Effects of divergent water colours on Amazon fish evolution

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To

My bases – my family

Contents

Abstract

Amazon has the highest freshwater fish fauna in the world with more than 3000 species formally described, inhabiting black-, clear- and white- waters. Many hypotheses considering speciation through allopatry have been developed. However, the Amazon aquatic environment highly varies in physical and chemical parameters, affecting the sensory systems of the aquatic biota. Explored Amazon water types' impact on fish sensory evolution (vision, chemical, mechanical, electrical reception). Black waters with high organic carbon alter pH and bias light towards red. Clear waters exhibit neutral pH and no color biases. White waters have basic pH, restricting light transmission. These distinct water features likely drive sensory system evolution, potentially leading to reproductive isolation through local adaptations. I used the sailfin tetra *Crenuchus spilurus*, an Amazon small fish species, that lives in forest stream waters (known as *igarapés*), as a model to investigate how divergent underwater lighting condition drives population apart through behavioural and genetic experiments. The sailfin tetra is a sexually dimorphic fish species in which males possess dorsal and anal fins conspicuously ornaments in red and yellow colours. These ornaments are used during an elaborated courtship behaviour. The sailfin tetra is composed of two phylogenetic lineages, one inhabiting black and the second one inhabiting clear waters. These lineages are reproductively isolated. I first tested how the divergent underwater lighting condition of the black and clear waters could affect male ornaments colouration and female mating choice. Black water light increased the perception of the males ornaments red colours. However, it also decreased the among-individual variation of the ornament red colouration. Females living in clear waters were more likely to accept larger ornamented mates and under black water lighting conditions. On the other hand, females living in black waters were more likely to accept mates as they showed larger ornaments, but their mate choice was not affected by the different lighting conditions. I suggest that the lower among-individual variation in male ornaments colouration due to the environmental lighting condition under black water represents a potential fitness cost to females living in such waters. I then investigated female colour preference through environmental colour choice. Females from black waters and clear waters preferred red-lightened environments. However, this red colour preference was stronger in the females from clear than in those

from black waters. This preference can be related to several ecological processes, and more importantly, such stronger preference for red colour in females living in clear waters may relate to their colour preference in mating behaviour. Finally, I investigated genetic divergence, expected maximum absorbance wavelength (λmax) and expression levels of the long wavelength sensitivity genes (LWS) -responsible for sensing red colours - in populations living in black and clear waters. I found five copies of the LWS genes in all samples, that were genetically similar and their expected λmax did not differ among the populations. The expression levels of most of the LWS genes were similar for all populations; however, it could be expected since individuals were kept under similar captivity conditions before the acquisition of the genetic samples. Because of the high environmental variation in Amazon igarapés, I propose that the visual adaptations may occur in the gene expression profile rather than the direct correlation between the LWS gene and environmental conditions. Here, I suggest that the Amazon water types affect the evolution of morphologic, genetic and behavioural traits in an Amazon fish species.

Chapter 1

A review on fish sensory systems and Amazon water types with implications to biodiversity

1.1 Introduction

Fish comprise the most diverse group of vertebrates, with nearly 35.635 extant species (as listed in Catalog of Fishes in October 2020, Fricke et al., 2020). Of that total, almost 18.000 species are found only in freshwater ecosystems (Fricke et al., 2020). The Neotropical region contributes the largest part of this astonishing diversity, harboring over 6000 species (Albert et al., 2011; Reis, 2013). Most Neotropical freshwater fish diversity occurs in the Amazon basin, where circa 3000 valid species occur (Dagosta & Pinna, 2019). Many hypotheses considering different factors as evolutionary drivers of the Amazon fish biodiversity have been developed (Dagosta & Pinna, 2018). Alfred Russell Wallace (Wallace, 1853) was the first to hint possible ecological reasons for Amazonian fish diversity (Pires et al., 2018). Wallace (1853) highlighted the differences of Amazon fish composition varied according to a classification based on water colour (later known as water types) and suggested that those environmental differences could represent an important component of fish diversification. Later, Roberts (1972) proposed that colourful fish species are more abundant in clear and black water types than in white water type, suggesting an effect of water colour in shaping the evolution of the Amazon fish colouration. Segregation of ecological niches driven by divergence in ecomorphological traits (e.g. adaptation to divergent feeding niches) has also been suggested as an important component in shaping Amazonian fish diversity (Kullander, 1986). Despite such early efforts in documenting ecological features that could be related to biodiversity, focus later shifted to contrasting sympatric and allopatric speciation (Albert et al., 2011; Albert & Reis, 2011; Bernardi, 2013; Géry, 1984), a framework that fails to specify mechanisms of organismal divergence (Nosil, 2012). This occurred in the light of important advancements in the understanding of landscape evolution of the Amazon basin, which includes how the uplift of the Andean cordilleras altered landscape features. Time is ripe for blending older and newer approaches, and pave the way for the

development of newer hypothesis that can be tested and contrasted. Here, I focus on the diversity of aquatic environments in an attempt to provide a stepping stone in the inclusion of how divergent selection can contribute an important component in shaping Amazon's freshwater biodiversity.

Compared to air, water is a much better conductor of chemical compounds, electric fields and mechanical waves; consequently, most research on the importance of these environmental characteristics in driving diversity has been conducted on aquatic animals (Cummings & Endler, 2018). Despite the contribution of research on aquatic organisms to our understanding of the linkage between environmental conditions, sensory systems and speciation, such studies are nearly absent for Amazon freshwater organisms. For instance, although the sensory drive hypothesis has long established a link between natural selection acting on a tuning between sensorial systems and characteristics of the environment (Boughman, 2002; Endler, 1992), a search for the keywords "Amazon", "fish", and "sensory drive" on Google Scholar returned only 138 studies (searched on 1st May 2020). Only one of these studies actually investigated the sensory drive hypothesis among Amazonian fish species (Pires et al., 2019), and none explicitly investigated any aspect of divergence in their sensory systems. By contrast, the importance of sensory drive in promoting diversification has been suggested for several other taxa in other aquatic environments, especially for African rift lakes organisms.

Individuals use sensory systems to perceive the world surrounding them and respond accordingly. Sensory systems can be divided into four modalities: (I) mechanoreception, (II) electroreception, (III) chemoreception and (IV) photoreception (Atema et al., 1988). Although most organisms use multimodal information to make decisions, the relative contribution of each modality to how animals perceive their environment differs among taxa. For instance, many electric fish rely more heavily on disturbances in the electric field than on vision or other sensory modalities. In this sense, many environmental components in the Amazon basin can represent sources of divergent selection and potentially drive sensory adaptations. Amazon aquatic habitats and microhabitats vary highly in several important ecological aspects such as the composition of the substrate, openness of the riparian forest canopy, composition of the surrounding forest and presence of floating meadows in river-floodplain systems. I acknowledge the importance of all of these aspects in driving adaptations of fish sensory systems; however,

here I focus on how limnologic differences between Amazon water types may have resulted in local adaptation and contributed through divergent selection processes to Amazon fish diversity.

1.2 Limnologic characteristics of Amazon aquatic systems

Amazon water types were originally described according to their apparent colouration as seen from above the water into black, blue (= clear; Sioli, 1984), and white (Wallace, 1853) (Figure 1.1). These differences in colouration stem from variable amounts of suspended particles, the degree of organic material leaching from the surrounding forests, and from the historical geomorphological characteristics of the drainage system (Leenheer, 1980). Soil types in the Amazon are highly diverse, especially due to the different degrees of weathering observed between relatively recent geological formations (such as the Andean cordilleras) and older cratonic regions (such as the Guiana and Brazilian shields) (Quesada et al., 2011).

Figure 1.1. Amazon water types. **(A)** Black, **(B)** clear and **(C)** white water types. Photo credits: A – Elio Borghezan; B – Jansen Zuanon; C – Tiago Pires.

Table 1.1 summarizes the main physical and chemical characteristics of white, black, and clear Amazon waters types. Most whitewater rivers have their headwaters on the Andean cordilleras and carry a high concentration of suspended sediments (Goulding et al., 2003) and dissolved salts (Furch, 1984). The Andean sediments increase the concentration of nutrients such as sodium, potassium, magnesium and calcium in the water (Furch, 1984; Table 1.1), resulting in high biological productivity and a muddy appearance. The high ionic concentration also increases electrical conductivity to a typical range of 68.8 and 93.3 μS/cm (Küchler et al., 2000). The high turbidity constrains

vertical light transmission (Muntz, 1978), with a typical local reach of a maximum of 0.50 m deep (Sioli, 1984; Table 1.1). The surrounding soil types, mostly latosols and oxisols, favor a more complete decomposition and absorption of organic matter from the surrounding forests (Leenheer, 1980), resulting in lower concentration of dissolved organic carbon (DOC). Because of the low DOC concentration, the pH of whitewater rivers is close to neutral or slightly alkaline, ranging from 6.2 to 7.2 (Sioli, 1984; Küchler et al., 2000). On the other hand, black and clear water rivers have their headwaters on the old (cratonic) Brazilian and Guiana shields (Goulding et al., 2003). Because of their ancient origin and the long process of lixiviation, these terrains lack fine sediments as well as several minerals (Furch, 1984), resulting in a higher water transparency. Therefore, black waters have lower concentrations of nutrients compared to white waters (Furch, 1984; Table 1.1). Although more transparent than white waters, black and clear waters are remarkably different in DOC composition. Clear waters have their origin in latosolic soils, which absorb soluble DOC (i.e. humic and fulvic acids), resulting in high transparency ranging from 1.10 to 4.30 m (Leenheer, 1980; Sioli, 1984). On the other hand, black waters have lower transparency values ranging from 1.3 to 2.9 m (Sioli, 1984). Black waters are stained by fulvic and humic acids and tannins leached from decomposing vegetation (Leenheer, 1980; Sioli, 1984), biasing light transmission toward long wavelengths (Costa et al., 2013; Mendonça et al., 2005). High concentrations of DOC make black waters remarkably acidic, with pH ranging from 3.80 to 4.90, whereas the near absence of DOC in clear waters results in circum-neutral pH ranging from 4.50 to 7.80 (Sioli, 1984). Black and clear waters also have low electrical conductivity, ranging from 8.80 to 28.60 μS/cm (Küchler et al., 2000; Ríos-Villamizar et al., 2013).

Table 1.1. Limnologic characteristics of Amazonian white, black and clear water types. Values presented here were combined from data presented by Sioli (1984), Furch (1984), Küchler et al. (2000) and Ríos-Villamizar et al. (2013).

	White	Black	Clear
pH	6.20 ~ 7.20	3.80 ~ 4.90	4.39 ~ 7.80
Electrical conductivity (μS/cm)	68.80 ~ 93.30	8.80 ~ 28.60	14.33 ~ 59.90
Transparency (m)	0.10 ~ 0.50	1.30 ~ 2.90	1.10 ~ 4.30
Sodium (mg/l)	1.60 ~ 2.50	0.33 ~ 0.38	1.50 ~ 3.88
Potassium (mg/l)	0.90 ~ 1.40	0.31 ~ 0.32	0.43 ~ 2.39
Calcium (mg/l)	4.30 ~ 8.60	0.18 ~ 0.21	<0.02 ~ 2.85
Magnesium (mg/l)	0.70 ~ 1.40	0.09 ~ 0.11	0.06 ~ 0.71
Chloride (mg/l)	2.00 ~ 3.10	1.70 ~ 1.80	0.53 ~ 2.50

The aforementioned differences in Amazon water characteristics set the stage for natural selection to drive local adaptations in the sensory systems of aquatic organisms. This could have important bearing in Amazonian fish biodiversity, as adaptations to divergent pressures imposed by different habitat conditions can drive populations apart and contribute to the speciation process (Servedio & Boughman, 2017). In the following text, I highlight the role of local adaptations to population divergence by the main sensory modalities (visual, chemical, mechanical, and electrical) through the sensory drive hypothesis (Boughman, 2002; Servedio & Boughman, 2017) in the context of Amazon water types.

1.3 Vision

Light is conceptualized as packets of energy (photons) with varying wavelengths that can be perceived by organisms (Cronin et al., 2014). Ranges of wavelengths represent discrete colours and can elicit different responses from organisms with light-sensitive organs, most notably the eyes. In the eyes, light excites rhodopsin, a photosensitive molecule presents in the retina. Rhodopsin is composed of a protein called opsin, and a

non-protein part derived from vitamin A (Nelson & Cox, 2014). Rhodopsin can be found in both cone and rod-type retinal cells. There are four spectrally distinct classes of opsins found in cone cell types across all vertebrate groups: long-to-middle wavelength class (LWS), sensitive to orange and red wavelengths (from 490 to 570 nm); middle wavelength class (RH2), sensitive primarily to green (from 480 to 535 nm); short wavelength class two (SWS2), sensitive to blue-violet (about 410 to 490 nm); and short wavelength class one (SWS1), sensitive to violet and ultraviolet (355 to 440 nm) (Bowmaker & Hunt, 2006). In addition to the four cone opsin types, there is a rod class of pigment (usually denoted Rh) that is used in dim-light vision perception (Bowmaker & Hunt, 2006).

Genetic analyses have shown that opsin structures differ in individuals living under different lighting conditions (Seehausen et al., 2008; Terai et al., 2006; Van Nynatten et al., 2015). Such differences in opsin structure represent adaptations of the protein to best absorb prevalent wavelengths in the environment (Bowmaker & Hunt, 2006; Partridge et al., 1988). Differences in wavelength transmission among aquatic environments are affected by the presence of particles and dissolved substances in the water column (Endler, 1991). Water molecules absorb long wavelength colours such as red and yellow, while short wavelengths such as violet and ultraviolet are filtered out as depth increases, as observed in the deep African rift lakes (Seehausen et al., 2008). Light can also be scattered by solid particles (Muntz, 1978), reducing the amount of environmental light. Additionally, compounds (usually DOC) present in the water can bias the prevalent colour in some environments (Endler, 1991; Mendonça et al., 2005). Such bias in wavelength transmission can affect the evolution, form and structure of light-sensitive organs, resulting in differences in light perception between organisms.

Populations can diverge in visual acuity, i.e. the level of details that individuals can discriminate. This ability is primarily influenced by the number of photoreceptors in the retina (cone and rod cells), which is related to eye size and cell density (Collin & Pettigrew, 1988a, 1988b, 1989). A trade-off exists between acuity and sensitivity, as cones allow for greater visual acuity than rods, but rods require less environmental light to function properly. Thus, adaptation to living under low light intensity typically results in lower visual acuity such as seen among deep-sea fishes and nocturnal animals (Warrant, 2004). Visual acuity also varies among individuals that occupy different ecological niches

and thus have to undertake different visual tasks (Parker et al., 2017). Predators usually have higher visual acuity than herbivores/omnivores (Parker et al., 2017) because of the need to target mobile and sometimes elusive prey.

Amazon water types are spectrally diverse and vary in their optical characteristics (Costa et al., 2013). The high amount of suspended solids in white waters result in high turbidity, which strongly constrains light transmission (Muntz, 1978) and can hamper visual communication. Therefore, it would be expected that individuals living in such conditions would have larger eyes or higher sensitivity to compensate for the low availability of light. By the same token, fishes inhabiting white water systems would be expected to have a higher number of rod cells compared to fish inhabiting clear and black water types. However, when light is mostly absent, the visual system of organisms adapting to such environments can reduce, which can even result in the loss of functional eyes (Soares & Niemiller, 2013). Therefore, either an increased eye size and higher density of rods, or a reduction of the visual system could be expected to occur in white waters depending on fish life-history traits (which also depend on evolutionary history). In any case, low visual acuity and poor vision due to a higher proportion of rod cells are expected to prevail in white water organisms. The lower visual acuity in white water organisms may be compensated by information acquired from other senses, such as chemical and mechanical. On the other hand, black and clear waters are more transparent than white waters (Muntz, 1978), increasing the importance of vision and allowing the development of adaptions of the visual system in relation to other sensory modalities. This is aligned with the aforementioned early observation of Roberts (1972), who proposed that most colourful Amazon fish species are found in black and clear water. However, clear and black water environments differ strongly in wavelength transmission (Muntz, 1978) so that further nuances in the evolution of fish colouration could be expected. Due to the absence of DOC and suspended particles, clear waters have no colour bias and are thus better at transmitting blue light (Fuller, 2002) than black waters that are biased toward yellow and red (Muntz, 1978).

The photo sensitive protein, opsin, is composed of about 350 amino acids, and divergences at amino acid positions 164, 181, 261 and 269 for LWS; 86 and 90 for SWS1 and; 118 and 269 for SWS2 have been shown to drive divergence in peak light sensitivity in terrestrial mammals (Bowmaker & Hunt, 2006), which may also be important for fishes

and other aquatic organisms. Additionally, changes in other amino acid positions can also result in divergent spectral tuning, as observed among cichlid fishes (Carleton et al., 2005; Seehausen et al., 2008). A similar divergence imposed by natural selection on Amazonian fishes would change the protein structure and light absorbance peak to best absorb the most prevalent wavelengths in black and clear water systems (Bowmaker & Hunt, 2006). The highest difference in colour peak sensitivity is expected to occur in the SWS1, SWS2 and LWS opsin genes, which are responsible for UV, violet, blue and red colour sensitivity.

The effect of water type on colour perception among species, populations or demes can also affect the evolution of body colouration, physiological/neutrally controlled colour changes, and sexual ornamentation, especially among sexually dimorphic and dichromatic species (e.g. Pinto et al., 2020; Pires et al., 2019; Figure 1.2). Additionally, because colour plays an important role in mating behaviour, especially among sexually dimorphic species, differences in colour peak sensitivity and sexual traits may mediate reproductive isolation among populations exposed to different water types, resulting in speciation and increasing biodiversity. Although interesting and promising, these hypotheses have been poorly explored with regard to the origin and diversification of the fish fauna in the Amazon basin.

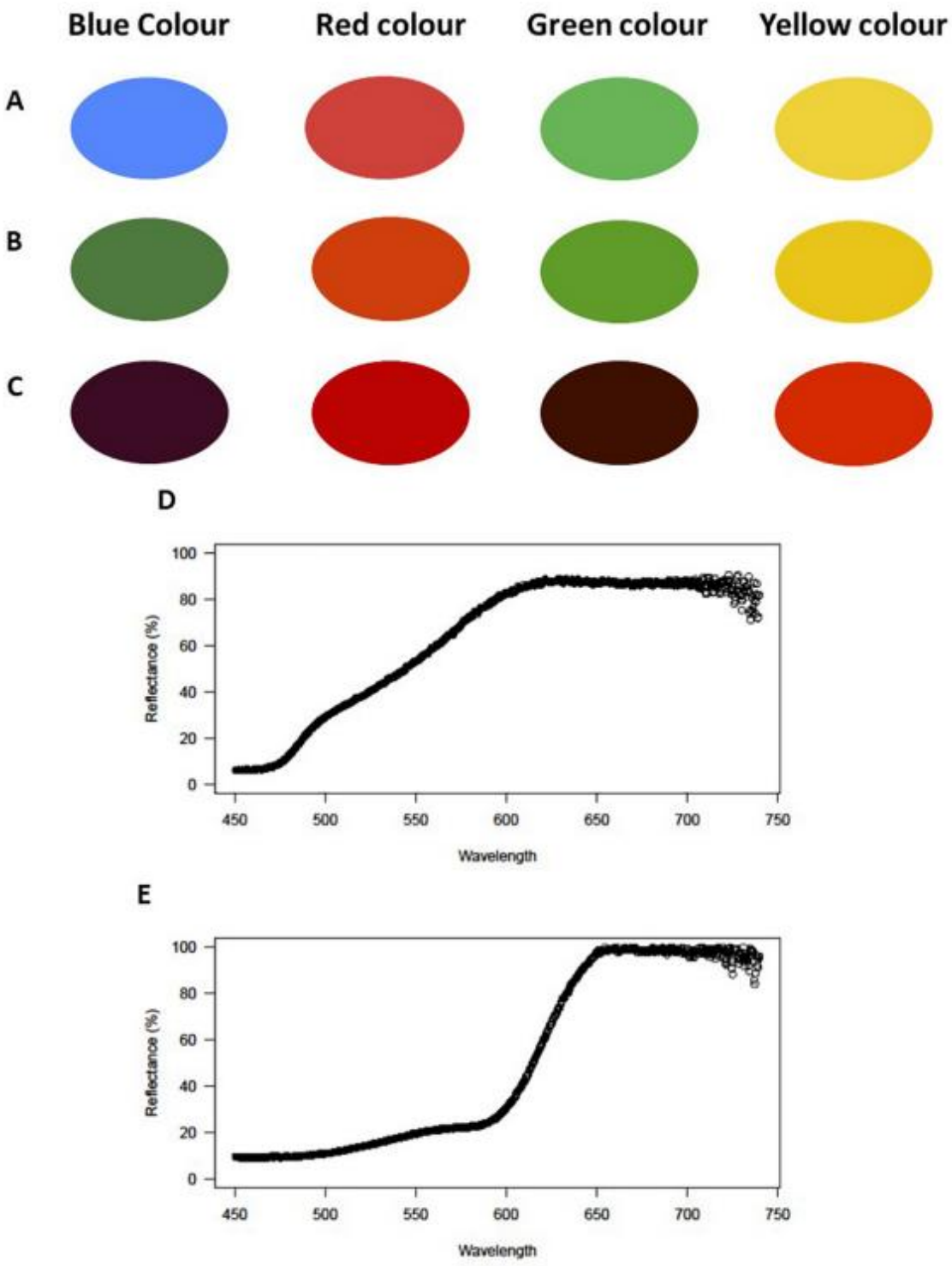

Figure 1.2. Simulated effect of water colour on light transmission across the water column for different wavelengths (blue, red, green, and yellow colours) as observed. **(A)** without wavelength bias (e.g. in clear water), **(B)** through an orange light filter (Figure 1.2D) simulating the black water light condition at ~40 cm deep, and **(C)** under a red light filter

(Figure 1.2E) simulating the light condition at ~150 cm of depth in a black water environment. Reflectance by wavelengths with **(D)** an orange light filter to simulate a black water light condition at ~40 cm deep, and **(E)** with a red light filter to simulate a black water light condition at ~150 cm deep.

In a recent study, Fabrin et al. (2017) compared LWS genes of 10 Neotropical and 19 African cichlids species. The authors suggested that LWS gene diversity in the Neotropics may surpass those found in the cichlids of African rift lakes. However, analyses considering synonymous and non-synonymous nucleotide modifications that are needed to assert whether such higher nucleotide diversity implies increased diversity in visual perception are still pending. Although an interesting subject, the results of Fabrin et al. (2017) do not allow comparisons among Amazon water types, as only one sample site in the Amazon was included in the study. Another investigation involving Amazon cichlids performed by Escobar-Camacho et al. (2017) evaluated a set of opsin genes, including SWS1, SWS2, RH2 and LWS, among three Amazonian cichlid species and concluded that opsin gene expression profiles were similar across species. Although sampling sites in that study did include black and seasonally mixed (black and white, due proximity of the meeting of the Amazon and Negro Rivers) water types, the authors did not conduct an explicit comparison of genetic divergence between water types. Another investigation of molecular evolution of light-sensitive protein was performed by Van Nynatten et al. (2015), which showed that the South America (including Amazon) freshwater environments have shaped the evolution of the rhodopsin and its spectral sensitivity among lineages of marine-originated anchovies (Clupeiformes: Engraulidae). However, that study does not consider any of the differences observed among Amazon water types.

Combining microspectrophotometry of fish retina and genetic analysis targeting opsin genes and structure is a promising approach for understanding how light perception varies among species and populations occupying different water types (as reported for other environments by Carleton et al., 2005 and Seehausen et al., 2008). Behavioural experiments, such as mate choice and male-male contests under different light conditions could also contribute to our understanding of how environmental light affects interactions among individuals when exposed to different water types and behavioural contexts

(Dijkstra et al., 2005; Seehausen et al., 2008).

Retinal analysis focused on estimating photoreceptor and ganglion cell densities could help with understanding differences in visual acuity (Collin & Pettigrew, 1989; Nakamura, 1968; Parker et al., 2017). Additionally, behavioural experiments could elucidate how differences in retinal topography affects an individual's acuity during behavioural tasks. Because sexually dichromatic species are particularly dependent on visual communication during mating, studies focusing on the interplay of lighting environment and colour peak sensitivity of fish populations exposed to different water types could contribute to our understanding of speciation via the sensory drive and sensory exploitation hypotheses (Boughman, 2002; Endler, 1992). Additionally, some Amazon fish species are morphologically very similar, differing only in some aspects of colouration (e.g. Pires et al., 2019), and knowledge of the level of detail a fish can see may help us understand how subtle morphological divergences affect reproductive isolation and species diversification.

1.4 Chemoreception

Chemical signals that transmit information among individuals are often referred to as pheromones. Pheromones can mediate many aspects of intra- and inter-specific communication, such as sociability (Ratchford & Eggleston, 1998), mate recognition and choice (Plenderleith et al., 2005), parental care (Keller-Costa et al., 2015) and predator avoidance (Griffiths & Richardson, 2006; Wisenden, 2000). Some fish innately recognize chemical signals released by predators, and others can learn to associate chemicals with predation risk (Griffiths & Richardson, 2006; Wisenden, 2000). Fish in the Superorder Ostariophysi have evolved an important mechanism to identify dangerous situations, termed "alarm reactions" ("*Schreckreaktion*") (Frisch, 1942). Individuals that have been injured (possibly after a predator attack) release chemical alarm signals into the water, inducing behavioural responses in conspecifics that contribute to predator avoidance and increase their probability of survival (Brown et al., 2012; Griffiths & Richardson, 2006; Wisenden, 2000). However, not only Ostariophysian fishes can use chemicals released by injured conspecifics to detect predators; since metabolic products like urea may also trigger similar alarm reactions (Brown et al., 2012).

Pheromones can also mediate aggregation among individuals (Ratchford & Eggleston, 1998), possibly requiring previous contact with such substances (i.e. learning, Keller-Costa et al., 2015). Changes in the chemical environment leading to perturbations of the learning process can reverse sexual preference, and even result in a preference for heterospecifics over conspecifics (Verzijden & Rosenthal, 2011). Chemosensory learning and signaling can be constrained by acidic water conditions, since some fish pheromones can be degraded and reduced to the point that no learning can occur (Brown et al., 2002; Leduc et al., 2007). Additionally, acidic conditions can trigger ion regulatory disturbances (via uptake of Na^+ and Ca^+) (Steinberg et al., 2006), which could affect chemical communication. Knowledge of whether fish in blackwater environments have evolved adaptations in sensory system to cope with the prevailing strongly acidic waters and resulting limitations imposed by the environment is virtually absent.

Sexual pheromones are hydrophobic or amphiphilic molecules (Stacey et al., 2003). As such, these pheromones can either dissolve in water or bind to suspended (DOC) or deposited organic matter (Mesquita et al., 2003). Chemical signals attached to DOC can be altered in ways that are unrecognizable to chemical receptors, precluding behavioural responses (Hubbard et al., 2002; Mesquita et al., 2003). Compared to clear and white water types, blackwater environments have lower pHs and higher concentrations of DOC and humic and fulvic acids. As such, intra- and inter-specific chemical communication among fishes from highly acidic black waters may be constrained (Figure 1.3). The influence of high concentrations of humic acids in chemical communication for reproduction has been demonstrated for the swordtail (*Xiphophorus birchmanni*; Cyprinodontiformes: Poeciliidae), whose females showed a strong preference for conspecific males' chemical cues, but failed to show any preference for the same chemicals in the presence of high concentrations of dissolved humic acids (Fisher et al., 2006). This illustrates how differences in chemical environments may favor hybridization by weakening or even completely suppressing species recognition mechanisms, which could ultimately affect speciation processes. However, even in the presence of high DOC concentrations, swordtail females expressed their preference for conspecific males when concomitantly exposed to males' visuals cues (Fisher et al., 2006). In this case, when chemical cues were constrained by environmental conditions, individuals used combined sensory modalities to make decisions. Based on these results,

it could be expected that organisms living in Amazon blackwater systems would primarily rely on vision to perceive their surroundings, with chemoreception being used as a supporting sensory mechanism.

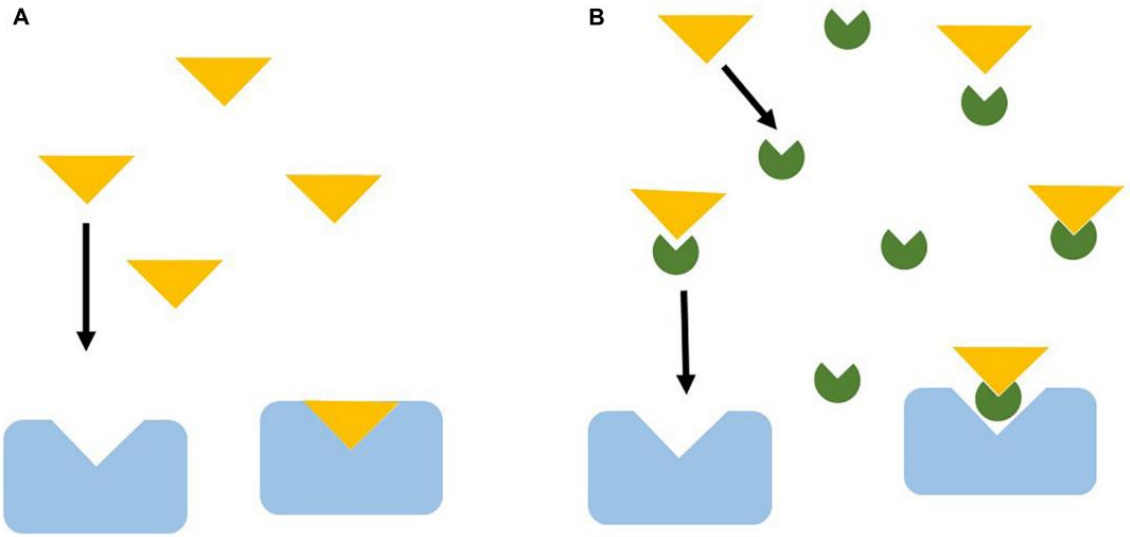

Figure 1.3. Scheme showing how chemical communication can be impaired by DOC. Blue boxes = chemical receptors, Yellow triangles = pheromones, Green semi-circles = DOC. **(A)** Pheromones flow through the water column and reach chemical receptors, **(B)** DOC bind to pheromones, DOC + pheromones complex fails to attach to chemical receptors, not eliciting a behavioural response.

When visual communication is constrained by environmental characteristics, chemical cues may be more relevant to intra- and inter-specific communication (Dodson et al., 1994). However, as far as I know, the relative importance of chemical communication among species that predominantly inhabit black and white Amazon water types has yet to be investigated. Studies of chemical communication among species that perform seasonal migrations between contrasting water types, such as species of the genus *Semaprochilodus* (Characiformes: Prochilodontidae) (de Brito Ribeiro & Junior, 1990), may help us understand how fish adjust their osmotic balance and sensory systems when exposed to diverse environmental conditions. While thought-provoking, research on these topics in the Amazon is lacking.

1.5 Mechanoreception

Mechanoreception in fishes includes hearing, touch, and perception of water movement through the lateral line. Fish use mechanoreception to sense the abiotic environment as well as for intra- and inter-specific interactions. Although all of these forms of mechanoreception can be used to detect mechanical vibrations, they differ in how the vibrations are detected and are used for different purposes. Therefore, they will be divided into three subsections and explored individually.

1.5.1 Sound production and hearing

Fish have the ability to produce and detect sounds (Ladich, 2014). However, due to the lack of a specialized vocal organ (such as the larynx in anurans, reptiles and mammals and the syrinx in birds, Bradbury & Vehrencamp, 2011), fish species that are able to produce sounds do not have large repertoires (Amorim, 2006; Dos Santos et al., 2000). Sounds in fishes are produced in different ways and can be classified in two main mechanisms: sounds produced through 1) the swim bladder; and by 2) stridulatory mechanisms (Kasumyan, 2008). Most sounds produced by fish are created in the swim bladder using drumming (or sonic) muscles that are either attached to the bladder wall or positioned outside (Ladich, 2014; Thorson & Fine, 2002). Contraction of these specialized muscles causes changes in the volume of the swim bladder and in the pressure of the gas inside (Zelick et al., 1999), causing the swim bladder to vibrate like a loudspeaker. Fish can also produce sounds by vibrating the bladder indirectly, via broad tendons or bones without any direct attachment to the swim bladder wall (Ladich, 2014). Stridulatory sound production occurs mainly in catfish; sounds are generated when a heavily ossified first pectoral-fin ray is pressed against a series of grooves in the shoulder girdle. Some species can even use a sonic muscle to vibrate their entire pectoral girdle (Ladich, 1989, 2014).

Sounds produced by fishes are generally used for intimidation, defense, territorial advertisement, courtship, or mating (Dos Santos et al., 2000; Ladich, 1989; Myrberg et al., 1986). These sounds are detected by the inner ear and by additional peripheral structures that improve hearing (Braun & Grande, 2008; Ladich, 2014; Popper & Fay, 2011). Inner ears are composed of three semicircular canals (utricle, saccule,

lagena) and by otoliths (sagittae, lapilli, asterisci) in the semicircular canals. These otoliths are calcareous (calcium carbonate) structures that lie on top of a bed of sensory hair cells (Ladich, 2014). When a sound is produced, it causes a vibration in the water, which moves the otoliths and stimulates the hair cells. Fishes of the Superorder Ostariophysi have an additional hearing structure called the Weberian apparatus that consists of a skeletal modification (a series of small bony elements derived from the first vertebral elements) that connects the swim bladder to the inner ear (Braun & Grande, 2008). This structure acts as an amplifier of the sound waves transmitted through the water to the inner ear (Braun & Grande, 2008).

Deposition of minerals in otoliths are highly dependent on the physical and chemical characteristics of the water, including the presence of different chemical elements (minerals) and pH (Morales-Nin, 2000; Walther & Thorrold, 2006). As such, otoliths formations in fishes living in blackwater systems may be very different from fishes originating in whitewater environments. Black water fish may have otoliths with lower mineral density than those found in white water fish, which may result in lower hearing ability (Oxman et al., 2007; Rossi et al., 2016; Simpson et al., 2011) and affect auditory responses.

