FLÁVIA BADUY VAZ DA SILVA

TOLERÂNCIA AMBIENTAL E COMPORTAMENTO SOCIAL E REPRODUTIVO DE *Australoheros facetus* (CICHLIDAE): QUE MECANISMOS FISIOLÓGICOS E MOLECULARES PERMITEM A ADAPTAÇÃO DESTA ESPÉCIE INVASORA EM PORTUGAL?

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Doutoramento em Ciências Biológicas
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UNIVERSIDADE DO ALGARVE

Faculdade de Ciências e Tecnologia

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2017

Tolerância ambiental e comportamento social e reprodutivo de *Australoheros facetus* (Cichlidae): que mecanismos fisiológicos e moleculares permitem a adaptação desta espécie invasora em Portugal?

Declaração de autoria

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências.
I declare to be the author of this work, which is original and unpublished. Authors and works consulted are duly cited in the text and are included in the list of references.

Flávia B V. Silva

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“Todos têm direito a um ambiente de vida humano, sadio e
ecologicamente equilibrado e o dever de o defender.”
(Artigo 66, Parágrafo 1, da Constituição da República Portuguesa)

“Todos têm direito ao meio ambiente ecologicamente equilibrado,
bem de uso comum do povo e essencial à sadia qualidade de vida,
impondo-se ao poder público e à coletividade o dever de defendê-lo
para as presentes e futuras gerações.”
(Artigo 225 da Constituição da República Federativa do Brasil)

Com meus pés fora da minha zona de conforto!

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Resumo

As espécies não-nativas invasoras estão reconhecidamente entre as principais causas de declínio da biodiversidade nativa, podendo levar a uma drástica homogeneização de fauna e/ou flora em determinados locais. Cada vez mais surgem novos estudos a fim de se conhecer melhor os mecanismos (fisiológicos e comportamentais) envolvidos no sucesso que determinadas espécies apresentam após sua introdução em novos ambientes. Tais conhecimentos são de grande importância para o desenvolvimento de programas de controlo e manejo de espécies não-nativas que sejam específicos para determinado taxon e assim produzam pouco ou nenhum impacto nas espécies nativas. Levando-se em conta a composição da ictiofauna de água doce em Portugal (pouco diversa e com altos níveis de endemismos, além da presença de um grande número de espécies não-nativas), e adicionando a crescente preocupação em conservação e proteção das espécies nativas (incluindo sugestões na alteração da legislação vigente sobre espécies não-nativas e invasoras), esta tese teve como principal objetivo traçar um perfil fisiológico e comportamental do único ciclídeo encontrado na natureza em Portugal, o chanchito *Australoheros facetus*. Para tal, realizou-se um estudo sobre a possível pressão que *A. facetus* possa estar produzindo na comunidade de peixes local, através de análises ecológicas das as quantidades e proporções entre nativas e não-nativas em duas bacias distintas ao sul de Portugal (Guadiana e Arade) (Capítulo 2). Além disso, de acordo com possíveis cenários de alterações climáticas, principalmente relacionados ao aumento de períodos de extrema seca ou chuvas torrenciais, esta tese avaliou as capacidades adaptativas de *A. facetus* frente a mudanças na temperatura (Capítulo 3) e salinidade (Capítulo 4) da água. Desta forma tentou-se traçar uma predição acerca da possibilidade de sobrevivência e/ou colonização em águas de alto estuário, ou utilização dos mesmos como formas de se alcançar corpos de água doce até então sem a presença de *A. facetus*. Como forma de se conhecer os comportamentos-chave ligados a organização social e reprodução da espécie, foram utilizados estudos etológicos em laboratório, com análises de perfil hormonal, que levaram a construção do etograma da espécie, inexistente até o momento (Capítulo 5). Um importante aspeto acerca da organização social nesta espécie está ligado a forma como animais territoriais (dominantes) e não-territoriais (subordinados) comunicam seus estados e assim evitam possíveis conflitos desnecessários. Desta forma, procedeu-se com uma investigação inicial sobre comunicação química, através de pistas químicas lançadas pelo fluido

intestinal e ácidos biliares (Capítulo 6). Para finalizar, procedeu-se a uma avaliação do estado atual de *A. facetus* em Portugal no que concerne a legislação ambiental vigente sobre espécies não-nativas e invasoras, utilizando dos resultados obtidos durante todo o desenvolvimento desta tese (Capítulo 7). Como principais resultados, no Capítulo 2 encontramos importantes valores de abundância e biomassa de *A. facetus* nas ribeiras estudadas. No Capítulo 3 descrevemos a grande amplitude térmica suportada por esta espécie com especial atenção aos valores mínimos os quais permitem sua sobrevivência durante o inverno português, e no Capítulo 4 demostramos a possibilidade de utilização de corpos de água salobra como pontes entre corpos de água doce. No Capítulo 5 vêm descritos os altos valores de cortisol em indivíduos subordinados (demonstrando um estado de estresse social) bem como a validação da ausência de estradiol-17β em machos de *A. facetus.* No Capítulo 6 incidimos sobre uma possível comunicação do estatuto de dominante através de liberação de sinais químicos. Acima de tudo, um importante contributo é a sugestão da alteração de estado de 'espécie não-nativa' para 'espécie invasora' em Portugal, desta maneira restringindo o manuseio e utilização desta espécie e evitando impactos ainda maiores na ictiofauna nativa. Com base nos conhecimentos sobre a biologia de *A. facetus*, um dos caminhos futuros sugeridos é um aprofundamento sobre a comunicação química entre machos e fêmeas a fim de se desenvolver uma ferramenta química específica (e.g. uma isca para aprisionamento de machos) que possa resultar em uma disrupção de sua reprodução nas ribeiras. Iniciativas de educação ambiental também devem ser reforçadas, visto que facilmente se encontram informações *on-line* sobre a possibilidade de aquisição de exemplares de *A. facetus* para criação em aquários. As espécies não-nativas e exóticas apresentam um problema de proporções globais, assim, o controle local de espécies que ainda não apresentam populações com grande distribuição geográfica, reduz o investimento financeiro necessário e aumenta a probabilidade de sucesso de tais programas.

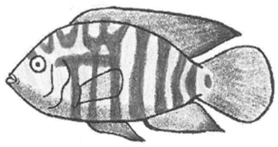
Abstract

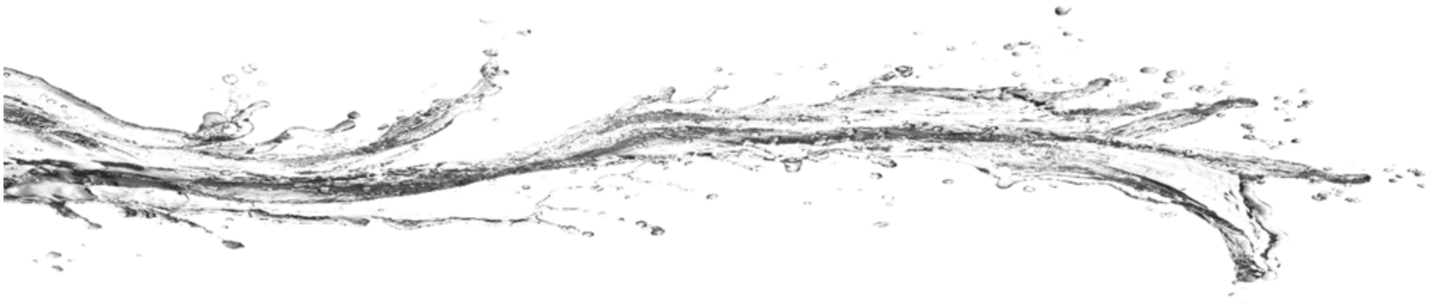
Invasive species are recognized as the main causes of decline of native biodiversity, leading to a drastic homogenization of fauna and flora in certain places. Increasingly new studies are emerging to better understand the mechanisms (physiological and behavioural) involved in success of a species to become established in a new environment. Such knowledge is of great importance for the development of control and management programs of non-native species that are taxon-specific and thus have produced few or no impact on native species. Considering the composition of freshwater ichthyofauna in Portugal (poor and highly endemic, with the presence of many non-native species), and adding an increasing concern for the conservation and protection of native species, the main objective of this thesis is to establish a physiological and behavioural profile of the only cichlid found in Portugal, the chanchito *Australoheros facetus*. For this, a study was carried out on a possible pressure of *A. facetus* on local fish community, through ecological analyses between quantities and proportions between native and non-native species in two distinct basins in southern Portugal (Guadiana and Arade) (Chapter 2). According to the climatic scenarios, mainly related to the increase of periods of extreme drought or torrential rains, we evaluated the adaptive capacities of *A. facetus* coping with changes in temperature (Chapter 3) and salinity (Chapter 4), and a subsequent prediction was made about the possibility of survival and / or colonization in the upper estuary, or the use of them as ways to reach new freshwater bodies. To learn about key behaviours linked to social organization and reproduction of the species, ethological techniques and hormonal profiling led to a construction of the ethogram of the species (Chapter 5). Another important aspect about social organization of this species is the way in which territorial (dominant) and non-territorial (subordinate) animals communicate their status and thus possibly avoid escalation of conflicts. Therefore, an initial research was carried out on chemical communication, through chemical cues released in the intestinal fluid and bile acids (Chapter 6). Finally, we propose a re-assessment of the current status of *A. facetus* in Portugal with regard to legislation on non-invasive and invasive species, using the results obtained during the development of this thesis (Chapter 7). Our main results include also in Chapter 2 the great values of abundance and biomass of *A. facetus* in the studied streams, as well as the great thermal amplitude supported by this species with special attention to the minimum values and the possibility of survival during the

winter, described in Chapter 3. The use of brackish water bodies as bridges between freshwater bodies is demonstrated in Chapter 4. In Chapter 5 we show high levels of cortisol in subordinate animals suggesting a state of social stress, as well as the validation of the absence of estradiol-17β in *A. facetus* males. Chapter 6 focuses on chemical communication of the dominant status through bioactive fluids. Nonetheless, one of the main results from the present work is the suggestion of changing the status of 'non-native species' to 'invasive species' in Portugal, thus restricting its use and avoiding even greater impacts on native ichthyofauna. Non-native and exotic species present a problem of global proportions, thus, local control of species that do not yet have populations with large geographical distribution, reduces the necessary financial investment and increases the probability of success of management programs.

CHAPTER ONE

GENERAL INTRODUCTION





GENERAL INTRODUCTION

1. Non-native species and Invasion Ecology

One important aspect while working with environmental management is to define the concepts well to avoid misunderstandings. Invasion Ecology has a recognized starting point with the publication of "The ecology of invasions by animals and plants" by Elton (1958). However, the terminology of "invasive species" has been an object of discussion, both because of a lack of clarity about the impacts caused, whether negative or positive (reviewed by Rejmánek et al., 2002), and the different points of view caused by divergent categorization. As an example, when defining an invasive species in policymaking and wildlife management reports, a political space is usually used, such as a country; whereas in scientific studies, ecological spaces are used, such as biogeographic or climatic regions (Boonman-Berson et al., 2014). Other important aspect to consider is if the target of discussion is an individual or a population, especially because for just one individual to pass through an environmental filter and persist is less complex than the filters that a population has to overcome to become self-sustained (Blackburn et al., 2011).

The International Union for Conservation of Nature and Natural Resources (IUCN) suggests that the definition of introduced, exotic, alien, non-native, non-indigenous and allochthonous species have the same biological significance and correspond to any species transported and released by man, intentionally or not, out of its natural distribution (IUCN, 1999; Shine et al., 2000). The term invasive species used in this thesis is based in the approach proposed by Colautti and MacIsaac (2004) that suggests that invasion is a process and that a potential invader has to pass through a series of filters or bottlenecks (transport and release, environmental survival, reproduction, dispersal and community suitability) until it becomes widespread and sometimes dominant – Stages IVa and V (Figure 1). In addition, it was added the potential negative impact that the species can cause in the novel environment (Mack et al., 2000; Shine et al., 2000). In this way '*invasive species*' refers to *a species introduced by humans, intentionally or not, that established a self-sustained population, spread and that can eventually negatively impact on other species or ecosystem.*

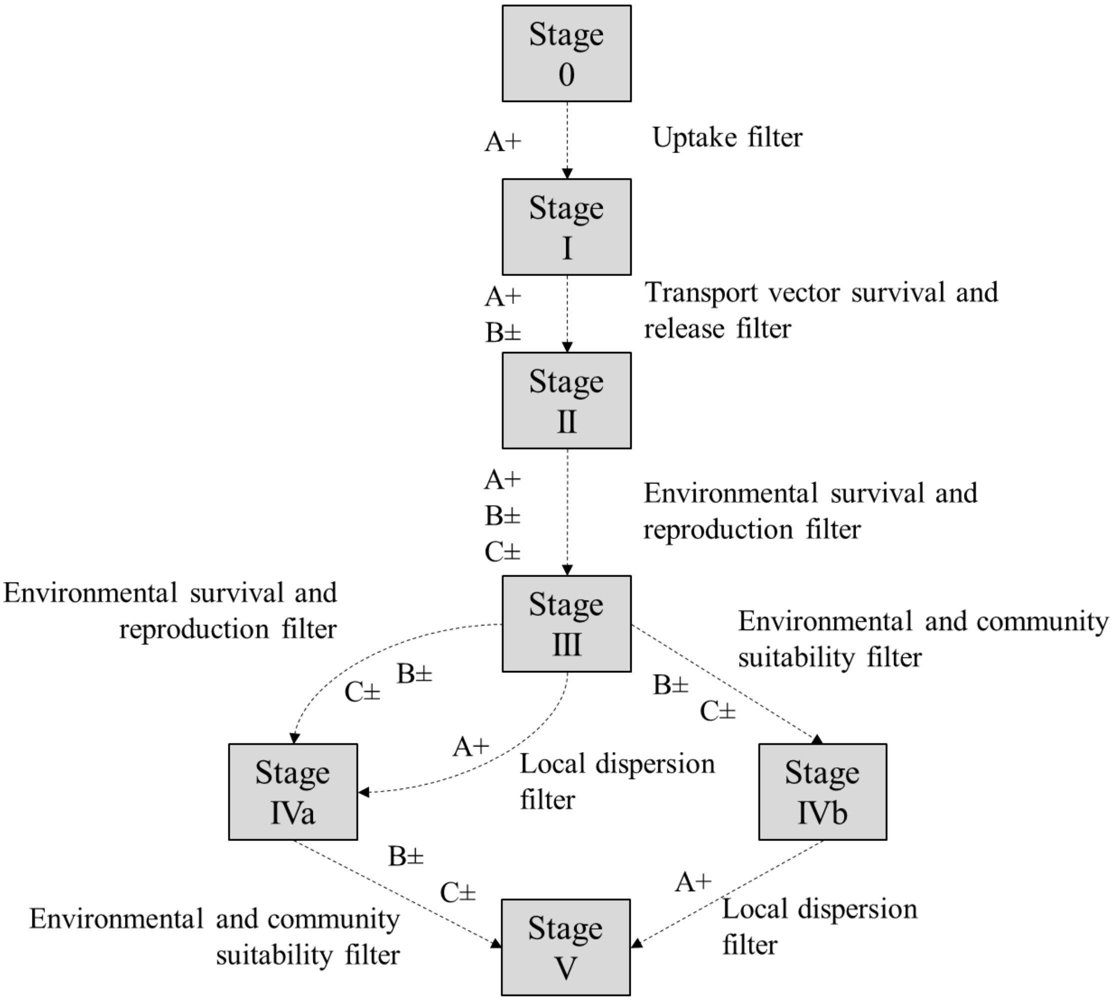


Figure 1. Framework suggested by Colautti and MacIsaac (2004) for defining terms in invasion studies; where (A) is the propagule pressure, (B) are the physic-chemical requirements of the potential invader, and (C) are the community interactions. Determinants may positively (+) or negatively (–) affect the number of propagules that successfully pass through each filter. Under this framework, a non-native species may be: localized and numerically rare (stage III); widespread but rare (stage IVa); localized but dominant (stage IVb); or widespread and dominant (stage V).

Examples of the variability in the taxonomy and life history characteristics of introduced fish and the human interests behind the introduction are those ranging from the small mosquitofish (*Gambusia* spp) introduced for mosquito control, to the piscivorous largemouth bass (*Micropterus salmoides*) and common carp (*Cyprinus carpio*) introduced for sport fishing and food supply (García-Berthou, 2007; Rahel, 2007), or for ornamental purposes as the 775 marine species sold online in the United States, that are usually purchased by customers as juveniles, while some of them can become excessively big as adult and be released in wild, instead of be euthanized (Holmberg et al., 2015).

However, dispersion of species mediated by the human activities to places beyond its original distribution has been identified as one of the majors causes of global biodiversity decline and consequent homogenization of fauna (Clavero & Garcia-Berthou, 2005; Filipe et al., 2010; Rahel et al., 2008; Wilson et al., 2009). In addition, it can change properties as fundamental as the ecological dominance of species and the physical characteristics of an ecosystem (Mack et al., 2000; Vitule et al., 2009). Usually, three main reasons for the lack of consensus on the impacts caused by the introduction of aquatic species can be cited: a) the lack of preliminary studies to invasion; b) anthropogenic habitat changes both before and after the introductions and c) inexistence of monitoring programs to assess their impacts (Crivelli, 1995).

Most studies related to non-native species have focused on distribution and life cycle characteristics (Ribeiro & Collares-Pereira, 2010; Ribeiro et al., 2008; Vila-Gispert et al., 2005), tolerance to environmental variations (Gutierre et al., 2014; Jud et al., 2014; Schofield et al., 2009) and propagule pressure (Lockwood et al., 2005; Rius et al., 2014; Wilson et al., 2009). However, an efficient management of invasive species requires a multidisciplinary approach (García-Berthou, 2007; Leung et al., 2002; Ricciardi & MacIsaac, 2011), and requires attention to various aspects including physiology (Lennox et al., 2015), behaviour (Carere & Gherardi, 2013; Fogarty et al., 2011), ecology (Peterson, 2003), genetics (Prentis & Pavasovic, 2013; Prentis et al., 2008) and economy (Tassin & Kull, 2015).

Some features are key elements in the successful establishment of a non-native species, especially among fish: short lifetime but with rapid growth and sexual maturation, high fertility rates and wide physiological tolerance or genetic variability and phenotypic plasticity (García-Berthou, 2007; Kates et al., 2012; Ribeiro & Collares-Pereira, 2010; Ribeiro et al., 2008). In addition, it is suggested that climate change could facilitate the invasion of non-native species increasing its impact on natural communities, favouring their reproduction and recruitment, by changing the discharge of rivers, increasing the water temperature, increasing the frequency of floods and severe droughts (Britton et al., 2010; Copp, 2006; Hellmann et al., 2008; Rahel et al., 2008). In temperate regions, where fish communities are dominated by species adapted to colder waters, a warm water can act facilitating the entry and establishment of species from warmer locations (Rahel et al., 2008). Also, invasive species have a greater probability of success in environments already disturbed when the native ones were adversely affected or even eliminated (Moyle & Light, 1996; Ward & Blum, 2012). As an example, under laboratory conditions, a native and an introduced freshwater fish species of *Cyprinella* were exposed to bisphenol A (BPA), a recognized endocrine-disrupting chemical that is used in the manufacture of plastics and resins, and the results showed an increase of the likelihood of hybridization by weakening sexual isolation between congeners (Ward & Blum, 2012).

Niche displacement, competitive exclusion, hybridization, behaviour shifts or extinctions are some of the negative impacts that invasive species could bring to the new environment and to native species (see reviews from Mooney & Cleland, 2001; Ribeiro et al., 2009). The invasive Nile tilapia (*Oreochromis niloticus*) and the native redspotted sunfish (*Lepomis miniatus*), that are likely to co-occur in many estuaries of northern Gulf of Mexico, were challenged with habitat choice and identification of behavioural interactions, showing that in the presence of *O. niloticus*, *L. miniatus* spent less time in a structured habitat than when alone, leading to a conclusion of a possible competitive exclusion and niche displacement of the native species (Martin et al., 2010). It is important to note that the negative impacts of invasive fishes are not exclusively on other fishes. In a study comparing two lakes in Spain, in the presence of the invader common carp (*Cyprinus carpio*) and after its posterior eradication, it was shown that in the presence of the carp the lakes had lower richness and abundance of diving ducks and other waterbirds, attributed mainly to the pressure that this carp puts on the macrophyte bed and macroinvertebrate abundance, leading to a loss of suitable habitats for the waterbirds (Maceda-Veiga et al., 2017). Furthermore, increase of floodwaters predicted to occur in climate change scenarios, can facilitate the spread of non-native species (dispersal opportunities) by, for example, escaping from ponds or fish-farms, and subsequently spreading downstream reaching waterbodies with different hydrological features (Diez et al., 2012). Also, severe droughts are expected to increase in frequency, trapping fish in pools with high temperatures and low dissolved oxygen. In both cases, when facing a new or changing environment, species with broader environmental tolerance can be at advantage over native species with narrower homeostatic intervals (Diez et al., 2012; Gutierre et al., 2014; Stauffer & Gray, 2004).

The types of fish management techniques available to managers to implement eradication programs can include chemicals, harvest regimes, physical removal, or biological control (Donaldson & Cooke, 2016; Ricciardi, 2013). However, these are invasive measures, susceptible to cause widespread ecological impacts. Only a few approaches take in account species-specific methodologies, such as using chemical cues as pheromones (Aquiloni & Gherardi, 2010; Johnson et al., 2009), which are less likely to have deleterious effects for the environment and human health. In this way, to better address the issue of invasive species it is necessary to incorporate physiological with ecological and behavioural data, so as to identify habitats that may provide higher fitness to them, and to develop better strategies to control invasive populations (Holway & Suarez, 1999; Lennox et al., 2015).

In other words, the identification of these species, their origin and current distribution, the trophic position the behaviors they exhibit, are key issues to the understanding of invasion processes and for the decision-making relating to control and mitigation of the possible negative impacts (García-Berthou, 2007).

In this sense, the present thesis assessed the principal mechanisms present in the neotropical cichlid *Australoheros facetus* that allowed its adaptation to Portuguese streams, mainly through evaluation of its environmental tolerance and social behaviour.

2. Environmental Tolerance

Environmental conditions are the major driver of fish distributions. The physiological processes of aquatic organisms are influenced by the surrounding water conditions, having to adjust to changes in physical-chemical conditions (salinity, temperature, pH, dissolved oxygen, among others) to keep homeostasis. A simple definition of environmental tolerance is the way organisms respond to their environment (behaviourally, morphologically and physiologically) and maximize fitness in face of both changing and variable environments (Lynch & Gabriel, 1987), in a window of abiotic conditions where a species function correctly (Angilletta, 2014).

The variation in the responses to the environment observed from one individual to the consequences at population-level could be due to genetic polymorphisms or plasticity (Figure 2). The main differences between genetic polymorphisms and plasticity can be summarized as:

a) Phenotypic plasticity is the production of different phenotypes by a single genotype in response to variation in **environmental conditions** during an individual lifetime (Forsman, 2015; Lema, 2014) and ultimately is an evolutionary factor that results in morphological diversification (Wimberger, 1991). Phenotypic plasticity can be (at least) divided in 1) developmental plasticity and 2) acclimation (Beaman et al., 2016; Forsman, 2015). Developmental plasticity happens when an irreversible phenotypic variation in traits occur during development and usually remains fixed after maturity; acclimation (or reversible phenotypic plasticity) refers to intra-individual reversible and repeatable changes such as metabolic and endocrine switches or behavioural traits (Beaman et al., 2016; Forsman, 2015).

b) Genetic polymorphisms are allelic variations in some determined coding loci (Forsman, 2015), being well studied, for example, in relation to colour morphs (Gray & McKinnon, 2007; Hugall & Stuart-Fox, 2012; Sowersby et al., 2015). One example is the discrete phenotypic classes in the cichlid fish *Neochromis omnicaeruleus* in which females can display three distinct colour morphs, showing a linear and sustained behavioural dominance among the colour morphs (Dijkstra et al., 2009), in other words, one female of one of the colour morphs will dominate the other two morphs, even under different contexts.

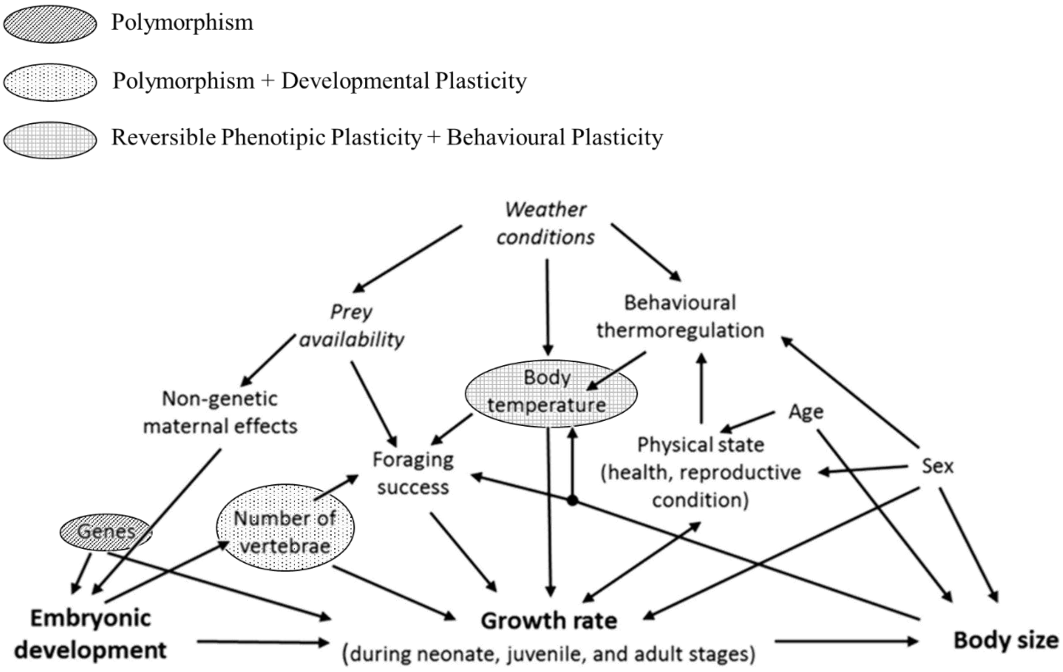


Figure 2. Schematic representation showing that measuring variation in one single trait, as, in the example, growth rate in snakes, can be a reflection of different causes, such as developmental plasticity, acclimation, genetic polymorphism and external environmental conditions (for details see review by Forsman, 2015).

It is important to highlight that phenotypic plasticity or genetic polymorphisms, although usually focussed on morphological characters, as in the cichlids' head and jaw morphology (as example see Stauffer & Gray, 2004), or the length and position of the fins in native and non-native populations of the pumpkinseed *Lepomis gibbosus* (Yavno & Fox, 2013), can also be related to molecular physiology and behaviour. As an example of behavioural plasticity, under similar laboratory conditions, nest-holder males of the peacock blenny *Salaria pavo* from different populations, express similar rates of courtship behaviour (Saraiva, J. L. et al., 2011), while in wild this species can express 10 fold higher courtship rates depending on different environmental conditions in which the assessed population is found (Saraiva, João L. et al., 2011). A study of genetic differences in *L. gibbosus* with native (North American) and non-native populations (Iberian Peninsula), showed that the introduced population has a reduced allelic diversity compared to native populations, attributed to the bottlenecks that non-native species experience in a new environment (Detta, 2011). Regardless of the fact that bottlenecks could reduce allelic diversity (that could only reflect the loss of rare alleles), genetic variance of quantitative traits (many of them linked to fitness-related traits) may not decline during demographic bottlenecks (Dlugosch & Parker, 2008). This may be due to the fact that quantitative traits depend on the cumulative action of several genes and the environment, and are characterized by distributions rather than discrete traits values (Dlugosch & Parker, 2008).

It is hypothesized that colonising species possessing a high degree of phenotypic plasticity and/or polymorphisms may have better chances to pass through different environmental filters and have an advantage over native species because of the ability to exploit different resources in different habitats that present ecological opportunities (Figure 3) (Burress, 2014; Stauffer & Gray, 2004). On one hand, developmental plasticity may favour the establishment and invasiveness of non-native species, allowing species to develop phenotypes appropriate to the conditions of the new environment, without the need to undergo genetic changes due to natural selection (Forsman, 2015; Huey et al., 2003). On the other hand, if the time lag between the introduction in a new environment and the induced response is significant, selection can drive the introduced population to extinction before the necessary time for the appropriate phenotype has been realized (Wennersten & Forsman, 2012).

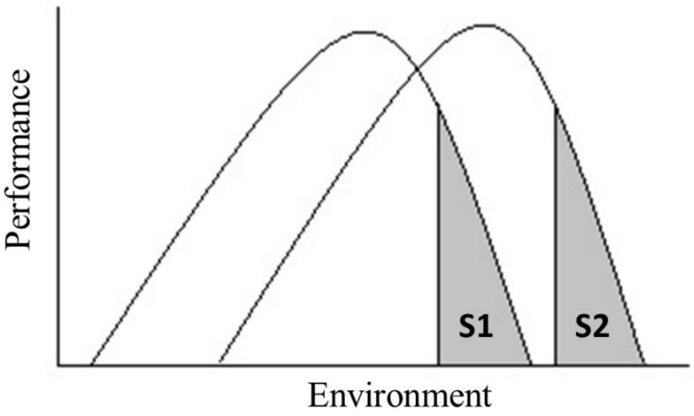


Figure 3. Phenotypic plasticity can shift the performance curve of an organism such that an environment that was previously experienced as stressful (S1) no longer results in declines in performance, and instead a new range of environments can trigger stress response (S2). From Schulte (2014).

2.1. Thermal tolerance

Among abiotic factors, temperature is perhaps the most important in shaping species distributions, ecology and evolution (Portner, 2001; Rius et al., 2014), since all the physiological processes have an optimum range of temperatures to operate. Outside this range, proteins' structure is affected and a cascade of processes will be disrupted (Somero, 2010). As exemplified by Somero (2010) and Dong and Somero (2009), small changes in cytosolic malate dehydrogenase (cMDH) sequence – an enzyme that plays a crucial role in many important metabolic pathway including the tricarboxylic acid cycle, the glyoxylate bypass, amino acid synthesis, gluconeogenesis and facilitation of exchange of metabolites between cytoplasm and subcellular organelles, reviewed by Musrati et al. (1998) – may lead two congeners of limpets (*Lottia digitalis and L. austrodigitalis*) to temperature-adaptive changes that can be reflected in their biogeographic distribution, where the recent shift in distribution of these two limpets may be a reflection of the warming trend observed in coastal Central and Northern California (Figure 4).

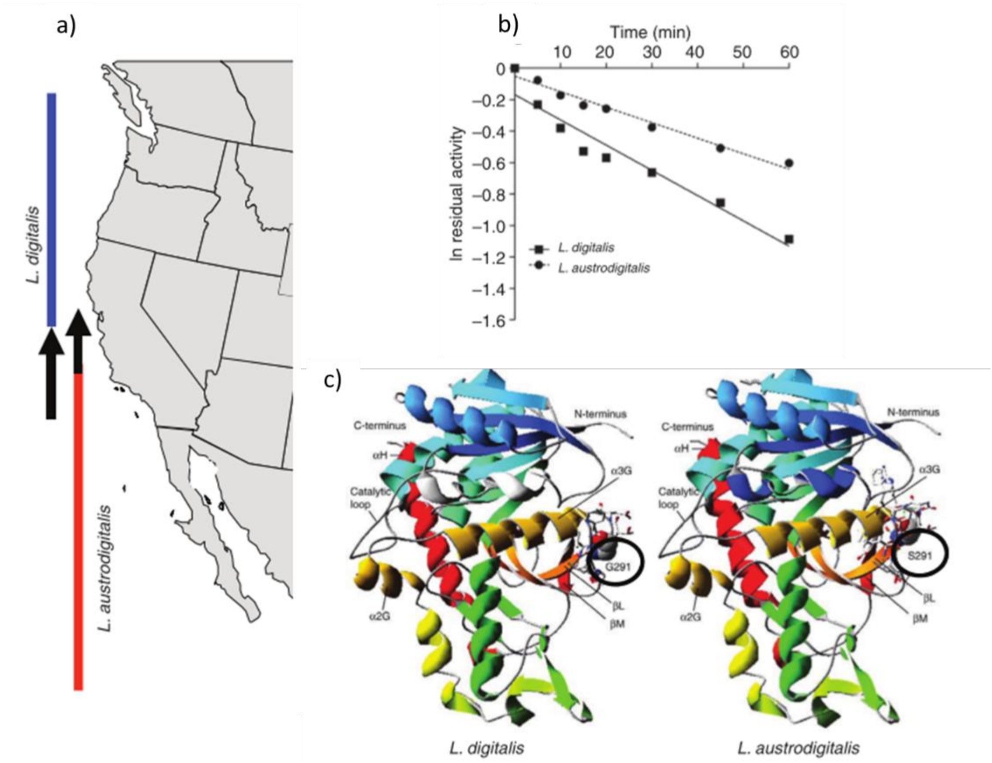


Figure 4. Biogeography and characteristics of cMDHs of *Lottia digitalis* and *L. austrodigitalis*. (a) Biogeographic distributions, where black arrows show contraction of the southern range of *L. digitalis* and expansion of the northern range of *L. austrodigitalis*. (b) Loss of activity during heat at 42ºC for cDMH; denaturation rate of cMDH for *L. digitalis* is significantly higher than that of the *L. austrodigitalis* ortholog. (c) Three-dimensional structures and the single amino acid replacement (site 291) for cMDHs. Images adapted from Somero (2010) and Dong and Somero (2009).

Thermal performance curves (Figure 5) plot the body temperature (or environmental temperature, which are identical when referring to poikilotherms) against quantitative measures of the individual’s performance (for review see Dowd et al., 2015). Usually a peak is found in the ‘optimal’ temperature in a static environment (T_{opt}), optimized oxygen supply to tissues are located between low and high pejus temperatures (T_p), whereas to the right or the left of this peak lie the critical temperature, where the organisms become dependent of an anaerobic metabolism (Portner, 2010). If the temperature continues to increase/decrease, an extreme hypoxaemia develops which leads to a deficiency in ATP synthesis, reaching the critical temperature maximum and minimum (CTMax and CTMín, respectively) where the performance is close to zero (Dowd et al., 2015; Portner, 2002; Portner et al., 2010). To determine the CTMax and CTMín, a dynamic method is usually applied that involves changing temperature at a constant rate, heating or cooling an animal from a starting temperature (acclimation temperature) until physiological processes start to fail (visually a predefined sublethal endpoint such as loss of equilibrium – LOE or onset of spasms) (Becker & Genoway, 1979; Beitinger et al., 2000).

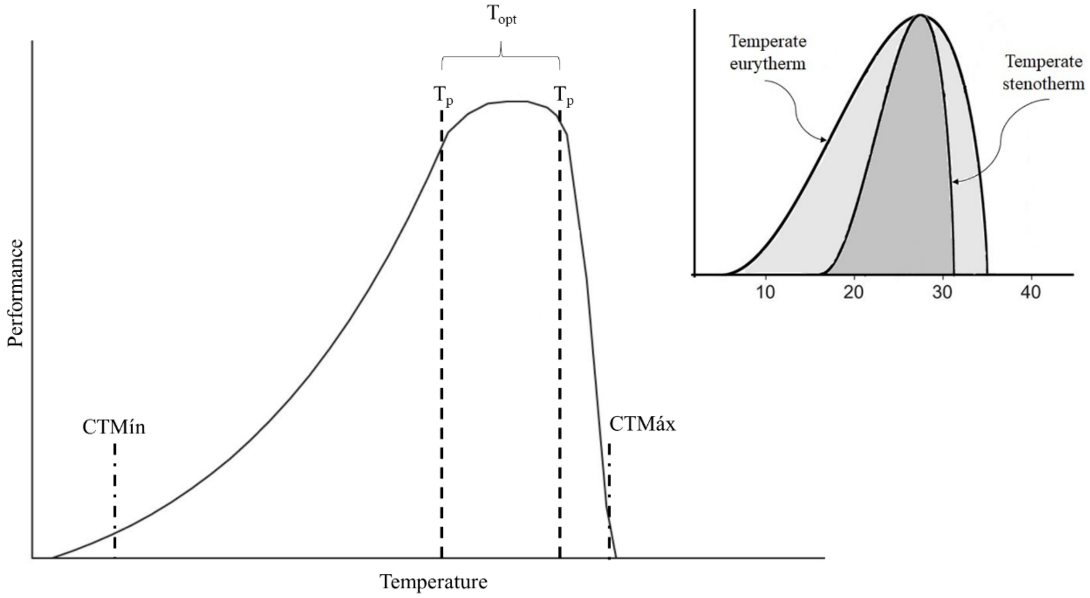


Figure 5. Example of a thermal performance curve adapted from the conceptual model of Dowd et al. (2015). Usually, the curve is asymmetric, such that performance increases relatively slowly up to T_{opt}, but decreases rapidly above T_{opt}. Eurythermal organisms have a relatively large thermal breadth, while stenothermal organisms have a narrower breadth.

In the wild, some organisms can be found with mean body temperatures below their T_{opt} observed in the laboratory. This *suboptimal-is-optimal strategy* could be adaptive as it keeps a safety margin, reducing the risk of experiencing high temperatures that could lead to the rapidly descending part of the curve, and to severe damages at physiological level (Dowd et al., 2015; Martin & Huey, 2008).

There are different approaches to evaluate how temperature can influence fitness in an organism, as well to define reliable biomarkers of critical temperatures. One of these approaches is the expression of genes linked to regulation of energy metabolism, and protection and repair of proteins. When challenged with increased temperature, two congeneric fish *Squalius carolitertii* and *S. torgalensis* showed increased expression of many heat-shock protein (HSP) genes, whereas *S. torgalensis* also showed downregulation in genes responsible for cell division and growth, reflecting an energy saving strategy during high temperatures (Jesus et al., 2016). Other responses assessed may rely in haematological parameters, as energetic substrates in plasma (such as glucose and lactate), which are expected to increase during rapid increases in temperature, as observed for the shortnose sturgeon *Acipenser brevirostrum*, suggesting that an increased metabolic rate associated with thermal stress could trigger an anaerobic metabolism to balance the energy demand (Zhang & Kieffer, 2014). The stress hormone cortisol is also used as a biomarker for thermal stress, showing a rapid increase in genetically improved farmed tilapia *Oreochromis niloticus* until 24h post cold shock treatment, and, despite decreasing after, remaining higher than fish that were not subjected to the cold-shock, which could be linked to enhancement of non-specific immune function and stress resistance (He et al., 2015).

2.2. Salinity tolerance

Depending on their habitat or life cycle, some fish species can experience either abrupt or gradual changes in salinity. In addition to catadromous or anadromous species, the most notorious examples are estuarine species, which can be exposed daily or seasonally to a wide range of salinities (Henriques et al., 2017; Vilar et al., 2017), while other species, although not inhabiting such ecosystems, can still tolerate important changes in salinity, perhaps due to ancestral and inherited abilities, as is the case of some cichlids (Gutierre et al., 2014; Moorman et al., 2016).

Because freshwater fishes are continually gaining water by osmosis (their internal media is hypertonic when compared to surrounding water), they have to excrete large volumes of diluted urine and use active ions transporters, mainly in gills, to replace the solutes lost (Figure 6) (Marshall & Grossel, 2005; Moyle & Cech, 1996). In contrast, marine fishes are continually losing water across the gills and must drink water; as the environmental water is rich in ions, they have to actively secret the ions across the gill epithelia. In addition, their kidney exhibits very low glomerular filtration (or even with absent glomeruli) and produce very low volume of isotonic urine (Figure 6) (Marshall & Grossel, 2005). To cope with such energy demands due to the differences in osmotic pressure between their internal and external media, the organisms can use a mixture of energetic substrates (like carbohydrates and proteins), usually controlled by molecular and endocrine mechanisms (Bœuf & Payan, 2001; Moyle & Cech, 1996).

