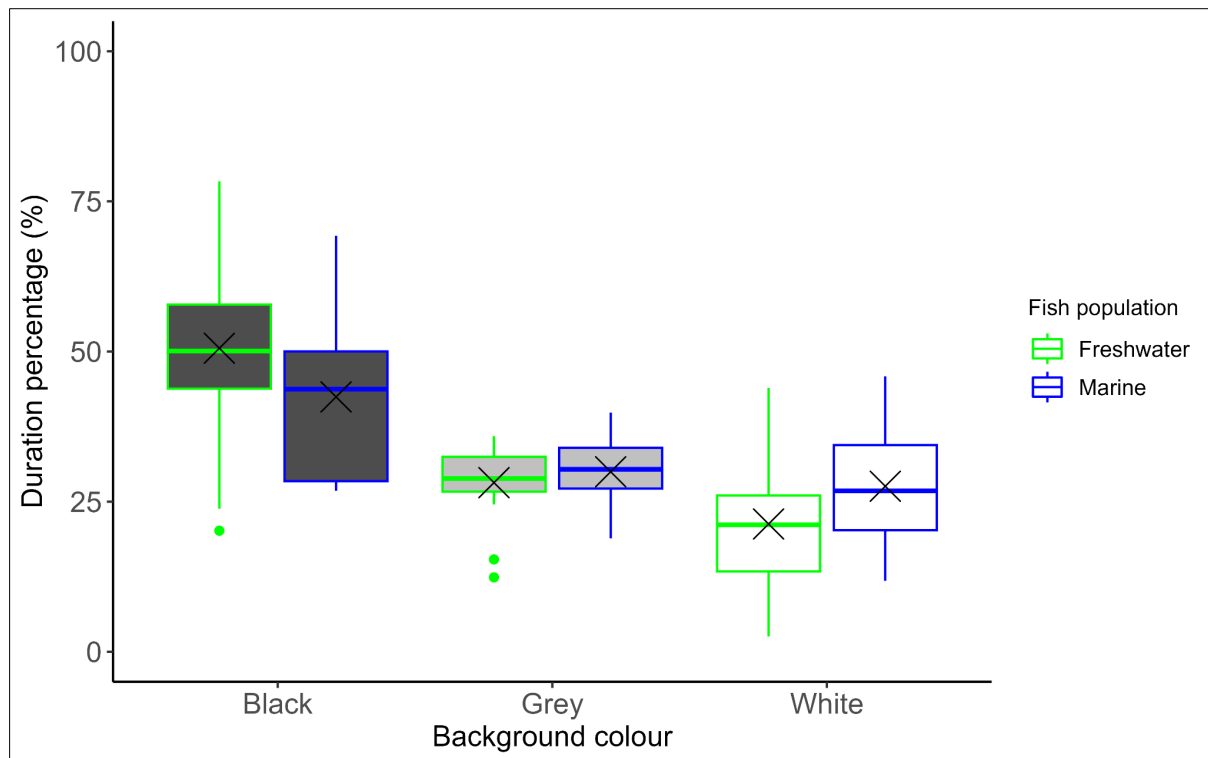


Figure 4.2: The proportion of time spent on each background type by the freshwater (green) and marine individuals (blue). The horizontal lines represent the medians, and the black crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The dots represent the outliers.

Luminance change

The fish did not exhibit any significant change in luminance in either the freshwater (T-test: $t(16) = 1.3203$, $p = 0.2053$) or marine populations (T-test: $t(13) = 0.9957$, $p = 0.3376$) (Figure 4.3).

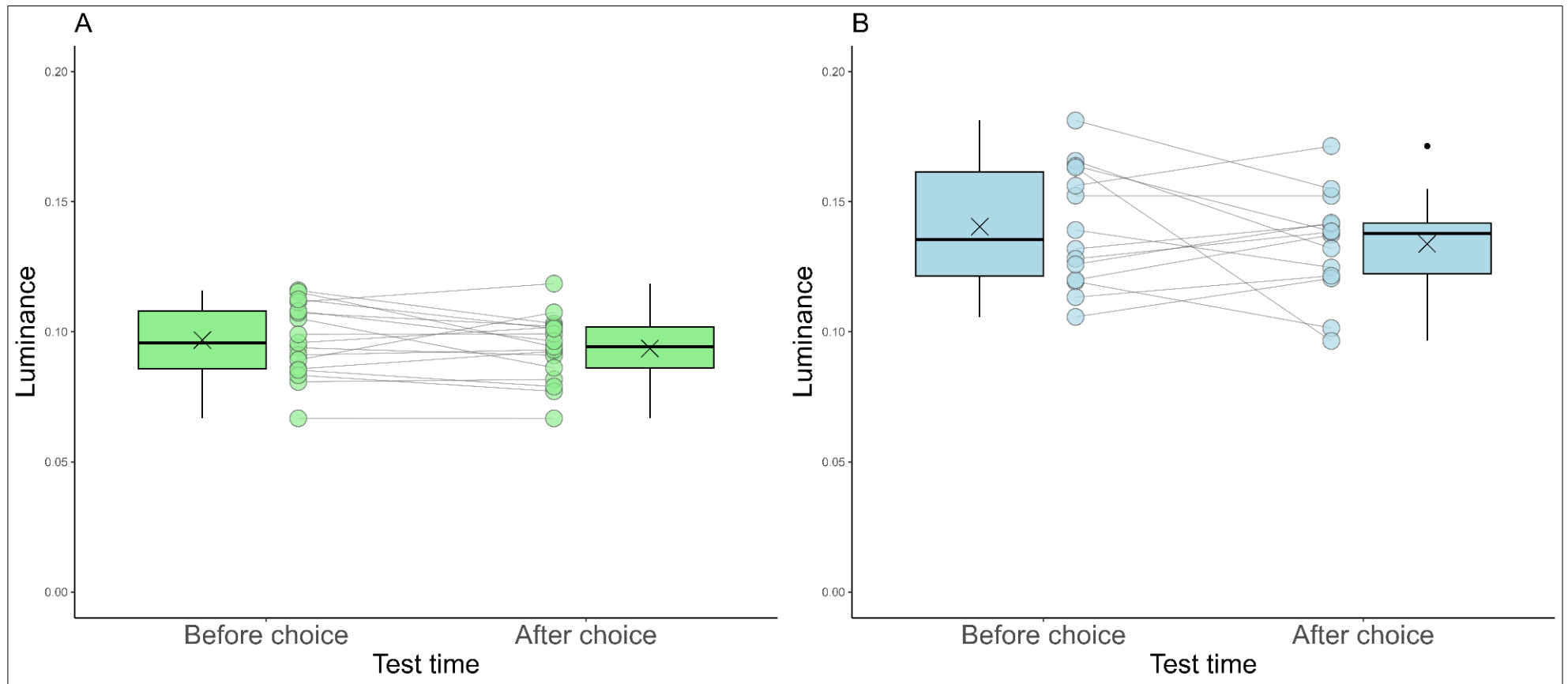


Figure 4.3: Changes in the luminance (perceived brightness) before and after the choice trials in individuals from (A) freshwater and (B) marine populations. The black horizontal lines represent the medians, and the black crosses represent the means. The slopes show the changes in luminance values for each individual (circles) after the choice experiment. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The black dot represents an outlier.

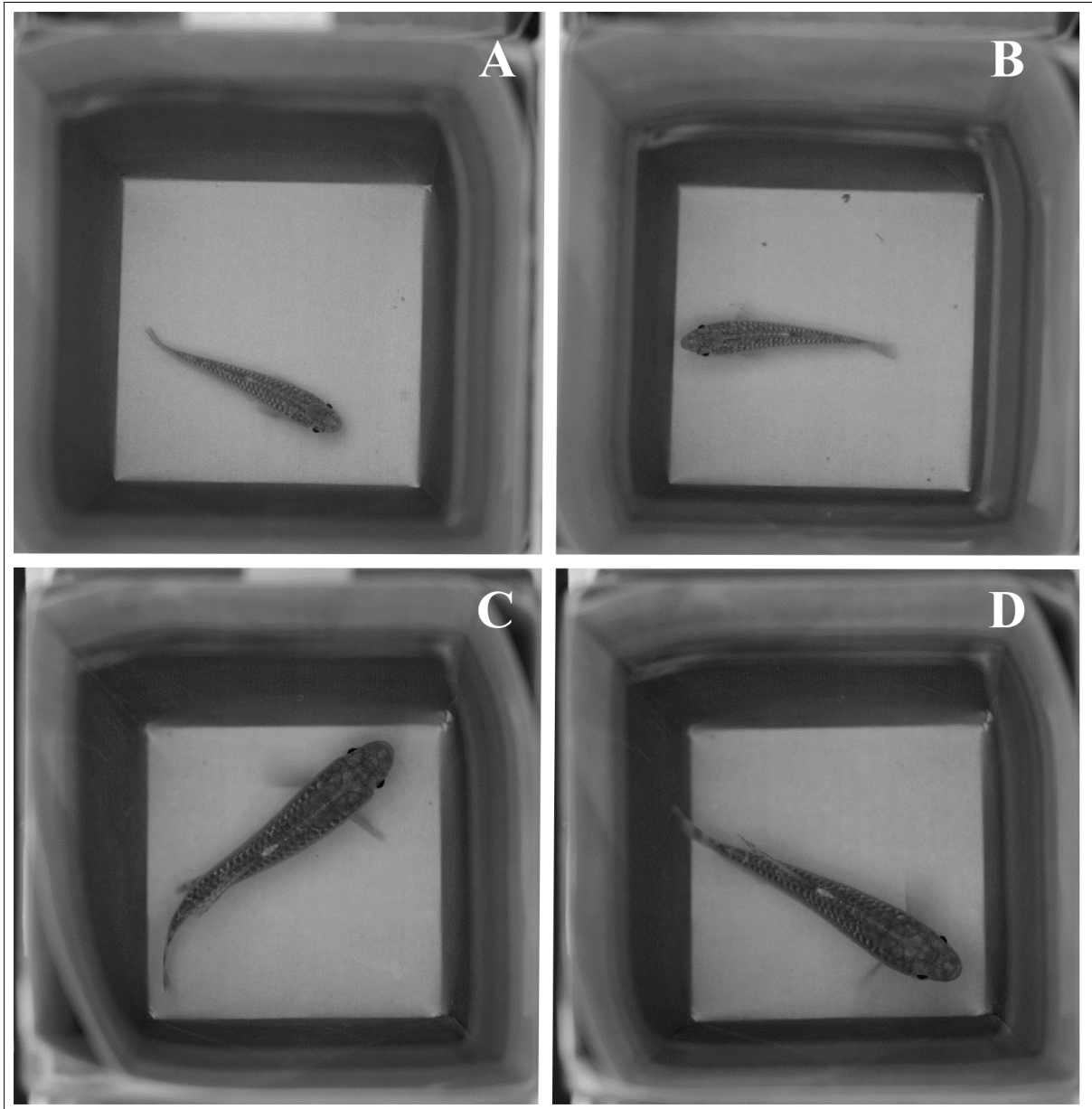


Figure 4.4: Unmodelled (RGB images in grey mode) illustrating the brightness of an Arabian killifish from the marine population Jeddah before (A) and after (B) the choice experiment, as well as an individual from the freshwater population Nawan before (C) and after (D) the choice experiments.

Luminance camouflage

Camouflage did not differ within backgrounds before and after choice trials in freshwater (LMM: $F(2, 96) = 0.596$, $p = 0.5533$) and marine populations (LMM: $F(2, 78) = 0.821$, $p = 0.4440$), and here, I only report camouflage after the choice trials. Camouflage differed significantly between background types in the freshwater population (LMM: $F(2, 96) = 565.154$, $p < 0.0001$) (Figure 4.5A), by which camouflage values before and after the choice

experiment were similar on the grey backgrounds (5.81 and 5.97 JNDs, respectively) and on the black backgrounds (6.15 and 5.99 JNDs, respectively). Higher JNDs values were observed on the white backgrounds (10.9 and 11.06 JNDs, before and after the choice trials respectively). Differences in camouflage were also observed in the marine population (LMM: $F(2, 78) = 255.598$, $p < 0.0001$) (Figure 4.5B), where JNDs values were the lowest on the grey backgrounds (4.24 and 4.47 JNDs, before and after the choice experiments, respectively) followed by the black backgrounds (7.82 and 7.59, before and after the choice experiments, respectively) and higher on the white backgrounds (8.84 and 9.07 JNDs, before and after the choice experiments, respectively).

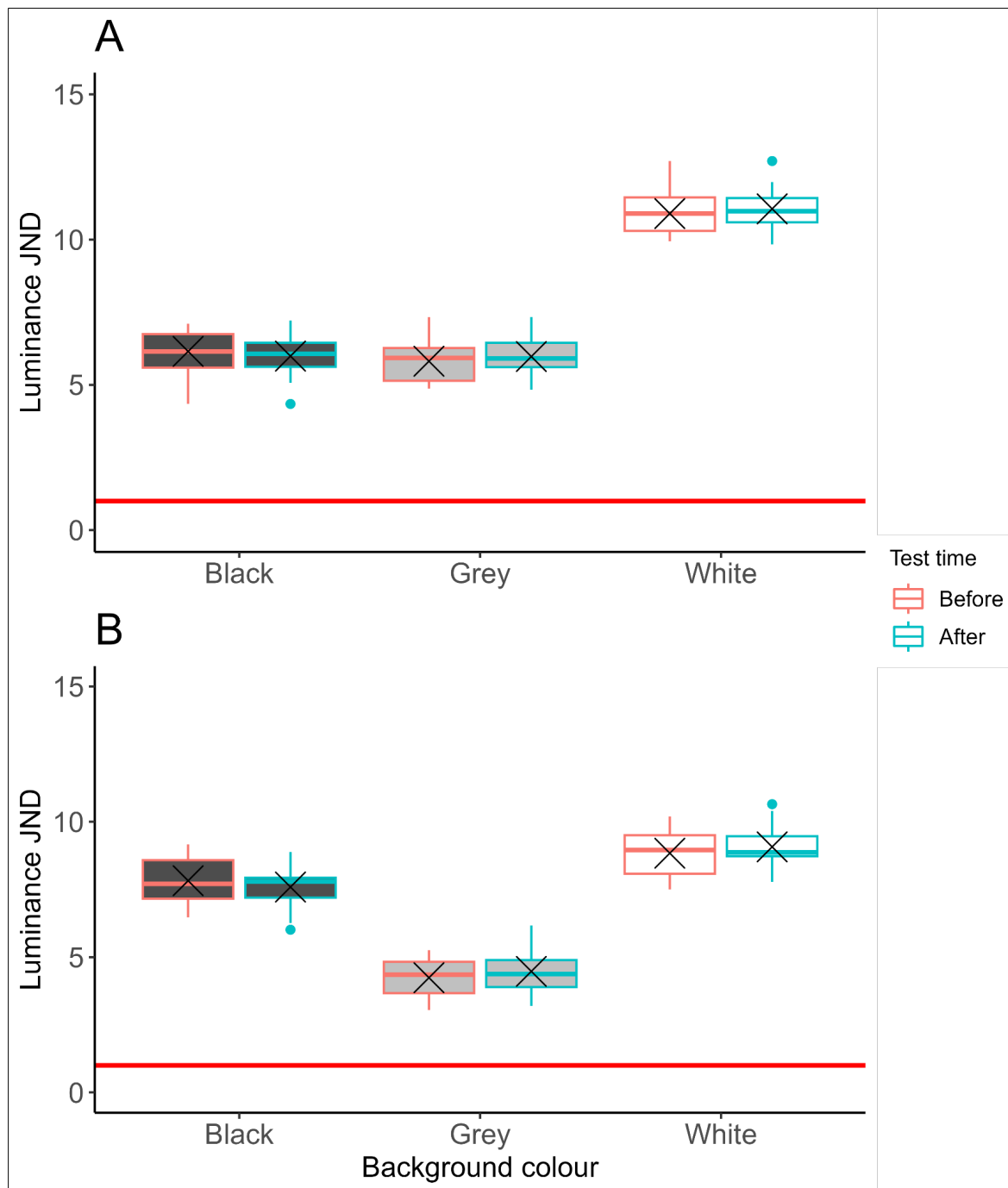


Figure 4.5: Differences between the individuals' camouflage (luminance JNDs) against the black, grey and white backgrounds before (red) and after (blue) the choice trials for (A) the freshwater and (B) marine populations. The horizontal lines in boxes represent the medians, and the black crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The dots represent the outliers. The horizontal solid red lines represent the detection threshold of luminance contrast at 1 JND by the avian model.

Discussion

Behavioural choice and colour change have mostly been studied separately, and research that has studied them together has generally focussed on how reversible changes in animals' body colouration mediate their behavioural preferences of backgrounds. This has been mostly done by acclimating animals to two contrasted backgrounds and running choice trials afterwards (e.g., Rodgers et al., 2013; Ryer et al., 2008; Smithers et al., 2018). Here, I focus on investigating the counter effects, that is, how the availability of different habitats mediates colour change. In this study, I aimed to examine how behavioural choice of substrates interacts with rapid colour change and how killifish from different ecosystems integrate these two strategies. I found that fish from both populations have similar background preferences. They mostly chose black backgrounds, which might have attenuated the killifish need to change colour.

The findings indicated that fish from both populations tend to strongly choose darker backgrounds over light backgrounds, where they spent the least of their time. The ability to change colour can be either costly or limited (reviewed in Alfakih et al., 2022). Hence, animals are expected to choose backgrounds that they can easily match by means of colour change (Smithers et al., 2018). In the previous chapter, I found that fish from both populations were able to change from bright to darker levels more easily within one minute, but the pronounced change toward the brighter levels on white backgrounds required more time (8–15 mins). Therefore, the strong choice of the black background in this fish, and probably many other species, is likely tailored and linked to the ability of the fish to change colour. Explicitly, fish can assess and select the surrounding backgrounds based on the easier direction of their colour change ability. If this is the case, then I predict to see more variation in the choice of substrates at the individual level in response to the morphological (e.g., pigment abundance) and physiological (e.g., energy) states of animals.

White backgrounds were shown in the study species to be more challenging to change to (Chapter 3; Figure 3.6)—probably due to morphologically constrained ability or associated costs of colour change. Here, fish showed significant avoidance of the white backgrounds compared to the neutral grey and the black substrates. This might have occurred because very bright backgrounds are not common in the wild, as shown in rock gobies (*Gobius paganellus*) (Smithers et al., 2018), where they inhabit rock pools that tend to be darker (Smithers et al., 2018; Stevens et al., 2014a), or they may increase detection by visually guided predators. Alternatively, adjusting body colouration to highly bright colouration may require a shift in the colour change mechanism from physiological to morphological colour change, which is a prolonged and time-constrained process (Alfakih et al., 2022). However, when compared to darker backgrounds, the lack of change to white is not surprising because reflectance in natural environments is much lower than the artificial white in the experiment, so it has no ecological significance for fish survival (Smithers et al., 2018).

Although I did not acclimate the fish to the contrasting colours in the experiments (i.e., black and white) and instead used intermediate grey acclimating backgrounds, I do not anticipate that the acclimation within the experimental timeframe will have an influence on the background selection. This is related to the colour-changing mechanism where rapid changes (physiological) are generated by the motility of the chromatophores, and therefore, alteration of colour is expected in any direction. Acclimation had no effect on fish choice in the rapidly colour-changing rock goby, as both groups of fish acclimated for 30 minutes in black and white pre-treatment tanks were shown to spend more than three-quarters of their time on the black background (Smithers et al., 2018). In contrast, guppies (*Poecilia reticulata*) did show a strong preference for black and neutral backgrounds when acclimated for 1–2 weeks to black and white backgrounds respectively (Rodgers et al., 2013), which probably underwent morphological colour change (changes in pigments and chromatophores quantity).

One of the important potential advantages that colour change and habitat choice share in common is that they both confer crypsis to animals in spatially and temporally changing environments. Therefore, operating the two strategies is beneficial (Boyle & Start, 2020) and acts to compensate for each other's inherent shortcomings, and a simple prediction is that animals will change colour during motility between microhabitats (Smithers et al., 2018). However, in real scenarios, this may be highly physiologically costly (Alfakih et al., 2022), and habitat selection may be favoured over plasticity or evolved as a supplementary mechanism to enhance plasticity (Edelaar et al., 2017). Counterintuitively, fish preferences

from both populations were not found to be linked to either their initial or final luminance. Despite the fact that fish mostly preferred black backgrounds, I found no significant changes in fish luminance after trials, and they tended to retain their initial luminance. In contrast to the findings from this chapter, Garcia and Sih (2003) found that the larvae of the salamander (*Ambystoma barbouri*) selected dark backgrounds regardless of their appearance, but they darkened their colour after selecting the black background, implying cryptic benefits.

This raises the question: why did killifish not change luminance to dark when choosing dark backgrounds? and why is the choice for dark backgrounds significant while not conferring high protective benefit (i.e., camouflage)? Generally, choosing dark or grey backgrounds is common in animals (e.g., Garcia & Sih, 2003), including fish species (Bradner & McRobert, 2001; Kjernsmo & Merilaita, 2012; Rodgers et al., 2013; Smithers et al., 2018) where these dark environments are probably more common in nature. Therefore, choosing dark substrates might be an adaptive response related to predation avoidance (Kjernsmo & Merilaita, 2012), by which visual predators may face difficulties in distinguishing dark prey in dark environments. In this chapter, the fish were highly mobile, with freshwater and marine individuals spending an average of ~12.5 s and ~8 seconds on the black backgrounds at any one time, respectively. Thus, alternatively, this non-changing response in colour could be due to the physiological cost of continuously changing, which seems challenging to many species (Alfakih et al., 2022), particularly when energy-depleting tasks such as locomotion are involved. However, it is difficult to acquire a clearer overview of the impacts of movement on colour change because the majority of individuals in both populations were extremely mobile (Appendix 3; Figures S4.1, S4.2). Future research is required to investigate the connection between colour change and movement using a larger sample size.

However, the interaction between colour plasticity and behavioural choice of habitats for crypsis is more complicated and is susceptible to influences from a host of factors (Green et al., 2019). Indeed, changing colour may require other information and cues in order to be effectively initiated, such as increased predation risk or disturbance. For example, studies confirm that the enhanced changes in colour in the three-spined stickleback (*Gasterosteus aculeatus*) (Tibblin et al., 2020) and the strong preferences for matching backgrounds in the least killifish (*Heterandria formosa*) (Kjernsmo & Merilaita, 2012) occurred when animals were faced with predation risks. In the experiments, I did not include a threat or risk treatment, which might influence the colour change in the presence of multiple backgrounds. Therefore, predator cues may potentiate the colour change toward dark levels, coupled with a concurrent strong preference for black substrates. Another factor that balances colour

change and habitat choice is the availability of refuge (Garcia & Sih, 2003) or shelter such as shade, which reduces the need for both strategies.