Boyle et al. (2015) evaluated morphological structures associated with sound production and sound characteristics in three Amazon doradid catfishes, *Acanthodoras cataphractus*, *Platydoras hancockii* and *Agamyxis pectinifrons*, revealing the existence of differences in waveform and amplitude modulation. Although distinguishable, the differences in sound parameters between species were not explained by any of the analyzed anatomic features (swim bladder size, muscle anatomy or muscle length), but instead were suggested to be modulated by means of differences in neural activation of sonic muscles (Boyle et al., 2015). Because sound production may be regulated by neural processes, it arguably plays an important role in intra- and inter-specific communication. On the other hand, the Amazon sailfin suckermouth catfish *Pterygoplichthys pardalis* does not display any behavioural responses to conspecific stridulation sounds, which seem to only be used to deter predators (Slusher, 2018).

Studies evaluating the possible effects of water types on otoliths' mineral deposition and associated auditory responses among Amazon fishes seems to be completely lacking. Notwithstanding, the production of sounds by several fishes just (or

mostly) during reproductive activities (courting and mating) indicates its potential importance for correct species pairing during spawning (Amorim et al., 2003). However, the relative importance of audial communication by fish in the different Amazon water types and its possible role on reproductive isolation remains to be verified.

1.5.2 Touch

Among fishes, tactile reception occurs through free nerve endings, Merkel cells and Rohon-Beard cells and their associated innervation. Rohon-Beard cells are usually present only during the larval phase, while Merkel cells persist throughout the entire life cycle. The main tactile organs of fish are barbels; free rays; filaments of pectoral, dorsal and caudal fins; rostrums; and breeding tubercles. These organs play an important role in reproductive stimulation, exploration, social behaviour such as schooling and shoaling, defensive behaviour, and foraging (Soares & Niemiller, 2013; Windsor et al., 2008).

Tactile organs are especially well developed among cavefish and other species that inhabit environments with dim or no lighting. To compensate for a lack of the vision, some fishes have enhanced mechanosensory systems. Cavefish species usually have elongated fins, particularly pectoral fins, which are used to touch objects and allow perceiving structures in the environment (Soares & Niemiller, 2013; Windsor et al., 2008). For instance, it has been observed that blind cavefish frequently touch surfaces with their pectoral fins while swimming (Baker & Montgomery, 1999; Windsor et al., 2008), and during rheotaxis (Baker & Montgomery, 1999).

Many Amazonian catfishes have small eye size and elongated fins filaments that likely play an important role for sensing the environment through the mechanosensory system. Such organs may differ depending on water type not only in size, but also in the density of Merkel cells, or even in cell sensitivity. Interestingly, a higher diversity of Amazon catfishes occurs in white waters (Queiroz et al., 2013; Dagosta & Pinna, 2019) and in the deeper portions of the Amazon's aquatic habitats (e.g. Beltrão et al., 2019) where underwater light is scarce, further stressing the importance of the mechanosensory system in this group. It is therefore unfortunate that the role of touch among species that inhabit black, clear, and white water types has not been investigated.

1.5.3 Mechanoreception by the lateral line system

The lateral line system acts as a "touch-at-a-distance" sense. It provides information about water currents, aiding in prey detection, predator avoidance, hydrodynamic imaging, and courtship communication (Coombs & Montgomery, 1999; Montgomery et al., 2001). The lateral line system is usually externally visible at the body surface and consists of sensory cells called neuromasts, which are found on the skin or underneath the skin surface in fluid-filled canals that are in contact with the water through a series of pores (Bleckmann, 1986, 2006; Coombs, 2001). Neuromasts on the skin are sensitive to low-frequency vibrations, such as water motion and flow velocity, while those found inside the canals are sensitive to higher frequencies, such as pressure and tactile information (Coombs & Montgomery, 1999; Montgomery et al., 1995). Neuromast sensitivity depends on the apical ciliated bundles where the mechanotransducer channels are located (Kazmierczak & Müller, 2012). When water flows over the hair bundles, it causes changes in ion influx, activating signal transduction in the ciliated cells (Fettiplace, 2009; Sand, 1975). Although potassium (K^+) and sodium (Na^+) ions can flow into the neuromasts, calcium ions (Ca^{+2}) play a central role in the activation of transduction (Fettiplace, 2009; Sand, 1975).

The number of neuromasts found in the peripheral lateral line system of a fish can vary from 100 to over 1000, and are distributed on the head and along the body (Coombs, 2001). Not only the number of neuromasts but their distribution, size and morphology vary between species (Maruska, 2001). These differences in neuromast structural features could affect their sensitivity, since variations in physical characteristics may confer to the neuromasts the capacity to respond to a specific range of stimuli frequencies (Kroese & Van Netten, 1989).

The number and size of neuromasts varies in response to a range of environmental conditions. In fishes living under very low light conditions, as is the case for the blind cavefish *Astyanax mexicanus*, the mechanosensory system may be oversized to compensate for lack of vision. Enlarged neuromasts in cavefish can confer a higher sensitivity and a higher range of stimuli than the neuromasts found among surface relatives (Yoshizawa et al., 2014). Conversely, other environmental conditions may impair the development of the lateral line system, also affecting its sensitivity. In zebrafish (*Danio rerio*) embryos, acidic conditions reduced the number and apical area

width of neuromasts along with the size of hair bundles, ultimately reducing neuromast functionality (Lin et al., 2019), but it is still unclear if such impairment lasts throughout an individual's life. Such morphological effects are expected to occur due to the internal pH balance that may regulate hair cell development and functioning (Lin et al., 2019). Interestingly, exposition to acidic water might affect the pH of the internal body fluids and the dome of the lateral line hair cells, disrupting pH-sensitive processes. Additionally, aquatic environments with low amount of calcium may also impair the activation of the signal transduction in the ciliated cells (Fettiplace, 2009). Black waters are remarkably acidic, with very small amounts of dissolved salts, especially calcium, compared to white waters (Sioli, 1984; Furch, 1984). Although the pH of clear waters can reach neutral/slightly alkaline values, their calcium concentrations are comparable to those observed among black waters (Table 1.1). Therefore, the lateral line system of individuals living in black and clear water systems is expected to have fewer neuromasts, with their hair bundles slightly shortened and the apical area of the neuromasts also shortened compared to individuals from white waters (Lin et al., 2019; Figure 1.4). Such effects of water quality on neuromast characteristics may restrict their functionality. However, it is still unclear if and how divergences in the lateral line system could affect individuals' communication.

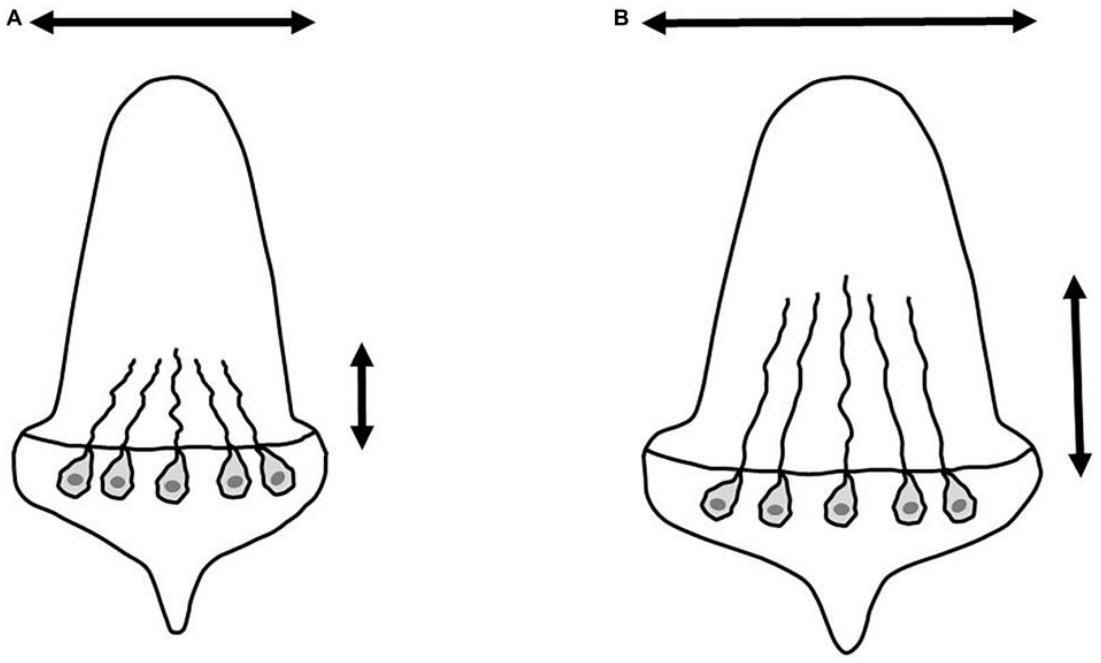

Figure 1.4. Expected morphological differences in fishes' neuromasts under acidic and neutral/alkaline environments according to Lin et al. (2019). **(A)** Fishes under acidic environments may show short hair bundles and the apical area of the neuromasts shortened. **(B)** On the other hand, fishes from neutral/alkaline aquatic environments may show longer hair bundles and larger apical area of the neuromasts. Arrows mark the expected differences in neuromasts shapes.

Determining the distribution, number and sensitivity of neuromasts in fish populations exposed to different water types could shed light on how divergent Amazon waters may have contributed to shaping fish diversity through differences in lateral line system. The scanning ion-selective electrode technique (SIET) can be used to survey the transport of various ions in biological membranes. Accessing the calcium entry in differently shaped neuromasts *in vivo* through SIET can show us the sensitivity of the hair cells (Lin et al., 2019). This can be especially important for species that use mechanical communication for accessing mating partners and for triggering spawning events (e.g. Pires et al., 2016) and for species that live in poorly-lit environments (such as Amazon white waters), since their visual system may be naturally constrained by low light availability.

1.6 Electroreception

Based on morphological similarities, electroreceptors are thought to be derived from mechanoreceptors of the lateral line system (Bleckmann, 1986; Crampton, 2019). Electroreceptors can be classified in two groups according to their morphological and physiological characteristics: ampullary or tuberous receptors (Bullock, 1982; Zakon, 1988). Ampullary receptors are sensitive to low electrical frequencies (0.1-50Hz) and are used in passive electrolocation and prey detection, while tuberous receptors are sensitive to high-frequency signals (50-2000Hz) and are used for active electrolocation and electrocommunication (Bleckmann, 1986; Bullock, 1982). Ampullary receptors are found on the fish's skin and are filled with mucus, whereas tuberous receptors are covered by the fish's skin and piled up in "packages".

In the Amazon, two closely related orders, Siluriformes and Gymnotiformes,

have ampullary receptors. However, only Gymnotiformes are able to both detect and produce electric fields. Such electric fields are generated by electric organs that are mostly derived from muscle tissue (Carlson & Gallant, 2013; Kramer, 2009). These organs are controlled by the brain and are characterized by a greater amplitude, intensity and higher temporal and spatial stability when compared to other electric body signals such as those that control heart rate (Kramer, 2009). Electric organ discharges (EODs) are used for sensing the environment and foraging (Feulner et al., 2009a). EODs are also used for species-specific communication and play an important role in individual recognition and mating activities (Feulner et al., 2009a), which have been suggested to promote speciation among African weakly electric fishes (Mormyridae) (Feulner et al., 2009a, 2009b; Tiedemann et al., 2010). Electric organ discharges can be classified according to the waveform as wave-type or pulse-type. Wave-type electric discharges are continuously produced and generate nearly sinusoidal waveforms. Pulse-type discharges are interrupted by relatively long and sometimes irregular "silent" intervals (Zakon, 1988).

Electric discharges and waveforms can be affected by characteristics of the aquatic environment, such as water temperature and electric conductivity. The EOD rate, frequency (in Hertz) and amplitude (in millivolts) increase with water temperature (Dunlap et al., 2000). Differences in water conductivity can even change the waveform of male individuals to be "female-like" and vice versa (Baier, 2008; Bratton & Kramer, 1988). However, these changes might not interfere in species recognition (Baier, 2008) since some species show a remarkable ability to physiologically acclimate to the changing water conductivity, returning their waveform to close to the original shape within 48 hours (Bell et al., 1976), which may be an adaptation to deal with natural variations in water conditions (Bratton & Kramer, 1988). Although EOD is involved in mate choice (Curtis & Stoddard, 2003; Feulner et al., 2009a), it is not clear how changes in waveform and EOD due to changes in water conductivity affect intrasexual communication. An interesting example on how conductivity can influence sensory information has been provided by MacIver et al. (2001), who showed that the highest prey detection distance occurred in low conductivity and the lowest in high conductivity in *Apteronotus albifrons* (Gymnotiformes), a weakly electric knifefish. Similarly, increased water conductivity may result in a decreased communication range as reported for the African *Brienomyrus niger* (Mormyriformes) (Squire & Moller, 1982).

Differences in water conductivity of Amazon water types may affect the performance of the electrogenic-electrosensory system of electric fishes. Fishes in white water (high conductivity) may use their electric organs to generate low-voltage, high-current electric fields, whereas fishes inhabiting black and clear waters (low conductivity) may generate high-voltage, low-current electric fields, adequate for mediums with high resistance (i.e. the ability of water to resist an electrical current, which is negatively related to the amount of dissolved salts in the water; water with a high concentration of dissolved salts will have a low resistivity, and vice versa) (Kramer, 2009). Ampullary and tuberous electroreceptors are affected by water conductivity in a similar way, and several studies have reported differences in behavioural responses occasioned by changes in electroreception ability (Baier, 2008; MacIver et al., 2001; Squire & Moller, 1982). Fishes that live in environments with high water conductivity possess longer ampullary (sensitive to low frequency) canals, longer receptor cells and more receptor cells than fishes inhabiting low-conductivity waters. Ampullary pores also tend to be larger in species living in high water conductivity (Gauthier et al., 2015). Therefore, electric fishes adapted to white waters may have more numerous and longer receptor cells, as well as larger pores of the ampullary organ than fishes adapted to black and clear water types (Figure 1.5). Water conductivity may also affect electroreception by tuberous organs (sensitive to high frequency). Tuberous electroreceptor sensitivity is tuned to the same frequency as the electric organ discharge of the species (Hopkins, 1976), and behavioural thresholds to high-frequency stimuli increase with decreasing water conductivity (Knudsen, 1974). Therefore, electric fishes inhabiting whitewater environments may be more sensitive to high-frequency electric signals than those inhabiting black and clear water environments. Although speculative, this represents a simple yet underexplored adaptive hypothesis on the evolution of electroreception in relation to water types.

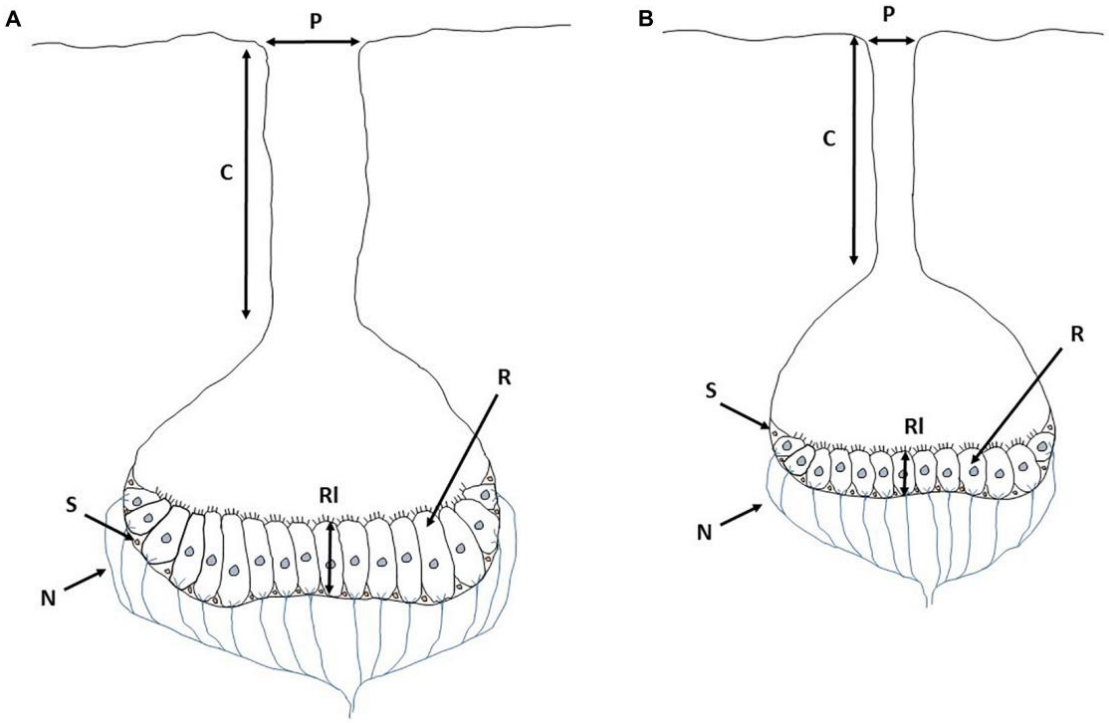

Figure 1.5. Expected morphological divergence in ampullary organ under **(A)** high (white water type) and **(B)** low (Amazon black and clear water types) electric conductivity, according to Gauthier et al. (2015). C = Ampullary canal, P = Pore, R= Receptor cells (cell with gray nucleus in this scheme), Rl = Receptors length, S = Supporting cell (cell with light pink nucleus in this scheme), N = Nerve. Draws based on images found in Collin & Whitehead, (2004) and Gauthier et al. (2015).

Divergence in water conductivity may also shape the evolution of electric organ morphology. Fishes adapted to high conductivity waters usually have shorter and thicker caudal filaments containing electrocytes arranged in five or more rows (Hopkins, 2009). On the other hand, fishes adapted to low water conductivity environments possess narrower caudal filaments with only three longitudinal electrocytes arranged in parallel columns (Hopkins, 2009). These morphological adaptations allow fishes to produce electric discharges that are tuned to the limnological characteristics of different aquatic environments.

Investigations regarding how an electric fish from one kind of habitat respond to variations in water conductivity are relatively common (e.g. MacIver et al., 2001). However, only a few studies focused on how fishes inhabiting different water

conductivity environments respond to reciprocal changes in water conductivity (e.g. Baier, 2008). Performing this kind of experiment on fish from Amazon black, clear, and white water types would reveal how characteristics of the electric organ and EOD vary depending on the water conductivity of the habitat of origin and habitat of transplantation, shedding light on how electric conductivity may affect the fish ability to communicate between divergent environmental conditions. Amazon aquatic habitats exhibit seasonal variation in electric conductivity related to river flood cycles and annual rainfall distribution (Lowe-McConnell, 1987), however the ability of the electrosensory system to adapt to such natural changes in electric conductivity is largely underexplored.

Adaptations to different water conductivities could affect several aspects of fitness. MacIver et al. (2001) found that fishes in aquatic environments outside the natural range of their species performed poorly in prey detection and predator avoidance. These differences in food intake efficiency could result in additional effects on condition-dependent traits (Greenway et al., 2016). Fish with low body condition due to occupying unfavorable environments may be rejected by potential mating partners. Additionally, differences in waveform could also result in mate rejection, at least in the short term. Therefore, the influence of different Amazon water types on the number, morphology and sensitivity of electroreceptors could result in reproductive barriers and ecological speciation.

1.7 Hypotheses to test in this study

It has been suggested that the strikingly different limnological characteristics of the rivers, streams and other water bodies that comprise the Amazon basin are important sources of the divergent selection pressures that may have originated its remarkable fish diversity (Amado et al., 2011; Beheregaray et al., 2015; Cooke et al., 2012b, 2012a, 2014; Pires et al., 2018). Although some authors have suggested the importance of speciation processes mediated by Amazon water types (Cooke et al., 2012b, 2012c; Beheregaray et al., 2015), as far as I know only one study directly evaluated the potential for reproductive isolation among populations exposed to divergent water quality characteristics (Pires et al., 2018). Previous reviews focused on the relative importance of allopatric vs sympatric speciation but did not explicitly consider mechanisms of speciation, being mostly

restricted to the biogeography of speciation. More recently, it has been suggested that differences in lighting environment have shaped the evolution of traits related to visual communication in an Amazonian stream fish species (Pires et al., 2019). However, none of the previous investigations directly evaluated any aspect of the targeted species' sensory systems (Amado et al., 2011; Cooke et al., 2012a, 2012b, 2014; Beheregaray et al., 2015; Pires et al., 2018, 2019).

The sensory drive hypothesis focuses on how sensory/communication systems may adapt to local environments, predicting that divergences in communication systems will arise among populations experiencing contrasting environmental conditions. Such adaptations to communication systems may involve any of the sensory systems: visual, auditory, chemosensory and even electrosensory. Although some limnologic parameters of the Amazon water bodies vary seasonally (Lowe-McConnell, 1987; Costa et al., 2013), the main characteristics that differentiate the main water types are maintained, arguably sustaining divergent selective pressures that can drive apart sensory systems. Although most previously suggested hypotheses consider ecological distinction of the Amazon water types and strongly focus on speciation via allopatry (Albert et al., 2011; Albert & Reis, 2011; Bernardi, 2013), here I investigate some potential mechanisms of divergence mediated by limnologic characteristics, particularly physical characteristics, that could promote ecological speciation in the aquatic realm of the Amazon basin (Boughman, 2002; Servedio & Boughman, 2017). Different characteristics of the Amazon water types may drive adaptations on fishes' sensory systems that do not necessarily result in conspicuous morphological differences among closely related species. However, these fine-tuned sensory adaptations could have a significant impact on intraspecific communication and eventually result in reproductive isolation among populations or even groups of individuals in the same environment (e.g. Cummings & Endler, 2018; Seehausen et al., 2008; Terai & Okada, 2011). Indeed, several studies have highlighted the occurrence of cryptic species within and among Amazon water types (Cooke et al., 2012a; Pires et al., 2018), but without proposing mechanisms related to water types and the speciation process.

Here, I investigated how divergent water colouration may drive populations apart, resulting in the speciation process. Specifically, I am interested in evalutating the role of Amazon water colours in male sexual traits evolution, particularly the colouration

of sexual traits, and female mating acceptance. Such evolutionary process can help us better understand how populations become pre-mating isolated, finally resulting in speciation. Also, I investigated the female colour preference for red and full-spectrum colours, which are those mostly dominant in the black and clear water tyes. Finally, I investigated the molecular basis of the colour vision in an Amazon fish species. By investigating such points, I expect to fdind different genetic, morphological and behavioural adapatations for different coloured Amazon water types, and their effects on leading populations apart. Per instance, black water colouration on males ornaments can have a particular effect that may drive female mating behaviour. Such effect may be associated to their red colour preference and have a molecular basis, which generally is associated to the fish colour vision.

1.8 Species model

In this study I used the sailfin tetra *Crenuchus spilurus* Günther, 1863 (Characiformes: Crenuchidae) an Amazon fish species that lives in small pounds in black and clear water forest streams, also known as *igarapés*. This is a small-sized fish species that may reach around 6 cm of standart length. They live in ponds with slow water flow that is surrounded by the buriti palm tree, which they also feed on their fruits. Their diet is composed by several items, including insects and their larvae (aquatic and terrestrial one, which may fall from the trees in the water body), fruits and alge. The sailfin tetra is composed for two main lineages, one inhabiting black and the second one inhabiting clear water types. Population living in different water types are reproductively isolated (Pires et al., 2018). Interestingly, some populations, as the case of the CJ1 population, live in black water types, but genetically belong to the clear water lineage. Also, the male ornament colour pattern assemble to those males of the other populations of the clear water lineage, suggesting that they recently (in a geological scale) colonized this black water *igarapé*. Here, I have a great opportunity to investigate the convergent evolution of the mating behaviour and visual adaptations to black water underwater lighting condition in Amazon using the CJ1 population.

The mating behaviour of the sailfin tetra is complex. The information here was summarized from Pires et al. (2016), Borghezan et al. (2019) and Pires et al. (2021), and

based on my personal observations in the captivity conditions and during naturalistic observations. For more detailed information, please consult the above-mentioned studies. Females receptive to courtship behaviour often show a darkened abdominal region. Also, the abdominal region may become dark during the courtship behaviour. When a male sexually approaches a female, the male spreads his dorsal and anal fins. The male then swims in circles above and/or surrounding the female, a movement that is conspicuously faster than ordinary swimming movements. The male also touches the female body with his snout during the courting process. When receptive, females adopt a sinusoidal (S-shaped) position with the caudal fin positioned in the opposite direction of the male, ending the movement with her body straight in a quick movement. The male then swims towards the nesting site. In some cases, the female promptly follows the courting male; however, if not followed, the male usually repeats the movements. Under natural conditions, the courtship behaviour is often paused by disturbances such as the approach of a larger fish, the presence of predatory species nearby, objects falling on the water surface, and especially, intervention by other (usually larger) males. Therefore, a couple may court for several consecutive days until the spawning event. Isolated similar-sized couples, which show higher mating probabilities, under captivity usually spawn at least 4 days after the first courtship movement is observed. Also, under captivity conditions, I have seen a female spawning with the same male in a 16 days interval (observed in three independent couples). After the spawn, males undertake paternal care for around a week, the time in which larvae reach the free-swimming phase. During the parental care phase, males stay inside their nests for the duration of the parental care. However, under captivity conditions, I have seen that some males may go out of the nesting site if there is another male around. I have little evidence of polygyny for this species, as only one clutch found in the field contained eggs at two different developmental stages; however, it is unclear if that particular male switched from the parental phase to the courting phase, or if a second female was attracted by the presence of eggs inside the male nest. Therefore, it is unclear if females of the sailfin tetra apply a mate copying strategy.

I have also described an alternative reproductive tactic in the sailfin tetra that is dependent on physical aggression. Larger and dominant males usually occupy and stay in the available nesting site or the best nesting site. Smaller males on the other hand, often court females leading them to the occupied nesting site. At this moment, the larger male

takeover the female and reproduce with her. By doing this, dominant males benefit from the courtship effort of such subordinate males. However, in some cases, smaller males may inspect occupied nesting sites facing the larger male. Under such cases, males may compete and physically interact for the nesting site. By losing the aggressive contests smaller males ceased courtship. In such instances, the courtship effort of dominant males is often higher, suggesting that dominant males modulate their courtship effort according to the effort of subordinate males.

Male display their colored anal and dorsal fins and perform an elaborated courtship behaviour towards females. The courtship behaviour involves displaying males ornaments, surrounding females vertically and horizontally, body touch, assuming a body position that is like a “S” and moviments that may lead female to the nesting site. Nest sites are usually small cavities such as the curled leaves of the buriti palm tree, which assembles a PVC pipe (Pires et al., 2016). Indeed, PVC pipes have been used for spawning in captivity conditions (Borghezan et al., 2019). Specific details of the sailfin tetra will be given in each of the following chapters as they are needed for understanding their background.

Chapter 2

Effect of light bias on male mating signal and female mate choice in a sexually dimorphic Amazon fish

2.1 Introduction

Female mate choice favours traits that are reliable indicators of direct and/or indirect fitness benefits (Andersson, 1994; Johnstone, 1995; Kokko et al., 2006). Colourful ornaments are usually honest indicators of male quality since they pose energetic and predation risk costs (Andersson, 1994; Giery & Layman, 2015; Weaver et al., 2017). Indeed, highly ornamented males have been reported to possess better foraging ability (Møller et al., 2000) and increased levels of resistance to parasites (Andersson, 1994; Milinski & Bakker, 1990). Therefore, it is expected that more intensely coloured males should be chosen as mating partners (Andersson, 1994). However, how ornaments are perceived by choosers varies significantly according to the environment, especially in aquatic environments (Endler, 1992; Endler & Basolo, 1998; Hunt et al., 2004). Clear waters do not bias the underwater colouration; however, black waters bias the colouration towards yellow and red colours (Borghezan et al., 2021). Therefore, the value of colouration as a sexual signal can be affected by the environment (Greenfield & Rodriguez, 2004). Consequently, sexual preference for a particular trait under a given environmental condition may become fruitless in another environmental condition (Candolin et al., 2007). This can lead to environmentally-dependent trait preferences and sexual selection (Heuschele et al., 2009).

Females can easily assess potential mates when mating signals vary among individuals, which may facilitate mate choice (Giery & Layman, 2015; Hunt et al., 2004). However, environmental conditions can bias and limit the available information (Endler, 1992), making it harder for females to correctly assess the quality of potential mates. Also, by biasing the available information, the environmental conditions can reduce the reliability of a particular sexual trait, a phenomenon known as “signal-masking” (Hunt et al., 2004; Reimchen, 1989). The precise mechanisms through which the environment affects signal transmission and its reliability, particularly by reducing perceived variation

among individuals, remain relatively unexplored.

Amazon forest streams (locally named *igarapés*) can be classified as black water or clear water depending on their apparent colouration as seen from land (Sioli, 1984; Wallace, 1853). These water types differ markedly in their physical and chemical characteristics (Sioli, 1984; reviewed in Borghezan et al., 2021), especially with regard to the amount of dissolved organic carbon (DOC), which biases lighting transmission towards long wavelengths. Black waters are yellow/red-biased environments, while clear waters are mostly transparent and nearly unbiased (Costa et al., 2013; Mendonça et al., 2005).

The sailfin tetra *Crenuchus spilurus* is a small-sized and sexually dimorphic fish species that occurs in forest streams across a wide geographical range of the Amazon, including black and clear waters (Pires et al., 2016). Males possess dorsal and anal fins conspicuously ornamented in red and yellow (Figure 2.1). A previous study has shown that the sailfin tetra is composed of two phylogenetically distinct main lineages that inhabit black (Rio Negro lineage) and clear (Amazonas lineage) water types and are reproductively isolated (Pires et al., 2018). Individuals do not migrate or disperse their young over long distances (Pires et al., 2016) and therefore, have contact only with their native water type. Some populations, however, as the case of the CJ1 population, are genetically part of the clear water lineage but currently live in a black water *igarapés* of a restricted geographical area of the Solimões River in Brazil (Pires et al., 2018). The species lives in ponds with low water flow that are frequently surrounded by buriti palm tree stands (*Mauritia flexuosa*). The sailfin tetra is omnivorous and feeds on algae, fruits and insect larvae, including the fruits of the buriti palm tree and Chironomidae larvae (Pires et al., 2016), which are red-coloured food items. Therefore, the red colour may play an important role in the sailfin tetra (more information about the ecology of the sailfin tetra can be found in Appendix 2.5.1).

Male ornaments colour pattern, eye size and red colour intensity in dorsal fin ornaments differ between the sailfin tetra lineages, which have been suggested to be shaped by water colour (Pires et al., 2019). Black water lineage males have larger red and dark areas on the dorsal fin and larger yellow anal fin spots than clear water lineage males (including males from the CJ1 population). Meanwhile, clear water lineage males have more and smaller anal fin spots than black water lineage males. These coloured spots may

function as a mate attractor and stimulate female mating behaviour, as observed in other fish species, particularly in mouth-brooding cichlids (Wickler, 1968). The shape of the dorsal fin is also clearly different between the two lineages. Black water lineage males have a flame-shaped dorsal fin whereas the upper outline of the dorsal fin of clear water lineage and CJ1 population individuals is more evenly convex (Figure 2.1). The red area of the dorsal fin is more intensely pigmented in black water than in clear water lineage males. However, no difference was found for the red-coloured portion of the anal fin. Black water lineage individuals also showed larger eyes when compared to the clear water lineage, a difference that is readily observed in larger individuals. Such difference in eye size possibly compensates for the decreased light intensity in black waters (Pires et al., 2019), and may represent a visual adaptation to processes such as finding food resources and avoiding predators. Interestingly, although the CJ1 population lives in black water, all ornament characteristics and eye size were similar to those of other clear water lineage populations (Pires et al., 2019). Such similarity between the CJ1 population and other clear water lineage populations suggests that the CJ1 population has undergone secondary and relatively recent contact with black waters, which is also supported by the genetic proximity between CJ1 and other populations from the clear water lineage (Pires et al., 2018). Here, the CJ1 population provides a good opportunity to evaluate the convergent evolution of male ornaments colouration and their interaction with water colouration and female mate choice to black water conditions.

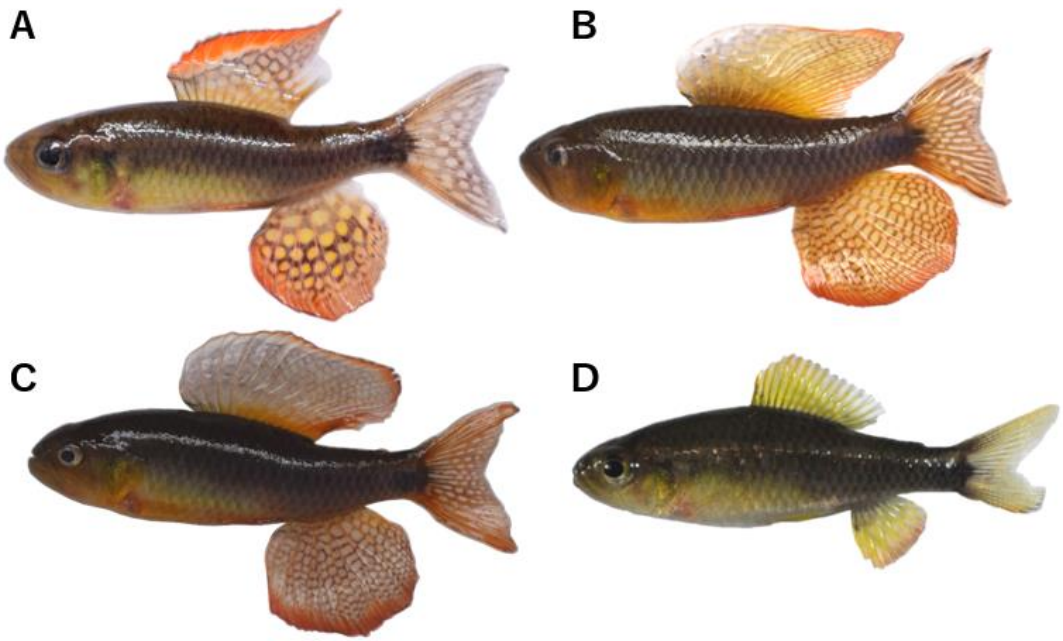

Figure 2.1. Morphological differences among males from different lineages and a female of the sailfin tetra *Crenuchus spilurus*. Males from **(A)** black water lineage, **(B)** clear water lineage, **(C)** CJ1 population and **(D)** a black water lineage female. Black water lineage males have larger areas of the red and black parts on the dorsal fin and larger eyes than clear water lineage males (including males from the CJ1 population). They also have larger and fewer anal fin spots than clear water lineage and CJ1 males. There was no difference between clear water lineage males and CJ1 population males in anal fin spot number and size. Morphological results were taken from Pires et al. (2019).