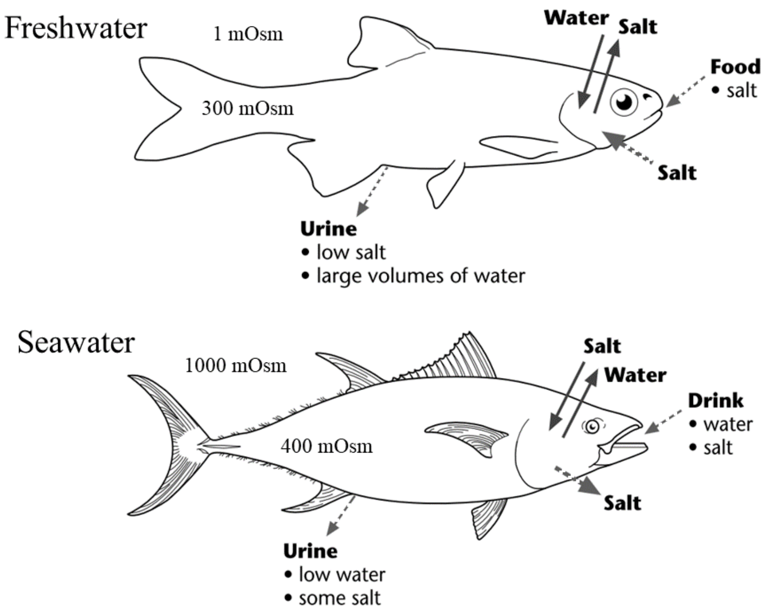


Figure 6. Mechanisms of osmoregulation by teleost fishes. Solid arrows represent passive movements of salt and water, and dashed arrows represent active pathways of osmoregulation. Adapted from Evans (2008); Evans (2003).

While euryhaline fishes can survive some changes in environmental salinity, they present an extreme case of osmoregulation capacity usually with modification of ion transport processes mainly through changes in the activity of sodium–potassium pumps ($Na^{+/}K^{+}$-ATPase) in gills and kidneys, production and release of cortisol and reallocation of energetic substrates (Gonzalez, 2012; Gonzalez et al., 2005; Yan et al., 2013). Some cichlids, for example, can withstand salinity challenges while maintaining osmotic homeostasis, as the case of the ‘California’ Mozambique tilapia *Oreochromis mossambicus x O. urolepis hornorum* (Sardella & Brauner, 2008) that presented little changes in plasma osmolality or muscle water content in salinities below 60 ppt; or the mayan cichlid *Cichlasoma urophthalmus* that when acclimated at 25ºC and 30 ppt, reached 90% of survival when salinity was increased until 37 ppt (Stauffer & Boltz, 1994). However, the cost of keeping homeostasis in such situation can cause a reduction of their fitness, such as growth or reproduction (Sokolova, 2013; Zhao et al., 2013). One of the hypotheses for the different degrees of euryhalinity found in cichlids is that they are re-invaders of the freshwater biotope, since the Perciformes are a marine group (Gutierre et al., 2016; Gutierre et al., 2014)

3. Social Behaviour

Social behaviour consists of a set of interactions among individuals of the same species, including aggression, reproduction, parental care, cooperation and social organization (Banks & Heisey, 1977). These actions rest on a set of physiological mechanisms related to reception, processing and responding to social and environmental stimuli, mainly through synthesis and release of hormones (Hoar, 1962; Rubenstein & Hofmann, 2015).

Aggressive interactions among conspecifics usually ensure the winner the possession of territories and resources, at least for a certain period of time, which can lead to the establishment of dominance hierarchies (Johnsson et al., 2006; Maruska, 2015; Smith, 1974; Smith & Price, 1973). It is common that individuals of different status within a hierarchy signal their position in several modes (behavioural, morphological or chemical), probably avoiding energy-consuming fights, both for dominants as for subordinate animals (Briffa & Elwood, 2004; Keller-Costa et al., 2015; Miyai et al., 2011; Rubenstein & Hofmann, 2015). It is hypothesized that bigger animals should show best fitness and best capacity of defend a territory and offspring, in this way, the size of the fish seems to be the major driver to the dominance within a group (Alonso et al., 2011; Dechaume-Moncharmont et al., 2011).

In the social cichlids studied so far, reproduction is tightly controlled by social status. Dominant individuals usually have exclusive access to mates (Enquist & Leimar, 1983; Parker, 1974) and undergo profound physiological processes aimed at communicating and maintaining status and reproductive success. These processes range from e.g. colouration changes within minutes after acquiring a territory, increase in circulating androgens in the subsequent hours, to gonad maturation after few days (Keller-Costa et al., 2015). Subordinates, on the other hand, either refrain from reproduction (Bell et al., 2014) or engage in alternative reproductive tactics such as cooperative breeding, helping or sneaking (Saraiva et al., 2013; von Kuerthy et al., 2016), and also undergo physiological changes in the reverse sense of dominants (Almeida et al., 2012; Culbert & Gilmour, 2016).

Parental behaviour (i.e. the care of progeny, from eggs to the juvenile stages, lasting from some days for several weeks, depending on the species) occurs in many cichlid fishes (Budaev et al., 1999; Keenleyside, 1991; Snekser & Itzkowitz, 2009). The most common system of parental care is maternal mouth brooding, where the female picks the eggs after spawning with her mouth, which could be fertilized before in the substrate or after inside its mouth, as occurs in *O. mossambicus* (Coward & Bromage, 2000). Biparental care, when both female and male take care of the eggs and larvae, is usually observed in substrate brooders species as described for *Cichlasoma dimerus* (Alonso et al., 2011) or in *Coptodon zillii* (McConnell, 1959), although some mouth brooding species can also perform biparental care such as *Sarotherodon galilaeus* (Iles & Holden, 1969). While paternal care was documented for very few cichlids as *S. melanotheron*, in which the male perform mouth brooding of eggs after fertilization (McConnell, 1959; Trewavas, 1983). Among Neotropical cichlids, biparental care in substrate spawner species is the most common system, meaning that not just both female and male are involved in protect the offspring, but also defending the territory (Keenleyside, 1991).

It has been suggested that especially during the establishment phase of a non-native species in a new environment, parental care is an advantage, since it could increase the survival of offspring in variable environments and under high predation pressure (Grabowska & Przybylski, 2014; Marchetti et al., 2004). In the same way, aggressiveness has been observed as an important feature among invasive species (Hudina et al., 2014), since it confers competitive advantageous on resource utilization, as for food or quality habitats (Martin et al., 2010; Vorburger & Ribi, 1999), as well as enhances predation (Janssen & Jude, 2001).

3.1. Chemical communication

Communication is one important barrier among species that will lead ultimately to reproduction between individuals of a same species, and can be defined as expression, transmission and reception of signals in the visual, acoustic, chemical or electric modalities (van der Sluijs et al., 2010). Individual recognition is especially important for species that live in groups, and visual signals are some of the most studied systems of communication (for examples see Carleton et al., 2005; Levine et al., 1980; Miyai et al., 2011). However, there is increasing evidence that chemical communication is involved during hierarchy establishment to signal social status i.e. to stabilize the group, and thus decrease aggression (reviewed in Keller-Costa et al., 2015). Nile tilapia *O. niloticus* of different social status had a reduction in aggressiveness when placed in separate compartments, visually connected, and with water exchange between the compartments, than when in same conditions but in absence of water exchange

(Giaquinto & Volpato, 1997). Similar results were found for *O. mossambicus* with the observation that dominant males have a large and more muscular bladder (Keller-Costa et al., 2012), which could be an adaptation for urine storage to enable increase of urinary frequency during aggressive interactions (Barata et al., 2007), since it was shown that urine can be used as a chemical signal (Almeida et al., 2005; Keller-Costa, 2014; Saraiva et al., 2017).

Intra-specific chemical communication through pheromones can be defined as "an odour or mixture of odorous substances, released by an individual (the sender) and evoking in conspecifics (the receivers) adaptive, specific, and species-typical response(s), the expression of which need not require prior experience or learning" (Sorensen & Stacey, 2004). Since a pheromone can be a mixture of substances, evolutionarily it is expected that the conspecific receivers develop a specialized olfactory system, making this kind of chemical communication species-specific (Sorensen & Stacey, 2004). The first chemical identification of a cichlid sex pheromone was recently published and it seems to prime the conspecific female endocrine system of *O. mossambicus*, enabling spawning synchrony (Keller-Costa et al., 2014). Additionally, the urine of dominant males of *O. mossambicus* have some chemical information signalling dominance that decreases aggression in dyadic fights (Saraiva et al., 2017). It is important to emphasize that chemical communication in fish may not be restrict to urine, as already shown for the flatfish *Solea senegalensis* (Velez et al., 2007) and the European eel *Anguilla anguilla* (Huertas et al., 2007) that have an olfactory epithelia sensitive to bile fluid, intestinal fluid and mucus of conspecifics.

4. A physiological approach to invasive species

It is assumed that less than 10% of introduced species become established (Williamson et al., 1986; Williamson & Fitter, 1996), although in Portugal the non-native fish establishment was higher (58%, Ribeiro et al., 2009). For a species to become established in a new environment, it must go through a series of biotic and abiotic filters, and this is only possible if the potential invader is physiologically capable of persisting (Lennox et al., 2015; Marchetti et al., 2004). Thus, studying the physiological mechanisms that allow non-native species to become established in a new environment may help to predict their spread, reproduction and invasiveness, and consequently improve management and tools for their control (Schofield & Schulte, 2016).

Two of the common methodologies employed to control and/or eradicate non-native and invasive fish species are direct fisheries techniques or using a piscicide, the most popular being a rotenone-derived from plants. However, these are neither easy nor safe alternatives, mainly because the negative impacts on non-target organisms (Dalu et al., 2015; Ribeiro et al., 2015; Tate et al., 2003).

As an alternative to control non-native and invasive fish species, some techniques using physical-chemical barriers for preventing their spread have been used. Examples are air bubble curtains, acoustic, electric or carbon dioxide barriers, among others, or combinations, with variable results (Kates et al., 2012; Noatch & Suski, 2012; Zielinski et al., 2014). However, the mechanisms that usually prevent the dispersion of the majority of fish, native or non-native, and the studies behind the application and monitoring of these systems have been mostly focused on their behavioural responses, such as avoidance behaviour, rather than on their physiological impact. Barriers may be useful to prevent new colonisations or access to suitable reproductive/spawning grounds, but will not work for areas already impacted.

The use of pheromones has been widely documented against pest insects (Witzgall et al., 2010; Wyatt, 2003), and an increasing number of studies have been made with other *taxa*, including fish, usually to attract animals to traps (Aquiloni & Gherardi, 2010; Johnson et al., 2009; Sorensen & Stacey, 2004). However, to achieve and develop a control protocol with pheromones, it is important to understand the mechanisms underlying the life-cycle of the target species, in particular related to its reproduction (hormonal profiles, mate choices, chemical communication, among others).

Therefore, carrying out ecophysiological studies to understand the causes and ecological consequences of individual variability in physiological traits (Metcalfe et al., 2016), can not only be used to make predictions about the future distribution of invasive species in case of climate change or human induced movements (Braby & Somero, 2006; Christiansen et al., 2015; Lehmann et al., 2015), but also to enhance actual efforts for trapping or biological control (Lennox et al., 2015). In addition, the physiological data (such as stress mechanisms or environmental tolerance) are even more useful when applied with other approaches including population dynamics, ecology and behaviour.

5. Target species

The chameleon cichlid, Acará-camaleão, locally called chanchito (or chanchita, or castanhola), *Australoheros facetus*, was first described in 1842 by Leonard Jenyns, and until recently known as *Cichlasoma facetum*. The genus name is a conjunction of 'australis' meaning southern, and Heros, after the nominotypic genus of the heroini tribe. The species name 'facetus' means clever or witty. It belongs to the Class Osteichthyes > Sub-class: Actinopterygii > Order: Perciformes > Family: Cichlidae > Sub-Family: Cichlasomatinae > Tribe: Heroini > *Australoheros facetus*.

Cichlids are one of the most diverse lineages of freshwater fishes with more than 1600 species (Kullander, 2003; McMahan et al., 2013). One of the hypothesis about the origin of the Cichlids, place them in the lowland tropics with a geographical distribution that conforms the Gondwanan pattern which dismembered about 120 Million years ago (Ma.) (Keenleyside, 1991). However, the oldest known fossils are dated around 35-55 Ma. and molecular analyses have placed their origin around 57-96 Ma., already after the Gondwanan fragmentation, (Burress, 2014; Keenleyside, 1991; McMahan et al., 2013), leading to new hypothesis about their dispersal, distribution and diversity (as examples see Friedman et al., 2013; Rícan et al., 2013).

An accepted hypothesis for the great diversity of Cichlids is the exploitation of novel habitats and environments combined with high competition among fishes in highly variable environments (Wagner et al., 2012). Competition can lead to resource partitioning, facilitating coexistence among competitors. However for this to happen, morphological and physiological adaptations may occur, leading to resource specializations, decreasing the competition pressure (Arbour & Lopez-Fernandez, 2016; Burress, 2014), and after sexual selection and assortative mating, additional phenotypic diversity occurred, leading them to their great radiation and speciation (as reviewed in Burress, 2014; Stauffer & Gray, 2004).

Australoheros facetus (Jenyns, 1842) is originally distributed in South America, specifically in Paraná, Paraguay and Uruguay basins, including coastal drainages (Bruno et al., 2011; Rícan & Kullander, 2006, 2008). It was first described as *Chromis facetus* Jenyns 1842 during the famous *HMS Beagle*'s voyage (Ringuelet et al., 1967). Since then, this species has received several names and descriptions, as *Heros jenynsii* Steindachner 1870, for specimens collected in Montevideo or *Acara faceta* Steindachner 1874, in Rio Paraná, and in 1905 was first named *Cichlasoma facetum* by

Regan (Ringuelet et al., 1967). The confusion with names and descriptions prolonged until 1983, when Kullander (1983) recognized that *Cichlasoma facetum* was a species complex and suggested the use of commas, in addition to the use of “group” after the specific name ('*Cichlasoma*' *facetum* group), while species of this group were not properly identified (Kullander, 1983, 1998).

In 2006, Rican and Kullander, tried to define a species delimitation for '*Cichlasoma*' *facetum* group using made phylogenetic analyses. They used two approaches for morphological characters – a character-based (morphological diagnostics characters that show differences between the supposed species) and tree-based delimitations (which through parsimonious solutions of character distribution can show monophyletic groups) – and with sequences of mitochondrial cytochrome *b*. From this work, a new monophyletic genus was described: *Australoheros,* with five distinct species and suggestion of at least another five species. Their results strongly support the monophyly of the genus *Australoheros* and locate them as a Heroine cichlid genus. Despite *Australoheros* having a South American distribution, it shows phylogenetic affinity with the Central American Heroini (Lopez-Fernandez et al., 2010; Rícan & Kullander, 2006). The type species of this genus is *Australoheros facetus* (Rícan & Kullander, 2006) and its official description was published by Rícan and Kullander (2008).

Since then, new studies with description of species have emerged and today there are about 20 species described for this new genus (as example see Ottoni, 2013; Ottoni & Costa, 2008; Ottoni et al., 2008; Rícan & Kullander, 2008; Rícan et al., 2011). It is important to note that even after the work of Rican and Kullander (2006), some reports, especially in the Iberian Peninsula, still addressed this species as *Herichtys facetum*, despite this no longer being a valid name (Ilheu et al., 2014; Matono et al., 2012).

Australoheros facetus is a freshwater fish common in streams, rivers, marshes and lakes, with a preference to inhabit small pools, especially under rocks and branches or roots of aquatic or riparian vegetation (Rícan & Kullander, 2008; Ruiz et al., 1992). It has certain tolerance to changes in salinity and can be found in some coastal brackish waters where salinity can reach ~15ppt (Bruno et al., 2011; Gómez & Naya, 2007; Perazzo et al., 2010; Pereira et al., 2011). It occupies waters with pH between 6.5 and 7.0, in subtropical climates with air temperatures between 25 and 30 ºC (Riehl & Baensch, 1991). Its maximum total length is between 18-19 cm (Andrade & Braga, 2005; Kullander, 2003). *A. facetus* is considered a diurnal opportunistic fish, eating detritus, plant material and preying on small aquatic animals, including fish, in its original (Bastos, 2002; Yafe et al., 2002) and invaded range (Ribeiro et al., 2007; Ruiz et al., 1992). The breeding season in South America is in summer (December to April), with high temperatures and elevated pluviosity (Andrade & Braga, 2005). This species lay their eggs on stones or pieces of wood, exhibiting biparental care for the eggs and fry (Axelrod, 1993; Ruiz et al., 1992).

Australoheros facetus in Portugal is defined as an introduced species by the Decreto Lei nº 565/99. Despite its wide distribution and high numbers in certain spots (as discussed in chapter 2 of this thesis) it is not considered an invasive species, which only discriminate *Lepomis gibbosus* and *Gambusia holbrooki* as invasive among the non-native freshwater fishes in Portugal. However, some of its physiological and behavioural traits (that will be discussed in detail in this thesis) led us to suggest *A. facetus* to be recognized as an invasive species in Portugal.

6. Aim and outline of the thesis

This study is especially needed to provide knowledge about the biology of *A. facetus* and its invasive potential as the single cichlid found in natural Portuguese freshwater ecosystems. This species was first referenced in Portugal by Helling (1943), describing a specimen found in Praia de Mira, although this finding in an improbable location is being evaluated (Ribeiro, pers. comm.). More certain is its occurrence in southern Portugal, where the species revealed excellent acclimatization to national rivers and lakes, where it is relatively low abundant along their course/perimeter, despite occasionally found in considerable numbers and high population densities (for examples see Godinho et al., 1997; Pires et al., 2010; Ribeiro & Collares-Pereira, 2010).

The thesis aimed at evaluating the physiological tolerance of *Australoheros facetus* to environmental conditions (as salinity and temperature), so as to gather information about their preferred habitat and the likeliness of spreading into other regions along their distribution in current and future climate conditions. We also aimed to characterize the social and reproductive behaviour and associated physiological mechanisms, and whether such relationships are achieved and maintained by any form of chemical communication.

The policy aim is that this information can be used as future tools to manage the species populations. This research is not only valuable in a local perspective, as it is the first long-term study of this species, but can also create a novel framework to approach conservation efforts of sensible areas. Knowledge on physiology and behaviour of non-native organisms can indeed facilitate the creation of management guidelines, by providing tools to disrupt reproduction or predict the dispersion of invasive species to new habitats, in order to limit the spread of the non-native invasive species as stated by IUCN (IUCN, 2000).

The thesis was divided in eight chapters:

The present **chapter one** is a general introduction of the theme of non-native and invasive species and current knowledge about prevention and control actions. It introduces the bases of physiological plasticity and environmental tolerance that could drive the establishment and spread of non-native species as well the importance of behavioural studies and chemical communication. The use of physiological knowledge to improve the programmes and protocols for management, control and eradication of target species is addressed. Finally, the actual status of *A. facetus* in Portugal, as information about its biology and taxonomic position is given.

Chapter two briefly addresses the presence and abundance of *A. facetus* in inland waters of Portugal, incorporating an ecological description of the fish fauna in two distinct basins from where the individuals of *A. facetus* were collected. This information is discussed in the frame of a review of the literature showing the presence and impact of *A. facetus* and other non-native species in Southern Portugal.

Chapter three addresses the responses and mechanisms that *A. facetus* utilizes to cope with water temperature changes, as one of the most important abiotic factors for fishes. Groups of fish were acclimated at different temperatures found within the geographical and seasonal ranges and submitted to chronic and acute thermal stress by gradually changing the water temperature. The main objectives were to determine the critical maximum and minimum temperatures viable for *A. facetus* and to analyse its response to temperature by analysing usual physiological and molecular stress markers.

Chapter four was based on predictions of climate change for the Mediterranean basin which indicate more frequent and strong flash floods and longer and more intense drought events in the future, creating new possible pathways of distribution of *A. facetus* in Portugal, as fish may be dragged downstream from their current territories. To assess the invasive potential of *A. facetus* in novel brackish water environments, fish groups were submitted to different exposure periods in several salinities. Growth, behaviour and stress markers (as cortisol and energetic metabolites) were critically analysed and used to discuss the physiological potential to reach, survive and colonize new areas as upper estuaries.

Chapter five studied the behavioural traits of *A. facetus* given the importance that behavioural traits can have in invasion biology. As with other cichlids, *A. facetus* forms groups show social hierarchy which leads to stable pair formation and the couple engages in parental care. Observations of hierarchy, pair formation and parental care were utilized to create an ethogram for this species. To search for environmental and physiological cues leading to this behaviour, the hormonal profiles of territorial and non-territorial animals were evaluated before and after hierarchy formation, during different months.

Chapter six was a first approach to understand if social individuals of *A. facetus* communicate with each other during hierarchy formation using chemical cues, as already demonstrated in other cichlids. The involvement of putative odorants present in intestinal and bile fluids of territorial and non-territorial males was analysed using an electro-olfactogram (EOG). The exposure of naïve fish to fluids from fish of both statuses indicated that fluids from territorial males evoked the stronger EOG responses.

Chapter seven devised a freshwater Fish Invasiveness Scoring Kit (FISK) and an Aquatic Species Invasiveness Scoring Kit (AS-ISK) based on the new information described in this thesis. The objective was to review the species status, updating the information provided by Almeida et al. (2013), and briefly discussing the validity and the strengths and weaknesses of these methods to evaluate invasive potentials.

Chapter eight summarized and discussed the main results from the thesis in light of the previous and novel information, and made final considerations for future research and suggestion of control measures.

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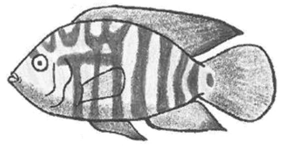
CHAPTER TWO

PRESENCE OF THE NON-NATIVE CICHLID *Australoheros facetus* (JENYNS 1842) IN SOUTHERN PORTUGAL



Presence of the non-native cichlid A*ustraloheros facetus* (Jenyns 1842) in southern Portugal

Abstract

Invasive species are known as one of the main cause of native fauna decline worldwide, not just because of the potential predation or competition, but also because of the changes in habitats that those species can cause. The aim of this work is to demonstrate the notable increase in the presence of the neotropical freshwater cichlid *Australoheros facetus* in Southern Portugal, in abundance, biomass and dominance in fish assemblages. According to the current environmental legislation, this cichlid is classified as 'non-native' in Portugal, but in some areas, it receives the same status as the native ones (Decreto-Lei nº 565/1999). To achieve this, we conducted electrofishing in Arade and Guadiana basins, in October/2014 and November/2015. All fishes were identified and weighted. As result a total of 1990 fishes were collected, being *A. facetus* the most collected species with 1433 individuals, with a ponderal index varying from ~69% to ~98%. The data presented here, however, suggests this fish to be an invasive freshwater species due its strong presence in the streams analysed. From this perspective, new protocols for control of this species should be implemented as soon as possible.

Key-words: Iberian Peninsula, assemblage structure, community ecology, abundance, Biodiversity

1. Introduction

In the middle of 19th century, international transfers of fish species in Europe increased quickly, especially for sports purposes and additional supply of food. After the end of World War II, the number of introductions of non-native fish species increased even further, aided by the development of advanced artificial reproductive techniques (Elvira, 2001; Ribeiro et al., 2009). In addition, it is suggested that climate change facilitates the invasion of non-native species, by changing the discharge of rivers, increasing water temperature and frequency of floods and severe droughts, increasing its impact on natural communities (Rahel et al., 2008; Rius et al., 2014). Torrential rains and floods can additionally create connections between rivers and non-native fish farms, thus increasing the propagule pressure of such species (Copp, 2006; Fobert et al., 2013). The propagule pressure is recognized as a major factor for the establishment of a non-native species and it is expressed by the number of individuals used in the introduction and how many times the species was introduced to remain self-sufficient in the new environment (Colautti & MacIsaac, 2004).

Certainly, endemic species are among the most affected by the introduction of new species since, by definition, its occurrence is restricted geographically and usually are at risk against stochastic events (Bonn et al., 2002; Godinho et al., 1997; Pires et al., 1999). Therefore, the entry of a new species, creating new competition and predation pressure, and the possibility of contact with new diseases and parasites, can lead to the decline of endemic species.

The Mediterranean bioclimatic region in Europe has circa 91 freshwater fish species, being 17 non-natives (Ferreira et al., 2007). The Iberian Peninsula, as part of that region, has a unique freshwater ichthyofauna, mostly because its biogeographic isolation by the Atlantic Ocean and the Pyrenees, characterized by the low number of families and the highest percentage of endemic fishes in Europe, that is almost 30% of native species (Almeida et al., 2013; Oliveira et al., 2012). Furthermore, it has a long history of non-native fish introductions, currently accounting for around 23 species, mainly with ornamental or fishery purposes (Leunda, 2010; Ribeiro & Leunda, 2012). In the Mediterranean-type rivers of the Iberian Peninsula, one of the factors most strongly linked to the presence and dominance of non-native species is the highly variable water regime (Bernardo et al., 2003; Collares-Pereira et al., 1998; Pires et al., 1999). The diversity of the fish fauna is often related to hydrological variability, especially the annual rainfall and habitat change after hydrological alterations, particularly current velocity and substrate type (Matono, Bernardo, Oberdorff, et al., 2012).

In Portugal circa 34 freshwater fish species have been recorded, of which 36% are endemic and 23.5% are non-natives (Ferreira et al., 2007). Most rivers in Portugal, especially in the south, are temporary and intermittent during the dry season, often losing their connectivity and directly influencing the composition and structure of the fish assemblage (Godinho et al., 1998; Godinho et al., 1997; Vila-Gispert et al., 2005). Still, the smaller the size of the river, the more it will be affected by hydrological changes. Some studies have shown that drastic reductions in river flows pose major negative impacts on fish populations, often making them susceptible to dominance by non-native species (Bernardo et al., 2003; Gehrke & Harris, 2001).

Australoheros facetus in Portugal

Although several reports point out that most of the introduced fishes found in Europe are native from North America (Marr et al., 2010; Ribeiro & Collares-Pereira, 2010; Ribeiro et al., 2009; Ribeiro et al., 2008), *Australoheros facetus* (Jenyns, 1842) is a neotropical Perciform cichlid native from South America, specifically in Paraná, Paraguay and Uruguay basins, including some coastal drainages (Bruno et al., 2011; Rícan & Kullander, 2006, 2008), and occurring also as a non-native species in Chile (Ruiz et al., 1992).

Australoheros facetus is a freshwater fish with bentho-pelagic habits, diurnal and opportunistic feeding behaviour and laying their eggs on stones or pieces of wood, exhibiting biparental care for the eggs and fry (Axelrod, 1993; Baduy et al., 2017; Ribeiro et al., 2007; Ruiz et al., 1992).

Most probably the principal cause of the dispersion n of *A. facetus* in the wild rivers of the Iberian Peninsula is translocation by human hands (Ribeiro et al., 2009). The first record places the species in the Vouga River drainage in 1943 (Helling, 1943), 300km north of its current distribution, despite no new records of this species were done there. The species is currently found in the Guadiana rivers and streams, (Collares-Pereira et al., 2000; Elvira & Almodovar, 2001; Hermoso et al., 2008; Ribeiro et al., 2009). Its presence in Mira basin is cited in Decreto-Lei nº 565/1999, however no scientific data was found to corroborate this location (Figure 1). However, its presence in Foupana and Guadiana was reported by locals to date at least from the 1970s, when it was fished using baskets placed in shallowed waters and fried for human consumption (personal observation). *A. facetus* was also recorded in Alqueva reservoir in 2003 (Ribeiro et al., 2006), and in 1997, it was recorded in the Odelouca stream (Pires et al., 2004; Pires et al., 2010).

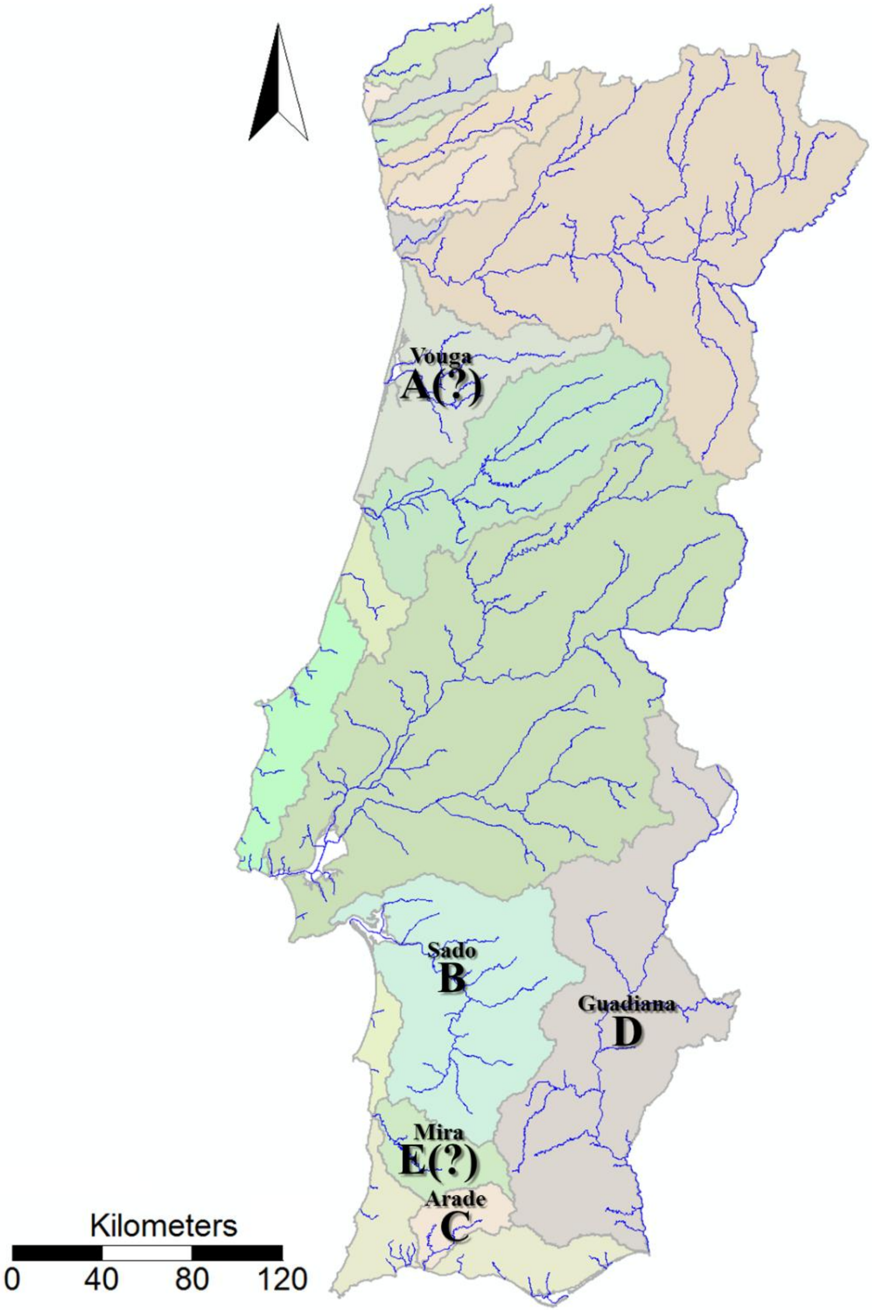


Figure 1. Distribution of *A. facetus* in Portugal per basin: A) Vouga Basin (Helling, 1943); B) Sado Basin (Alexandre et al., 2012; APA, 2012; CIMAL; Decreto-Lei, nº 565/1999; Matono, Bernardo, Oberdorff, et al., 2012; Oliveira et al., 2007); C) Arade Basin (INAG, 2009; Pires et al., 2004; Pires et al., 2010); D) Guadiana Basin (Bernardo et al., 2003; Decreto-Lei, nº 565/1999; Godinho et al., 1997; Matono, Bernardo, Oberdorff, et al., 2012; Oliveira et al., 2007; Pires et al., 1999; Ribeiro & Collares-Pereira, 2010); E) Mira Basin (Decreto-Lei, nº 565/1999). Map adapted from: *Sistema Nacional de Informação de Recursos Hídricos* (SNIRH/Portugal).

In Portugal, besides the initial problems with ascribing the correct taxonomy to actual species that was introduced, Almaça (1995), after analysis of Portuguese samples stored at Museu Bocage and samples of *Cichlasoma facetum* stored at Muséum National d'Histoire Naturelle (Paris), changed the species found in Portugal, earlier described as *Cichlasoma severum* (= *Heros spurius*) to *Cichlasoma facetum* (valid name: *Australoheros facetus* (Jenyns 1842), Eschmeyer et al. (2017)).

It is important to note that even after the work by Rícan and Kullander (2006), that described the *Australoheros* genus, some reports in Iberian Peninsula still refer to this species as *Herichtys facetum* (Ilheu et al., 2014; Matono, Bernardo, Ferreira, et al., 2012; Matono, Bernardo, Oberdorff, et al., 2012).

Currently, the legal status of *A. facetus* in Portugal is as non-native species (Decreto-Lei nº565/99, published in Diário da República – I Série-A).

The main goal of the present study is to show the increasing presence of *A. facetus* in Southern Portugal. To achieve this purpose, field collections were carried out in two distinct basins in Southern Portugal (Arade and Guadiana), during the autumns of 2014 and 2015. In addition, we performed of a literature review on the composition and abundance of fish assemblages in Portugal, focusing on the regions with known presence of *A. facetus*.

2. Material and Methods

2.1. Study area

Sampling took place at Guadiana Basin (localized NE of Portugal, 37.528472º, -7.523783º as sampling point P1 – Vascão River, and 37.387361º, -7.527000º as P2 – Foupana River), and two points at Arade Basin, both at Odelouca River (37.22700º, -8.505611º, as P3; and 37.237139º, -8.486917º, as P4), during October/2014, (corresponding to the end of the dry season) and November/2015, already in the wet season (Figure 2).

For all analysis P1 and P2 were summed and from this point will be identified as Vascão microbasin (VAS, Figure 3a and b) and P3 and P4 will be identified as Odelouca microbasin (ODE, Figure 3c and d). Both these regions are influenced by the Mediterranean climate, with an irregular annual hydrological regime that can induce severe droughts during summer and flash floods during autumn and winter (Pires et al., 2004; Pires et al., 2008; Ribeiro & Collares-Pereira, 2010).

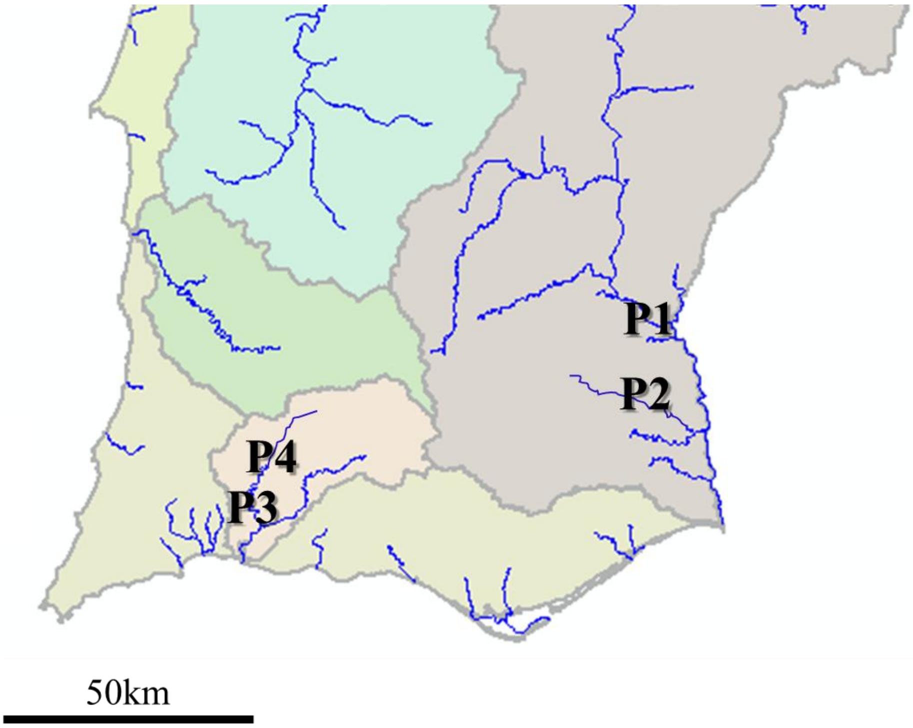


Figure 2. Sampling points: P1 and P2 in Guadiana Basin (VAS), P3 and P4 in Arade Basin (ODE). Adapted from *Sistema Nacional de Informação de Recursos Hídricos* (SNIRH).

The Guadiana Basin has a total drainage area of 66960 km^2, and is sparsely populated. The basin geology is highly impermeable and flow is strongly dependent on the seasonal variation of rain (Bernardo et al., 2003; Ribeiro & Collares-Pereira, 2010), although the construction of the Alqueva dam, completed in 2002, introduced some sort of flow regulation. The Vascão and Foupana Rivers are tributaries of the Guadiana, downstream of the dam and their flow is highly variable throughout the year. Vascão is the longest Portuguese rivers without any artificial interruptions and the part of its course and surroundings is a ‘Ramsar site’ for the protection of Wetlands of International Importance.

The Arade Basin drains an area of 987 km^2, and its tributary, the Odelouca River is moderately influenced by human activities but with some pristine habitats and with heterogeneous riparian vegetation (Pires et al., 2004; Pires et al., 2010). The Odelouca river is also constrained by a dam, which was closed in 2009, and some habitats were altered to build flood safety areas and banks.

Both the Guadiana and the Arade have important estuarine areas before they merge with the sea.