The possibility that fish changed colour during the trials cannot be ruled out. There was variation between individuals in the direction and extent of colour change. The video recordings were not adequate for colour quantifications, and it is possible that the Arabian killifish underwent a significant colour change during the trials but was not observed at the end of the experiments. A study on sticklebacks found that fish changed colour for 20 minutes in an oscillatory-like trend but eventually retained a similar colour to that at the beginning of the trials (Clarke & Schluter, 2011).

Animals are prone to experience many changes in their habitats during their lifecycle (Boyle & Start, 2020), including changes in the visual complexity within and between patches. Therefore, to determine how a species might effectively use the colour change and behavioural choice of habitats in tandem, studies are needed over multiple time points because heterogeneity may vary temporally within environments. Visual complexity is suggested to impede detection (Kjernsmo & Merilaita, 2012; Merilaita, 2003) and may lower the need to change colour. However, the multiple substrates available in the experimental setup might be the reason why the Arabian killifish from both populations did not significantly change colour in this experiment, and thus, I predict that the change in colour will be triggered by the lack of multiple backgrounds. I acknowledge that the relationship between habitat visual complexity and the use of colour change with behavioural choice can be species-specific. To illustrate, a study on a rapidly colour-changing fish belonging to *Galaxias sp.* found contrasting findings to the findings in this chapter, and rather than selecting a type of background when these fish were given a choice between uniform and complex substrates, they did not show a strong preference for any background (Magellan & Swartz 2013). Individuals' non-changing responses may also be linked to the fish's mobile lifestyle, which may render them generalists in habitat use (Briolat et al., 2021; Duarte et al., 2016), but may also mean they are compromisingly camouflaged depending on the momentary situations in their natural habitats (Hanlon et al., 1999).

The choice of the Arabian killifish for the black backgrounds did not fully impede detection from the avian predator in the experiments. The majority of fish from all populations showed JND values above 4, which means that they were highly detectable on all the types of backgrounds, including the black backgrounds where the fish spent most of their time on. The lack of optimal camouflage in the killifish could have resulted from the improved visual

sensitivity of the avian model at discriminating luminance, whereas luminance might be observed differently by the Arabian killifish. Behavioural preferences may allow the maintenance of dynamic habitat matching in highly mobile species (Green et al., 2019). In real scenarios, in highly mobile species as seen in the study species and other *Aphaniops* congeners (Al-Daham et al., 1977), compromised camouflage in heterogeneous habitats might be effective in impending visual detection and advantageous in maximising exploitation of the habitats and utilising the resources available.

Although the choice of black backgrounds conferred some degrees of improved camouflage to the freshwater fish when compared to white, this does not apply to the marine population, where they were more camouflaged on neutral grey backgrounds. However, fish from all populations were mostly highly detectable on all types of backgrounds, which contrasted with the anticipated benefit of being on dark backgrounds. Although it is hard to explain the occurrence of this pattern of choice in the lack of crypsis, here are several potential but non-mutually exclusive explanations. (1) The choice of the black substrates may be fixed at the species level (Stevens & Ruxton, 2019) and, within a real biological context, would confer protection, taking into account other attributes such as visual acuity of predators, water turbidity and movement. (2) The visual ability of the killifish to discriminate between brightness levels may differ from the avian predators. (3) The early experience of fish in the wild might have contributed to the observed behavioural decision for black backgrounds, and there is evidence that experiencing morphotype-matched conspecifics affects shoaling decisions (Gómez-Laplaza, 2009).

Animals can assess their body colours and the visual properties of their habitats to make decisions about which substrates are suitable for their specific scenario (Garcia & Sih, 2003; Gómez-Laplaza, 2009). Therefore, it is more likely that the killifish chose the black backgrounds based on their ability to change colour more rapidly to black. I uphold the proposal of linking behavioural choice to the ability to colour change (Magellan & Swartz, 2013; Smithers et al., 2018; Tyrie et al., 2015) and emphasise the need for more studies to validate this in a wide range of taxa. However, I take this opportunity to redefine this relationship: regardless of the expressed colours or patterns by means of colour change, animals would select habitats that suit their expected ability of colour change.

Future work should address how shoaling interacts with colour change and the behavioural preference of backgrounds in the Arabian killifish and other socially and dynamically colour-changing taxa. The individuals of the Arabian killifish swim in shoals (Al-Daham et al., 1977),

and this might dispense with the other behavioural choices or plasticity by diluting the risk of predation or increasing the confusion effect to the observer while in motion (Murali et al., 2019). Fish can avoid predation risks by shoaling (Magurran, 1990) and choosing matched over mismatched conspecifics, as in rainbowfish (*Melanotaenia australis*) (Rodgers et al., 2010), angelfish (*Pterophyllum scalare*) (Gómez-Laplaza, 2009) and guppies (Rodger et al., 2013). There is also evidence that fish prefer to shoal with their matched conspecifics on contrasting backgrounds over staying on matching backgrounds (Bradner & McRobert, 2001), and this may occur in colour-changing animals as well.

Crypsis does not always explain the choice of specific microhabitats or substrates. For example, black individuals of the seahorse (*Hippocampus erectus*) showed a strong avoidance of black backgrounds, which may be in favour of enhancing food search over crypsis (Li et al., 2022). Moreover, black newts (*Lissotriton boscai*) were shown to stay on mismatching white gravel when cues of predators were added, which might be inferred as an escape response from predators (Polo-Cavia & Gomez-Mestre, 2017).

I found no evidence of differences in the use of colour change and habitat preference between marine and freshwater populations, despite the fact that they live in ecosystems with vastly different predator diversity, and marine fish may face greater predation risk than freshwater fish (Chapter 1). One possible explanation for the prevalence of those same strategies among populations may be attributed to the habitat use where both populations reside in shallow water with generally low substrate brightness. Therefore, I expect to see pronounced differences in habitat preference coupled with colour change if I compared either population to another population with more diverse substrates, such as those killifish that inhabit coral reefs in the Red Sea (Freyhof et al., 2020). However, natural habitats vary spatially and temporally, and the use of the two strategies (i.e., colour change and habitat choice) might change under environmental changes.

It is well known that animals benefit from the capacity of colour change and the selection of microhabitats to keep up with spatial or temporal changes in their habitats (Caro et al., 2016; Stevens & Ruxton, 2019). However, colour change is suggested to allow animals to colonise novel habitats that differ in visual properties even in the lack of genetic divergence (Whiteley et al., 2009). *A. dispar* and generally other members of the Aphanidiidae are widespread and inhabit different and extreme ecosystems (Bonzi et al., 2021; Schunter et al., 2021). The lack of differences in the observed use of both strategies in the Arabian killifish populations suggests that may be species-specific. I found that these populations are closely related by

mitochondrial genetic distance (Chapter 2). The pattern observed in the use of colour change and habitat preference might have allowed the killifish to occupy multiple habitats and expand their distribution. It is evident that the populations of the Arabian killifish in some areas of Saudi Arabia are geographically clustered in a latitudinal manner (Bonzi et al., 2021; Schunter et al., 2021), meaning that freshwater populations are temporarily connected to marine populations with the facilitation of seasonal flash floods (Bonzi et al., 2021; Schunter et al., 2021) or via egg dispersal by predators (Silva et al., 2019). This pattern of connectivity may be sufficient to sustain gene flow, which in turn may constrain local adaptation to certain habitats (Storfer et al., 1999).

Conclusion

In this chapter, I investigated the interaction between luminance change and behavioural choice of multiple achromatic substrates (black, neutral intermediate grey and white) in the Arabian killifish from freshwater and marine populations and examined how killifish from the different ecosystems integrated these two strategies. I found that fish from both populations used luminance change and background preference similarly, where they mainly selected the black backgrounds over the other background types. Additionally, individuals from both populations showed no significant luminance change after the choice experiments despite being easily detectable by the avian visual model on all the types of backgrounds, including on the most chosen backgrounds (i.e., black). These patterns of nonchanging in luminance and selecting the black backgrounds might indicate that the choice could be species-specific, and the fish selected the black backgrounds because they were easier to match by means of luminance change—compared to more physiologically challenging backgrounds such as white, by which changes in luminance to become lighter can be difficult and take longer time (Chapter 3). Therefore, selecting the black backgrounds, which are easier to match via rapid luminance change (Chapter 3), might have attenuated the killifish need to change their luminance in the experiments of this chapter.

Chapter 5: General Discussion

Summary of the thesis findings

The capacity to change colour appears to vary intricately throughout taxonomic levels (Cadena et al., 2017), where it is susceptible to opposing selection pressures (e.g., sociosexual, natural and thermoregulatory) (Kang et al., 2016; Stuart-Fox, 2014; Stuart-Fox & Moussalli, 2009), which may have led to the observed variation in this capacity among closely related species from different families (e.g., John et al., 2023; Stuart-Fox and Moussalli, 2009). However, the geographical variation in colour change has not been thoroughly investigated, and it has been challenging to establish how this variation has arisen between species. Therefore, it is necessary to investigate the variation in colour change among populations of a particular taxon to shed more light on its evolution. Only recently have studies looked at the interpopulation variation in colour change, and the literature presents a few instances of this (Chapter 3). However, the processes that drive the evolution of this interpopulation variation remain unclear. Furthermore, how any variation in colour change interacts with essential behaviours such as habitat choice is almost nonexistent in the literature.

This thesis aimed to examine the geographical variation in achromatic (luminance) and chromatic (hue and saturation) colour change (Chapter 3) and the related behaviours, particularly habitat choice (Chapter 4). I approached this by studying the euryhaline Arabian killifish (*Aphaniops dispar sensu lato*) from freshwater and marine populations from the Saudi Arabian Red Sea basin. Briefly, *A. dispar* was hypothesised to constitute all the *Aphaniops* populations in and around the Arabian Peninsula. However, recent molecular research disproved this hypothesis and confirmed the existence of nine distinct species within the genus *Aphaniops*, seven of which have been treated mostly as *A. dispar*. Nevertheless, the taxonomic status of the Red Sea populations of *A. dispar* in Saudi Arabia remained unstudied. Therefore, this thesis also sought to ascertain whether marine and freshwater populations of *A. dispar* from the Red Sea basin of Saudi Arabia were the same species or belonged to distinct entities (Chapter 2).

It has been assumed that the marine populations of *A. dispar* display continuous gene flow, whereas landlocked freshwater populations are prone to genetic isolation, potentially representing independent species (Freyhof et al., 2017). Indeed, several populations in

Saudi Arabia have been identified as landlocked (Krupp, 1983). By utilising morphometrics, colour pattern analysis, and molecular methods, in Chapter 2, it was revealed that the freshwater populations were closely related to the marine populations, indicating they are not separate species. However, one of the significant outcomes of this thesis is that it identified two distinct molecular operational taxonomic units (MOTUs). The first MOTU was *dispar* MOTU, which comprised all the closely related freshwater and marine populations from the central-southern and northern Red Sea. The freshwater population from Khaybar is the most isolated in Saudi Arabia from other freshwater and marine populations (Krupp, 1983). It possessed a unique colour pattern, which would be speculated to be my study's most genetically distinct population. Unexpectedly, the Khaybar population was grouped with most populations in the Red Sea basin and displayed low genetic divergence from the northern marine population. Counterintuitively, the southernmost marine population from Gizan was genetically distinct from all other Red Sea basin populations and is referred to as Gizan MOTU. This population potentially represents a new cryptic species because it shares similarities in morphology and colour pattern with other populations of the *dispar* MOTU found in the Red Sea. The thesis treated Gizan MOUT as a population rather than a separate species and named it throughout as *A. cf. dispar*.

In Chapter 3, it was found that freshwater and marine populations of the Arabian killifish *A. dispar* from the *dispar* MOTU and the marine Gizan population *A. cf. dispar* within the Gizan MOTU can change colour rapidly within a few minutes. This is the first documented evidence of this ability to change colour in the Eurasian killifish family Aphaniidae, which contains around 45 species. All the studied populations showed increased ability for achromatic colour change (luminance) in response to the black and white substrates, compared to chromatic colour change in response to the beige, brown and green substrates. It was found that the achromatic change in response to the black backgrounds was faster and more pronounced than to the white backgrounds. There were significant differences among populations in each type of the achromatic backgrounds. Interestingly, the freshwater population from Khaybar displayed almost no change in response to the white backgrounds. The achromatic changes were found to improve the fish's luminance similarities to their respective backgrounds (luminance JND). In contrast to the achromatic backgrounds, all populations exhibited a reduced ability to change colour chromatically (hue and saturation), but marine populations were generally better at changing than freshwater populations. The responses to the chromatic backgrounds were more complex than the responses to the

achromatic backgrounds, whereby each population displayed different responses among the given backgrounds, and some were likely maladaptive responses.

In Chapter 4, it was observed that there were high similarities between the freshwater population, Nawan, and the marine population, Jeddah, within the *dispar* MOTU in terms of their (1) preference for the achromatic backgrounds (black, neutral intermediate grey, and white), (2) achromatic change during the choice experiments, and (3) achromatic camouflage (luminance JND) before and after the choice experiments. Fish from both populations significantly preferred the darker backgrounds by spending most of the time on them and displayed increased avoidance of the white backgrounds. Both populations showed no significant luminance change when directly comparing their luminance before and after the choice experiments. Finally, the analysis of the achromatic camouflage revealed that the fish from both populations were highly detectable (i.e., had high luminance contrast) against all three backgrounds before and after the choice experiments.

Colour change at the population level

The variation in colour change between disconnected populations in terrestrial (Batabyal & Thaker, 2017; Cadena et al., 2017; Choi & Jang, 2014; Munguia et al., 2013; Spadavecchia et al., 2023; Stuart-Fox et al., 2006; Stuart-Fox & Moussalli, 2009; Whiting et al., 2022) and aquatic environments (Pinto et al., 2020; Westley et al., 2013; Whiteley et al., 2009, 2011), gives an impression that no single factor or combination of factors apply to all colour-changing species. To illustrate, some species, such as the central bearded dragon (*Pogona vitticeps*) have evolved a colour-changing ability tailored to the visual properties of their local habitats, which vary from one area to another (e.g., Cadena et al., 2017). However, variation in colour change exists in other species regardless of habitat, as in the brown trout (*Salmo trutta*) (Westley et al., 2013). This incompatibility in the results between the available studies on this topic suggests that the processes that drive the evolution of population variation in colour change vary between and among taxa, and that they are poorly understood.

Colour change in some taxa, such as chameleons, is suggested to have evolved for conspicuous signals under social selective pressures (Stuart-Fox & Moussalli, 2008, 2009). However, within a prey-predator context, it is predicted that populations that experience stronger natural selection for camouflage than others will possess varying capacities to change colour (Stuart-Fox et al., 2006), and variation in habitat visual properties is paralleled

by interpopulation variation in the ability to change colour (Cadena et al., 2017). Additionally, animals inhabit environments where they encounter multiple predators, each possessing unique visual capabilities (Stuart-Fox & Moussalli, 2009). Thus, selection for increased colour change capacity may correspond to the diversity (Whiteley et al., 2011) and or abundance of visually guided predators (Stuart-Fox et al., 2006; Stuart-Fox & Moussalli, 2009).

The results presented in this thesis suggest that constraints (e.g., gene flow) might exist on the evolution of colour change in the studied populations regardless of the differences in factors that could influence it, such as predators and the visual characteristics of the habitat. The thesis findings lend support to Bonzi et al. (2021) and Schunter et al. (2021) on the hydrological connectivity between the freshwater and the marine populations and so contradict the suggestion of landlocked freshwater populations of the Arabian killifish (e.g., Freyhof et al., 2017; Krupp, 1983). The molecular results revealed that the populations in the Red Sea basin were geographically clustered latitudinally (Chapter 2). The maintenance of periodic gene flow between the counterparts from the two aquatic systems can be established with the facilitation of seasonal flash floods (Bonzi et al., 2021; Schunter et al., 2021) or presumably via egg dispersal by predators (Silva et al., 2019).

This connectivity pattern may constrain local adaptation to particular habitats due to the sustained gene flow (Storfer et al., 1999) and favour phenotypic plasticity. The genetic findings (Chapter 2) and the observed variation in the achromatic and chromatic colour change (Chapter 3) are in tandem with the persistence of phenotypic plasticity. The marine population from Jeddah and the freshwater population from Nawan within the *dispar* MOTU showed general similarities in the achromatic response, but they differed significantly in the chromatic response (Chapter 3) despite sharing a low genetic distance and being clustered in the same lineage. These results align with similar studies among populations of other fish species, where variation in colour change was observed despite being genetically connected or inhabiting similar habitats within short distances (Westley et al., 2013; Whiteley et al., 2009). Ultimately, constraints on local adaptation are predicted to be less in strongly disconnected populations (e.g., Cadena et al., 2017; Munguia et al., 2013; Whiting et al., 2022). I found that the freshwater Khaybar within the *dispar* MOTU, which is the most isolated population in the study, showed a clear adaptive response only to the black background. However, the marine Jeddah population is more comparable to the marine population of Gizan in terms of the achromatic and chromatic responses, morphology, and

colour patterns. In the present study, inferring the similarity in colour-changing ability as convergence is challenging due to the suggested connectivity (cf. Jeddah vs. Nawan) and gene flow continuum (Schunter et al., 2021). Instead, proximate factors like learning (Hatamoto & Shingyoji, 2008) or carryover effects due to prolonged exposure to predators potentially influence the chromatic responses (Chapter 3).

Habitat choice at the population level in colour-changing animals

The relationship between phenotype and environment in various species of animals is influenced by habitat preference and colour plasticity (Boyle & Start, 2020). These strategies can complement each other to enhance survival (Smithers et al., 2018). Previous research on colour-changing animals has mainly focused on the impact of the acclimation to a background on the behavioural choice for different given backgrounds. However, except from this thesis and the work of Allen et al. (2010) and Nakajima et al. (2022), the free choice between different backgrounds and its effect on colour change has proven challenging to find elsewhere. Additionally, one area for improvement in studies on habitat preference in colour-changing animals is the lack of measuring colour and camouflage in conjunction with choice trials (Stevens & Ruxton, 2019). This thesis addressed these gaps in knowledge by incorporating the above considerations.

Animals that seek to maximise crypsis in their environments are predicted to select (1) complex (Kjernsmo & Merilaita, 2012; Merilaita, 2003) and/or matching habitats (Edelaar et al., 2008) to reduce their need for colour change (Green et al., 2019; Polo-Cavia & Gomez-Mestre, 2017; Rodgers et al., 2013), (2) substrates that facilitate their capacity for colour change if they cannot adapt to the rapid pace of environmental changes (Polo-Cavia & Gomez-Mestre, 2017; Smithers et al., 2018), and (3) display compromised colour or patterns that approximately match multiple visual habitats (Hanlon et al., 1999). The second prediction was met in this thesis; the Arabian killifish were shown to select dark substrates (Chapter 4) that suited their ability to change colour (Chapter 3), possibly because they could not keep up with the rate of changes due to very contrasting backgrounds (Chapter 4). Low-reflecting substrates, often preferred by different fish species, might impair predator vision, reducing predation potential on fish, as evidenced by some studies on dark substrates (Fairchild & Howell, 2004; Sumner, 1934, 1935a, 1935b).

Not only did the fish select darker backgrounds, but in contrast to prediction three, they exhibited dorsal colours that did not match any of the given achromatic substrates. Therefore, an additional testable prediction was drawn: animals will show conspicuous colour in the absence of reliable signals that induce colour change. The dispensation of being cryptic against all backgrounds, along with the decision to choose darker backgrounds instead of lighter ones, may reduce the potential physiological cost of rapid colour change, specifically pigment aggregation, which could be more expensive than pigment dispersion (Alfakih et al., 2022). Becoming lighter on a white background was observed to be also challenging in different freshwater species from different families of Cyprinodontiformes (Cox et al., 2009), and for many taxa, it might only be pronounced through pigment apoptosis during the shift to morphological colour change (Alfakih et al., 2022; Sugimoto et al., 2005).

As mentioned earlier, there was no significant difference in the background choice of fish from freshwater and marine environments, suggesting a potential species-specific preference for dark backgrounds (Stevens & Ruxton, 2019). This behavioural choice for backgrounds that are easy to match, along with the use of colour change under unfavourable situations (i.e., changing when induced to), might have allowed the Arabian killifish to occupy multiple habitats, so expanding their distribution. Interestingly, among other euryhaline species inhabiting the Red Sea (Freyhof et al., 2020), only *A. dispar* colonised wadis and became a secondary freshwater fish (Krupp, 1983). Colour plasticity was suggested to facilitate colonising novel deglaciated habitats, such as in the coastrange sculpin (*Cottus aleuticus*) (Whiteley et al., 2009; 2011).

Geographical variation and evolution of colour change beyond *A. dispar*

When Nardo in 1827 described the genus *Aphanius* (including *Aphaniops*), there was no mention of the meaning of this generic name. The name *Aphanius* comes from Greek, which means inconspicuous (Esmaeili et al., 2020; Wildekamp, 1993). In this thesis, I provided evidence that the Arabian killifish fish can be visually inconspicuous by means of background matching via rapid colour change. This inconspicuousness may be widespread among Aphaniidae, which might have led Nardo to choose the genus name, *Aphanius*. The Aphaniidae family is well known for adapting to various environmental factors, especially salinity and temperature, which appear to be conserved traits among many species in the family (Freyhof et al., 2020; Kottelat & Freyhof, 2007).

Whether colour change persists among the Aphaniidae members is yet to be determined. To the best of my knowledge, there are only two studies that mentioned the variation in colouration (i.e., brightness, not the patterns on the flanks) among populations of two species, the Mediterranean killifish (*Aphanius fasciatus*) (Cavraro et al., 2021) and the Arabian Gulf killifish (*Aphaniops stoliczkanus*) (Bidaye et al., 2023) by which the variation has been loosely interpreted as an interspecific variation due to habitat differences and not a plastic response to the different habitats (i.e., via colour change). The results presented in this thesis are not in agreement with that interpretation because freshwater and marine populations of *A. dispar* within the *dispar* MOTU and *A. cf. dispar* within the Gizan MOTU do have the ability to change colour. It is possible, therefore, that *A. fasciatus* and *A. stoliczkanus* also possess the ability to change colour, which may be a conserved trait among other Aphaniidae members.

Given the aforementioned, it would, therefore be intriguing to study the evolution of colour change among and within members of the Aphaniidae family. To date, comparative phylogenetic studies to investigate the evolution of colour change seem limited only to Chamaeleonidae (Dollion, 2019; Stuart-Fox & Moussalli, 2008). Several molecular studies on the Aphaniidae have covered most members, especially those that utilised mitochondrial DNA (e.g., Esmaeili et al., 2020). Therefore, a comparative phylogenetic analysis utilising a time-calibrated phylogeny approach would strengthen the study of the evolution of colour change in Aphaniidae.