Ornaments in male *C. spilurus* are yellow and red coloured. These colours probably come from carotenoids present in the fish's diet, especially from buriti palm tree (*Mauritia flexuosa*) fruits (Pires et al., 2016). Since carotenoids come from the fish's diet, it can be expected that males with redder ornaments are healthier (Andersson, 1994) and better at foraging (e.g. Møller et al., 2000). Also, the expression of colours can be genetically dependent, in which case more intensely coloured males would show a better genetic quality (Andersson, 1994). Additionally, the red colour of the male fins does not change quickly, which may increase the reliability of the colourful traits when assessing potential mates (Weaver et al., 2017). However, male ornaments colouration and how

they are perceived by females are expected to be affected by water colour (Borghezan et al., 2021), but the effects of black water light conditions on ornament colouration are still unknown. A comparison of male ornaments colouration between lineages under light conditions simulating those in their natural habitats can help us to understand how water colouration can drive female mate choice for male ornaments colouration.

Based on experiments under captive conditions, black water lineage female prefer males with larger ornaments (Pinto et al., 2021); however, the role of male ornament size for clear water lineage, and of ornament colouration on female mate choice for both lineages, are still unknown. Considering the characteristics of black water and its effect on achromatic cues, I expect that, under black water lighting conditions, the red colouration of male ornaments would be perceived even brighter and more intense (Endler, 1993; Endler & Houde, 1995). Therefore, it is also expected that female mating acceptance will be higher under black water lighting conditions. However, since strong red-light bias limits colour transmission, it can reduce individual variation perceived by females. Thus, I investigated whether the individual variation of the red colour in male ornaments would be lower under black water conditions. If black water "spectrally masks" the red colouration of male ornaments while reducing their variability, it may subsequently reduce the reliability of assessing male quality through red colouration. This is due to the potential drawbacks faced by females when predominantly selecting males based on a trait that is both poorly variable and subject to spectral masking. Females would need more energy to detect small differences in the trait and could choose low-quality males that appear like high-quality ones (Hunt et al., 2004). Such a strategy could prove costly and pose risks for female mate choice decisions. In contrast, clear water does not affect the perceived ornament colouration and females inhabiting this water type can rely on males' ornament colouration to assess male quality. Here, I investigated the effect of water colouration on the perceived colouration of male ornaments and male and female mate choice between the sailfin tetra lineages to understand the evolution of male ornaments and mate choice signals used by females.

2.2 Material and Methods

2.2.1 Fish sampling and housing

I collected sailfin tetra individuals from populations that represent the two main lineages (Rio Negro - black water, and Amazonas - clear water) as well as the CJ1 population (Pires et al., 2018). Black water lineage individuals were sampled from an igarapé inside an urban forest fragment in Manaus, Brazil (3°06'22.8"S; 59°58'40.5"W); clear water lineage individuals were sampled from a small forest stream near Iquitos, Peru (4°0’46.20”S; 73°27’47.70”W), and CJ1 population individuals from a small stream on the outskirts of the Coari municipality, Brazil (4°07'09.9"S; 63°04'29.9"W). Fish were sampled using seines and hand nets. Fish were housed in 92 L aquariums containing natural and artificial plants and PVC pipes to provide shelter. Individuals were separated by population and sex for at least 30 days prior to experimentation. A more detailed description of the housing methods can be found in the Ethical Note (see below). All individuals used in the experiments were adults since the smallest individual had a standard length (the length of a fish measured from the tip of the snout to the posterior end of the last vertebra) of 3.23 cm, 0.65 cm larger than the estimated size at maturity for this species (2.57 cm, Pires et al., 2016). All experiments took place at the National Institute of Amazonian Research – INPA, Manaus, Brazil.

2.2.2 Simulating natural lighting conditions for courtship experiment

I simulated natural lighting conditions in the laboratory to investigate the effect of red-biased water colouration on the male ornament and to investigate female mate choice under different light conditions. I chose to simulate black water instead of using the black water itself aiming to isolate the effects of the lighting conditions from the chemical differences of the water types. I initially sampled water from Rio Negro and from the other three black water *igarapés* where the sailfin tetra has been collected by our team (see Appendix 2.5.2 for geographic coordinates). Also, based on naturalistic observations during snorkelling, I noted that sailfin tetra individuals usually court in the water column at nearly 40 cm below the water surface (Pires et al., 2016); therefore, I measured the water transmittance of a 40 cm water column from Rio Negro and the three black water *igarapés*. Given variations in the transmittance of black waters (see Appendix

2.5.3), our study aimed to assess the impact of water colour on male ornamentation and female mate choice, comparing these effects across different underwater lighting conditions rather than emphasizing absolute differences. Based on these measurements, I placed a set of yellow and orange colour filters between a full-spectrum light source and the water surface of the aquarium so that the resulting light transmittance fell within the range of our sampled water. Light transmittance results of the water samples and black water filter used in our courtship trials can be found in the Appendix 2.5.3.

2.2.3 Measuring male ornaments' size and colouration

To assess male ornament size and colouration, males were photographed immediately after the mating trials. Males were anaesthetized in a solution containing Eugenol (125 mg/L), placed on a smooth and moist whiteboard and had their dorsal and anal fins gently spread using a wet soft bristle paintbrush (Pires et al., 2019). The fins were spread as much as possible while avoiding damage to the inter-radial membranes. Fins were pinned using entomological needles; a reference scale and tags were positioned on the picture frame. Photos were taken under two different light conditions, full-spectrum light and black water filter, representing the two most extreme conditions in terms of light bias. The black water filter was also used in our mating trails. All pictures were taken inside a white photo studio with no influence from the room light, and using a Nikon D3300 camera. A full-spectrum LED light (ADA, Japan) was positioned inside the photo studio box and was used as a light source. Ornament measurements were taken from these digital images using the software ImageJ. Based on pictures of the males, we measured the standard length and the area of the dorsal and anal fins using the software ImageJ. Similarly, the red, green and blue (RGB) values from nine different sample pixels were taken from the red area of the dorsal fin, the red area of the anal fin, the yellow spots of the anal fin and the brown background of the anal fin (Appendix 2.5.4). Individuals were moved to an oxygen-rich aquarium right after the photographs. No fish died from this procedure and the minor damages to the fins were fully recovered after about five days.

2.2.4 Female choice trials

Because the sailfin tetra males and females prefer and show a higher likelihood of spawning with similar-sized mates (Borghezan et al., 2019), I selected pairs of individuals (mates) that matched in size as much as possible (average difference between sexes 1.70 ± 1.99 mm standard deviation). Males and females had their standard length measured with a digital calliper and were separated in individual tanks (40 x 30 x 30 cm) for five days prior to the experiment. Each tank contained white sand substrate and an artificial plant similar to those found in their natural habitat.

A male and female were simultaneously inserted into an experimental tank (40 x 30 x 30 cm) that was identical to the individual housing tanks. I recorded interactions with a video camera (Sony Handycam CX405) attached to a tripod positioned 60 cm away from the experimental tank. A black cloth that contained a small hole for the camera lens prevented the fish from seeing the observer. All trials lasted one hour and were conducted between 1600h and 1800h, to match the period when most courtship behaviour was recorded during direct field observations and under laboratory conditions (EAB and THSP personal observations; Pires et al., 2016). Mating trials were performed under two different lighting conditions, full-spectrum light and black water light. Two different full-spectrum LED lights (ADA, Japan) provided 250 lux of light to the experimental tanks. For the black water light condition, I used a full-spectrum light source filtered out by a red-biased colour filter (Appendix 2.5.3). The experimental tank, light source, and recording equipment were sheltered within a dark chamber to prevent any interference from external light during mating trials. The aquarium was rinsed and the water was completely renewed after each trial to avoid chemical cues from influencing subsequent trials. Each couple was used only once. Female mate choice was assessed through acceptance of male courtship behaviour following Pires et al. (2016). Female acceptance was assumed when females started following males, performed “S” body shape position (sexually interacting with the courting male) and showed darkening of the ventral region of the belly. The number of pairs used per lineage under each lighting condition can be found in Appendix 2.5.5.

2.2.5 Statistical analysis

2.2.5.1 Male ornament colouration

The effect of different light conditions on the perceived male ornament colouration was evaluated using RGB values (Montenegro et al., 2019). RGB values can be a good indicator to evaluate how ornaments are perceived by females since the sailfin tetra has only opsins genes that are sensitive to blue, green and red spectrum colours (Escobar-Camacho et al., 2020). Additionally, under red-biased environments, the values of the blue channel of the RGB decrease, similar to what happens with the short wavelengths (e.g. blue colour) under black waters (i.e. red-biased environment). Therefore, the RGB red colour index may reflect the expected differences in the red colour brightness between clear and black underwater lighting conditions. The red intensity of the anal and dorsal fins and the yellow spots in the anal fin were measured separately and compared between light conditions and fish lineages. The average of the nine RGB values for each measurement was used to create a red colour index following the equation:

$$\text{Red colour index} = ((R-G) + (R-B)) / ((R+G) + (R+B)),$$

Where, R, G and B represent red, green and blue values. I also measured the contrast between the yellow spot and the brown background of the anal fin using the following equation:

$$\text{Colour contrast} = (Rs - Rb) + (Gs - Gb) + (Bs - Bb),$$

Where Rs = Red value in the yellow spot; Rb = Red value in the background; Gs = Green value in the yellow spot; Gb = Green value in the background; Bs = Blue value in the yellow spot; Bb = Blue value in the background. Higher values of this equation mean higher contrast between the yellow spot and the anal fin background. I took photos of all tested males under full-spectrum and black water lighting conditions. To evaluate how ornament colours varied among lineages under each lighting condition and because of unequal variation, I performed four independent Kruskal-Wallis tests using the (I)

Dorsal fin red index, (II) Anal fin red index, (III) Yellow spot on anal fin red index, and (IV) Anal fin spot contrast as dependent variables, and lineage as our independent variable. I also ran post-hoc Wilcoxon tests to evaluate the difference among lineages.

I also evaluated the effects of the black water lighting condition on male ornament colouration by comparing how each ornament colouration differed between lighting conditions for each lineage. For that, I fitted several mixed-effects models to examine the effects of different light conditions (full-spectrum or black water light) on each of our dependent variables (e.g. Anal fin red index) for each sailfin tetra lineage. Because I took photos of all males under both lighting conditions, our model required accounting for the repeated measures design. To appropriately handle the repeated measures aspect of the data, I included male identity as a random factor in the mixed-effects models. I also controlled for the unequal variation (heteroscedasticity) between the two lighting conditions in our data set.

I also evaluated the among-individual variation in each of the four dependent variables (i.e. colour trait in male ornaments) for each lineage between both lighting conditions. Because of our small sample size (Appendix 2.5.5), I analysed if the variation in our dependent variables differed between lighting conditions per lineage through Levene's test. Levene's test performs an ANOVA test over the absolute deviations from each group's average or the absolute deviations from each group's median to evaluate whether the samples have equal variances or not. Finally, I evaluated the relationship between male ornament size index and the red colouration on the dorsal and anal fins under the full spectrum light (no colour bias). For that, I performed two linear models per lineage, (I) using the anal fin red index and (II) the dorsal fin red index as dependent variables, and male ornament size index as independent variable. The whole data set was used for this analysis.

2.2.5.2 Male ornament size index

Because sailfin tetra females prefer males with larger ornaments (Pinto et al., 2021), I also included the size of male ornaments in my analysis. Also, because larger males have exponentially larger ornaments, I could not use the absolute ornament size as my variable. Therefore, to assign the degree of ornamentation in males, I took the

residuals from the Linear Mixed-Effects Model (*lme4* package, Bates et al., 2014) using the sum of the areas of dorsal and anal fins (square root transformed, to reach linearity) as the dependent variable, male standard length as the factor and lineage as a random factor. Positive values thus represent a higher degree of ornamentation than expected for the size of the fish. Conversely, negative values represent a lower degree of ornamentation than expected for a given standard length (Pinto et al., 2021). The whole data set was used for this analysis.

2.2.5.3 Male mate choice

I assessed if males were more likely to sexually display toward females under a particular light treatment and if the male mate choice differed between groups (lineages) or based on ornaments size. I fitted a glm model using the presence or absence of male sexual display (binomial, 0 or 1) as our dependent variable and light condition (full-spectrum or black water), sailfin tetra lineage (black water, clear water, or CJ1) and male ornament size index as factors. I included the interaction between all terms in our analysis. Because our dataset was unbalanced (see Appendix 2.5.5), I fitted an ANOVA type II to evaluate the significance of each factor and their interactions in our glm model. The whole data set was used for this analysis.

2.2.5.4 Females mate choice

I fitted a glm model using female acceptance (binomial, 0 = rejection of the courting male, 1 = acceptance of the courting male) as the dependent variable and light condition (full-spectrum or black water), sailfin tetra lineage (black water, clear water, or CJ1) and male ornament size index as factors. I included the interaction between all terms in the analyses. I also fitted an ANOVA type II to evaluate the significance of each factor and their interactions in my glm model. Only those trials in which the male attempted to court the female were considered in my statistical analysis.

2.2.6 Ethical note

Fish were sampled using seines and hand nets. Fish collections in Brazil were carried out under IBAMA permanent licence SISBIO #10199-1 to Jansen Zuanon, one of my collaborators. Once in the laboratory, fish were housed in 92 L aquariums separated by population and sex for at least 30 days prior to experiments. To simulate the natural environment, natural and artificial plants were added to the tanks and over 10 pieces of PVC pipes (10 cm long, 25 mm in diameter) to provide shelter. This species does not actively swim around the tanks, and its territory is only defended at a very close range near PVC pipes; thus, aggressive interactions were rarely observed.

Each tank contained a filter and an air pump. Water changes (25%) were performed weekly to maintain water quality. Windows that covered over half of the wall of the laboratory provided indirect natural light and photoperiod (12:12 h light:dark cycle). I used dark cloth on the windows to control the amount of light that reached the laboratory, effectively simulating the natural canopy-shaded condition of an Amazon forest stream. An air conditioning system maintained the laboratory at a constant temperature of 24 °C, simulating natural temperature conditions of Amazon forest streams. All individuals were fed ad-libitum with high-quality ornamental fish food once a day.

Experiments were conducted following Brazilian federal law 11.794/2008. This study was approved by the Ethical Committee for Animal Use in Experiments of Kyoto University (WRC-2019-009A). No fish died during or immediately after the experiments. After the experiments, fish were maintained in the laboratory for other studies.

2.3 Results

2.3.1 Colour differences among lineages under full spectrum light

The red index of the red area of the dorsal fin differed among lineages (Kruskal-Wallis: $H(2) = 26.004$, $p < 0.0001$, $\eta^2 = 0.2017$): it was higher for black water than clear water (Wilcoxon rank sum test: $W = 1795$, $p < 0.0001$, $r = 0.510$) and CJ1 (Wilcoxon rank sum test: $W = 863$, $p = 0.001$, $r = 0.366$), with no significant difference between clear water and CJ1 (Wilcoxon rank sum test: $W = 538$, $p = 0.272$, $r = 0.127$) (Appendix 2.5.6 Figure A2.6.1A). In contrast, the red index of the red area of the anal fin did not

differ among the three lineages (Kruskal-Wallis: $H(2) = 3.662$, $p = 0.160$, $\eta^2 = 0.0139$) (Appendix 2.5.6 Figure A2.6.1B). The red index of the yellow spot on anal fin differed among lineages (Kruskal-Wallis: $H(2) = 46.419$, $p < 0.0001$, $\eta^2 = 0.3732$): it was higher in black water than in clear water (Wilcoxon rank sum test: $W = 1985$, $p < 0.0001$, $r = 0.656$) and CJ1 (Wilcoxon rank sum test: $W = 1010$, $p < 0.001$, $r = 0.569$), without significant difference between clear water and CJ1 (Wilcoxon rank sum test: $W = 570$, $p = 0.459$, $r = 0.086$) (Appendix 2.5.6 Figure A2.6.1C). The anal fin spot contrast also differed among lineages (Kruskal-Wallis: $H(2) = 7.294$, $p = 0.026$, $\eta^2 = 0.0444$). It was higher for black water than clear water (Wilcoxon rank sum test: $W = 1469.5$, $p = 0.010$, $r = 0.262$), but without significant difference between black water and CJ1 (Wilcoxon rank sum test: $W = 758$, $p = 0.061$, $r = 0.221$) or between clear water and CJ1 (Wilcoxon rank sum test: $W = 606.5$, $p = 0.738$, $r = 0.039$) (Appendix 2.5.6 Figure A2.6.1D).

2.3.2 Colour differences among lineages under black water lighting condition

Under the simulated black water lighting condition, the red index of the red area of the dorsal fin differed among lineages (Kruskal-Wallis: $H(2) = 25.856$, $p < 0.0001$, $\eta^2 = 0.2004$): it was higher in black water than in clear water (Wilcoxon rank sum test: $W = 1784$, $P < 0.0001$, $r = 0.502$) and CJ1 (Wilcoxon rank sum test: $W = 877$, $p = 0.001$, $r = 0.385$), without significant difference between clear water and CJ1 (Wilcoxon rank sum test: $W = 547$, $p = 0.319$, $r = 0.116$) (Appendix 2.5.7 Figure A2.7.1A). On the other hand, the red index of the red area of the anal fin did not differ among the three lineages (Kruskal-Wallis: $H(2) = 2.313$, $p = 0.314$, $\eta^2 = 0.0026$) (Appendix 2.5.7 Figure A2.7.1B). The red index of the yellow spot on anal fin differed among lineages (Kruskal-Wallis: $H(2) = 64.088$, $p < 0.0001$, $\eta^2 = 0.5217$): it was higher in black water than clear water (Wilcoxon rank sum test: $W = 2155$, $p < 0.0001$, $r = 0.785$) and CJ1 (Wilcoxon rank sum test: $W = 1053$, $p < 0.0001$, $r = 0.629$), without significant difference between clear water and CJ1 (Wilcoxon rank sum test: $W = 526$, $p = 0.218$, $r = 0.143$) (Appendix 2.5.7 Figure A2.7.1C). The anal fin spot contrast also differed among lineages (Kruskal-Wallis $H(2) = 18.617$, $p < 0.0001$, $\eta^2 = 0.1396$); it was higher for black water than clear water (Wilcoxon rank sum test: $W = 1683$, $p < 0.0001$, $r = 0.425$) and CJ1 (Wilcoxon rank sum test: $W = 830$, $p = 0.006$, $r = 0.321$), without significant difference between clear water

and CJ1 (Wilcoxon rank sum test: W = 540, p = 0.282, r = 0.125) (Appendix 2.5.7 Figure A2.7.1D).

2.3.3 Differences between lighting conditions

In all lineages, the simulated black water light condition significantly increased the red index of the red area in the dorsal fin (for black water lineage, ANOVA: $F(1,45)= 836.973$, $p < 0.0001$, $\eta^2 = 0.0667$; for clear water lineage, ANOVA: $F(1,48)= 1650.776$, $p < 0.0001$, $\eta^2 = 0.2842$; for CJ1 population, ANOVA: $F(1,25)= 325.7402$, $p < 0.0001$, $\eta^2 = 0.1460$) (Figure 2.2A) and those of the red area and yellow spot on the anal fin (Red area in anal fin: for black water lineage: ANOVA: $F(1,45)= 1018.35$, $p < 0.0001$, $\eta^2 = 0.0596$; for clear water lineage: ANOVA: $F(1,48)= 629.975$, $p < 0.0001$, $\eta^2 = 0.0290$; for CJ1 population: ANOVA: $F(1,25)= 325.595$, $p < 0.0001$, $\eta^2 = 0.0543$), and the yellow spots in anal fin: for black water lineage: ANOVA: $F(1,45)= 2389.419$, $p < 0.0001$, $\eta^2 = 0.1022$; for clear water lineage: ANOVA: $F(1,48)= 3461.201$, $p < 0.0001$, $\eta^2 = 0.2571$; for CJ1 population: ANOVA: $F(1,25)= 927.5013$, $p < 0.0001$, $\eta^2 = 0.2872$) (Figure 2.2B and 2.2C). In contrast, the simulated black water lighting condition decreased the anal fin spot contrast for all lineages (for black water lineage: ANOVA: $F(1,45)= 62.2059$, $p < 0.0001$, $\eta^2 = 0.1269$; for clear water: ANOVA: $F(1,48)= 149.0221$, $p < 0.0001$, $\eta^2 = 0.2375$; for CJ1 population: ANOVA: $F(1,25)= 927.5013$, $p < 0.0001$, $\eta^2 = 0.0732$) (Figure 2.2D).

The mean and standard deviation of each measurement for each lineage under full-spectrum and black water light can be found in Appendix 2.5.8. Interestingly, in all lineages the among-individual variation of the red index of the red area of the dorsal and anal fins was much smaller under the simulated black water lighting condition than it was under full-spectrum light (Levene's Test for Homogeneity of Variance: dorsal fin: black water lineage: $F(1,90) = 23.421$, $p < 0.0001$; for clear water lineage: $F(1,96) = 17.152$, $p < 0.0001$; for CJ1 population: $F(1,50) = 6.0748$, $p = 0.0171$; anal fin: for black water lineage: $F(1,90) = 17.458$, $p < 0.0001$; for clear water lineage: $F(1,96) = 22.917$, $p < 0.0001$; for CJ1 population: $F(1,50) = 15.474$, $p = 0.0002$) (Figure 2.2A and 2.2B). The simulated black water light condition decreased the variation of the red index of the yellow spot on the anal fin of the black water lineage (Levene's Test for Homogeneity of

Variance: $F(1,90) = 17.299$, $p < 0.0001$). However, it did not change the variation of the red index of the yellow spot on the anal fin for both clear water (Levene's Test for Homogeneity of Variance: $F(1,96) = 2.8248$, $p = 0.096$) and CJ1 (Levene's Test for Homogeneity of Variance: $F(1,50) = 0.549$, $p = 0.462$) (Figure 2.2C). Finally, the simulated black water lighting condition decreased the variation of the anal fin spot contrast for black water (Levene's Test for Homogeneity of Variance: $F(1,90) = 6.902$, $p = 0.010$) and clear water males (Levene's Test for Homogeneity of Variance: $F(1,96) = 4.2741$, $p = 0.041$), but not for CJ1 (Levene's Test for Homogeneity of Variance: $F(1,50) = 1.2779$, $p = 0.263$) (Figure 2.2D).

It is important to highlight that the red part of the black water lineage male ornaments varies among individuals, similarly to the clear water lineage males when seen under the full spectrum water. However, under natural conditions, for the black water lineage males such variation is spectrally masked and had its among-individual variation decreased by the black water lighting condition.

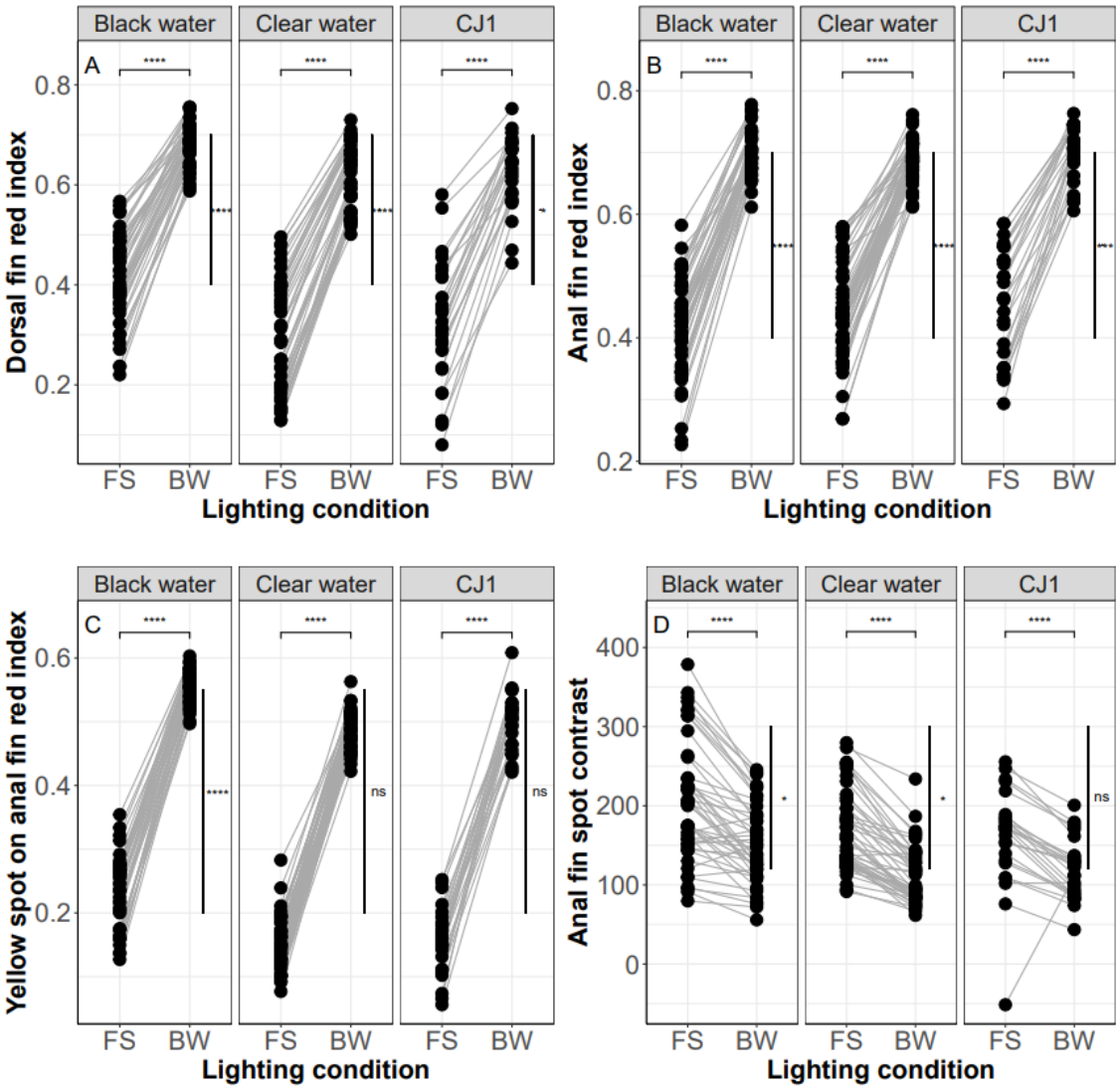

Figure 2.2. Differences in the value and among-individual variation of the colouration index between the simulated black water and full-spectrum lighting condition. **(A)** red area of the dorsal fin, **(B)** red area of the anal fin, **(C)** yellow spot on the anal fin, and **(D)** anal fin spot contrast under simulated black water light. Points connected by lines represent the same individual data under both lighting conditions. The horizontal lines show the comparisons between lighting conditions, while the vertical lines show the comparisons of the variance between lighting conditions. Indicates: **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns = non-significant. FS = full-spectrum; BW = black water.

2.3.4 Male mate choice

The sample size for each lineage and light condition and the full factorial statistical analysis results can be found in the Appendix, Appendix 2.5.5 and Appendix 2.5.9 respectively. The willingness to perform sexual displays towards females was affected by the male ornament size index (ANOVA II: DF = 1, LR = 5.093, p = 0.024, VPC = 0.489), but did not change among lineages (ANOVA II: DF = 2, LR = 1.435, P = 0.487, VPC = 0.494) or lighting conditions (ANOVA II: DF = 1, LR = 3.307, p = 0.068, VPC = 0.492). Males with larger ornaments were more likely to court females than those less ornamented (Figure 2.3). Also, there was no significant relationship between the ornament size index and the intensity of the red colouration on the anal fin for the three lineages (Clear water lineage: Linear model: F(1,23) = 0.4658, R2 = 0.0198, p = 0.5018; Black water lineage: Linear model: F(1,23) = 0.1801, R2 = 0.0077, p = 0.6752; CJ1: Linear model: F(1,14) = 0.1437, R2 = 0.0101, p = 0.7103). Similarly, no significant relationship between the ornament size index and the intensity of the red colouration on the dorsal fin was found (Clear water lineage: Linear model: F(1,23) = 0.5983, R2 = 0.0253, p = 0.4471; Black water lineage: Linear model: F(1,23) = 2.661, R2 = 0.1037, p = 0.1164; CJ1: Linear model: F(1,14) = 0.4699, R2 = 0.0324, p = 0.5042) (Appendix 2.5.10). After checking the plot of the probability of males performing sexual displays towards females in relation to their own ornament size index (Figure 2.3), I checked the data for possible outliers. Despite finding some outliers, the results of the analysis remained the same after treating them (Appendix 2.5.11).

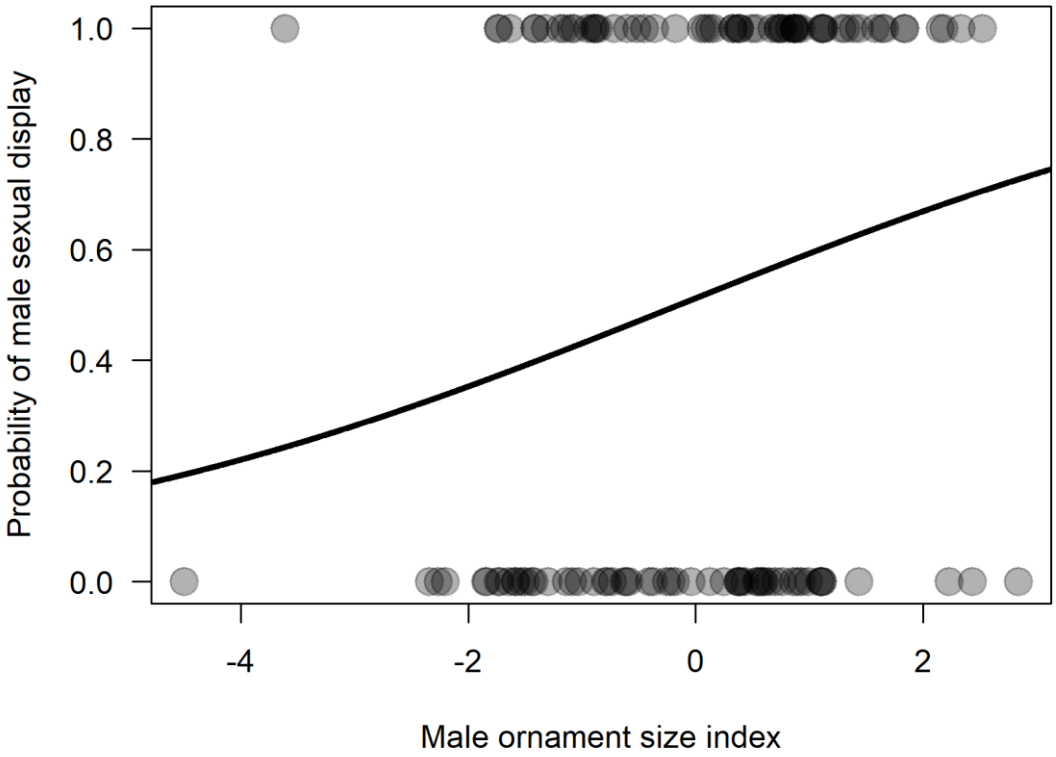

Figure 2.3. Relationship between the occurrence of male sexual display and male ornamental size index. Points represent each male used in the mating experiment.

2.3.5 Female mate choice under different light conditions

The sample size for each lineage and light condition and the full factorial statistical analysis results can be found in the Appendix 2.5.5 and Appendix 2.5.12. respectively. The relationship between female acceptance and male ornament size index was statistically significant (ANOVA II: DF = 1, LR = 4.671, p = 0.030), indicating that females of the three sailfin tetra lineages are more prone to respond to males with larger ornaments (Figure 2.4). The interaction between lighting condition (full-spectrum or black water light) and lineage (black water, clear water or CJ1) was also statistically significant (ANOVA II: DF = 2, LR = 6.994, p = 0.030), suggesting that lighting conditions had different effects on female mating acceptance in females from different

lineages. Mate acceptance by females from black water lineage was not influenced by lighting condition (GLM: DF = 19, Resid. Dev = 26.17, p = 0.187) (Figure 2.5A). I also did not find any significant influence of lighting condition on the mate acceptance by females from the CJ1 population (GLM: DF = 11, Resid. Dev = 15.589, p = 0.498) (Figure 2.5B). On the other hand, females from clear water lineage were more willing to accept males under black water lighting (GLM: DF = 26, Resid. Dev = 34.666, p = 0.045) (Figure 2.5C). Interestingly, five out of 10 clear water lineage males that were accepted under black water lighting condition (which spectrally masked their red colouration) had smaller ornaments than expected by their respective body size (Appendix 2.5.13). After checking the plot of the probability of female acceptance and male ornament size index (Figure 2.4), I checked the data for possible outliers. Again, despite finding some outliers, the results of the analysis remain the same after treating them (Appendix 2.5.11).