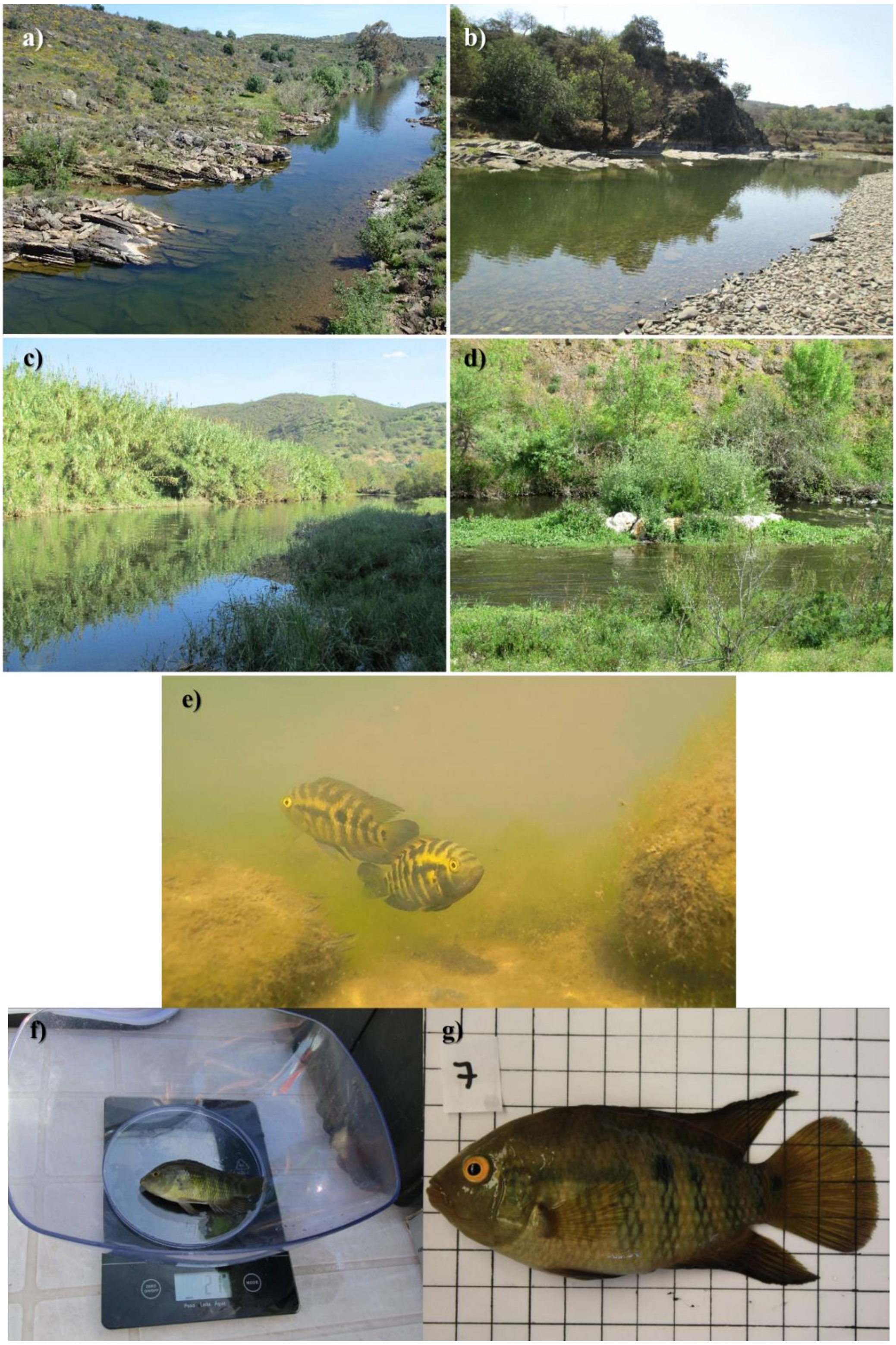


Figure 3. Collecting sites (a) and (b) Vascão River; (c) and (d) Odelouca River; (e) couple protecting their fry in wild; (f) and (g) measurements.

2.2. Abiotic data

Measurements of environmental data were performed in situ: water temperature, salinity, pH, DO (dissolved oxygen) and conductivity were measured with a ThermoFisher Scientific Orion Star^{TM} A329, water depth was measured with a graduated scale and superficial water velocity was measured by means of the displacement velocity of a floating object – to create a profile of each sampling site. All the procedures were repeated three times in order to create a mean value (Table 1) for each site. Data for rainfall in the region during the sampling and historical mean for the months was collected from *Sistema Nacional de Informação de Recursos Hídricos, Portugal* (available at http://snirh.apambiente.pt).

2.3. Fish sampling

The collections were performed by electrofishing, using a Hans-Grassl ELT60II generator 300/500 V and 1300 watts pulse DC were used, with the same field crew operating across sites (capture licenses numbers 403, 404, 405 and 406/2015/CAPT, issued by the ICNF). Stop-nets were stretched across the stream to prevent fish from swimming out of the sampling area and the sampling was done in an upstream direction. In narrow streams (relative to the span of the electrodes), fish are captured efficiently and absolute measures of abundance may be generated (Kietzmann et al., 2002; Mazzoni et al., 2000).

The native species were identified, counted and weight and then returned alive to the river. The specimens of *A. facetus* (Figures 3e, f and g) and other non-native species were weighed, measured and placed in ventilated containers and transported to the experimental facilities of the Centre for Marine Sciences (CCMAR) at the University of Algarve, in Campus de Gambelas. CCMAR facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal) in accordance to the three 'R' policy and national and European legislation.

2.4. Data analysis

To better characterize the dynamics of the fish assemblages related to abiotic conditions and sampling sites, Pearson correlations and a canonical correspondence analysis (CCA) were used. This method was designed to extract synthetic environmental gradients, that are used to describe and to visualize habitat preferences via an ordination diagram (ter Braak & Verdonschot, 1995). The model was tested with a Monte Carlo test (500 permutations). A non-linear ordination method was selected as a preliminary detrended fluctuation analysis showed that the species' responses to the environmental gradients had a non-linear distribution. For the Pearson correlation and CCA analysis, the data was transformed as $y = Ln\ (y + 1)$ to remove the effect of the different scales of the abiotic data, and to reduce the influence of species in extremely high abundance we used the NPUE value transformed as $y = Ln\ (y + 1)$ (NPUE: number of individuals per unit effort, details of how it was calculated are described below). For the CCA, species that occurred in less than 3 sites were removed, in pairs of abiotic variables that had $r > 0.8$ and $p < 0.05$ one of the data was excluded. Salinity was also excluded because the variability found among the sampling sites was biologically negligible. For sequential Pearson correlations among the species and abiotic variables, the obtained p-value were adjusted using the Bonferroni correction.

Fish assemblages were analysed through number of species, composition, richness and abundance. The total number of species in each sampling point is represented by *S*. To compare the number of species of the different assemblages it was used the rarefaction method. The rarefaction (*E(S)*) is the number of species expected in a random sample of size *n* individuals as the sum of probabilities that each species will be included in the sample (Ludwig & Reynolds, 1988). For the rarefaction, the equation used was: $E(S) = \sum_{i=1}^{S} \left\{ 1 \left[\binom{N - ni}{n} / \binom{N}{n} \right] \right\}$, where N is the total number of individuals in the sample, ni is the number of individuals of a species i and n is the standard number of individuals (the minimum number of individuals among the samples to be tested) (Hurlbert, 1971). This index is usually used to give a better way to compare samples with different sizes if they had the same number of individuals.

The Shannon's diversity index (*H'*) is a measure to analyse the α-diversity of an assemblage and was used to show the numerical participation of the species in the assemblage indicating its uniformity (Odum & Barrett, 2005). Shannon's diversity gives more weight to rare species and the equation is as follows: $H' = -\sum_{i-1}^{s}(Pi \times LnPi)$, where i: species i; Pi: importance of each species (% of abundance). To test for differences in *H'* between sampling sites and years was used a Student t-test described by Magurran (1988). The increase in the species' number and uniformity of species' abundance increases the diversity.

The maximum diversity expected to occur in an assemblage was calculated by: $H'_{MAX} = LnS$ (Odum & Barrett, 2005). The Shannon's equitability index (E) serves as a benchmark for assessing the value of a diversity index. In its calculation, the calculated value of diversity is compared with the theoretical maximum value. The greater the difference between the calculated value and the theoretical value, less equitable is the sample. The calculation follows the equation: $E = \frac{H'}{H'_{MAX}}$. Equitability assumes a value between 0 and 1 with 1 being complete evenness. Equitability will be low when there are few highly dominant species among many rare species.

To characterize the differential diversity, i.e. how much the sampling points are different from each other in relation to their species composition and/or abundance, the Jaccard's similarity index and the Bray-Curtis percent of similarity index were applied.

The Jaccard's similarity index (*J*) is based on presence/absence of species between sampling sites and years and followed the equation: $J_{XY} = \frac{a}{a+b+c}$; where *a*: the number of species occurring in both samples; *b*: number of species occurring in sample *X* only; *c*: number of species occurring in sample *Y* only (Beeby & Beeby, 2013).

Furthermore, the Bray-Curtis percent of similarity index (PS) based in abundance of species was applied, using the equation: $PS_{XY} = \left\{\frac{(2\times\sum_{i=1}^{S}[MIN(ix.iy)])}{(\sum_{i=1}^{S} ix+\sum_{i=1}^{S} iy)}\right\} \times 100$, where *ix* is the abundance of species *i* at point *X* and *iy* is the abundance of species *i* at point *X*. If the pair of samples to be tested have identical species abundances, their similarity is complete, i.e. *PS* = *100%* (Ludwig & Reynolds, 1988).

One way to measure the abundance of fish sampled is catch per unit effort CPUE, that standardizes the abundance per fishing effort, in this case volume of water sampled by electrofishing per hour. It was calculated for the abundance of each species (NPUE: number of individuals of each species/m^3/hour) and for the biomass of each species (BPUE: total weight of each species in g/m^3/hour).

The ponderal dominance index establishes which species have outshined in each sampling (Resende et al., 2014), considering the contribution of the number of individuals captured and the corresponding biomass. It was calculated by: $ID(\%) = \frac{(Ni\times Wi)}{\sum(Ni\times Wi)} \times 100$, where Ni is the number of individuals in each species and Wi is the total weight of each species.

All analysis were carried out with SigmaPlot 12.5, Microsoft Excel and XLStat-Ecology software.

3. Results

Rainfall was higher in the second year, which in turn increased the flow velocity of the water (Table 1). October of 2014 in Southern Portugal had levels of rainfall below the historical mean for the region (October/14: VAS = 8.3 mm, October historical mean = 58 mm; ODE = 0.3 mm, October historical mean = 87 mm). While in Vascão microbasin the rainfall was below the historical mean in November of 2015, in Odelouca microbasin the rainfall was above the historical mean (November/15: VAS = 36.7 mm, November historical mean = 75 mm, ODE = 143.6 mm, November historical mean = 102 mm).

A great difference was also observed in water temperature that decreased from October/2014 to November/2015 (Table 1). However, no significant correlation was found among species, among abiotic variables, or among the species and the abiotic variables (Pearson correlations, $p > 0.05$).

Table 1. Mean values of the abiotic data for the sampling points. Rnf: rainfall; Vel: superficial water velocity; Temp: water temperature; Sal: salinity; DO: dissolved oxygen; Cond: conductivity. Mean values are presented.

Year	Point	Rnf mm	Vel cm/s	Temp ºC	Sal ppt	pH	DO		Cond µS/cm	Depth m	Time h	Area m^2
							mg/L	%				
2014	VAS	8.3	2.0	24.8	0.20	7.5	8.7	105	404	1.0	2.0	600
	ODE	0.3	2.6	23.8	0.20	7.2	8.3	115	347	0.8	1.1	654
2015	VAS	36.7	6.3	13.8	0.19	7.8	10.6	101	312	0.3	0.9	435
	ODE	143.6	8.6	17.1	0.17	7.0	8.5	87	257	0.8	1.0	605

Rainfall data from *Sistema Nacional de Informação de Recursos Hídricos, Portugal* (SNIRH).

Due to the distribution of the data, the CCA-PLS method was selected (ter Braak & Verdonschot, 1995). The results showed that the constrained inertia explained little of the variation found (11.63%). The Monte Carlo test (pseudo-F = 0.26, p = 0.83) corroborating the preliminary detrended fluctuation analysis, showed that the species are not linearly related to the abiotic variables.

A total of 1990 fishes were collected, distributed in *S* = 12 species, being *A. facetus* (1433 individuals) the most collected species followed by *Cobitis paludica* (113 individuals) and *Lepomis gibbosus* (105 individuals), accounting for 6 non-natives and 6 native species (Table 2). The highest total abundance in 2014 and 2015 was observed in Odelouca, with 592 and 633 individuals collected, respectively (Vascão had 317 in 2014 and 448 in 2015).

Table 2. Fish composition and abundance during collections in 2014 and 2015. E: non-native; N: native.

Species	Code	Status	2014		2015		Total
			VAS	ODE	VAS	ODE	
Australoheros facetus	AFA	E	183	474	287	489	1433
Cobitis paludica	CPA	N	0	30	13	70	113
Lepomis gibbosus	LGI	E	94	4	7	0	105
Luciobarbus sp;	LUC	N	15	2	86	0	103
Squalius sp	SQU	N	5	43	31	8	87
Anguilla anguilla	AAN	N	7	22	20	33	82
Unidentified nases	BOG	N	0	3	4	27	34
Liza ramada	LRA	N	7	10	0	6	23
Carassius auratus	CAU	E	0	4	0	0	4
Micropterus salmoides	MSA	E	4	0	0	0	4
Cyprinus carpio	CCA	E	2	0	0	0	2
*Gambusia holbrooki**	-	E	-	-	-	-	-
			317	592	448	633	1990

*Due to the high amount of the non-native *Gambusia holbrooki* during all samplings, this species was excluded from the analysis.

The rarefaction, standardized for n = 317, presented a very similar number of species expected of that observed during sampling (Table 3).

Considering all species, the Shannon’s diversity index was far below of that expected for each point (Table 3). Vascão had higher diversity than Odelouca, in 2014 and in 2015 (VAS2014 and ODE2014 Student t-test = 4.00, dF = 771.41, $p < 0.001$; VAS2015 and ODE2015 t = 4.67, dF = 985.05, $p < 0.001$; VAS2014 and VAS2015 $p > 0.05$; ODE2014 and ODE2015 $p > 0.05$).

The Shannon’s equitability index, considering all species, showed that VAS had a fish assemblage more balanced between fishes’ species and their abundances than ODE (Table 3). But, when analysing the assemblages excluding the non-native species, there were no differences among the assemblages, and the equitability of each assemblage increased, highlighting the disruptive effect the non-native species ($p > 0.05$; Table 4).

Table 3. Ecological indexes of the sampling points with all species considered. *S*: number of species; *E(S)*: rarefaction method; *H'*: Shannon's diversity index; H'_{MAX}: maximum Shannon's diversity index expected; *E*: Shannon's equitability. Asterisks denote significant differences, Student t-test between *H'* of Vascão and Odelouca microbasins ($p < 0.001$).

	S	*E(S)*	*H'*	H'_{MAX}	*E*
2014					
VAS	8	8.00	1.14	2.08	0.55
ODE	9	8.59	0.83*	2.20	0.38
2015					
VAS	7	6.99	1.13	1.95	0.58
ODE	6	5.98	0.82*	1.79	0.46

Table 4. Ecological indexes of the sampling points excluding the non-native species. *S*: number of species; *E(S)*: rarefaction method; *H'*: Shannon's diversity index; H'_{MAX}: maximum Shannon's diversity index expected; *E*: Shannon's equitability.

	S	*E(S)*	*H'*	H'_{MAX}	*E*
2014					
VAS	4	4.00	1.29	2.08	0.93
ODE	6	5.18	1.43	2.20	0.80
2015					
VAS	5	4.60	1.22	1.95	0.76
ODE	5	4.70	1.30	1.79	0.80

The Jaccard's similarity index based just in the presence/absence of species showed highest similarity between the sampling of ODE/14 and ODE/15, similarly when taking in account the abundance of species, using the Bray-Curtis percent of similarity index, ODE/14 and ODE/15 also had the highest similarity (Table 5). Excluding the non-native species Jaccard's similarity rank had a slight increase but considering the abundance of the species the similarity between sites increased (Table 6)

Table 5. Jaccard's similarity index (*J*), based on the presence or absence of species between sampling points, and Bray-Curtis percent of similarity (*PS*), based on the species' abundance.

	J: presence/absence			*PS*: abundance (%)	
↑ Similarity ↑	ODE/14 x ODE/15	0.67	↑ Similarity ↑	ODE/14 x ODE/15	90.29
	VAS/15 x ODE/15	0.63		VAS/15 x ODE/15	79.74
	VAS/14 x ODE/14	0.60		VAS/14 x VAS/15	64.58
	VAS/14 x VAS/15	0.50		VAS/14 x ODE/14	55.23

Table 6. Jaccard's similarity index (*J*), based on the presence or absence of species between sampling points, excluding the non-native species, and Bray-Curtis percent of similarity (*PS*), based on the species' abundance.

	J: presence/absence			*PS*: abundance (%)	
↑ Similarity ↑	ODE/14 x ODE/15	0.83	↑ Similarity ↑	VAS/15 x ODE/15	91.95
	VAS/15 x ODE/15	0.67		VAS/14 x ODE/14	75.00
	VAS/14 x ODE/14	0.67		VAS/14 x VAS/15	54.26
	VAS/14 x VAS/15	0.50		ODE/14 x ODE/15	44.09

The number of individuals per effort (NPUE) was higher for AFA through all samplings (NPUE VAS/14 = 0.31 individuals/m^3/hour; ODE/14 = 0.87; VAS/15 = 2.03; ODE/15 = 0.98; Figure 4A). Another important species was LUC, in VAS/15, with 0.61 individuals/m^3/hour (Figure 4A). The total NPUE for sampling sites was higher in 2015 than in 2014 (VAS/14 = 0.53 individuals/m^3/hour; ODE/14 = 1.08; VAS/15 = 3.17; ODE/15 = 1.27).

Considering the species biomass (BPUE), the highest value in VAS/14 was for LUC with 4.57 g/m^3/hour, while AFA had the highest values in the other samples (BPUE VAS/14 = 2.31 g/m^3/hour, ODE/14 = 5.38; VAS/15 = 6.50 and ODE/15 = 5.11; Figure 4B). LUC had the second biggest BPUE at ODE/14 with 1.08 g/m^3/hour and at VAS/15 with 4.98 g/m^3/hour; LRA had the second biggest value at ODE/15 with 3.11 g/m^3/hour (Figure 4B). The highest total BPUE recorded occurred at VAS/15 with 15.47 g/m^3/hour (VAS/14 = 10.34 g/m^3/hour; ODE/14 = 8.78; ODE/15 = 10.62).

The ponderal index pointed out the importance of AFA in the assemblages with the highest values (VAS/14 = 69.4%; ODE/14 = 98.1%; VAS/15 = 79.3% and ODE/15 = 95.7%, Figure 4C). LGI was the second most important species in VAS/14 with 17.4%, followed by LUC with 11.2. In VAS/15 LUC was the second most important species with 18.2%. The other species all presented a ponderal index lower than 2% each (Figure 4C).

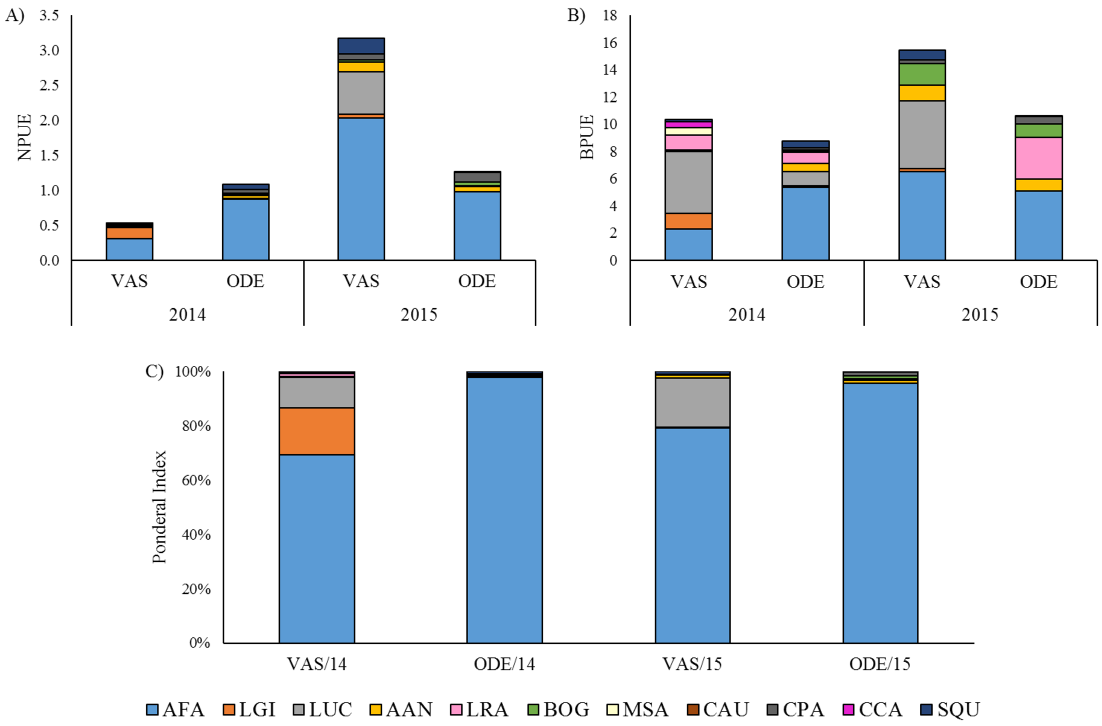


Figure 4. Catch per unit of effort: (A) NPUE: number of individuals of each species/m^3/hour; (B) BPUE: total weight of each species/m^3/hour, and (C) ponderal dominance index of each species.

4. Discussion

This study analyzed the main ecological indexes used in community ecology to describe the freshwater fish assemblage of two different basins in Southern Portugal. The main results highlight the increasing abundance of the non-native cichlid *A. facetus.*

The freshwater bodies of Iberian Peninsula have long been affected by human activities such as irrigation, sand extraction, urbanization, tourism and introduction of non-native species (Godinho et al., 1997). In the region studied these impacts had an addition after the construction and now operation of the Alqueva dam, in Guadiana River (circa 107 km from the nearest derivation to P1, located 1.4 km upstream from the mouth into Guadiana, and 133 km the derivation to P2, or in a straight line 74 km and 90 km respectively), and the smaller Odelouca dam (circa 13.5 km from P3 and 8.3 km from P4, which are 9.2 and 14.4 km respectively from the mouth into Arade river). The decrease in the river flow caused by dams affects directly the aquatic community (e.g. reducing the availability and quality of habitats for food or reproduction), or indirectly, favoring the introduction, and enhancing the propagule pressure and spread of non-native fishes (Alexandre et al., 2012; Hermoso et al., 2011; Hermoso et al., 2012).

In fact, the presence of non-native fish has been reported to be related to flow, as well as high water temperature, conductivity and depth (Pires et al., 1999). Nonetheless, in the present study, no correlations were observed among the species abundance (NPUE), non-natives or natives, and the abiotic variables tested, although this result could be a reflection of insufficient data collected.

The Iberian fish fauna is rich in endemism but the number of native fish species per site is usually low (Ilheu et al., 2014; Matono, Bernardo, Ferreira, et al., 2012). The number of species found in the present study, even after the rarefaction method that did not show differences from the expected *vs*. the observed number, is in accordance with other studies performed in Southern Portugal: usually 5 to 12 native species and from 0 to 8 non-native species (Bernardo et al., 2003; Godinho et al., 1997; Mesquita et al., 2006; Pires et al., 2008).

Considering the non-native species, Vascão had a greater Shannon’s diversity index than Odelouca (VAS around 1.13 and ODE around 0.82). However, excluding the non-native species there was no difference between sites, showing the great impact that non-native species can have in these fish assemblages, since the diversity index was biased by the high abundance of *A. facetus* in both sites. Few studies in the region used this index, which could be useful to compare different ecosystems and better adjust management protocols developed somewhere else. In other study for Guadiana tributaries, the Shannon’s diversity index varied from 0.3 to 1.9 (Pires et al., 1999), and it was around 0.3 for Arade basin (Matono, Bernardo, Ferreira, et al., 2012). However, these studies did not refer the equitability of the samples. It is important not just to know how many species and individuals occur in determined site but also how the number of individuals are distributed among the species and how far from the expected diversity the site is. This was especially true in this study, where the non-native *A. facetus* and *L. gibbosus* had high abundances, and where we observed a great increase in equitability when the non-native species were removed from the analysis.

The two measures of differential diversity, Jaccard’s similarity index (based on presence/absence of species) and Bray-Curtis percent of similarity (based on abundance of each species), gave some quite different responses, but with similarities between Vascão and Odelouca that were above 55%. These results were to be expected when considering that the few species that differed between the sites were in low abundance

(*Carassius auratus* with four individuals in Odelouca; *Micropterus salmoides* with four individuals in Vascão and *Cyprinus carpio* with two individuals in Vascão), and in a general way the fish fauna described here is in accordance with other publications for Southern Portugal, as cited below.

While working with environmental drivers and human pressure related to the presence and abundance of non-native species, in 380 sampling sites in continental Portugal, Ilheu et al. (2014) found 10 non-natives species within the 41 species in total, and just a minor presence of *A. facetus* (named as *Herichthys facetum*), being the most collected fish the non-native and invasive *Lepomis gibbosus* (with 0.89 individuals/m^2). An important difference from *A. facetus* to all others non-native species in the cited study, was that *A. facetus* was not correlated with disturbed or non-disturbed sites, while the other non-native species were more abundant from medium to maximum disturbed sites. Similar results were found by (Matono, Bernardo, Oberdorff, et al., 2012), that collected eight species of non-native fish within the 19 collected in Guadiana and Sado basins, and also observed that non-native fishes occurred mainly in the most disturbed areas by human activities, being dominant in number of individuals in some sampling points; *A. facetus* was in low frequency of occurrence, unfortunately the authors did not inform about its abundance.

In the present study, *A. facetus* was the most collected species with a total number of 1433 individuals, 12-fold more than the second most collected species. The great number found could be related to the fact that familiar groups aggregate in relatively small areas, being easy to catch dozens of young individuals with less than 3 cm of body length at each time (personal observation). Ribeiro and Collares-Pereira (2010), found a greater abundance of non-native than native species, with *L. gibbosus* in the top (with 65% of total fish collected in Guadiana river, 112 individuals/100m^2), and with some marked presence of *A. facetus*, especially in Ardila river (19 individuals/100m^2); while in other study performed by Ribeiro et al. (2007) in Vascão river, *A. facetus* were most abundant the non-native species (13 individuals/100m^2).

There are some inconsistencies in literature about the dominance (presence and abundance) of non-native fishes in Portugal. On one hand, for example, Matono, Bernardo, Ferreira, et al. (2012), analyzing fish assemblages of continental Portugal found that less than 2% of fish density collected was from non-native species. On the other hand, among the five most caught species (CPUE) by Godinho et al. (1997) in the lower Guadiana basin, three were non-native and *A. facetus* was the fifth most collected with 209 individuals in total (CPUE mean of 6.8 individuals/30 minutes).

Fish assemblages in streams under Mediterranean climate influence, are usually composed rather by the ability of resilience of each species (migration, recolonization) than the local features of the habitats (Magalhaes, Beja, et al., 2002). The dominance of native or non-native species of freshwater fish in Portugal seems to be related to season of the year as also the interannual variation of pluviosity (Alexandre et al., 2012; Bernardo et al., 2003). Usually the dry seasons/years are dominated by non-native fishes (Godinho et al., 1997; Ribeiro & Collares-Pereira, 2010), while wet seasons/years are dominated by native species (Magalhaes, Batalha, et al., 2002; Matono, Bernardo, Ferreira, et al., 2012; Pires et al., 2004). This could happen because during the dry seasons, non-native species that typically have an opportunistic diet can have some advantage with the reduction of available habitats (Pires et al., 1999; Ribeiro et al., 2007). During the dry seasons, the fish assemblage is kept confined in pools, with low flow and loss of connectivity, increasing the pressure of competition and predation (even with some terrestrial organisms and the avian fauna). In addition, abiotic conditions are more extreme, with increased water temperature and reduced dissolved oxygen. If the site is already disturbed by human-induced changes, the presence of non-natives can be enhanced, since they are known to be tolerant to various chemical-physical changes in environment (Atwood et al., 2003; Carveth et al., 2011; Gutierre et al., 2016 and chapters 3 and 4 of this thesis). So, in addition to the stress caused by these environmental changes, the native fish have to face the pressure caused by the non-native species, and this impact is increasing with the presence of species that are not yet officially recorded, as the case of several juveniles of *Sander lucioperca* observed in Vascão River (specifically in Monte Vascão, personal observation). In other words, if there is a synergy among dry seasons/years, the presence of non-native species and human-induced environmental changes, the native fish fauna can be severely impacted. As showed in other studies, the high abundance of *A. facetus* in addition to its parental care (Baduy et al., 2017) and plastic environmental tolerance (Chapters 3 and 4 of this thesis) can become a threat to native species with narrower tolerances and limited or no care at all of offspring.

Excluding the non-native species, the Jaccard's similarity rank kept the same trend, with a slight increase in the indexes values. But, considering the species' abundances, the interannual similarity decreased and the similarity between the sites increased. It is expected that the presence of non-native widespread species lead to an increase in similarity of native species between sites or a decrease in β-diversity, which means in short words, homogenization of fauna (Hermoso et al., 2012). It is important to highlight that, independently of presence of non-native species, generally the similarities indexes were high. It was already suggested that the native fish fauna of Mediterranean streams can be resilient to habitat perturbations (as floods, droughts or human pressure) and recover in absence of non-native species, but they are quite sensitive to interactions with non-native species (Hermoso et al., 2012).

As a territorial and aggressive fish that forms complex hierarchies and have high degree of parental care (Baduy et al., 2017), it is reasonable to suppose that this degree of organization places *A. facetus* as a successful competitor against other species that do not show this kind of behaviour. This can lead to the hypothesis that they can severely injury other fish that approach their offspring leading to a local displacement of the native fish fauna. The importance of parental care is also reflected in the high survival rates observed in juveniles, which were an important fraction of the fish collected. Moreover, as *A. facetus* is a generalist feeder (Kottelat & Freyhof, 2007; Ribeiro et al., 2007), its ability to adapt to new environment may be doubled in case of nutriments lack during hard and dry periods. The results from NPUE, BPUE and ponderal index emphasize the notable presence of *A. facetus* in Southern Portugal, especially at Odelouca microbasin with a ponderal index above 95%. As *A. facetus* is characterized as a limnophilic species (Alexandre et al., 2012) and resilient to changes in temperature and dissolved oxygen in water (see chapter 3 of this thesis), the formation of pools during the dry seasons can bust their abundance, even from few individuals as they have an intensive parental care, allowing their offspring to survive. Despite the second sampling performed here (November/2015) occurred in the beginning of the rainy season, there was an increase in absolute and NPUE number of *A. facetus*, while the other exotic species showed a reduction. Unfortunately, this study is just a snapshot of the actual situation and samplings throughout the year and in several other sites are necessary to clarify if this species can keep some stability in its population structure or suffer with the strong rains and intense river currents that can occurs during winter in the region.

Australoheros facetus is qualified as invasive by Leunda (2010) because its predation impact, based mainly in the results published by Ribeiro et al. (2007) related to its feeding habits as a generalist and opportunistic species. Curiously, Ribeiro et al.

(2007) highlight that from their work it is not possible to ascertain about competition or predation risks for the native species. In addition, in an early application of FISK (freshwater Fish Invasiveness Screening Kit) for *A. facetus*, while calibrating the kit for Iberian Peninsula, Almeida et al. (2013) reached the FISK score of 19.3 ± 5.8, classifying it with a medium risk of becoming invasive (with a calibration threshold of 20.25). See chapter 7 in this thesis for further details.

Because it is difficult to eradicate an invasive species, successful management against non-native species should focus on methods to prevent the introduction of new individuals and monitor existing populations, either through campaigns or the development of techniques that inhibit the reproduction of the invasive species into the environment. Data on transport, sale, release and escape of freshwater fish into the wild are needed. Unauthorized and illegal fish introductions should receive more attention from both academic research (e.g. frequency, source and types of transport and releases) and government agencies (García-Berthou, 2007). In addition, new studies should be made in order to confirm the apparent rapid spreading of *A. facetus* in rivers and streams of Southern Portugal.

The data from this study presents direct evidence of a dramatic increase in numbers of *A. facetus* in southern streams. Together with results about its generalist diet (Ribeiro et al., 2007), aggressiveness (Baduy et al., 2017) and high environmental tolerance (see chapters 3 and 4 of this thesis), we suggest an update of the freshwater Fish Invasiveness Screening Kit, and possible inclusion of this species in the list of invasive species in Portugal.

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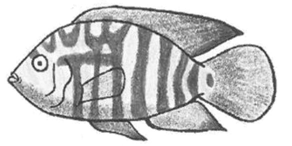
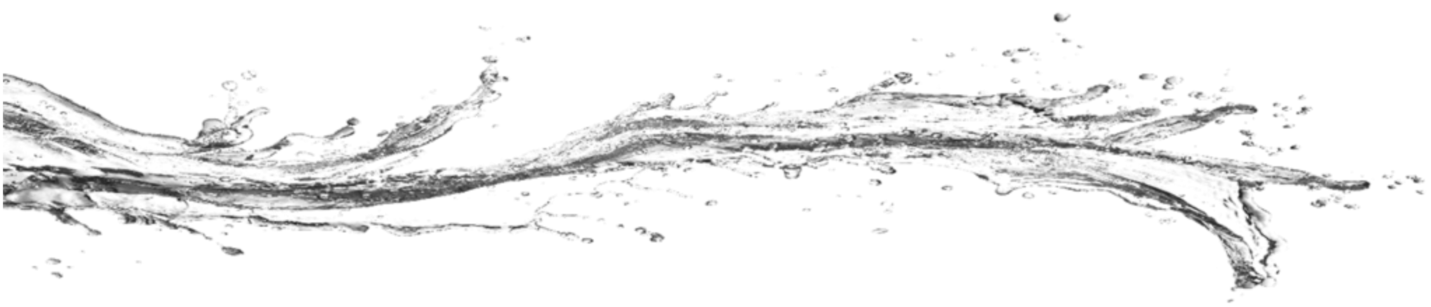
CHAPTER THREE

THERMAL ENDURANCE MECHANISMS IN *Australoheros facetus*, A NON-NATIVE FRESHWATER FISH IN HIGHLY VARIABLE STREAMS OF SOUTHERN PORTUGAL





Thermal endurance mechanisms in *Australoheros facetus,* a non-native freshwater fish in highly variable streams of southern Portugal

Abstract

Environmental temperature is a major determinant of geographic range of ectotherms, as it is responsible for the rate of most of their physiological processes. Thus, most species thrive within a specific thermal range and struggle beyond its limits. Invasive fish may survive in novel territories and expand their range through thermal tolerance mechanisms that allow them to endure severe temperature variations. In this work, we determined the critical thermal maximum (CTMax) and critical thermal minimum (CTMin) for *Australoheros facetus,* a neotropical cichlid found in Southern Portugal, and assessed the temperature effect on physiological mechanisms and stress indicators. Fish initially at 18ºC were placed at 7ºC, 12ºC, 18ºC and 24ºC during 15 days (chronic exposure) to simulate approximate seasonal averages. During this chronic exposure, plasma osmolality was lowest at 7ºC and did not differ among the other temperature groups. Blood cortisol was also significantly lower at 7ºC but, interestingly, circulating glucose, but not lactate, was highest at this temperature. Expression levels of *hsp70* and *hif-1α* genes were highest at 7ºC. Additional groups were heated or cooled from 12ºC and 24ºC at a rate of 3ºC per hour until loss of equilibrium for at least 50% of fish (acute exposure). CTMax and CTMin values were 36.5ºC and 4.5ºC respectively for the 12ºC-acclimated group, and 39.1ºC and 5.8ºC for the 24ºC-acclimated group. Osmolality, glucose and lactate greatly increased upon rapid heating. Surprisingly cortisol showed the opposite pattern, with slightly lower plasma levels in heated *vs* control fish and higher in rapidly cooled groups. Expression levels of both *hsp70* and *hif-1α* genes were also greatly increased by the temperature rise and only slightly modified by water cooling. Taken together these results can be used as indicators for the prediction of fish seasonal and geographical distribution.

Key-words: cichlid, thermal tolerance, stress response, acclimation temperature

1. Introduction

The body temperature of fishes is directly affected by the temperature of their environment (Hasnain et al., 2013), which has direct impacts on their physiology and lifecycle (Brown et al., 2004). Thermoregulatory behaviours can help fish to avoid or find refuge when the environment is not adequate (Sauer et al., 2016). However, when fish are trapped in a pool, as it frequently occurs in the hot and dry summer of Mediterranean regions (Gasith & Resh, 1999), they must rely on thermal regulation to adjust or protect their physiological processes. This is a energetically-demanding process, which influences metabolism and can be reflected in fitness, growth and ultimately survival (Brown et al., 2004).

High environmental tolerance has obvious advantages during invasion and colonization of new habitats (Colautti et al., 2017; Valladares et al., 2014). Addressing physiological markers at different temperatures of acclimation can indicate temperatures more favourable for invasive species to expand their distribution. For example, in marine fish, the latitudinal warm boundary of a species is closely related to the critical temperature observed in laboratory experiments (Payne et al., 2016). The critical temperature (Tcrit) is usually defined as the temperature at which an organism becomes dependent of anaerobic metabolism, mainly due to the reduce availability of dissolved oxygen in water and blood (Ern et al., 2016; Portner, 2010). Above the Tcrit, the continuous increase in temperature can lead to a critical deficiency of ATP (Ern et al., 2016).

The critical thermal maximum and minimum (CTMax and CTMin) are physiological limits used to quantify the upper/lower thermal tolerance, i.e., a measure of the thermal point at which physiological processes start to break down and collapse (Vinagre et al., 2015). They are determined using constant linear temperature changes upward or downward from the acclimation temperature (Beitinger et al., 2000; Cox, 1974). The physiological endpoint usually chosen to determine CTMax and CTMin is the loss of equilibrium (LOE, the inability to maintain itself in the water column.), as it can reflect the temperature at which an organism is unable to escape from predators and forage (Beitinger et al., 2000). In addition, lethal temperatures can also be used as ecological indicators (Gutierre et al., 2016).

Physiological responses to environmental stressors can be classified in three main groups: a) primary response, that include neuroendocrine responses (the production and release of hypothalamico-hypophyseal hormones, catecholamines and corticosteroid hormones); b) secondary response, including physiological responses such as haematological, osmoregulatory, metabolic and differentiated expression of heat shock or stress proteins; c) tertiary response, reflected in changes in performance as growth, reproduction, behaviour and survival (Barton, 2002; Kindle & Whitmore, 1986).

Cortisol is a general stress-response hormone and responsible for the mobilization of metabolic substrates and health balance (Martínez-Porchas et al., 2009; Mommsen et al., 1999), while plasmatic lactate and glucose are an indirect way to measure energy metabolism (Gladden, 2004; Kroon et al., 2017). When the water temperature rises, the oxygen becomes less soluble, hence less available, i.e., the environment becomes hypoxic. The increase in plasma lactate indicates a shift from aerobic to anaerobic metabolism when the temperature increases, which enables the production of ATP at low oxygen (Axenov-Gribanov et al., 2014; Zhang & Kieffer, 2014).

Heat shock proteins (HSPs) act as chaperones, stabilizing not only denatured polypeptides but also new proteins, mediating, formatting and preventing occurrences of cytotoxic aggregates (Basu et al., 2002; Feder & Hofmann, 1999). They are a conservative and ubiquitous group of proteins with constitutive and inducible expression (Feder & Hofmann, 1999; Iwama et al., 1998). Proteins tend to be denatured at high temperature, therefore an increase in hsp70 expression may be an adaptive mechanism for keeping the protein structure intact during chronic and/or acute warming (Iwama et al., 1998; Portner, 2010). At low temperature an increase in the synthesis of metabolic enzymes can be expected to compensate the decrease in the catalytic rate of the enzymes, and this increase may elevate the demand for HSPs (Teigen et al., 2015).

The hypoxia-inducible factor 1 (hif-1) is a transcription factor responsible for inducing the expression of many genes during hypoxia such as those involved in angiogenesis, erythropoiesis, and glycolysis (Koblitz et al., 2015). During normoxia, hif-1α is continuously degraded. However, in the absence of oxygen the degradation of hif-1α is blocked, which allows its accumulation and enabling the transcription of downstream genes (Rytkonen et al., 2011). The mRNA expression levels for *hif-1α* may indicate a limitation of the oxygen available, since this gene is linked to a hypoxia response processes, reflecting the Tcrit (Portner, 2010).

Australoheros facetus, or chanchito, is a neotropical cichlid native to rivers and streams in south-eastern Brazil, Paraguay, Uruguay and Argentina (Rícan & Kullander, 2006). In the Iberian Peninsula, the species was introduced in the wild probably via aquarists and is now found in streams of the Sado and Guadiana basins, in the Arade and Odelouca rivers and in related dams of Southern Portugal (Almaça, 1983, 1995; Doadrio, 2002). Interestingly, in Portugal this species was first reported in the Vouga River drainage in 1943 (Helling, 1943), 300km north of its current distribution.