Limitations

The thesis applied a zigzag sampling pattern to ensure disconnectivity between populations covering a large distance, and it utilised only one mitochondrial DNA marker. Thus, these approaches did not fully clarify the assumption of gene flow effects on colour-changing ability and the determination of a new Red Sea species. Due to time limitations resulting from the COVID-19 pandemic, the thesis selected only four populations; thus, the outcomes may not be generalised and applied to other populations. Future research should use other nuclear markers in a paralleled pattern of sampling, as that of Schunter et al. (2021) from multiple locations of the Red Sea coast and its adjacent wadis, to understand the rule of population connectivity on colour change.

Chapters 3 and 4 and similar studies on colour-changing animals are limited by the absence of ecological data pertaining to both measurements of the visual natural habitats and/or predator accounts, which are extremely challenging to fulfil and require high expenditure on materials and prolonged time (e.g., Smithers et al., 2018; Spadavecchia et al., 2022; Westley et al., 2013). Although the backgrounds used in Chapters 3 and 4 covered a broad range of brightness and multiple hues, they were not precise replicates of those substrates in the Arabian killifish's natural habitats, potentially limiting or altering their responses. Future research may benefit from utilising natural backgrounds to determine how colour change might operate in natural environments.

Future directions

One difficult aspect to study is the effect of predator diversity and richness on colour-changing animals. Such effects have not been adequately addressed and are only marginally acknowledged in the limited research attempting to incorporate this effect (see Magurran et al., 1993; Stuart-Fox & Moussalli, 2009; Whiting et al., 2022). Before the pandemic, my plans had included conducting experiments to measure the physiological cost of colour change. I aimed to study the costs in two directions in both types of colour change ((1) physiological: dispersion versus aggregation, and (2) morphological: pigment production versus apoptosis). These experiments would have provided valuable insights into the energetic effects on colour change, such as the observed between-treatment variation in the white and black backgrounds in Chapter 3. Variation in colour change is not caused only by differences in visual habitats and predators. Physiological and morphological constraints such as energetic costs expended on colour change or limitation in pigments may cause varying colour changes at different levels (Alfakih et al., 2022). Only a few studies have examined the physiological cost of colour change (e.g., Polo-Cavia & Gomez-Mestre 2017; Radovanović et al., 2023), and several questions remained unanswered (reviewed in Alfakih et al., 2022). Due to the time limitation in the thesis, I focused only on physiological colour change, and the variation between populations in the initial colour metrics suggests an involvement of morphological colour change, which might contribute to the instantaneous (i.e., temporary) geographical variation observed among populations. Moreover, the way in which different types of chromatophores operate concurrently is not well known (Bagnara & Hadley, 1973). Therefore, the widely used photography techniques for measuring colour change do not touch on aspects of the histological changes involved in colour change; thus,

histological studies are required to describe any differences in chromatophore configuration during colour change.

To the best of my knowledge, all of the studies conducted so far on interpopulation differences in colour change have been limited to a single period of time. It remains unknown how colour change will be performed over different times (Stevens et al., 2014a). Therefore, it is crucial to prioritise the study of colour change dynamics and seasonality in future research. Colour change has been extensively studied in terms of its mechanisms, triggers and functions, but such widespread and accessible plasticity that can be easily studied remains poorly understood. Most studies on colour change have focused on a single population, which has left a gap in our knowledge pertaining to the evolution of colour change. Thus, examining colour change variation across populations of a taxon can shed light on how it evolves. This thesis attempted to expand the knowledge on the geographical variation in colour change and its influence on habitat preference, and throughout it identified several areas to address gaps in knowledge. The geographical variation in colour change can be investigated by utilising unified, non-invasive approaches such as digital photography. This will allow for the easy expansion of the studies to more populations across countries and at the species level, which ultimately will help gain a bigger picture of the geographical variation and evolution of colour change.

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Appendix 1A – Supplementary materials for Chapter 2: Additional statistical tests for the raw standardised morphometric data of males and females

Methods

Statistical analysis

After conducting principal component analysis (PCA) on the scaled data from twenty-one morphometric traits, I observed significant differences among populations in PC2 for males and PC1 for females (see Results). There were three variables that exhibited high loadings on PC2 for males (Table 2.3). Similarly, three variables displayed substantial loadings on PC1 for females (Table 2.3). Therefore, I conducted statistical tests (ANOVA or Kruskal-Wallis rank sum test) on the raw standardised data for both males and females. The choice of test depended on two factors: (i) the homogeneity of variance, determined by Levene's test for homogeneity of variance, and (ii) the normality of the ANOVA residuals, determined by the Shapiro-Wilk normality test. If the data met the assumptions of homogeneity of variance and normality of the ANOVA residuals, I reported the results from the one-way ANOVA test. If the assumptions were unmet, I used and reported a Kruskal-Wallis rank sum test (hereinafter Kruskal-Wallis test). After conducting the one-way ANOVA and Kruskal-Wallis tests, a post-hoc analysis was carried out. For the one-way ANOVA, the Tukey honest significant differences test (Tukey test) was used, while for the Kruskal-Wallis test, the Wilcoxon rank sum test (Wilcoxon) with Bonferroni adjustment of p values was employed. All analyses were performed in R version 4.2.3 (R Core Team, 2023).

Results

Males

There was a significant difference among populations in SNL (Kruskal-Wallis test: $\chi^2 = 14.256$, $df = 3$, $p = 0.0026$), where the Jeddah population differed from the Nawan populations (Wilcoxon: $p = 0.0006$), and no significant differences occurred between the rest (Figure S2.1). Also, there were highly significant differences among populations in CPL (Kruskal-Wallis test: $\chi^2 = 33.046$, $df = 3$, $p < 0.0001$), where each of the freshwater populations (Khaybar and Nawan) differed from each of the marine populations (Gizan and Jeddah) (Wilcoxon: $p < 0.001$) and no significant differences occurred between Gizan and Jeddah or between Khaybar and Nawan (Figure S2.2). Additionally, there were highly

significant differences among populations in BD (ANOVA: $F(3,76) = 16.02$, $p < 0.0001$), whereby each population from the freshwater habitats (Khaybar and Nawan) differed significantly from each of the marine populations (Gizan and Jeddah) (Tukey test: $p < 0.001$), and no significant differences occurred between Gizan and Jeddah or between Khaybar and Nawan (Figure S2.3).

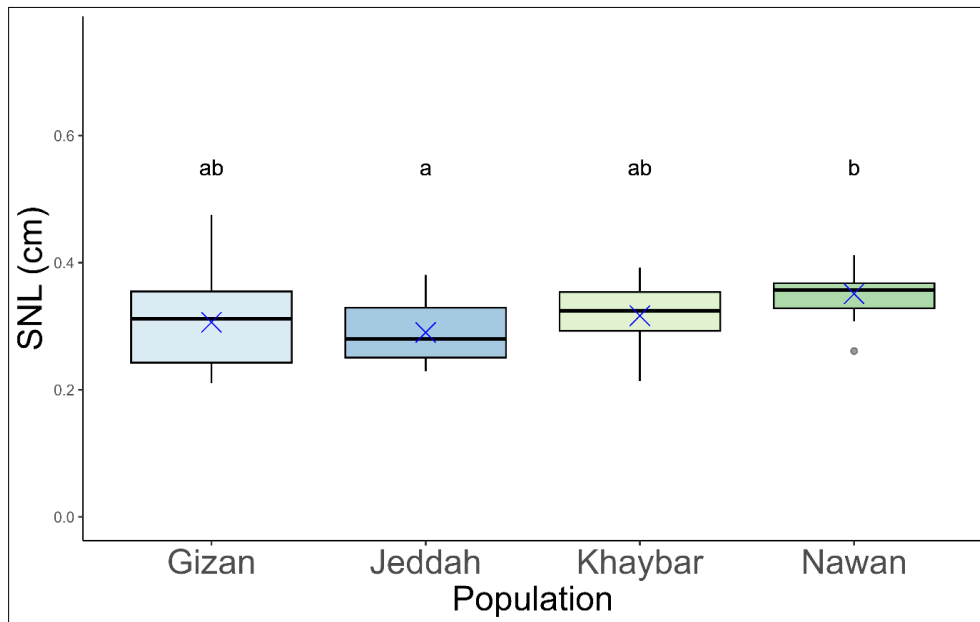


Figure S2.1: Box plot showing the differences in the snout length of males of *A. dispar* among populations. The black lines represent the medians, and the blue crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The grey dots represent outliers. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations.

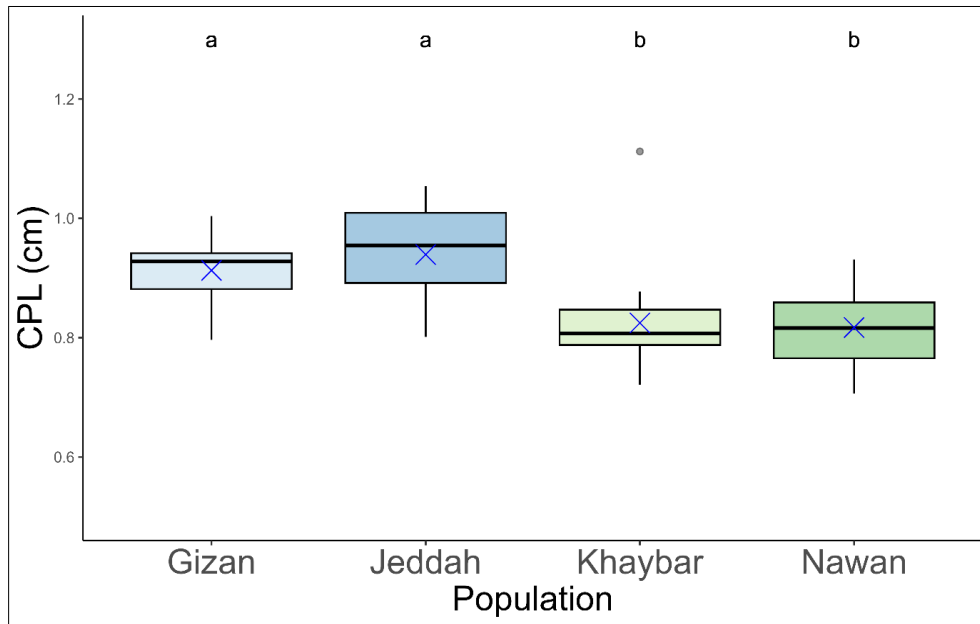


Figure S2.2: Box plot showing the differences in the caudal peduncle length of males of *A. dispar* among populations. The black lines represent the medians, and the blue crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The grey dots represent outliers. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations.



Figure S2.3: Box plot showing the differences in the body depth of males of *A. dispar* among populations. The black lines represent the medians, and the blue crosses represent the

means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The grey dots represent outliers. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations.

Females

There were highly significant differences among populations in HL (ANOVA: $F(3,74) = 6.622$, $p = 0.0005$), where the Jeddah population differed from all the populations (Tukey test: $p < 0.01$), and no significant differences occurred between the rest (Figure S2.4). Furthermore, there were highly significant differences among populations in CPL (ANOVA: $F(3,74) = 18.79$, $p < 0.0001$), where the Jeddah population differed from all the populations (Tukey test: $p < 0.0001$), and no significant differences occurred between the rest (Figure S2.5). Additionally, there were highly significant differences among populations in DCP (Kruskal-Wallis test: $\chi^2 = 30.386$, $df = 3$, $p < 0.0001$), whereby all populations differed from each other (Wilcoxon: $p < 0.01$), but no differences were observed between Nawan to Gizan and to Khaybar (Figure S2.6).

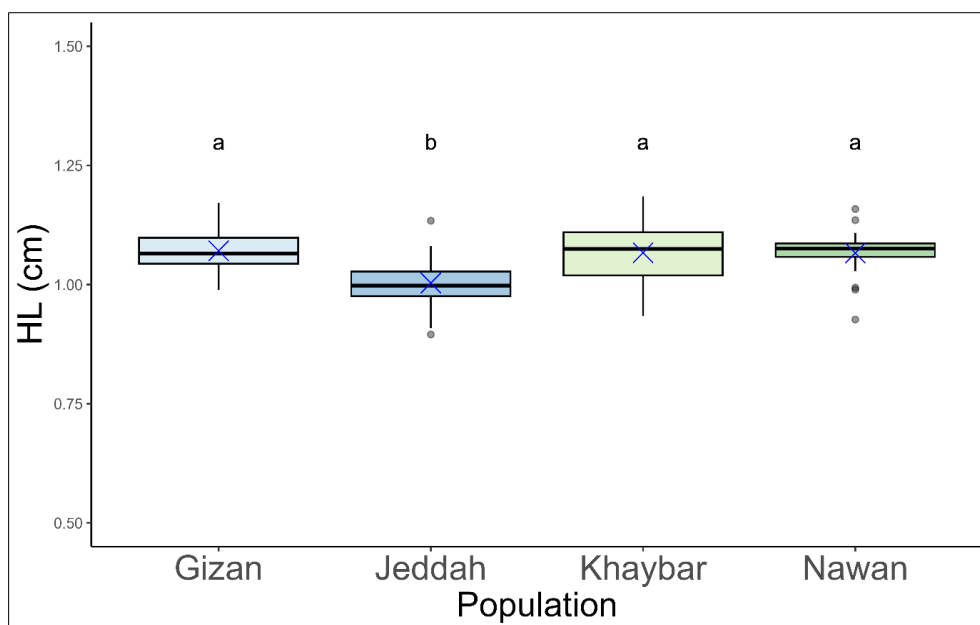


Figure S2.4: Box plot showing the differences in the head length (cm) of females of *A. dispar* among populations. The black lines represent the medians, and the blue crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The grey dots represent

outliers. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations.

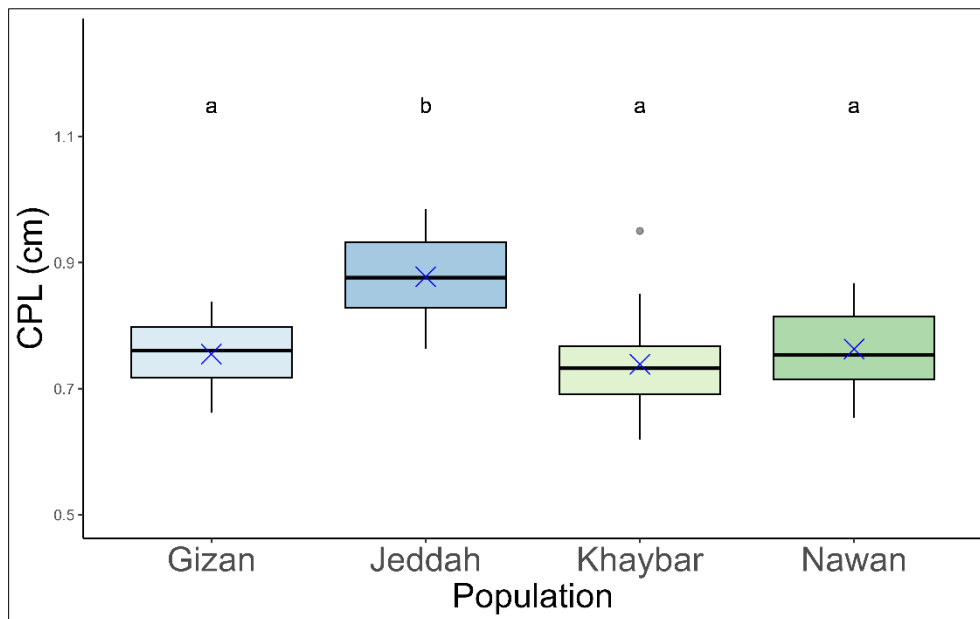


Figure S2.5: Box plot showing the differences in the caudal peduncle length (cm) of females of *A. dispar* among populations. The black lines represent the medians, and the blue crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The grey dots represent outliers. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations.

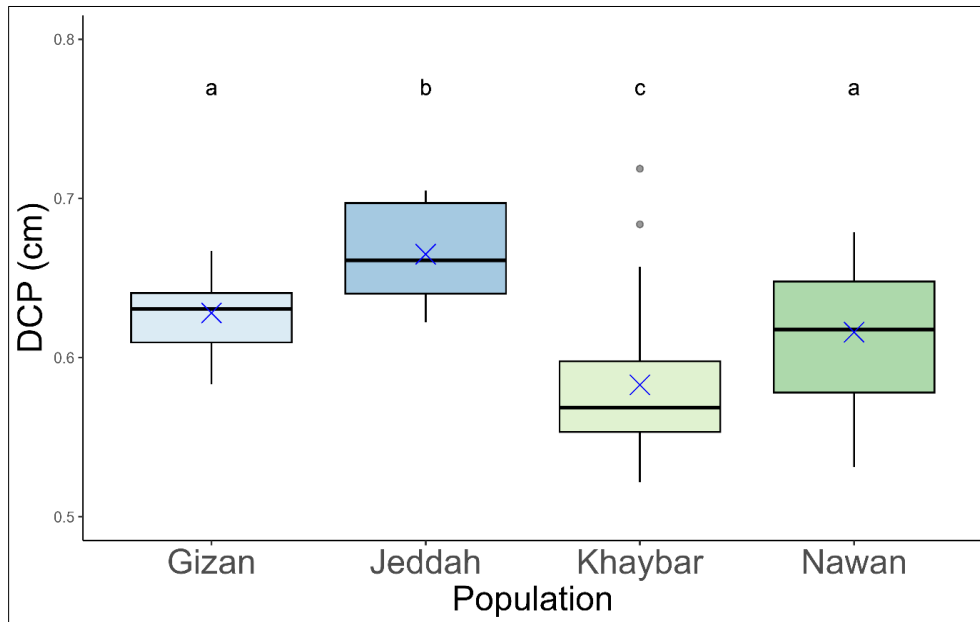


Figure S2.6: Box plot showing the differences in the caudal peduncle depth (cm) of females of *A. dispar* among populations. The black lines represent the medians, and the blue crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The grey dots represent outliers. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations.

References

R Core Team. (2023). *R: a language and environment for statistical computing* (4.2.3) [Computer software]. R Foundation for Statistical Computing. <https://www.r-project.org/>

Appendix 1B – Supplementary materials for Chapter 2: Additional methods for analysing male colour patterns, and additional statistical tests for the raw male colour pattern data, and female vertical stripes

Methods

Colour pattern analysis

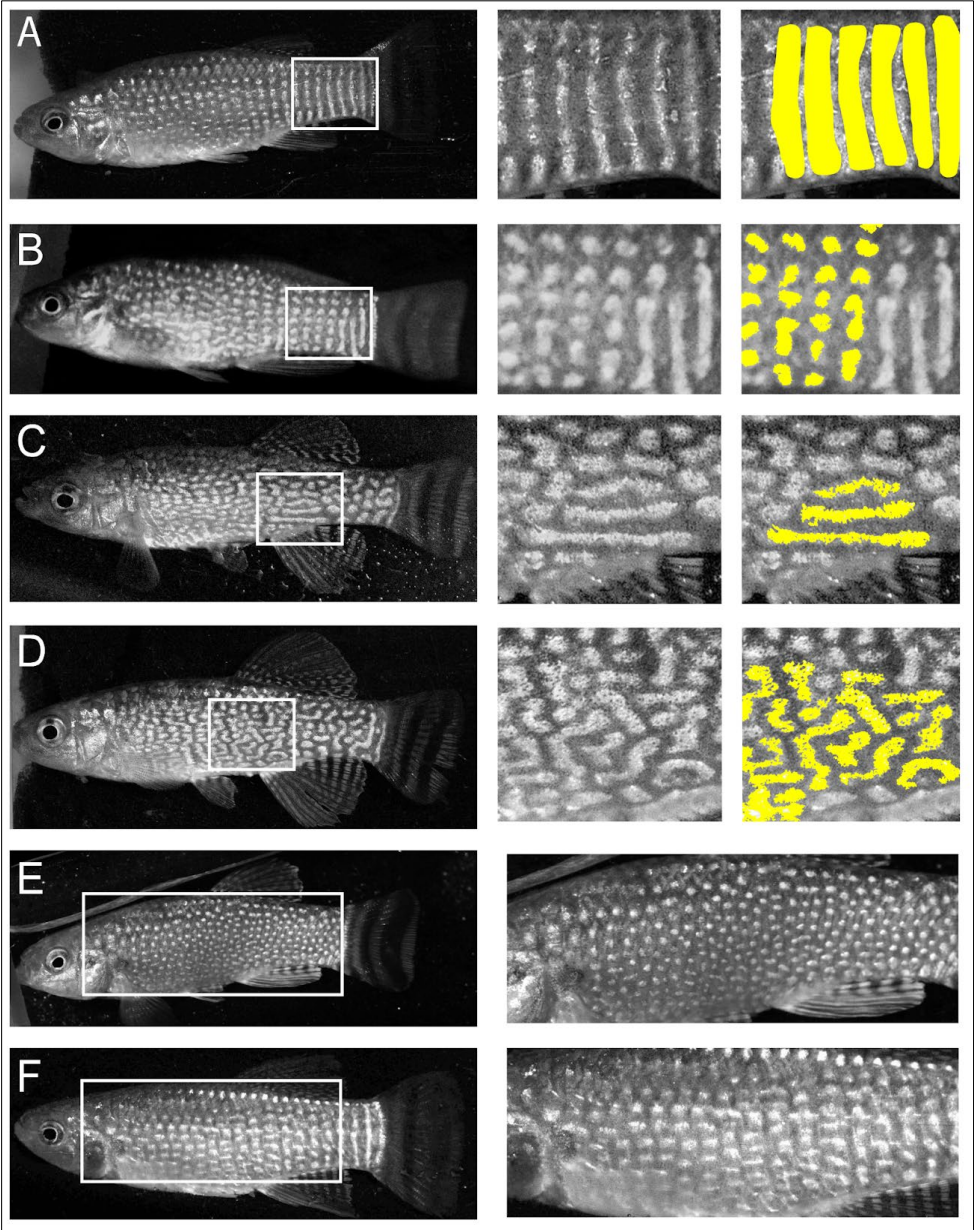


Figure S2.7: Examples of the scoring methods used in colour pattern analysis for scoring of characteristics 1–5 as per the criteria in Table 2.2. The left column shows random representative individuals with selected regions. The right columns show the magnified selected areas from the left column, where the top four are further highlighted in yellow to specify the characteristics of interest from other characteristics. (A) A male from the Gizan

population possesses 6 bars at the caudal peduncle. (B) A male from the Jeddah population shows arranged blotches at the caudal peduncle, highlighted in yellow, and three elongated vertical patterns to the right. (C) A male from the Khaybar population exhibits elongated horizontal patterns. (D) A male from the Khaybar population displays vermiculation. (E) A male from the Nawan population shows scattered blotches across the flank, and (F) a male from the Gizan population displays no scattered blotches, which possesses arranged blotches across the flank.