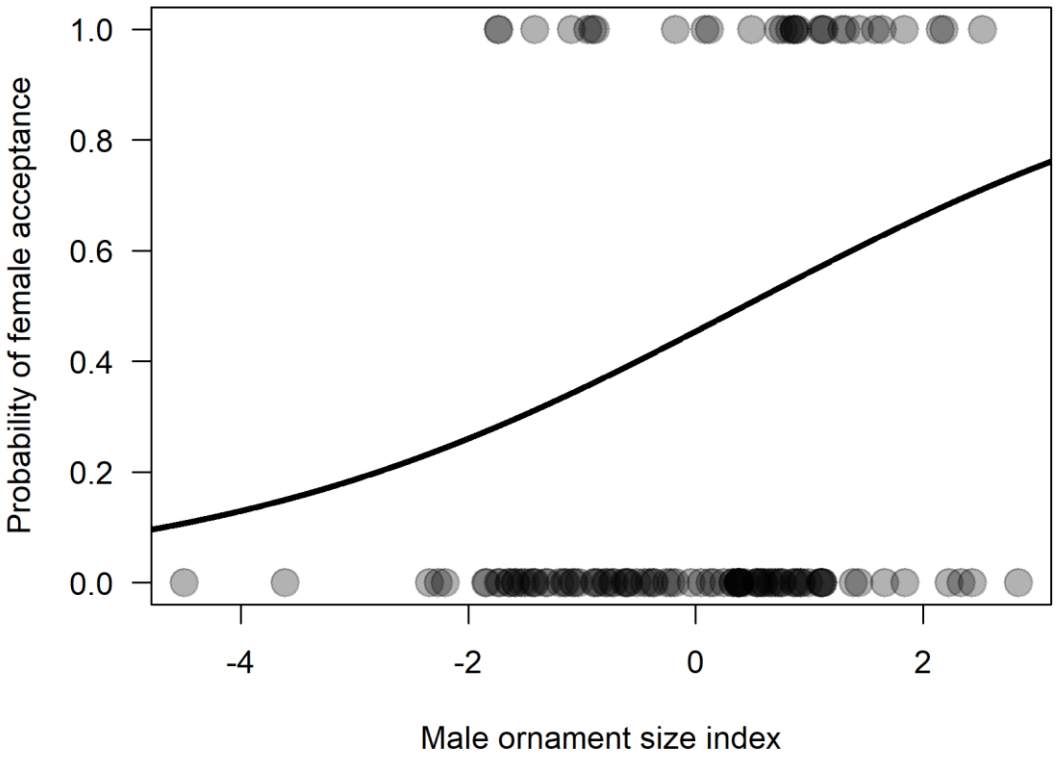

Figure 2.4. Relationship between female acceptance and male ornamental size. Points represent each male used in the mating experiment.

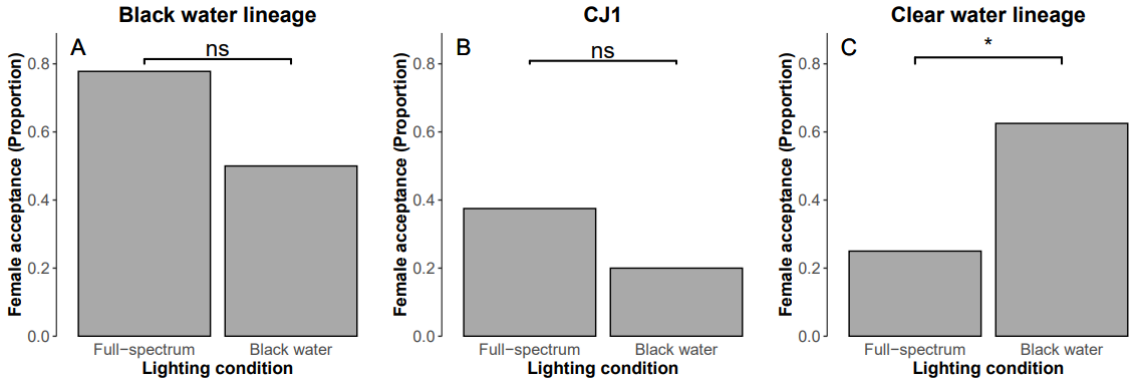

Figure 2.5. Proportion of mate acceptance under full-spectrum and black water lighting conditions by females of **(A)** black water lineage, **(B)** CJ1 population and **(C)** clear water.

2.4 Discussion

Under simulated strong red-biased lighting conditions, I observed an increase in the apparent intensity of the red colour in the dorsal and anal fins and the red colour index of yellow spots in the anal fin of sailfin tetra males. On the other hand, the perceived among-individual variation in the red colour index of the dorsal and anal fins significantly decreased under black water light, irrespective of the sailfin tetra lineage. Black water light biases the transmission of long wavelengths, and therefore, it is expected that red colour is perceived brighter in black waters (Endler, 1993). Similarly, yellow and orange colours would appear not only brighter but also long-wavelength shifted, appearing "red-like colour" in black waters. Such changes in perceived colours are expected, since shorter wavelength colours, such as blue and green, are filtered out under black water lighting conditions. As a result of such colour bias, the perceived variation of the red colouration in male ornaments would decrease under black waters.

In mate acceptance experiments, males with larger ornaments were more likely to sexually display towards females, and females from all lineages preferred males with larger ornaments as mating partners. Male ornament size may be a reliable indicator of mate quality since it is usually an honest signal; it is energetically costly, can be related to foraging performance and health condition, and represents a good indicator of the genetic background of the potential mate (Andersson, 1994; Milinski & Bakker, 1990; Weaver et al., 2017). Indeed, female sexual preference for males with larger ornaments

has been reported for other species (Andersson, 1982; Andersson, 1994; Bischoff et al., 1985). Furthermore, the perception of the size of the ornaments in the sailfin tetra may not be directly influenced by colour bias or the instability of the underwater lighting conditions. This is especially true because the courtship behaviour in the sailfin tetra is carried out at a close distance (Pires et al., 2016). Therefore, male ornaments size can be used in both clear and black water lighting conditions to assess male quality. On the other hand, ornament colouration is directly affected by underwater light.

Female sexual response to the different underwater light conditions differed among lineages. Only females from clear waters (Amazonas lineage) showed a significant increase in the probability of male acceptance under black water light, suggesting that they mainly used the red colouration of male ornaments for their mate choice. In contrast, the mate acceptance of females from black water lineage and CJ1 population did not differ in function of lighting conditions, suggesting that they did not use the red colouration of male ornaments as the main criteria for their mate choice. These results suggest that females living in black waters might have shifted the main mating signal used for their mate choice from male ornament colour to other signals, such as ornaments size.

The evolution of signal reliability is related to the environment in which such process occurs (Heuschele et al., 2009); and therefore, sexual preference for a particular trait under a given environmental condition may become fruitless in another environmental condition (Candolin et al., 2007). For the sailfin tetra, such process has probably occurred in a non-biased lighting environment for the clear water lineage individuals. Therefore, under the given environmental condition, clear water lineage females had contact with males that show their ornament colouration varying according to their genetic/health condition. Because males' ornaments colouration varied according to their quality, such trait may represent a reliable trait for the clear water lineage individuals. Since males' ornament colouration arguably indicates foraging ability, genetic quality and general health condition (Andersson, 1994; Milinski and Bakker, 1990; Møller et al., 2000), females in clear waters may benefit from choosing mates based on their colouration. On the other hand, black water lineage females had contact with spectrally-masked traits and with small among-individual variation in male's ornaments colouration. Under such situation, it could be more difficult for females in black waters to correctly assess mate quality based only on male ornament colouration. Moreover, the

energetic investment needed for females to discriminate between individual differences in male colouration in black waters might be too high to be maintained in the population. In that case, females living in black waters could mistakenly choose low-quality mates if they choose based only (or mainly) on the red colour of male ornaments (Hunt et al., 2004). Therefore, the ornament colouration for those individuals living in black waters may not be as reliable as it is for the clear water lineage individuals. Then, females living in black waters may assess males' quality mainly through other traits, such as male ornament size. Finally, these results suggest that the importance of the male ornament colouration for mating assessment is dependent on the environmental conditions (in this case, the lighting environment).

Another important point is that the colouration of sexually selected traits can evolve to maximize the contrast between the signal and the environmental background (Fleishman et al., 2022; McDonald et al., 1995), rather than evolving to increase the total brightness of the trait colouration for conspecific attraction. In such cases, higher contrast between signal and environmental background can favour the perception and attraction of potential mates. For the sailfin tetra, I have not measured the colour contrast between males' ornament colouration and their natural habitat's colour background. However, red and black areas on the dorsal fin are typically larger and broader in black water lineage males. Additionally, the red area on the dorsal fin is more intense in black water lineage males (Pires et al., 2019). The higher expression of the most abundant wavelengths in an environment increases brightness (Endler, 1993), which, together with the higher expression of black colour, suggests increased contrast and conspicuousness of the dorsal fin of black water lineage males under red-biased lighting conditions. However, future studies could directly address the role of the colour contrast between males' ornaments and their natural environment and its relation to female mating acceptance between the sailfin tetra lineages.

In Amazon small forest streams, two non-exclusive factors could potentially drive female mate preference for male ornament colouration: the effects of the environmental lighting conditions (i.e. water type) that I have discussed, and the instability of the overall environmental lighting conditions. The underwater environment of Amazonian small forest streams can change unpredictably as a consequence of daily rainfall, which can reach around 130 mm (Espírito-Santo et al., 2017). The rainfall brings

sediments and organic matter to the stream channel, increasing turbidity, changing the amount of DOC, and therefore, the water's lighting red bias. However, due to the differences in soil types between black and clear water river basins (Leenheer, 1980; as reviewed in Borghezan et al., 2021), such variation in the water colouration occurs mainly in black water systems, while clear water streams change mostly with regards to turbidity. Therefore, the importance of the male ornaments' red colouration for mate assessment may decrease in individuals living in black waters. In such quick-changing environments, the benefit of the strict preference for a particular trait that is directly affected by unpredictable environmental fluctuations can be impaired, especially when it disrupts the link between signal value and individual quality (Candolin & Heuschele, 2008). Consequently, females from the black water lineage and the CJ1 population may have shifted the main criteria used for mate choice from the ornament's red colouration to traits that are more reliable under such situations, such as male ornament size. Interestingly, the red colour signal-masking in the Amazon black water was similar to the observed in other black water environments. Such environmental effects on male colourations are suggested to drive the loss of red nuptial colour in males of the three-spined sticklebacks, independent of the presence or absence of predators (Reimchen, 1989); and a lower preference for the red colour (Boughman, 2001) in females of living in stained waters (red-shifted environment). On the other hand, in clear waters, light transmission is not biased and the among-individual variation of the red colours of the dorsal and anal fins was high, and may represent a better indicator of health and other signals of genetic quality (Andersson, 1994; Møller et al., 2000).

In some haplochromine cichlids, males have egg spots in their fins that are used by females during mate assessment, and females select mates based on males' egg spots (e.g. Egger et al., 2011; Hert, 1989). Such egg spots often mimic eggs laid by other females and are used to attract mates in polygynous species (Wickler, 1968). The sailfin tetra males also have yellow egg spots on their anal fin (Figure 2.1) of which the red colour index, contrast, and variance are affected by ambient lighting conditions. Our results, however, did not show clear effects of the yellow spots on female mating acceptance under the two lighting conditions in any of the three lineages. For instance, I did not find a higher female mating acceptance under full-spectrum light in any lineage, though the anal fin spot contrast was higher under such conditions in all lineages.

Similarly, I found a higher mating acceptance under black water light only for clear water lineage, though the red index of the anal fin yellow spot was higher under black water light in all three lineages. These results suggest that the importance of the colouration and/or contrast of the anal fin yellow spots as possible signals triggering female mate choice may differ among lineages. However, I cannot discard the possibility that conspicuous yellow spots on the anal fin of sailfin tetra males may be used for other types of signals, such as guiding females along the way to oviposition sites. Again, studies focusing on the contrast of such colourful markings and the environmental background colour of the sailfin tetra natural habitat could be insightful.

Alternatively, the yellow spots on the anal fin of sailfin tetra males may be more related to intrasexual (male-male interactions) than to intersexual communication, as it has been previously reported for other species (e.g. Theis et al., 2012). The number and size of the anal fin egg spots, which differ between sailfin tetra lineages (Pires et al., 2019), could also be relevant to such communication because the ornaments in the anal fin are only displayed during social interactions. However, future investigations on the role of the yellow spot on the male's anal fin of sailfin tetra are needed to test such hypotheses.

Under intersexual selection, if a trait is not used by females choosing mates, I can expect this trait to regress and become less and less conspicuous among males. Contrary to this expectation, black water lineage males were observed to have redder dorsal fins than clear water lineage males. This pattern was also found in previous studies using spectrophotometers (Pires et al., 2019). This suggests that even though redness is no longer used as the main sexual signal it still has functions in black waters. For example, redness could increase in black water to emphasize the outline and the size of the dorsal fin. The redness of the dorsal fin may also function as a communication signal among males (i.e. intrasexual selection) because the dorsal fin is constantly displayed during their regular swimming, and thus, it can broadcast information on the dominance status among males. The male signaling for broadcasting information on dominance can occur at longer distances, compared to the courtship behaviour, and may be even more affected by the environmental condition (black waters). Therefore, the evolution of redder parts on the male dorsal fin can be expected under black waters, where receiving a visual signal over long distances can be more difficult than in clear waters. However, our study was not designed to specifically test for such interesting points, and future investigations can

address them.

Another interesting point is that the red area of the dorsal fin, which is constantly displayed, can act firstly as a sexual lure, attracting females at a distance. This mechanism would prove more useful in black waters where observing a male over long distances can be more difficult than in clear waters, also favouring the evolution of the redder colour in the male dorsal fin. Indeed, the shape of the red area on the dorsal fin resembles a chironomid larvae, a red-coloured food item present in the sailfin tetra diet (Pires et al., 2016). Also, the sailfin tetra colour opsin gene profile is mostly composed of LWS (red colour sensitivity) genes (Escobar-Camacho et al., 2020), highlighting the potential importance of the red colour for feeding, social and sexual behaviour.

I could expect that at some evolutionary point in the sailfin tetra history, males might have had smaller and less intense red parts on their ornaments, and I may hypothesize their evolution based on the sensory drive (Endler, 1992; Boughman, 2002), sensory exploitation and emancipation from exploitation hypothesis (Arnqvist, 2006). Because of the individual high sensitivity for red colours (Escobar-Camacho et al., 2020) that may also be used for finding food resources, males developed red colours in their fins that attract females. Furthermore, red colours in male ornaments would evolve to be more intense in black waters (which favours the transmission of the long wavelength) than clear waters (Endler, 1992). However, because of the costs associated with choosing mates based on the male red colours (due to the spectrally-masking effect), females shifted the main criteria on which they assess mates from the male ornament colouration to the ornament size (which is known as resistance to the male stimuli, Arnqvist, 2006), allowing females to disentangle reproductive responses from other types of responses (as feeding behaviour and predator avoidance). However, future investigations may address such interesting issues.

Although the CJ1 population belongs to the clear water lineage based on genetic nuclear markers (Pires et al., 2018), this population currently inhabits a black water drainage system. All four male ornament colouration indexes measured in this study were similar between clear water lineage and the CJ1 population, consistent with the results of a previous study (Pires et al., 2019). The genetic and morphological similarities found between the CJ1 population and other clear water lineage populations suggest that the CJ1 population has only recently had contact with black water conditions. However, CJ1

females had a response to the different lighting conditions in the mate acceptance experiment that was similar to black water lineage females. The similar mating acceptance results for the CJ1 population and black water lineage (shifting the main mate-choice signal from red colour intensity to ornament size) suggest convergent evolution in black water environments. These results also suggest that behavioural adaptations can evolve faster than morphological ones. Also, as far as I know, this is the first report suggesting convergent evolution to black waters in Amazon. It may be possible that several species went throught a convergent evolution to different coloured Amazon waters; and therefore, future studies can investigate such interesting evolutionary process among other species. The result of the CJ1 population in my mate acceptance experiment, however, should be interpreted with caution because the sample size of the CJ1 population was small (Appendix Table A2.5.2).

Female mate choice can drive the reproductive isolation between groups if their mating preferences diverge (Andersson, 1994). Changes in mating preference can be expected in lineages that are exposed to divergent ecological pressures (Boughman, 2002; Cummings & Endler, 2018). According to my results, clear water lineage females would be more willing to accept black water lineage males, since they have more intense red colour in their dorsal fin ornament in either clear water or black water lighting condition. On the other hand, females of black water lineage and CJ1 population would not accept clear water lineage males in higher proportion in either clear water or black water lighting condition because they do not choose mates based primarily on male ornament colouration. Therefore, it is possible that reproductive isolation between the sailfin tetra lineages occurred due to differences in mate choice signals used by females adapting to black water lighting conditions (Table 2.1). However, several factors can act simultaneously to affect female mate choices and the reproductive event (Rosenthal et al., 1996). For instance, when sailfin tetra couples from different lineages were subjected to limnological condition similar to black waters (pH = 5.5 and conductivity at 20 µS cm-1, but without DOC) in captivity, all pairs with at least one clear water lineage individual were reported to show significantly lower spawning success (Pires et al., 2018). The authors suggested that the water type and its chemical condition affected the fitness and contributed to reproductive isolation between lineages of the sailfin tetra. Therefore, the reproductive isolation between lineages of the sailfin tetra may have been driven by

physical (e.g. light) and chemical (e.g. pH) parameters of the water.

My results suggest that black water lineage and CJ1 females shifted from using red colouration intensity as a mating signal due to environmental impairment of the signal's reliability in black water lighting conditions. Since the biased lighting conditions of black waters fundamentally result from DOC enrichment, these results support the hypothesis of chemical and physical factors jointly influencing the evolution of mating signal preferences in the sailfin tetra. This set of ecological conditions may have also contributed to the reproductive isolation and speciation of other Amazonian visually oriented and dimorphic black water fishes, such as the dwarf cichlids of the genus *Apistogramma* in the highly biodiverse Rio Negro, which should be investigated.

Table 2.1. Differences in environmental conditions, male ornamental traits, the expected effects of lighting conditions on male ornaments and mating signals used by females during mating assessment for black water lineage, clear water lineage and the CJ1 population.

Environmental condition	**Black water lineage**	**Clear water lineage**	**CJ1 population**	**Reference**
Water type	Black water	Clear water	Black water	Pires et al., 2016; Pires et al., 2018
Habitat stability	Highly unpredictable	Relatively stable	Highly unpredictable	Espirito-Santo et al., 2017; Borghezan et al., 2021
Male traits				
Intensity of the red colour in dorsal fin	Higher	Lower	Lower	Pires et al., 2019; Present study
Area of the red part in dorsal fin	Larger	Smaller	Smaller	Pires et al., 2019
Shape of dorsal fin	Flame-shaped	Curve-shaped	Curve-shaped	Pires et al., 2019
Intensity of the red colour in anal fin	Similar	Similar	Similar	Pires et al., 2019; Present study
Egg spot size	Larger	Smaller	Smaller	Pires et al., 2019

Egg spot number	Fewer	Higher	Higher	Pires et al., 2019
Anal fin spot contrast	Higher	Lower	Lower	Present study
Yellow spot redness index	Higher	Lower	Lower	Present study
Effects of natural water colour in ornaments				
Red colour in dorsal fin	Higher	Lower	Higher	Present study
Red colour in anal	Higher	Lower	Higher	Present study
Red colour in dorsal fin variation	Lower	Higher	Lower	Present study
Red colour in anal fin variation	Lower	Higher	Lower	Present study
Yellow spot redness in natural	Higher	Lower	Higher	Present study
Yellow spot contrast in natural	Lower	Higher	Lower	Present study
Female mate choice				
Similar-sized individuals	Present	Possible	Possible	Borghezan et al., 2019; Present study
Larger ornaments	Present	Present	Present	Pinto et al., 2021; Present study
Redder ornaments	Absent	Present	Absent	Present study

2.5 Appendix

Appendix 2.5.1

Feeding behaviour

The sailfin tetra lives in pounds with low water flow that are surrounded by the buriti palm tree (*Mauritia flexuosa*). This species feeds on several items, such as algae, terrestrial insects that fall into the forest stream and aquatic insects like Coleoptera, Trichoptera, Ephemeroptera and Diptera (Chironomidae), dead tadpoles of *Hypsiboas geographicus*, flowers of *Thurnia sphaerocephala*, Ostracoda, detritus, fruits, including the buriti palm tree fruit (Pires et al., 2016). Some of these food items are red-coloured, like Chironomidae larvae and buriti fruits.

Predation risk

The most common predators of the sailfin tetra are *Crenicichla* spp. (which exhibits an active hunting strategy), *Erythrinus erythrinus* and *Hoplias malabaricus* (which exhibits a passive hunting strategy - ambush). *Crenicichla* spp. are diurnal and visually oriented predators (Fraser et al., 2006). The sailfin tetra may minimize the predation risk during courtship by shifting visually conspicuous displays to times of day when visual predation is minimal, usually between 1600 and 1800, in which the light intensity is lower (especially because the forest stream is partially covered by the forest) and relatively safer from predators, as reported to other fish species (Endler, 1987). Males of the sailfin tetra usually swim with the anal fin folded or partially folded (Pires et al., 2016), which may reduce the attractiveness to predators and display the anal fin only during courtship and male-male interactions. Interestingly, *Crenicichla* spp. were reported to occur in the Western basins (black waters) of the Reserva Florestal Adolfo Ducke (Manaus, Brazil), but not in the Eastern basins (clear waters) (Mendonça et al., 2005). Interestingly, the black water lineage males show redder ornaments in the dorsal fin than clear water lineages (Pires et al., 2019; present study), suggesting that the evolution of the red colouration in the dorsal fin may be driven by social/sexual behaviour despite of the higher predation risk.

Aspects of the black waters

The classification of “black water” in the Amazon encompasses a wide range of

waters that show high amounts of dissolved organic carbon (DOC) that bias the underwater light condition toward long wavelengths (i.e red colours). However, such classification does not consider differences in the DOC concentration between rivers and forest streams. Such difference in the DOC concentration results in different levels of red biased waters (as observed in the Figure 2.1 and Figure A4.6.3).

Appendix 2.5.2

Table A2.2.1. Geographic coordinates in which water samples were collected.

Water samples	Geographic coordinates
Igarapé Acará	2°57'06.4"S 59°57'31.9"W
Igarapé Bolívia	2°59'03.0" S 59°55'57.0"W
Igarapé Jiboia	3°06'22.8"S 59°58'40.5"W
Rio Negro	3°02'52.6"S 60°07'32.1"W

Appendix 2.5.3

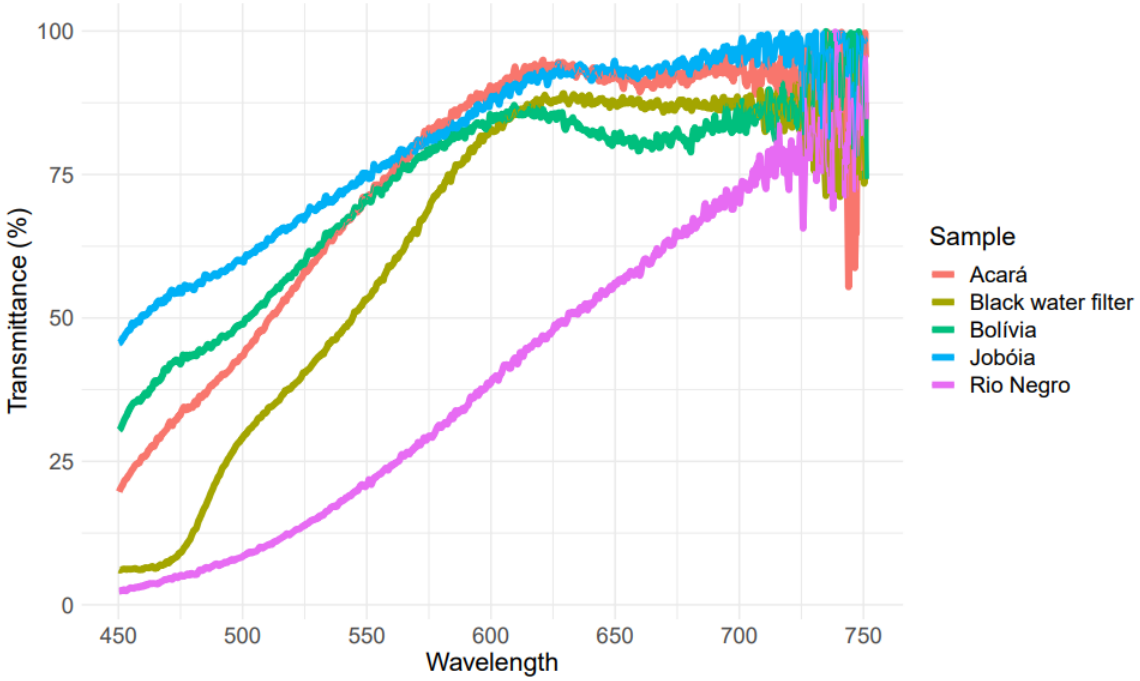

Figure A2.3.1. Transmittance (%) of the Rio Negro and other three black water *igarapés* and the black water filter used during the mate choice experiments and for measuring the effects of black water lighting conditions on male ornaments colouration.

Appendix 2.5.4

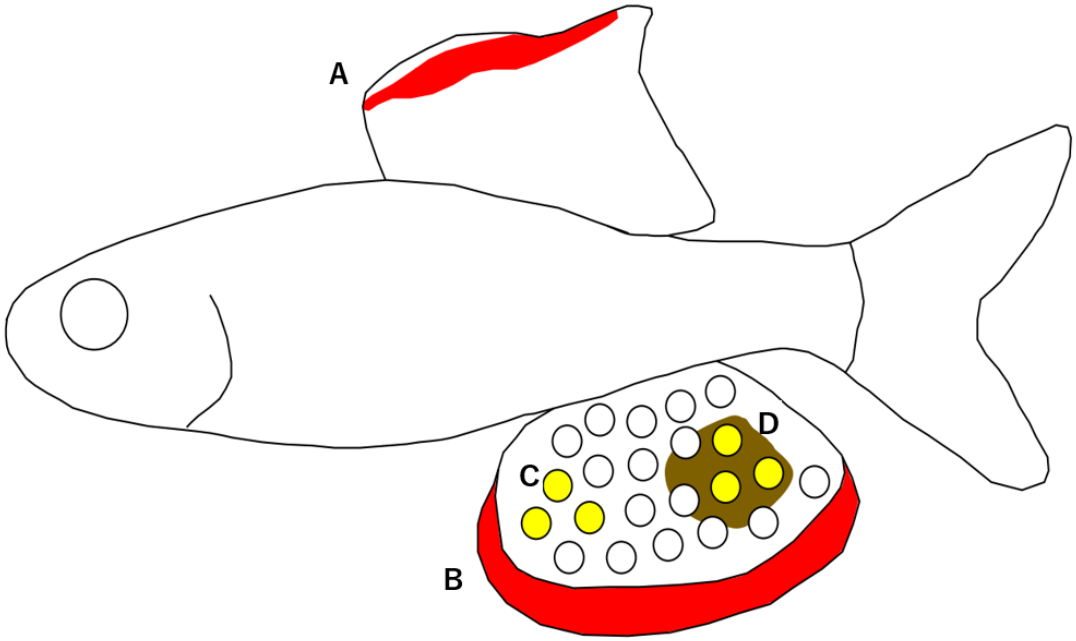

Figure A2.4.1. Schematic representation of the sailfin tetra male and the colourful measurements taken. **(A)** the red area of the dorsal fin, **(B)** the red area of the anal fin, **(C)** the yellow spots of the anal fin and, **(D)** the brown background of the anal fin.

Appendix 2.5.5

Table A2.5.1. Number of males of each lineage that did not perform any sexual display (i.e courtship behaviour) towards females under, and number of males that sexually displayed towards females under clear and black underwater lighting conditions. FS = Full-spectrum light condition. BL = Black water light.

	Total trials		No courtship behaviour		Courtship behaviour	
Lineage	FS	BL	FS	BL	FS	BL
Black water	25	21	16	9	9	12
Clear water	25	24	13	8	12	16
CJ1	13	13	8	5	8	5

Appendix 2.5.6

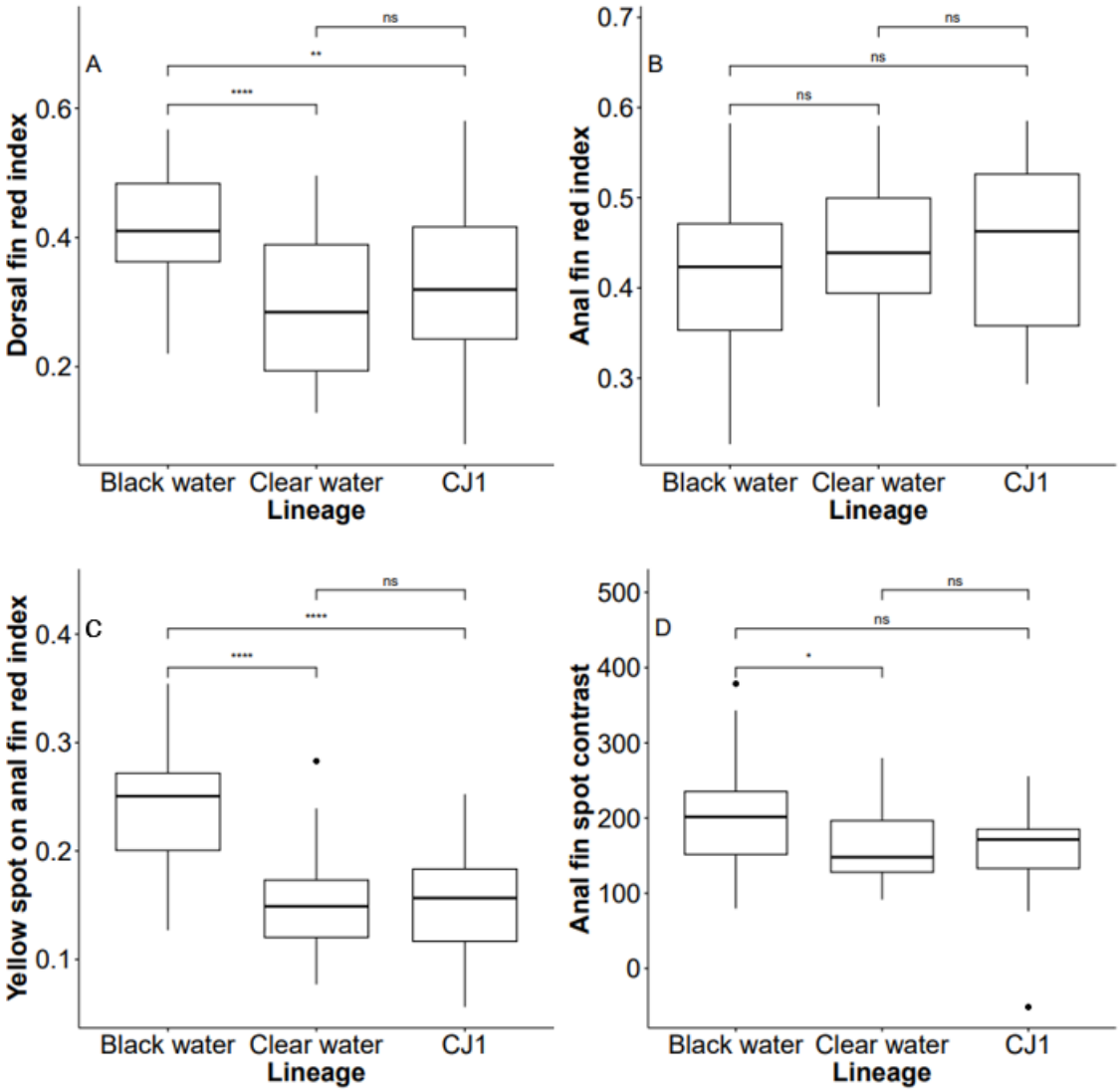

Figure A2.6.1. Comparison of the **(A)** red area of the dorsal fin, **(B)** red area of the anal fin, **(C)** yellow spot on the anal fin, and **(D)** anal fin spot contrast among lineages of the sailfin tetra under full-spectrum light. Indicates: **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns = non-significant.

Appendix 2.5.7

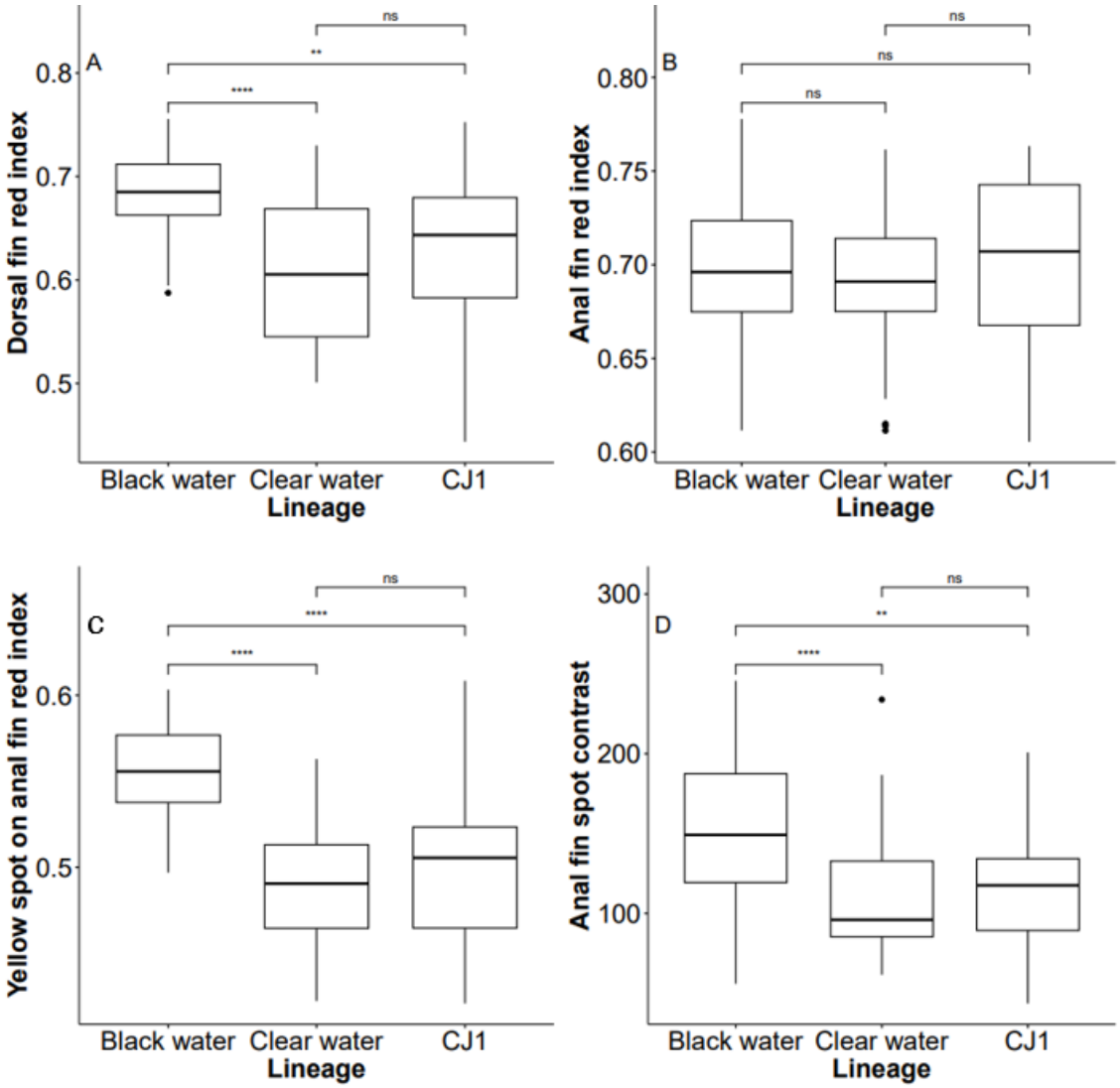

Figure A2.7.1. Comparison of the **(A)** red area of the dorsal fin, **(B)** red area of the anal fin, **(C)** yellow spot on the anal fin, and **(D)** anal fin spot contrast among lineages of the sailfin tetra under simulated black water light. Indicates: **** $p < 0.0001$; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns = non-significant.