These riverine systems display wide seasonal amplitude in temperature and flow regimes, a likely deterrent for a sub-tropical fish. It is hypothesized that the main reason why the species survived and established in Mediterranean habitats is its better tolerance to low winter temperatures but also to warm waters during hot dry summers, when compared to native fishes. If true, this invasive fish may use thermal tolerance mechanisms to further expand its geographical distribution, which may constitute an advantage in future climate scenarios.

The main objective of this study was to assess the thermal tolerance of *Australoheros facetus*. We analysed 1) the primary response to heat stress through circulating cortisol levels; 2) the secondary response through plasmatic parameters and gene expression related to energy metabolism and heat-shock responses; and 3) the tertiary response through observations of behaviour and survival.

2. Material and Methods

2.1. Experimental fish

Fish were captured by electrical fishing in the Vascão (37º31'43.38"N and 7º31'26.05"O) and Odelouca (37º13'37"N and 8º30'20"O) rivers during spring and summer of 2015 (capture licence numbers 403, 404, 405 and 406/2015/CAPT), and transported to the experimental facilities of the Centre for Marine Sciences (CCMAR) at the University of Algarve, Campus de Gambelas. Fish were maintained outdoors in three ~2000 L community tanks for at least one-month prior to the experiments, under natural temperature and photoperiod and fed *ad libitum* every morning. Dissolved oxygen and ammonia were monitored and each tank was fitted with an independent biological filter.

Environmental enrichment, acclimation periods, anaesthesia and the three 'R' policy were followed to minimize discomfort. CCMAR facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal) in accordance with national and European legislation.

2.2. Chronic thermal acclimation

Selected fish were transferred to experimental tanks (tank volume = 30 l) in a closed system maintained at the same temperature of the stock tanks, and remained undisturbed during 3 days for acclimation to the new conditions. Changes in water temperature were then performed at a rate of 3ºC/day using a 600 W aquarium heater or a Hailea HC-500A chiller, placed in a separate container with water flow to the tanks, until the designated acclimation temperatures, chosen to simulate seasonal averages, were reached (T_A groups: 7ºC, 12ºC, 18ºC and 24ºC).

An annual mean and amplitude of water temperature was estimated based in personal observations, personal communication (Dr. Filipe Ribeiro, Marine and Environmental Sciences Centre – MARE-FCUL) and data from National Water Resources Information System – Portugal (available in http://snirh.pt/), for Vascão and Odelouca rivers, and National Water Resources Information System – Brasil (http://snirh.gov.br) for the Paraguay basin (where *A. facetus* is native). For the calculations, we used the available data between 2000 and 2016. The annual mean water temperature is 18.6ºC in Vascão (range 6.6 to 31.8ºC), and 18.6ºC in Odelouca (range 10.9 to 26.4ºC), while in Paraguay Basin it is 18.6ºC (range between 11.0 to 28.4ºC).

Photoperiod in the new tanks was set constant at 12L:12D. The acclimation period at each T_A was 15 days and two replicates (tanks) for each group (treatment) were used (N = 20 fish per tank, totalling 40 fish per group). The size of the fish did not vary among experimental tanks (weight = 14.83 ± 0.63 g, Kruskal-Wallis H = 3.57, p = 0.98; standard length = 6.42 ± 0.10 cm, Kruskal-Wallis H = 4.91, p = 0.93).

After 15 days of acclimation, 10 fish per group (5 from each replicate) were anesthetized in 3 L of water from the same tank where they were collected using 0.33% MS-222 and 0.33% sodium bicarbonate. Blood samples were taken using heparinized 1-ml syringes fitted with 26 G needles centrifuged at 5000g for 5 minutes and the plasma collected and stored at -20ºC. Fish were euthanized by cervical section and tissues collected for gene expression analysis were snap frozen in dry ice.

2.3. Acute thermal stress and CTMax and CTMin trials

Additional groups in duplicate (N = 20 fish per tank, N = 160) similarly acclimated during 15 days at 12ºC and at 24ºC were cooled (groups A and B) or heated (groups C and D) at a rate of 3ºC per hour until loss of equilibrium (LOE) (Figure 4). At 50% LOE, 12 fish per group were anesthetized and sampled as described above. The cooling/heating continued in the experimental tanks until the remaining fish showed no more movements, even after physical stimuli. Oxygen levels were monitored throughout the experiment.

CTMax was defined as the temperature of 50% fish LOE above acclimation conditions and CTMin as the temperature of 50% fish move only after physical stimulation below acclimation conditions (adapted from Jobling, 1981).

2.4. Plasma parameters

Cortisol was measured using a radioimmunoassay. Briefly, plasma samples were diluted in phosphate buffer containing 0.5 g l^{-1} gelatine, pH 7.6, and heat-denatured at 70ºC for 30 min. Samples were incubated overnight with fixed amounts of antisera and tritiated cortisol, [1,2,6,7-3H(Hydrocortisone)] (PerkinElmer). Bound and free phases were separated with charcoal and the remaining beta-activity measured with scintillation cocktail (Ultima Gold, PerkinElmer) in a Microbeta Trilux Detector (PerkinElmer). The cross-reactions for the cortisol radioimmunoassay are described in Rotllant et al. (2005)and Guerreiro et al. (2006).

Plasma glucose and lactate were measured in duplicate by enzymatic colorimetric methods and chloride by a chemical colorimetric assay using commercial kits from Spinreact (Glucose-Ref. 1001190; Lactate Ref. 1001330; Chloride-Ref. 1001360; Barcelona, Spain) adapted to 96-well microplates and using a standard curve to compute mean values for each individual. Osmolality was measured with a Vapor Pressure Osmometer, based on a 10 µl sample.

2.5. Isolation and sequencing of candidate genes

Liver samples (n = 6 per group of all groups) were immediately frozen in dry-ice after collection and stored at -80ºC. RNA was extracted from 50mg of each sample following an adapted protocol of Maxwell 16 Total RNA Purification kit (Ref. AS1050). The quantity and quality of isolated RNA was determined by absorbance at 260 and 280 nm using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). RNA samples were treated with DNase (DNA-free kit, Ambion, UK) and cDNA synthesis was carried out in 20 µL reactions containing 500 ng of DNase-treated RNA, 200 ng of random hexamers (Jena Biosciences, Germany), 100 U of RevertAid (Fermentas, Thermo Fisher Scientific, USA) reverse transcriptase and 8 U of RiboLockRNase Inhibitor (Fermentas). Reactions were incubated for 10 min at 25ºC and 60 min at 42ºC, followed by enzyme inactivation for 10 min at 70ºC, and storage at 20ºC until use. cDNA was then amplified by reverse transcription-polymerase chain reaction (RT-PCR) in 20 µL containing 2 µL of cDNA, 0.4 µL of each primer, 0.4 µL of dNTPs and 0.08 µL DreamTaq DNA Polymerase (5 u/µL, Fermentas), in 2 µL DreamTaq buffer. Cycling conditions were 5 min at 95ºC, 40 cycles at 10 s at 95ºC, 10 s at the optimized annealing temperature for each primer pair and 20 s at 72ºC, followed by 5 minutes at 72ºC.

Primers for genes of interest were designed based on conserved regions of multisequence alignments of the cichlids *Cichlasoma dimerus*, *Oreochromis niloticus* and *Haplochromis burtoni*, when available (Table 1). PCRs were performed on liver cDNA and electrophoresed on 2% agarose gel. The gel bands were purified using GFX PCR DNA and Gel Band Purification Kit (28-9034-70) and sequenced in a ABI 3130xl sequencer. The identities of the sequences were verified using BLAST searches against the NCBI nr database with an E-value cut-off of $1E^{-10}$.

Table 1. Sequences of primers used in this experiment.

Target gene	Primer sequence (5´- 3´)
18S	F: TGACGGAAGGGCACCACCAG
	R: AATCGCTCCACCAACTAAGAACGG
β-Actin	F: GCCGTGACCTCACAGACTAC
	R: CCATCTCCTGCTCGAAGTCC
hsp70	F: TCATCTTGGTGGGGAGGACTT
	R: CGACGAACAGCCCTCTTGTT
hif-1α	F: TCGTGTGGGTGGAAACACAG
	R: TCACATCTGCGGTCTGCTTC

2.6. Quantitative Real-Time PCR

Transcript levels of the target genes in liver were measured by real-time PCR (qPCR) using a Bio-Rad iClycler iQ5 qPCR thermocycler and SYBR Green dye following the manufacturer's recommendations. The reaction mixtures were 10 µL, containing 2 µL cDNA sample, 5 µL EvaGreen Supermix (Bio-Rad Laboratories, USA), 2.4 µL DEPC water, 0.3 µL of each 10mM forward and reverse primers. The qPCR conditions were as follows: 95°C for 30 s, then 40 cycles at 95°C for 5 s and optimized annealing temperature for 10 s, finishing with 60ºC for 10s and then 95ºC 0.5C. All samples were run in duplicate and negative controls were included in each plate to ensure lack of contaminating DNA. A standard curve derived from the gel band purified for each gene was run on a separate plate. Additionally, a cDNA pool was used as internal control between plates for the same gene. After finishing the program, the threshold cycle (Ct) values were obtained for each sample. Melt curve analyses were performed to verify amplification of a single product in each reaction. Amplicon size was analysed by 2% agarose gel electrophoresis. The expression of each target gene was normalized over the geometric mean of *18S* and *β-actin*. qPCR efficiency ranged between 96.1% and 96.5% with $R^2 > 0.98$.

2.7. Data analyses

All statistics were computed with SigmaPlot 12.5. Data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene’s test.

To test for differences in the physiological parameters among fish of control groups a one-way ANOVA or Kruskal-Wallis, when appropriate, was performed. To analyse for influence of T_A and treatment (control, cooled and heated) a two-way ANOVA was used, with T_A and treatment as factors. To test for differences in CTMax and CTMin, as well as the lethal endpoint, for fish acclimated at 12ºC or 24ºC, a Student t-test was performed.

Statistical significance was established at $\alpha = 0.05$ for all tests and data is presented as mean ± standard error of mean (SEM). Two-tailed tests were used throughout.

3. Results

3.1. Chronic thermal acclimation

No mortality was observed in any of the acclimation temperatures. Observations of *A. facetus* behaviour during acclimation showed that fish acclimated to 24ºC were extremely active and fed voraciously. At the other extreme, fish acclimated to 12ºC and 7ºC remained on the tank bottom and exhibited reduced activity. These behaviours however, were not systematically characterized and quantified at this instance.

Analysis of plasma parameters showed that after 15 days of acclimation cortisol levels were at their lowest in fish exposed to 7ºC. Cortisol concentration was five-fold higher at 12ºC and 24ºC than at 7ºC and two-fold higher than at 18ºC (one-way ANOVA F = 47.28, $p < 0.001$; Figure 1A). Plasma glucose was higher at 7ºC, while no difference was observed between 12ºC, 18ºC and 24ºC (one-way ANOVA F = 4.51, p = 0.009, Figure 1B). Lactate was significantly higher at 18ºC (one-way ANOVA F = 16.50, $p < 0.001$, Figure 1C) than at any other temperature.

Plasma osmolality was lowest in fish at 7ºC, with a mean value of ~290 mOsmol/kg, and was slightly but significantly higher at the other three temperatures, averaging between 310 and 315 mOsmol/kg (Kruskal-Wallis H = 18.31, $p < 0.001$, Figure 1D). Circulating chloride levels were not significantly different between groups (Figure 1E).

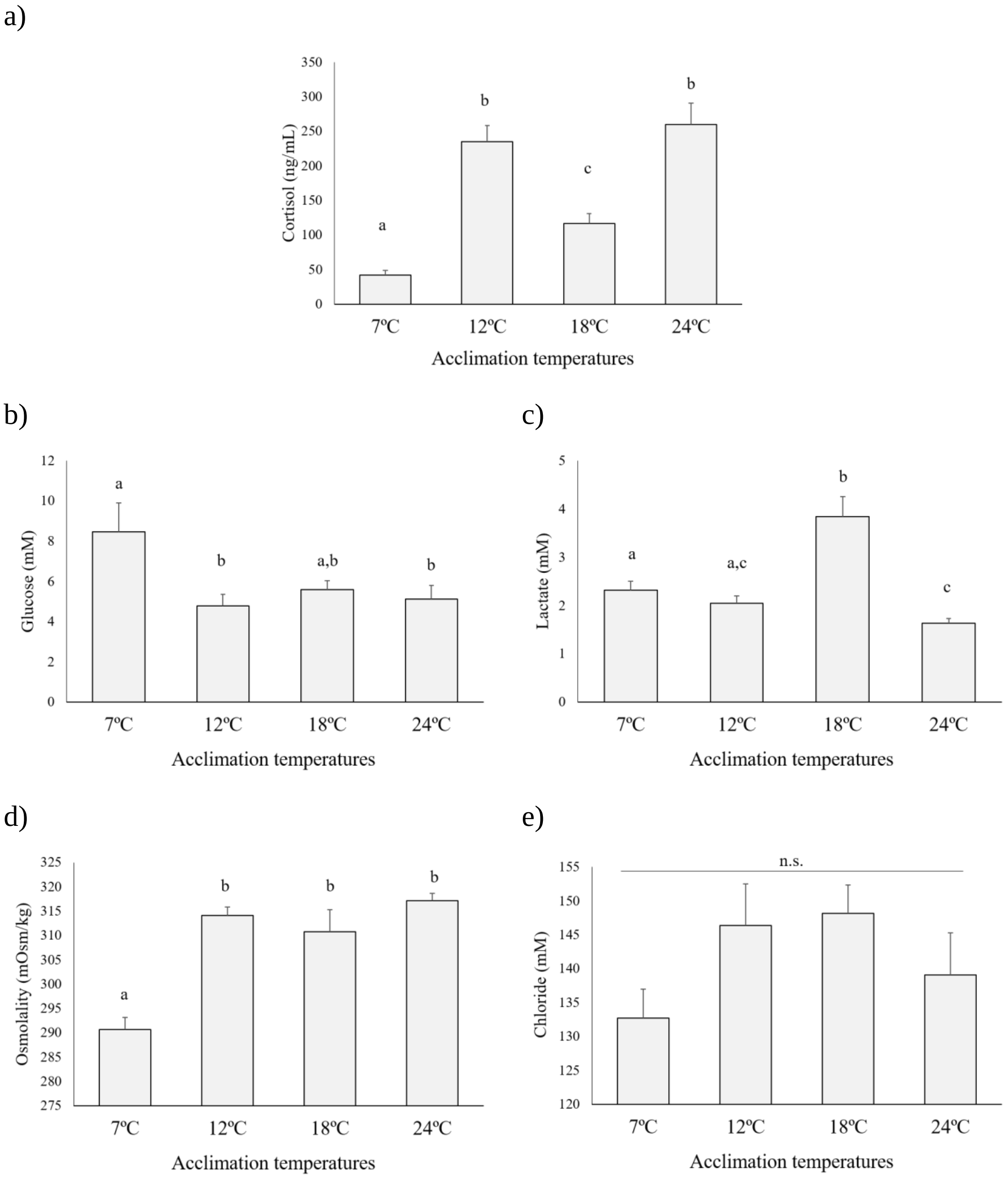


Figure 1. Circulating physiological markers of *A. facetus* acclimated to different tempertaures: a) cortisol, b) glucose, c) lactate, d) osmolality, e) chloride. Letters denote significant differences, one-way ANOVA, $p < 0.05$.

The relative expression of *hsp70* in liver was higher in fish in colder water and decreased with increasing temperature (one-way ANOVA F = 22.74, $p < 0.001$, Figure 2A). Similarly, *hif-1α* expression was high at 7ºC, with twice the relative number of transcripts than fish at 12ºC (Kruskal-Wallis H = 10.47, $p = 0.01$, Figure 2B). No differences were seen between 12ºC, 18ºC and 24ºC.

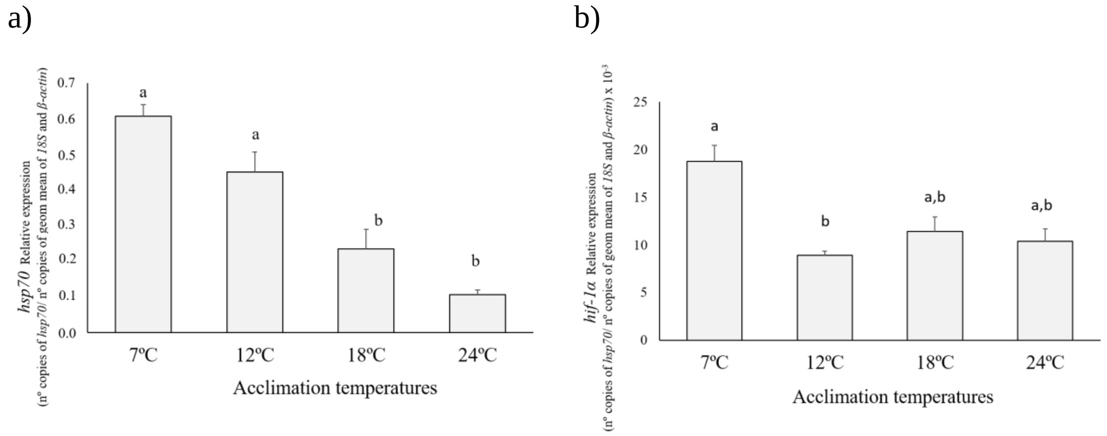


Figure 2. Relative gene expression in *A. facetus* acclimated to different acclimation temperatures (number of copies of target gene divided by the geometric mean of number of copies of *18S* and *β-actin*). a) *hsp70* and b) *hif-1α*, letters denote significant differences, one-way ANOVA, $p < 0.05$.

3.2. CTMax and CTMin experiments

The peak of dissolved oxygen was 10.3 ± 0.06 mg/L with saturation of 101.25 ± 1.63% at 16ºC, while the lowest value was 4.58 ± 0.54 mg/L with saturation of 70.25 ± 5.63% at 39ºC (Figure 3). The concentration and saturation of dissolved oxygen were not linearly related to the increase in temperature.

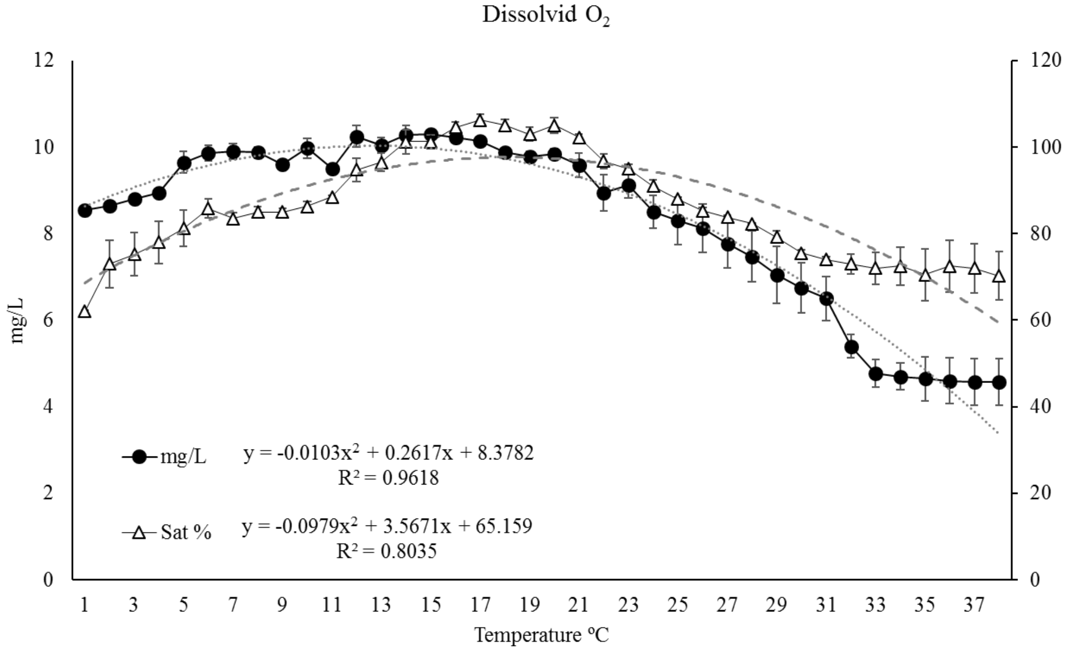


Figure 3. Concentration (mg/L) and saturation (%) of dissolved oxygen during trials. Data presented as mean ± SEM.

The CTMax values for *A. facetus* were 36.5 ± 0.1ºC for the group acclimated at 12ºC and 39.1 ± 0.1ºC for the group acclimated at 24ºC (Students t-test = -7.72, p<0.001, Figure 4). The CTMin were 4.5 ± 0.1ºC for the group acclimated at 12ºC and 5.8 ± 0.1ºC for group acclimated at 24ºC (t-test = -4.92, p<0.01, Figure 4).

Mortality occurred at different points for groups acclimated at different temperatures: 37.2 ± 0.3ºC for fish acclimated at 12ºC and 39.4 ± 0.1ºC for fish acclimated at 24ºC (t-test = -4.77, p<0.01, Figure 4). Defining a minimum temperature point was complex as fish were able to recover even from minute-long exposure to 0ºC. The endpoint was initially set to when neither opercular movements nor any response to physical stimuli were visible, and this occurred at 3.0 ± 0.5ºC for the 12ºC acclimated group, and 4.7 ± 0.3ºC for the fish acclimated at 24ºC (Students t-test = -2.60, p = 0.04, Figure 4). However, fish were able to recover from lower temperatures after being placed in warmer water.

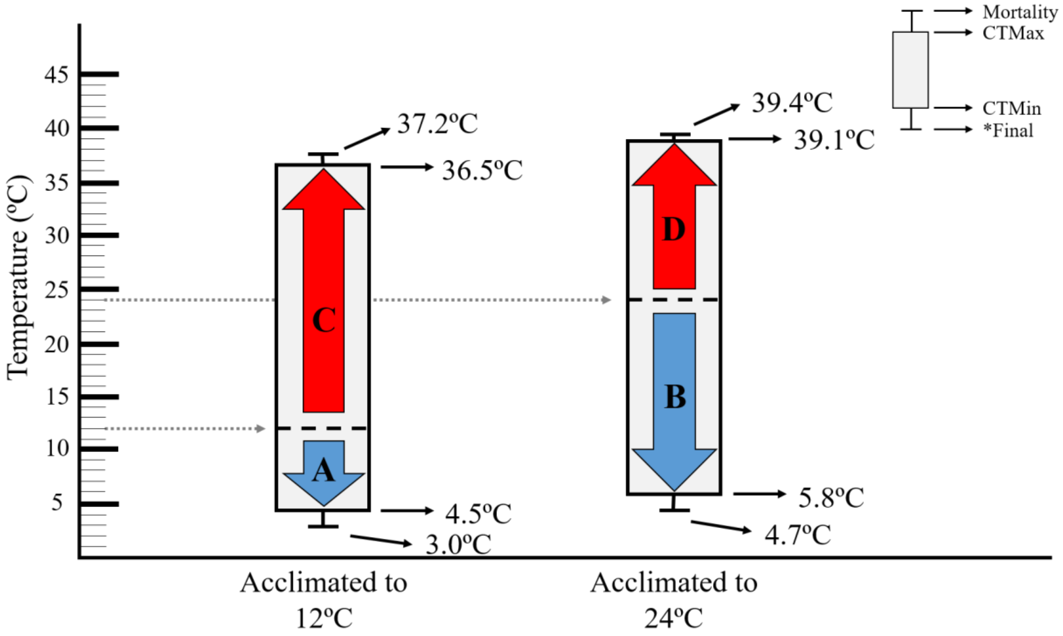


Figure 4. Endpoints (CTMax and CTMin: 50% of LOE and mortality) after gradual increase and decrease of temperature. *Final: 100% of fish without any reaction to physical stimuli but recovering after warmed. Blue groups cooled from 12ºC (A) and 24ºC (B), red groups, heated: from 12ºC (C) and 24ºC (D).

3.3. Acute thermal stress

In this trial, no significant differences were found between the initial control situations at 12ºC and 24ºC for any of the parameters tested, with exception of the expression of *hsp70*. There was no mortality in either condition before the onset of the temperature ramps.

For fish initially at 12ºC, the cooling treatment evoked a significant increase in cortisol levels, while for fish at 24ºC, this rise was not so evident (two-way ANOVA,

T_A: F = 0.37, p = 0.55, treatment: F = 15.46, $p < 0.001$, interaction between T_A and treatment: F = 1.64, p = 0.21; Figure 5a).

Although acclimation to the different T_A had no influence on glucose levels after 15 days of exposure, the rapid heating of the tank water increased glucose levels in both the 12ºC and 24ºC acclimated fish. Cooling also increased glucose concentration, but it was only significant in fish acclimated at 12ºC (two-way ANOVA, T_A: F = 0.02, p = 0.88, treatment: F = 45.29, $p < 0.001$, interaction between T_A and treatment: F = 1.35, p = 0.27) (Figure 5b).

Plasmatic lactate increased, up to 4-fold influenced by the rapid increase in water temperature in groups departing from both 12ºC and 24ºC, and was only slightly reduced by lower temperatures. T_A influenced the concentration of plasmatic lactate when fish were subjected to an increase in water temperature, which was higher for fish acclimated to 24ºC; at the same T_A, heating the water lactate increased 3-5 fold when compared to the control groups and more than 10 fold when comparing to the cooled groups (two-way ANOVA, T_A: F = 2.30, p = 0.13, treatment: F = 312.57, $p < 0.001$, interaction between T_A and treatment: F = 5.79, $p < 0.01$, Figure 5c).

As for the other parameters, plasma osmolality was not influenced by long-term T_A, but significantly responded to the rapid increase in water temperature in both groups (two-way ANOVA: T_A: F = 0.96, p = 0.33, treatment: F = 20.50, $p < 0.001$, interaction between T_A and treatment: F = 0.22, p = 0.80, Figure 5d). T_A and treatment did not have statistically significant effects on chloride values (two-way ANOVA, $p > 0.05$, Figure 5e).

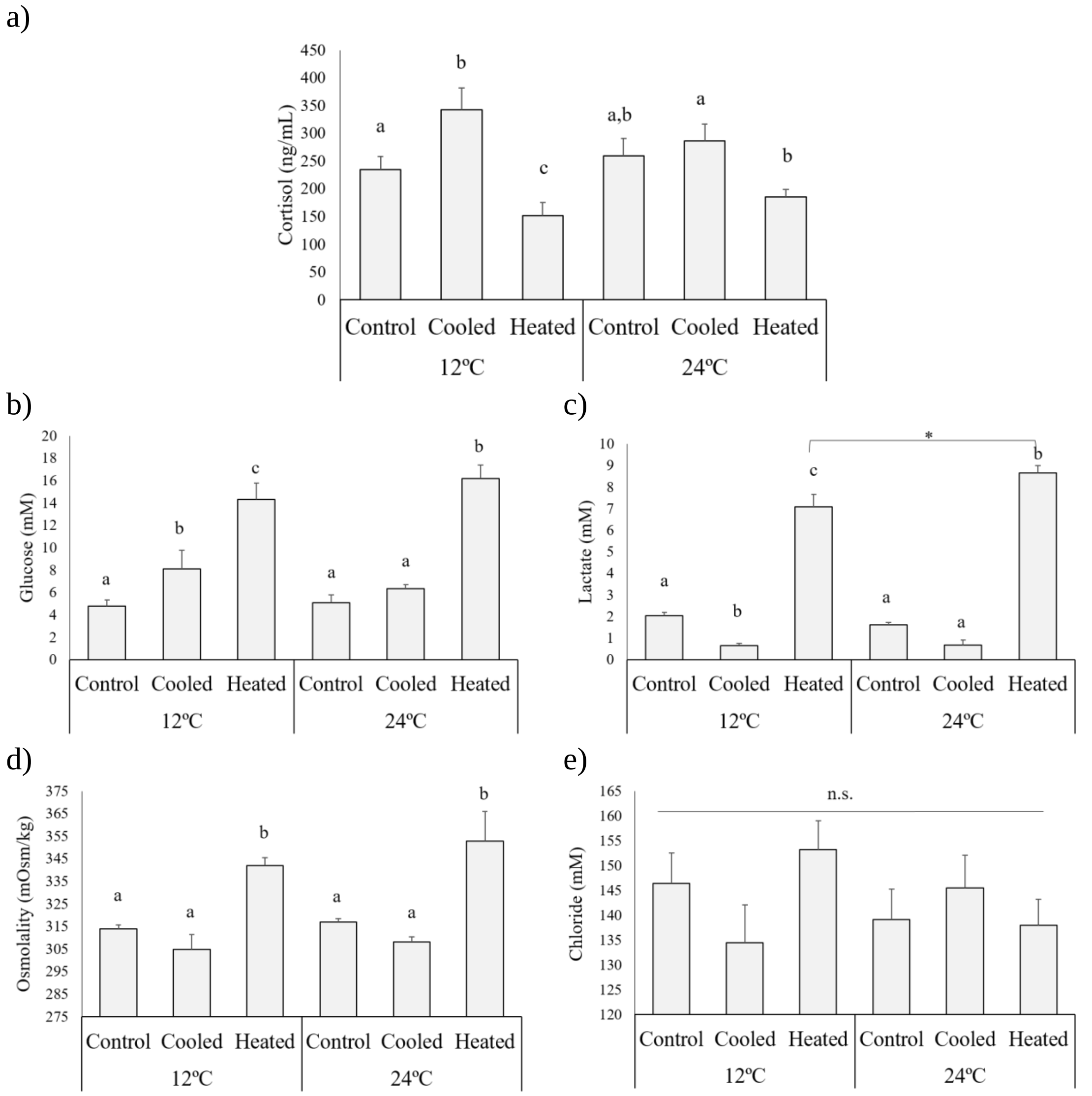


Figure 5. Circulating markers of *A. facetus* acclimated to different tempertaures: a) cortisol, b) glucose, c) lactate, d) osmolality, e) chloride among 12ºC and 24ºC acclimated groups. Letters denote significant differences among treatments (control, cooled or heated) at same acclimation temperature. Asterisks denote significant difference in one same treatment, between 12ºC and 24ºC. Two-way ANOVA, planned comparisons, $p < 0.05$.

Expression of *hsp70* was significantly lower in fish at 24ºC than in those at 12ºC, but in both cases it increased significantly when water was heated, especially for fish acclimated at 24ºC and no differences were observed between control or cooled groups. There was thus a statistical difference between warmed and cooled fish regardless of the initial acclimation temperature (two-way ANOVA: T_A: F = 9.62, $p < 0.01$, treatment: F = 21.05, $p < 0.001$, interaction between T_A and treatment: F = 4.15, p = 0.03, Figure 6a).

Heating the water led to a 3-4 fold increase in the expression of *hif-1α* in fish from 12ºC or 24ºC. Expression of this gene was also elevated in response to lower temperature but only in fish previously acclimated to 24ºC. There were also significant differences in the expression levels between warmed and cooled fish at 12ºC and 24ºC (two-way ANOVA: T_A: F = 12.23, p = 0.001, treatment: F = 25.73, p < 0.001, interaction between T_A and treatment: F = 2.64, p = 0.08, Figure 6b).

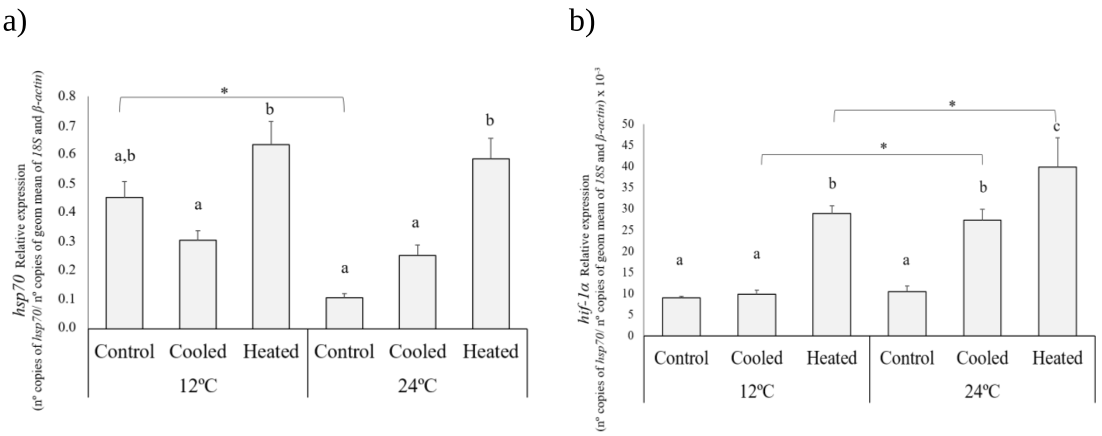


Figure 6. Relative gene expression in *A. facetus* acclimated to different temperatures (number of copies of target gene divided by the geometric mean of number of copies of *18S* and *β-actin*). a) *hsp70* and b) *hif-1*α show differences among treatments (control, cooled or heated); letters denote significant differences among treatments at same acclimation temperature and asterisks denote significant difference in one same treatment, between 12ºC and 24ºC. Two-way ANOVA, planned comparisons, p < 0.05.

4. Discussion

The temperature tolerance of *Australoheros facetus* is related to acclimation temperature, as previously reported for other temperate fishes (Beitinger & Bennett, 2000; Dülger et al., 2012; Ford & Beitinger, 2005). Most importantly, we have defined the CTMax and CTMin to be quite wide, and well beyond the average temperature in the current habitats. Such amplitude (from 4.5ºC to 36.5ºC for the 12ºC acclimated group and from 5.8ºC to 39.1ºCfor the 24ºC acclimated group) may confer the *A. facetus* a large advantage in Mediterranean streams.

Differences in CTMax and CTMin related to different T_A probably reflect a cellular metabolic adaptation providing the fish with a safety margin for extreme events in relation to its habitat seasonal optima, as annual fluctuations usually afford sufficient time for acclimatization (Payne et al., 2016). The amplitude of abiotic factors where an organism can occur (i.e. physiological tolerance) reflects adaptation to that place (Angilletta, 2014). In the present case, we showed these variations to be within only 1-

3ºC of the thermal limits despite an acclimation difference of 12ºC, which shows that this species has remarkable thermal plasticity regardless of the temperature it is seasonally adjusted to.

Cichlids occupy many niches in tropical and subtropical areas, and it is expected that those from higher latitudes will be able to withstand wider variations in their environmental conditions. The Oscar cichlid *Astronotus ocellatus,* native to the rivers from the Amazon basin, when acclimated to 25ºC showed a lethal thermal maximum at 41ºC and a minimum at 9ºC (Gutierre et al., 2016). Similarly the Mayan cichlid, *Cichlasoma urophthalmus,* native of Central America, or the Sub-Saharan Jewel cichlid *Hemichromis letourneuxi* acclimated at 24ºC and submitted to decreasing temperatures, showed a lower cold tolerance than here described for *A. facetus* (Schofield et al., 2009). In that study, both fish reached LOE at ~11.7ºC and mortality at ~9.4ºC, which highlights the unusual resistance to cold in *A. facetus*. When those species were evaluated in the wild, during cold storms, similar limits were found (Schofield et al., 2009), which highlights the usefulness of laboratory trials to predict behaviour and survival in nature, or even to forecast future distribution of species in a climate change scenario.

4.1. Chronic thermal acclimation

Temperatures of 12ºC and 24ºC triggered the highest cortisol response possibly for different reasons. At 24ºC fish start to reproduce, which can elevate the cortisol of subordinate animals (Baduy et al., 2017). As in social groups the subordinate animals are the majority, this can skew the means of cortisol towards higher values. Therefore, high values of cortisol at 24ºC are probably more related to social interactions than to temperature *per se*.

High levels of cortisol were observed at 12ºC acclimated fish and also reported in fishes that were subjected to cold exposure until a minimum of 11.5ºC (Kindle & Whitmore, 1986) and could reflect a failure to compensate during cold water stress. Conversely, fish acclimated at 7ºC had the lower cortisol values. Possibly the continuous stress can down-regulate the hypothalamus-pituitary-interrenal axis because of the negative feedback by cortisol, attenuating its response (Mommsen et al., 1999).

Decreasing the water temperature until 7ºC increased glucose levels in *A. facetus*. This hyperglycaemia upon cold exposure was already observed for *Oreochromis niloticus* after a slow decrease in water temperature of 0.5ºC/day (Atwood et al., 2003) and *O. aureus* after a decrease of 1ºC/day (Kindle & Whitmore, 1986). Hyperglycaemia may represent a form of thermal acclimation by supplying substrate for essential reactions while also helping to maintain osmolality in freshwater fish during exposure to low temperature (Kindle & Whitmore, 1986). Other hypothesis can be due the increased expression of *hif-1α* (discussed below). As HIF-1 has a consensus sequence that resembles that of some glucose-responsive elements, it is hypothesized that *hif-1α* can cross-talk within the glycolytic enzyme L-PK (Kietzmann et al., 2002). The enzyme L-PK catalyses the formation of pyruvate and ATP from phosphoenolpyruvate and ADP, and thus it might be possible that HIF-1 can interfere with the glucose-dependent-induction of the L-PK gene or that glucose can influence a HIF-1-dependent promoter (Kietzmann et al., 2002)

Osmoregulatory mechanisms are often down-regulated during temperature stress (McCormick et al., 1996; Sigholt & Finstad, 1990). In the present case, the chronic cold stress may be responsible for decreasing the activity of ion-regulating enzymes, such as the Na^+/K^+-ATPase, responsible for the driving force for most ion-exchanging mechanisms, and which is dependent on temperature (Esmann, 1988; Handeland et al., 1998). Change in membrane permeability is also an hypothesis (Barnes et al., 2014; McKinley & Hazel, 2000) as explained by the homeoviscus adaptation theory. This theory predicts an increase in long-chain polyunsaturated fatty acids at low temperatures (Podrabsky & Somero, 2004), making membranes more fluid, allowing the entrance of water by osmosis and resulting in loss of electrolytes to the environment (Atwood et al., 2003; Kindle & Whitmore, 1986). This process could lead to the low value of plasma chloride (despite no significant, it could indicate a tendency) and the drop in osmolality observed in *A. facetus* acclimated at 7ºC. Similar results were observed in stressed goldfish *Carassius auratus* that had lower chloride levels when acclimated at 10ºC than when acclimated at 32ºC (Umminger & Gist, 1973). Furthermore, in *O. niloticus* the expression of a class of genes associated with ion-transport, including Cl^- transporters, was significantly below the control levels under low-temperature stress (Yang et al., 2015). So, it is possible that not only the fluidity of the membranes but also the activity, quantity and composition of ion transport channels are affected by cold.

Increases in plasma lactate are usually related to change from aerobic to anaerobic metabolism, namely after high intense activity (Driedzic & Hochachka, 1978; Sola-Penna, 2008). Somewhat surprisingly, we have not seen a linear increase in plasma lactate with increasing temperature, but a higher value at 18ºC, remaining at similar levels among the other tested temperatures. Although we have not specifically quantified fish activity, swimming, feeding and even aggression were influenced by increasing temperature, and as already observed for *A. facetus*, social and reproductive interaction are triggered with temperatures above 20ºC and evident at 24ºC (Baduy et al., 2017). So, if there was increased metabolism at 18ºC, activity was even higher in fish at 24ºC, which did not show such high lactate levels. Thus, the reasons for this change in lactate remain to be identified.