Statistical analysis

After conducting principal component analysis (PCA) on scaled data from six pattern traits, I observed significant differences among populations in PC1 and PC2 (see Results). I noted that all variables were heavily loaded on the two major principal components, PC1 and PC2 (Table 2.4). Therefore, I conducted statistical analyses on each of the six raw data. I used a Kruskal-Wallis rank sum test (Kruskal-Wallis test) for APS, SB and NB. The tests for APS and SB were followed by the Wilcoxon rank sum test (Wilcoxon) with Bonferroni adjustment of p values. The test for NB was followed by Dunn's test with Bonferroni adjustment of p values, as I had count data. ABP, EP, and V were binary data, and I performed contingency Chi-square tests on each trait. For the vertical stripes of females, I used a Kruskal-Wallis rank sum test (Kruskal-Wallis test), followed by Dunn's test with Bonferroni adjustment of p values, as I had count data. All analyses were performed in R version 4.2.3 (R Core Team, 2023).

Results

Males

There were highly significant differences among populations in the average pattern size (Kruskal-Wallis test: $\chi^2 = 35.025$, $df = 3$, $p < 0.0001$), whereby all populations differed significantly from each other (Wilcoxon: $p < 0.01$), but no significant differences occurred between Gizan and Khaybar and between Nawan and Jeddah (Figure S2.8).

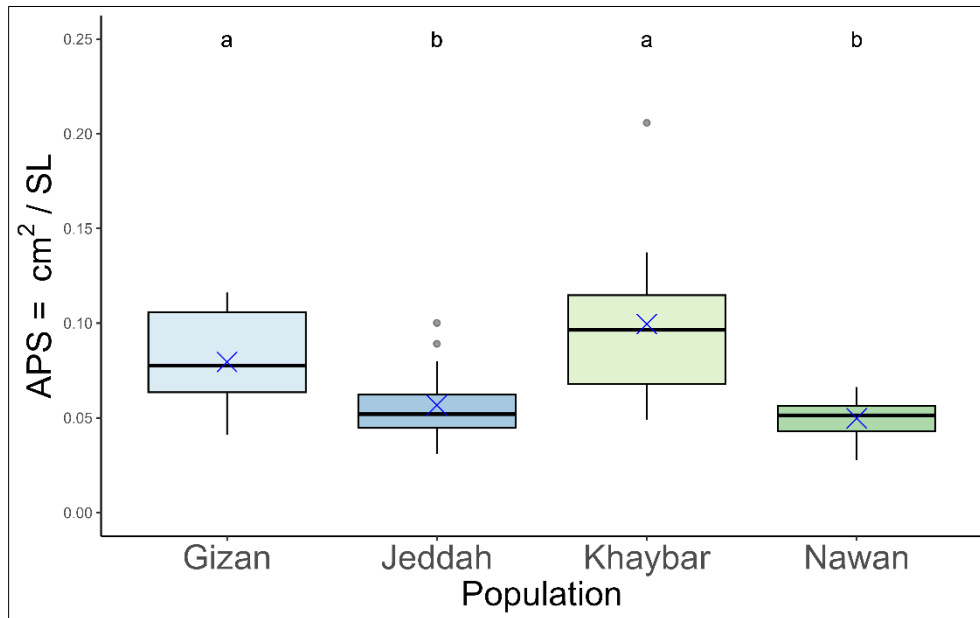


Figure S2.8: Box plot showing the differences in the average pattern size on males of *A. dispar* among populations. The black lines represent the medians, and the blue crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The grey dots represent outliers. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations.

There were highly significant differences among populations in the number of bars (Kruskal-Wallis test: $\chi^2 = 48.823$, $df = 3$, $p < 0.0001$), whereby all populations differed significantly from each other (Dunn's test: $p < 0.05$), and no significant differences occurred between Nawan and Khaybar (Figure S2.9).

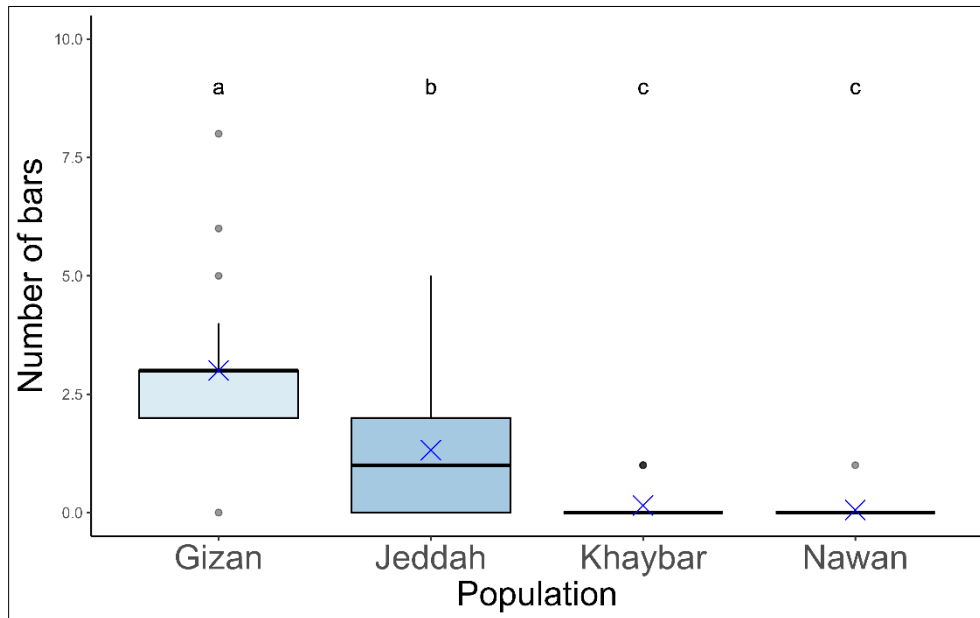


Figure S2.9: Box plot showing the differences in the number of bars on males of *A. dispar* among populations. The black lines represent the medians, and the blue crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The grey dots represent outliers. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations.

Moreover, there were highly significant differences among populations in the arrangement of blotches at the caudal peduncle (Chi-square test: $\chi^2 (3, N = 78) = 44.685, p < 0.0001$), where each of the freshwater populations (Khaybar and Nawan) had fewer arranged blotches at the peduncle than the marine populations (Gizan and Jeddah) (Figure S2.10).

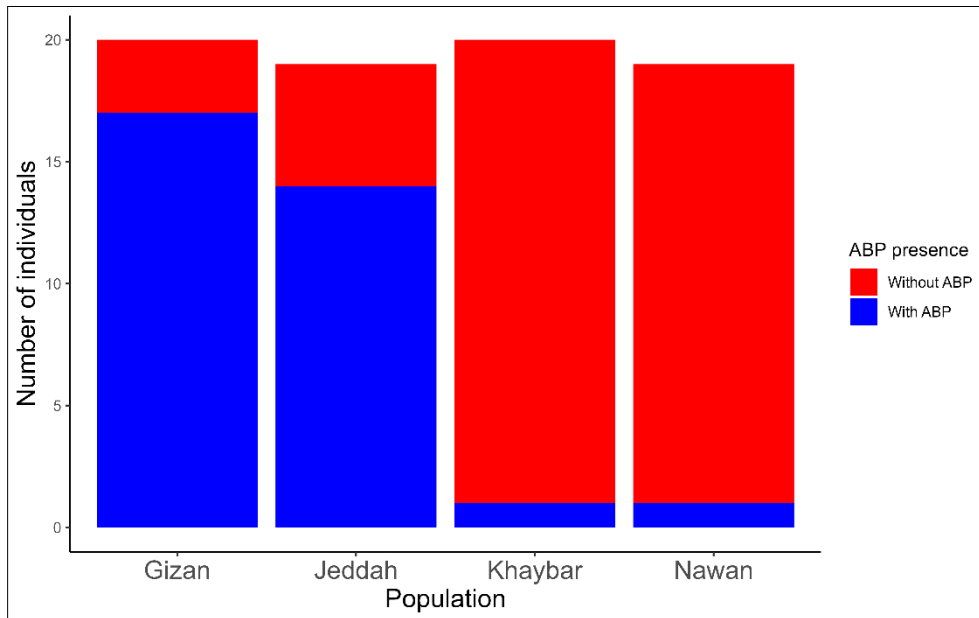


Figure S2.10: The differences between populations in the number of *A. dispar* male individuals with and without arranged blotches at the caudal peduncle.

In addition, there were highly significant differences among populations in the elongated patterns (Chi-square test: χ^2 (3, $N = 78$) = 49.81, $p < 0.0001$), with the freshwater population Khaybar having more elongated patterns than the other populations (Figure S2.11).

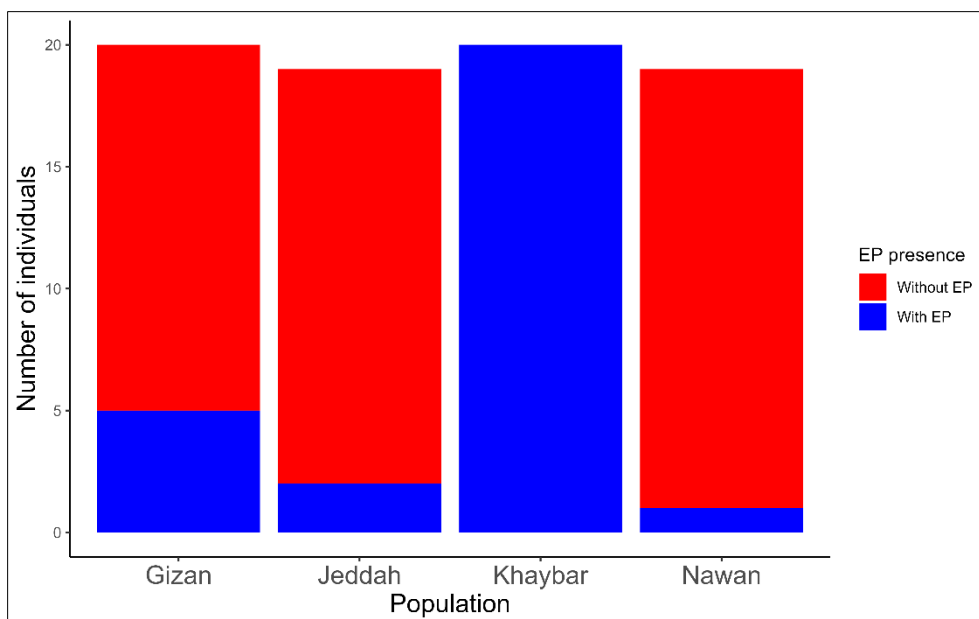


Figure S2.11: The differences between populations in the number of *A. dispar* male individuals with and without elongated patterns.

Moreover, there were highly significant differences among populations in the vermiculation (Chi-square test: $\chi^2 (3, N = 78) = 53.28, p < 0.0001$), with the freshwater population Khaybar having more vermiculation patterns than the other populations (Figure S2.12).

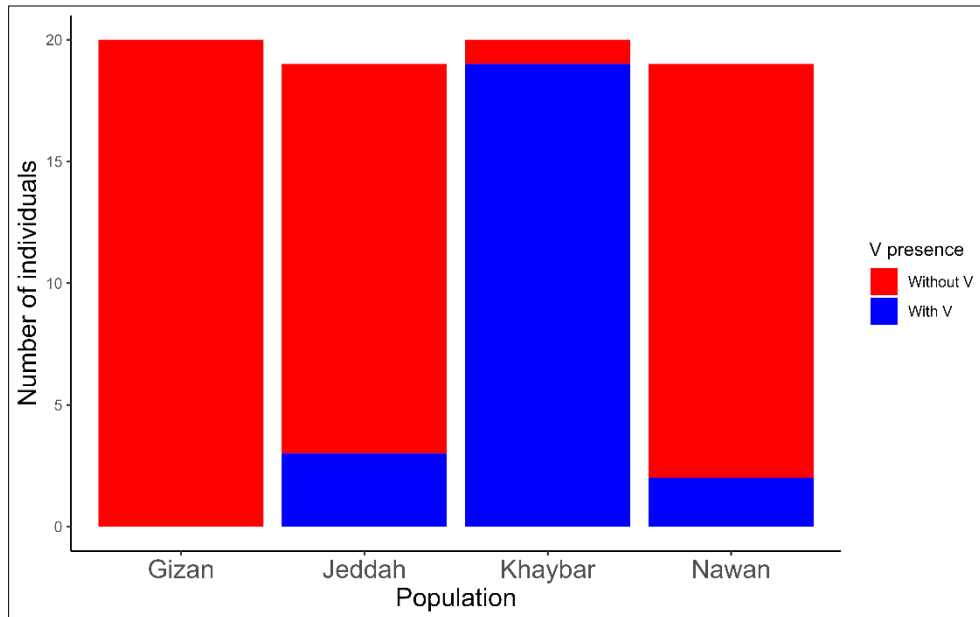


Figure S2.12: The differences between populations in the number of *A. dispar* male individuals with and without vermiculation.

Finally, there were highly significant differences among populations in scatter of blotches (Kruskal-Wallis test: $\chi^2 = 45.461, df = 3, p < 0.0001$), where each of the freshwater populations (Khaybar and Nawan) differed from each of the marine populations (Gizan and Jeddah) (Wilcoxon: $p < 0.001$), and no significant differences occurred between Gizan and Jeddah or between Khaybar and Nawan (Figure S2.13).

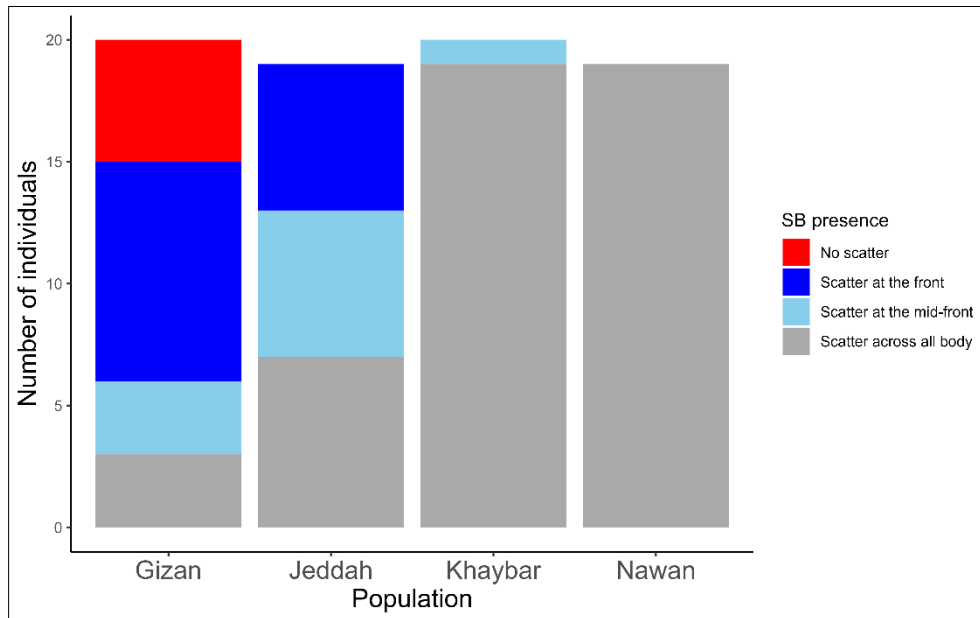


Figure S2.13: The differences between populations in the number of *A. dispar* male individuals with scattered blotches on the front flank, at the mid-front flank, across the body and without scattered blotches.

Females

There was a highly significant difference among populations in the number of vertical stripes (Kruskal-Wallis test: $\chi^2 = 48.062$, $df = 3$, $p < 0.0001$), whereby all populations differed significantly from each other (Dunn's test: $p < 0.05$), and no significant differences occurred between Gizan and Jeddah or between Jeddah and Nawan (Figure S2.14).

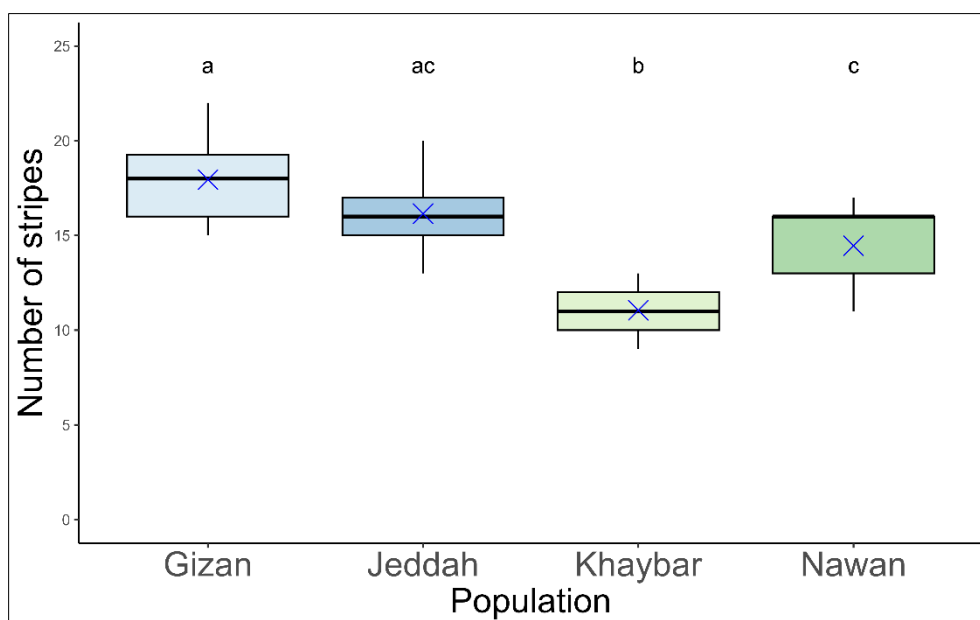


Figure S2.14: Box plot showing the differences in the number of vertical stripes on females of *A. dispar* among populations. The black lines represent the medians, and the blue crosses represent the means. Each box represents the interquartile range (IQR) of the data, and the whiskers represent the minimum and maximum values within $1.5 \times \text{IQR}$. The different characters indicate statistical significance between populations at $p < 0.05$, while the similar characters indicate no statistical significance between populations.

References

R Core Team. (2023). *R: a language and environment for statistical computing* (4.2.3) [Computer software]. R Foundation for Statistical Computing. <https://www.r-project.org/>

Appendix 1C – Supplementary materials for Chapter 2: additional data for the molecular analyses

Table S2.1: Names, coordinates and dates of the visited freshwater and marine locations on the western coastline of Saudi Arabia. The table contains the sample identifications, sex and the GenBank accession numbers for materials used in the molecular analyses. The locations' names in boldface were involved in all analyses, including the morphometric, colour pattern and molecular analyses.

Sample ID	Sex	Location name	Location coordinates		Habitat	Collection date	Accession No.
A1	Male	Wadi Al Arj (Arj)	20°27'13.9"N	40°48'55.7"E	Freshwater	14-Aug-2020	OR863925
A2	Male						OR863926
A3	Female						OR863927
A4	Female						OR863928
D1	Female	Deba Beach (Deba)	27°21'03.7"N	35°41'56.6"E	Marine	24-June-2021	OR863929
D2	Female						OR863930
D3	Female						OR863931
D4	Female						OR863932
G1	Male	Al Marjan Beach (Gizan)	16°50'07.9"N	42°34'34.4"E	Marine	26-Aug-2020	OR863933
G2	Male						OR863934
G3	Female						OR863935
G4	Female						OR863936
H1	Male	Wadi Hali (Hali)	18°46'07.9"N	41°34'24.9"E	Freshwater	01-March-2021	OR863937
H2	Male						OR863938
H3	Female						OR863939

H4	Female						OR841348
J1	Male						OR863940
J2	Male	Jeddah Beach	21°29'24.9"N	39°10'58.9"E	Marine	04-Apr-2021	OR863941
J3	Female	(Jeddah)					OR863942
J4	Female						OR863943
K1	Male						OR863944
K2	Male	Al Jammah spring	25°44'38.6"N	39°15'19.3"E	Freshwater	07-Aug-2022	OR863945
K3	Female	(Khaybar)					OR863946
K4	Female						OR863947
N3	Female	Wadi Nawan (Nawan)	19°39'25.1"N	41°16'57.7"E	Freshwater	20-Aug-2020	OR863948
Q1	Male						OR863949
Q2	Male	Wadi Qanuna (Qanuna)	19°15'03.5"N	41°28'51.4"E	Freshwater	15-Jul-2020	OR863950
Q3	Female						OR863951
Q4	Female						OR863952

Table S2.2: Requirements for preparing 500 ml of the Digsol buffer and Low TE.

DIGSOL 500 ml	Low TE (Tris 10 mM EDTA 0.1 mM) 500 ml
20 ml of 0.5 M EDTA (pH 8.0)	5 ml 1 M Tris-HCl (pH 8.0)
3.425 g NaCl	100 µl 0.5 M EDTA (pH 8.0)
25 ml of 1 M Tris-HCl (pH 8.0)	495 ml ddH ₂ O
430 ml ddH ₂ O	Autoclaved
Autoclaved at 15 psi for 20 min.	
Then 25 ml of 20% SDS was added.	

Table S2.3: The estimates of the evolutionary divergence between the molecular operational taxonomic units (MOTUs), and between the *dispar* MOTU populations using the Kimura 2-parameter (K2P) model. The table shows the mean (black) and the standard error (blue) of the K2P distance. The information of the MOTUs is detailed in table S2.4.