Appendix 2.5.8

Table A2.8.1. Mean and standard deviation (X±SD) values for the four colour measurements taken from the sailfin tetra male ornaments under simulated black water and full spectrum light. BW = Black water lineage; CW = Clear water lineage; RA = Red part of the anal fin; RD = Red part of the dorsal fin; YS = Yellow spot on anal fin; AFSC = Anal fin spot contrast; FSL = Full-spectrum light condition; BWL = Black water light condition.

	RA		RD		YS		AFSC	
	FSL	BWL	FSL	BWL	FSL	BWL	FSL	BWL
BW	0.414 ±0.080	0.700 ±0.037	0.413 ±0.090	0.681 ±0.042	0.235 ±0.055	0.555 ±0.026	204.4±76.8	151. 9 ±49.8
CW	0.443 ±0.079	0.689 ±0.033	0.287 ±0.110	0.611 ±0.066	0.149 ±0.041	0.488 ± 0.029	165.4 ±49.5	110.5 ±35.3
CJ1	0.453±0.090	0.698±0.047	0.324±0.127	0.627 ±0.073	0.156±0.052	0.498 ±0.044	160.6 ±62.2	118.3 ±37.3

Appendix 2.5.9

Table A2.9.1. Complete factorial statistical analysis result for the glm model considering the male sexual display (binomial) as the dependent variables, and male ornament size index, light condition and lineage as independent variables. Bold lines show those factors that significantly drove male sexual display.

	LR Chisq	DF	P-value
Male ornament size index	**5.0931**	**1**	**0.0240**
Light condition	3.3076	1	0.0689
Lineage	1.4352	2	0.4879
Male ornament size index:Lineage	0.4147	2	0.8127
Male ornament size index:Light condition	0.0770	1	0.7814
Light condition:Lineage	0.3293	2	0.8481
Male ornament size index:Light condition:Lineage	0.9576	2	0.6195

Appendix 2.5.10

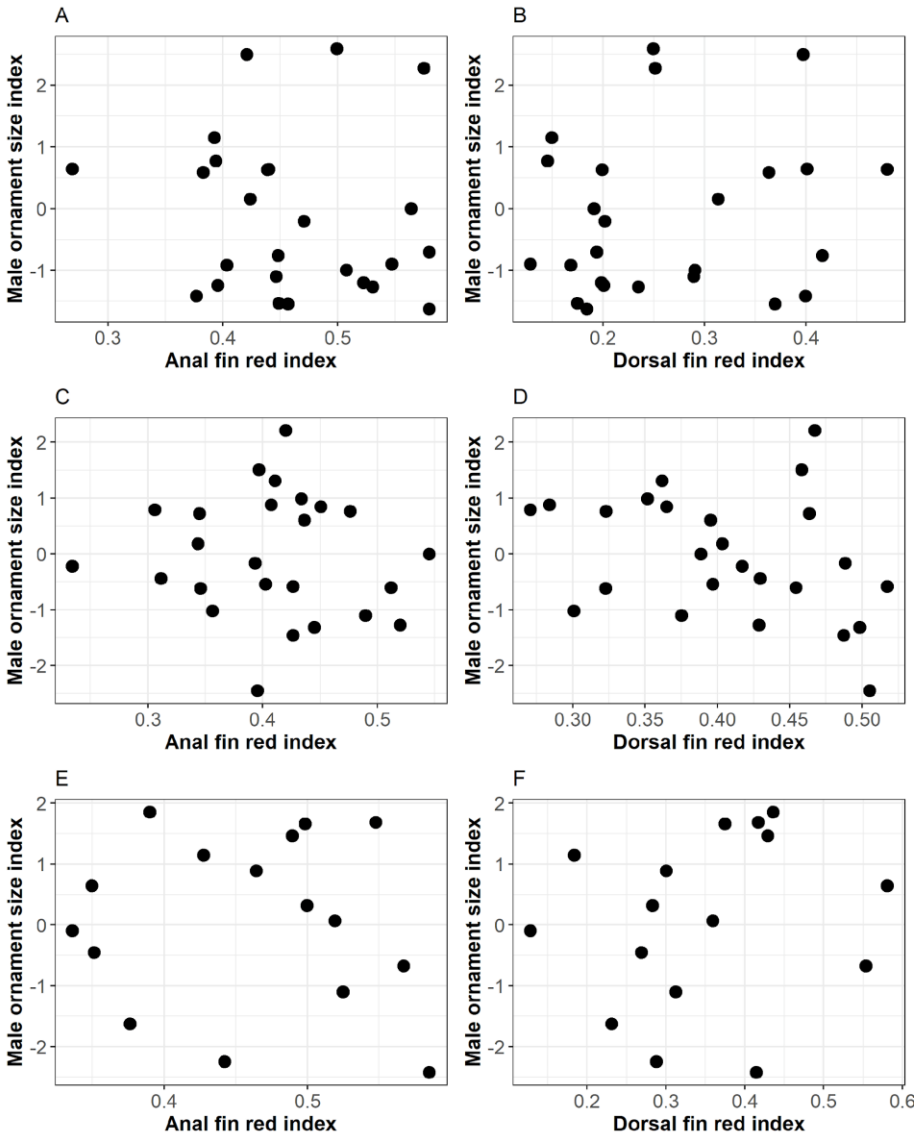

Figure A2.10.1. Relationship between male ornament size index and the intensity of the red colouration on the anal and dorsal fins, respectively, for Clear water (A-B), Black water (C-D) and CJ1 (E-F) lineages, under full spectrum light.

Appendix 2.5.11

I checked the newer ornament size index data for possible outliers (through Cook distance). I found four outliers for the analysis addressing the factors that drove male sexual display, and three cases in the analysis addressing the factor that drove female mating acceptance (e.g. a male with larger ornaments that was rejected by a female; or a male with small ornaments that was accepted by a female). In order to not reduce the sample size, I performed the flooring and capping strategy of handling outliers. Capping is replacing all higher side values that exceed the upper control limit (UCL) by the UCL value itself; flooring is replacing all values that fall below the lower control limit (LCL) by the LCL value itself. I considered and calculated the 5^{th} and the 95^{th} percentiles and replaced the outliers with their respective percentile value. The 5^{th} and 95^{th} percentile were chosen since they would make smaller changes in the data set and treat the outlier simultaneously. We checked for the outliers for each analysis (male sexual display and female mating acceptance), independently (i.e. an outlier in one analysis could not be considered an outlier in the other). The statistical models showed similar results before and after treating the outliers: larger-ornamented males were more likely to sexually display towards females (Table A2.10.1); females were more likely to accept males with larger ornaments as mating patterns, and the light condition affected female mating acceptance differently among lineages of the sailfin tetra (Table A2.10.2). Therefore, because (1) results did not change after treating outliers and (2) there might not be any biological reasons to remove such cases, I decided to keep the analysis with the untreated points in the main text.

Table A2.10.1. Complete factorial statistical analysis results for the glm model considering the male sexual display (binomial) as the dependent variables, and male ornament size index, light condition and lineage as independent variables after treating the outlier points. Bold line shows that factor that significantly drove male sexual behaviour.

	LR Chisq	DF	P-value
Male ornament size index	**8.3453**	**1**	**0.0038**
Light condition	3.2764	1	0.0702

Lineage	1.5977	2	0.4498
Light condition:Lineage	0.2773	2	0.8705
Light condition:Male ornament size index	0.0743	1	0.7851
Lineage:Male ornament size index	2.5376	2	0.2811
Light condition:Lineage:Male ornament size index	1.0833	2	0.5817

Table A2.10.2. Complete factorial statistical analysis results for the glm model considering the female mate acceptance (binomial) as the dependent variables, and male ornament size index, light condition and lineage as independent variables after treating the outlier points. Bold lines show those factors that significantly drove female mate choice.

	LR Chisq	DF	P-value
Male ornament size index	**4.9446**	**1**	**0.0261**
Light condition	0.0426	1	0.8363
Lineage	4.2319	2	0.1205
Light condition:Lineage	**6.9505**	**2**	**0.0309**
Light condition:Male ornament size index	0.2599	1	0.6102
Lineage:Male ornament size index	3.8848	2	0.1433
Light condition:Lineage:Male ornament size index	3.6675	2	0.1598

Appendix 2.5.12

Table A2.12.1. Complete factorial statistical analysis result for the *glm* model considering the female mate acceptance (binomial) as dependent variables, and male ornament size index, light condition and lineage as independent variables. Bold lines show those factors that significantly drove female mate choice.

	LR Chisq	DF	P-value
Male ornament size index	**4.6719**	**1**	**0.0306**
Light condition	0.0483	1	0.8259
Lineage	4.1569	2	0.1251
Male ornament size index:Lineage	4.1229	2	0.1272
Male ornament size index:Light condition	0.2609	1	0.6094
Light condition:Lineage	**6.9940**	**2**	**0.0302**
Male ornament size index:Light condition:Lineage	3.5998	2	0.1653

Appendix 2.5.13

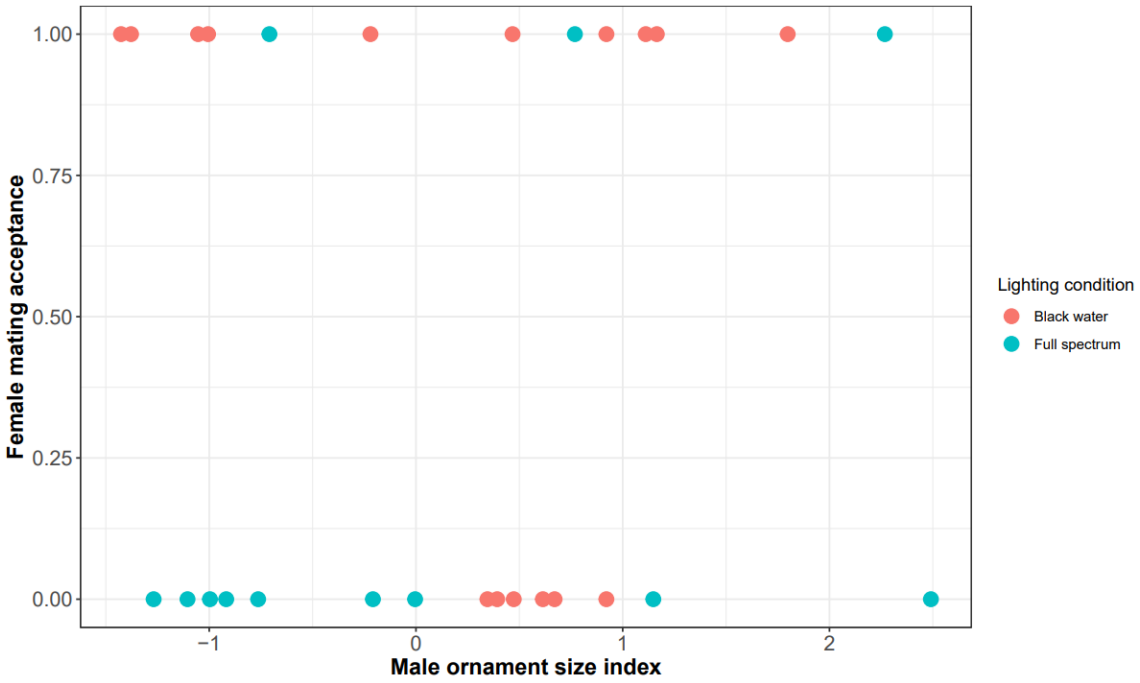

Figure A2.13.1. Relationship between female acceptance and male ornamental size index for the clear water lineage under both full spectrum (blue points) and black water (red points) lighting conditions. Points represent each male used in the mating experiment. Please note the five males whose ornament size index (X-axis) is below zero, but were still accepted by the females (Y-axis) in the black water lighting condition.

Chapter 3

Females of the sailfin tetra prefer red-lightened environments

3.1 Introduction

Colours and their preferences have profound impacts on individuals' life and fitness of visually-oriented species. Environmental colouration can modulate physiological and behavioural responses, such as feeding (Duray et al., 1996), growth (Downing & Litvak, 2000), reproduction (Volpato et al., 2004) and aggression (Höglund et al., 2002). Aquatic environments highly vary in colouration, affecting individual colour sensitivity and possibly driving individual and habitat/environment colour preferences.

Colour preference can evolve through a pre-existing bias in the sensory system (Luchiari et al., 2007; Spence & Smith, 2008). For instance, the Nile tilapia *Oreochromis niloticus* individuals strongly preferred the yellow environmental colour, which is suggested to be driven by the colour sensibility and the yellow pigments in the fish eyes maximizing photon capture (Luchiari et al., 2007). Similarly, the preference for orange colours is associated with the feeding behaviour in the guppy *Poecilia reticulata* (Rodd et al., 2002). Therefore, the colour preference can be driven by the colour peak sensibility. On the other hand, colour preference can be disassociated from the opsin gene expression and consequently colour vision (Johnson et al., 2013), indicating that lighting environment rather than the opsin genes expression (and colour vision) may be more relevant to the colour preference.

The preference for a particular colour and a specific environmental lighting condition can be more important to sexually dimorphic and coloured species since colours play an important role in intra- and interspecific communication (Boughman, 2001; Endler & Houde, 1995). Amazon waters can be classified into three different types depending on their apparent colouration as seen from land: black, blue (= clear, *sensu* Sioli, 1984), and white (Wallace, 1853). These water types highly vary in their physical and chemical characteristics (Sioli, 1984; reviewed in Borghezan et al., 2021). Such water classification was conceived based on observations of the main river channels in the Amazon. However, small forest streams (locally termed *igarapés*) can only be classified

into two of these categories: black and clear waters. Black and clear waters show high light transmission, especially in shallow water bodies; however, due to the abundance of dissolved organic carbon (DOC), black waters are yellow/red biased environments, while clear waters are mostly transparent (Mendonça et al., 2005; Costa et al., 2013).

The sailfin tetra *Crenuchus spilurus* is a small-sized and sexually dimorphic Amazon fish species. In this species, males possess dorsal and anal fins conspicuously ornamented in red and yellow colours, and such colourful ornaments are used during an elaborated courtship behaviour (Pires et al., 2016). Female sexual preference for the male colourful traits differs according to the underwater lighting environment individuals live in. Females that live in black water conditions do not rely primarily on the colouration of the males' ornaments, but instead, prefer males with larger ornaments as mating partners. On the other hand, females living in clear water environments prefer those males with redder ornaments colouration as well as those males with larger ornaments (Borghezan et al., 2023). Such divergence in female mating signal preference is suggested to be driven by the perception of the males' ornaments colouration. Black water increases the red colouration on males' ornaments; however, it also decreases the perception of the variation among males' ornaments colouration (Borghezan et al., 2023). Therefore, selecting males based primarily on a trait with intense signals and low perceptual variation may be costly to females from the black water lineage, since they could choose low-quality males that would appear like high-quality ones due to the environmental condition (Hunt et al., 2004). However, red colours are still important for other processes, such as finding several red-coloured food resources such as fruits of the buriti palm tree *Mauritia flexuosa* and Chironomidae larvae (Pires et al., 2016). Therefore, outside the mating context, I can expect both lineages to show a preference for red colours; however, this preference could be more intense in those individuals living in clear waters, since they are also attracted by the red colours in male ornaments (i.e. inside the mating context). Here, I investigated the female preference for red colour through environmental colour choice. This experiment was used to test female colour preference since environmental colours can affect fish physiology, behaviour and the details individuals can discriminate in the environment, thereby providing them with various advantages, including feeding, defence and mating opportunities (Luchiari et al., 2007; Yokoyama, 2000).

3.2 Material and Methods

3.2.1 Ethics statement

Ethical statement

Fish collections in Brazil were carried out under IBAMA permanent licence SISBIO #10199-1 to Jansen Zuanon, one of my collaborators. Experiments were conducted following Brazilian federal law 11.794/2008. This study was approved by the Ethical Committee for Animal Use in Experiments of Kyoto University (WRC-2019-009A). No fish died during or immediately after the experiment.

3.2.2 Fish sampling and housing

I collected the sailfin tetra individuals from populations that represent the two main lineages (Rio Negro - Black water, and Amazonas lineages - Clear water) and CJ1 population. CJ1 population phylogenetically belongs to the Clear water lineage (Pires et al. 2018). Also, the colour pattern and red colour intensity in CJ1 population males ornaments are similar to those individuals from other populations from clear water lineage (Pires et al., 2019; Borghezan et al., 2023). However, CJ1 population individuals live nowadays under black water condition. Females mating preference for male ornaments red colours is similar to those from black water lineage females (Borghezan et al., 2023). Therefore, this population provide a good opportunity to evaluate the convergent evolution of female red colour preference to black waters. Black water lineage individuals were sampled in an *igarapé* (= upland forest stream) inside an urban forest fragment in Manaus (3°06'22.8"S; 59°58'40.5"W), Amazonas state, Brazil. Clear water lineage individuals were sampled in a small stream near Iquitos (4°0’46.20”S; 73°27’47.70”W), Peru, and CJ1 population individuals were collected in a small stream in the outskirts of the municipality of Coari (4°07'09.9"S; 63°04'29.9"W), Amazonas state, Brazil. At the laboratory, fishes were housed in 92 L aquariums separated by lineage and sex for at least 30 days prior to the experiment. The majority of sailfin tetra individuals are between 3 and 4.5 cm long (standard length). To simulate the natural environment, natural and artificial plants were added to the tanks and over 10 pieces of PVC pipes (10 cm long, 25 mm in diameter) to provide shelter. This species does not actively swim around the tanks,

and its territory is only defended at a very close range near PVC pipes; thus, aggressive interactions were rarely observed.

Aquariums were filled using artesian well water, with limnological conditions similar to black waters (pH = 5.5 and conductivity at 20 µS cm-1, but without DOC). Aquariums were kept under 24°C, simulating natural conditions. Windows covering over half of the laboratory's wall provided indirect natural light and photoperiod (12:12 h light:dark cycle). I used dark cloth on the windows to control the amount of light that reached the laboratory, effectively simulating the natural canopy-shaded condition of an Amazon forest stream. Water changes (25%) were weekly performed. Fishes were fed commercial pelleted food for aquarium fishes *ad libitum* once a day. All individuals used in the experiments were adults since the smallest individual measured 34.04 mm of standard length (minimum = 34.04 mm, maximum = 46.77 mm, average = 40.84 mm, ± 3.20 mm SD), 8.34 mm larger than the estimated size at first maturity for this species (25.7 mm, Pires et al., 2016).

3.2.3 Experimental setup

I used an aquarium (60x15x15 cm) to evaluate the environmental colour preference. The aquarium was half-enlightened by a red light and half by a full spectrum light source (Figure 3.1). Such colours simulate the general tendency of clear and black water colours in Amazon *igarapés*. I used the same full spectrum light source for both, full spectrum and red light, treatments; however, for the red light treatment, the full spectrum light was filtered out by a red colour gelatin filter. The position of the light source, red biased and full spectrum light, was randomized among the trials to avoid side bias. Such procedures in my experimental setup was established to avoid any possible side bias in the study. The intensity of light sources was equalized to 250 lux on both sides of the experimental aquarium; therefore, the red biased treatment contains larger amount of red-spectrum than the full spectrum condition. On each side of the aquarium were introduced six artificial plants (similar to those found in their natural environments) that could potentially be used as shelters. I used water from the same source throughout all trials. The water used during the trials was similar to those found in the black water basin (pH at 5.5 and conductivity at 20 µS cm^{-1}) with the exception of high DOC

concentration. Because I used the same water source, I evaluated only the effect of the underwater lighting condition on the female colour choice.

3.2.4 Experimental procedure

The experiment was conducted as follows: a fish was gently caught in the housing tank and introduced into a small container (around 1L) and then transferred to the experimental aquarium. This was done to reduce possible stress to the testing individual due to the handling and transferring of testing individuals between tanks. The fish was kindly introduced in the middle of the experimental aquarium and I recorded its movement for 46 minutes. The first 6 minutes of each record were considered acclimation time and were removed from the analysis. I measured the time spent in each environmental colour (in seconds) for 40 minutes.

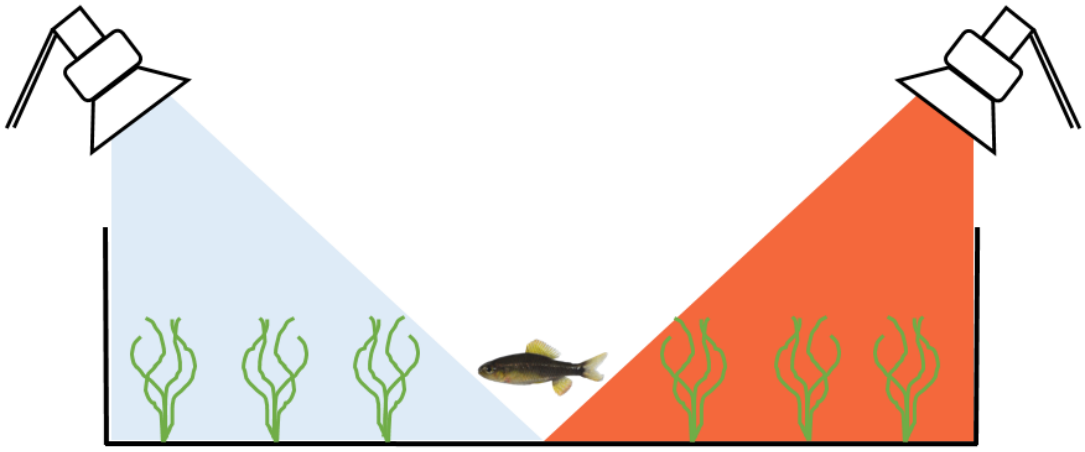

Figure 3.1. Schematic representation of the experimental aquarium in lateral view. The blue light on the left side represents the full spectrum light/clear water environment, while the orange light on the right side represents the red light/black water environment. The green lines represent artificial plants.

Each fish was tested only once. A total of 51 trials were performed. Individuals

that have not contacted at least the edge of both enlightened environments (excluding the moment of fish insertion in the experimental tank) within 10 minutes of the experimental time were excluded from the analysis. This was done to prevent possible noises in the results due to the individuals that potentially failed to express their environment colour preference (cases of stress, possibly due to handling). Ten individuals failed to contact the edge of the two lightened environments within 10 minutes and were excluded from the analysis. Therefore, 41 trials were used to fit the statistical model. I assessed the environment choice of 15 females from black water lineage, 9 from CJ1 population and 17 from clear water lineage. Individuals from black water lineage and CJ1 population live under black water type, while those from clear water lineage live under clear water type.

3.2.5 Statistical analysis

I first calculated the proportion of the time individuals spent in the red enlightened environment (i.e total time spent in the red environment divided by the total experimental time, in seconds). Values higher than 0.5 indicate a preference for the red colour, conversely, values lower than 0.5 indicate a preference for the full spectrum environment. I then fitted a one-sample t-test comparing the mean proportion against the null hypothesis value of 0.5 for each lineage. However, I fitted a Wilcoxon signed rank test, which is the equivalent non-parametric test, for the clear water lineage, in which data failed to show a normal distribution (Shapiro-Wilk normality test, $W = 0.6235$, p-value < 0.001). I also performed an additional test for each lineage to investigate if the environment colour preference was influenced by the first enlightened environment (red or full spectrum environments) females swam to right after the insertion of the female in the experimental aquarium. I fitted a Welch's t-test for the black water lineage, and a Wilcoxon test for the clear water lineage, using the proportion of the time individuals spent in the red enlightened environment as my dependent variable and the first lightened environment females swam to as my independent variable. Because in seven out of nine cases, individuals from CJ1 population swam to the red enlightened environment, a statistical model was not possible to be fitted.

I also compared the strength of the red colour preference among lineages. The proportion of time spent in the red environment was used as the dependent variable and

lineages (black water, clear water and CJ1 population) as the independent variable in a Kruskal-Wallis test. The Kruskal-Wallis test was used since the proportion of time spent in the red environment was not normal (Shapiro-Wilk normality test, W = 0.896, p-value = 0.001). I checked for pairwise comparisons using Wilcoxon rank sum test with the Bonferroni correction. All analysis was performed in R version 4.2.2.

3.3 Results

Females from black water lineage spent longer in the red enlightened environment than in the full spectrum enlightened environment (One-sample t-test: p-value = 0.0008) (Figure 3.2A). Similarly, females from the clear water lineage (Wilcoxon signed rank exact test: V = 144, p-value = 0.0005) (Figure 3.2B) and CJ1 population (One-sample t-test: p-value = 0.0414) (Figure 3.2C) spent longer in the red enlightened environment than in the full spectrum enlightened environment.

There was no significant difference between the proportion of the time individuals spent in the red illumined environment when they first swam to red or full spectrum enlightened environments for black water lineage (Welch Two Sample t-test: t = 0.49259, df = 12.433, p-value = 0.6309), and for the clear water lineage (Wilcoxon rank sum test: W = 28, p-value = 0.6462). However, females from the CJ1 population spent a higher proportion of the time in the red enlightened environment when they first swam to the red enlightened environment (7 out of 9 cases) (Figure 3.3).

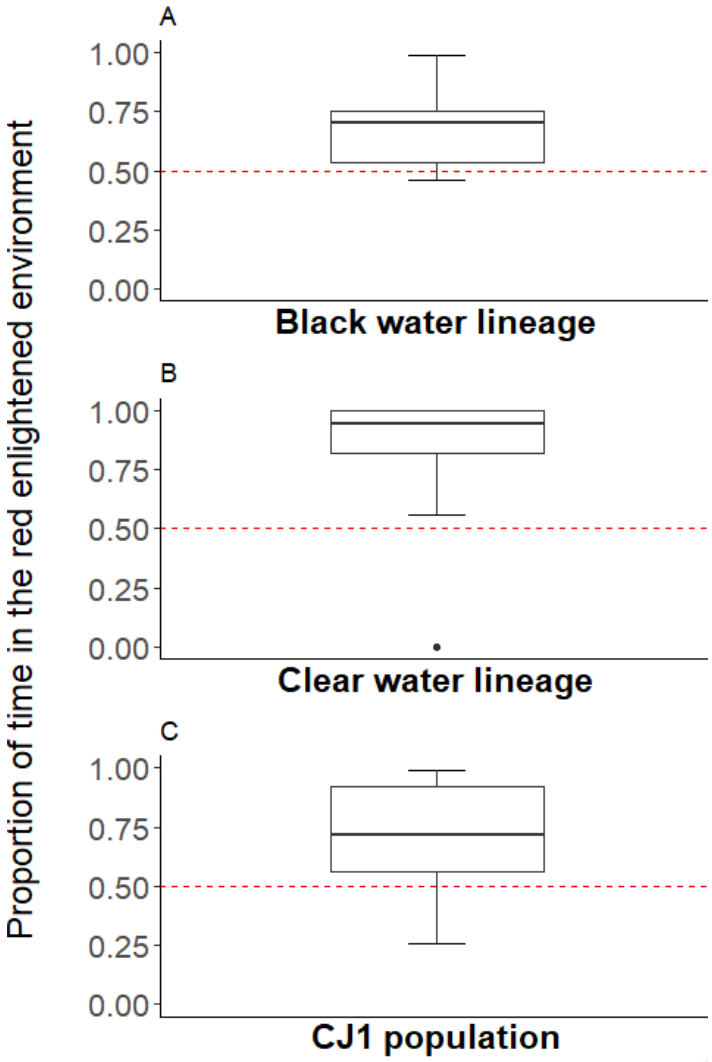

Figure 3.2. Proportion of the time females spent in the red enlightened environment for the three lineages of the sailfin tetra. **(A)** Black water lineage, **(B)** Clear water lineage, and **(C)** CJ1 population. The red dashed line at 0.50 marks the point where no colour preference is expected (i.e when the time individuals spent in red enlightened environment is equal to the time spent in the full-spectrum one). The box in the boxplot represents the interquartile range (Q1-Q3) and the whiskers represent the minimum and maximum values.

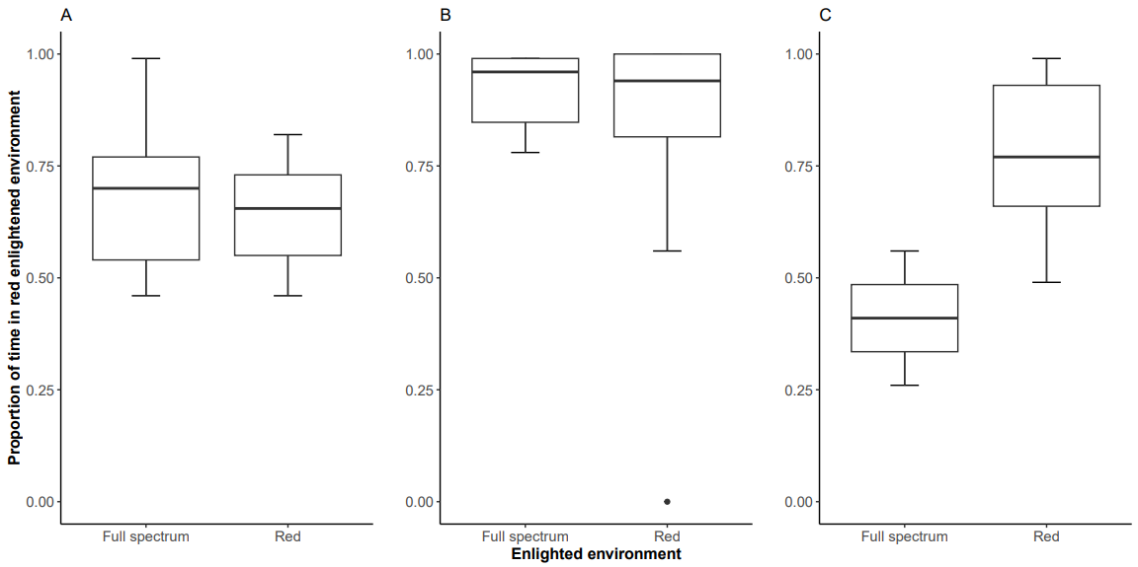

Figure 3.3. Proportion of the time females of the sailfin tetra spent in the red enlightened environments when they swam firstly to full spectrum and red enlightened environments. **(A)** Black water lineage, **(B)** Clear water lineage, and **(C)** CJ1 population. The box in the boxplot represents the interquartile range (Q1-Q3) and the whiskers represent the minimum and maximum values.

The proportion of the time spent in the red environment differed among lineages (Kruskal-Wallis chi-squared = 12.177, df = 2, p-value = 0.002). The comparison of the red colour preference among lineages suggested that the red colour preference is stronger in females of the clear water lineage than for females from black water lineage (Pairwise comparisons using Wilcoxon rank sum, p-value = 0.002) and CJ1 population (Pairwise comparisons using Wilcoxon rank sum, p-value = 0.042) (Figure 3.4). There was no significant difference between the strength of the red colour preference between females of the black water lineage and CJ1 population (Pairwise comparisons using Wilcoxon rank sum, p-value = 0.675).

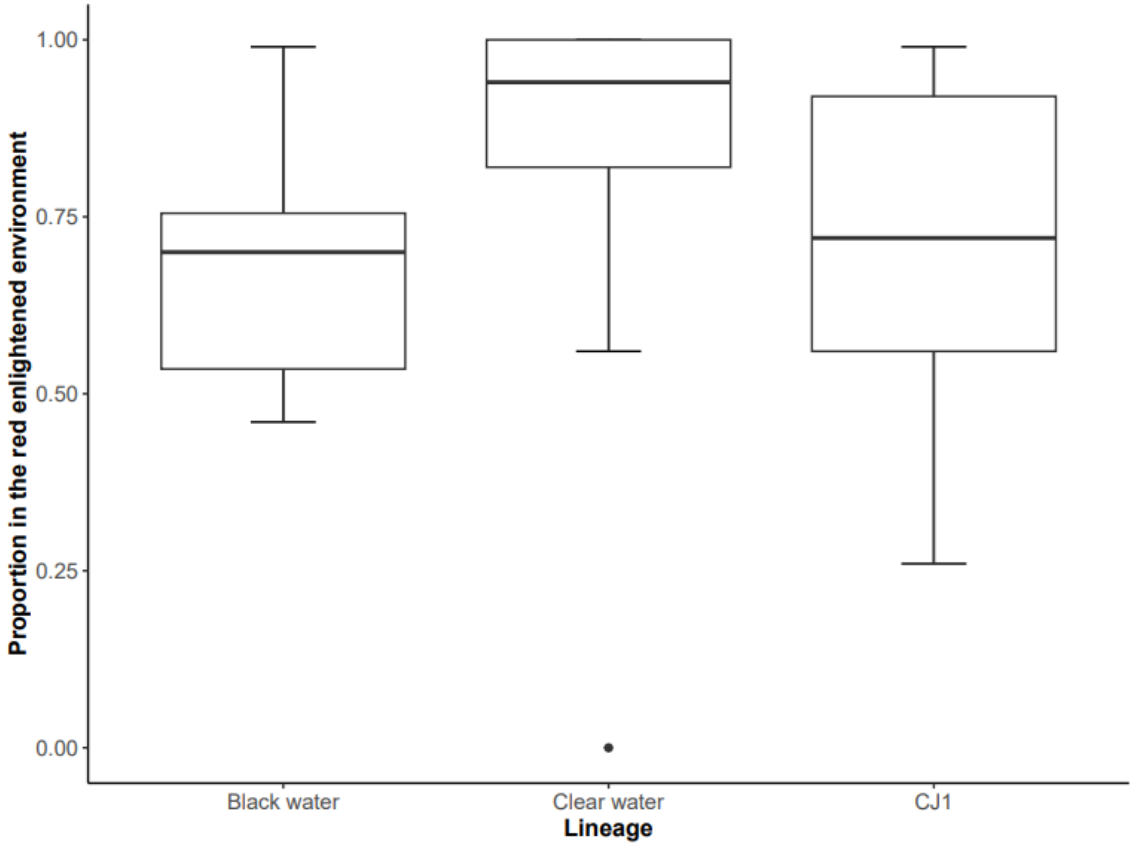

Figure 3.4. Proportion of the time spent in the red environment by females of the Black water, Clear water lineages and CJ1 population. The box in the boxplot represents the interquartile range (Q1-Q3) and the whiskers represent the minimum and maximum values.