In agreement with our results in liver, high expression of the heat-shock protein *hsp70* at low temperature has been reported in kidney of rainbow trout (Verleih et al., 2015) and could be related to an up-regulation of proteasomes, such as 26S proteasome, increasing the capacity for protein degradation during cold acclimation (Podrabsky & Somero, 2004). This enhanced capacity together with a decrease in metabolic reaction rates due to low temperatures, may increase protein synthesis and therefore the demand for HSPs to assist with targeting of damaged proteins and aggregations and folding of newly synthesized proteins (Dias et al., 2010; Storch et al., 2005; Teigen et al., 2015). Furthermore, HSPs have been linked to long-term adaptation during prolonged environmental stress and thermal tolerance (reviewed in Lindquist & Craig, 1988; Verleih et al., 2015). Thus, it is likely that *A. facetus* cold acclimation involves apoptosis and remodelling as found in gills of the zebrafish *Danio rerio* and Nile tilapia *Oreochromis niloticus,* submitted to a cold stress of 8ºC, which is the lower temperature limit for the tilapia (Hu et al., 2016).

The Hif-1α has been described as a master regulator of hypoxic adaptive response, which is usual in warming waters (Lushchak, 2011; Rytkonen et al., 2011). Here, we have not seen a temperature induction of *hif-1α* despite the 17ºC difference between the lowest and the highest temperatures. The high levels seen during cold adaptation rather may reflect a lack of oxygen in tissues than in water, as the concentration of dissolved oxygen in water was never below 4.0 mg/L and around 10.0 mg/L at the lowest temperature. One hypothesis is that during chronic stress, some organism can reduce their standard metabolic rate and enter in a phenomenon called 'metabolic depression', which means a decrease in oxygen consumption rate at the animal, tissue, cellular and mitochondrial levels (Guderley & St-Pierre, 2002), that is reflected at a cellular level by a reduced ATP turnover (Boutilier & St-Pierre, 2000; Brand et al., 2000), and a reduction in mitochondrial aerobic capacity (Barnes et al., 2014; St-Pierre & Boutilier, 2001; Stuart et al., 1998), so a deficit in oxygen delivery to tissues and cells is possible to happen.

4.2. Acute thermal stress

The relatively fast increase or decrease in water temperature evoked substantial responses on stress hormones and energetic substrates. Increasing temperature is a particularly important factor mainly due to an increase in demand of metabolic oxygen while simultaneously the oxygen availability decreases (Jenny et al., 2016; Salin et al., 2015). Aquatic organisms must therefore respond quickly to maintain homeostasis.

Changes in temperature are a major factor stimulating cortisol release in fish. In the present study, we found higher cortisol in fish acclimated at 12ºC and even higher in the abrupt cooled groups rather than in warmed fish. Likewise, it was observed a peak of cortisol in gilthead sea bream after 24h following an abrupt transfer to 9ºC, (Rotllant et al., 2000), and in *O. aureus* at 12ºC (Chen et al., 2002). This could mean that a drop in water temperature can be more stressful than rapid warming for these species (He et al., 2015). These results were the opposite pattern observed for the Senegalese sole (*Solea senegalensis*) (Benitez-Dorta et al., 2017) and for the common carp (*Cyprinus carpio*) (van den Burg et al., 2003), where the cortisol levels increased with increase in water temperature, and was attributed to the effects that cortisol has in the energetic metabolism (Mommsen et al., 1999). So, it is possible that these discrepancies in the responsiveness of cortisol to increase or decrease of water temperature may be more related to the evolutionary history of the species.

Biochemical reactions are greatly affected by temperature. Our observed increases in glucose and lactate when temperature rose could reflect a change in energy utilization. Several studies place lactate as the most important substrate for gluconeogenesis (for review see Gladden, 2004). The lactate synthetized by anaerobic glycolysis in the muscle travels to the liver through blood stream where it is converted in pyruvate and then in glucose by the gluconeogenesis cycle. All these processes can explain the high circulating glucose and lactate found in the present study. The same pattern of increased glucose and lactate after heat shock was also observed in shortnose sturgeons (*Acipenser brevirostrum*) (Zhang & Kieffer, 2014). However, if the accumulation of lactate continues, a decrease in the production of glucose in liver can occur, since lactate can function as a regulator of intracellular concentration of important glycolytic intermediates such as 6-phosphofructose-1-kinase (Sola-Penna, 2008). Peaks in plasma lactate are expected to be associated with acute stress rather than with chronic stress because during recovery from an intense or prolonged muscular activity there is a net uptake of lactate from blood by muscle (Gladden, 2004). For obvious reasons, most animals cannot maintain an anaerobic metabolism indefinitely.

Our results did not show a significant difference in plasma chloride levels between control and cooled groups acclimated at 12ºC. However, lower values for plasma osmolality and chloride were observed in *O. mossambicus* subjected to a rapid decrease in temperature (Allanson et al., 1971). The lack of change found in our experiment could be explained by the fact that during rapid cooling the gill membrane was not able to adjust, passing from a sol state to a gel state, inhibiting interactions among its proteins and decreasing chloride transport, as suggested by Malone et al. (2015). Similarly, we did not find differences in fish acclimated at 24ºC. Considering that the point where they were sampled in the 24ºC acclimated group (5.8ºC) was slightly higher than the 12ºC acclimated group (4.5ºC), it is possible that 5ºC is a threshold for proper function of membranes. Although we did not analyse catecholamine responses in the present study, it is known that they are also an important stress primary response (Mazeaud et al., 1977) and their action on gill function can increase its permeability to water (Mazeaud et al., 1977), lowering the osmolality in the cooled groups compared to control. On the other hand, osmolality was higher in warmed fish regardless of the initial acclimation temperature – this may relate to increased amount of solutes such as glucose and lactate, or other catabolic products related to the increased activity at high temperatures. Blood appeared more viscous and collection was slower at high temperatures (personal observation).

As for the long term exposure experiments, *hsp70* was highly expressed liver of fish exposed to 12ºC when compared to those at 24ºC, which may indicate a more permanent protective effect at lower temperature. This appears to be confirmed by the fact that expression increases substantially upon heating and not in the cooling treatment. However, the high level of expression in the 12ºC control group could be partially masking the rise after the rapid heating, leading us to suggest that at 12ºC, and below, the fish are already in a compensatory state. In addition, the high levels of cortisol after a rapid decrease in water temperature could be mediating the *hsp70* responses, as suggested by Basu et al. (2001) and Basu et al. (2002). Nonetheless, as the induction of HSPs are usually rapid and intense during heat-shocks (Basu et al., 2002; Lindquist & Craig, 1988), it was not surprising to see a rise after sudden increase in temperature. Similarly, *hsp70* in liver, fins and muscle was up-regulated during a rapid water temperature increase, from 18ºC to 30ºC, in two Iberian freshwater fishes (Jesus et al., 2016), and a sudden exposure of *Channa striatus* to heated water also promoted a significant increase in *hsp70* expression when compared to exposure to cold water (Eid et al., 2016).

As indicated hif-1 is an important regulator of energy metabolism and quickly responds to decreases in oxygen availability (Metzen et al., 2005; Rytkonen et al., 2011). Even with constant aeration in tanks, the abrupt temperature increase lowered the dissolved oxygen in water (although never below 4 mg/L), which was enough to trigger a response in the expression of *hif-1α*, not seen during long term exposure to milder temperatures. Interestingly, salmonids present high values of *hif-1α* proteins in normoxia. In these fish, the oxygen tension in tissues is similar or lower than the venous oxygen tension allowing these proteins to accumulate (Nikinmaa & Rees, 2005). There are however few studies about the direct effect of temperature on *hif-1* expression (as discussed by Nikinmaa & Rees, 2005), as most studies focus on the indirect influence of changing temperatures in dissolved oxygen availability.

Conclusions

The results presented herein can partially explain the current distribution patterns of *A. facetus*, and more importantly, they can be used as indicators for the seasonal and geographical distribution, and as tools for the management of this invasive species. The metabolic markers used in this study indicate deteriorating overall condition in chronic and acute exposure to temperatures below 7ºC with thresholds for maintaining normal swimming close to 5ºC (CTMin). On the other hand, a rapid increase in water temperature (and consequent oxygen reduction) appear to lead to the shift from aerobic to anaerobic metabolism, increasing the mobilization of energetic substrates, as glucose and lactate. This is a situation in which usually organisms cannot stand for long periods. However, the increased expression of *hif-1α* and *hsp70* suggest a rapid response and probably represent a coping mechanism for *A. facetus.*

Considering this species' plastic physiology and broad temperature tolerance, it is not a surprise that it can cope with large variations in water temperature in the wild, either in its original distribution range or in invaded areas in Southern Portugal, where the minimum values were 6.6ºC and the maximum were 31.8ºC. Still, it remains to be seen how these fish fare at temperatures close to the highest values recorded for the streams where they were collected (ca. 32ºC). In a climate change scenario where floods can increase in frequency and severity, distinct water bodies can be connected, creating new pathways or habitats for exotic or invasive species. Based on our results, water temperatures varying from ~5ºC (mean CTMin of the 12ºC and 24ºC acclimated groups) to ~37ºC (mean CTMax of the 12ºC and 24ºC acclimated groups) will not be a constraint for the presence of *A. facetus* individuals. However, one must take into account the effects of temperature on long term physiology, and, specifically on this species, on behaviour and reproduction, which may hinder the establishment of populations in conditions too far from their temperature optima or preferendum.

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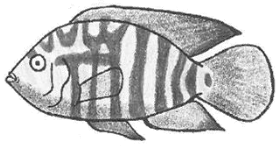
CHAPTER FOUR

OSMOREGULATORY POTENTIAL OF THE CICHLID *Australoheros facetus*, A NON-NATIVE SPECIES IN STREAMS OF SOUTHERN PORTUGAL



Osmoregulatory potential of the cichlid *Australoheros facetus*, a non-native species in streams of southern Portugal

Abstract

Australoheros facetus is a freshwater neotropical cichlid and a non-native species currently found in several river basins of Southern Portugal, where they occupy areas upstream of important estuarine zones. Some of these habitats, in typical Mediterranean-type streams, are still influenced by tidal water level changes, with little or no salinity intrusion, and seasonally exposed to flash floods and strong currents. The ability to adapt to different habitats and environmental conditions could be a key to the apparent success of this species, but whether *A. facetus* can withstand variations in water salinity is not known. Such ability would place them as likely winners if climate change scenarios increase salinization of the tidal water mass, or could allow fish carried by floods to colonise novel habitats in upper or middle estuaries. Here, fish were exposed to a range of salinities from freshwater (0 ppt) to estuarine (18 ppt) during short and long-term trials. Important variations in physiological parameters such as plasma osmolality and osmoregulatory-related enzymatic activity occurred in fish at higher salinity. Higher salinity also evoked a cortisol rise in the short term trial, but not in the long trial. These modifications were accompanied with changes in activity and metabolism, and a reduction in growth and social behaviour. The results indicate that *A. facetus* is able to live and show normal behavioural and metabolic features at salinities from zero to near its isosmotic point, but it is greatly affected by increased salinity and incapable to mount a successful hypoosmoregulatory response.

Key-words: salinity tolerance, cichlid, invasiveness, plasticity, osmoregulation

1. Introduction

Non-native species are a growing concern for habitats worldwide. One of the challenges of understanding the ecological consequences of non-native species is the difficulty in identifying the mechanisms that allow these species to become established in the new habitat (Mack et al., 2000). The behavioural and physiological ability to cope with changes in the environment, can determine which species can expand their occurrence in areas with different features. In freshwater environments salinity can act as a barrier for the expansion of non-native teleosts and the interaction of salinity with other variables such as temperature and oxygen, may limit even further such expansion (Clavero et al., 2016; Rehage et al., 2015).

Salinity tolerant species, however, may use estuaries as bridges to reach other freshwater bodies (Brown et al., 2007; Gutierre et al., 2014), or even acclimatize and use these areas as novel habitats. Salinity has nonetheless, major influences on fish growth, reproduction and survival and in general, adjustments to salinity require energy partitions that may compromise or have deleterious effects on these processes (Bœuf & Payan, 2001; Qiang et al., 2013). As showed for *Oreochromis niloticus* that grew faster and had higher feed efficiency at a combination of 28.9ºC and 7.8 ppt (Qiang et al., 2013), while some reproductive parameters (number of oocytes and gonadosomatic index) were reduced at salinities >30 ppt (Schofield et al., 2011).

The osmotic balance in fish is held mainly by chloride cells located in gills, which use the iono-electrical gradient created by the ATP-driven Na^+/K^+-ATPase pump to, directly or indirectly, exchange ions and water with the environment and drives many other transporters (McCormick, 1995; Saoud et al., 2007). Indeed, the activity of these ion pumps and exchangers in branchial epithelium is considered the main component through which fish adapt to different salinities, due the gill large surface permanently in contact with the water (Mayer-Gostan & Lemaire, 1991). However, there is a metabolic cost related to osmoregulation (Altinok & Grizzle, 2001; Bœuf & Payan, 2001; Ern et al., 2014), that could be reflected in less energy spent in growth (Wang et al., 1997) or social activities (St Mary et al., 2001).

Osmoregulation in teleosts is dependent on the interaction of a variety of hormones, including cortisol. In addition to being a general stress response hormone, cortisol plays an essential role in the metabolism of carbohydrates and health balance, and favours the adaptation to saline environment, mainly by stimulation the gill Na^+/K^+-

ATPase pump, lowering thus the osmolality of blood (Evans, 2008; McCormick, 1990; Mommsen et al., 1999) or by mediating the proliferation of chloride cells (McCormick, 2001). Cortisol may also have an important behavioural role as it responds to the social environment, usually rising in situations of social stress (Alcazar et al., 2016; Baduy et al., 2017; Baker et al., 2013). This is the case of *Australoheros facetus*, that has an elaborate social behaviour with cortisol levels higher in subordinate animals (Baduy et al., 2017).

In species with complex social behaviour, as in the case of many cichlids, aggressive interactions can occur in an attempt to monopolize resources, as food, territory or mates (Keenleyside, 1991). These social interactions are an important step leading to reproduction, and eventually allowing the species to colonize new habitats. However, these energetically demanding behaviours may collide with other physiological processes such as osmoregulation. Despite its potential significance, there are still few studies that look for differences in social behaviour among different salinities. Exceptions include male-male interactions and social displays of the euryhaline sand goby *Chlamydogobius eremius* (Lehtonen et al., 2016), evaluation of aggressiveness with mirror test of the neotropical *Herichthys cyanoguttatus* (Lorenz et al., 2015), parental care and reproductive success in Florida flagfish *Jordanella floridae* (St Mary et al., 2001), and rates of aggression of *Cichlasoma urophthalmus* (Schofield et al., 2009).

Some cichlids are known to be euryhaline (i.e. capable to maintain relatively constant the ionic composition and osmolality of their internal fluids when exposed to environmental salinity changes), as is the case of the ‘California’ Mozambique tilapia *Oreochromis mossambicus x O. urolepis hornorum* (Sardella & Brauner, 2008) or the mayan cichlid *Cichlasoma urophthalmus* (Stauffer & Boltz, 1994). One of the hypotheses for the different degrees of euryhalinity found in cichlids is that they are re-invaders of the freshwater biotope, since the Perciformes are a marine group (Gutierre et al., 2016; Gutierre et al., 2014).

The chanchito, *Australoheros facetus*, is a neotropical cichlid that is non-native in Southern streams of Portugal (Almaça, 1995). These small rivers are habitats with striking seasonal variations in hydrological regimes and are subjected to flash floods (Gasith & Resh, 1999) that can drag the fish downstream towards the estuary and potentially colonize it. This study aims to assess the species’ physiological potential of intrusion in brackish waters, through measurements of Na^+/K^+-ATPase in kidney and gills, plasma osmolality and circulating levels of cortisol, metabolic substrates and behaviour at different salinities. While of interest from a biological perspective, our aim was also to help provide an understanding of possible new pathways for the spreading and possible invasion of new habitats by this species in Southern Portugal. The study was divided in four trials, with different duration, fish size, temperature and photoperiod in order to test the possible interactions with developmental stage.

2. Material and Methods

2.1. Fishing and animal housing

Fish were caught using an electrofishing apparatus Hans-Grassl ELT60II generator 300/500V and 1300 watts, pulse DC, in Vascão river (37º31'43.38"N and 7º31'26.05"O), upstream of the Guadiana estuary, but under influence of tidal volume, and in Odelouca River (37º13'37"N and 8º30'20"O), upstream of the Arade estuary and without any tidal influence (capture licence numbers 403, 404, 405 and 406/2015/CAPT).

Upon capture, fish were kept in aerated containers and transported to the experimental facilities of the Centre for Marine Sciences (CCMAR). The animals were maintained in three ~2000 L outdoor community tanks for at least one-month prior to the experiments, under natural temperature and photoperiod and fed *ad libitum* every morning. Environmental enrichment, acclimation periods and anaesthesia were implemented to minimize distress. CCMAR facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal) in accordance to the three 'R' policy and national and European legislation.

2.2. Experimental conditions

Before each trial, fish were initially separated into experimental tanks (details about group and tank size below). The target salinity was achieved by slowing replacing the tank volume with diluted seawater previously prepared in separate containers to avoid osmotic shock. The rate of change was identical to all groups and whenever salinity was altered for one or more treatments, the water was changed in all experimental tanks to maintain similarity of handling disturbance across treatments. Water quality (pH, NH_3/NH_4, NO^-_2) was checked every two days with commercial kits.

Salinity and dissolved oxygen were measured with a calibrated portable meter Thermo OrionStar A329. Fish were fed twice a day with commercial cichlid food at 2% of the total biomass of each tank. One hour after feeding the tanks were siphoned to remove food residues and organic waste. Since this species can be very aggressive, everyday groups were observed for signals of aggression, injured animals or evidences of reproductive behaviour. Animals that presented important injuries or potential infection focus were removed and humanely sacrificed.

2.3. Sampling procedure and assays

At the end of each trial the animals were anesthetized with 2 g of MS-222 plus 2g of sodium bicarbonate in 3 litres of water and sacrificed via rapid cervical transection. Blood samples were collected by puncture of the caudal vein using heparinized syringes. Blood was centrifuged (5000 *g*, 5 min) and plasma was stored at -20ºC until assays were performed. Samples of gill and kidney tissues were placed in SEI buffer (50 mM Imidazole, 10 mM $Na_2EDTA*2H_2O$, 150 mM sucrose) and snap frozen on dry ice. Additional tissue samples were collected into RNAlater.

The measurement of the enzymatic activity of Na^+/K^+-ATPase pump followed a protocol adapted from McCormick (1993). In brief, the tissue frozen in SEI buffer was rapidly thawed and homogenized, and the homogenate centrifuged at 5000 g for 30 s at 4°C. Then, 10 µl supernatant were added to four wells of a 96-well microplate on ice, and 200 µl of assay mixture (with or without 0.5 mM ouabain) added to each well. Na^+/K^+-ATPase activity was determined by subtracting the oxidation rate of NADH to NAD (measured at 340 nm) in the presence of ouabain from the oxidation rate in the absence of ouabain at 25°C for 5 minutes. Absorbance was measured using a Benchmark microplate reader (Biorad). Total protein in 10 µl supernatant was determined with a Biorad Bradford assay. Activity was expressed as µmol ADP mg protein^{-1} h^{-1}.

Cortisol was measured by radioimmunoassay (RIA) following the methodology by Scott et al. (1982). The RIA cross-reactions for cortisol are described in Rotllant et al. (2005). Plasma osmolality of was measured with a Vapour Pressure Osmometer 5520, based on a 10 µl sample. To determine glucose, lactate and chloride plasmatic concentrations, Spinreact commercial enzymatic colorimetric kits (Barcelona, Spain) were used and adapted to microplates. Total protein was measured using a Bio-Rad kit

(Bio-Rad Laboratories, Hercules, CA) using a bovine serum albumin (BSA) standard curve.

Muscle water content (MWC - %) was determined by subtracting the wet weight from dry weight in a sample of epaxial muscle (~1 g) dried at 60ºC until constant weight.

A summary of each trial is presented in Table 1 and described in detail below.

Table 1. Summary of trials presented in this chapter.

Trial	Days	W ± SEM (g)	SL ± SEM (cm)	Photoperiod L/D	Temperature ºC
1 (Pilot)	30	5.24 ± 0.66	4.96 ± 0.15	12/12	18
2	60	8.42 ± 0.16	*	10/14	24
3 – I	90	2.84 ± 0.12	3.80 ± 0.06	8/16	20-24
3 – II	90	1.52 ± 0.05	3.10 ± 0.04	8/16	20-24
4 – Baseline	5	50.43 ± 1.46	9.74 ± 0.08	12/12	20
4 – Behaviour	5	50.43 ± 1.46	9.74 ± 0.08	12/12	24

*Missing data.

Trial 1 (Pilot)

Twenty-five juveniles were distributed in three 30 litres glass aquaria with aerator and a biological filter. The standard length (SL) and weight (W) of animals did not vary among treatments (SL_{mean} = 4.96 ± 0.15 cm, one-way ANOVA, F = 0.33, $p > 0.05$; W_{mean} = 5.24 ± 0.66 g, one-way ANOVA, F = 0.21, $p > 0.05$).

The photoperiod was 12L:12D and temperature 18 ± 1ºC. After a 10 days period of acclimation, an aquarium was kept at 0 ppt salinity (control) and the others had their salinity gradually increased to 5 ppt every three days, until the 5 ppt and 15 ppt were reached. After three days of acclimation to the new salinity, the experimental period started and lasted for 30 days.

In this trial, the activity of Na^+/K^+-ATPase pump in gills and plasma osmolality and cortisol were measured, as described above.

Trial 2

We selected 128 fish, divided them among four groups of treatments, in duplicate (0 ppt = 16 x 2; 6 ppt = 16 x 2; 12 ppt = 16 x 2; 18 ppt = 16 x 2). Weight of fish did not vary among tanks (weight: 8.42 ± 0.16 g, one-way ANOVA, F = 0.55, $p > 0.05$).

Three groups of fish were acclimated with an increase of 3 ppt each three days until reaching 6 ppt, 12 ppt and 18 ppt, and one group was maintained at 0 ppt (control). The experimental period lasted 60 days. Tanks were kept at ~24 ºC and the photoperiod was 10L:14D.

Fish were weighted every 15 days, and blood samples were taken for plasma osmolality, cortisol, glucose, lactate and chloride 30 (T30) and 60 days (T60) after the beginning of the experiment.

Trial 3

Two groups were used (I and II). In both groups salinity was increased by 3 ppt each three days the concentration of the tanks. Photoperiod was kept constant at 8L:16D and temperature varied with the environment from 20ºC to 24ºC.

In group I, 168 fish were divided among three treatments (0 ppt, 6 ppt and 12 ppt, with 56 fish each group, divided in two replicates) in 30 L tanks each with independent mechanical filters but with (pseudo)replicates sharing the same biological filter. Weight of fish was 2.84 ± 0.12 g and standard length of 3.80 ± 0.06 cm. Size of fish did not vary among tanks (weight: one-way ANOVA, F = 0.88, $p > 0.05$; standard length: one-way ANOVA, F = 0.89, $p > 0.05$).

In group II, 64 fish were divided between two treatments (0 ppt and 18pp, with 32 fish each group, divided in two replicates) in 15 L tanks. Weight of fish was 1.52 ± 0.05 g and standard length of 3.10 ± 0.04 cm. Size of fish did not vary among tanks (one-way ANOVA weight, F = 0.85, $p > 0.05$; standard length, F = 1.24, $p > 0.05$).

Fish were measured three days after the target salinities were achieved, (time zero: T0). The next measurements were made at 30 days (T30), and thereafter every 15 days until 90 days of experiment (T90).

In this trial growth (variation of W and SL along time) was measured for both groups. Additionally, behavioural analysis was performed in group II after the end of the trial when fish started to show social and aggressive interactions. The total number of aggressive interactions (*AI*: bite, chase, mouth fighting and tail beating as described in Baduy et al. (2017)) was recorded during five minutes per group for 5 consecutive days.

Trial 4

Fish (n = 189, 50.43 ± 1.46 g and 9.74 ± 0.08 cm of SL) were selected and distributed among three stock tanks. These stock tanks had constant aeration and independent physical and biological filters. Photoperiod was 12L:12D and temperature averaged 20 ± 1ºC during the duration of the experiment.

After one week acclimation at 0 ppt, two fish of each stock were randomly selected and sampled for blood and tissues as described above. Salinity was then increased to 6 ppt in all tanks at a rate of 3 ppt/day so as to minimize osmotic shock, and the fish were kept in those conditions for one week (Figure 1).

The process was repeated for 12 ppt and 18 ppt. Since fish were showing abnormal behaviour after 3 days at 18 ppt (the majority grouped quietly in the bottom, while a few stayed next to the air-stone), fish were sampled at this time-point (18 ppt 3d) and a last sampling after 5 days (18 ppt 5d) (Figure 1).

Concomitantly, after one week of acclimation period, 1-2 fish of each stock were randomly selected to set up a behavioural observation group. Four groups with five fish each for each salinity were formed (0 ppt, 6 ppt and 12 ppt) at 24ºC. As this species does not show evident sexual dimorphism, sex-ratio of social groups was not defined *a priori*. Fish were anesthetized for blood sampling as described above, and this time-point will be thereafter named - initial phase (before the formation of a hierarchy). A hierarchy is achieved when is possible to distinguish territorial from non-territorial animals or a territorial pair is formed, as described by Baduy et al. (2017). Social groups were observed as described below and at the end of the week (final phase), fish were anesthetized again for blood sampling and biometric measures and sacrificed for gonad inspection (Figure 1).

Behaviour was recorded with an underwater camera (GoPro Hero 4) daily from 9 a.m. during 4 days. The first 5 min of each session was considered as conditioning period and discarded. Behavioural observations were made with each fish as a focal individual during 5 minutes, totalling 1200 minutes of observation. The frequency the of aggressive interactions of the focal fish against the other members of the group was measured. The social hierarchy was determined by an interaction matrix. A dominance index (DI) was calculated for each individual from this matrix as: $DI=\Sigma(Wi/Ti)/N$, where Wi is the number of interactions won by individual i, defined as biting or chasing,

Ti is the number of interactions with the individual i, and N is the total number of opponents (Alonso et al., 2012).

The activity of Na^+/K^+ATPase pump in gills, plasma osmolality, cortisol, glucose, lactate, total protein and chloride and muscle water content were measured as described above.

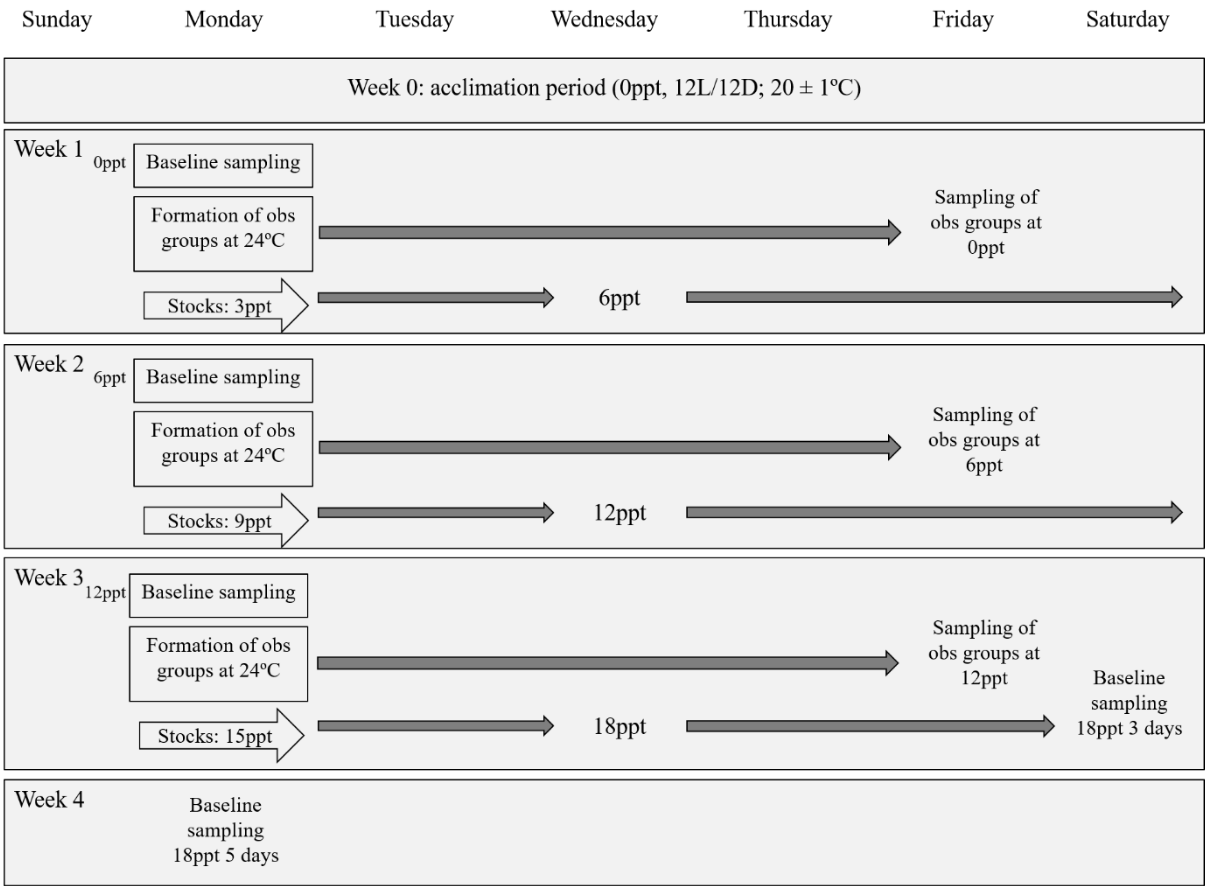


Figure 1. Time series for *trial 4* sampling for baseline values and observational groups (obs groups).

2.4. Data analyses

SigmaPlot 12.5 Software was used for statistical analysis. Data were tested for normality and homogeneity of variances using Shapiro-Wilk and Levene’s test. When these assumptions were not met, the first approach was to log10 transform data. If parametric assumptions were not met after transformation, non-parametric statistics were used. Statistical significance was established at $\alpha = 0.05$ level for all tests and data is presented as mean ± SEM. Two-tailed tests were used throughout. Further details are provided for each trial below.

Trial 1. To assess plasmatic parameters and activity of Na^+/K^+-ATPase pump in gills it was applied a one-way ANOVA, followed by a Holm-Sidak method or a Kruskal-Wallis test followed by Dunn's method.

Trial 2. To assess plasmatic parameters among salinities it was used a one-way ANOVA for each time (T30 and T60), followed by a Holm-Sidak method or a Kruskal-Wallis test followed by Dunn's method. It was used a t-test or a Mann-Whitney Rank Sum Test to test for difference in each salinity over time.

Trial 3. To test for differences in growth among salinities, it was used a one-way ANOVA followed by Holm-Sidak test or a Kruskal-Wallis teste followed by Dunn's method. To test for difference in aggressiveness between 0 ppt and 18 ppt a t-test was performed.

Trial 4. To assess plasmatic parameters and activity of Na^+/K^+-ATPase pump in gills among salinities it was used a one-way ANOVA, followed by a Holm-Sidak method or a Kruskal-Wallis test followed by Dunn's method. The values of dominance index (DI) were transformed by arcsine square root. Pearson correlations were estimated between DI and size (standard length and weight), and between DI and plasmatic parameters. For this purpose, the biometric data was normalized to avoid bias related to differences among groups (normalized standard length SL': standard length of an individual divided by the mean standard length of the group, normalized weight W': weight of an individual divided by the mean weight of the group). To test for the effects of salinity and phase (initial or final) or status (territorial or non-territorial) on plasma parameters (cortisol, glucose and lactate), a 2-way ANOVA was used, followed by the Holm-Sidak method.

3. Results

Trial 1

No mortality was observed at any salinity. Plasma osmolality was significantly elevated at the highest salinity (0 ppt = 312.00 ± 12.26 mOsm/kg; 5 ppt = 321.67 ± 14.49 mOsm/kg; 15 ppt = 360.75 ± 7.20 mOsm/kg; one-way ANOVA F = 5.90, p = 0.01, Figure 2a).

Cortisol peaked at 15 ppt (0 ppt = 42.75 ± 8.80 ng/mL; 5 ppt = 30.76 ± 10.37 ng/mL; 15 ppt = 226.11 ± 61.29 ng/mL; Kruskal-Wallis H = 11.34, p = 0.003; Figure 2b).

Gill Na$^+$/K$^+$-ATPase activity was significantly higher at the highest salinity (0 ppt = 1.13 ± 0.14 µmol ADP mg protein^{-1} h^{-1}; 5 ppt = 1.03 ± 0.07 µmol ADP mgprotein^{-1}h^{-1}; 15 ppt = 2.11 ± 0.23 µmol ADP mg protein^{-1} h^{-1}; Kruskal-Wallis H = 11.18, p = 0.004, Figure 3a), but its activity in the kidney did not differ among salinities (one-way ANOVA F = 2.76, p > 0.05, Figure 3b).

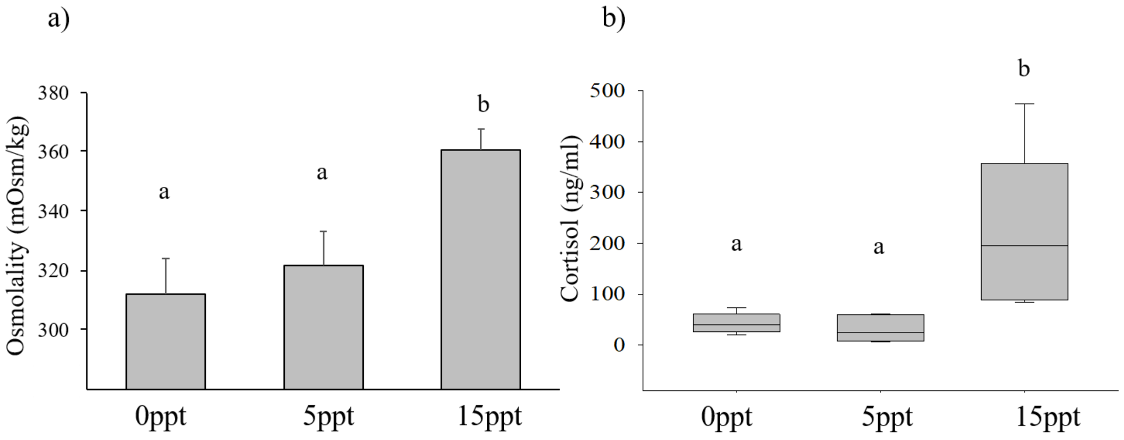


Figure 2. Values of (a) osmolality (mean ± SEM), and (b) cortisol (median, interquartile range, maximum and minimum) of *A. facetus* among treatments. Different letter denotes significant difference.

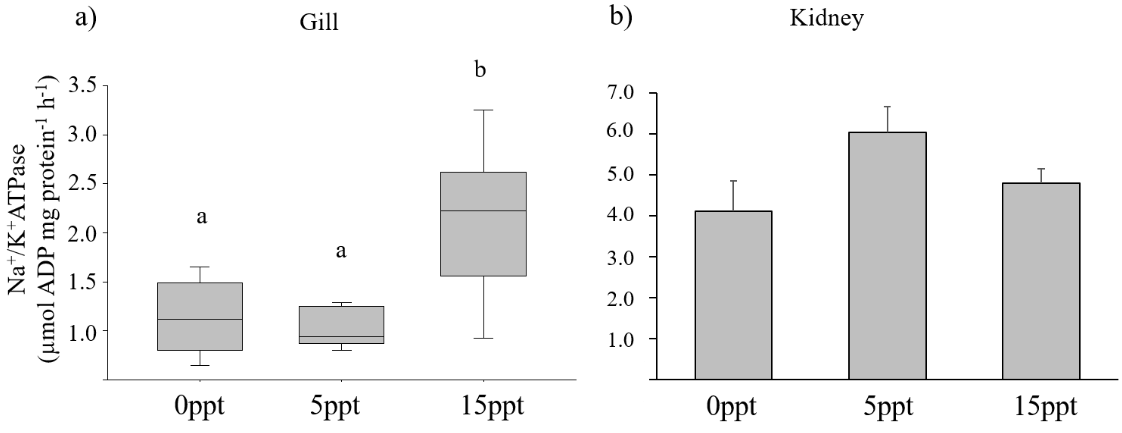


Figure 3. Activity of Na$^+$/K$^+$-ATPase in gills (median, interquartile range, maximum and minimum) and kidney (mean ± SEM) of *A. facetus* among treatments. Asterisks denote significant difference against control (0 ppt).

The gill Na^+/K^+-ATPase activity returned a weak but positive correlation with osmolality (Spearman correlation r = 0.49, $r^2 = 0.24$; p = 0.03, n = 19). However, there was no significant correlation between osmolality and the activity of kidney Na^+/K^+-ATPase (Spearman correlation r = 0.17, $r^2 = 0.03$; p > 0.05, n = 18). The correlation between osmolality and cortisol levels was positive (Spearman correlation r = 0.66, $r^2 = 0.44$; p = 0.001, n = 19) and mainly driven by the correlation at 15 ppt (Spearman correlation r = 0.92, $r^2 = 0.85$, p = 0.001, n = 8, Figure 4). Other correlations were not significant.

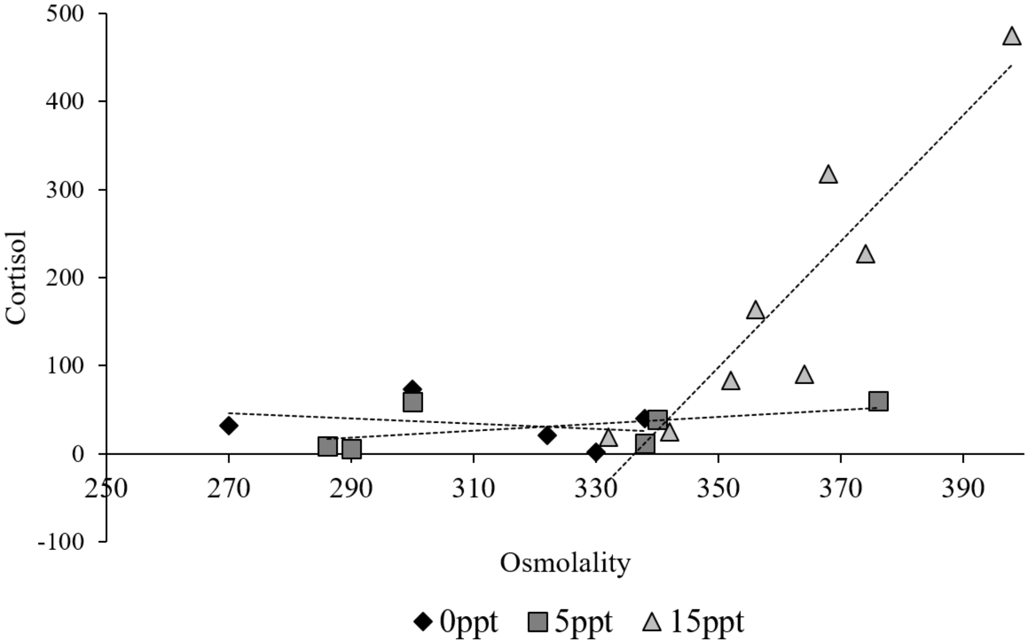


Figure 4. Correlation between osmolality (mOsm/kg) and plasma cortisol (ng/mL) in *A. facetus* subjected to acclimation at different salinities (0 ppt, 5 ppt and 15 ppt).

Trial 2

At 0, 6 and 12 ppt, the mortality observed was due to injuries caused by aggression. No social behaviour was observed at 18 ppt, and the mortality observed was related to the salinity (43.8% of mortality at 18 ppt after 15 days of acclimation, 53.1% after 30 days, 65.6% after 45 days and 65.6% after 60 days).