MOTU	1	2	3	4	5	6	7	8	9	10
1		0.006856	0.016364	0.016006	0.173266	0.009882	0.040694	0.013986	0.011231	0.017614
2	0.018459		0.015083	0.013699	0.142281	0.00868	0.036656	0.012435	0.011004	0.015846
3	0.062372	0.055498		0.014051	0.116537	0.017318	0.048211	0.017711	0.01519	0.010328
4	0.060097	0.053429	0.05319		0.139534	0.013321	0.045395	0.015541	0.012382	0.013081
5	0.594592	0.50286	0.430485	0.510097		0.16956	0.106503	0.158406	0.159768	0.137995
6	0.034365	0.029219	0.066001	0.050018	0.585983		0.044571	0.014184	0.010861	0.017019
7	0.159966	0.147259	0.193189	0.187123	0.404222	0.172222		0.047701	0.042288	0.040262
8	0.055368	0.04629	0.068528	0.06063	0.552555	0.053021	0.193309		0.004214	0.017684
9	0.041253	0.040854	0.058699	0.047511	0.557443	0.039087	0.171395	0.008822		0.015119
10	0.067598	0.060531	0.036161	0.050573	0.491474	0.064703	0.162747	0.067203	0.057613	
11	0.058335	0.051781	0.024122	0.042321	0.459522	0.055749	0.170438	0.057999	0.049044	0.020052
12	0.016648	0.027334	0.02511	0.031063	0.476635	0.030356	0.147897	0.043015	0.030055	0.022843
13	0.019811	0.015462	0.056995	0.061934	0.573749	0.035756	0.164875	0.051139	0.037561	0.062213
14	0.018467	0	0.055529	0.053458	0.503394	0.029233	0.147367	0.046314	0.040875	0.060564
15	0.046619	0.047488	0.097521	0.085884	0.700818	0.059338	0.228243	0.073755	0.057404	0.098998
16	0.016652	0.012492	0.053256	0.057274	0.57256	0.031441	0.15599	0.046933	0.033843	0.057595
17	0.02086	0.020622	0.064218	0.060197	0.587653	0.036967	0.152078	0.048254	0.040043	0.069505
18	0.016511	0.005164	0.052084	0.04533	0.515999	0.026531	0.151474	0.043873	0.033637	0.056422
19	0.018704	0.014428	0.053073	0.057146	0.561161	0.031982	0.151001	0.046858	0.033775	0.058229
20	0.016598	0.012453	0.053039	0.057052	0.576985	0.031935	0.156646	0.04676	0.033725	0.058125

Table S2.3 (Continued)

MOTU	11	12	13	14	15	16	17	18	19	20
1	0.015265	0.006151	0.007146	0.006861	0.012281	0.006537	0.006853	0.006477	0.006922	0.006515
2	0.013839	0.008591	0.005625	0	0.01262	0.004966	0.006747	0.003123	0.005405	0.004948
3	0.007887	0.008284	0.015327	0.015093	0.024641	0.014563	0.016796	0.01415	0.014509	0.014498
4	0.011328	0.009435	0.015708	0.013707	0.02171	0.014726	0.015079	0.012149	0.014695	0.014659
5	0.12232	0.133141	0.167728	0.142502	0.215207	0.166576	0.169811	0.145404	0.162204	0.16826
6	0.014972	0.009295	0.010518	0.008685	0.015072	0.009416	0.010429	0.008004	0.009612	0.009592
7	0.041937	0.03672	0.042859	0.0367	0.058984	0.040043	0.038699	0.038287	0.03849	0.0402
8	0.015591	0.012251	0.013565	0.012443	0.01808	0.012593	0.012529	0.011846	0.012568	0.012534
9	0.013227	0.009476	0.010629	0.011012	0.01421	0.009768	0.010907	0.009583	0.009743	0.009729
10	0.006479	0.007678	0.015894	0.015856	0.024294	0.014997	0.017651	0.014727	0.015197	0.015161
11		0.005958	0.01393	0.013847	0.019932	0.013255	0.0153	0.012875	0.013225	0.013202
12	0.016501		0.008162	0.008597	0.013445	0.007422	0.00798	0.007791	0.007407	0.007396
13	0.053301	0.024275		0.005628	0.013144	0.001843	0.004824	0.005379	0.002639	0.001837
14	0.051809	0.027347	0.015468		0.012625	0.004968	0.006751	0.003124	0.005409	0.004951
15	0.081869	0.05116	0.049656	0.0475		0.012434	0.011475	0.01242	0.013102	0.012406
16	0.049688	0.020955	0.00255	0.012496	0.045349		0.004159	0.004764	0.001673	0
17	0.060101	0.025389	0.012723	0.020629	0.044269	0.009816		0.006484	0.004617	0.004146
18	0.048597	0.022975	0.01355	0.005165	0.044924	0.010631	0.018623		0.005172	0.004754
19	0.04958	0.020914	0.004285	0.014434	0.048144	0.001678	0.011711	0.012531		0.001669
20	0.049499	0.020885	0.002542	0.012458	0.045254	0	0.009788	0.010608	0.001674	

Table S2.4: The names and GenBank accession numbers for the 89 materials used in the molecular analyses, including the MOTUs used in the genetic distances in Table S2.3.

Number of MOTU in Table S2.3	Name of MOTU	Name of sample and accession number
1	<i>Aphaniops dispar</i> MOTU East Mediterranean	<i>Aphaniops cf. dispar</i> MF918576, <i>Aphaniops cf. dispar</i> MF918577, <i>Aphaniops cf. dispar</i> MF918589, <i>Aphaniops cf. dispar</i> MF918588
2	<i>Aphaniops dispar</i> MOTU Saudi Arabia Thulbah	<i>Aphaniops cf. dispar</i> MF918581, <i>Aphaniops cf. dispar</i> MF918582, <i>Aphaniops cf. dispar</i> MF918583
3	<i>Aphaniops ginaonis</i> MOTU	<i>Aphaniops ginaonis</i> MF918592, <i>Aphaniops ginaonis</i> MF918590, <i>Aphaniops ginaonis</i> MF918591
4	<i>Aphaniops kruppi</i> MOTU	<i>Aphaniops kruppi</i> MF918598, <i>Aphaniops kruppi</i> MF918597, <i>Aphaniops stoliczkanus</i> MF918610, <i>Aphaniops kruppi</i> MF918593, <i>Aphaniops kruppi</i> MF918594, <i>Aphaniops kruppi</i> MF918595, <i>Aphaniops kruppi</i> MF918596
5	<i>Paraphanius mento</i> (Outgroup)	<i>Paraphanius mento</i> KJ552373, <i>Paraphanius mento</i> KJ552492
6	<i>Aphaniops richardsoni</i> MOTU	<i>Aphaniops richardsoni</i> MF918600, <i>Aphaniops richardsoni</i> MF918601, <i>Aphaniops richardsoni</i> KJ552638, <i>Aphaniops richardsoni</i> MF918599, <i>Aphaniops richardsoni</i> KJ552571, <i>Aphaniops richardsoni</i> KJ552395, <i>Aphaniops richardsoni</i> KJ552643, <i>Aphaniops richardsoni</i> KJ552736
7	<i>Aphaniops sirhani</i> MOTU	<i>Aphaniops sirhani</i> MF918602, <i>Aphaniops sirhani</i> MF918603, <i>Aphaniops sirhani</i> KJ552717, <i>Aphaniops sirhani</i> MF918604, <i>Aphaniops sirhani</i> KJ552402
8	<i>Aphaniops cf. dispar</i> MOTU3 Horn of Africa	<i>Aphaniops cf. dispar</i> MF918579, <i>Aphaniops cf. dispar</i> MF918580
9	<i>Aphaniops cf. dispar</i> MOTU2 Horn of Africa	<i>Aphaniops cf. dispar</i> MF918578
10	<i>Aphaniops stoliczkanus</i> MOTU2	<i>Aphaniops stoliczkanus</i> MF918613, <i>Aphaniops stoliczkanus</i> MF918614, <i>Aphaniops stoliczkanus</i> MF918611, <i>Aphaniops stoliczkanus</i> MF918612
11	<i>Aphaniops stoliczkanus</i> MOTU1	<i>Aphaniops stoliczkanus</i> MF918624, <i>Aphaniops stoliczkanus</i> MF918616, <i>Aphaniops stoliczkanus</i> MF918615, <i>Aphaniops</i>

		<i>stoliczkanus</i> KJ552732, <i>Aphaniops stoliczkanus</i> KJ552389, <i>Aphaniops stoliczkanus</i> MF918621, <i>Aphaniops stoliczkanus</i> MF918622, <i>Aphaniops stoliczkanus</i> MF918620, <i>Aphaniops stoliczkanus</i> MF918619, <i>Aphaniops stoliczkanus</i> MF918618, <i>Aphaniops stoliczkanus</i> MF918617, <i>Aphaniops stoliczkanus</i> MF918609, <i>Aphaniops stoliczkanus</i> MF918608, <i>Aphaniops cf. kruppi</i> MF918587, <i>Aphaniops stoliczkanus</i> MF918605, <i>Aphaniops stoliczkanus</i> MF918606, <i>Aphaniops stoliczkanus</i> MF918607, <i>Aphaniops cf. kruppi</i> MF918584, <i>Aphaniops cf. kruppi</i> MF918586, <i>Aphaniops cf. kruppi</i> MF918585
12	<i>Aphaniops stoliczkanus</i> MOTU3	<i>Aphaniops stoliczkanus</i> MF918623
13	<i>Aphaniops dispar</i> MOTU Saudi Arabia, Arj	<i>Aphaniops dispar</i> Saudi Arabia Arj OR863926, <i>Aphaniops dispar</i> Saudi Arabia Arj OR863927, <i>Aphaniops dispar</i> Saudi Arabia Arj OR863925, <i>Aphaniops dispar</i> Saudi Arabia Arj OR863928
14	<i>Aphaniops dispar</i> MOTU Saudi Arabia, Deba	<i>Aphaniops dispar</i> Saudi Arabia Deba OR863929, <i>Aphaniops dispar</i> Saudi Arabia Deba OR863930, <i>Aphaniops dispar</i> Saudi Arabia Deba OR863931, <i>Aphaniops dispar</i> Saudi Arabia Deba OR863932
15	<i>Aphaniops cf. dispar</i> MOTU1 Saudi Arabia, Gizan	<i>Aphaniops cf. dispar</i> Saudi Arabia Gizan OR863933, <i>Aphaniops cf. dispar</i> Saudi Arabia Gizan OR863934, <i>Aphaniops cf. dispar</i> Saudi Arabia Gizan OR863936, <i>Aphaniops cf. dispar</i> Saudi Arabia Gizan OR863935
16	<i>Aphaniops dispar</i> MOTU Saudi Arabia, Hali	<i>Aphaniops dispar</i> Saudi Arabia Hali OR863938, <i>Aphaniops dispar</i> Saudi Arabia Hali OR863939, <i>Aphaniops dispar</i> Saudi Arabia Hali OR863937, <i>Aphaniops dispar</i> Saudi Arabia Hali OR841348
17	<i>Aphaniops dispar</i> MOTU Saudi Arabia, Jeddah	<i>Aphaniops dispar</i> Saudi Arabia Jeddah OR863940, <i>Aphaniops dispar</i> Saudi Arabia Jeddah OR863941, <i>Aphaniops dispar</i> Saudi Arabia Jeddah OR863942, <i>Aphaniops dispar</i> Saudi Arabia Jeddah OR863943
18	<i>Aphaniops dispar</i> MOTU Saudi Arabia, Khaybar	<i>Aphaniops dispar</i> Saudi Arabia Khaybar OR863944, <i>Aphaniops dispar</i> Saudi Arabia Khaybar OR863945, <i>Aphaniops dispar</i> Saudi Arabia Khaybar OR863946,

		<i>Aphaniops dispar</i> Saudi Arabia Khaybar OR863947
19	<i>Aphaniops dispar</i> MOTU Saudi Arabia, Nawan	<i>Aphaniops dispar</i> Saudi Arabia Nawan OR863948
20	<i>Aphaniops dispar</i> MOTU Saudi Arabia, Qanuna	<i>Aphaniops dispar</i> Saudi Arabia Qanuna OR863949, <i>Aphaniops dispar</i> Saudi Arabia Qanuna OR863950, <i>Aphaniops dispar</i> Saudi Arabia Qanuna OR863951, <i>Aphaniops dispar</i> Saudi Arabia Qanuna OR863952

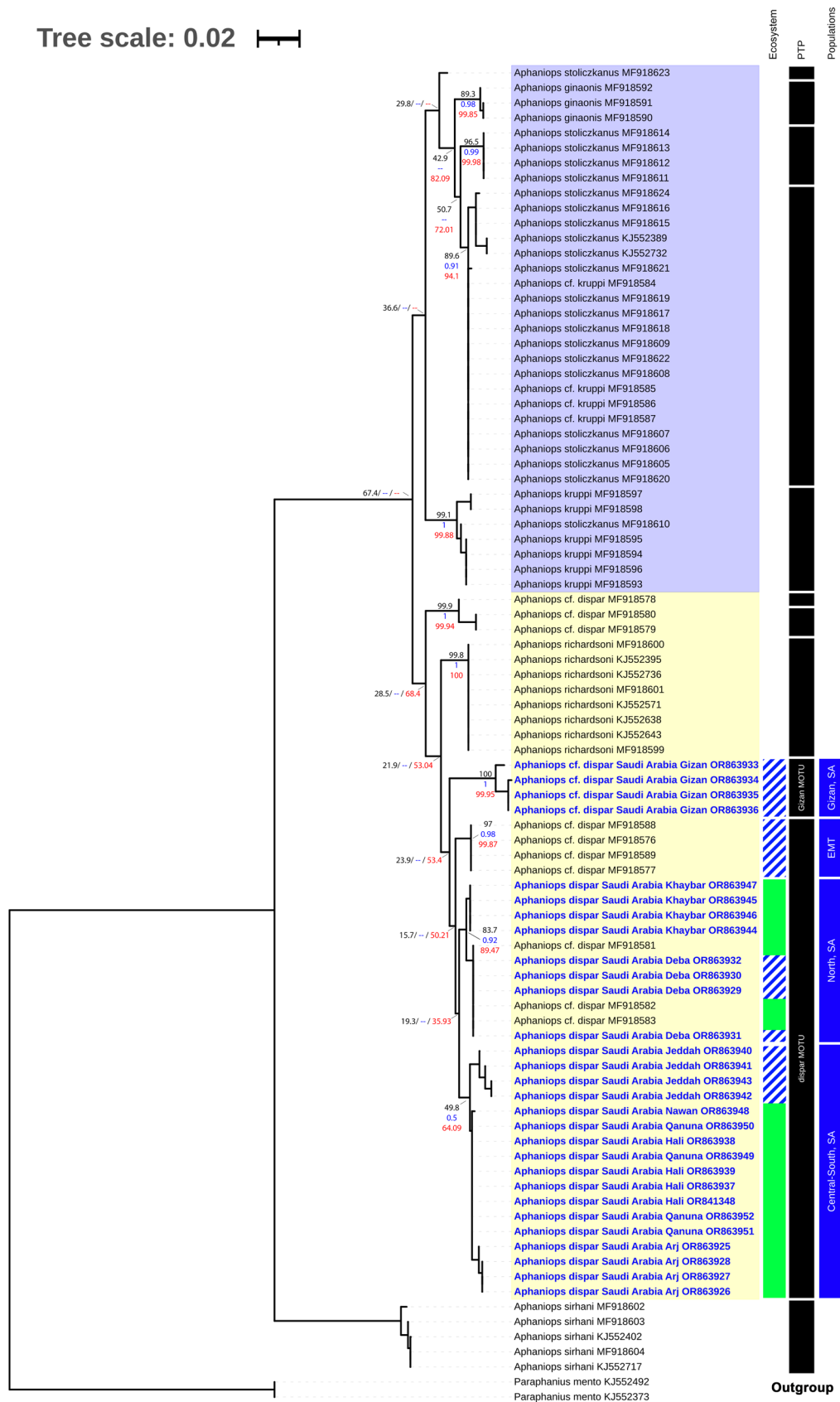


Figure S2.15: Phylogenetic relationships inferred from the mitochondrial COI barcode region using the consensus maximum likelihood tree. The tree was constructed using the HKY model with a Gamma distribution (4 classes), a G parameter of 0.1490 and a

transition/transversion value of 7.70693. Numbers on nodes as ordered from left to right or top to bottom represent support values obtained from 1000 bootstrap replicates using the ML method (black), posterior probability values from BI (blue), and bootstrap values obtained from 10000 bootstrap replicates using the NJ method (red). The Arabian Gulf clade is collapsed and highlighted in blue, while the Red Sea clade is highlighted in yellow. The solid black bars represent the 11 delimited MOTUs using the PTP approach. The solid blue bars represent the Saudi Arabian (SA) and East Mediterranean (EMT) populations within the *dispar* MOTU, as well as the Gizan MOTUs from Saudi Arabia. The solid green bars represent the freshwater populations within the *dispar* MOTU. The blue diagonal striped bars represent the marine populations within the *dispar* and Gizan MOTUs. Labels in blue boldface represent the samples collected in this study from Saudi Arabia. The scale bar indicates 0.02 substitutions per site.

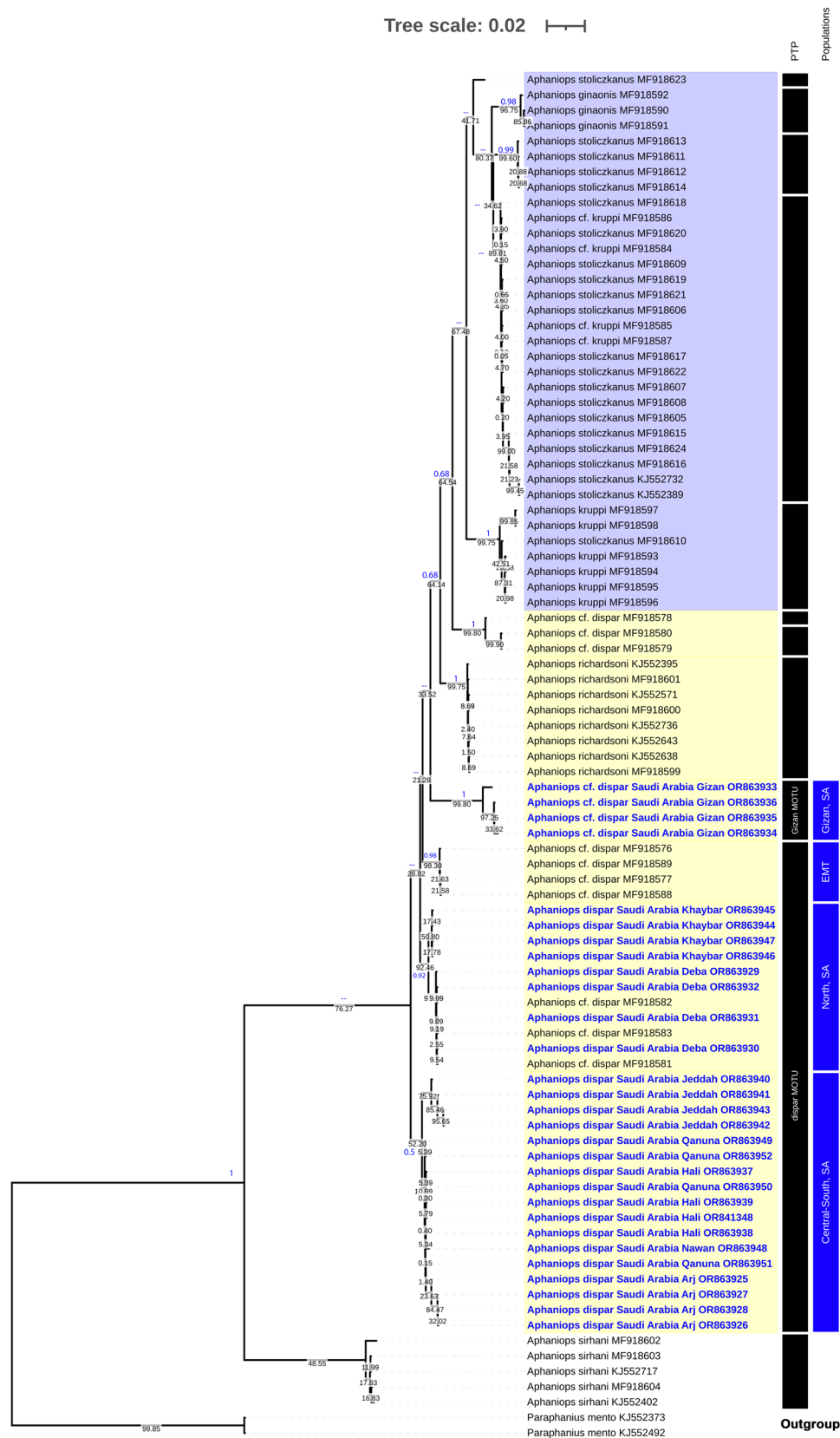


Figure S2.16: Phylogenetic relationships inferred from the mitochondrial COI barcode region using the constructed consensus Bayesian tree from the original 2002 BI raw trees. The original BI tree was constructed using the HKY model with a Gamma distribution (4 classes), a G parameter of 0.1490 and a transition/transversion value of 7.70693. Numbers

on nodes as ordered from left to right or top to bottom represent posterior probability values obtained from the majority rule of the original BI tree (blue), and bootstrap values obtained from 2002 raw BI trees using the Consensus tree builder plugging in Geneious Prime (black). The Arabian Gulf clade is highlighted in blue, while the Red Sea clade is highlighted in yellow. The solid black bar represents the 11 delimited MOTUs using the PTP approach. The solid blue bar represents the Saudi Arabian (SA) and East Mediterranean (EMT) populations within the *dispar* MOTU, and the Gizan MOTUs from Saudi Arabia. Labels in blue boldface represent the samples collected in this study from Saudi Arabia. The scale bar indicates 0.02 substitutions per site. *P. mento* is an outgroup species.

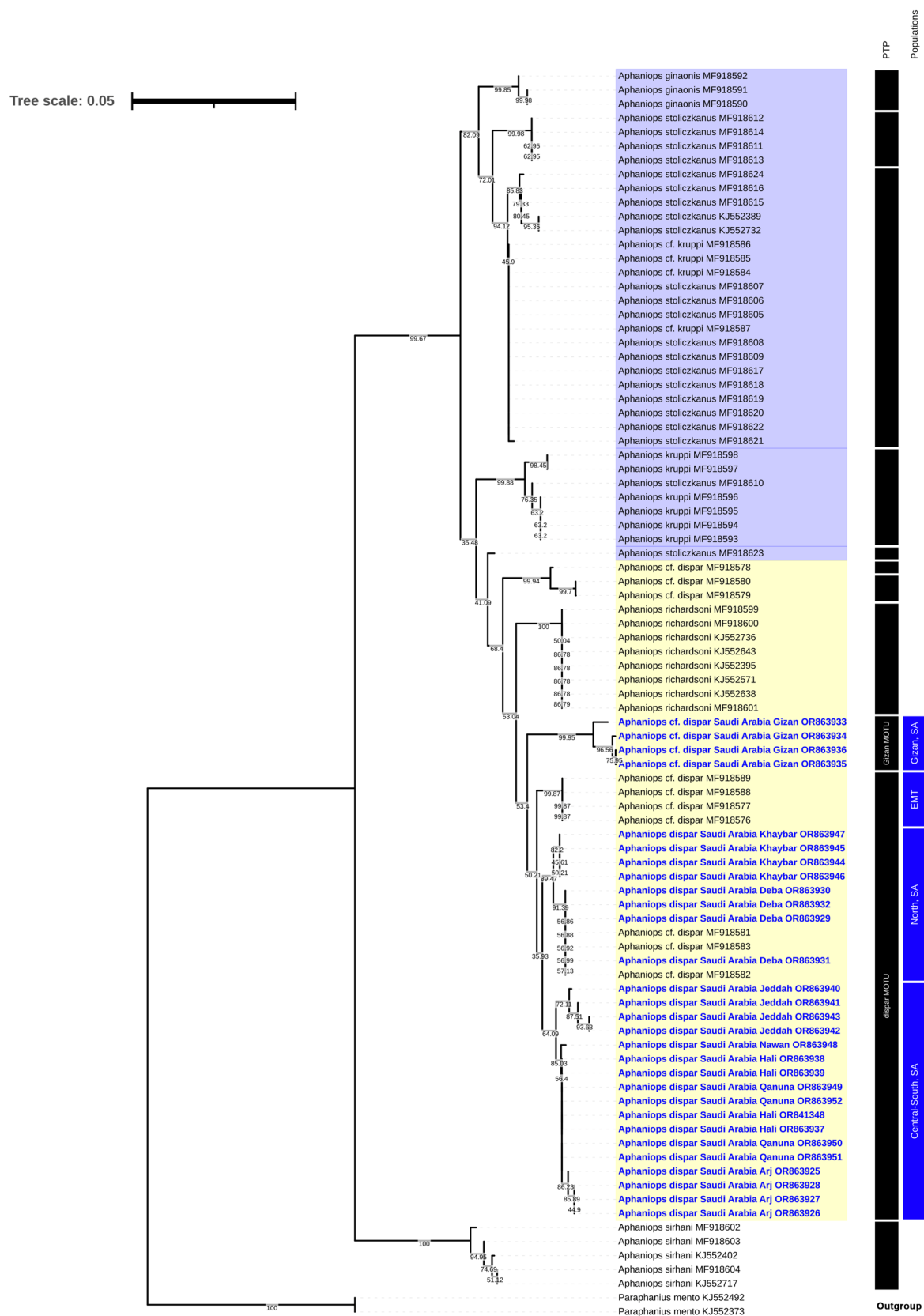


Figure S2.17: Phylogenetic relationships inferred from the mitochondrial COI barcode region using the consensus neighbour joining tree. The tree was constructed using the HKY model. Numbers on nodes represent support values obtained from 10,000 bootstrap replicates. The Arabian Gulf clade is highlighted in blue, while the Red Sea clade is

highlighted in yellow. The solid black bar represents the 11 delimited MOTUs using the PTP approach. The solid blue bar represents the Saudi Arabian (SA) and East Mediterranean (EMT) populations within the *dispar* MOTU, and the Gizan MOTUs from Saudi Arabia. Labels in blue boldface represent the samples collected in this study from Saudi Arabia. The scale bar indicates 0.05 substitutions per site. *P. mento* is an outgroup species.