3.4 Discussion

My results show that females from all three groups strongly preferred red colours. Such colour preference seems not to be affected by the first contact individuals had with both red or full-spectrum enlightened environments for the black and clear water lineage. However, females from the CJ1 population spend a higher proportion of the time in the red enlightened environment when they had the first contact with this environment. However, it is important to highlight my small sample size for the CJ1 population (N=9). Also, the strength of the red environment colour preference was higher for clear water lineage females than for those females living in black waters.

The colour preference can be related to the colour peak sensitivity (Luchiari et

al. 2007). In my previous analysis of the red colour peak sensitivity, I did not find any difference between the long wavelength sensitivity (LWS) genes that are responsible for sensing red colours among lineages. However, differences in the red colour sensitivity may be achieved by the differential expression level of each LWS gene. Indeed, I suggested that the expression of different LWS genes and the colour peak sensitivity may be environmentally driven (Borghezan et al., 2023). However, the LWS genes account for more than 90% of the gene expression profile in the eyes of the sailfin tetra individuals (Escobar-Camacho et al., 2020), highlighting the potential importance of the red colours for the sailfin tetra. Therefore, even though all individuals tested in my experiment were kept under similar laboratory lighting conditions, it is likely that the colour preference in both lineages of the sailfin tetra is driven by their red colour sensitivity.

The attraction for red colour and red colour sensitivity are important components of the sailfin tetra diet and sexual and social behaviours. Such colour preference may be associated with the acquisition of food resources. Per instance, red food items may look more intense coloured under black than clear water. Therefore, individuals may detect and be more attracted to red coloured food items in black waters. The sailfin tetra feeds on several red-coloured items such as buriti palm tree *Mauritia flexuosa* fruits and Chironomidae larvae (Pires et al., 2016). Most of the ripe fruits are orange/red coloured, and such colouration may indicate higher levels of carbohydrates and less unpalatable or potentially toxic secondary compounds. Therefore, the red colour preference for food items may be observed in several Amazon fish species. The ornaments of the sailfin tetra males are coloured in red colours. Such ornaments are used during sexual behaviour (i.e courtship behaviour) and play an important role in male-male interactions, especially during contests (Pires et al., 2016; personal observation). The colours pattern and red colour intensity in the male ornaments may mediate individuals' recognition and maintenance of the hierarchical structure. However, it is still unclear how the red colour preference found in the present study can affect the preference for different coloured food items. Also, the effects of different lighting conditions on potential male fighting ability and hierarchical structure are still to be explored. Future investigations can address such interesting points and shed light on how colours affect different social behaviours.

The sailfin tetra lineages live under clear and black water types. These water types differ in colouration and in chemical composition (reviewed in Borghezan et al.,

2021). Interestingly, individuals of the sailfin tetra can assess and actively choose environments that chemically resemble *igarapés* over the main drainage basin water (Stefanelli-Silva et al., 2019). Similarly, couples under *igarapés* waters showed higher spawning success than those under the main black- or white- basin river water types (Stefanelli-Silva et al., 2019), suggesting that they are sensitive to the chemical and physical limnologic characteristics of the water. The red biased light in black water systems can decrease individuals' stress levels (measured as plasma cortisol concentrations), resulting in individuals being bolder (i.e the exploratory behaviour) and more active behaviour when compared to those exposed to the white light (Owen et al., 2010). Such increased fish activity can also increase their feeding behaviour under red light conditions (Volpato et al., 2013). Such effects of the red light on individuals' physiological status may also make them more comfortable and less prone to diseases. Therefore, the red environmental colour preference in the sailfin tetra females may also represent a choice for environments that favour their physiological condition. Finally, the reproductive isolation of the sailfin tetra lineages is hypothesized to be shaped by differences in osmoregulation (Pires et al., 2018). Therefore, the chemical composition of the aquatic environment must play an important role in the fitness of the sailfin tetra individuals. Here, I acknowledge the importance of the chemical composition of the different water types; however, I tested the isolated influence of a physical composition (lighting bias) of the different water types on the female environment preference.

The red colour preference was stronger for the individuals living under clear waters than for those living in black waters. Such stronger preference for red colour in clear waters individuals may relate to the colour preference in mating behaviour (Borghezan et al., 2023). Therefore, stronger colour preference in females may indicate a higher relevance of such trait for mating decisions. As the importance of colours for mating choice varies according to predation risk (Godin & Briggs, 1996), the importance of colours for mating choice may vary according to the strength of the colour preference. Indeed, the sexual preference for a colourful trait has been hypothesized to be driven by a preliminary preference for such colouration (Taylor & Hunter, 2016). Future studies can investigate how the strength of the colour preference may correlate with female mating preference.

The colour preference outside the mating context has also been reported, as in

the case of the orange colour preference by female guppies *Poecilia reticulata* (Rodd et al., 2002), and the purple colour preference in the female bowerbirds (Madden & Tanner, 2003). Here, even though I did not directly investigate the mechanisms of the colour preference, I suggest that the females of the sailfin tetra are attracted to red colours even outside of the sexual context. Interestingly, the colour preference often matches the male sexual colourful traits in several species (Rodd et al., 2002; Madden & Tanner, 2003). However, female mating preference in relation to the male ornament colouration differs in relation to the lighting conditions of the environmental colouration individuals live in, with females from black water preferring red environments but with no effects of different lighting conditions on female mate acceptance (Borghezan et al., 2023). Therefore, because of the observed differences in the female preference for red colours under a mating context (Borghezan et al., 2023) and during non-reproductive activities for the sailfin tetra populations living in black waters, I suggest that the relevance of colours and their preference can vary according to the context and are shaped by environmental conditions.

Chapter 4

Unstable environmental condition constrains the fine-tune between opsin sensitivity and underwater light in an Amazon forest streams fish

4.1 Introduction

Natural selection drives individuals' sensory systems adaptation to best fit their environments. For visually-oriented species, the structure of visual system and its colour sensitivity are crucial to engaging a suite of activities such as searching for food and mates (Boughman, 2002; Melin et al., 2014; Seehausen et al., 2008). There are four spectrally distinct classes of opsins in cone cell types among higher vertebrates: long-to-middle wavelength class (LWS) sensitive to orange and red (approx. 490-570 nm) wavelengths, middle wavelength class (RH2) sensitive primarily to green (approx. 480-535 nm), short wavelength class two (SWS2) sensitive to blue-violet (approx. 410-490 nm), and short wavelength class one (SWS1) sensitive to violet and ultraviolet (approx. 355–440 nm) (Bowmaker & Hunt, 2006). Therefore, it is expected that the light-absorbing and sensitive part (chromophore) of photoreceptive proteins` peak to be adjusted according to the prevalent environmental lighting conditions of the habitat the individuals live in.

Several mechanisms can cause changes in individual light sensitivity, including shifts in chromophore usage (A1 or A2), evolution of opsin sequences, and changes in opsin gene expression (Carleton & Kocher, 2001; Hofmann & Carleton, 2009; Seehausen et al., 2008; Terai et al., 2017; Whitmore & Bowmaker, 1989). For instance, Lake Victoria cichlids live in different water depth, and the spectral composition of environmental light of their habitat influenced the amino acid sequence of LWS gene, and divergent spectral compositions caused divergent evolution of the cichlid`s LWS gene set (Seehausen et al., 2008). Visual adaptations to divergent underwater light conditions can evolve not only through opsin amino acid substitution, but also by which derivate of the vitamin A the amino acid chain is bounded with. The amino acid chain can be bounded with either *11-cis*-retinal (a derivative of vitamin A1) or 11-*cis* 3,4-dehydroretinal (a derivative of vitamin A2 chromophores) (Hofmann & Carleton, 2009). Their usage allows individuals

to enlarge their spectral sensitivities since A2-derivate pigments shift individual spectral sensitivity towards long wavelengths and have been shown to play an important role in visual adaptation to divergent lighting conditions (Escobar-Camacho et al., 2019; Terai et al., 2017). Fishes living in red-biased environments usually express more A2-derived visual pigments than those living in non-biased environments (Terai et al., 2017; Escobar-Camacho et al., 2019). Furthermore, differential spectral sensitivity can also arise from the expression level of different opsin genes, even when these are nearly identical among species inhabiting different lighting conditions (Carleton & Kocher, 2001). Differential gene expression levels allow individuals to use those genes that would best fit their environmental condition. Finally, individuals can modulate gene expression level and A1/A2 usage to maximize their sensitivity according to the environmental lighting condition (Escobar-Camacho et al., 2020).

Freshwater fishes belonging to the order Characiformes have undergone a whole genome duplication event around 300-450 MYA (Meyer & Van de Peer, 2005; P. Taylor et al., 2001), with some genes passing through subsequent duplication events, resulting in many species having multiple copies of the same gene (Escobar-Camacho et al., 2020). Natural selection exhibits diverse effects on multiple copies of opsin genes, encompassing alterations or mutations that modify individual sensitivities, while also manifesting instances of functional loss within certain gene copies (Marques et al., 2017; Torres-Dowdall et al., 2017). Most of the Characiformes inhabit the Neotropical region, with more than 3,700 valid species (Fricke et al., 2020), with the highest diversity in Amazon rivers and small forest streams (Dagosta & Pinna, 2019). Amazon forest streams, also known as *igarapés*, can be classified into black and clear water types (Fig. 4.1A,B), which are remarkably different in their chemical and physical conditions, with divergent underwater lighting conditions (reviewed in Borghezan et al., 2021). Black waters are red-biased environments while clear waters are mostly transparent and do not show any apparent colour bias (e.g. Mendonça et al., 2005).

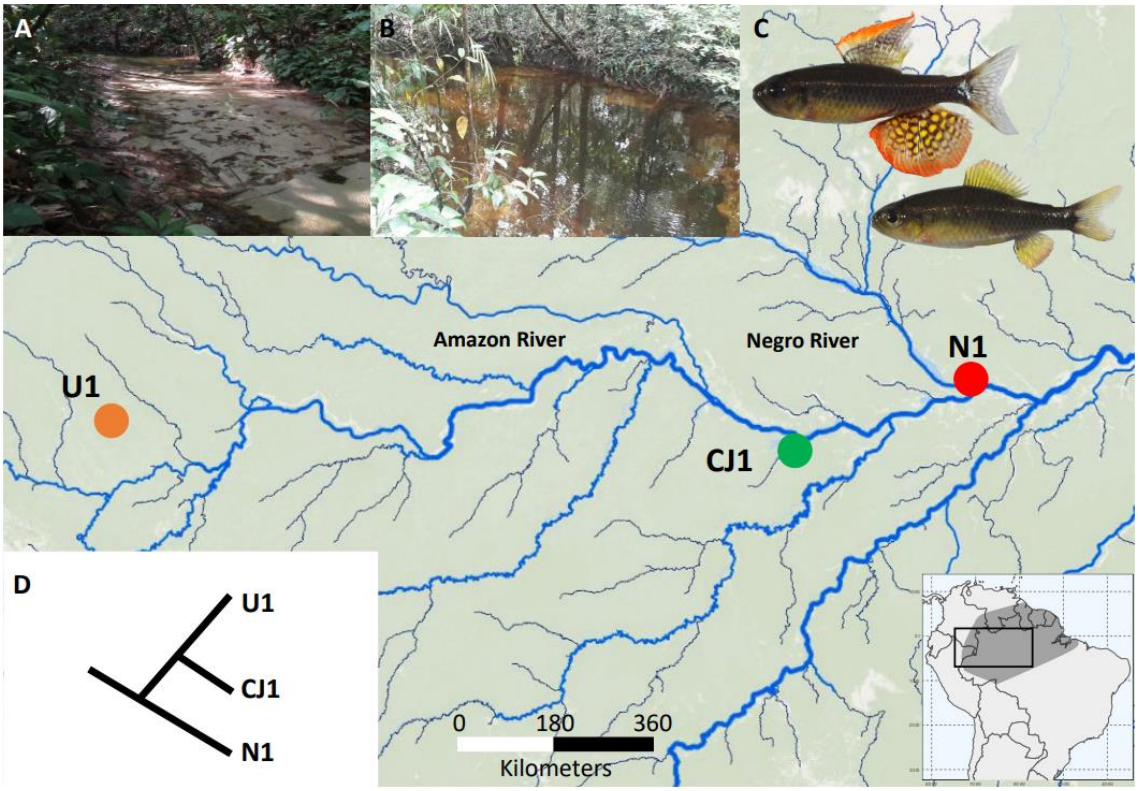

Figure 4.1. Localities where the three sailfin tetra *C. spilurus* populations were sampled. Examples of **(A)** clear, and **(B)** black water *igarapés*. **(C)** Male (above) and female (below) of the sailfin tetra. **(D)** Simplified phylogenetic relationship among the three populations used in the present study. Detailed phylogenetic relationships among populations can be found in Pires et al. (2018) and in Appendix 4.5.1. For the sake of clarity, U1 belongs to the clear water lineage; CJ1 genetically belongs to the clear water lineage but lives under black water condition; N1 belongs to the black water lineage. The insert in the right lower corner shows a part of the South America map (Pires et al., 2016), the distribution of the sailfin tetra in dark grey and the amplified map with the populations used in the present study in the rectangle.

The sailfin tetra *Crenuchus spilurus* Günther, 1863 (Characiformes: Crenuchidae) is a sexually dimorphic species that is native to Amazonian *igarapés* (Pires et al., 2016). Males possess hypertrophied dorsal and anal fins conspicuously ornamented in red and yellow (Figure 4.1C) that are used in an elaborate courtship behaviour (Pires et al., 2016). This species is composed of two reproductively isolated and phylogenetically distinguished lineages, one inhabiting black water *igarapés* (Rio Negro

lineage) and the second one inhabiting mostly clear water *igarapés* (Amazonas lineage) (Pires et al., 2018). The sailfin tetra does not migrate and does not disperse their eggs/larvae (Pires et al., 2016), which may difficult the gene flow among populations. Interestingly, some populations, such as the case of CJ1, genetically belong to the clear water lineage but currently live in black water conditions (Figure 4.1D) (Pires et al., 2018; Appendix 4.5.1). A previous study has shown that Characiformes may adapt their visual system to different Amazon water types by amino acid chain variation, differential expression of opsin genes A1/A2 to maximise light absorption (Escobar-Camacho et al., 2020). However, this study evaluated several species exposed to either clear or murky waters but did not focus on a single species inhabiting different lighting conditions. Red colours are important for the sailfin tetras during foraging activity (i.e. finding insect larvae or fruits of the buriti palm tree - *Mauritia flexuosa*) and for attracting mates (Borghezan et al., 2023; Pires et al., 2016; Chapter 2), suggesting that LWS genes play an important role in the adaptation of these to the water colour of their habitat and main resources. Indeed, the sailfin tetra has three copies of the *LWS1* gene, which is red sensitive and a single copy of the *LWS2* gene, which is green sensitive (Escobar-Camacho et al., 2020). On these bases, I hypothesise that the sailfin tetras have (I) LWS genes with different sensitivities among those populations living in different water types (cf. Seehausen et al., 2008), and/or (II) their visual adaptations for different coloured water types arise from the expression of different visual opsin genes, and therefore, the expected sensitivity of their LWS proteins would be similar among populations inhabiting different water types (Carleton & Kocher, 2001).

Here, I analysed the number of copies, nucleotide sequences, expected maximum spectral absorbance (λmax), and expression levels of the LWS opsin genes of black and clear water lineages in sailfin tetras to test for these hypotheses. I also evaluated the gene expression level of the SWS2 (i.e. blue light sensitive) and a gene (*Cyp27c1*) associated related to chromophore usage. I mainly focused on the LWS opsin genes, since the *SWS1* and *RH2* genes are not present in the sailfin tetra (Escobar-Camacho et al., 2020), and for the given biological relevance of red hues for the sailfin tetra. My results can help understanding the ways through which Amazon fishes have adapted to different aquatic environments, as well as shedding some light on the processes that have allowed the evolution on the astounding fish diversity of the Amazon basin.

4.2 Material and Methods

4.2.1 Ethics statement

Fish collections in Brazil were carried out under IBAMA permanent license SISBIO #10199-1 to Jansen Zuanon, one of my collaborators. All experiments were approved by Kyoto University Ethics Committee under protocol number WRC-2019-010A. Experiments were carried out following all regulations on Animal Experimentation at Kyoto University.

4.2.2 Sampling individuals

The sailfin tetra individuals were sampled from three different populations: three individuals from N1 (an urban forest fragment in Manaus), representative and herein called black water lineage (3°06'22.8"S; 59°58'40.5"W); three individuals from U1 (a small stream water near Iquitos), representative and herein called clear water lineage (4°0’46.20”S; 73°27’47.70”W); and four individuals from CJ1 population (in a small stream in the outskirts of the Coari municipality) (4°07'09.9"S; 63°04'29.9"W) (Fig. 3.1). Individuals were collected using seine and hand nets following local regulations, hence brought to the laboratory of the National Institute for Amazonian Research - INPA (Manaus, Brazil). The majority of the collected sailfin tetra individuals were between 3 and 4.5 cm long (standard length). To simulate the structures of their natural environment, natural and artificial plants were added to the tanks and 10 - 12 pieces of PVC pipes (10 cm long, 25 mm in diameter) to provide shelter. This species does not actively swim around the tanks, and its shelter is only defended at a very close range; thus, aggressive interactions were rarely observed. Individuals were kept separately for each collecting location in tanks filled using artesian well water, with limnological conditions similar to black waters (pH = 5.5 and conductivity at 20 µS cm-1), but without the usually high amount of dissolved organic carbon - DOC. It is important to highlight that the water colouration (i.e spectral composition) where the individuals live differs among sampling sites and more importantly, differs from my housing tanks, which is more similar to the

clear water. Aquariums were kept under 24°C, simulating the temperature of their habitats. Windows covering over half of the laboratory's wall provided indirect natural light and photoperiod (12:12 h light:dark cycle). I used dark cloth on the windows to control the amount of light that reached the laboratory, effectively simulating the natural canopy-shaded condition of an Amazon forest stream. Water changes (25%) were weekly performed to avoid the accumulation of detritus and dissolved organic waste. Fishes were fed commercial pelleted food for aquarium fishes *ad libitum* once a day. Individuals were kept in captivity for around five months before being transported to the Wildlife Research Center of Kyoto University (Kyoto, Japan).

4.2.3 DNA and RNA extraction, sequencing and genome assembling of the sailfin tetra

I created a genome library using the DNA and RNA samples. I extracted total genomic DNA (gDNA) from a dorsolateral muscle sample of one individual from the black water lineage using the DNeasy Blood and Tissue Kit (QIAGEN - Tokyo, Japan). Paired-end sequencing libraries with 350 bp and 550 bp insert sizes were prepared using the TruSeq DNA PCR-Free Sample Prep kit (Illumina - Tokyo, Japan) and their libraries were sequenced on an Illumina NovaSeq6000 platform with a sequencing length of 2x151 bp. Details of the sailfin tetra genome assembly constructed in this study are provided in Appendix 4.5.2. I also extracted a total RNA from the retina of the same individual using the RNeasy Minit Kit (QIAGEN). I measured the quality (A260/A280 and A260/A230) and the concentration (ng/ul) of the gDNA and all RNA samples using a NanoDrop 2000 (Thermo Scientific) and a Qubit 3.0 Fluorometer (Life Technologies), respectively. All samples had at least 40 ul (volume) and 33.9 ng/ul (concentration) of RNA/gDNA. The quality and concentration of the gDNA and all RNA samples were latter on confirmed during the sequencing process. The extracted RNA was used to construct a paired-end sequencing library using the TruSeq standard mRNA Sample Prep kit (Illumina) and sequenced on an Illumina NovaSeq6000 platform (2x101 bp). PLATANUS_trim (Kajitani et al., 2014) with default settings was employed to remove low quality regions and adapters. Trimmed gDNA reads were used to construct contig assembly using PLATANUS allee (Kajitani et al., 2019). Scaffolds were constructed based on the contig

assembly with the trimmed RNA-seq data using the P_RNA_scaffolder (Zhu et al., 2018). The age of the fish individuals included in the genetic analysis is unknown. Nonetheless, they were confirmed as adults since they surpassed the species' estimated size at maturity of 2.57 cm (Pires et al., 2016), with all individuals in my study exhibiting a standard length exceeding 3.0 cm. Furthermore, all individuals selected for the experiment were females, evidenced by the sexual ornaments (hypertrophied dorsal and anal fins) in males and their absence in females (Figure 4.1C). The sailfin tetra genome assembly and RNA-seqs of all individuals obtained in this study are available in the GenBank with the BioProject accession PRJDB13548.

4.2.4 Identification of the LWS genes repertoire from the sailfin tetra genome assembly

I identified the LWS genes in my sailfin tetra genome assembly using the known conspecific LWS1 (GenBank accession numbers MT310728.1 – MT310730.1), LWS2 (accession number MT310742.1) and SWS2 (accession number MT310768.1) genes (Escobar-Camacho et al., 2020) as queries and employing the BLASTN function in BLAST+ v2.6.0 (Camacho et al., 2009) with E-value cut-off of 1e-10. *Cyp27c1* gene is responsible for the red-shifts in the spectral sensitivity of photoreceptors by converting vitamin A1 into A2 (Enright et al., 2015). Therefore, I did the same procedure to annotate the *Cyp27c1* gene, using the *Danio rerio* amino acid sequence (accession number AAI54633.1) as query. I also searched for the SWS1 and RH2 genes in my genome assembly, using the *Danio rerio SWS1* gene (accession number KT008399.1) and the *Piabucina panamensis RH2* (accession number MT310781.1) as queries. These genes were not present in my genome assembly, as also found in other study (Escobar-Camacho et al., 2020). Since the sailfin tetra possesses four copies of the LWS gene (Escobar-Camacho et al., 2020), I performed one BLASTN search for each gene. Sequences were compared with the known opsin genes to assess their identity, and to single out the amino acid coding regions. Because I have found two copies of the *LWS2* gene, to assure the accuracy of my gene annotation, I also fitted a phylogenetic relationship with the genes I have found in one of my black water lineage samples with those previously identified for the sailfin tetra and other Characiformes species (Escobar-Camacho et al., 2020). This

was mostly done to access the *LWS2_2* gene identity, since such gene was not found in a previous study that included the sailfin tetra (Escobar-Camacho et al., 2020). The phylogenetic relationship among genes can be found in Appendix 4.5.3.

4.2.5 RNA sample extraction, identification of heterozygote and expression of LWS opsin genes

Retina RNA of the individuals used in this study was extracted as soon as the individuals arrived at Kyoto University. Therefore, individuals stayed around 72 hours dark acclimated (during shipment) prior to the RNA sample extraction. I extracted RNA from three individuals from the black water lineage, three from the clear water lineage and four from the CJ1 population. The total RNA from the left eyeball was isolated using a RNeasy Mini Kit (QIAGEN). A paired-end sequencing library was prepared from the extracted RNA of each individual using the TruSeq standard mRNA Sample Prep kit (Illumina) and sequenced on an Illumina Novaseq6000 platform (2x101 bp). RNA-seq reads were aligned to the sailfin tetra reference genome using HISAT2 v2.2.1 (Kim et al., 2019). I converted the alignment SAM to BAM file using Samtools v1.12; hence, I extracted the LWS gene nucleotide sequences. I also used the BAM file to visually identify heterozygotes sites among all samples using IGV v2.11.3. I calculated the synonymous/non-synonymous nucleotide substitution ratio (dS/dN) per each gene and within each lineage.

Genes expression levels were calculated in FPKM (fragments per kilobase per million mapped reads) using Cufflinks v2.2.1 (Trapnell et al., 2010). I additionally employed the -u function in Cufflinks, a feature designed to initiate an estimation procedure. This procedure aims to enhance the accuracy of read weighting for sequences that map to multiple locations within the genome. Comparisons between the RNA samples and the genomic sequence unveiled exon/intron boundaries in the genomic sequence. These boundaries were precisely defined by the conventional GT … AG splicing junctions, situated at locations consistent with the introns found in visual pigments of other organisms (e.g., Carleton & Kocher, 2001). I also compared the gene expression level of the LWS genes present in my samples with those of the sailfin tetra from Coropina Creek, a black water river in the Para district of Suriname (Escobar-

Camacho et al., 2020).

4.2.6 Determining the LWS genes expected maximum wavelength sensitivity

To determine the expected light peak sensitivity of the LWS genes, nucleotide sequences were firstly converted into amino acid sequences and then aligned with those of the bovine rhodopsin (NCBI access number: NP_001014890.1) and some teleost fishes. Previous researches have determined the molecular basis of spectral tuning in LWS pigments where five amino acid changes (S164A, H181Y, Y261F, T269A, A292S; following the bovine rhodopsin residue numbering system) can shift λmax up to 50 nm (Yokoyama, 2008; Yokoyama et al., 2008). Therefore, I looked for substitutions that fall into the five key spectral tuning sites to determine the expected maximum wavelength sensitivity. Positions 177, 194, 274, 282 and 305 in the three *LWS1* genes in my samples were equivalent to those 164, 181, 261, 269 and 292 in the bovine rhodopsin. Positions 174, 191, 271, 279 and 302 in the *LWS2_1* gene in my samples were equivalent to those 164, 181, 261, 269 and 292 in the bovine rhodopsin. Positions 175, 192, 272, 280 and 303 in the *LWS2_2* gene in my samples were equivalent to 164, 181, 261, 269 and 292 in the bovine rhodopsin. Since the amino acid chain can be combined with A1 or A2 (Hofmann & Carleton, 2009; Wald, 1968), I evaluated the expected sensitivity for both chromophores. A1 (λA1) is determined by the amino acid composition in the five key sites (Yokoyama et al., 2008), while that of its combination with A2 (λA2) is estimated by the following equation (Whitmore & Bowmaker, 1989):

$$\lambda A2 = (\lambda A1/52.5)^{2.5} + 250$$

I also compared the expected maximum wavelength sensitivity for A1 (λA1) and A2 (λA2) in my samples with the results previously found for the sailfin tetra from Coropina Creek, a black water river in the Para district of Suriname (Escobar-Camacho et al., 2020).

4.2.7 Statistical analysis

I first extracted the FPKM values of each *LWS* gene, *SWS2* and *Cyp27c1* genes using Cufflinks (v2.2.1). I then calculated the sum of the FPKM values for all six opsin genes (*LWS1_1*, *LWS1_2*, *LWS1_3*, *LWS2_1*, *LWS2_2*, and *SWS2*). Subsequently, I determined the proportion of each opsin gene for each individual by dividing the FPKM value of each gene by the total sum of all opsin genes. I then compared the gene expression profile (in proportion of each gene) in my samples with the Suriname population. Finally, I fitted a set of Kruskal-Wallis tests, one for each gene, to evaluate the differential gene expression level among lineages of the sailfin tetra. I used a one-factor design (levels: Black water lineage, Clear water lineage and CJ1 population) to analyse all samples. After identifying significant differences in gene expression levels among lineages, I proceeded to conduct a series of Wilcoxon rank sum exact tests. I applied the Holm method to adjust the p-values in order to explore pairwise differences in gene expression levels among the lineages. All analyses were done in R (version 4.3.1).

Upon finding differences in mean opsin expression in the sailfin tetra lineages, I estimated how they translated into differences in spectral sensitivity. I calculated a spectral sensitivity curve (400-700 nm) for each lineage based on its average relative expression of the LWS opsin genes and using the absorbance templates from (Govardovskii et al., 2000). Because I could not estimate the light peak sensitivity for the SWS2 protein in my samples, I did not include such opsin in the spectral sensitivity curve. This model assumes that opsin expression contributes additively to spectral sensitivity; at this point in time, it is a necessary simplification as I still lack empirically informed models that describe and generalize any potential inhibitory interactions among opsins during signal integration and interpretation (Rennison et al., 2016). Because I could not determine the chromophore ratio and more importantly, which gene was bounded to A1 and A2, I estimated the spectral sensitivity of the sailfin tetra lineages using three different ratios representing the extremes: 100% A1; 50% A1 and 50% A2; and 100% A2.

4.3 Results

4.3.1 The repertoire of opsin genes, heterozygous sites, and their expected maximum wavelength sensitivity

I found three copies of the *LWS1*, two copies of the *LWS2* and a copy of *SWS2* genes in the sailfin tetra genome assembly, with these six opsin genes being expressed in all individuals analysed in this study. Nucleotide sequences and heterozygous sites varied among lineages and genes (Table 4.1). Heterozygous sites were mostly observed in the CJ1 population. On the other hand, black water lineage samples genes were more conserved and showed fewer heterozygous sites. *LWS1_3* was the most variable gene in the black and clear water lineages, whereas the *LWS1_1* was the most variable gene in the CJ1 population. *SWS2* was the most conserved gene in all three lineages. Some of these heterozygous sites resulted in amino acid changes (Table 4.1). Most of the amino acid changes were observed in the CJ1 population, whereas in black water lineage samples the amino acid chain was the most conserved. LWS1_3 protein chain was the most variable and SWS2 the most conserved. However, these amino acid changes among the LWS proteins did not encompass the five key sites and therefore are not expected to shift individual light peak sensitivity. Interestingly, the high dS/dN values suggest that the opsin genes in the sailfin tetra are under negative selection, where synonymous changes are favoured (Table 4.1).

Based on the comparison of the five key amino acid sites, all LWS1 opsin proteins have different λmax to each other in the sailfin tetra samples. However, the expected maximum absorbance wavelength of each LWS1 and LWS2 copy does not differ among lineages, including the comparison with the Suriname population, varying from 532 to 560 nm when the amino acid chain is bounded to A1 and from 577 to 622 nm when the amino acid chain is bounded to A2 (Table 4.2). However, I found that the expected light peak sensitivity of the LWS1_1 and LWS1_2 opsin proteins in those individuals from Suriname does not differ and it estimated to be at 560 and 622 nm when the amino acid chain is bound to A1 and A2, respectively. Also, the sensitivity of the LWS1_3 opsin protein differs from those expected in my samples and it is the same as expected to the LWS1_2 opsin protein in my samples. Therefore, individuals from Suriname do not have a peak sensitivity to 555 and 612 nm, when the amino acid chain

is bounded to A1 and A2, respectively; which are the sensitivities expected for the LWS1_3 opsin protein in my samples. The expected spectral sensitivity based on the absorbance templates from Govardovskii et al., (2000) of each spectrally distinct LWS opsin is shown in Figure 4.2.

Table 4.1. Nucleotide and amino acid variation (polymorphic sites) within each lineage of the sailfin tetra. Nucleotide changes do not include the stop codon, which was nonetheless included in the nucleotide length. BW = Black water lineage; CJ1 = CJ1 population; CW = Clear water lineage. In the dS/dN columns, the following notations are used: X indicates that all nucleotide changes did not result in any amino acid changes, implying synonymous substitutions. "-" signifies that there were no nucleotide sequence changes, resulting in an unchanged amino acid chain for the specific gene.

	Sequence	Nucleotide changes			Amino acid changes			dS/dN		
Gene	length (bp)	BW	CJ1	CW	BW	CJ1	CW	BW	CJ1	CW
LWS1_1	1074	0	18	8	0	5	3	-	6.557	6.953
LWS1_2	1077	0	9	12	0	2	4	-	14.705	6.598
LWS1_3	1077	7	16	18	3	3	6	3.384	15.346	5.723
LWS2_1	1059	0	6	6	0	0	0	-	X	X
LWS2_2	1062	2	10	4	0	2	1	X	19.745	14.657
SWS2	1068	0	1	0	0	0	0	-	X	-

Table 4.2. List of LWS genes, the amino acid found in the five key site positions and their expected λmax sensitivity for A1 and A2. All amino acid positions in the table below are following the bovine rhodopsin residue numbering system. S = Serine; A = Alanine; H = Histidine; Y = Tyrosine; F = Phenylalanine; T = Threonine. Baseline animo acid information from Yokoyama et al. (2008).