Growth halted after 15 days at 18 ppt and was consistently lower thereafter, while in the other salinities fish experienced continuous growth (Kruskal-Wallis T15: H = 16.95, p < 0.001; T30: H = 18.84, p < 0.001; T45: H = 17.07, p < 0.001; T60: H = 20.23, p < 0.001, Figure 5).

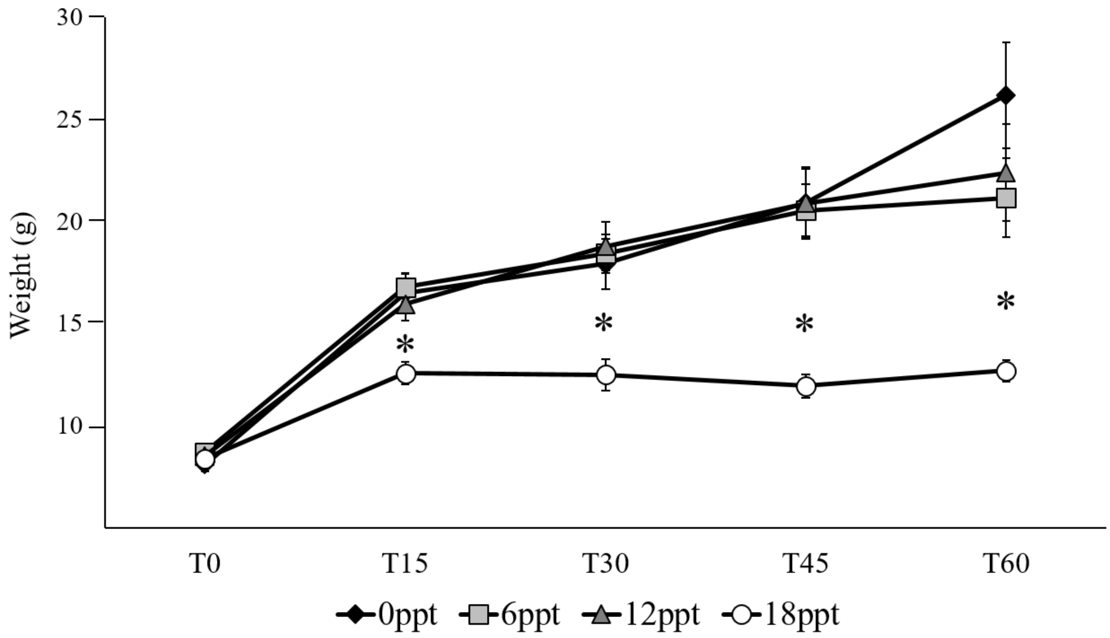


Figure 5. Weight (g) of *A. facetus* subjected to different salinities along time. Asterisks denote significant difference against control (0 ppt). Kruskal-Wallis test, $p < 0.05$, for each time frame, mean ± SEM.

Osmolality increased with the increase in salinity (Kruskal-Wallis: T30 H = 14.84, p = 0.002; T60 H = 28.71, p < 0.001, Figure 6a).

While no differences in cortisol were observed at T30, cortisol was lower at 18 ppt compared to 0 ppt at T60 (One-way ANOVA: T30 F = 0.92, p = 0.4; Kruskal-Wallis: T60 H = 17.83, p < 0.001, Figure 6b).

Fish at 18 ppt showed the highest values for chloride at both T30 and T60 (One-way ANOVA: T30 F = 9.06, p < 0.001; T60 F = 55.70, p < 0.001, Figure 6c).

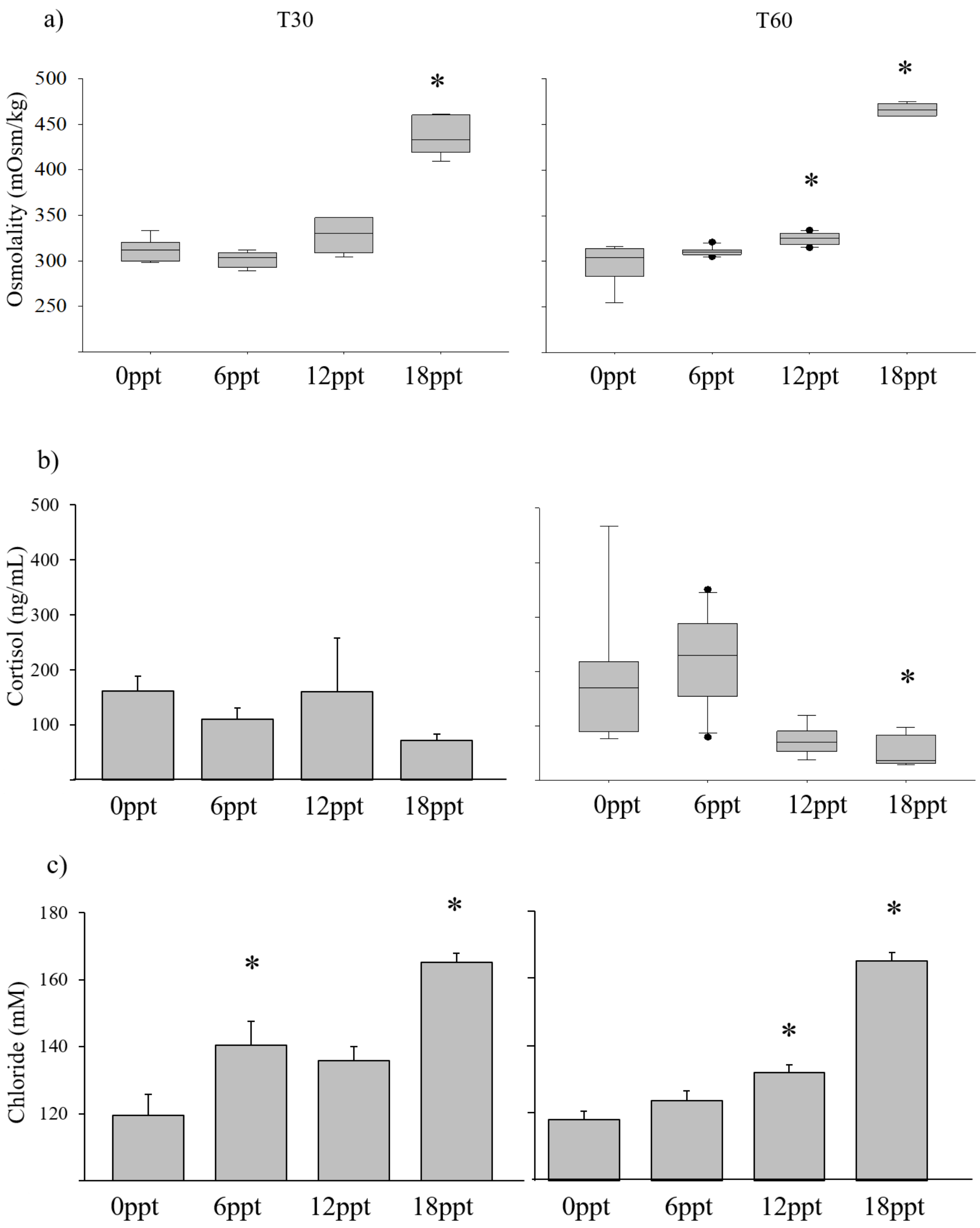


Figure 6. (a) Osmolality (T30 and T60: median, interquartile range, maximum and minimum), (b) cortisol (T30: mean ± SEM; T60: median, interquartile range, maximum and minimum), and (c) chloride (T30 and T60: mean ± SEM) of *A. facetus* subjected to acclimation at different salinities. Asterisks denote significant difference in salinity treatment against control (0 ppt) within the same time (T30 or T60). One-way ANOVA, $p<0.05$.

There was no difference in glucose levels neither at T30 nor T60 (One-way ANOVA, $p> 0.05$, Figure 7a). For lactate, differences among salinities occurred just at T60 (Kruskal-Wallis: T30 H = 2.96, $p > 0.05$; T60 H = 10.61, $p = 0.01$, Figure 7b).

At 6 ppt and 18 ppt, osmolality increased after 60 days, while only fish at 6 ppt showed an increase in cortisol (Table 2). Chloride decreased after 60 days in fish acclimated at 6 ppt (Table 2). For glucose, there was an increase in fish in the control group (0 ppt) and those acclimated at 12 ppt after 60 days; while for lactate, only fish in the control group had an increase after 60 days (Table 2).

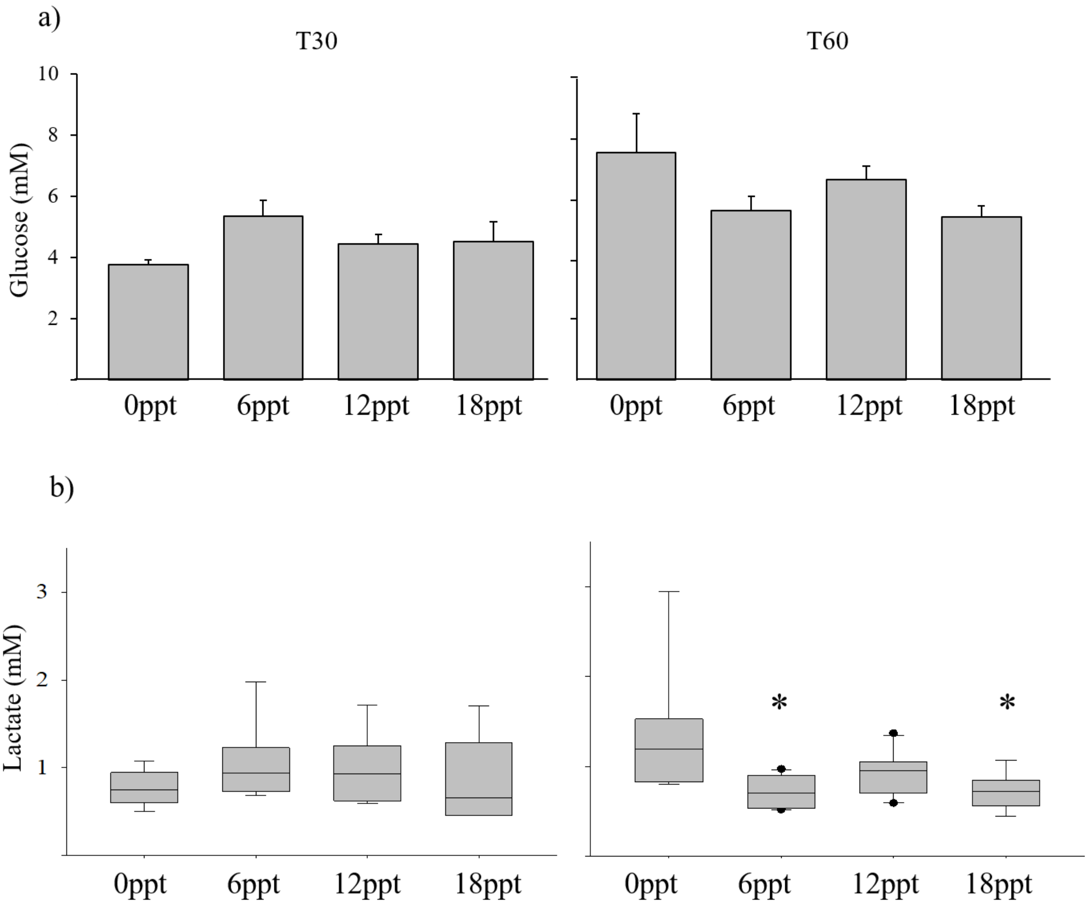


Figure 7. (a) Glucose (mean ± SEM), and (b) lactate (median, interquartile range, maximum and minimum) of *A. facetus* subjected to acclimation at different salinities. Asterisks denote significant difference in salinity treatment against control (0 ppt) within the same time (T30 or T60) ($p<0.05$).

Table 2. Mean values ± SEM of plasma parameters of *A. facetus* subjected to different salinities treatments after 30 days (T30) and 60 days (T60). Asterisks denote significant differences along time (t-test: t; Mann-Whitney test: U; p < 0.05).

	Salinity (ppt)	T30 Mean ± SEM (n)	T60 Mean ± SEM (n)	T30 *x* T60 Test value; p-value
Osmolality (mOsm/kg)	0	311.7 ± 4.71 (6)	297.4 ± 6.89 (8)	t = 1.48; 0.16
	6	301.7 ± 3.22 (6)	310.6 ± 1.39 (10)	t = -2.72; 0.02*
	12	328.4 ± 7.91 (5)	324.6 ± 2.07 (10)	U = 23.5; 0.90
	18	438.2 ± 8.76 (5)	465.9 ± 2.12 (8)	U = 4.0; 0.02*
Cortisol (ng/mL)	0	162.3 ± 26.89 (6)	185. 7 ± 47.38 (7)	U = 20.0; 0.94
	6	110.2 ± 20.39 (5)	219.4 ± 24.06 (10)	t = -2.75; 0.02*
	12	161.1 ± 97.07 (3)	72.9 ± 8.54 (8)	U = 9.0; 0.63
	18	72.2 ± 11.54 (4)	52.1 ± 11.79 (5)	U = 5.0; 0.28
Chloride (mM)	0	119.4 ± 6.23 (6)	118.2 ± 2.47 (7)	t = 0.17; 0.86
	6	140.5 ± 7.04 (6)	123.9 ± 2.79 (10)	t = 2.37; 0.03*
	12	135.8 ± 4.21 (5)	132.3 ± 2.24 (10)	t = 0.74; 0.47
	18	165.2 ± 2.85 (5)	165.7 ± 2.55 (8)	t = -0.11; 0.90
Glucose (mM)	0	3.75 ± 0.16 (6)	7.42 ± 1.28 (4)	U = 0.0; 0.01*
	6	5.35 ± 0.52 (6)	5.53 ± 0.47 (10)	t = -0.22; 0.83
	12	4.43 ± 0.32 (5)	6.53 ± 0.44 (10)	t = -2.97; 0.01*
	18	4.51 ± 0.66 (5)	5.32 ± 0.37 (8)	t = -1.06; 0.31
Lactate (mM)	0	0.77 ± 0.07 (6)	1.39 ± 0.26 (7)	U = 6.0; 0.03*
	6	1.04 ± 0.18 (6)	0.72 ± 0.05 (10)	U = 12.0; 0.06
	12	0.98 ± 0.15 (6)	0.92 ± 0.07 (10)	t = 0.37; 0.71
	18	0.83 ± 0.21 (5)	0.73 ± 0.06 (8)	t = 0.52; 0.61

Trial 3

In group I no mortality was observed and no difference was found neither for weight nor standard length among salinities (Kruskal-Wallis, p > 0.05, Figure 8).

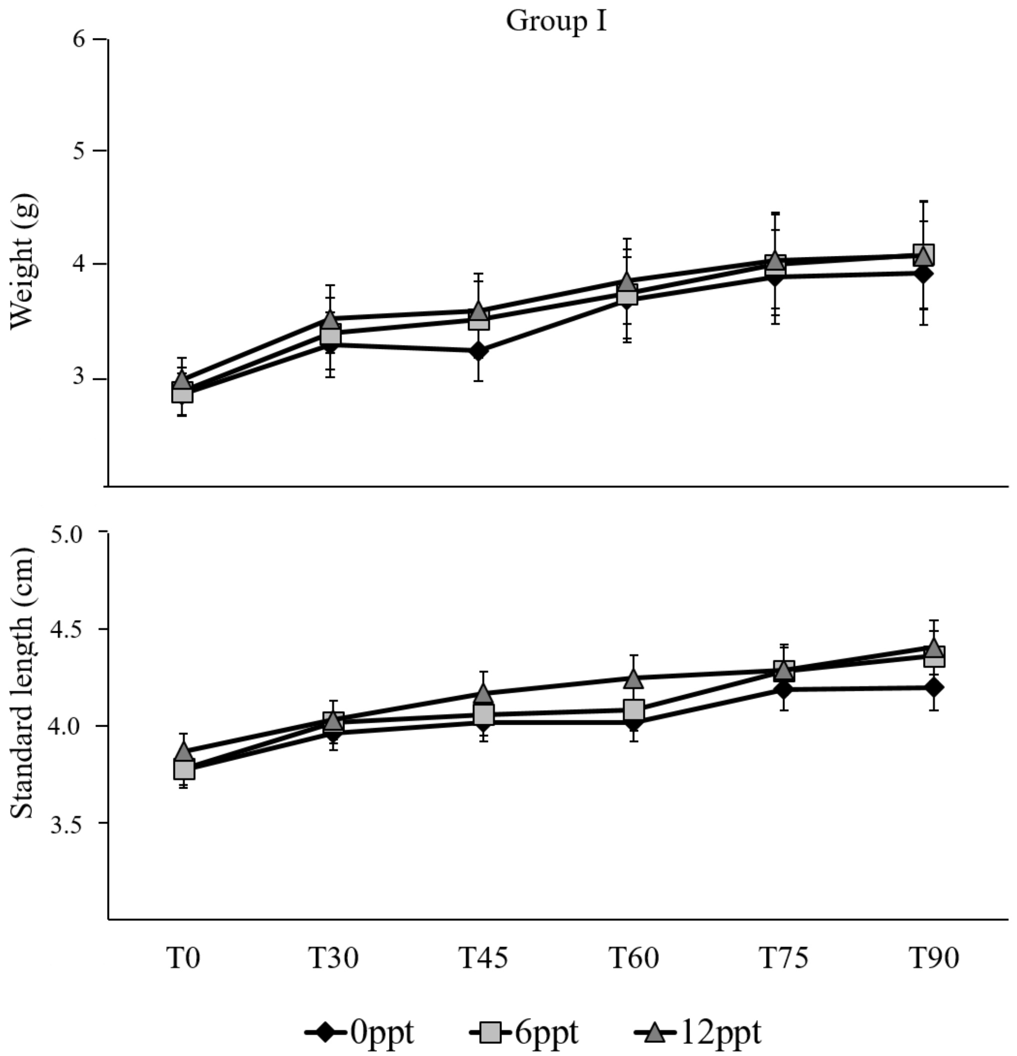


Figure 8. Weight and standard length of *A. facetus* subjected to different salinities along time. Kruskal-Wallis test for each time frame, $p > 0.05$; mean ± SEM.

In group II, 41% of fish died at 18 ppt while no mortality was observed at 0 ppt. No difference in size was observed between fish at control group and acclimated to 18 ppt at each time point, neither for weight nor for standard length (Mann-Whitney, $p > 0.05$, Figure 9).

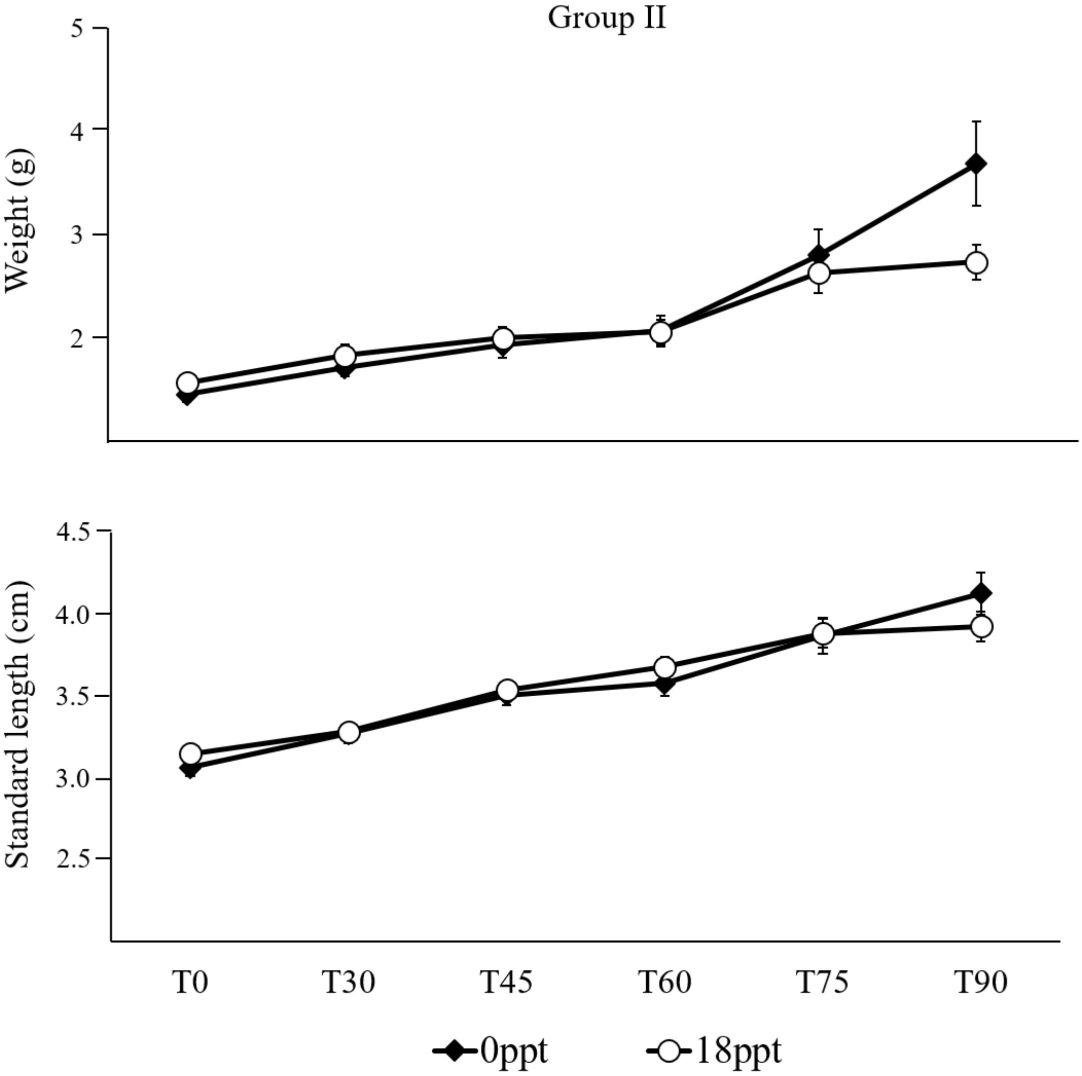


Figure 9. Weight and standard length of *A. facetus* subjected to different salinities along time. Kruskal-Wallis test for each time frame, $p > 0.05$, mean ± SEM.

Aggressive interactions were higher in control group than in fish acclimated to 18 ppt (0 ppt = 2.28 ± 0.29 AI/minute; 18 ppt = 1.24 ± 0.10 AI/minute; t-test = 4.01, p = 0.004, Figure 10).

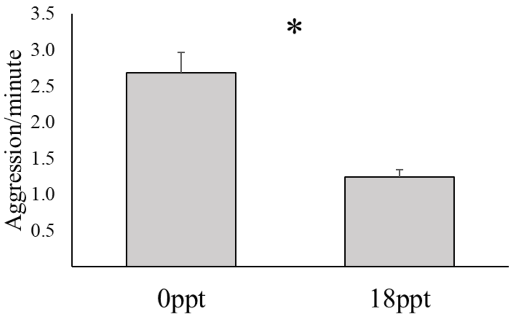


Figure 10. Quantity of aggressive interactions of *A. facetus* acclimated at 0 ppt and 18 ppt. Asterisks denote significant difference, t-test, $p<0.05$.

Trial 4

After 5 days at 18 ppt, 75% of fish died. After the acclimation period, the size of animals selected for behaviour and physiological analyses did not vary among treatments (Weight: $W_{0ppt} = 36.58 \pm 2.59$ g; $W_{6ppt} = 58.33 \pm 4.34$ g; $W_{12ppt} = 63.74 \pm 13.28$ g; $W_{18ppt3d} = 53.80 \pm 4.87$ g; $W_{18ppt5d} = 40.76 \pm 4.11$ g; one-way ANOVA, $F = 2.40$, $p > 0.05$; Standard length: $SL_{0ppt} = 8.88 \pm 0.23$ cm; $SL_{6ppt} = 10.38 \pm 0.2$ cm8; $SL_{12ppt} = 10.20 \pm 0.65$ cm; $SL_{18ppt3d} = 9.88 \pm 0.30$ cm; $SL_{18ppt5d} = 9.34 \pm 0.25$ cm; one-way ANOVA, $F = 2.35$, $p > 0.05$).

Baseline

Osmolality increased as salinity increased (one-way ANOVA, $F = 74.10$, $p < 0.001$, Figure 11a), while there were no differences in the activity of Na^+/K^+-ATPase pump in gills (one-way ANOVA, $F = 1.81$, $p = 0.16$, Figure 11b).

At 18 ppt chloride increased (Kruskal-Wallis, $H = 17.97$, $p = 0.001$) and muscle water content decreased (one-way ANOVA, $F = 14.71$, $p < 0.001$; Figure 11c and d, respectively).

Cortisol presented extremely high values when fish were kept at 18 ppt (Kruskal-Wallis, $H = 21.42$, $p < 0.001$, Figure 11e) whereas glucose did not show differences from control 0 ppt (Kruskal-Wallis, $H = 18.08$, $p > 0.05$, Figure 11f).

Lactate had a decrease after 5 days at 18 ppt (Kruskal-Wallis, $H = 21.19$, $p < 0.001$, Figure 11g). Total protein had a drop at 18 ppt (one-way ANOVA, $F = 22.24$, $p < 0.001$, Figure 11h).

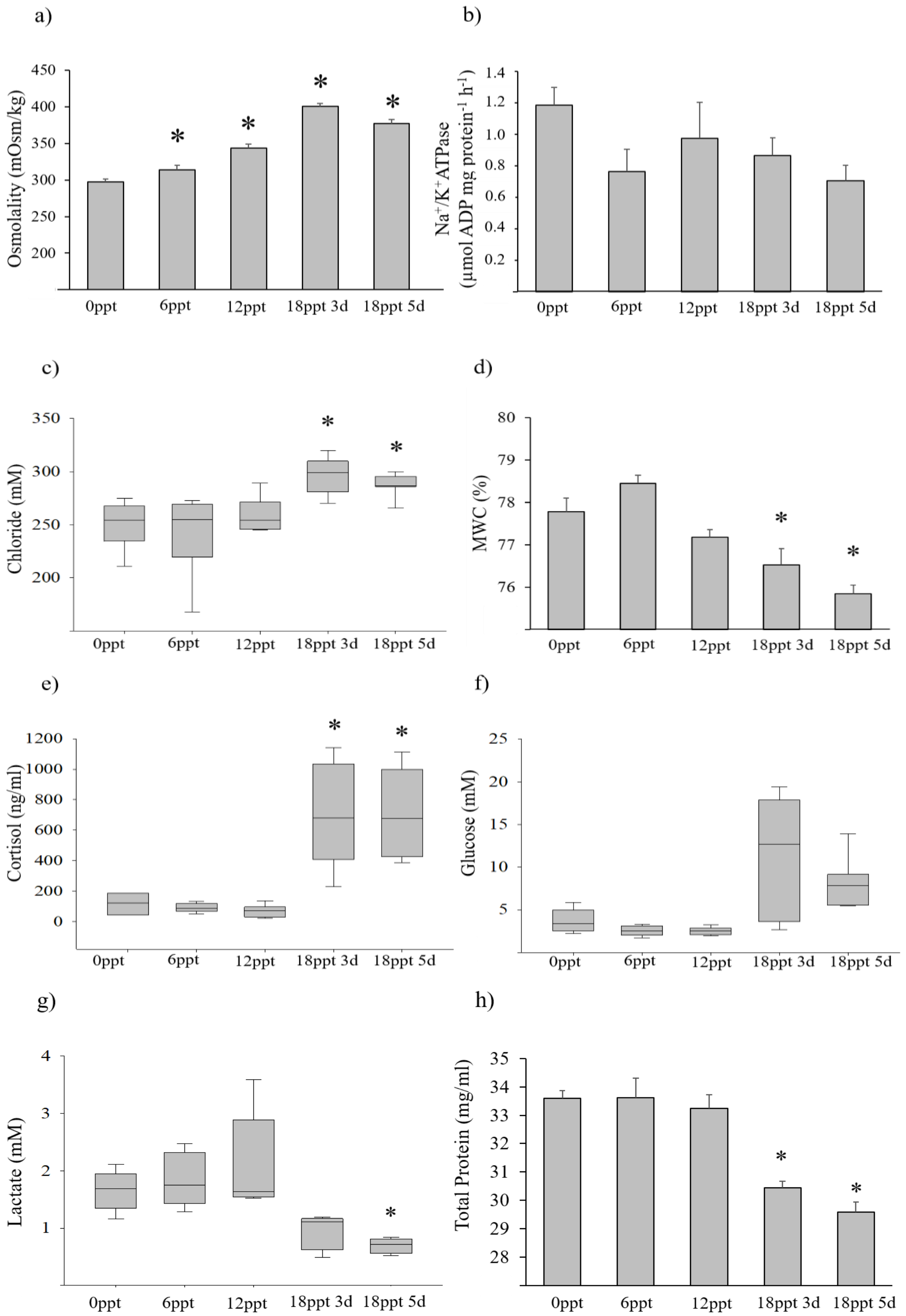


Figure 11. (a) Osmolality, (b) activity of Na+/K+-ATPase pump in gills, (c) chloride, (d) muscle water content, (e) cortisol, (f) glucose, (g) lactate, (h) total protein of *A. facetus* subjected to different salinities. Asterisks denote significant difference in salinity treatment against control (0 ppt) ($p < 0.05$). Mean ± SEM for osmolality, Na^+/K^+-ATPase pump in gills, muscle water content and total protein. Median, interquartile range, maximum and minimum for chloride, cortisol, glucose and lactate.

Behaviour

There was no difference in aggression among salinities (0 ppt = 0.65 ± 0.18 AI/minute; 6 ppt = 0.57 ± 0.14 AI/minute; 12 ppt = 0.53 ± 0.13 AI/minute; one-way ANOVA F = 0.16, $p > 0.05$). Unfortunately, it was not possible to carry out this evaluation at 18 ppt.

Within all salinities, size had a strong effect on DI for males (Table 3). However, in females only at 6 ppt the weight positively correlated with DI (Table 3).

Table 3. Pearson correlations between dominance index (DI) and weight (W) or standard length (SL) for *A. facetus* at different salinities. Asterisks mark significant differences (*p < 0.05; **p < 0.01).

		W (g)			SL (cm)		
		0ppt	6ppt	12ppt	0ppt	6ppt	12ppt
Females	r	-0.97	0.75*	-0.62	-1.00	0.48	-0.75
	n	3	8	6	3	8	6
Males	r	0.66**	0.81**	0.66*	0.72**	0.77**	0.65*
	n	17	12	13	17	12	13

No correlation was found between DI and cortisol, glucose or chloride at any salinity or phase ($p > 0.05$). But a significant negative correlation was found for lactate in males at 12 ppt, both at initial and at the final phases (initial phase: r = -0.57, n = 13, p = 0.04; final phase: r = -0.69, n = 13, p = 0.01).

There was no difference in cortisol levels among salinities before hierarchy formation (initial phase), but cortisol was higher at 12 ppt at the final phase (two-way ANOVA, salinity F = 2.42, p = 0.09, phase F = 3.38, p = 0.07, interaction F = 12.94, $p < 0.001$, Figure 12a).

The levels of glucose were dependent on both salinity and phase, with high levels of glucose at 0 ppt and 12 ppt at final phase (two-way ANOVA, salinity F = 11.41, $p < 0.001$, phase F = 30.81, $p < 0.001$, interaction F = 12.74, $p < 0.001$, Figure 12b). At the onset of the experiment, fish at 12 ppt had the highest levels of lactate, but at the end lactate did not show differences among salinities (two-way ANOVA, salinity F = 0.63, p = 0.53, phase F = 9.37, p = 0.003, interaction F = 0.96, p = 0.39, Figure 12c).

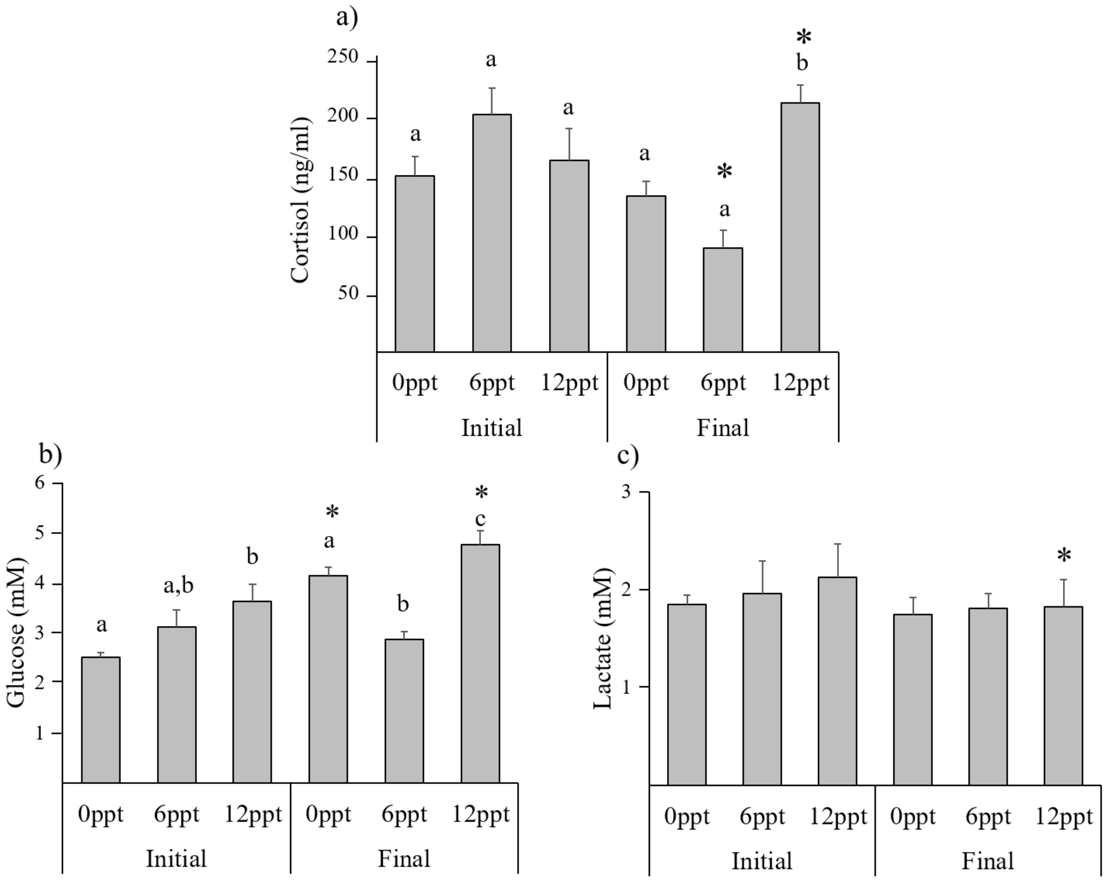


Figure 12. Plasmatic parameters assessed in *A. facetus* during hierarchy formation at different salinities. Different letters denote significant difference among salinity treatments within same phase (initial and final); asterisks denote significant difference of the same salinity-group over time at $p < 0.05$. Two-Way ANOVA, $p < 0.05$, mean ± SEM.

Salinity influenced the results of plasmatic parameters with cortisol and glucose having lower values when fish were at 6 ppt, regardless of social status, while lactate did not change (two-way ANOVA for cortisol: salinity $F = 12.35$, $p < 0.001$, status $F = 1.53$, $p = 0.22$, interaction $F = 0.04$, $p = 0.95$; for glucose: salinity $F = 15.54$, $p < 0.001$, status $F = 0.08$, $p = 0.77$, interaction $F = 0.02$, $p = 0.98$; for lactate: salinity $F = 0.03$, $p = 0.67$, status $F = 1.88$, $p = 0.17$, interaction $F = 0.26$, $p = 0.77$; 3) (Table 4).

Table 4. Plasmatic parameters assessed in *A. facetus* related to its social status. Letters denote differences among salinities for the same social status. No differences between status were observed. (n Territorial at 0 ppt = 4, 6 ppt = 4, 12 ppt = 5; n non-Territorial at 0 ppt = 16, 6 ppt = 16, 12 ppt = 14). Two-way ANOVA, $p < 0.05$, data is presented as mean ± SEM.

	Salinity (ppt)	Territorial	non-Territorial
Cortisol (ng/mL)	0	115.9±23.50 ^{a,b}	135.39±14.52 ^{a}
	6	68.5±24.99 ^{a}	92.4±17.44 ^{a}
	12	185.7±6.08 ^{b}	219.2±19.54 ^{b}
Glucose (mM)	0	4.06±0.14 ^{a,b}	4.16±0.21 ^{a}
	6	2.76±0.14 ^{a}	2.89±0.19 ^{b}
	12	4.76±0.41 ^{b}	4.76±0.33 ^{a}
Lactate (mM)	0	1.69±0.04 ^{a}	1.76±0.10 ^{a}
	6	1.68±0.12 ^{a}	1.84±0.10 ^{a}
	12	1.62±0.14 ^{a}	1.90±0.11 ^{a}

4. Discussion

The present work showed that the freshwater neotropical cichlid *Australoheros facetus*' tolerance to salinity ranges between 0 and 12, while some individual fish can tolerate up to 18 ppt, with this upper limit already causing important physiological disturbances, growth arrest and significant mortality. Analysis of physiological parameters in response to osmotic challenges clear demonstrate attempts to maintain internal homeostasis beyond 12 ppt, although some variations occur between the different trials performed, possibly depending, among others, on fish individual tolerance, fish size or water temperature.

Overall, there were no major differences in physiology and behaviour of fish acclimated from 0 ppt to 12 ppt, suggesting *A. facetus* is a freshwater fish with some degree of euryhalinity. Within these salinities plasma osmolality was kept between 300-340 mOsmol/Kg, but it rose significantly above 15 ppt, reaching over 450 mOsmol/kg when acclimated 18 ppt. This indicates the species has poor hypoosmoregulatory capacity above the isotonic conditions, although osmolality and cortisol levels, as well as gill Na^+/K^+-ATPase activity were highly variable among individual fish in these higher salinities, thus suggesting some variation in the individual coping ability, and the possibility that some fish may successfully acclimate. Nonetheless, we observed a clear breakdown point at 18 ppt for the fish from *trial 4* (largest fish analysed, ~50.5 g), that were not able to withstand that salinity despite a 3-week acclimation period, while smaller fish could withstand the same challenge during longer periods (*trial 3*, group II: ~1.5 g, 90 days), with different degrees of physiological and behaviour impairments.

In a previous study, acute toxicity tests recorded a maximum incipient lethal level of salinity for a south American population of *A. facetus* (*Cichlasoma facetum* in the cited study) of 20.2 ppt (Gómez & Naya, 2007), but noting that they were able to resist at 35 ppt for circa 80 minutes (50% of mortality). In the present study, no mortality related to salinity changes from 0 ppt to 12 ppt occurred - the mortality observed in these groups was related to aggression between fish, associated to confinement and temperature. Salinity related mortality was first identified in *trial 2* at 18 ppt, being above 40% after two weeks of exposure, and then in *trial 3* using small fish and in *trial 4* using adults. Indeed, at 18 ppt, the proportion of mortality increased with fish size (41% in fish with ~1.5 g; ~66% of fish with ~8.4 g and 75% of fish with ~50.5 g). It is nevertheless important to note that the larger fish were exposed to 18 ppt for 5 days, while the smaller were kept at 18 ppt at least for 60 days. These results, where larger individuals appear more susceptible than smaller ones, were opposite of that found for some species of sturgeons, where the adult fish were able to withstand salinities above 20 ppt better than juveniles (Altinok et al., 1998; LeBreton & Beamish, 1998; McEnroe & Cech, 1985). However, it has also been shown for other species that different life stages respond differently to varying environmental conditions (Banks et al., 1991; Glover et al., 2012; Jensen et al., 2015). The reason why adults should fare worst is, however, still elusive. As osmoregulatory capability is intrinsically linked to the activity of the Na^+/K^+-ATPase pump it is important to highlight that gill Na^+/K^+-ATPase activity has been related to size and allometric growth of the gill (Gibbs & Somero, 1990). Gibbs and Somero (1990) hypotesized that larger individuals could have relatively smaller gills or that gills in larger animals could have lower specific activity. Interestingly, in *trial 1* (fish with ~5.2 g) after 30 days showed a significant increase in the gill Na^+/K^+-ATPase activity at 15 ppt, while in *trial 4* (fish with ~50 g), after 5 days of acclimation did not show any difference in their gill Na^+/K^+-ATPase activity at any salinity.