Figure S3.1: A combination of images shows the visual substrates at different marine locations where the Arabian killifish were observed along the Saudi Arabian Red Sea. Photo credits: Ateah Alfakih.



Figure S3.2: A combination of images shows the visual substrates at different freshwater locations where the Arabian killifish were observed in the western wadis of Saudi Arabia. Photo credits: Ateah Alfakih.

Methods

Apart from the molecular analysis in Chapter 2, the research of this thesis mainly relied on photography-based fieldwork and needed to be controlled, which could not be done in situ due to multiple factors, including environmental (high temperature 40–50 °C, high UV indices, humidity, summer floodings, wind and water turbidity), or ex situ overseas due to logistic limiting factors (transportation of over 500 living fish to the UK). Therefore, I established transportable and cost-effective husbandry and photography setups to ensure that the experiments were controlled across all the study locations. Fish were collected in batches (batch per background per location) using cuboid-shaped nylon traps (L 54 × W 25 × H 25 cm) (Figure S3.3A); each batch contained around 40 fish that were placed into two tanks (Figure S3.3B). The fish were transported by car from the collection sites to husbandry rooms into 12 L tanks filled with water from the collection sites, oxygenated using portable oxygen pumps powered by a 12-volt electricity inverter (Pililong). Fish were then placed into plastic bags containing the water from their original sites and placed into the tanks to acclimate to the new water temperature gradually and then transferred by nets to the tanks; the same process was applied when returning the fish to their original locations. Tanks were washed thoroughly and dried after each batch was released.

The husbandry consisted of a 2-tier shelving unit (D 60 × W 90 × H 90 cm) made of steel and medium-density fibreboards, with a load capacity of 175 kg per shelf (Yaheetech, UK) and two custom-made tanks (L 50 × W 30 × H 40 cm) (Figure S3.3B), with each tank containing around 20 fish. Each tank had filtered dechlorinated fresh water supplied from local desalination and filtration stations. The salinity for the marine Gizan and Jeddah populations was controlled by gradually mixing filtered dechlorinated freshwater with Red Sea salt mix (Red Sea), which was allowed to fully dissolve and measured with a Seawater refractometer (Red Sea). The lighting conditions were maintained at 12: 12 hours from 6:30 AM to 6:30 PM using LED aquarium light sources, and the water temperature was set to 28±1 °C using aquarium heaters with built-in thermometers that were regularly monitored with a temperature controller (Inkbird ITC-306A). Each tank had a collection of hand nets, and the fish were fed commercial fish flakes (Siso Bio-tech CO., LTD).

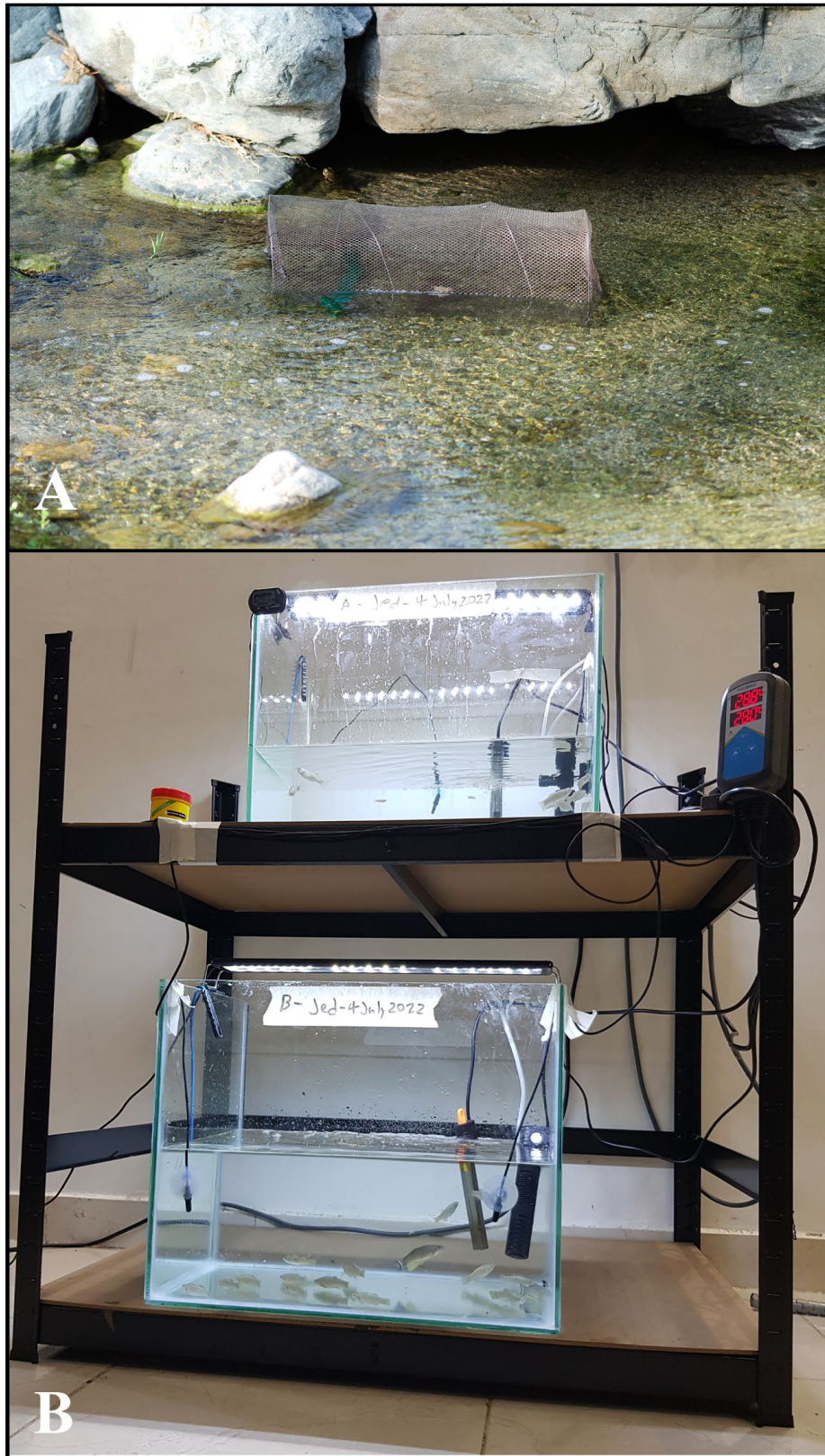


Figure S3.3: The cuboid-shaped nylon trap used for collecting fish submerged in a shallow freshwater stream (A) and the husbandry setup used across the study (B).

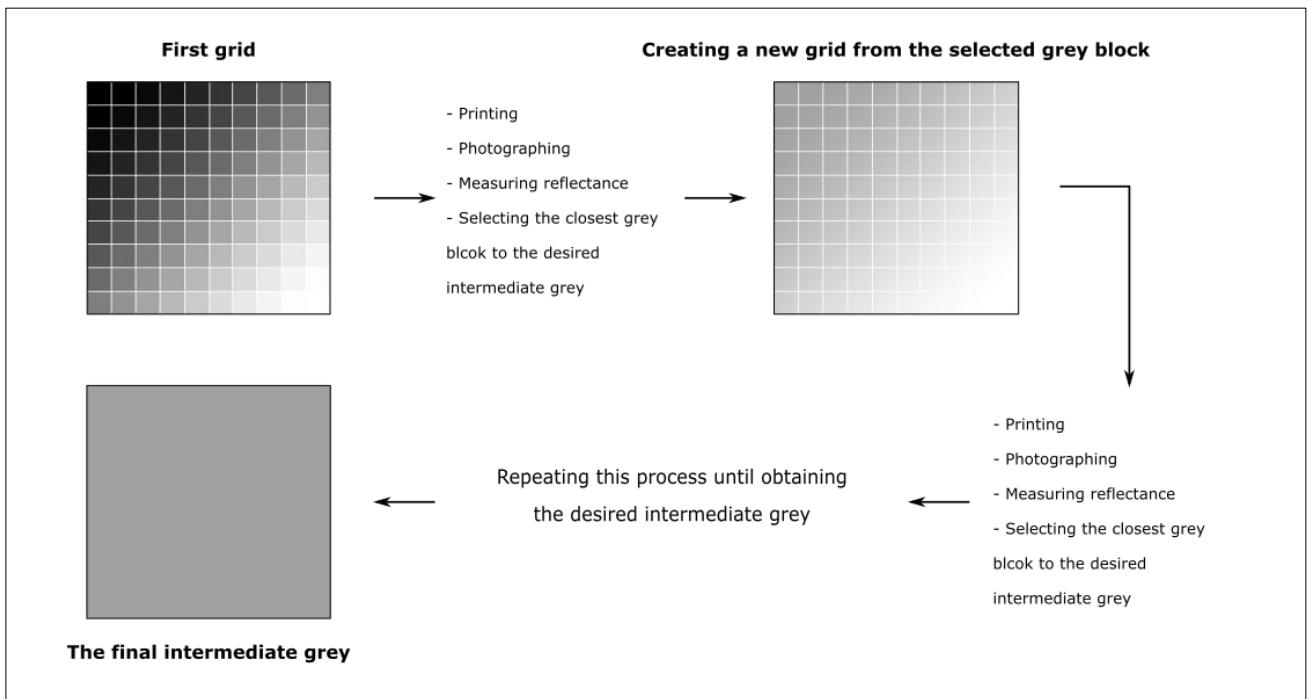


Figure S3.4: Schematic diagram illustrating the process of creating the midpoint grey colour.

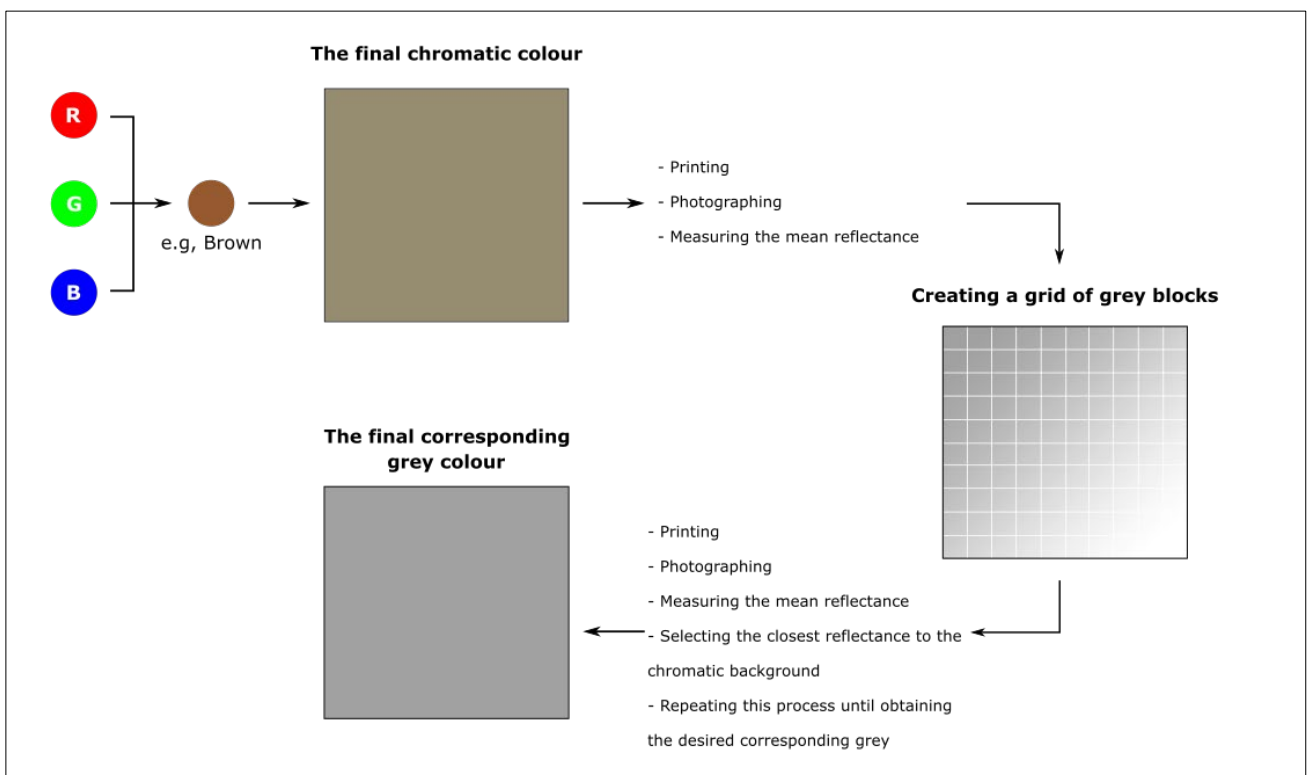


Figure S3.5: Schematic diagram illustrating an example of creating the chromatic and the corresponding achromatic backgrounds with uniform reflectance.

Table S3.1: The RGB values, mean reflectance of the beige, brown and green backgrounds used for the chromatic experiments, and the RGB of the corresponding grey value of these backgrounds as well as their mean reflectance.

Background colour	RGB values of the printed background	Mean reflectance of the printed background	RGB values of the corresponding grey background	Mean reflectance of the printed corresponding grey background
Beige	169; 153; 117	32.4%	132; 132; 132	32.38%
Brown	124; 104; 070	18.91%	106; 106; 106	18.93%
Green	107; 108; 026	16.15%	096; 096; 096	16%



Figure S3.6: A collection of images illustrating the photography setup for Chapters 3 and 4. The top left image shows the EyeColor Arc MT70D lamp next to the custom-built tent. The images of the top right and bottom show the Nikon camera over the photography containers.

Results

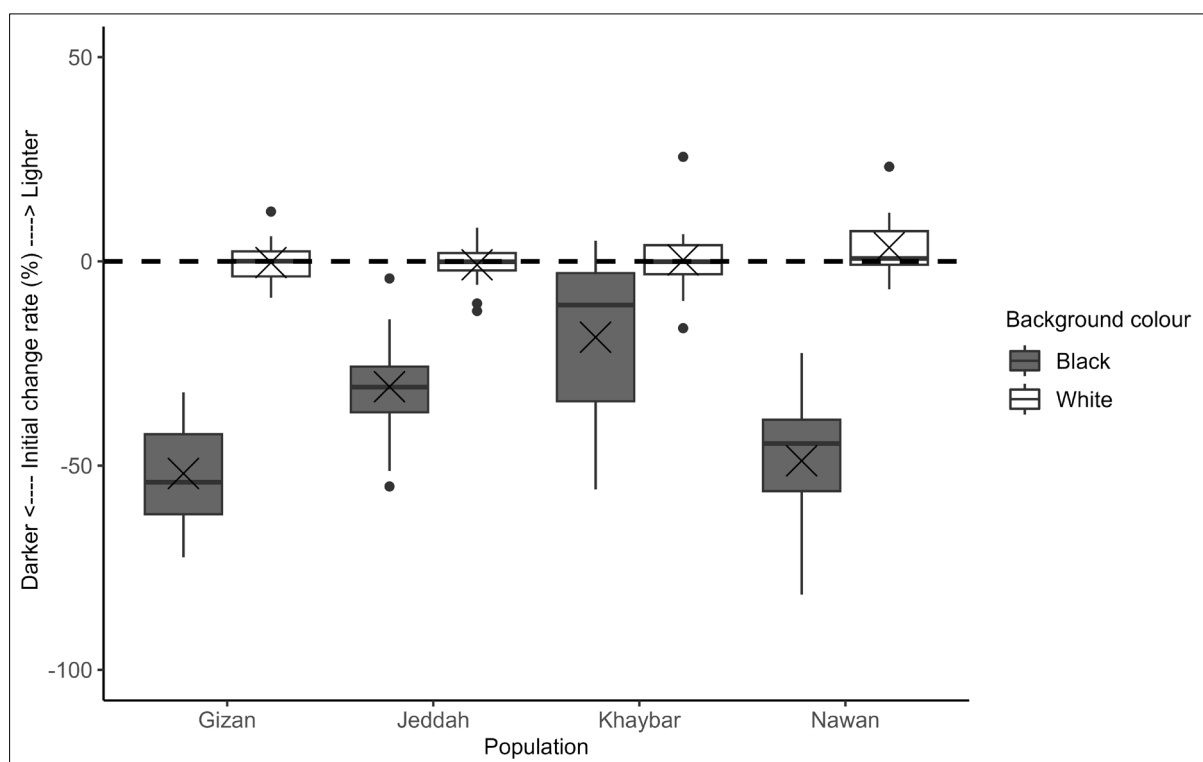


Figure S3.7: Differences among populations in the initial relative change rate $[((Minute\ 01 - Minute\ 00) \div Minute\ 00) \times 100]$ in the achromatic black and white background experiments. Boxes show means (black crosses), medians (black lines) and interquartile ranges, whiskers represent the lowest and highest values within 1.5 of the interquartile ranges, and dots represent outliers. The horizontal black dashed line represents the starting point (0 %) where positive changes indicate lightening responses and negative changes indicate darkening responses.

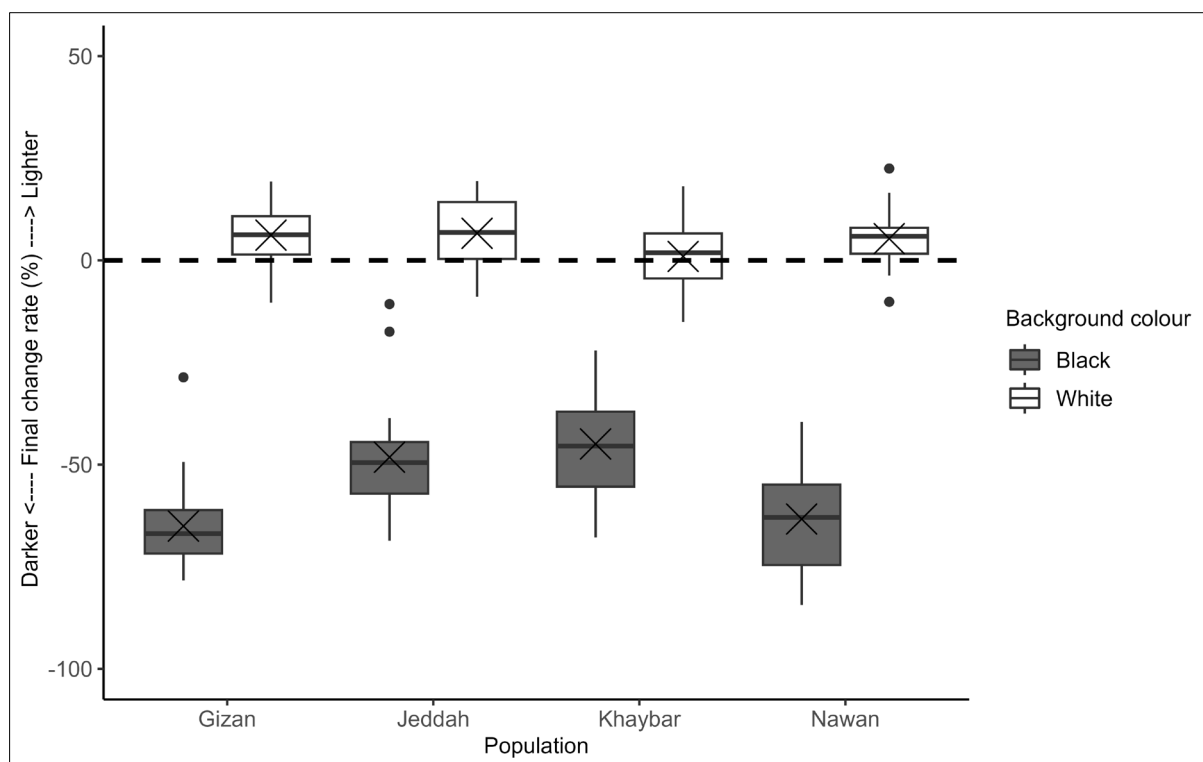


Figure S3.8: Differences among populations in the final relative change rate [$((\text{Minute } 15 - \text{Minute } 00) \div \text{Minute } 00) \times 100$] in the achromatic black and white background experiments. Boxes show means (black crosses), medians (black lines) and interquartile ranges, whiskers represent the lowest and highest values within 1.5 of the interquartile ranges, and dots represent outliers. The horizontal black dashed line represents the starting point (0 %) where positive changes indicate lightening responses and negative changes indicate darkening responses.

Results of fish length effects as an interaction term on luminance, luminance JND and hue

When I added the fish length as an interaction term with population Colour metrics $\sim \text{TestTime} * \text{FishPopulation} * \text{FishLength} + \text{FishSex} + (1|\text{FishID})$, I did not find significant effects of body size on changes in the metrics mentioned above ($\text{TestTime}:\text{FishLength}$). However, I found some near significant effects on the changes over time per population ($\text{TestTime}:\text{FishPopulation}:\text{FishLength}$) for luminance in the beige background experiment ($\log(\text{LMM}): F(9,216) = 1.918, p = 0.0506$). In addition, I found significant effects on the changes over time per population in the green background experiment ($\text{LMM}: F(9,215.028) = 3.035, p = 0.0020$), as well as in luminance JND in the beige background experiment ($\text{LMM}: F(9,216) = 2.076, p = 0.0325$) and in the green background experiment ($\sqrt{\text{LMM}}: F(9,215.030) = 3.226, p = 0.0010$). I also found significant effects of body length on the

changes over time per population for hue in the beige background experiment (LMM: $F(9,216) = 2.792$, $p = 0.0041$). These findings suggested within-population variation in changing abilities due to size differences (Table S3.2). However, due to the small size ($n = 20$ fish per background/population), it is difficult to determine the effects of size on colour changes.