Gene	Sample	164	191	261	269	292	Expected λA1 (nm)	Expected λA2 (nm)
-	Baseline	S	H	Y	T	A	560	622
LWS1_1	BW	.	.	.	.	.	560	622
LWS1_1	CJ1	.	.	.	.	.	560	622
LWS1_1	CW	.	.	.	.	.	560	622
LWS1_1	Suriname	.	.	.	.	.	560	622
-	Baseline	A	H	F	T	A	545	597
LWS1_2	BW	.	.	.	.	.	545	597
LWS1_2	CJ1	.	.	.	.	.	545	597
LWS1_2	CW	.	.	.	.	.	545	597
LWS1_2	Suriname	S	.	Y	.	.	560	622
-	Baseline	A	H	Y	T	A	555	613
LWS1_3	BW	.	.	.	.	.	555	613
LWS1_3	CJ1	.	.	.	.	.	555	613
LWS1_3	CW	.	.	.	.	.	555	613
LWS1_3	Suriname	.	.	F	.	.	545	597
-	Baseline	A	H	F	A	A	532	577
LWS2_1	BW	.	.	.	.	.	532	577
LWS2_1	CJ1	.	.	.	.	.	532	577
LWS2_1	CW	.	.	.	.	.	532	577
LWS2_1	Suriname	.	.	.	.	.	532	577
LWS2_2	BW	.	.	.	.	.	532	577
LWS2_2	CJ1	.	.	.	.	.	532	577
LWS2_2	CW	.	.	.	.	.	532	577

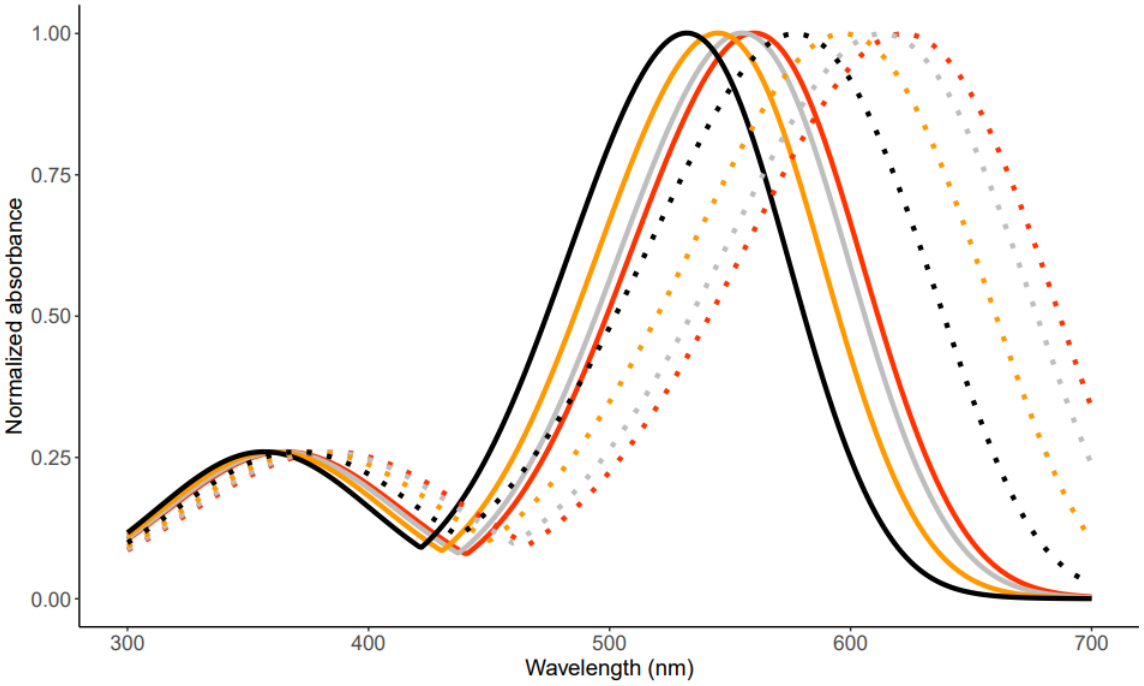

Figure 4.2. Estimated spectral sensitivity of each spectrally distinct LWS opsin gene in the sailfin tetra. Solid lines represent the estimated spectral sensitivity for A1 and dotted lines for A2. LWS1_1 = red; LWS1_2 = orange; LWS1_3 = grey; and LWS2_1 and LWS2_2 = black in my samples of the sailfin tetra.

4.3.2 Gene expression

Spectral sensitivity in my samples of the sailfin tetra is based mainly on the expression of *LWS1_2* and *SWS2* genes with around 60 and 20%, respectively (Figure 4.3A, 3.3B and 4.3C). However, the opsin expression determined from RNA-Seq data showed considerable variation amongst individuals (Fig. 4.3D). *LWS1_1* and *LWS1_3* accounted for <6% of expressed cone opsins in all samples. *LWS1_2* was the most highly expressed visual pigment in 9 out of 10 samples. All samples expressed the same opsins (Fig. 4.3D). The *LWS2_1* accounted for around 12% of the gene expression in black water lineage, whereas in clear water lineage and CJ1 population this gene represented around 1% of their gene expression profile. An interesting observation is the marked disparity in the opsin gene expression profile between the samples and the Suriname population. The population of Suriname expressed mainly the *LWS1_2* and *LWS1_3*, with these genes representing more than 80% of the total opsin gene expression (Figure 4.3D). Also, the

SWS2 accounted for less than 5% of the total opsin gene expression. On the other hand, the *SWS2* gene accounted for around 20% of the opsin gene expression in most of the samples, irrespective of the fish lineage (Figure 4.3D). The normalized sensitivity of all lineages of the sailfin tetra was estimated to be dominant by the *LWS1_2* gene, with the light peak sensitivity at 545 and 597 nm when bound to A1 and A2, respectively (Appendix 4.5.4).

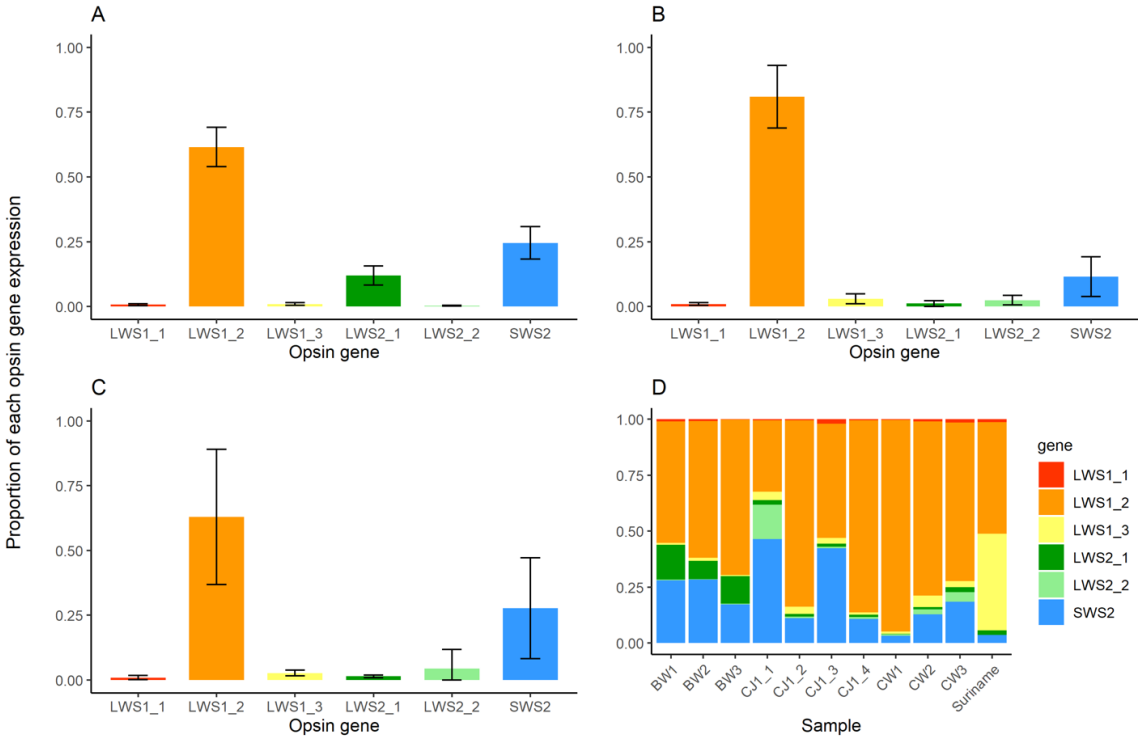

Figure 4.3. The proportion of the FPKM values of each opsin gene among **(A)** Black water lineage, **(B)** Clear water lineage and, **(C)** CJ1 population. Error bars represent one standard deviation. **(D)** Proportion of the raw read count of each opsin gene for each sample. The Suriname represents the gene expression level in the Suriname population as estimated from Escobar-Camacho et al., (2020).

By comparing each gene expression level among lineages, I found no significant difference in the expression of the *LWS1_1* (H = 3.7545, df = 2, P = 0.153) (Figure 4.4A). I found a marginally significant difference in the expression level of the *LWS1_2* among lineages (H = 5.7273, df = 2, P = 0.0570). While the observed difference may not reach strict statistical significance, it is noteworthy that the clear water lineage displayed a

tendency to exhibit higher expression of the *LWS1_2* gene in comparison to the black water and CJ1 population samples (Figure 4.4B). On the other hand, we found a significant difference in the *LWS1_3* gene expression level among lineages (H = 6.7455, df = 2, P = 0.0343). However, when performing Wilcoxon rank sum exact tests to evaluate the pairwise comparison among the lineages, I found no significant difference between the *LWS1_3* gene expression between black water and clear water lineages (W = 0, P = 0.1); black water and CJ1 population (W = 2, P = 0.2286); and only a marginally significant difference between clear water lineage and CJ1 lineages (W = 12, P = 0.0571) (Figure 4.4C).

I found a marginally significant difference in the expression level of the *LWS2_1* gene among lineages (H = 5.7909, df = 2, P = 0.0552). While the observed difference lacks statistical significance, it is worth noting that the black water lineage exhibited a tendency to express higher levels of the *LWS2_1* gene when compared to the clear water and CJ1 population samples (Figure 4.4D). There was a significant difference in the expression level of the *LWS2_2* gene among lineages (H = 6.7455, df = 2, P = 0.0343). Similarly, I found no difference between the *LWS2_2* gene expression between black water and clear water lineages (W = P = 0.1); a marginally significant difference between black water and CJ1 population (W = 0, P = 0.0571); and no difference between clear water lineage and CJ1 lineages (W = 10, P = 0.2286) (Figure 4.4E). I found no difference in the *SWS2* (H = 1.1818, df = 2, P = 0.5538) (Figure 4.4F) and *Cyp27c1* genes expression level among lineages (H = 3.6091, df = 2, P = 0.1645) (Figure 4.4G).

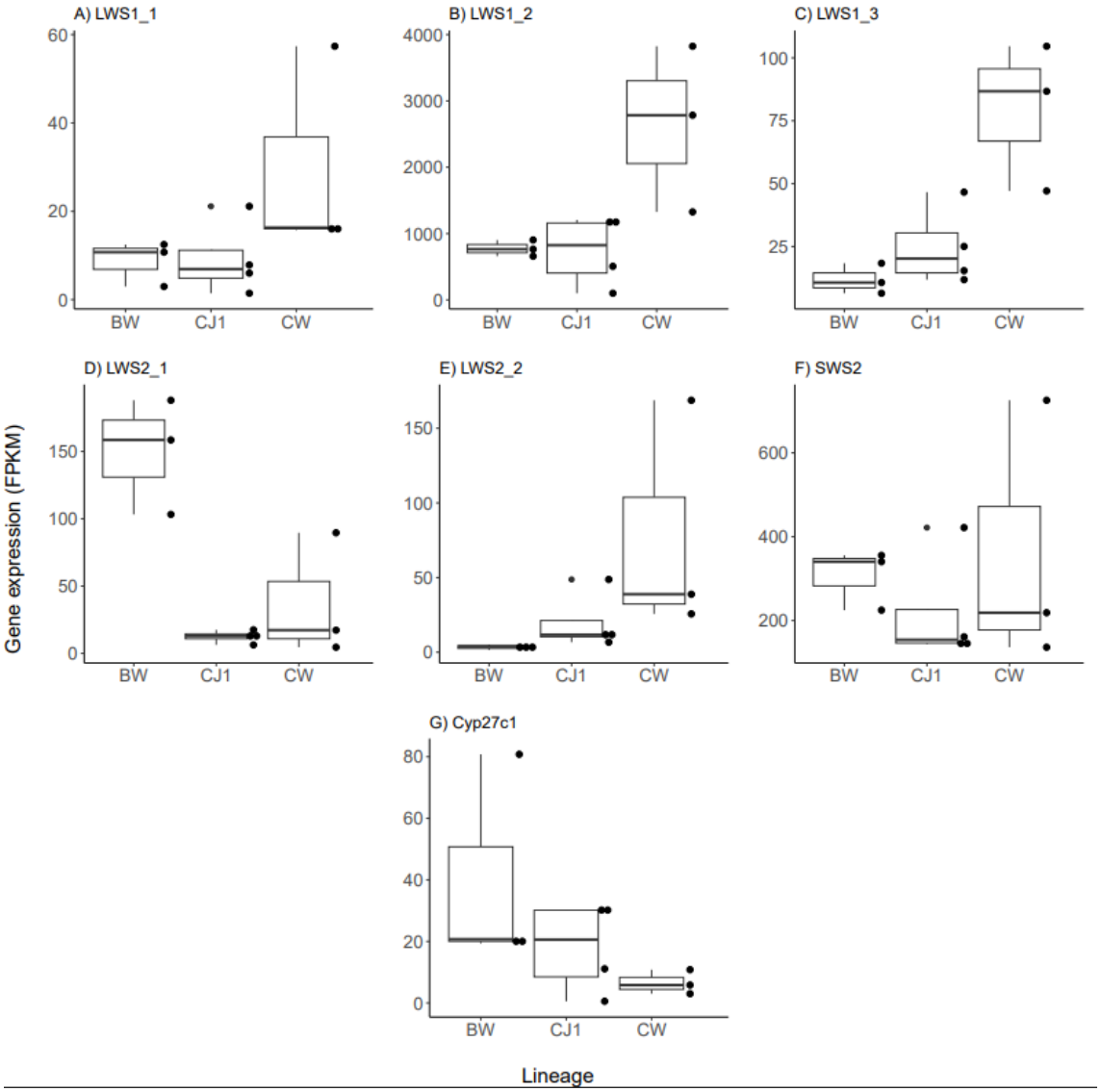

Figure 4.4. Gene expression level among black water, clear water lineage and CJ1 population for **(A)** *LWS1_1*, **(B)** *LWS1_2*, **(C)** *LWS1_3*, **(D)** *LWS2_1*, **(E)** *LWS2_2*, **(F)** *SWS2*, and **(G)** *Cyp27c1* genes based on the PPKM values. The points on the right side of each box show the raw read count value for that particular sample. Please note the differences on the Y-axis scale.

4.4 Discussion

The amino acids at the five critical spectral tuning sites in the LWS genes (Yokoyama et al., 2008) exhibit variability within the LWS1 gene copies but remain uniform across the two LWS2 gene copies. Interestingly, these key sites in each LWS

ortholog remain consistent among populations representative of sailfin tetra lineages. This suggests that the differences in their visual adaptations to the divergent underwater lighting conditions may arise from variations in opsin gene expression (Carleton & Kocher, 2001). Despite the predominance of the LWS1 class in the sailfin tetra opsin gene expression, I observed differences in the expression level of the *LWS2_2* genes among lineages, with a higher expression in samples from the black water lineage. Notably, the two copies of the LWS2 gene share identical λmax, suggesting that the expression of one gene over the other may not confer significant visual adaptation. In this context, the mechanisms driving gene expression may reflect the individuals' evolutionary history more than a local adaptation to divergent underwater lighting conditions. Even though all individuals were kept under similar conditions in captivity for approximately five months before RNA extraction, the similarity in the expression of the *LWS2_2* gene in most CJ1 population and clear water lineage samples (Figure 4.3D) suggests that their phylogenetic proximity plays a significant role in determining *LWS2_2* gene expression. This relationship supports the idea that genetic proximity likely influences gene expression. However, the consistent high expression levels of the *LWS1_2* gene and the *SWS2* gene among all samples (Figure 4.2D) imply that colour vision, especially red and blue sensitivity, can be influenced by environmental factors. Thus, the interplay between genetic background and environmental lighting conditions probably shapes the light sensitivity of sailfin tetra individuals, determining their colour vision.

Differences in gene expression can be caused by environmental characteristics. Fishes from clear waters usually have a higher expression level of SWS genes in their retinas than those from stained waters, which usually express higher levels of LWS genes (Fuller et al., 2003, 2004). Controlled experiments have shown that this difference among populations was predominantly driven by environmental conditions, rather than genetic background (Fuller et al., 2005; Shand et al., 2008). Such a relationship between the opsin gene expression and environmental conditions has been observed in several fish species (Carleton & Kocher, 2001; Hofmann & Carleton, 2009; Fuller et al., 2003). Indeed, adult fishes can change their gene expression profile due to changing environmental conditions within one month or a few weeks (Luehrmann et al., 2018). The LWS gene expression level in the samples differs from the only record of the sailfin tetra gene expression level in their natural environment (Escobar-Camacho et al., 2020). Fishes from Coropina Creek,

a black water river in the Para district of Suriname, have approximately 1% of *LWS1_1*; 50% of *LWS1_2*; 43% of *LWS1_3* and 2% of *LWS2* and 3% of *SWS2* in contrast with approximately 1% of *LWS1_1*; 61% of *LWS1_2*; 1% of *LWS1_3*, 12% of *LWS2* and 24% of *SWS2* in my black water samples. Also, the similarities in the *SWS2* gene expression level among all samples kept under similar underwater lighting conditions for around five months may suggest that the gene expression level in the sailfin tetra is environmentally driven. In this case, I might be missing substantial differences between populations. It can be expected since populations living in different and changing water colourations may express those genes that would best fit the current environmental light condition (Hofmann & Carleton, 2009; Nandamuri et al., 2017; Shand et al., 2008). Based on this prediction, I could also expect different red light peak sensitivities, driven by the differential expression of LWS genes, within the same population throughout the year (Whitmore & Bowmaker, 1989), which could be driven by different gene expression profiles among the sailfin tetra lineages. This situation could particularly occur between the rainy and dry seasons in Amazonia, which witness an increase and a reduction in the volume of the daily rainfall and DOC transport from the forest into the *igarapé*, respectively.

On the other hand, gene expression profiles may be primarily genetically determined, but due to local adaptation, they exhibit significant variation across native environments (Novales Flamarique et al., 2013; Rennison et al., 2016). Also, changes in the gene expression profile may occur mainly during the early phases of ontogenetic development (Härer et al., 2017). Therefore, because the sailfin tetra individuals sampled for this study were adults, it is possible that phenotypic plasticity could be weak or absent in the study. So, my results would suggest that individual's visual capabilities do not differ as expected between populations inhabiting clear and black waters. Although the most significant changes in gene expression may be typically observed in the young (Härer et al., 2017), it is possible that the adult fish in the study may have regulated their gene expression. Therefore, the observed gene expression profile in the results could have been influenced because the individuals were kept in similar laboratory conditions for approximately 5 months before the RNA sample extraction, and only the analysis of newly sampled individuals can shed light on how individuals may visually adapt to different Amazon water colours.

Fish may also adapt their blue light sensitivities according to the environment. For instance, the threespine stickleback living in black waters has an allele with two amino acid changes responsible for the red-shift in their *SWS2* gene (Marques et al., 2017). This kind of genetic changes and convergent adaptation have appeared in species inhabiting different underwater lighting conditions (Marques et al., 2017; Seehausen et al., 2008; Torres-Dowdall et al., 2017). Interestingly, the five key sites that are expected to drive the changes in λ max in LWS proteins did not differ in my samples, also, I have found no amino acid substitution in the *SWS2* gene in all of my samples from black and clear waters, suggesting the role and flexibility of gene expression may generate adaptive visual adaptation and diversification among the sailfin tetra lineages (Carleton & Kocher, 2001; Torres-Dowdall et al., 2017; Torres-Dowdall et al., 2021).

Previous studies have investigated, through mutagenic experiments, the role of amino acid changes and LWS protein sensitivity (Yokoyama, 2008; Yokoyama et al., 2008). They have proposed that five amino acid changes (S180A, H197Y, Y277F, T285A and A308S) can explain the entire range of λ max (510–560 nm) of M/LWS (middle to long wavelength) pigments. I found no difference in the five key sites for the same gene across the sailfin tetra lineages. However, there is a slight difference between the expected protein sensitivity between Suriname population and samples analysed in the present study. Based on the expected λ max analyses, individuals from Suriname have lost the light peak sensitivity to 555 and 613 nm; however, drivers other than the five key sites may underlie protein sensitivity. For instance, one amino acid change in the position Y203F (following the bovine rhodopsin residue numbering system) between M3 and P alleles among cichlid species is responsible for shifting the protein sensitivity in 9 and 4 nm when the amino acid chain is bounded to A1 and A2, respectively (Terai et al., 2017; Appendix 4.5.5). Additionally, the importance of A2-derived pigments for visual adaptation to underwater lighting environments in freshwater fish has been demonstrated (Escobar-Camacho et al., 2019; Terai et al., 2017). Differences in lighting conditions have been shown to change the ratio of A1 to A2-based visual pigment (Whitmore & Bowmaker, 1989; Terai et al., 2017). For instance, the peacock bass *Cichla monoculus*, a predatory Amazon cichlid, can modulate the A1/A2 ratio in relation to water colour (turbid and clear waters); individuals living in turbid waters had higher proportions of vitamin A2, shifting sensitivities to longer wavelengths, than fish living in clear waters

(Escobar-Camacho et al., 2019). Although not statistically significant, I have found that the black water lineage and CJ1 population individuals showed a tendency to express higher levels of *Cyp27c1* gene, suggesting that they may use more A2 derivate pigments than clear water lineage. The use of a higher proportion of A2 pigments can be an adaptation to red-shifted clack waters (Terai et al., 2017; Escobar-Camacho et al., 2019). However, only the evaluation of the retinal light peak sensitivity through micro spectrophotometry (MSP) can help us understand the actual range of individuals' sensitivities.

Under the framework of natural selection, it is expected that an individual's colour sensitivity would align with the prevalent environmental colour (Endler, 1992; Boughman, 2002). As a result, a correspondence between opsin sensitivity and environmental lighting conditions would be expected. This alignment is thought to play a role in driving the speciation of African cichlids (Seehausen et al., 2008). African lakes are conceivably more ecologically stable compared to Amazon *igarapés* in terms of water colour fluctuations, which could facilitate the evolution of the correlation between opsin gene sensitivity and environmental light. Indeed, the genetic differentiation observed between differently coloured species correlates with the marginal differences in ambient light conditions (Seehausen et al., 2008). Moreover, disruptions in sexual communication among African cichlid species have predominantly arisen from recent human disturbances (Seehausen et al., 1997), underscoring the stability of underwater lighting under natural circumstances. In contrast to the relatively constant conditions of African lakes, Amazon black water *igarapés* experience a degree of unpredictable variability (Appendix 4.5.6). This stochastic environmental variability over short time scales could hinder the development of the alignment between the peak sensitivity of light-sensitive proteins (i.e., amino acid chains) and the prevailing environmental light conditions. Conversely, clear water *igarapés*, attributed to their specific soil characteristics, exhibit greater stability and consistency in colouration (i.e. spectral composition) (Mendonça et al., 2005; Borghezan et al., 2021). Under such circumstances, the expression of different opsin genes that would best fit the transitory underwater lighting condition would be favoured. This in turn, may favour the potential phenotypic plasticity as a mechanism of visual adaptation in itself, especially among species that have several copies of the same gene, as the case of the sailfin tetra. However, future investigations may test what I have

suggested here.

Even though clear and black waters *igarapés* differ in terms of environmental stability, the estimated λmax based on the five key sites (Yokoyama, 2008; Yokoyama et al., 2008) of the opsin sequences among the sailfin tetra lineages inhabiting these two divergent environments are not expected to differ. This can be expected when there is a gene flow among populations. However, the hypothesis proposing significant gene flow among the examined populations appears improbable for two primary reasons. Firstly, these populations inhabit separate drainage systems, with a minimum spatial separation of 360 km in a straight line. Secondly, the sailfin tetra does not engage in migration or egg/larval dispersal (Pires et al., 2016), factors that would constrain gene flow among populations, a conclusion corroborated by genetic analyses (Pires et al., 2018). Therefore, the visual adaptations in the sailfin tetra lineages inhabiting different water types may arise from factors other than the fine-tune between the opsin amino acid sequence and the underwater light condition. An adaptive strategy to uphold optimal sensitivity in such an unstable environment might involve the utilization of multiple genes that exhibit varying expression patterns contingent on transient environmental conditions, even if none of the alleles perfectly adapts to the local conditions throughout the whole year. Moreover, distinct black water *igarapés* demonstrate varying levels of DOC and red bias. These dissimilarities in redness among black water *igarapés* could conceivably lead to divergent levels of gene expression within populations of the sailfin tetra black water lineage. Ultimately, these genes may be associated with A1 or A2 opsins in distinct manners, thereby optimizing the array of potential sensitivities individuals could employ in response to prevailing environmental conditions. Again, future studies can address these interesting issues raised here.

The colour vision has a direct impact on animal behaviours. Guppies (*Poecilia reticulata*) that grew under orange light showed a higher expression of LWS genes and a higher sensitivity to 600 nm light than those individuals grown under green light. Such differences in colour sensitivities were also related to the behavioural sensitivities to long wavelengths (Sakai et al., 2016). For the sailfin tetra, a previous study investigating female environmental colour preference, showed that females from black and clear water lineages, including CJ1 population, prefer red over full spectrum illuminated environments (Chapter 3). Such red colour preference in the sailfin tetra may be

associated to a high LWS gene expression, which encompasses most of the opsin gene expression in fish retina as observed in the present and previous studies (Escobar-Camacho et al., 2020). Therefore, the differential gene expression level under natural environmental conditions may play an important role in food acquisition and predator avoidance, since their colours transmission is also modulated by the water column colour spectrum, thus, plastic changes in sensitivity to background light environments might contribute to survival in variable light environments (Sakai et al., 2016).

Previous studies have stressed the role of water chemistry, especially the differences in relation to pH and conductivity, as an important source of divergent selection in driving biodiversity across Amazon water types (Cooke et al., 2012a, 2014; Beheregaray et al., 2015; Pires et al., 2018). Recently, I have highlighted the role of the Amazon waters colouration in driving local diversity. The ornament colouration pattern, red colour intensity in the dorsal fin and eye size differ between lineages of the sailfin tetra and were suggested to be shaped by water colours (Pires et al., 2019). Additionally, Amazon water colouration affects the perceived variation of the red colour in male ornaments and is suggested to have driven the female mating preference for this colouration and size depending on the underwater lighting condition of the habitat sailfin tetra individuals live in (Borghezan et al., 2023). Here, I suggest that Amazon waters can also mediate the evolution of colour vision in the sailfin tetra. Overall, Amazon water types may have driven the evolution of the reproductive isolation among the lineages of the sailfin tetra (Pires et al., 2018), and have contributed to fish biodiversity with both their chemical and physical conditions (Borghezan et al., 2021). For instance, the difference in the expression level of the *LWS1_3*, which is expected to confer 555 and 613nm wavelength sensitivity, may affect the ability to detect food resources, potential mating patterns and predators. However, the potential implications of such differential red colour sensitivity and its effects on feeding, social and sexual behaviours remain unclear, since reflectance values of food resources, mating patterns, potential predators and their interaction with the different underwater lighting conditions (clear and black waters) are still unknown. Therefore, future studies using newly sampled individuals from the natural environment (or specifically designed experimental studies) to assess gene expression levels and light peak sensitivity through MSP can test what I have suggested here. Additionally, studies correlating the red light peak sensitivity to the ornaments' red

colouration may give us a better understanding of how individuals may socially and sexually communicate and if the expression of certain genes may make lineages better adapted to their underwater lighting conditions and mediate such interactions. Finally, studies modelling the underwater lighting condition in captivity may also help us better understand how individuals can adjust their gene expression level and the differential usage of A1 and A2 in response to fast-changing environmental conditions (Shand et al., 2008).

Amazon *igarapés*, unlike the main river channels that show a predictable seasonality (Bittencourt & Amadio, 2007), are unpredictable environments in the short run, changing according to daily rainfall (Espírito-Santo et al., 2017). Such unpredictability can impair the evolution of opsin amino acid sequences to a long-lasting match of specific underwater lighting conditions. Therefore, here I suggest that in highly variable environments and in species yielding multiple copies of the same gene, visual adaptations may occur mostly in the gene expression profile and/or in the ratio of A1 to A2-based visual pigments rather than in the fine-tune between environmental light and protein light sensitivity.

4.5 Appendix

Appendix 4.5.1

The chronological age of the sailfin tetra lineages remains unknown. Nevertheless, both mitochondrial markers (Figure A4.1.1), known for their comparatively accelerated mutation rate, and nuclear markers, which exhibit greater stability and a slower mutation rate, demonstrate a distinct molecular signal and lineage differentiation. These genetic patterns align with the native water types in which the lineages live in (Pires et al., 2018). The CJ1 population exhibits both genetic and morphological characteristics consistent with the clear water lineage (Pires et al., 2018; Pires et al., 2019). However, it currently resides in a black water *igarapé*, indicating a relatively recent colonization (in geological terms) of this black water system.

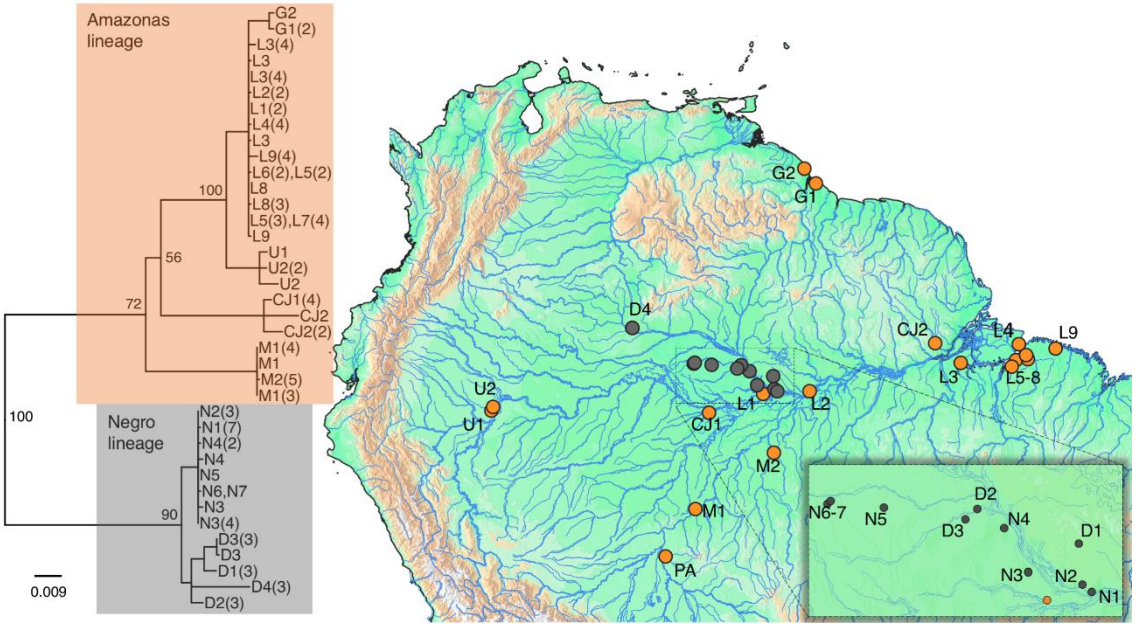

Figure A4.1.1. Maximum-likelihood phylogeny of the sailfin tetra *Crenuchus spilurus* and a map of northern South America with sampling points taken from Pires et al. (2018). Dots and codes represent sampling sites colour-coded to represent the two main lineages defined *a posteriori* (orange: Amazonas (clear water) lineage; grey: Rio Negro (black water) lineage). Please note the position of the N1, U1 and CJ1 populations, which are the sampled locations for the black and clear water lineages for the study.

Appendix 4.5.2

Table S4.2.1. Details of the sailfin tetra genome assembly, with the platform used, library, and the amount of the base pair sequenced.

Sequences used for assembling the genome

Platform	library	sequencing amount
Illumina Novaseq6000	gDNA 150bpx2PE, 350bp insert	54.1G [bp]
	gDNA 150bpx2PE, 550bp insert	54.0G [bp]
	retinaRNA 100bpx2PE	5.0G [bp]
Statistics of the genome assembly		
Total length		1,135,247,105 [bp]
No. of scaffolds		94312
Scaffold N50		27744
Contig N50		12968
GC content		38.9 [%]

Appendix 4.5.3

I conducted a phylogenetic with the LWS and SWS2 genes found in one of my black water lineage samples with those previously found in the sailfin tetra by Escobar-Camacho et al. (2020). I also added the *Hoplias microlepis* and *Serrasalmus rhombeus* LWS opsin genes in this analysis. I rooted the phylogenetic tree in the SWS2 genes for easier visualization. Phylogenetic relationships were inferred by using the Maximum Likelihood method based on the Tamura-Nei model.

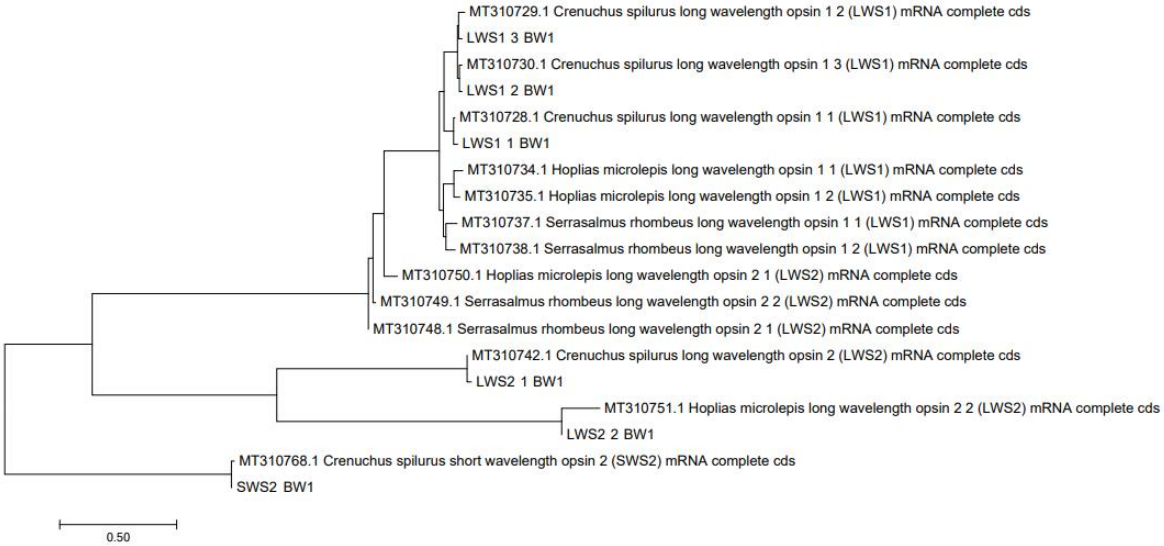

Figure A4.3.1. Phylogenetic relationship between the LWS and SWS2 opsin genes found in one of my black water lineage samples and those found by Escobar-Camacho et al. (2020).