This inability to mount a branchial osmoregulatory response, paralleled by evident disturbances in blood substrates, may explain the high mortality in large fish. In general, however, the Na^+/K^+-ATPase activities measured was low, with average values around 1.2 µmol ADP mg protein^{-1} h^{-1}, and rising to 2.5 µmol ADP mg protein^{-1} h^{-1}. These are about 10 fold lower than those recorded for juvenile sea bream of the same size in similar salinities acclimated during two weeks (Laiz-Carrion et al., 2005), despite not so different from the pearl spot *Etroplus suratensis* acclimated at 15 ppt during 15 days (3.28 ± 0.16 µmol ADP mg protein^{-1} h^{-1}) (Chandrasekar et al., 2014). The lack of differences of the Na^+/K^+-ATPase activity in kidney, in *trial 1*, could be related to the suggestion that this organ is more related to the excretion of divalent ions (McCormick, 2011), in a way that it would be better to assess the Ca^{2+}-ATPase activity in kidney (Marshall & Grossel, 2005), or even the vacuolar H^+-ATPase (V-ATPase) that regulates the acid-base balance (Perry et al., 2003).

These small changes in branchial Na^+/K^+-ATPase activity proved inefficient to maintain osmotic homeostasis even in the smaller animals. In addition to significant changes in plasma osmolality, which follow similar trends of plasma chloride, fish tissues also suffered significant dehydration. The decrease in muscle water content of fish subjected to 18 ppt reflect the osmoregulatory failure. The limitations of anisosmotic extracellular regulation trigger mechanisms of anisosmotic intracellular regulation, and increases in extracellular salt concentration beyond the capacity for cell volume regulation can lead to significant loss of cell water (Freire et al., 2008). This is of course valid for most cell types, thus contributing for overall physiological impairment.

Previous studies have shown that exposure to hypertonic environments, leading to ingestion of salts and the consequent osmoregulatory activity, is energetically expensive (Altinok & Grizzle, 2001; Bœuf & Payan, 2001), with possible consequences on growth and reproduction (St Mary et al., 2001; Wang et al., 1997). We have recorded growth arrest in juvenile fish (~8.4 g) exposed to 18 ppt in *trial 2*, which were followed for 60 days, and size were significantly reduced from 15 days onwards, but no changes were seen between fish at 0, 6 or 12 ppt. Surprisingly no significant weight and length differences were observed between 0 ppt and 18 ppt, even after 90 days of acclimation in small fish (*trial 3*, ~1.5 g). As indicated, fish acclimated at 0 ppt, 6 ppt or 12 ppt did not show differences in growth, irrespective of their initial size/age (*trials 2* and *3*) or time of exposure. Rearing fish near their isosmotic point, i.e. around 300-340 mOsm/kg and equivalent to approximately 10 ± 2 ppt (De Boeck et al., 2000; Evans, 2008), should have an energy saving effect since it reduces the energy expenditure to osmoregulation, consequently promoting growth (Bœuf & Payan, 2001). However, as an example, the common carp exposed to a salinity of 10 ppt had a decreased growth rate, observed after 10 days of acclimation, with a drop in food intake by 70% probably because the potential salinity-related alterations in appetite (De Boeck et al., 2000). Martinez-Palacios et al. (1990) working with *C. urophthalmus* observed a final body weight and specific growth rate higher in brackish and saline water than in freshwater, in agreement with the fact that it is an euryhaline species that inhabits from freshwater to estuaries and coastal marshes.

In parallel with increased osmoregulatory activity, *A. facetus* in higher salinity showed significant changes in plasma substrates such as glucose, lactate and total protein, when compared to fish between 0 and 12 ppt. These substrates were dramatically altered in adult fish within 3 and 5 days of exposure to 18 ppt. Lactate, which is suggested as a secondary energetic source that along with glucose serves as an oxidative fuel for elevated neuronal energy metabolism used to restore ionic balance through ATPase pumps (Gladden, 2004), was substantially reduced., which could be related to its depletion, moving it from the plasma to the gills, for example. In juveniles, a drop in lactate occurred in all but the 0 ppt group after 60 days. Additional analyses of enzyme activities for glycolysis in gills, as pyruvate kinase (PK) or markers of oxidative metabolism, could help to understand this dynamic process. Glucose is an essential fuel for several tissues (Kroon et al., 2017; Martínez-Porchas et al., 2009) and despite the non-significant increase in plasma levels at 18 ppt for bigger fish despite the high individual variability, it is possible that those animals had to complement the energetic demand of osmoregulation using the circulating protein in plasma (Kroon et al., 2017). *Trial* 2 (with smaller fish) also did not show differences in glucose levels among all salinities tested, both after 30 days and 60 days of exposure. Similar results were found for juvenile shortnose sturgeon *Acipenser brevirostrum*, that after 10 weeks acclimated to different salinities did not show differences in its plasmatic glucose (Jarvis & Ballantyne, 2003).

Cortisol is a general stress-response hormone and responsible for the mobilization of metabolic substrates (Martínez-Porchas et al., 2009; Mommsen et al., 1999). This appears to fit our data, since elevated plasma cortisol was found in the fish at the higher salinity, which constitutes a stressful situation, also showing increased enzymatic activity and in general a trend in increased glucose. However, cortisol was also shown to act in the hydromineral balance, namely in the adaptation to hyperosmotic environments in several fish, including cichlids (Aruna et al., 2012; Dean et al., 2003), stimulating the Na^+/K^+-ATPase pump in gills, thus adding to lower blood osmolality (Laiz-Carrión et al., 2002; McCormick, 1990, 2001; Mommsen et al., 1999). Our data showed increases in Na^+/K^+-ATPase activity (2.11 ± 0.23 µmol ADP mg protein $^{-1}$ h^{-1}) and cortisol (175.07 ± 52.33 ng/mL) in juvenile fish at 15 ppt in *trial 1*, but in *trial 4*, despite the extreme peak in cortisol (712.07 ± 82.48 ng/mL) at 18 ppt, the gill Na^+/K^+-ATPase activity in adults was at basal levels (~0.79 ± 0.08 ADP mg protein $^{-1}$ h^{-1}), likely reflecting a breakdown point for osmoregulation ability. Interestingly, in *trial 2*, juvenile fish showed no marked differences in cortisol within 30 days of exposure, but cortisol was apparently higher in the 0 and 6 ppt groups. Taking into consideration the higher water temperature during this trial and the difference in fish sizes this may be related to the increase activity in social behaviours (Baduy et al., 2017) not seen at higher salinities.

As already recorded for *A. facetus*, the limiting resource while in a conspecific group is territory and mate and they usually show a strong size response when kept in groups, the territorial animals being the largest (Baduy et al., 2017). Fish groups can show a size-hierarchy effect, which means that some fish within a group can grow faster than the others either due to genetic differences or social interactions driven by competition triggered by a limited resource (Zimmerer, 1983). A study tested this effect at different salinities for three fish species and found that within intra-specific groups of two fish species the size-hierarchy effect was present, but when the fish were reared isolated, eliminating the competition for food, the size-hierarchy effect was not apparent (Zimmerer, 1983). The author concluded that salinity itself had no effect on the size-hierarchy effect, but salinity can modify the degree of size-hierarchy effect through competition for food. At salinities below 12 ppt the individual variation in growth of *A. facetus* could be a reflection of this. At 18 ppt, even with non-significant responses to salinity (as in the case of *trial 3*), the individual variation in weight and standard length of animals were lower than fish kept at 0 ppt. This could be an indication of the cost and trade-off among social/reproductive behaviour and osmoregulation.

The behavioural results (*trial 4* – behaviour) suggest that size strongly influences male rank as already described (Baduy et al., 2017), and salinity ranging from 0 ppt to 12 ppt did not influence the quantity of aggressive interactions within a group. In contrast, small fish acclimated at 18 ppt (*trial 3*, group II) showed less aggressive interactions than fish at 0 ppt. Additionally, *trial 4* showed that social status did not influence cortisol, glucose and lactate, but salinity did for cortisol and glucose. To our knowledge, this is the first study evaluating the process of hierarchy formation at different salinities, highlighting differences in stress markers before and after the hierarchy formation and between social status. The results achieved here could indicate that at salinities below 12 ppt *A. facetus* seems to not have severe constraints for hierarchy formation. In addition, in a few occasions in *trial 2*, we observed spawning and successful hatching at 6 ppt, and normal pair formation and reproductive behaviour at 12 ppt, despite the small tanks.

In *trial 4*, salinity and phase (before and after hierarchy formation) influenced glucose levels, which increased with salinity before the hierarchy formation. After the hierarchy formation, glucose did not show a clear pattern related to salinity, but was higher in the groups at 0 ppt and 12 ppt. The lack of differences in amount of aggressive interactions among salinities, suggests that other kinds of behaviour or reproductive status drove the changes in glucose. Altogether with the results for cortisol, these results can be a reflection of the social stress during the hierarchy formation, since for cortisol only the interaction between salinity and hierarchy formation was significant.

In conclusion, these results suggest that *A. facetus* can occur in brackish waters, at least for a short period of time, at the expense of increased energy for osmoregulation to keep the balance between external and internal ion concentrations at salinities equal to or higher than 15 ppt. At higher salinities, this species may be not able engage in typical social behaviours, which will hinder reproduction. However, if *A. facetus* colonizes the upper and middle reaches of the Guadiana estuary (0 ppt to 12 ppt), probably it won’t have problems regarding its social dynamics and most of the physiological parameters evaluated here. In addition, despite the low survival at higher salinities, not all individuals showed abnormal physiological indicators, which may suggest some individual variation can give the species plasticity to survive at hypertonic environments. This information may be relevant to assess ecological implications, either establishing the upper estuaries as corridors to other freshwater bodies or as potential new habitats.

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CHAPTER FIVE

SOCIAL ORGANIZATION AND ENDOCRINE PROFILES OF *Australoheros facetus*, A NON-NATIVE FRESHWATER FISH IN SOUTHERN PORTUGAL

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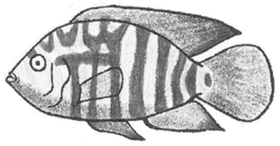

Social organization and endocrine profiles of *Australoheros facetus*, a non-native freshwater fish in southern Portugal

Abstract

Australoheros facetus is a neotropical cichlid and an exotic species in the Guadiana and Odelouca basins (Southern Portugal). In this research, we aimed to characterize the main behavioural patterns and circulating hormones, 17β-estradiol (E_2) for females, and testosterone, 11-ketotestosterone (11KT) and cortisol for both sexes during the formation of social groups, a crucial step in the life-history of *A. facetus.* A pair-breeding strategy with territorial behaviour and aggressive interactions was found, with a positive correlation between dominance and size for both sexes. There were no significant differences between non-territorial and territorial individuals in the baseline levels of hormones, but 11KT was higher in males when they became territorial, as E_2 for territorial females, while there was no clear pattern for testosterone. In contrast, cortisol was higher in non-territorial males and correlated negatively with social dominance. These results suggest that size is the main driver for social dominance and that formation of stable hierarchies result in higher circulating 11KT and lowers stress in territorial males. Related to *A. facetus*' behaviour, aggressiveness and biparental care may be an advantage during the invasion process of *A. facetus*, facilitating colonization of new habitats.

Key-words: non-native fish, aggressiveness, hormonal profile, hierarchy formation, Cichlid

1. Introduction

In nature, animals often compete for resources such as food and territory. If the benefits stemming from the resource are larger than the risks or energy spent to defend it, competition will likely take the form of aggressive interactions (Johnsson et al., 2006). When aggressive interactions occur intra-specifically, they can lead to the formation of social hierarchies with different degrees of dominance (Colleter & Brown, 2011; Dewsbury, 1982; Morse, 1974; Ramallo et al., 2015). In many animals, including fish, size is the main cue for the establishment of the hierarchical position although dynamic social interactions can also influence the rank of an individual within a hierarchy (Beacham, 1987; Chase et al., 2002).

Hierarchy related conflicts, however, can generate a large energy cost and antagonists risk physical injuries and even death (Neat et al., 1998). To avoid these costs, some fish species have developed morphological, physiological and behavioural responses to affirm status of an individual within a hierarchy, such as colour change, chemical signals and/or corporal displays, integrating the social information with their internal physiology to show an adequate behavioural response (Keller-Costa et al., 2016; Miyai et al., 2011; O'Connell et al., 2013).

It has been suggested that territoriality can influence androgen responsiveness with an increase in response to territorial intrusion and that the response of androgens to certain behaviours can be greater in periods of social instability (Almeida, Goncalves-de-Freitas, et al., 2014; Hirschenhauser et al., 2004; and reviwed in Oliveira et al., 2002; Tibbetts & Crocker, 2014). Accordingly, the “challenge hypothesis” predicts that an unclear pattern in high values of circulating androgens may be more related to male-male aggression than to the expression of mating behaviour or secondary sexual characters (Tibbetts & Crocker, 2014; Wingfield et al., 1990). Among the fish androgens, testosterone is normally present in high concentrations in blood plasma and is generally viewed as the precursor of the potent androgen 11-ketotestosterone (11KT) and 17β-estradiol (E_2). 11KT is produced in the testis where it promotes spermatogenesis and it has also been found to be associated with resolution of social conflicts (O'Connell et al., 2013; Oliveira, 2005; Schulz et al., 2010). However, some recent studies also suggest a dissociation between 11KT and aggressive behaviour or hierarchical changes, and that hormones might not be directly responsible for the expression of aggressive behaviours but rather have a moderator role, modifying the frequency and intensity of aggressive territorial and mate-guarding behaviours, even in the absence of the testis (Almeida, Canario, et al., 2014; Hau & Goymann, 2015; Maruska, 2015; Wingfield et al., 1990).

E_2 is the classical female hormone which mediates vitellogenesis and oogenesis in non-mammalian vertebrates, and feeds back on hypothalamus and pituitary to regulate gonadotrophins (Lubzens et al., 2010). Among the described behavioural effects of exposure to estrogens (including environmental estrogens) on dominant males is a reduction in aggression (Bell, 2001; Colman et al., 2009; Filby et al., 2012; Majewski et al., 2002) and induction of feminization or sex reversal on fish populations (Falahatkar et al., 2014; Wang et al., 2008). Aggression has also been reported to correlate positively with aromatase activity in the brain pre-optic area in the African cichlid fish *Astatotilapia burtoni* (Huffman et al., 2013), although no correlation was found between dominance and brain aromatase activity status in *Oreochromis mossambicus* males (Oliveira & Canario, 2000).

In fish, as in other vertebrates, circulating levels of corticosteroids are associated with stress. An increase in cortisol leads to an increase in blood glucose concentration and elevated blood pressure, necessary to cope with the energy demanded during a stressful situation (Mommsen et al., 1999). Thus, cortisol levels rise dramatically during demanding events and reflect not only the status (e.g. territorial or non-territorial, reproductive or non-reproductive), but also the stability of the hierarchy itself (Fox et al., 1997; Mommsen et al., 1999). Higher cortisol levels have also been found in subordinate fish suggesting high stress levels (Bender et al., 2008; Fox et al., 1997).

Exotic species are increasingly frequent, either due to direct human activities or to changes in climate that shift environmental conditions, habitats and the organisms inhabiting them (Rahel et al., 2008). When exotic species proliferate, spread, and persist in a new habitat, they start to impact negatively the native community, at which stage they are considered invasive (Mack et al., 2000). Research on invasive success has been focused on identifying environmental tolerance, propagule pressure and life-history traits of invasive organisms (Lennox et al., 2015; Thomaz et al., 2014). However, despite the fact that behavioural traits can play a key role in successful invasive episodes, studies on this subject are scarce (exceptions include Chapple et al., 2012; Holway & Suarez, 1999). Parental care and aggressiveness, for example, can be crucial to the establishment of invasive species as they may enable the species to dominate, to displace or even to locally extinguish the native community through competitive and/or predatory interactions (Drake, 2007; Pereira et al., 2014). Thus, detailed knowledge about the choice and defence of territories, parental care and reproductive tactics can be essential tools for the control and management policies of invasive species (Holway & Suarez, 1999).

The neotropical cichlid *Australoheros facetus*, (commonly known as chanchito) is originally distributed in river drainages of Argentina, Paraguay, Uruguay and Brazil (Rícan & Kullander, 2006) and is exotic in the southern regions of Portugal since *circa* 1940 (Doadrio, 2002). This species has a generalist and opportunistic diet in the lower Guadiana drainage and in South America (Ribeiro et al., 2007; Yafe et al., 2002), and it is a substrate spawner with biparental care (Ruiz et al., 1992). There is a lack of information about its reproductive tactics on its native range, however, its reproduction seems to be triggered by water temperature (personal observation during laboratorial and field works). In general cichlids have complex social behaviours with different patterns of aggressiveness (Keenleyside, 1991) which may be linked to invasive success. The present work aimed at establishing the detailed behavioural repertoire and correlated hormonal profile of *A. facetus* from southern Portugal in social and reproductive contexts, with the view of identifying factors that may contribute to its success as an invasive freshwater fish.

2. Material and methods

2.1. Experimental animals

The *Australoheros facetus* individuals were captured in multiple sessions during the year, by electrical fishing in the Vascão (37º31'43.38"N and 7º31'26.05"O) and Odelouca (37º13'37"N and 8º30'20"O) rivers (capture licence numbers 403, 404, 405 and 406/2015/CAPT), and transported to the experimental facilities of the Centre for Marine Sciences (CCMAR) at the University of Algarve, Gambelas campus. Fish were maintained outdoors in three ~2000 L community tanks for at least one-month prior to the experiments, under natural temperature and photoperiod and fed *ad libitum* every morning (average water temperature measured with a digital thermometer during February: 13.5ºC, May: 24.5ºC, August: 26ºC and November: 14ºC).

Environmental enrichment, acclimation periods and anaesthesia were implemented to minimize discomfort. CCMAR facilities and their staff are certified to house and conduct experiments with live animals ('group-1' license by the Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal) in accordance to the three 'R' policy and national and European legislation.

2.2. Experimental setup

Randomly selected animals from community tanks were anesthetized with 1g of MS-222 and 1 g of sodium bicarbonate in 3 L of water. Standard length (SL, mm) and body mass (W, g) were measured and fish were colour tagged. Blood samples were collected upon tagging (initial phase) to quantify monthly hormone levels under natural photoperiod and temperature, by puncture of the caudal vein using heparinized syringes. Blood samples were centrifuged at 10,000 g for 5 minutes and the supernatant plasma (~ up to 100 µl/animal) collected and stored at −20°C until assayed. Social groups of 4 to 6 individuals were assembled in 250 L tanks with a glass window and an empty flower pot to serve as shelter or nest. Different groups were settled during August and November 2014 and February and May 2015 (Table 1). The groups were formed with individuals of similar size to minimize the coefficient of variation (CV%) (Table 1). As this species does not have evident sexual dimorphism, the sex-ratio of social groups was not defined *a priori*. Experiments were carried out at 24±1ºC under natural photoperiod.

Table 1. Morphometric characteristics of the experimental groups. Number of individuals within a group (n), density, ratio of females (%), weight (W) and standard length (SL), of the social groups of *A. facetus*. (SE: standard error, CV: coefficient of variation).

Month	Group	n	Density (g/L)	Females (%)	W Mean ± SE(g)	W CV	SL Mean±SE (cm)	SL CV
Aug/2014	1	4	2.41	25	150.35±7.13	0.09	13.75±0.12	0.02
	2	6	1.20	33.3	50.03±3.50	0.17	10.18±0.22	0.05
	3	5	2.28	40	114.18±9.68	0.19	13.00±0.49	0.08
	4	6	0.36	33.3	14.91±2.14	0.35	7.00±0.29	0.10
Nov/2014	5	4	2.18	25	136.25±10.02	0.15	13.25±0.28	0.04
	6	5	1.42	60	71.13±7.08	0.22	11.10±0.46	0.09
	7	5	1.06	100	53.20±5.11	0.21	10.40±0.26	0.06
	8	5	2.09	60	104.55±6.38	0.14	12.30±0.30	0.06
	9	6	1.74	100	72.51±2.59	0.09	10.92±0.14	0.03
Feb/2016	10	5	2.49	40	124.75±14.36	0.26	12.90±0.43	0.08
	11	5	1.81	80	90.49±11.68	0.29	11.90±0.38	0.07
May/2015	12	5	1.19	20	59.62±5.83	0.22	10.72±0.31	0.06
	13	6	1.06	16.7	46.15±6.21	0.33	9.73±0.40	0.10
	14	6	0.73	0	30.51±1.78	0.14	8.87±0.22	0.06
	15	5	0.72	20	36.25±3.24	0.20	9.16±0.36	0.09

The observations started the day after the fish were transferred to the experimental tanks. A total of 78 individuals were observed for behaviour in this study. Behaviour was recorded daily from 9 a.m. with an underwater camera (GoPro Hero 4). The first 5 min were considered the habituation period and observations were discarded. This was followed by 5 min behavioural observations with each fish as a focal individual, totalling 20 to 30 min per group per day depending on the number of fish in a tank, every day, during 7 days. These observations are summarized in the ethogram in Appendix I, Table 1. One of the striking features of the behaviour of this species is the formation of territorial pairs. When a reproductive pair was formed and started to defend a territory, the social hierarchy was determined by an interaction matrix from which a dominance index (DI) was calculated for each individual as $DI=\Sigma(Wi/Ti)/N$, where Wi is the number of interactions won by individual i, defined as biting or chasing (Appendix I, Table 1), Ti is the number of interactions with the individual i, and N is the total number of opponents (Alonso et al., 2012).

At the end of experiment (after the 7 days of observations), animals were anaesthetized as previously described, blood samples were taken again (final phase, repeating the procedures for the initial phase, as described above), and sacrificed via rapid cervical transection. Sex was assigned by inspection of the gonads. The gonadosomatic index (GSI) was calculated as gonad weight (g) / W x100. The hepatosomatic index (HSI) was calculated as liver weight (g) / W x100 to assess the overall physiological condition.

2.3. Hormone measurements

Steroids were measured by radioimmunoassay (RIA): cortisol from heat denatured plasma (80ºC for 1 hr) and 11KT, testosterone and E_2 from diethyl ether extracts of plasma as described by Canário and Scott (1989). The RIA cross-reactions for cortisol are described in Rotllant et al. (2005), for 11KT in Kime and Manning (1982), for testosterone in Mota et al. (2014) and for and E_2 in Guerreiro et al. (2002). In order to confirm the specificity of the 11KT and E_2 antisera towards *A. facetus* blood plasma, separate pools of blood plasma (1 mL) from females and males, were extracted, ran on thin-layer chromatography (TLC) and the resulting fractions assayed. Briefly, plasma samples of females and males were extracted with solid phase Sep-pak C18 cartridges using methanol according to the manufacturer's instructions (Waters, Inc., UK). Extracts were evaporated at 40ºC with nitrogen gas and applied to a pre-coated silica gel TLC plates (LK6DF - Silica Gel 60A - Whatman Inc., New Jersey, USA) using dichloromethane. The plates were developed at room temperature for 60 minutes, using as mobile phase chloroform:methanol 48:2. Tritiated 11KT and E_2 were used as reference in adjacent lanes and detected with a radiochromatograph Bioscan (Lablogic, Sheffield, England). Lanes were divided in 0.5cm strips, scraped off, eluted with dichloromethane:ethanol 80:20 and evaporated under nitrogen at 40ºC in a dry bath. The residue was re-suspended in 600 µL of RIA buffer for analysis.

2.4. Data analysis

Data were tested for normality and homogeneity of variance using Shapiro-Wilk and Levene's test. When the assumptions of the analysis of variance (ANOVA) were not met, data was transformed as detailed below. When the assumptions were not met even after transformation, non-parametric statistics were used. Monthly changes in hormone levels were tested by one-way ANOVA.

To test for differences in size (W and SL) between sex (female and male) or social status (territorial and non-territorial); differences in hormone levels between males and females and the ratio E_2/testosterone and 11KT/testosterone between territorial and non-territorial individuals were analysed with a Student's t-test or a Mann–Whitney U tests. To avoid bias related to size differences among groups the biometric data was normalized to the mean of the group (normalized standard length SL' = standard length of an individual divided by the mean standard length of the group; normalized weight W' = weight of an individual divided by the mean weight of the group).

The DI, GSI, HSI and proportion of victories (%victories) were arcsine square root transformed. Pearson correlations were tested between DI and size (SL or W) and between DI and GSI or HSI. The Student's t-test was used to test for differences in GSI or HSI between status and for aggressive interactions and victories between males and females of territorial pairs. Spearman correlations were calculated between aggressive acts and biomass density (g/L), W, SL and percentage of females and males in each group; between hormone levels, both at the initial phase and final phase; and between DI (and also GSI) and hormone levels (initial and final phases, separately).

Statistical significance was established at the $p<0.05$ level for all tests (two-tailed) and data is presented as mean ± SEM.

3. Results

A total of 25 hours of video from the 15 social groups and 5 hours of video from 5 mating pairs were analysed to build the *A. facetus* ethogram (Appendix I, Table 1). In our experiments, reproduction only occurred at temperatures above 24ºC under long photoperiod (light > dark). Reproductive pairs can be stable from weeks to months, and if larvae are removed they can continue spawning throughout the season at intervals of 15 to 20 days. Interestingly, we observed adoption of a brood by a neighbouring pair when the larvae of one pair (~1 week within the free swimming phase) crossed the net that separated the two pairs.

3.1. Behaviour

Agonistic behaviour

In agonistic interactions, the sequence of threats and attacks starts with frontal or lateral displays, and occasionally with tail beating. The dispute is normally resolved at this stage but if the encounter escalates they can engage in mutual striking, chasing and ultimately mouth fighting. Usually, both animals show dark stripes.

Territorial behaviour

The fish skin usually has a pale background colour with little visible marks when kept at temperatures under 20 ºC within a community tank (~2000L) (Figure 1a), where territorial behaviour was rarely observed. Circa 24 h after transferred to a smaller tank (~250L), with a new social group and at temperatures above 20ºC, a dominant fish, usually a male, starts to defend a territory. This territorial male (TM), stays at the bottom of the tank guarding the flower pot, chasing away the other fish when they approach.

The TM normally assumes a reproductive colouration, with dark stripes and bright background when temperature is above 24 ºC (Figure 1b). In two or three days, a female can join the territorial male, when it happens its colouration also change (Figure 1c). They both defend the territory and chase away the non-territorial (NT) fish (males or females). NT fish usually hover nearby in the water column.

When attacked, NT fish change their eye colour from pale grey to black. The eye colour change can be transitory or permanent, depending on the social rank of the individual. The fish that are intermediate in rank remain with a pale and opaque skin background and stripes or bars are not present, but the mid-corporal spot is visible (Figure 1d). If an individual is attacked frequently, it assumes a dark skin background (Figure 1e).

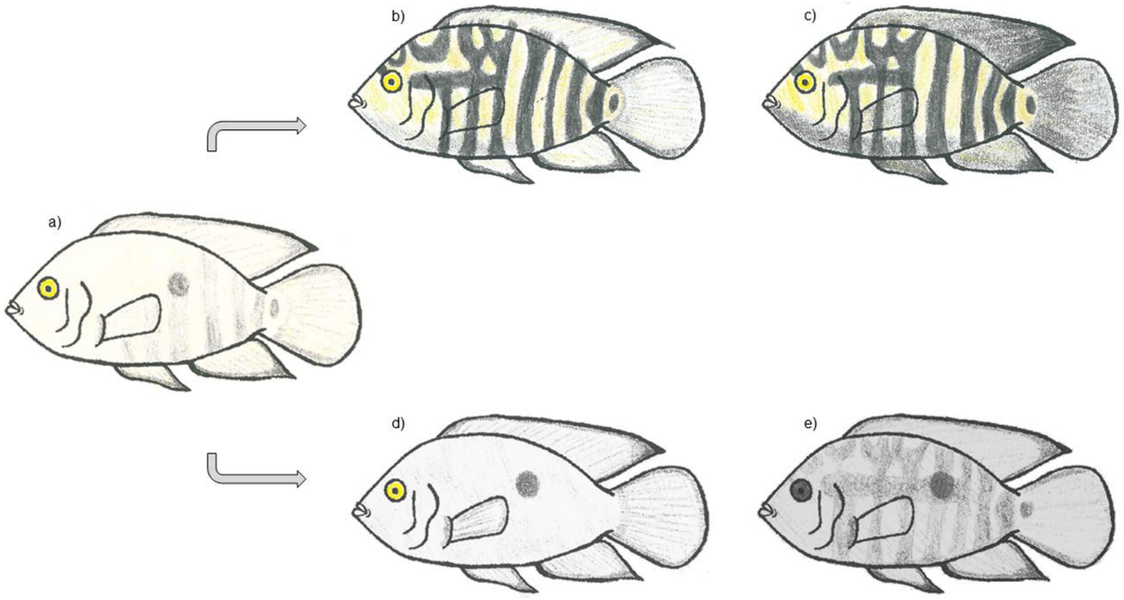

Figure 1. Colour pattern found in *A. facetus*. a) gregarious individual; b) territorial male; c) territorial female; d) non-territorial individual; e) lowest individual in hierarchy.

Social status and dominance hierarchy

The dominant individual in the group was usually a TM, followed by the territorial female pair (TF), both of which defended a territory and reproduced within a week of pairing. A linear hierarchy was found in groups in which lower ranking individuals were non-territorial males (NTM) and non-territorial females (NTF), with different degrees of DI.

In fifteen groups studied, eleven territorial pairs were formed, one of these being a female homosocial pair. In two groups, a territorial male assumed a dominant position but did not form a pair, despite the presence of females. The females of the pairs were approximately 10% smaller (SL': TF = 0.96 ± 0.01, TM = 1.04 ± 0.01, Student t-test = 4.47, $p<0.001$, $n_{TF} = 10$, $n_{TM} = 10$) and 20% lighter than the male with whom they paired (W': TF = 0.89 ± 0.03, TM = 1.10 ± 0.03, Student t-test = 3.87, p = 0.001, $n_{TF} = 10$, $n_{TM} = 10$). Territorial fish of both sexes were significantly more dominant (DI: TF = 0.67 ± 0.08, NTF = 0.28 ± 0.05, Student t-test = 3.424, p = 0.001, $n_{TF} = 14$, $n_{NTF} = 20$; TM = 0.90 ± 0.04, NTM = 0.32 ± 0.04, Mann-Whitney U = 10, $p<0.001$, $n_{TM} = 13$, $n_{NTM} = 31$,) larger and heavier than NT fish, with exception for SL' for females (SL': TF = 1.02 ± 0.02, NTF = 0.97 ± 0.01, Mann-Whitney U = 87, $p>0.05$, $n_{TF} = 14$, $n_{NTF} = 20$;

TM = 1.08 ± 0.01, NTM = 0.98 ± 0.01, Student t-test = -5.25, $p<0.01$, n_{TM} = 13, n_{NTM} = 31. W’: TF = 1.11 ± 0.07, NTF = 0.89 ± 0.03, Student t-test = -2.96, $p<0.01$, n_{TF} = 14, n_{NTF} = 20; TM = 1.23 ± 0.05, NTM = 0.92 ± 0.03, Student t-test = -5.65, $p<0.01$, n_{TM} = 13, n_{NTM} = 31).

Positive correlations were found between DI and SL’ (females Pearson correlation r = 0.62, $p<0.01$, $n_{females}$ = 34; males Pearson correlation r = 0.72, $p<0.01$, n_{males} = 44, Figure 2a) and DI and W’ (females Pearson correlation r = 0.76, $p<0.01$, $n_{females}$ = 34; males Pearson correlation r = 0.67, $p<0.01$, n_{males} = 44, Figure 2b).

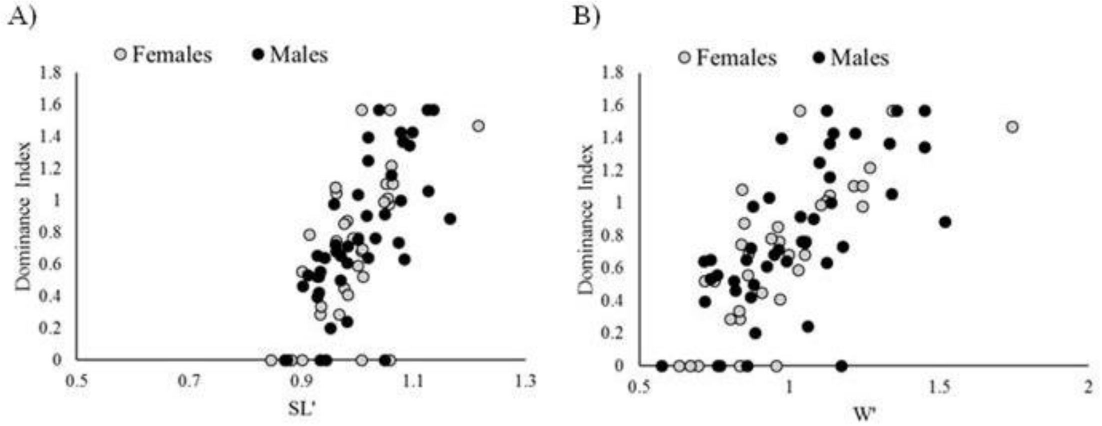

Figure 2. Correlations between dominance index (arcsine square root transformed) and (A) normalized standard length (SL’ Females: Pearson correlation=0.62, n=34, $p<0.01$, Males: Spearman correlation=0.72, n=44, $p<0.01$) and (B) normalized weight (W’ Females: Pearson correlation=0.76, n=34, $p<0.01$, Males: Pearson correlation=0.67, n=44, $p<0.01$).

The GSI and HSI were not associated with dominance index in males nor in females (HSI: TM = 2.39 ± 0.25, NTM = 2.19 ± 0.12, TF = 2.26 ± 0.78, NTF = 1.83 ± 0.37, $p>0.05$, n_{males}=36, $n_{females}$=26,). However, when separated by their status, the GSI was higher in TM than NTM (TM = 0.48 ± 0.05, NTM = 0.29 ± 0.03, Student t-test = -2.76, $p<0.01$, n_{TM} = 7, n_{NTM} = 31), but not between TF to NTF (TF = 2.64 ± 1.71, NTF = 2.13 ± 1.90, $p>0.05$, n_{TF} = 6, n_{NTF} = 20).

Among the territorial pairs, there was no significant difference in the number of interactions between males and females, but TM won those interactions more frequently than the TF (%victories: TM = 91.28 ± 4.60%, TF = 61.71 ± 8.57%, Student t-test = 2.63, $p<0.05$, n_{TM} = 10, n_{TF} = 12).

The acts of high aggression (bite, chase, strike, mouth fight and tail beating) were positively correlated to biomass density (Spearman correlation r = 0.68, $p<0.01$, n = 14), weight (Spearman correlation r = 0.70, $p<0.01$, n = 14) and standard length of fish (Spearman correlation r = 0.76, $p<0.01$, n = 14), but not with number of females or males (p>0.05) in a group. Group 4 was excluded from the analyses owing to the much low number of interactions and lower biomass density than in other groups.

3.2. Hormonal profiles

E_2 was detected only in females, while 11KT was detected in both sexes, as demonstrated by the immunoreactivity on the TLC fractions of blood plasma extracts coinciding with the radioactive reference standards (Figure 3).

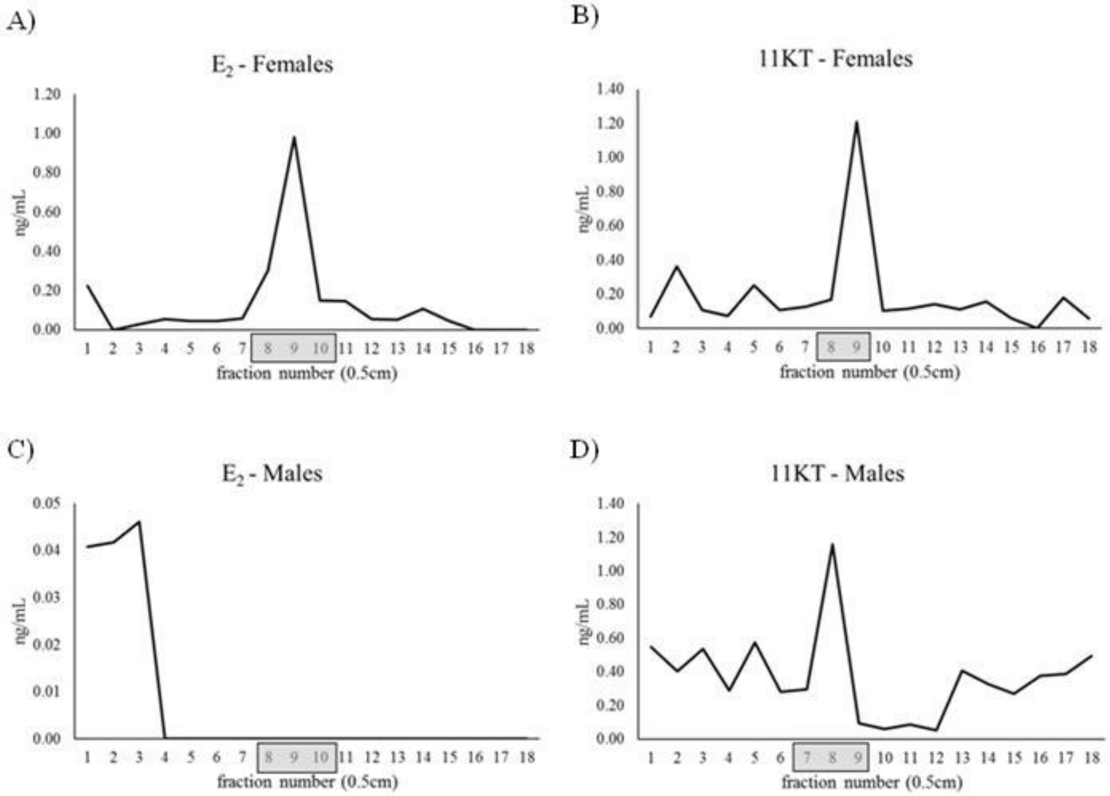

Figure 3. Thin layer chromatography (TLC) scan of immunoreactive steroids for a pool of samples of females and males of *A. facetus*, separately. Grey areas show elution positions of radioactive standards.

Males had higher levels of androgens (11KT and testosterone) than females in the community tanks (initial values, Table 3). The peak of plasma sex steroids in the fish kept in the community tanks was in February (E_2 females: one-way ANOVA F = 8.12, p<0.001; 11KT females: Kruskal-Wallis H = 22.86, p<0.001; 11KT males: Kruskal-Wallis H = 17.57, p<0.001; testosterone females: one-way ANOVA F = 9.08, p<0.001; testosterone males: one-way ANOVA F = 13.57, p<0.001, $n_{females}$: February = 6, May = 3, August = 7 and November = 18; n_{males}: February = 4, May = 19, August = 14 and November = 7, Figure 4a-c) and lower levels were observed in August and November. However, for cortisol it was the opposite pattern with highest values in

August to November (females: one-way ANOVA F = 4.14, p = 0.01; males: one-way ANOVA F = 9.21, p<0.001, Figure 4d).