Results of fish length (as a category) effects on luminance and hue change in the beige and green experiments

After finding significant effects on change in luminance, luminance JND and hue when adding the fish length (cm) as a numerical interaction term: Colour metrics ~ TestTime * FishPopulation * FishLength + FishSex + (1|FishID) as seen above, I further categorised the fish length into four size categories based on the standard length (S: < 3.0 cm, M: 3.0–3.99 cm, L: 4.0–4.99 cm and XL: > 5.0 cm). Next, I included the body size as a categorical interaction term: Colour metrics ~ TestTime * FishPopulation * FishSize + FishSex + (1|FishID) to see the effects of the fish length clearly while keeping the transformation of models as the original ones across the study. Post Hoc analyses followed the LMMs via the Tukey's honest significant difference tests to obtain the significant differences in the means of the colour metrics values between the time points within each size category within the populations, as described in the main methods. The results are summarised in Table S3.2.

Table S3.2: Summary of the effects of the fish length on luminance and hue changes within populations in the beige and green experiments. N.S. indicates that no significant change in colour metric values was observed, whereas NA indicates that the size category was absent in the tested population. Numerical values indicate the p values from the Tukey's tests for statistical significance by the end of the experiments, unless otherwise stated between specific minutes (Min).

Population	Fish Size				Treatment
	S	M	L	XL	
Gizan	N.S.	N.S.	N.S.	NA	Luminance in the beige experiments
Jeddah	N.S.	N.S.	$p = 0.0269$	N.S.	
Khaybar	Min 00–01: $p = 0.0496$	N.S.	NA	NA	
Nawan	N.S.	N.S.	N.S.	NA	
Gizan	N.S.	N.S.	$p = 0.0003$	NA	Hue in the beige experiments
Jeddah	N.S.	N.S.	N.S.	N.S.	
Khaybar	Min 00–01: $p = 0.0155$	N.S.	NA	NA	
Nawan	N.S.	N.S.	N.S.	NA	
Gizan	NA	Min 00–08: $p = 0.0018$	$p < 0.0001$	NA	Luminance in the green experiments
Jeddah	N.S.	N.S.	N.S.	Min 00–08: $p = 0.0101$	
Khaybar	N.S.	N.S.	N.S.	NA	
Nawan	N.S.	N.S.	N.S.	NA	

Appendix 3 – Supplementary materials for Chapter 4

Results

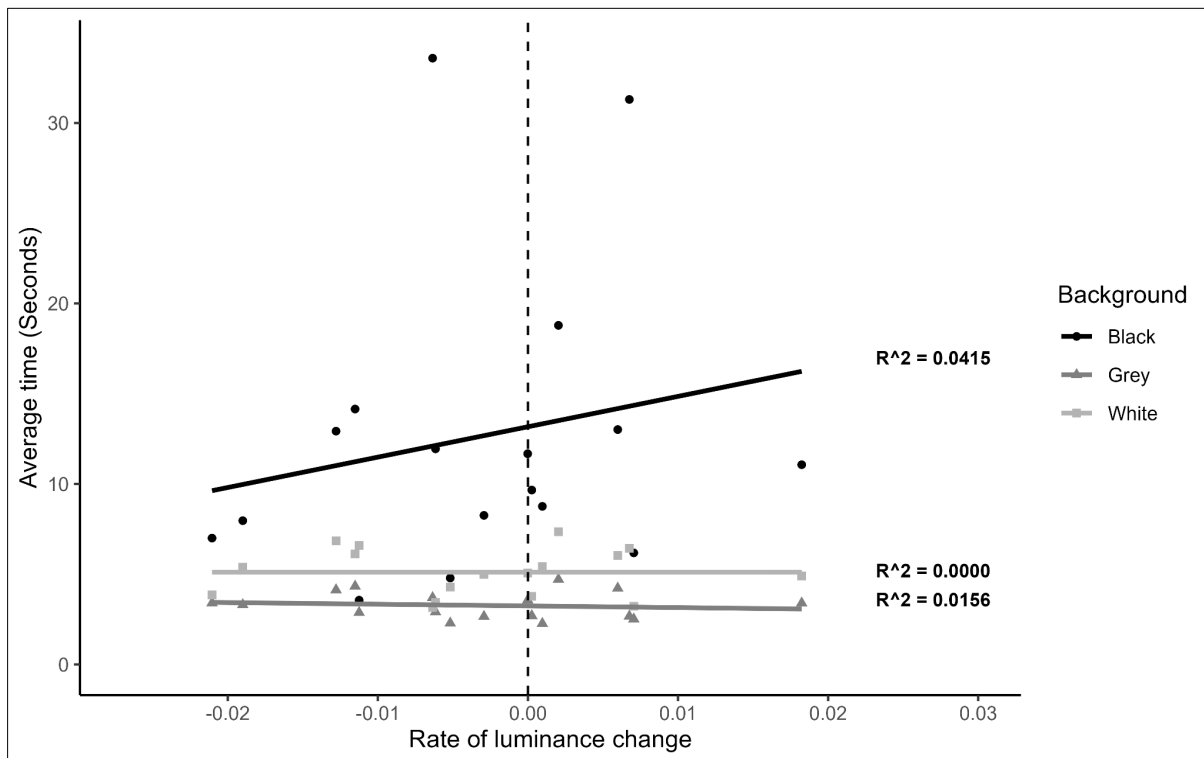


Figure S4.1: A scatter plot showing the relation between the average time spent (seconds) by the freshwater fish on the black, intermediate grey and white backgrounds and the rate of luminance change (luminance after the experiment – luminance before the experiment). Positive values on the x axis indicate the fish increased luminance, while negative values indicate a decrease in luminance. The vertical dashed line denotes the nonchanging point. The coefficient of determination R^2 is displayed alongside the regression lines. The figure shows that there is no strong correlation between the movement of fish and luminance change on all backgrounds ($p > 0.05$).

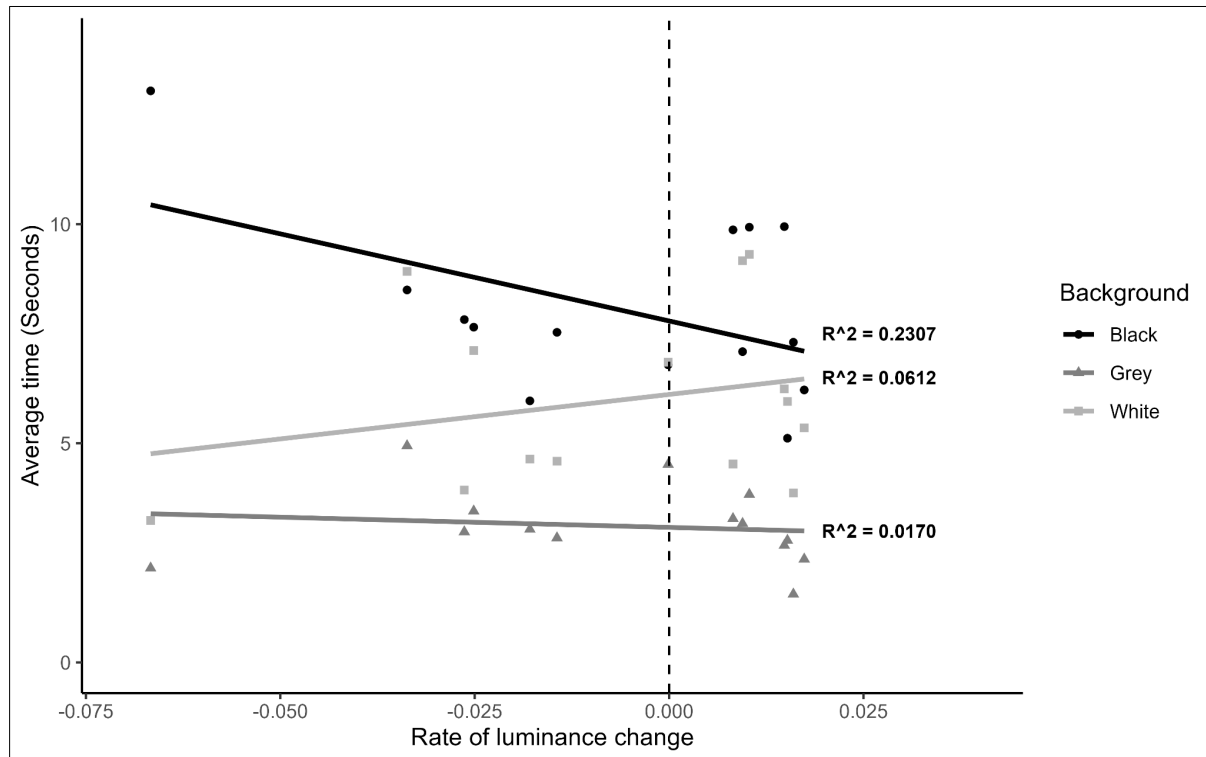


Figure S4.2: A scatter plot showing the relation between the average time spent (seconds) by the marine fish on the black, intermediate grey and white backgrounds and the rate of luminance change (luminance after the experiment – luminance before the experiment). Positive values on the x axis indicate the fish increased luminance, while negative values indicate a decrease in luminance. The vertical dashed line denotes the nonchanging point. The coefficient of determination R^2 is displayed alongside the regression lines. The figure shows that there is no strong correlation between the movement of fish and luminance change on all backgrounds ($p > 0.05$).

Appendix 4 – The physiological cost of colour change: Evidence, implications and mitigations

REVIEW

The physiological cost of colour change: evidence, implications and mitigations

Ateah Alfakih^{1,2,*}, Penelope J. Watt² and Nicola J. Nadeau²

ABSTRACT

Animals benefit from phenotypic plasticity in changing environments, but this can come at a cost. Colour change, used for camouflage, communication, thermoregulation and UV protection, represents one of the most common plastic traits in nature and is categorised as morphological or physiological depending on the mechanism and speed of the change. Colour change has been assumed to carry physiological costs, but current knowledge has not advanced beyond this basic assumption. The costs of changing colour will shape the evolution of colour change in animals, yet no coherent research has been conducted in this area, leaving a gap in our understanding. Therefore, in this Review, we examine the direct and indirect evidence of the physiological cost of colour change from the cellular to the population level, in animals that utilise chromatophores in colour change. Our Review concludes that the physiological costs result from either one or a combination of the processes of (i) production, (ii) translocation and (iii) maintenance of pigments within the colour-containing cells (chromatophores). In addition, both types of colour change (morphological and physiological) pose costs as they require energy for hormone production and neural signalling. Moreover, our Review upholds the hypothesis that, if repetitively used, rapid colour change (i.e. seconds–minutes) is more costly than slow colour change (days–weeks) given that rapidly colour-changing animals show mitigations, such as avoiding colour change when possible. We discuss the potential implications of this cost on colour change, behaviour and evolution of colour-changing animals, generating testable hypotheses and emphasising the need for future work to address this gap.

KEY WORDS: Physiological cost, Physiological colour change, Morphological colour change, Chromatophores, Behaviour, Phenotypic plasticity

Introduction

Colour change is a widespread strategy for many animals (Cott, 1940), which occurs during development (ontogeny) or throughout the animal's life as a form of phenotypic plasticity. Colour change performs several functions in changing environments, including thermoregulation (Smith et al., 2016), concealment (Duarte et al., 2017), signalling (Stuart-Fox and Moussalli, 2008; Ligon and McGraw, 2016) and protection from harmful ultraviolet (UV) radiation (Garcia et al., 2004). Colour change is generally mediated by colour-containing cells called chromatophores (see Glossary

(Bagnara and Hadley, 1973), and this change falls into two categories depending on the mechanism and speed (Bagnara and Hadley, 1973; Duarte et al., 2017). Morphological colour change is mainly defined by quantitative and qualitative changes in chromatophores (Thurman, 1988; Stuart-Fox and Moussalli, 2009), based on the synthesis and degradation of pigments (Bagnara and Hadley, 1973) occurring over long periods, ranging from days to months (Thurman, 1988; Umbers et al., 2014). In contrast, physiological colour change is the redistribution of pre-existing pigments in the integument and occurs over shorter time frames, from milliseconds to hours (Duarte et al., 2017) (Box 1). Both types of colour change can be under hormonal and neural control or a combination of the two (reviewed in Sköld et al., 2016; Duarte et al., 2017).

Growing interest in colour change has yielded a better understanding of the cues, mechanisms and benefits of this plastic trait. However, although plastic traits have clear benefits to organisms, through optimising their fitness across different environments, they also incur costs (DeWitt et al., 1998; Relyea, 2002). Generally, the costs of colour change have had minimal attention. The physiological cost associated with colour change was debated in the 1950s, but was restricted to the energy requirements of the mechanism pertaining to pigment dispersion and aggregation in the integument (see Horowitz, 1958, and references therein). Yet, the most discussed physiological cost of coloration in the literature is the cost of pigment and colour production, but not colour change per se (see Webster et al., 2018).

Recently, changing colour has been appreciated to carry some physiological (i.e. energetic and metabolic) costs based on a number of assumptions. First, there is a cost associated with pigment production, as synthesising pigments may require energy and can depend on dietary components (Sköld et al., 2016; Stevens, 2016), making pigment utilisation energy demanding or resource limited (Hill, 1996; 2000). Some pigments used for colour change, such as carotenoids, might be more costly than others (Hill, 1996) or may be used for other internal functions such as the immune system (Sköld et al., 2016; Duarte et al., 2017), and thus a cost emerges from trade-offs of allocating resources, which would ultimately affect the physiological state (Polo-Cavia and Gomez-Mestre, 2017). Furthermore, the catabolism of pigments is also suggested to carry some cost (Polo-Cavia and Gomez-Mestre, 2017). Second, intracellular translocation of pigments (Kang et al., 2016) and neuromuscular-mediated colour change (see Glossary) are considered energy-depleting activities (Sköld et al., 2012). Finally, maintaining physiological and morphological colour change can be a costly process, as it necessitates continuous monitoring of the environment and hence continuous optimisation of coloration depending on the changing circumstances, such as changes in visual properties (Hanlon et al., 1999; Stuart-Fox and Moussalli, 2009; 2011; Stevens and Ruxton, 2019).

Generally, colour change may drain energy during neural processing in rapidly changing animals, as encoding and

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Glossary**Chromatophores**

Colour-containing cells that are classified broadly according to the colour of the pigment they contain.

Neuromuscular-mediated colour change

A change in the chromatophore organs (not cells) found in cephalopods, which is regulated via a neural pathway, by which relaxation and contraction of radial muscles surrounding these chromatophore organs causes a colour change.

Melanophore

A type of chromatophore containing the pigment melanin, which is generally black or brown and packaged within organelles called melanosomes.

Background matching

The situation where an animal bears a general resemblance to the colour, brightness and patterns of the background.

Disruptive coloration

The situation where an animal possesses markings that generate false edges that make the observer unable to detect or recognise the animal.

Masquerade

The situation where an animal resembles the shape of an uninteresting or inedible object in its environment (such as a stick) to make the observer unable to recognise it.

transmitting information is expensive (Stevens, 2013), and hence a considerable amount of energy is depleted during neural signalling (Laughlin, 2001; Niven and Laughlin, 2008). Also, hormones are essential components in motility and the increase and decrease in chromatophore number in both types of colour change (Leclercq et al., 2009; Bertolesi and McFarlane, 2020; Yang et al., 2021), and thus production and maintenance might ultimately pose energetic costs. However, current knowledge of the physiological cost of colour change has not advanced beyond these assumptions, and a number of questions remain unresolved (Sköld et al., 2016; Stevens, 2016; Duarte et al., 2017). In this Review, we assess the direct and indirect evidence of the physiological cost of colour change, from the molecular to the population level, in a wide range of taxa. We also discuss the potential ecological and evolutionary implications of colour change being physiologically costly, generating testable hypotheses.

We limit our Review to animals that use chromatophores for colour change, meaning that the colour is contained within living cells and tissues (unlike in birds and mammals, where most colour is contained in dead integumentary structures of hair or feathers, and colour change requires moulting). We discuss evidence from a wide range of species including vertebrates, such as fish, amphibians and reptiles, as well as invertebrates, including crustaceans and cephalopods (see Table 1). Colour-change mechanisms differ between taxa, especially vertebrates and invertebrates (Umbers et al., 2014; Ligon and McCartney, 2016), and this may affect the associated costs, but we attempt to draw general conclusions that will be applicable across taxa.

Evidence for a physiological cost of colour change**Direct evidence**

There are a few lines of direct evidence supporting the hypothesis that colour change incurs a cost. On the cellular level, a few studies have stated that molecular motors such as myosin, kinesin and dynein, responsible for translocating pigment organelles within cells, at least in melanophores (see Glossary), depend on energy from ATP hydrolysis to move pigments from and towards the centre of the cells (reviewed in Aspengren et al., 2009; Sköld et al., 2016). For example, the depletion of ATP from fish melanophores has been

shown to cause complete cessation of pigment granule movement (Rodionov et al., 1998).

Rodgers and colleagues (2013) were the first to provide direct evidence of physiological costs associated with colour change from behavioural experiments on guppies (*Poecilia reticulata*). When fish were induced to change colour over a period of days, they were found to consume more food, which may imply compensation for the energy expended. Generally, diet per se acts as a constraint such that colour change is contingent upon the availability and acquisition of dietary-pigment sources. It may be that fish consumed more food to compensate for low pigment availability, where food represents a constraint (Duarte et al., 2017). If this is the case, we predict that fish that darken their body will require more food than individuals that lighten their bodies. However, it is also possible that the everyday alternation between black and white backgrounds in Rodgers et al.'s (2013) study might have caused a stress response (Duarte et al., 2017). It has been suggested that metabolic measurements would help quantify the energetic expenditure associated with colour change (Duarte et al., 2017), and using a waterborne hormone sampling approach to detect cortisol might be useful to determine whether a stress response occurs.

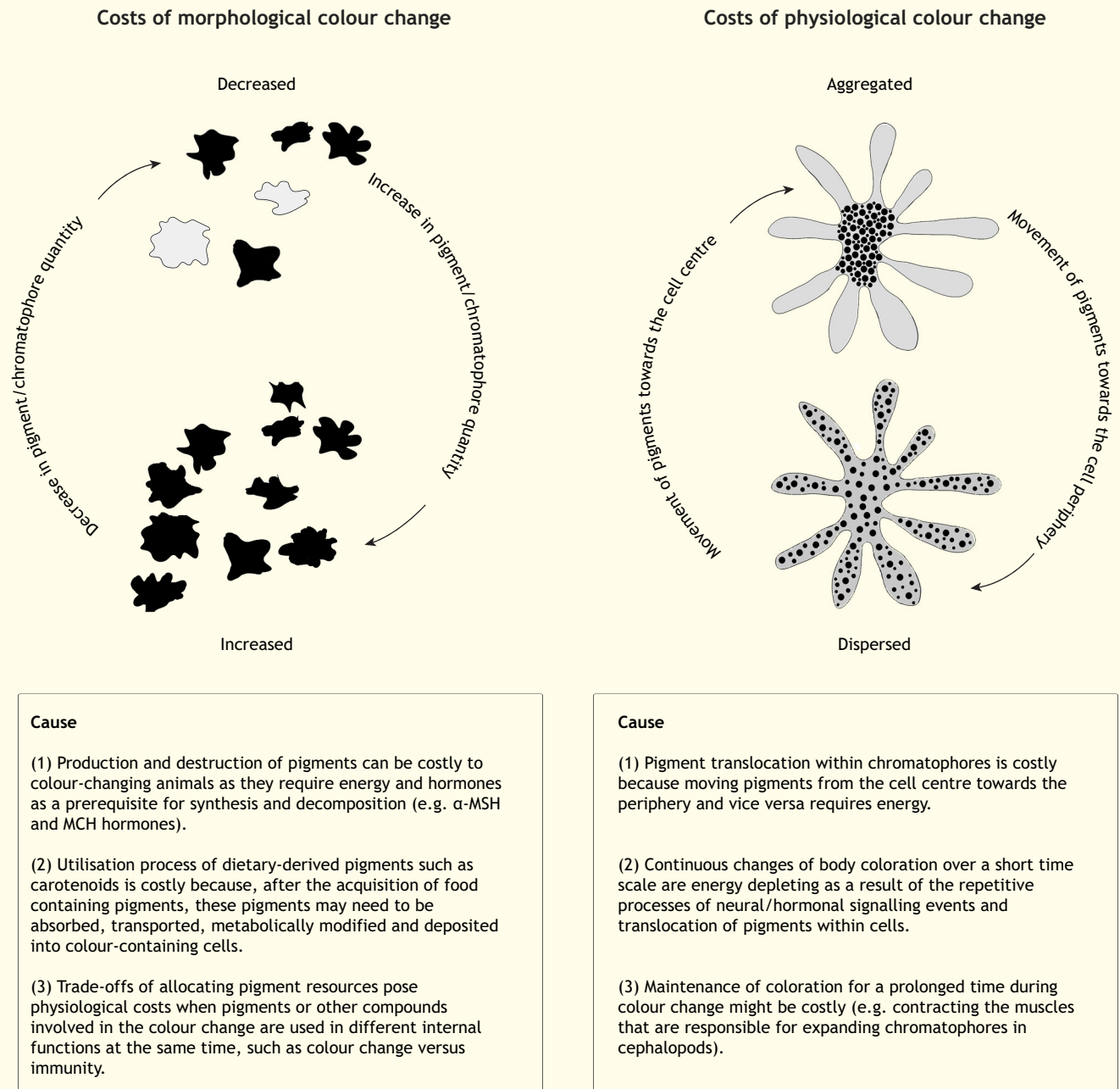
To the best of our knowledge, only one study has successfully determined a metabolic cost associated with colour change. Polo-Cavia and Gomez-Mestre (2017) showed that larvae of Bosca's newt (*Lissotriton boscai*) changed their colour with respect to the background on which they were placed over 2 weeks, and those that produced dark pigment when placed on black exhibited a higher metabolic rate than those placed on a white background, which reduced their pigment. This implies that changing colour in terms of producing pigments in the integument is accompanied by some physiological costs. However, this does not tell us about the costs of colour change per se, and it is still unknown whether the destruction of pigments during colour change poses a cost, or whether dispersion is more costly than the aggregation of pigments (Horowitz, 1958; Fan et al., 2014), though they both require energy (Iga and Bagnara, 1975; 1976). Thus, measuring metabolic rate in species changing colour morphologically and physiologically would be of help. For example, individuals could be assigned to a midpoint starting substrate with uniform colour and then allowed to change to dark and light backgrounds to demonstrate the costs in both directions.