Appendix 4.5.4

I estimated how the differences in the gene expression level translated into differences in spectral sensitivity. I calculated a spectral sensitivity curve (400-700 nm) for each lineage based on its average relative expression of the LWS opsin genes and using the absorbance templates from Govardovskii et al. (2000). I estimated the spectral sensitivity of the sailfin tetra lineages using three different ratios representing the extremes: 100% A1; 100% A2; and 50% A1 and 50% A2.

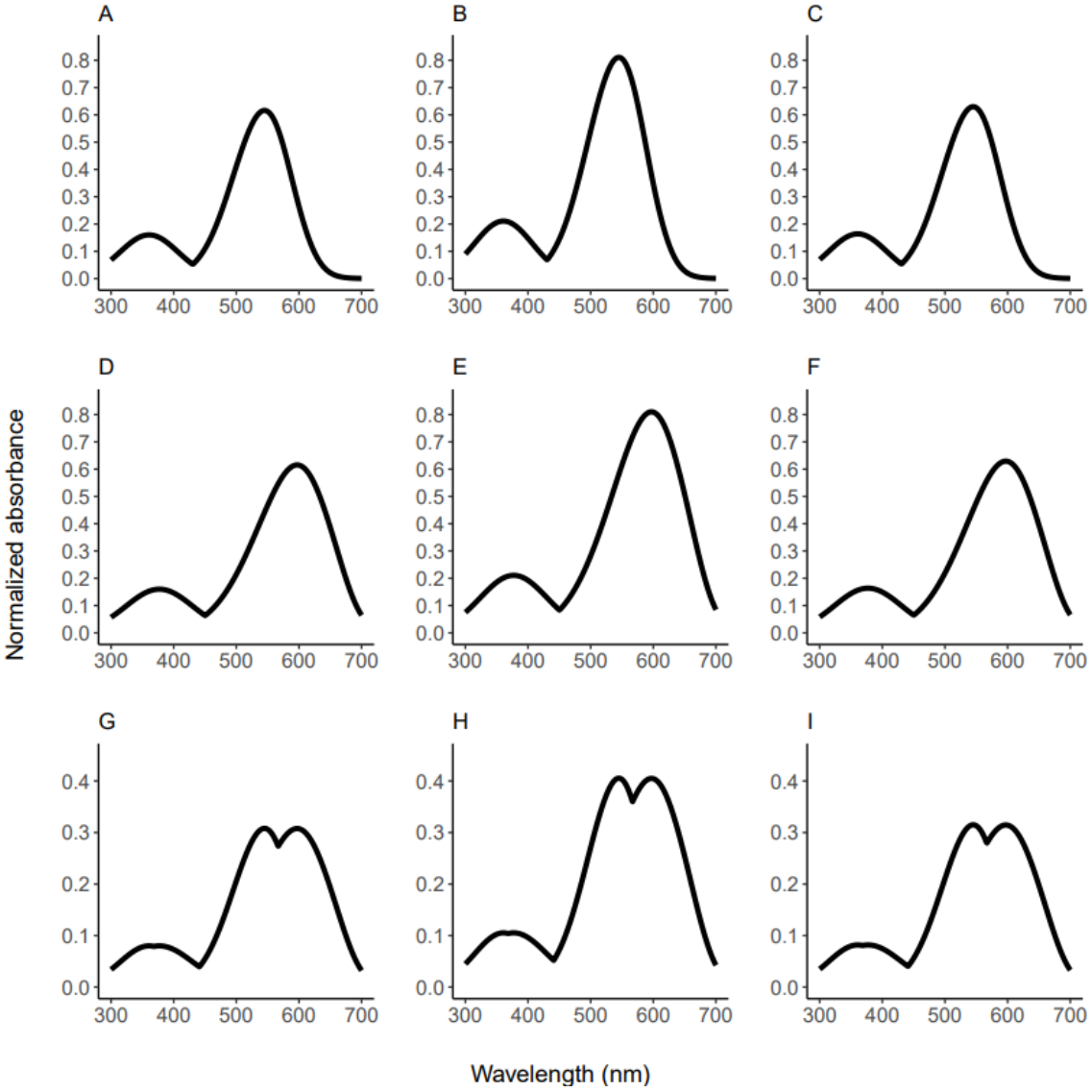

Figure A4.4.1. Expected normalized sensitivity of the sailfin tetra lineages. The first row shows the expected sensitivity of **(A)** black water lineage, **(B)** clear water lineage and **(C)** CJ1 population when the amino acid is bound only to A1. The second row shows the expected sensitivity of **(D)** black water lineage, **(E)** clear water lineage and **(F)** CJ1

population when the amino acid is bound only to A2. And finally, the third row shows the expected sensitivity of **(G)** black water lineage, **(H)** clear water lineage and **(I)** CJ1 population when the amino acid is bound to a 50% A1:A2 ratio. Please note the differences on the Y-axis scale.

Appendix 4.5.5

I performed a second comparison of the amino acid chain found among my samples to other amino acid changes that have been previously shown to change light peak sensitivity (Terai et al., 2017). Three alleles found in African cichlids had their sensitivity determined through MSP and the amino acid sequences found in my samples were compared to those previously described (Terai et al., 2017). Each allele is composed of 13 amino acids that are important in driving the light peak sensitivity. The amino acid positions 62, 131, 137, 168, 177, 179, 216, 222, 226, 227, 230, 275, and 282 in the LWS1 genes samples correspond to positions 49, 118, 124, 155, 164, 166, 203, 209, 213, 214, 217, 262, and 269 in bovine RH1. The amino acid positions 59, 128, 134, 165, 174, 176, 213, 219, 223, 224, 227, 272 and 279 in the LWS2 genes samples correspond to positions 49, 118, 124, 155, 164, 166, 203, 209, 213, 214, 217, 262, and 269 in bovine RH1. Amino acid positions 49, 118, 124, 155, 164, 166, 203, 209, 213, 214, 217, 262, and 269 in bovine RH1 correspond to those 60, 129, 135, 166, 175, 177, 214, 220, 224, 225, 228, 273 and 280 in the LWS2_2 genes.

This amino acid comparison of all LWS genes suggests that the λmax differs among all groups. When looking at the 13 amino acid positions, I found five different alleles for LWS1_1, two alleles for LWS1_2, two alleles for LWS1_3, one allele for LWS2_1 and one allele for LWS2_2. From those, one allele of LWS1_1 was found only in BW, one allele of LWS1_1 was found only in CW, and three alleles of LWS1_1 were found only in CJ1, and one allele of LWS1_2 was found only in BW (Table S4.5.1). All these alleles have not been previously described, therefore, I cannot estimate their protein light sensitivity.

Table S4.5.1. List of some of the LWS alleles found by Terai et al. (2017) and those found in my samples considering the 13 amino acid positions described by Terai et al. (2017). All amino acid positions in the table below are following the bovine rhodopsin residue numbering system. Positions 164 and 269 are two of the five key sites described by Yokoyama et al. (2008). ND = not determined.

Gene	Sample	Allele	49	118	124	155	164	166	203	209	213	214	217	262	269	A1 (nm)	A2 (nm)
LWS	Terai *et al.*, 2017	M3	V	S	A	A	A	V	Y	L	I	F	T	I	T	553	601
LWS	Terai *et al.*, 2017	P	V	S	A	A	A	V	F	L	I	F	T	I	T	544	597
LWS	Terai *et al.*, 2017	Sp	I	S	A	A	A	V	F	L	I	F	T	I	T	549	595
LWS	Terai *et al.*, 2017	D	V	S	A	A	A	V	Y	L	I	F	T	I	A	537	568
	BW	1	I	G	G	S	S	F	Y	I	I	I	A	I	T	ND	ND
LWS1_1	CW	2	A	A	G	S	S	F	Y	I	I	I	A	I	T	ND	ND
LWS1_1	CJ1	3	V	A	G	S	S	F	Y	I	I	M	A	I	T	ND	ND
LWS1_1	CJ1	4	V	A	G	S	S	F	Y	I	I	I	A	I	T	ND	ND
LWS1_1	CJ1	5	V	A	G	A	S	F	Y	I	I	I	A	I	T	ND	ND
LWS1_2	BW	6	V	A	G	A	A	F	Y	L	I	T	S	C	T	ND	ND
LWS1_2	CW, CJ1	7	V	A	G	A	A	F	Y	I	I	M	S	C	T	ND	ND

LWS1_3	BW, CJ1	8	V	A	G	A	A	F	F	I	I	I	S	I	T	ND	ND
LWS1_3	BW, CW, CJ1	9	V	A	G	A	A	F	F	I	I	I	S	C	T	ND	ND
LWS2_1	BW, CW, CJ1	10	I	S	A	A	A	I	Y	L	A	L	F	V	A	ND	ND
LWS2_2	BW, CW, CJ1	11	V	S	G	A	A	I	Y	L	I	L	F	V	A	ND	ND

Appendix 4.5.6

I measured the variation in the underwater lighting condition in black and clear water *igarapés*. The measurements were performed in two clear (Tinga and Ipiranga) and two black (Acara and Bolívia) water *igarapés* in Ducke Reserve (Manaus-Brazil). All measurements were performed using HOBO data loggers model MX2202. Two data loggers were attached to a PVC structure in each site, one positioned at 40 cm of water depth and the second one at 10 cm above the water surface. Both data loggers were positioned parallel to the water surface, following manufacturer instructions. The data loggers were set to take a measurement every day at 1200 hours. The underwater lighting condition was measured for a total of 18 days (from June 24th to July 11th 2022), except for one site (Acara *igarapé* – black water) that was measured for 13 days due to technical issues with the equipment. Field measurements were approved by the DISER-INPA under the process SEI nº 7903525.

Dissolved organic carbon (DOC) absorbs short wavelengths (i.e. blue colours), therefore, black waters are biased towards long wavelengths (i.e. red colours) due to the presence of the DOC. A higher amount of DOC absorbs a higher proportion of the short wavelengths, resulting in redder waters. Therefore, I involved the data logger sensor in a blue light filter to evaluate the variation in the redness of the black and clear waters. The blue light filter transmission has its peak at 446 nm (Fig. S4.6.1). Higher lux values of the blue light detected by the data logger are equal to a higher transmittance of blue and consequently less red environment. Conversely, lower values of the blue light detected suggest a higher blue light absorption by the water column, which is expected by the result of redder waters. However, the light intensity detected by the data loggers also depends on the environmental light, which varies according to the weather (e.g. cloudy, rainy, sunny), time (e.g. morning and afternoon) and the environmental condition (e.g. open area, shaded). Therefore, the among-site lighting condition needs to be interpreted with caution.

I divided the light intensity (in lux) detect underwater by the light intensity detected above the water (in lux). The result of such measurement returns the proportion of the blue light from above the water that reaches 40 cm of water depth. I fitted a set of linear models to evaluate the relationship between the transmission of the blue light

(dependent variable) along the days (independent variable).

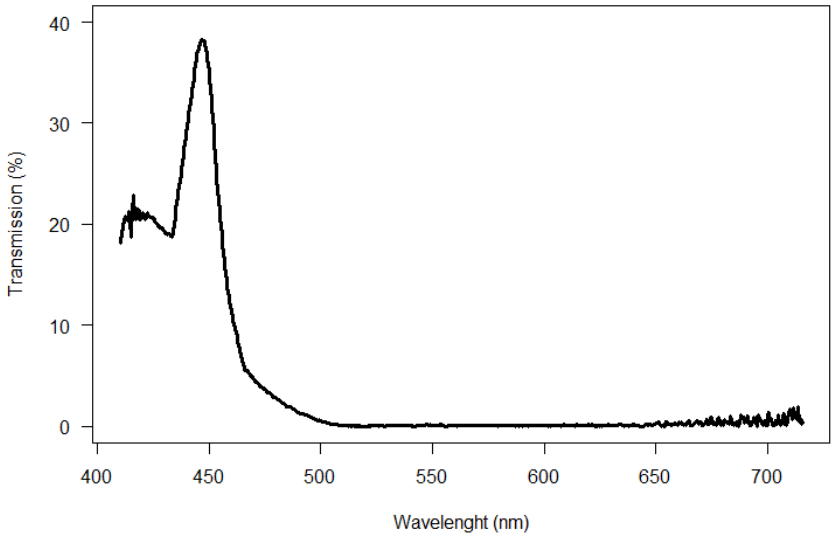

Figure A4.6.1. Transmittance of the blue light filter used to measure the variation in the underwater lighting condition in black and clear water *igarapés*.

We found a significant increase in the transmission of the underwater blue light along the days in Bolivia (black water) *igarapé* (linear model: $F_{1,16}$ = 7.43, Slope = 0.00196, Intercept = 0.05599, Adjusted R-squared: 0.2744, P = 0.0149) at 1200 hours (Fig. S4.6.2). I did not find any significant relationship with the other *igarapés*.

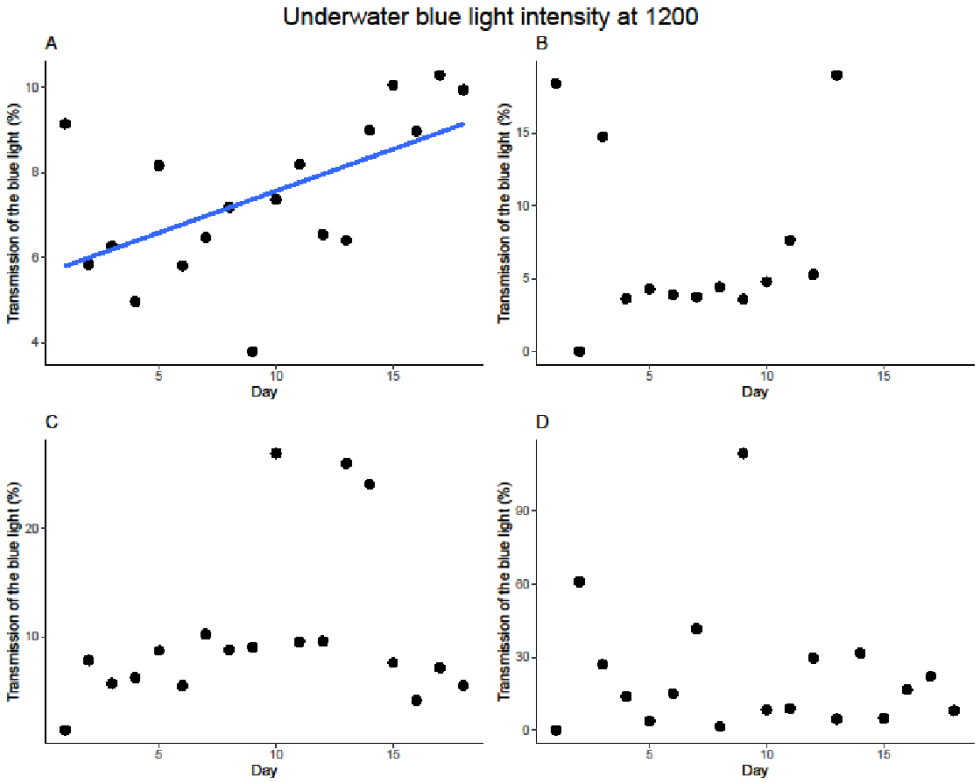

Figure A4.6.2. Proportion of the blue light reaching 40 cm of water depth at 1200 hours. **(A)** Bolívia *igarapé*. **(B)** Acará *igarapé*. **(C)** Ipiranga *igarapé*. **(D)** Tinga *igarapé*. The blue line represents the significant relationship between the increase in the proportion of underwater blue light over the days. Please note the differences in the Y-axis scale among sampling sites.

The data from the black water *igarapés* suggested the presence of a high underwater light colour variation, especially in Bolívia *igarapé*, which showed a significant increment of the blue light intensity over the days. This increment of the blue light transmission in this *igarapé* could be driven by the decrease of the rain volume, which brings less DOC into the water (and decreases the blue light absorption). Even though this tendency was not found for the Acara *igarapé*, I believe the values detected by the data logger's sensor are in agreement with the expected values for the underwater lighting condition in black water systems.

Under clear waters, I expect a higher proportion of blue light reaching the bottom of the *igarapé*. However, my data suggests that the blue light can be highly reduced by environmental conditions, such as shaded/opened areas, and soil and/or organic particles

in the water column. Such effect can be observed by the fluctuation in the transmission of blue light along the days in clear water *igarapés*, especially in Tinga *igarapé*. Interestingly, the blue light intensity has never reached high values in black water *igarapés*. It suggests that the black water highly constrains the blue light transmission; therefore, its variation seems lower in black water systems than in clear water systems.

The low values observed among clear water *igarapés* can be expected by several factors. Water refraction and the reflection from the water surface can reduce the light intensity detected by the data loggers' sensor set underwater. Additionally, the shaded area caused by the forest canopy reduces the intensity of the light on the water surface and also in the underwater environment. Such effects could affect both black and clear water *igarapés*. However, I found some semi- and mostly decomposed organic material, such as leaves and trunks, as well as soil particles, such as sand and clay, mostly on the underwater sensors set in the clear water *igarapés*. Such material could be transported by the water, and deposited on the data loggers' sensor, affecting the light intensity detected in the underwater environment, but not on the water surface. The result of such conditions reduces the blue light intensity detected by the underwater data loggers, highly decreasing the proportion of the detected blue light that reaches the bottom of the *igarapé*, as suggested by the low proportion of the blue light in my results. Such effects could not be controlled in the field measurements and potentially affected the light intensity. However, it does not necessarily mean that the transmission of the blue light was similar to those detected in black water *igarapés*. Therefore, future investigations may account for these technical issues and deliver more accurate data from the underwater colour variation among clear water *igarapés*.

Finally, the classification of "black waters" considers the waters that show a significant level of DOC (which biases light transmission), without distinguishing among the different levels of the DOC in the water column. Indeed, different black water *igarapés* show different amounts of DOC and different red bias levels (Figure A4.6.3). However, the difference between black and clear waters is clear for most of the *igarapés*.

Figure A4.6.3. Bolívia (black water), Acara (black water) and Tinga (clear water) *igarapés* water samples, from the left to the right, respectively. Note the difference in the water colouration among samples.

Chapter 5

General discussion

5.1 Amazon waters

I reviewed the physicochemical characteristics of the three Amazon water types. Black waters are rich in dissolved organic carbon (DOC), which decreases the pH and biases the underwater lighting condition towards long wavelengths (red colours), and are poor in most of the minerals. Clear waters are mostly transparent with no apparent light biases and are rich in carbonates and others minerals that buffer the water acidity. Finally, the white waters are rich in minerals and other particles that decrease the light transmission and increase the pH. I also reviewed the four main fish sensory modalities, mechanoreception, electroreception, chemoreception, and photoreception, and how these divergent water conditions could potentially drive individuals' sensory systems apart. For instance, visual signals are affected by aquatic features. Black waters are red biased environments and short wavelengths, such as violet and blue, are filtered out. Additionally, long wavelengths, such as orange and red, are perceived more brightly and intensely (Endler, 1992). On the other hand, clear waters do not bias the underwater lighting condition and white waters are overloaded with suspended sediments that constrain light transmission. Such contrasting underwater lighting conditions can drive divergence in the evolution of the visual system, as observed in some Amazon fish (e.g Escobar-Camacho et al., 2020). Similarly, all three Amazon water types have contrasting characteristics that also can drive the divergent evolution of the mechano-, electro-, and chemosensory systems of the aquatic biota. Because natural selection shapes the sensorial systems according to the environment, it can also lead to changes in sexually selected traits (Endler, 1991; Fuller & Noa, 2010). Therefore, the coupled evolution of sensory systems and sexual signals can promote diversification and possibly lead to speciation (Boughman, 2002), as it has been suggested for several taxa (Leal & Fleishman, 2002; Marchetti, 1993; Seehausen et al., 2008; Servedio & Boughman, 2017).

5.2 Effects of water colour on male ornaments and female mate choice

To actually investigate the role of the divergent water colour in the evolution of fish colouration, female mate choice and colour vision, I used the sailfin tetra *Crenuchus spilurus* as a model species. I have been using this fish species to investigate how divergent ecological pressures in Amazon waters may drive populations apart since my under graduation in Brazil (Pires et al., 2018; Pires et al., 2019; Pinto et al., 2020). The sailfin tetra is a sexually dimorphic species in which males possess exponentially large dorsal and anal fins conspicuously ornamented in red and yellow colours. These ornaments are used during an elaborated courtship behaviour that can last for several days (Pires et al., 2016). This species is composed of two phylogenetically distinct lineages, one inhabiting black waters and the second one inhabiting mostly clear waters *igarapés* (Pires et al., 2018). *Igarapés* are small forest streams that are extremely unstable aquatic environments. The daily rainfall can reach up to 130 mm, and *igarapés* water volume can change unpredictably (Espírito-Santo et al., 2017). Such daily rainfall brings organic matter and soil particles into the *igarapés*, changing their turbidity and colouration. However, even though the environment is highly changeable, characteristics that define black and clear water types still remain.

Because these lineages of the sailfin tetra inhabit divergent environmental conditions, I expected them to differ in visual traits and in how colours are used during the mating assessment. Interestingly, some populations, such as CJ1, genetically belong to the Clear water lineage, but nowadays live in the black water conditions. This gave us an opportunity to investigate a possible convergent evolution to the black water condition.

I measured the effects of the divergent underwater lighting condition (clear and black waters) on males' ornaments colouration, and female mate choice for both sailfin tetra lineages, including the CJ1 population. As expected, the red biased light increased the redness of the red parts in the dorsal and anal fins; however, the among-individual variation in the red colour decreases under red-biased light for all lineages. The small among-individual variation in males' ornaments colours can increase the difficulty of distinguishing different intensities of the red colour, decrease decreasing the reliability of ornaments colouration as a mating signal. Therefore, selecting mates primarily based on a trait with intense signal but with small among-individual variation can lead females to choose those males that show less coloured ornaments but that appear like red intense

ornamented ones. As a consequence of the small variation in the males' ornaments colouration, females mate acceptance for those individuals living under black waters was not directly affected by the light condition. On the other hand, clear water females mate acceptance was directly affected by the red-biased underwater lighting condition, highlighting the importance of red colours in males' ornaments in triggering female mating behaviour. I suggested that the lower variance in the red parts of the males' ornaments signaling posed by the black water lighting environment can represent a high cost to black water lineage females in assessing male mating quality based on ornaments colouration. Therefore, females from black waters may rely heavily on male ornaments size than ornaments colouration to choose males of best quality, whereas females from clear waters can still use male ornaments colouration as a reliable indicator of mate quality. Interestingly, CJ1 population ornaments colour did not differ from the Clear water lineage, but the female mate choice was similar to the Black water lineage. Such results suggest that the evolution of behavioural traits may be faster than morphological ones. Also, the behavioural adaptations to the different water colours seems not to be genetically determined, but instead, driven by the environmental condition. Additionally, females inhabiting black waters showed a similar pattern of mate choice, suggesting a convergent evolution to Amazon black waters. As far as I know, these are the first report demonstrating that Amazon water colours have shifted the signals used by females during mating assessment in close related lineages, and the convergent evolution in female mate choice to black waters (Chapter 2). Although the importance of the male body size and ornament size in the female mating acceptance of the Black water lineage were already reported (Borghezan et al., 2019; Pinto et al., 2021), this is the first report that suggests the importance of ornament size for the female mate choice in Clear water lineage and CJ1 population.

5.3 Red colour preference

Colour preference and sensitivity have an important impact on individuals' fitness. It may be even more important to sexually dimorphic and colourful species, in which colour plays an important role in social and sexual communication (Endler, 1993). So, because the sailfin tetra lives in divergent underwater lighting conditions (Pires et al.,

2016), I also investigate the female preference for red colour through environmental colour choice among the sailfin tetra lineages. I found that females from all lineages prefer red enlightened environments. Interestingly, the red colour preference was stronger for those females living in clear water than for those living in black waters. The red colour preference can be related to several processes. The high red colour sensitivity in the sailfin tetra, with LWS genes being dominant (Chapter 4) and accounting for more than 90% of the gene expression profile in the eyes of the wild caught sailfin tetra individuals (Escobar-Camacho et al., 2020) may explain the red colour preference found in the Chapter 3. Also, the sailfin tetra feeds on several red-coloured items, such as Chironomidae larvae and buriti palm tree fruits (Pires et al., 2016). Therefore, the red colour preference may also be explained by the foraging activity. Another interesting point is that the red colour may be qualitatively darker than the full-spectrum enlightened areas. Therefore, the red colour may potentially turn individuals less conspicuous than if they were under full spectrum light to potential predators, conferring a higher survival rate. Finally, the red colour, present in males' ornaments, plays an important role in sexual and social behaviours (Pires et al., 2016; Chapter 2). However, the red colour preference was stronger for the individuals living in clear waters than for those living in black waters (Chapter 2). I have previously found that females that live in black water conditions do not rely primarily on the colouration of the males' ornaments, but instead, prefer males with larger ornaments as mating partners. On the other hand, females living in clear water environments prefer those males with redder ornaments colouration as well as those males with larger ornaments. Such stronger preference for red colour in clear waters individuals found here may relate to the colour preference in mating behaviour. Even though I have not studied the direct mechanisms that drive female red colour preference, here I showed that they prefer red over full-spectrum enlightened environments. I suggest that the relevance of colours and their preference can vary according to the context and are shaped by environmental conditions.

5.4 Genetic study

I also investigated the genetic divergence and light peak sensitivity of the LWS (Long Wavelength Sensitivity) proteins (responsible for sensing red colours) among the

sailfin tetra lineages. Differently to most of the investigated species, as the case of the cichlid fish in African lakes that show only one copy of the LWS gene, the sailfin tetra showed several copies of the LWS gene. The genetic divergence among those individuals living in clear and black waters was relatively low. The light peak sensitivity differed among the LWS gene copies, but not among lineages (Chapter 4). I proposed that the highly variant environmental condition of the Amazon *igarapés* may constrain the evolution of the fine-tune between the light peak sensitive protein and the prevailing environmental light. I also suggest that under such highly variable environmental conditions, as the Amazon *igarapés*, visual adaptation may occur in the expression level of each gene and/or in the chromophore usage (A1 or A2) that can allow individuals to sense different light peaks according to the most transient environmental light.

The fine tune between the environmental condition and sensory systems, as observed in the African lakes, and the reliability of mating signals are dependent on the aquatic environmental stability. Indeed, the small differences in underwater light condition due to the lake depth constrains the genetic flow between different coloured species (Seehausen et al., 2008). However, differently to the African lakes, Amazon *igarapés* are highly unpredictable environments, with possible daily changes. Such instability of the environmental light condition may constrain the adaptation of visual system light proteins to a particular light peak sensitivity. Additionally, the instability of the environment may also affect the transmission of the signals, as colours. Therefore, female mate choice based on the ornaments colouration for those individuals living under black waters may be constrained. Here I showed that under highly unstable environments, the evolution of the fine-tune between environmental light conditions and individual light sensitivity, and males' ornaments colouration and female mating choice can be impaired by the environmental condition. Future studies using other species from Amazon *igarapés* can add more knowledge to what I have tested here.

5.5 New perspectives

Inittially, I was interested in seeing how the divergent water type could lead populations apart. As stated in the first Chapter, Amazon waters can be classified into three types. However, this classification does not take in account how the physical and

chemical components of the waters can change in a dialy and/or season basis; and more importantly, the effects of such changes in the individuals' traits. Per instance, I proposed that the instability of the black water colour, that can vary based on the daily rain fall, decreases the reliability of male ornaments red colouration as a mating signal. Such effect may have lead female to shift their criterias for correctly assess males of better quality in black waters (Chapter 2). I also proposed that the visual adaptations in the sailfin tetra may arise from the differential opsin gene expression. Such mechanism of visual adaptation can be more important to those species that possess several copies of the same gene and live in unstable environments, as the case of the sailfin tetra living in the Amazon black water *igarapés*. The role of the differential gene expression level as the main source of visual adaptation may be stressed by the negative natural selection (high dS/dN values suggest that the opsin genes in the sailfin tetra are under negative selection, where synonymous changes are favoured) on the LWS opsin genes (Chapter 4).

Therefore, my study brings a new perspective and drivers of the evolutionary mechanisms, the instability of the environmental conditions. However, it is important to highlight that, fortunately, the sailfin tetra had the best biological traits for finding such driver of the evolutionary. Per instance, the courtship behaviour of the sailfin tetra last for several consective days (Pires et al., 2016). Such long lasting courtship behaviour activity is thought to be responsible for the final egg maturation in females' tummy (Pires et al., 2021). Therefore, the underwater lighting condition may change along the day of the long lasting courtship activity, which may decrease the importance of the red colours for females living in black waters. Also, the sailfin tetra possesses five copies of the LWS genes, three of them that are red colour sensitive and differed in their expected maximum absorbance, but with no difference among lineages. Therefore, it is unclear how the instability of the environment may affect the mating behaviour and visual adaptations for those species that possess a single copy of each opsin gene class, such as chiclids. Similarly, it is unclear how the instable underwater lighting condition may affect the female mating preference in those species in which the courtship behaviour lasts for a couple of hours.

5.6 Future directions of research

Here I have shown that the relevance of the red colours differs between lineages for female mate choice (Chapter 2). However, the importance of other ornaments features, like the number and size of the egg spots in anal fin, is still unknown. Future studies may address such interesting research point and more importantly, how the differences in the number and size of the egg spots in the anal fin may affect female mate choice and contribute to the reproductive isolation between lineages. Per instance, the egg spots on male fins is thought to mimic eggs and drive female mating behaviour, as it is suggested among several cichlid species and gobies. The presence of eggs drive female spawning behaviour because especially in fish, males undertake exclusive parental care. Several hypotheses have been put forward to explain this behaviour, such as reduced risk of predation or cannibalism (the dilution effect), in which male may eat some eggs to gain energy as the parental care id energetic costly; increased parental investment, in which males with more eggs usually invest more energy in parental care when they have more eggs in their nests; and finally, mate copying, in which females may save energy evaluating tge male quality, which has been evaluated previously by another female. These are interesting hypothesis to test and investigate drives of female mating behviour in the sailfin tetra.

The evolution of male ornaments is often related to the intersexual selection (i.e females selecting the most attractive male) (Andersson, 1994). However, male ornaments can be also influence male-male interactions (i.e intersexual selection) (Berglund et al., 1996). Because the sailfin tetra males' ornaments are also used during male-male interactions, their colouration may also mediate male-male behaviour and hierarchical structure. However, it is still unknown the role of the sailfin tetra male ornament colouration in male-male interactions, as well as if the importance of male ornaments colourations differs among lineages, as I suggested for female mate choice (Chapter 2). Future studies can address such interesting questions and help us better understand how Amazon water colouration can drive the evolution of coloured ornaments and their importance to social and sexual behaviour.

I found that females of the black and clear water lineages, including the CJ1 population, prefer red colours. However, it is unclear which mechanisms may have driven such colour preference. Per instance, the sailfin tetra feeds on several red-coloured items;

however, the food colour preference is still unknown for the sailfin tetra.

The visual adaptations in the sailfin is suggested to be mediated by the differential gene expression mechanism. Such mechanisms may be particularly important in those highly imstable environments, such as the Amazon *igarapés*. However, it is still unknown the fish spectral sensitivity in their different coloured water types, and future studies using newly sampled individuals may help us understand their colour vision in their environments. Also, the CJ1 population still brings another interesting opportunity to investigate how individuals may visually adapt to the different water colours. Per instance, the gene expression level differed among the four samples of the CJ1 population, with two of them showing higher expression levels of the SWS2 gene than the other two, despite of being under similar captivity conditions. Such difference suggests that other genetic drivers can act on and regulate the gene expression. Per instance, the environmental conditions the youngs experience may drive their gene expression profile throughtout their lives, an effect known as epigenetic programming. Such effect as been suggested to drive the gene expression level among rats that had different maternal care (Weaver et al., 2004). Because the colour of the black water differs according to the rainfall; some individuals may experience darker or clearer underwater lighting conditions in the black water *igarapés*. Such difference in the water colouration in the black water system may have a profound effect on the basis and regulators of the gene expression profile that may be carried out throught the individuals' lives. If so, some indivudlas may be prone to to express a different set of genes than others. Therefore, future investigations may address such interesting mechanism of the visual adaptations among those individuals that live in black water systems.

Finally, here I have shown that the environment drives the evolution of senses (visual adaptions) and female mating preference. Also, because populations exposed to divergent *igarapés* water types differ morphologically (Pires et al., 2019; Chapter 2), behaviourally (Chapter 2 and 3), genetically (Pires et al., 2018), and potentially in their sensory systems (Chapters 1 and 4), different coloured *igarapés* can bear a huge hidden diversity. However, such diversity is not considered during the management of areas and species or conservation efforts. Thus, I also highlight the importance of understanding the mechanisms of diversification in the Amazon for better conservation efforts of the Amazon biodiversity, especially among different coloured *igarapés* and rivers. The

sailfin tetra is an ornamental fish species that is traded in several countries, including Japan. Individuals living in clear and black water types are considered the same species and have been traded indiscriminately. Similarly, other species as the case of the firehead tetra *Hemigrammus bleheri*; spotted tetra *Copella nattereri*; golden pencilfish *Nannostomus beckfordi*; marbled hatchetfish *Carnegiella strigata* and many others are captured in clear and black *igarapés* and are traded as a single species. However, based on my results, all those species may be more diverse than expected. Per instance, the distribution of different lineages/species of the discus cichlid (genus *Symphysodon*) is determined by differences in pH and conductivity of the water (Amado et al., 2011). Similarly, the distribution of the sailfin tetra lineages is modulated by differences in water types (Pires et al., 2018). Therefore, I encourage future investigations in Amazon *igarapés* biota to a better understanding of how the biota evolves and the drivers of such huge biodiversity. Finally, understanding the evolutionary mechanisms in Amazon fish fauna can help us to manage the actual biodiversity and propose optimal conservations efforts.

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