Indirect evidence

Despite the small number of studies pertaining to the physiological cost of colour change, there is more indirect evidence. Observations of rapidly colour-changing octopuses have found that these animals do not show highly cryptic body patterns at all times, and it has been suggested that maintaining maximum crypsis in synchrony with heterogeneous backgrounds is neurophysiologically expensive (Hanlon et al., 1999). Further, studies on dwarf chameleons uphold this suggestion, as several species (*Bradypodion* spp.), rather than exhibiting and maintaining highly camouflaging patterns, seem to facultatively respond only when predators are present (Stuart-Fox et al., 2006; 2008; Stuart-Fox and Moussalli, 2009).

Many colour-changing animals preferentially select visually similar habitats that match their own coloration. For example, the rockpool goby (*Gobius paganellus*) can change colour rapidly, within 1 min, but is better able to match certain backgrounds than others (Stevens et al., 2014a; Smithers et al., 2018). These gobies were also shown to display a strong preference for backgrounds that

Box 1. Schematic diagram of the chromatophore changes in morphological and physiological colour change, and the potential causes of the costs for each.



Under morphological colour change, there is a decrease or increase in the quantity of the pigments or chromatophores in the integument. Under physiological colour change, pigments within the chromatophore change from aggregated to dispersed states.

are easier to match using colour change (Smithers et al., 2018). Slowly colour-changing taxa, such as fish (Rodgers et al., 2013), amphibians (Polo-Cavia and Gomez-Mestre, 2017) and crustaceans (Green et al., 2019), also show habitat preference that presumably maximises their camouflage. These findings suggest that the strong preference for certain backgrounds diminishes the need for costly colour change. Alternatively, certain colours might be more costly to exhibit than others, or matching the visual properties of the backgrounds of certain colours and textures could be beyond the

ability of these animals and, therefore, using behavioural choice is a better option to improve their camouflage.

Some pigments needed for colour change cannot be synthesised *de novo*. Carotenoids, which vary in colour from red to yellow, are among the most common pigments found in plants, but most animals must obtain them through their diet (Bagnara and Hadley, 1973). These pigments can either be deposited in the integument directly or modified metabolically to other derivatives of carotenoids (Maoka, 2019). Important physiological costs

Table 1. Summary of the potential ways that animals can avoid or minimise the physiological costs of morphological and physiological colour change, with examples from different taxa

	How mitigation is achieved	Type of colour change	Example	Time to achieve colour change	Reference
Facultative colour change	Animals express body coloration that may or may not confer some degree of function but is less physiologically costly. Therefore, animals may change colour only when needed, such as when signalling for conspecifics or facing instantaneous threats.	Physiological	Octopus Fish Chameleons	Milliseconds Minutes Seconds	Hanlon et al., 1999 Burmeister et al., 2005 Stuart-Fox et al., 2006 Stuart-Fox et al., 2008 Stuart-Fox and Moussalli, 2009
Energy conservation	Animals can minimise the energetic costs resulting from colour change by:				
	(1) Changing to a less costly state in rhythmic patterns when the costly colours are not needed.	Physiological	Crabs Salamanders	Hours	Powell, 1962b Darnell, 2012 Stevens et al., 2013 Garcia and Sih, 2003 Garcia et al., 2004
	(2) Changing colour only in situations in which changing colour is affordable (condition dependent), causing variation in the colour change ability between and within individuals over time. However, the costly colour change may be selected against and lead to variation in the ability to change colour within populations.	Physiological Morphological	Shrimps Crabs Frogs Prawns Frogs	Hours Weeks	Siegenthaler et al., 2018 Stevens et al., 2014b Kang et al., 2016 Duarte and Flores, 2016 Wente and Phillips, 2003
	(3) Deploying strategies that functionally replace colour change but are less costly, such as burying or decoration.	Physiological Morphological (ontogenetic, not reversible)	Cuttlefish Shrimps Fish Crabs	Milliseconds Hours Seconds Weeks	Allen et al., 2010 Siegenthaler et al., 2018 Tyrie et al., 2015 Hultgren and Stachowicz, 2008 Hultgren and Stachowicz, 2009
Habitat selection	Animals may lessen the physiological cost of colour change by:	Physiological	Fish	Seconds	Smithers et al., 2018 Tyrie et al., 2015
	(1) Avoiding mismatched habitats to prevent the need to change colour. (2) Choosing substrates that grant less costly colour change (i.e. choosing dark backgrounds when changing to/ maintenance of dark colours is less costly). Selection of habitats is linked to the speed of colour change where slowly colour-changing animals show higher preference than rapidly colour-changing animals.	Morphological	Prawns Fish Newts	Weeks	Green et al., 2019 Rodgers et al., 2013 Polo-Cavia and Gomez-Mestre, 2017
Shift mechanism	The above approaches constitute short-term solutions to avoid or minimise the costs of colour change. Animals that use physiological colour change may shift to morphological colour change (pigmentation or depigmentation) if they are under a relatively long-term pressure that causes repetitive colour change for prolonged periods.	Physiological	Crabs Fish Frogs and salamanders	Hours	Powell, 1962b Bergstrom et al., 2012 Bertolesi and Mcfarlane, 2020 Babák, 1913 Bertolesi et al., 2016

associated with dietary-derived pigments are: (i) their acquisition (searching for and obtaining food), (ii) their utilisation (the internal processes from absorption to deposition in the skin) (Hill, 1996; 2000) and (iii) trade-offs with other processes that require these compounds (e.g. immune function) (Duarte et al., 2017).

Beyond the acquisition of pigment resources, which may require some energy, the utilisation process of these pigments may be more costly. The utilisation of pigments involves multiple stages, including absorption, transport, metabolic modification and deposition into colour-containing cells, each of which requires energy (Hill, 1996; 2000). The type of pigment derived from

different foods might vary in costliness in colour-changing taxa relying on diet. For example, red carotenoids might be physiologically more costly to obtain than others (Hill, 1996).

Chameleon prawns (*Hippolyte varians*) display slower colour change when shifting from green to red colour than from red to green (Duarte et al., 2017; Mynott, 2019), especially in larger individuals (Green et al., 2019). This might be due to the utilisation of costly red carotenoids, particularly in larger individuals that require more. Many carotenoids found in aquatic animals are metabolites of other types of carotenoids present in food sources (Maoka, 2019). Lee (1966a,b) successfully isolated the pigments

from green, brown and red individuals of two species of colour-changing isopods (*Idotea* spp.) and found evidence for the modification of red carotenoids; the final displayed red pigments in these taxa were derivatives of the original carotenoids found in the algal food source.

These examples together may imply that the red carotenoids involved in colour change are more energetically costly than the other types of pigments or could have pleiotropic effects on other internal functions. Thus, investigating non-changing and changing individuals in systems that rely on diet for colour change would highlight whether there is a pronounced differential physiological cost associated with the colour-change process or whether this is specific to one direction of colour change.

Implications

Depending on the time scale and frequency of colour change, there are several short- and long-term implications. At the individual level, these could be mitigated by physiological and behavioural strategies. At the population level, microevolutionary changes due to selection against costly colour change and in favour of alternative strategies could result in variation in colour change within and between populations and individuals (Table 1).

Facultative colour change

Changing colour is expected to be manifested only when the benefits, such as avoiding instantaneous threatening situations, outweigh the costs, including energetic (Stuart-Fox et al., 2008; Stuart-Fox and Moussalli, 2009) and social costs. Facultative colour change is stimulated by interspecific and intraspecific interaction between animals. As discussed, crypsis in octopuses and dwarf chameleons is not maximised at all times when predators are absent, which might be a less costly strategy compared with maintaining crypsis for long periods. Moreover, some species of dwarf chameleons adjust their coloration according to the perceived threat; for example, they respond to birds and snakes in a different way and invest more in colour change when faced with avian predators, which are thought to have better vision, by showing a better match to their backgrounds (Stuart-Fox et al., 2006; 2008; Stuart-Fox and Moussalli, 2009). Alternatively, although used for crypsis, changing colour facultatively might have evolved primarily to facilitate social interactions (Stuart-Fox and Moussalli, 2008) and can be used to signal to potential mates, or to avoid social costs such as receiving increased aggression from conspecifics when dishonestly signalling dominance, as in chameleons (Ligon and McGraw, 2016) and fish (Burmeister et al., 2005).

However, changing colour facultatively seems to be strategy dependent and might be confined to rapidly colour-changing organisms (i.e. those that change colour within seconds to minutes) faced with an immediate threat or higher social costs (Table 1), and is less relevant to slowly colour-changing animals. Studies on amphibians revealed that predator cues do not show clear effects on colour change (Garcia and Sih, 2003; Polo-Cavia and Gomez-Mestre, 2017). In theory, slow colour change for crypsis might have evolved to tackle temporally predictable fluctuations in microhabitats (Caro et al., 2016).

Energy conservation

One potential way to offset the physiological cost incurred during colour modification is to retract the process to a less costly state when pigments are not needed. This can be achieved via relaxing the muscles of chromatophore organs, as seen in cephalopods (Hanlon et al., 1999), or modifying the expressed colours, as seen in

chameleons (Stuart-Fox and Moussalli, 2009), to reduce energy expenditure when predators are absent (Stevens, 2016). Many animals display a rhythmic form of physiological colour change (Abbott, 1973; Thurman, 1988; Darnell, 2012; Duarte et al., 2017). The day–night circadian rhythm in colour change may reflect a physiological cost resulting from the maintenance of the chromatophores in a dispersed or aggregated state (Darnell, 2012), and changing colour at night could be a way to conserve energy (Stevens, 2016; Duarte et al., 2017).

The shore crab (*Carcinus maenas*) can change colour in accordance with brightness of the background within ~2 h (Powell, 1962a; Stevens et al., 2014b), but it has also been shown to undergo an endogenous rhythmic colour change, becoming darker during the day and lighter during the night (Powell, 1962b). A similar photoperiodic pattern was found in the subtropical fiddler crab (*Uca panacea*) (Darnell, 2012). The function of changing colour in these organisms may be to confer protection against potential harm from UV radiation during the day. For example, observations on salamander larvae (*Ambystoma* sp.) indicated that they are darker during the daytime and lighter at night (Garcia and Sih, 2003). When further examined, larvae were found to darken their body when exposed to UV radiation (Garcia et al., 2004). From these examples, blanching of pigments at night may offset the physiological cost of maintaining the dispersed state of colour cells when colour seemingly is not needed.

Although a darker appearance displayed during the day appears logical because it may protect animals against UV, rhythmic colour change is still perplexing because some animals show the opposite pattern. For instance, the horned ghost crab (*Ocypode ceratophthalmus*) becomes lighter during the day and darker during the night, a rhythmic change that might maximise camouflage over 24 h (Stevens et al., 2013). Regardless of what type of colour appears during the day or night, colour-changing species showing cyclic changes in colour are assumed to utilise this to save energy, but no studies have examined this (Stevens, 2016). However, some studies have argued over this point, with contrasting conclusions regarding whether dispersing or aggregating pigments is more energetically costly (Horowitz, 1958; Fan et al., 2014). Moreover, it remains undetermined whether chromatophores in a dispersed or aggregated state are active or passive (i.e. whether energy is required to maintain the chromatophores in a dispersed or aggregated state; see Parker, 1935).

Habitat choice

When colour change is likely to be physiologically costly, it is hypothesised that habitat choice will mitigate the potential cost by two means: (i) avoiding mismatched habitats to prevent the need to change colour (Rodgers et al., 2013; Polo-Cavia and Gomez-Mestre, 2017) and (ii) choosing habitats that meet the colour-changing ability of animals when forced to alter coloration (Polo-Cavia and Gomez-Mestre, 2017). Broadly, rapidly colour-changing animals are predicted to lack strong habitat preferences, as they can cope with the heterogeneity through fast colour change compared with slowly colour-changing animals (reviewed in Stevens and Ruxton, 2019).

To some extent, this prediction about habitat preference seems to be true when comparing dynamically colour-changing organisms that differ in their colour-changing ability as a consequence of morphological constraints (i.e. the types of patterns and colours they are able to produce). Cephalopods appear to lack a preference for particular habitats (Allen et al., 2010; Hanlon et al., 2011), and this may largely be the result of the repertoire of their patterns that serve

different camouflaging strategies, such as background matching, disruptive coloration and masquerade (see Glossary) (Hanlon et al., 2009; Chiao et al., 2011), but the continuous changes are considered to be neurophysiologically costly (Hanlon et al., 1999).

Allen and co-workers (2010) studied the physiological cost of colour change in cuttlefish (*Sepia officinalis*) through sets of behavioural experiments to determine whether different body patterns – uniform, mottle and disruptive – differed in cost. They allowed individuals to change their coloration and choose between backgrounds that provoked the three types of patterns or a mixture of them. The researchers did not find support for the preference of cuttlefish for one type of background or body pattern over the others and concluded that the physiological cost of colour change between the three types of camouflage patterns was negligible. However, the time frame in this study was relatively short, ranging from 8 to 12 min. Thus, it is possible that the maintenance of certain pattern types for longer periods is more costly than the expression itself. In cephalopods, the expansion and retraction of chromatophores is governed by contracting and relaxing radial muscles around them (Bagnara and Hadley, 1973), which is likely to be an energy-consuming mechanism. The cost in these systems may be minimised in the absence of habitat preference through displaying conspicuous or moderately camouflaging patterns and/or through hiding inside dens (e.g. octopus; Hanlon et al., 1999) or beneath sand (e.g. cuttlefish; Allen et al., 2010) to avoid detection.

However, many rapidly colour-changing animals lack the exceptional capabilities of cephalopods. For example, the peacock flounder (*Bothus lunatus*) can change its pattern within 3 s, yet still actively chooses certain backgrounds that match the patterns it is able to produce, such as sand, rubble and dead corals (Tyrie et al., 2015). Moreover, the rockpool goby, *G. paganellus*, can adjust its colours within minutes and yet has limited ability to match some backgrounds (Stevens et al., 2014a; Smithers et al., 2018) and, when given a choice, prefers substrates that are easier to align with through colour change (Smithers et al., 2018). This may imply that some types of colours and patterns in habitats are difficult to replicate and are limited by physiological and morphological constraints (Stuart-Fox and Moussalli, 2011) or that some colours or patterns are more costly to produce than others; therefore, animals may choose substrates that grant less costly colour change or substrates that may allow them to dispense with colour change such as soft sands (Tyrie et al., 2015; Siegenthaler et al., 2018).

Behavioural choice of colour-matching substrates in slowly colour-changing taxa is crucial because it aids the efficacy of colour change given its slower pace. Selecting the appropriate microhabitats requires continuous modification of the choice as the colour changes (Stevens and Ruxton, 2019). For example, the chameleon prawn, *H. varians*, which shifts between green and red coloration within weeks, strongly selects a substrate to match its colour when given the choice (Green et al., 2019). As previously mentioned, changing to the red morph is slower – probably because of the utilisation of the red carotenoids – which would increase the mismatch intervals, potentially leading to higher predation risk.

In other slowly colour-changing animals, there are varying patterns of bias towards which background is chosen (see Stevens and Ruxton, 2019, for further details), which may indicate differences in strategies employed by animals to mitigate the physiological cost within and between species. For example, when guppies, *P. reticulata*, acclimatised to black and white backgrounds, they displayed a preference for black substrates but no clear choice of white (Rodgers et al., 2013). In contrast, larvae of the newt *L. boscai* preferred white over black substrates, with the latter

proving more costly to change colour to (Polo-Cavia and Gomez-Mestre, 2017), as discussed earlier. Hence, the choices in these cases may be to mitigate the physiological costs incurred during the colour-changing processes, but in different directions.

However, there is still a need for studies to address how habitat preference would make up for the physiological costs incurred by colour-changing animals, in conjunction with measuring the metabolic rate, as done by Polo-Cavia and Gomez-Mestre (2017).

Individual variation

The physiological cost of maintaining a certain chromatophore state (e.g. fully dispersed pigments) in the integument may have an impact on the evolution of colour change. Variation in the capacity of colour change between individuals occurring in nature may be maintained by differential physiological costs within populations. Therefore, in a prey–predator context, the differences in cost may drive the evolution of other antipredator responses, such as having a fixed appearance with reliance on behavioural avoidance or other strategies. Intrapopulation variation in colour-changing ability has been documented in the Japanese tree frog, *Dryophytes japonicus* (Kang et al., 2016), and the shore crab *C. maenas*, which can change colour within an hour and over several hours, respectively, with some individuals easily changing colour and others showing almost no change (Stevens et al., 2014b).

The co-existence of colour-changing and non-changing morphotypes within populations has been documented in some species, such as Pacific tree frogs (*Pseudacris regilla*) (Wente and Phillips, 2003) and prawns (*Hippolyte obliquimanus*) (Duarte and Flores, 2016), and may possibly occur in other species (e.g. chameleon prawns, *H. varians*; Duarte et al., 2017). Although the literature seems lacking in studies on interpopulation variation in colour change, there is evidence of this from a few studies (e.g. Stuart-Fox et al., 2006; Whiteley et al., 2009; Cadena et al., 2017). Further investigation is warranted on whether and how physiological costs might maintain variation within and between populations. Euryhaline species, such as fish living in both saline and freshwater ecosystems, are excellent models for testing this given the substantial differences in abiotic and biotic components of their habitats.

There are two potential explanations for variation in colour-change ability within populations that would implicate physiological costs. It could be a consequence of opposing selection pressures for higher colour-changing capacity and reduced physiological cost (Kang et al., 2016). Alternatively, in the short-term, variation in colour change might be condition dependent (i.e. depend on energy availability), such that only individuals in optimal condition can undergo colour change (Stevens et al., 2014b). Equally, the presence of these changing and non-changing morphotypes and their frequencies may not necessarily reflect the involvement of a physiological cost of colour change as a selective pressure but, rather, they could be traded against other types of costs (e.g. opportunity cost) and linked to the locomotion patterns (i.e. mobile versus motionless) and other lifestyles such as habitat use (Duarte et al., 2016).

The extent to which these assumptions are met is not yet understood. There is evidence for trade-offs between anti-predator strategies in some closely related species of crabs (Hultgren and Stachowicz, 2008; 2009). These crabs can change colour according to the algal substrate over some weeks and can also camouflage themselves by utilising decoration within hours to days. Yet, there was a negative correlation between the magnitude of colour change

and decoration within and between the crab species: species that showed little decoration had a higher capacity for colour change, while those that were more decorated exhibited less capacity for colour change; species that were moderately decorated were intermediate in their colour-change capabilities. Presumably, the driver of intraspecific and interspecific variation in camouflage strategies in these species of crab is the difference in physiological cost between decoration and colour change, in the sense that both strategies may be costly and hence there is a trade-off, with resources being allocated to one or the other (Hultgren and Stachowicz, 2008; 2009).

Fitness cost

Direct or indirect physiological costs resulting from colour change (Sköld et al., 2016) would ultimately affect animal fitness. Currently, there is almost no support for this assumption, though at least one study on coastrange sculpins (*Cottus aleuticus*) found a non-significant trend of decreased fitness with colour change (Bergstrom et al., 2012). In this study, fish were placed in three groups and assigned to three background treatments for 6 months. The control group was on a constant background, the physiological colour-change animals had a 2 day alternated background (either white or black) and the morphological colour-change individuals had a 2 month alternated background. After 6 months, they found that fish that underwent slow (morphological) colour change had higher mortality than those that underwent rapid (physiological) colour change, followed by the control group. These findings may suggest that the production and destruction of chromatophores (morphological) is more costly than maintaining the dispersed and aggregated pigments within colour cells (physiological).

Arguably, physiological colour change should pose a more challenging cost than morphological colour change (Duarte et al., 2017). Morphological and physiological colour-change processes are interlinked. The prolonged exposure to constant backgrounds can lead animals to shift from physiological colour change to the morphological type (Sugimoto, 1993), as the latter is often preceded by the former (Bagnara and Hadley, 1973; Sköld et al., 2016). For example, the shore crab *C. maenas* can physiologically change its colour according to the background within 2 h (Powell, 1962a; Stevens et al., 2014b) and in an endogenous rhythmic way becomes darker during the day and lighter during the night (Powell, 1962b). These crabs, after long-term adaptation to a black background over 35 days, were found to increase their dark coloration during the night compared with those on neutral backgrounds (Powell, 1962b).

It is unknown why both types of colour change operate in the one species. On the one hand, the shift might be linked to the temporal pressures where animals face both predictable and short-term unpredictable changes in habitats. On the other hand, we suggest that the shift could help animals minimise the physiological cost of maintaining colours (physiologically) in a fully dispersed/aggregated state of the chromatophores for a prolonged period. However, it is predicted that this shift comes with a potential concomitant reduction in the animals' mobility and activity patterns and an increase in behavioural preferences for substrates.

Although the shift from physiological to morphological colour change is seemingly observable in some groups of animals (e.g. crabs: Powell, 1962b; amphibians: Babák, 1913; Bertolesi et al., 2016; fish: Bergstrom et al., 2012; Bertolesi and Mcfarlane, 2020), it is poorly known, and the mechanisms linking the two types of colour change (Bertolesi et al., 2016; Bertolesi and Mcfarlane, 2020) and their adaptive values are not well understood.

Conclusion

Although changing colour is clearly a successful strategy in many animals, its costs – specifically the physiological costs – have received limited attention. Previous studies have occasionally assumed that changing colour could be physiologically costly, but only a few studies have attempted to investigate these costs. There is evidence that changing colour does incur physiological costs from the processes of producing, translocating and maintaining pigments within chromatophores. Although both rapid and slow colour change are costly, rapid colour change appears more costly if continuously employed. Moreover, the costs of rapid and slow colour change can be mitigated behaviourally and physiologically via some shared and slightly different mitigation approaches depending on the change strategy (i.e. rapid or slow; Table 1), and can be different at the individual, population and species level as well.

However, the physiological cost of changing colour is still ambiguous across many genera and many substantive questions must be resolved to fully understand the role that such a cost has on colour change. First, are the costs of colour change equal in all directions? For example, is the dispersion of pigments within chromatophores more costly than their aggregation in rapidly colour-changing animals? Is the production more challenging than the destruction of chromatophores in slowly changing taxa? What explains the differentials, if they exist? Second, are the chromatophores in fully expressed states active or passive? Third, does the physiological cost explain interspecific and intraspecific variation in colour change? Fourth, what type of colour change is more physiologically costly? Does the physiological cost differ mechanistically between reversible and irreversible colour change and does the cost differ phylogenetically among related colour-changing taxa? Finally, how does morphological colour change interact with physiological change in reducing the physiological cost of colour change, and how would it affect the efficiency of physiological colour change? At what point is the shift to morphological colour change triggered and what is the adaptive benefit of it?

Competing interests

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