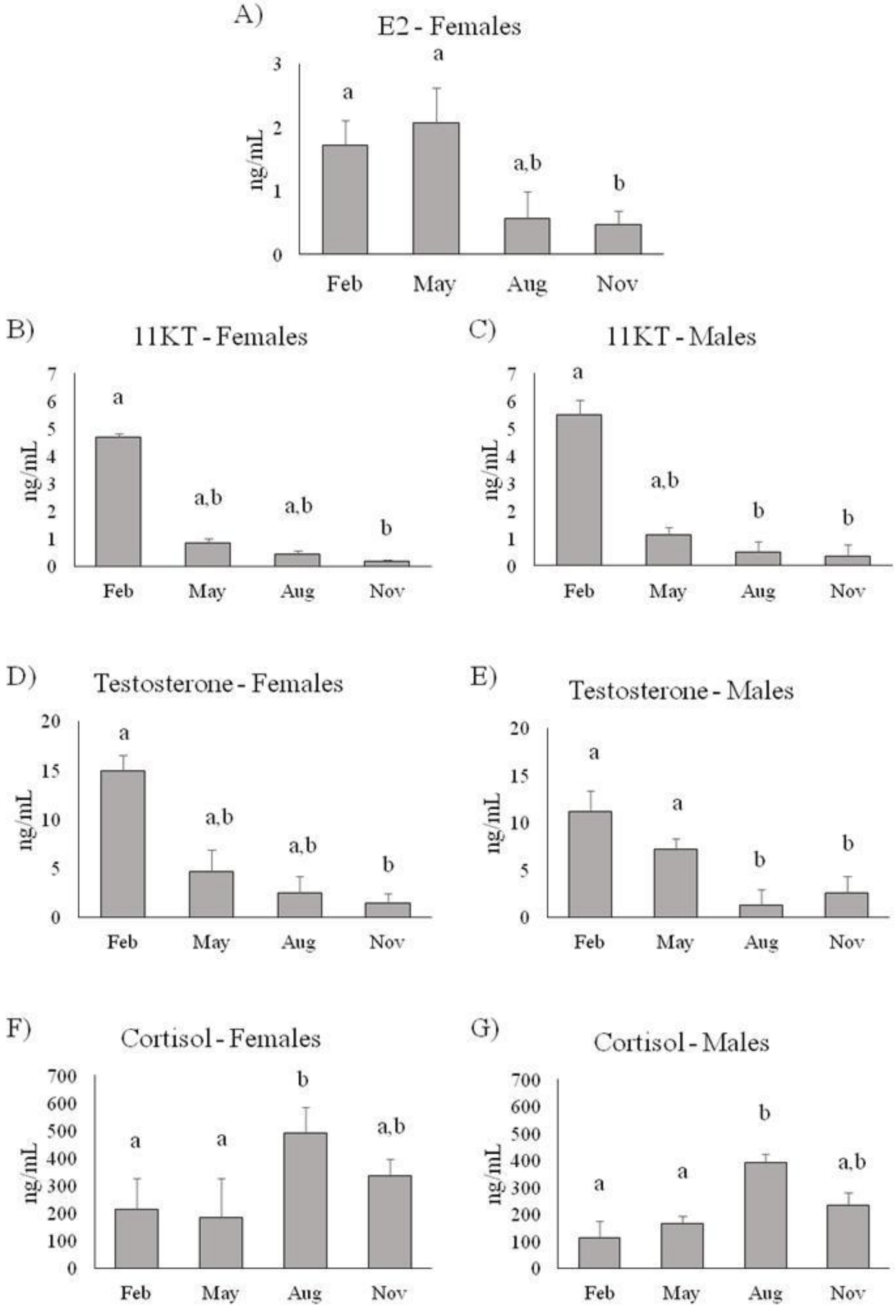

Figure 4. Sex hormones by month of *A. facetus* at the initial phase of the experiment. A) E_2, B) 11KT, C) testosterone, D) cortisol. Different small letters mark significant differences among months ($p<0.05$, one-way ANOVA).

Although at the beginning of group formation there were no differences between future TM and NTM, at the end of the observations (final phase) TM had higher 11KT levels than NTM (Appendix I, Table 2). A similar pattern was observed for E_2 in TF and NTF (Appendix I, Table 2).

The plasma E_2/testosterone ratio did not differ between NTF and TF at initial nor final phase ($p>0.05$), but the 11KT/testosterone ratio was higher for NTF at final phase (NTF = 1.47 ± 0.49; TF = 0.30 ± 0.09; Mann-Whitney U = 72.00, p = 0.04, $n_{NTF} = 18$, $n_{TF} = 14$), while no other differences were observed for females or for males.

Plasma cortisol levels were significantly higher in females than in males both at the beginning and at the end of the 7-day group observations (Appendix I, Table 2). TM and NTM had similar levels of cortisol at the beginning of the observations but at the end NTM had lower levels of cortisol than TM (Appendix I, Table 2).

For females, cortisol was not correlated with 11KT, neither at initial nor final phases ($p>0.05$), but it was negatively correlated with testosterone and E_2 (Table 2). While for males, cortisol was negatively correlated with sex hormones only at initial phase (Table 2). All sex hormones, regardless sex or phase, were strongly and positively correlated to each other (Table 2).

Table 2. Spearman's correlation between hormones of *A. facetus*.

	Cortisol	11KT	Testosterone
Females (initial phase)			
Cortisol	-	-	-
11KT	-0.2	-	-
Testosterone	-0.49**	0.72***	-
E_2	-0.53**	0.79***	0.82***
Females (final phase)			
Cortisol	-	-	-
11KT	-0.28	-	-
Testosterone	-0.41*	0.66***	-
E_2	-0.58***	0.67***	0.71***
Males (initial phase)			
Cortisol	-	-	-
11KT	-0.39*	-	-
Testosterone	-0.69***	0.67***	-
Males (final phase)			
Cortisol	-	-	-
11KT	-0.16	-	-
Testosterone	-0.29	0.57***	-

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

GSI was positively correlated with final sex hormones levels in NTM and NTF but not in TM and TF (with exception to 11KT for TF) (Table 3).

Table 3. Spearman's correlations between GSI and final hormone levels in *A. facetus*. (TF: territorial female, NTF: non-territorial female, TM: territorial male, NTM: non-territorial male).

	Cortisol	11KT	Testosterone	E_2
Females	-0.31	0.69***	0.54**	0.61**
TF	-0.37	0.75*	0.25	0.39
NTF	-0.22	0.66**	0.72**	0.77***
Males	-0.21	0.53**	0.64***	-
TM	-0.16	-0.16	-0.21	-
NTM	-0.12	0.54**	0.69***	-

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

The DI does not appear to be influenced by the basal levels of sex steroids in males or females before the group formation as no significant correlations were found between the initial levels of any hormone and DI ($p > 0.05$). After group formation, among the sex steroids only 11KT showed a positive correlation to DI ($r = 0.32$; $n = 41$; $p = 0.04$). However, this result should be considered with caution as there were only 4 samples analysed in February, when 11KT levels were highest, and they were all from mid to high DI, thus possibly biasing the distribution (Figure 5). As for cortisol, after group formation high DI fish of both sexes had lower cortisol levels (Pearson correlations, females: $r = -0.38$, $n = 30$, $p = 0.03$; males: $r = -0.48$, $n = 42$, $p < 0.01$; Figure 5).

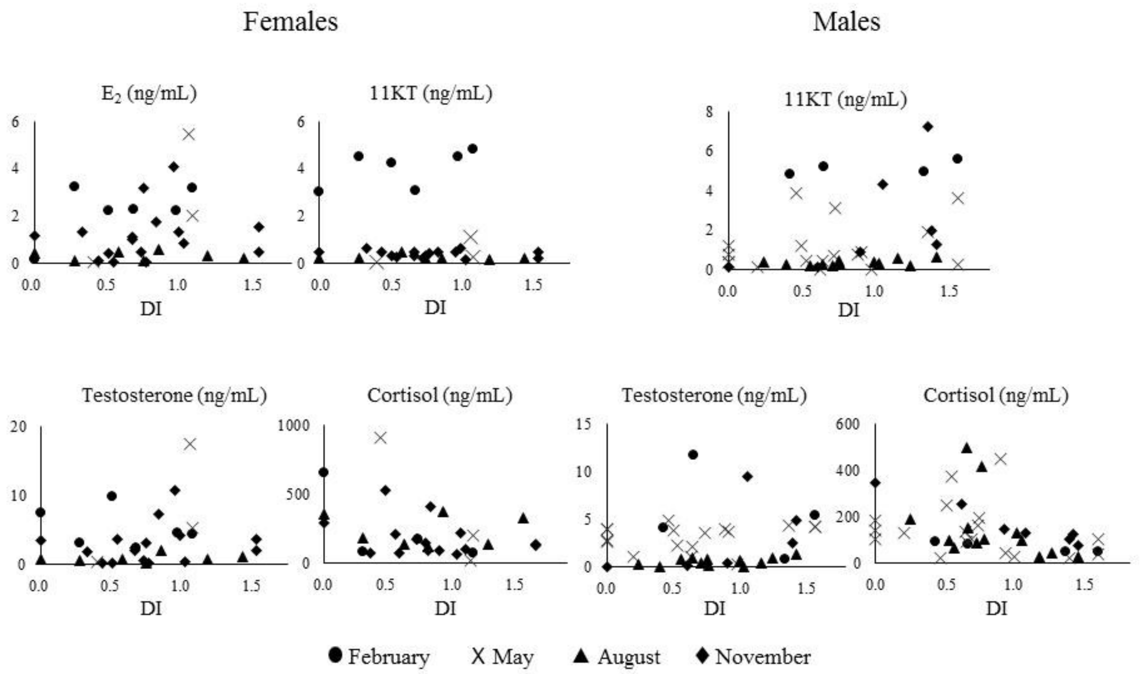

Figure 5. Correlations between dominance index (DI) and final hormones levels in *A. facetus* in both sexes.

4. Discussion

This is the first study describing the main behavioural patterns of *Australoheros facetus,* and it shows that high social status and dominance are linked with high levels of 11KT in males and females. Furthermore, our data shows that individuals of lower social status present higher levels of cortisol, suggesting influence of social stress on subordinates.

Territorial pairs dominate the social group through aggressive interactions with other individuals, as in other neotropical cichlids, such as in *Cichlasoma dimerus* (Ramallo et al., 2015). In most cases, the larger male starts to defend a territory less than 24h after group formation, chasing and attacking all individuals of the group (including the females and the future mate). Large body size commonly enhances male reproductive success and one hypothesis is that larger males are stronger and can held nests for longer periods of time (Johnson & Hixon, 2011). As result, NT individuals develop a specific body and eye colour pattern, which has a parallel in *Oreochromis niloticus* (Volpato et al., 2003) and in the neotropical cichlid *Geophagus brasiliensis* (Miyai et al., 2011) after dyadic interactions. This signalling may have a positive adaptive value in order to reduce attacks from dominants (Alonso et al., 2011; O'Connor et al., 1999). This suggests that vision could be important to establishment of hierarchies in this species, as indicated by the differences in size among territorial and non-territorial animals and perhaps body and eye colour. However, it is more likely that multiple sensory modalities mediate conflict decisions and mate choice (Fernald, 2014). For example, in *O. mossambicus*, *O. niloticus* and *Pimephales promelas*, urine seems to be the major channel of communication of social status (Hubbard et al., 2014; Keller-Costa et al., 2014; Martinovic-Weigelt et al., 2012). Whether *A. facetus* uses signals other than vision is not known although they have olfactory sensitivity to intestinal and bile fluids that seem to be correlated to position in the hierarchy (Hubbard et al., 2017). Interestingly, in an all-female group in our experiment a homosocial dominant pair also formed. This may interpreted as a result of the "prisoner effect", where captive animals in monosex groups during the breeding season exhibited homosexual behaviour (McGraw & Hill, 1999).

Australoheros facetus provides bi-parental care, with task specialization between members of the pair. Males usually protect the offspring from predators while the females remain very close to the fry, oxygenating and cleaning them as reported for other biparental cichlids (Annett et al., 1999; Keenleyside, 1985; Snekser & Itzkowitz, 2009; Teresa & Goncalves-de-Freitas, 2011; Wisenden, 1994). However, it is possible that this division of tasks can be associated with the relative size of the animals and not with sex, as observed for the cichlids *Julidochromis ornatus* (Awata & Kohda, 2004) and *Archocentrus nigrofasciatum* (Itzkowitz et al., 2005). Nevertheless, and in concert with observations by others, it was rarely observed offspring left alone at any stage of development (Annett et al., 1999; Itzkowitz et al., 2003).

TM and TF show an interesting synchronization in sex steroids (11KT and E_2, respectively) after the hierarchy is formed. High social status seems thus to enable reproduction for the dominant pair, while the remaining members of the group are probably socially inhibited from spawning.

Females showed the expected pattern of circulating E_2 with higher levels during ovarian growth. However, we could not detect E_2 in *A. facetus* males. This contrasts with what is reported for *C. dimerus* (Birba et al., 2015) and *Astatotilapia burtoni* (Maruska et al., 2013; O'Connell et al., 2013). The reason for the difference could be biological or could reflect methodological aspects. A common feature of the studies with *C. dimerus* and *A. burtoni* is that E_2 was assayed with ELISA kits with no validation of the assays reported, including determination of blood plasma cross-reactivity (for cautions about measuring steroid hormones see Scott et al., 2008). In the present study, a combination of thin-layer chromatography and RIA enable us to ascertain no cross-reactivity between the E_2 antibody and male blood plasma while there was cross-reaction in the expected position with the female blood plasma (Figure 3). The E_2 RIA system used in our study is very sensitive and would have detected E_2 levels well below 50 pg/ml, making it unlikely we would have missed significant amounts of hormone. Thus, in this species, where major sexually dimorphic features are lacking, circulating levels of E_2 can provide a diagnostic tool to distinguish adult females from males.

The GSI can be interpreted as the investment in reproduction of an individual to the breeding season. Usually after the formation of an hierarchy, dominant males that acquire a territory start to court available females and prepare to reproduction by increasing the size of the gonads (high spermatogenesis and sperm density) (Maruska, 2014; White et al., 2002). In our experiments, TM had larger GSI than NTM, although the short experimental period and seasonal effects may explain the lack of positive correlation between the GSI and DI. However, GSI and final sex hormone levels for NTM returned a strong and positive correlation, which could be related to some physiological mechanisms that can afford non-territorial animals to adjust for an opportunity to ascend in hierarchy (Alonso et al., 2012). Interestingly, both TF and NTF showed a positive correlation between GSI and 11KT which, based on recent work on another cichlid that showed a dissociation between 11KT and aggression (Almeida, Canario, et al., 2014), suggests a possible link to reproduction, although the mechanism needs to be investigated.

The HSI is used as indirect measure of wellbeing of animals, which can reflect the amount of reserve substances stored in body (Koporikov & Bogdanov, 2013). Assuming that the larger body size and aggressiveness provide an advantage to the dominant fish during feeding, it can be expected that a lower food intake by subordinate fish could influence the HSI (Culbert & Gilmour, 2016). However, as in the case of GSI the short experimental period and different physiological states related to the time of the year of the experiments may not have allowed enough differences to develop and be detected.

We found higher cortisol levels in NT compared to T individuals (Figure 1, Table 3) which is consistent to what was described for *C. dimerus* and *Astatotilapia burtoni*. It is possible that in *A. facetus* the fact that TM and TF cooperate to dispel NT causes heightened anxiety in the latter, which is reflected in the higher levels of cortisol. This social stress could inhibit reproduction of subordinate fish (Alonso et al., 2011; White et al., 2002). Overall, increasing cortisol was related to a decrease in sex hormones (Table 4). Cortisol has been reported to have negative effects on reproduction, decreasing synthesis and release of sexual hormones or altering the capacity of the gonads (Pankhurst & Van Der Kraak, 1997; Poursaeid et al., 2012). Curiously, in some cichlids the opposite pattern for cortisol levels was found and attributed to the high cost of maintaining a dominant position (Bergmuller & Taborsky, 2005; Mileva et al., 2009). In the other hand, all sex hormones were positively correlated to each other, which could be a reflection of the pathway of production of E_2 and 11KT through aromatization of testosterone.

The plasmatic ratio 11KT/testosterone was higher in NTF. In contrast, females of *Neolamprologus pulcher* that won or lost dyadic contests for territory did not show differences in 11KT/testosterone ratio, although females that won those contest showed positive correlations of 11KT/testosterone with submissive behaviours (Taves et al., 2009). Unfortunately, the authors did not find a reasonable explanation for that correlation. However, the results for *A. facetus* are similar with that found for *N. pulcher* in that, for females, agonistic behaviour can elicit an androgen response just enough to be noticed through analysis of the 11KT/testosterone ratio.

Sex steroids in the fish in the community tank peaked in February (11KT and T for both sexes and E_2 for females, Figure 4). This may indicate preparation for the breeding season probably with gonadal recrudescence as a result of increasing photoperiod and temperature (Bromage et al., 2001; Pankhurst & Porter, 2003). The testosterone peak in February may be related to its precursor function for both E_2 and 11KT necessary for reproduction and possibly to respond to social challenges (Oliveira, 2009; Wingfield et al., 1990). Although the breeding season for *A. facetus* in Portuguese streams is still unknown, the highest frequency of breeding pairs actively defending a territory and performing parental care to the young were observed between July and August, corresponding to the lowest levels of sex steroids which suggests that spawning may start in May/June. Interestingly, cortisol levels in fish from the community tank showed important individual variation, and average values for both sexes showed a seasonal profile, rising towards the summer and with higher values recorded in August and still elevated in November. The high levels of cortisol during the summer could reflect a stress response to the high temperatures they experience, with water temperature reaching 26ºC. However, a parallel between cortisol levels and the reproductive cycle has also been detected in several other fish species from cold and warm water (Bry, 1985; Lamba et al., 1983; Wingfield & Grimm, 1977) which indicates a reproduction related cycle of cortisol also in the case of *A. facetus*. The still high values of cortisol during November could be related to the decrease in water temperature, since some fish species can experience stress responses during cold (Chen et al., 2002; He et al., 2015).

There was a positive correlation between aggression and biomass density. Although we have not assessed directly the size of the territories, space could be an important limiting factor for this species, increasing competition and aggressive interactions. This could also influence the success of *A. facetus* in Southern Portuguese streams. This region is subjected to strong seasonal changes in hydrology, where most of the streams lose connectivity during the dry season and remain in a pool-like state (Collares-Pereira et al., 2000; Gasith & Resh, 1999) reducing available sites for reproduction and feeding and therefore increasing competition. The social organization, complex parental care and high aggressiveness could be an advantage for this species during colonization of new habitats in Mediterranean-type streams. In these areas, the native fish community is usually highly endemic, specialized and typically without any form of parental behaviour to protect their offspring (Hermoso et al., 2012; Marr et al., 2013).

In conclusion, this study provides a first behavioural and endocrine assessment of *A. facetus* (summarized in Figure 6). Sex steroids are synchronized in the dominant reproductive pairs and peak in February, anticipating the breeding season. Complex social skills may be advantageous for the invasive success of this species in Mediterranean-type streams.

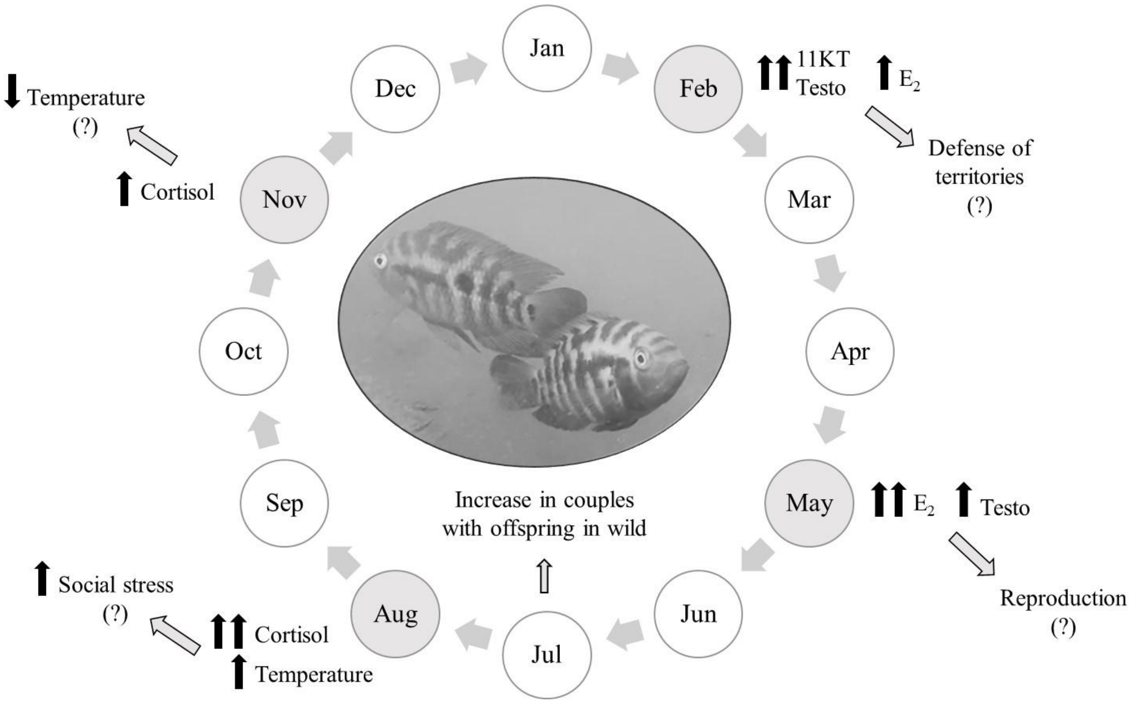

Figure 6. A graphical summary of the hormonal cycle of *A. facetus* throughout the months of sampling. Months marked in grey show when the groups used in this study were formed. In February, end of winter and approaching Spring, the sex steroids were high, probably reflecting the start of competition for mates and spawning territories. The high levels of E_2 in May could indicate the spawning season. During the middle of the summer, in July, it was observed in the wild a great number of couples with offspring, which could lead to the high levels of cortisol in August that summed with the high temperatures, could increase the stress in *A. facetus*. The decreased water temperature in November explains the elevated cortisol levels, since cold can trigger stress responses in fish.

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CHAPTER SIX

HIGH OLFACTORY SENSITIVITY TO CONSPECIFIC INTESTINAL FLUID IN THE CHAMELEON CICHLID *Australoheros facetus*: COULD FAECES SIGNAL DOMINANCE?

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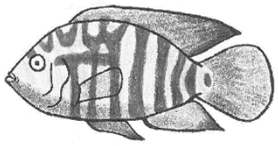

High olfactory sensitivity to conspecific intestinal fluid in the chameleon cichlid *Australoheros facetus*: could faeces signal dominance?

Abstract

The present study shows that the olfactory potency of intestinal and bile fluids taken from dominant male chameleon cichlids *Australoheros facetus* is greater than those from subordinate males. Thus, dominant status may be communicated by odorants released in the intestinal fluid and bile acids may contribute towards this.

Key words: behaviour; bile; chemical communication; intestinal fluid; olfaction; social status

1. Introduction

Chemical communication, mediated by the olfactory system, is an important but understudied facet of fish biology. Cichlids belong to a taxon characterized by a high degree of complex social behaviour, including the formation of social hierarchies and courtship displays. Investigation of the communication channels, however, has focused largely on visual and acoustic signals (Amorim et al., 2008; Barlow, 2000; Carleton et al., 2005; Escobar-Camacho & Carleton, 2015; Maan & Seehausen, 2010); chemical communication is less well studied, but there is abundant evidence that it is involved (Keller-Costa et al., 2015).

In several fishes, including cichlids, urine is a vehicle of pheromone release (Appelt & Sorensen, 2007; Keller-Costa, 2014; Yambe et al., 2006). Male African cichlids increase urine release during courtship and agonistic interactions with rival males (Barata et al., 2008; Barata et al., 2007; Maruska & Fernald, 2012). Whether or not other cichlid groups, such as the South American cichlids, use urine as a vehicle for chemical communication has not yet been investigated. In other fishes, evidence suggests that different body fluids, such as intestinal and bile fluids, may also convey cues as to the sex or physiological status of the donor (Hubbard et al., 2003; Huertas et al., 2010; Huertas et al., 2007).

The chameleon cichlid *Australoheros facetus* (Jenyns 1842) is a South American cichlid that has been invasive in the southern Iberian Peninsula since the 1940s (Doadrio, 2002). It is a close relative of the convict cichlid *Amatitlania nigrofasciata* (Günther 1867), a popular aquarium fish that has been used extensively in the investigation of alarm cues (Ferrari et al., 2010; Keller-Costa et al., 2015). *Australoheros facetus* is a benthic spawner with biparental care of eggs and larvae (Ruiz et al., 1992) and usually forms a complex hierarchy with different degrees of dominance achieved through aggressive interactions among all individuals within a group (Baduy et al., 2017) . A dominant male, typically the bigger individual, defends a territory and 1 or 2 days later, a dominant female joins it. Dominants and subordinates rapidly acquire the characteristic colouration of their respective status (Baduy et al., 2017). The use of chemical communication, however, in social interactions is unknown. Initial attempts to take urine samples by gentle squeezing of the abdomen were unsuccessful, suggesting that this species does not habitually store urine for release in specific contexts. It was decided, therefore, to investigate the possible involvement of other body fluids in communication by assessing the olfactory potency, using the electro-olfactogram (EOG), of intestinal and bile fluids from dominant and subordinate males that were being sampled as part of another study.

Bile salts and amino acids are common and potent odorants for fishes in general (Hara, 1994). As far as the authors are aware, this is the first time the olfactory system of A. facetus has been investigated, therefore the olfactory potency of some amino acids (L-serine, L-methionine and L-alanine) and bile acids (the C_{24} taurolithocholic and tauro-chenodeoxycholic acids and the C_{27} 5α-cyprinol sulphate) was also assessed.

2. Material and Methods

2.1. Formation of social hierarchies

Australoheros facetus were captured from the Odelouca River, Portugal (37º 13′ 37″ N; 8º 30′ 20″ W) by electro-fishing (licence number 403, 404, 405 and 406/2015/CAPT) and maintained in outside tanks (Centre for Marine Sciences – CCMar, University of Algarve) under natural photoperiod (36.6ºN) and temperature and fed once a day with commercial cichlid food until use in experiments. CCMar facilities and their staff are certified to house and conduct experiments with live animals and all procedures were conducted according to national and European legislation for animal experimentation (Veterinary General Directorate, Ministry of Agriculture, Rural Development and Fisheries of Portugal group-1 licence).

Australoheros facetus were selected from stock tanks to create eight groups with five A. facetus in each with minimal size differences among them [mean ± s.e.; standard length (LS) = 97 ± 1 mm; mass = 50 ± 2 g)]. *Australoheros facetus* were tagged (with coloured beads) and behaviour was recorded with an underwater camera (GoPro Hero 4; www.gopro.com) daily from 0900 hours and made with each *A. facetus* as a focal individual during 5 min every day for 5 days. The total number of aggressive interactions (bite, chase, mouth fighting and tail beating) of the focal *A. facetus* against the other members of the group was recorded. A dominance index (*ID*) was calculated for each individual in a social matrix as: $ID=\Sigma(WiTi^{-1})N^{-1}$,where *Wi* is the number of interactions won by individual *i*, defined as biting or chasing, *Ti* is the number of interactions with the individual *i* and *N* is the total number of opponents (Alonso et al., 2012). The mean frequency of aggressive interactions of those *A. facetus* designated as dominant was 0.22 min^{-1} and for subordinates was 0.01 min^{-1} (Mann–Whitney U-test U=1·500, n=15, P<0·05).

2.2. Intestinal and bile fluid samples

After 5 days of observation, *A. facetus* were anaesthetized with 200 mg l^{-1} MS222 (Sigma-Aldrich, Spain; www.sigmaaldrich.com) buffered with 400 mg l^{-1} $NaHCO_3$ and then sacrificed by rapid cervical section. Sex was determined through inspection of the gonads.

Bile fluid was taken directly from the gall bladder with a syringe, placed in small plastic tubes, immediately frozen on dry ice and then stored at -20ºC. Two pools of bile fluid were subsequently made, one using an equal volume (5 µl) of bile from four dominant males (ID: 0.98–1.00; LS: 99–109 mm; mass: 55–68 g) and another using 5 µl of bile from seven subordinate males (ID: 0.00–0.25; LS: 86–106 mm; mass: 39–66 g). These were then diluted 1:10 in distilled water, mixed, aliquoted and frozen until use for EOG recording.

Intestinal fluid was taken by squeezing intestinal contents from the last 10 cm of intestine into a small plastic tube and immediately frozen in dry ice and then stored at -20ºC. Samples were subsequently diluted 1:2 in distilled water, mixed thoroughly and centrifuged. Two pools of intestinal fluid were prepared with equal volumes of the supernatant (100 µl of the 1:2 dilution), one taken from seven dominant males (ID: 0.95–1.00; LS: 95–109 mm; mass: 44–68 g) and the other from six subordinate males (ID: 0.00–0.25; LS: 95–106 mm; mass: 45–66 g), then aliquoted and frozen until use. There were no significant differences in body-size between dominant and subordinate males sampled for either bile or intestinal fluid.

2.3. Recording the electro-olfactogram

Individuals (mass: 13–78 g) of both sexes, from a stock different from those above used for sampling of body fluids, were initially anaesthetized in dechlorinated water containing 200 mg l^{-1} MS222 buffered with 400 mg l^{-1} NaHCO3. They were then placed in a custom-built fish-holding box with water (containing 100 mg l^{-1} MS222 and 200 mg l^{-1} $NaHCO_3$) pumped over the gills *via* a tube placed in the mouth. The olfactory epithelium was exposed by surgically enlarging the nostril (cichlids have only one nostril per side) and irrigated with clean water (not containing anaesthetic) at *c.* 6 ml min^{-1}. Stimulus-containing solutions were introduced into this flow by a computer operated three-way solenoid valve (in 4 s pulses). The EOG was recorded as previously described for the Mozambique tilapia *Oreochromis mossambicus* (Peters 1852) (Frade et al., 2002). Briefly, the EOG is a D.C. field potential recorded immediately above the olfactory epithelium; glass micropipettes were used filled with 0.9% NaCl in 4% agar connected *via* a Ag–AgCl salt-bridge to a pre-amplifier and head-stage (NL102, Digitimer Ltd; www.digitimer.com). The signal was filtered above 50 Hz (NL125, Digitimer Ltd) and amplified (NL106, Digitimer Ltd) then digitized (DigiData 1440A, Molecular Devices; (www.moleculardevices.com) and displayed on a PC running Axoscope 10·2; Molecular Devices). Dilutions of the body fluids were made immediately prior to use in the same water used to irrigate the olfactory epithelium. Dilutions of amino acids were made from frozen aliquots (10−3M in distilled water) and of bile acids from 10−2M stock solutions (in ethanol kept at −20ºC), again immediately prior to use. The order in which different stimuli was varied, but each stimulus was given in order of increasing concentration. At least 1 min was allowed to elapse between successive stimuli (2 min in the case of the lower dilutions of body fluids). At the end of the recording, the *A. facetus* (still anaesthetized) was killed rapidly (cervical section and pithing) and the sex and size noted.

Prior to each group of experimental stimuli, the response to 10−5M L-serine and blank water (water treated in exactly the same way as that used for diluting the stimuli, other than without the addition of any stimuli) was measured. The amplitude of the blank response (<10% of the response to 10−5ML-serine), was subtracted from the amplitude of EOG responses to test stimuli. These were then normalized to the response to 10−5ML-serine (similarly blank-subtracted); all analyses were carried out on these normalized data (except for analysis for possible effects of size and sex on response amplitude).

2.4. Data analysis

Thresholds of detection were estimated by linear regression of log10 –log10 plots and calculating the intercept on the *x*-axis (Hubbard et al., 2011); in the case of intestinal fluid, only dilutions 1:10^7 to 1:10^4 were used, due to the sigmoidal shape of the concentration-response curve. Concentration-response curves of the body fluids were compared using two-way repeated-measures ANOVA with social status and dilution or sex of receiver and dilution (dominant intestinal fluid only) as the two factors, as appropriate. The thresholds of detection were compared using paired a *t*-test.

Pearson's product moment correlation was used to check for any correlation of response amplitude with body mass. Thresholds of detection of the body fluids were compared using a *t*-test and those of amino acids and bile acids were compared using one-way ANOVA with Tukey's *post hoc* test where appropriate (using log10-transformed data). In all cases, $P<0.05$ was taken to mean significance.

3. Results

3.1. Olfactory sensitivity to conspecific body fluids

Intestinal fluid proved to be an extremely potent olfactory stimulus, evoking large-amplitude EOG responses (up to 40 mV) in both male and female *A. facetus* (Figure 1a), with no clear differences depending on the sex of the receiver (two-way repeated measures ANOVA, $F_{1,47} = 4.227$, $P>0.05$), although males gave larger responses ($P<0.05$) to dilutions of $1:10^4$ to $1:10^2$. The intestinal fluid from dominant males, however, was more potent than that of subordinate males (two-way repeated measures ANOVA, $F_{1,95} = 35.751$, $P<0{\cdot}001$), which was more apparent at the higher, more ecologically relevant, dilutions. This was reflected in the lower thresholds of detection of intestinal fluid from dominant males ($1:10^{7.42\ (\pm0.17)}$ or 1 in 26.3 million) compared with that of subordinate males ($1:10^{6.93\ (\pm0.04)}$ or 1 in 8.5 million; paired *t*-test, $t_7 = -2.849$, $P<0.05$).

Conspecific bile fluid also proved to be a highly potent olfactory stimulus for *A. facetus* (Figure 1b). Owing to the lower volume available, only dilutions $1:10^7$ to $1:10^4$ were used. Although no apparent maximum was approached, bile fluid from dominant males evoked larger amplitude EOGs, but only at dilutions of $1:10^5$ and $1:10^4$, (two-way repeated measures ANOVA, $F_{1,63} =8.053$, $P<0.05$) and the calculated thresholds of detection (dominant, $1:10^{7.45\ (\pm0.05)}$ or 1 in 28.2 million; subordinate, $1:10^{7.40\ (\pm\ 0.06)}$ or 1 in 25.1 million dilution) were not statistically different (paired *t*-test, $t_7 = -0.749$, $P>0.05$).

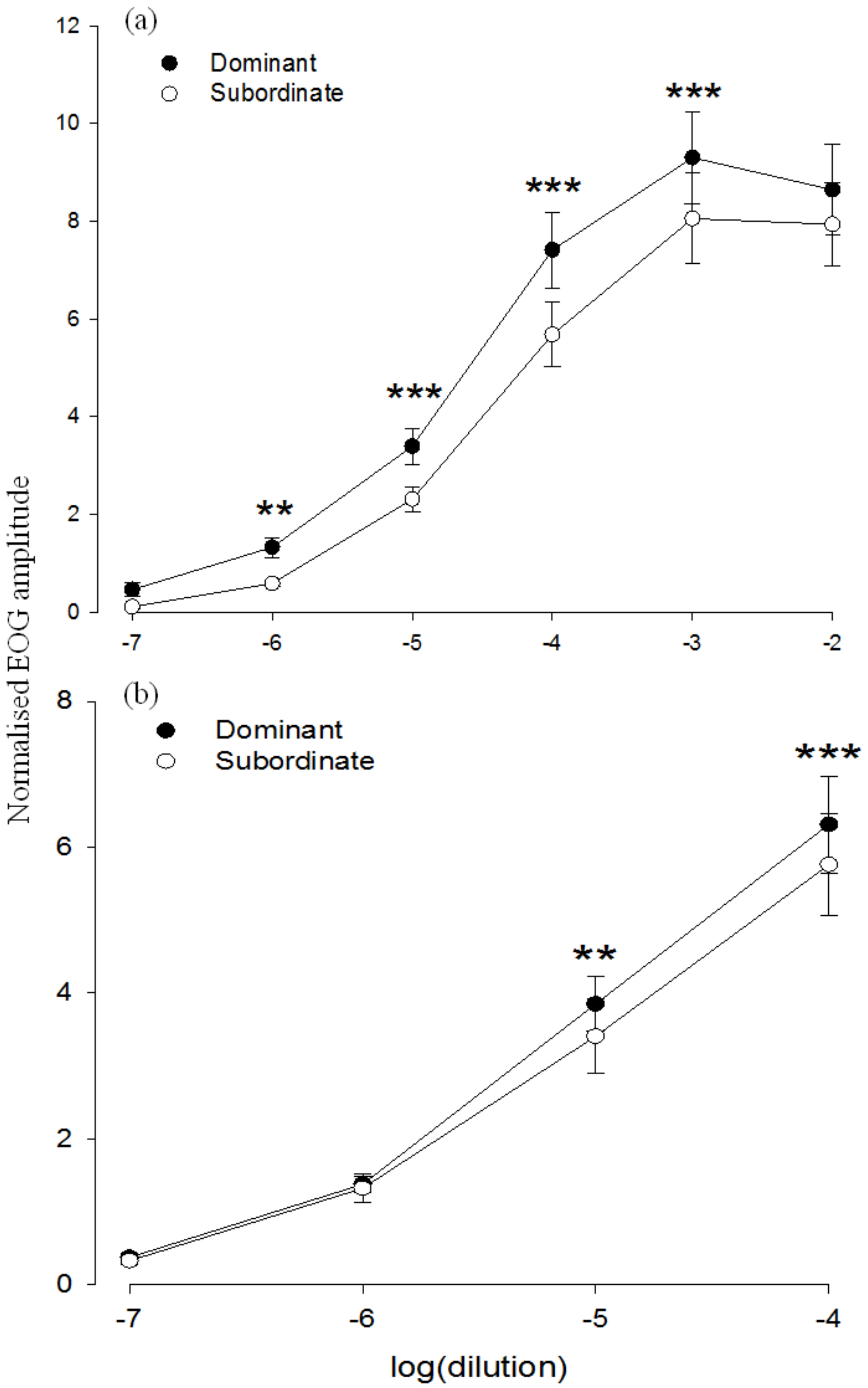

Figure1. Semi-logarithmic plots of pooled normalized electro-olfactrogram (EOG) amplitude in response to dilutions of (a) intestinal fluid and (b) bile fluid from dominant (●) and subordinate (○) males in *Australoheros facetus* with two-way repeated measures ANOVA, followed by Tukey's *post hoc* test ($n = 8$): **, $P<0.01$; ***, $P<0.001$.

3.2. Olfactory sensitivity to amino acids and bile acids

The standard stimulus, 10−5M L-serine, evoked EOG amplitudes of mean±s.d.= 3.77 ± 1.38 mV ($n = 16$). There was no apparent difference between the sexes (t-test, $t_{13} = -0.317$, $P>0.5$) and no correlation with body mass (Pearson's product moment correlation, $r = 0.024$, $P>0.05$). Increasing concentrations of L-serine, L-alanine and L-methionine produced typical exponential concentration-response curves when plotted semi-logarithmically (Figure 2a) with calculated thresholds of detection of $10^{-7.67\ (\pm 0.32)}$ M (L-serine), $10^{-7.61\ (\pm 0.25)}$ M (L-alanine) and $10^{-7.75\ (\pm 0.35)}$ M (L-methionine); there were no apparent differences in the thresholds among the three amino acids (one-way ANOVA, $F_{2,24} = 0.358$, $P>0.05$). The three bile acids tested proved to be more potent than the amino acids; larger amplitude EOGs were evoked by similar concentrations of the bile acids (Figure 2b) and the thresholds of detection were correspondingly lower. 5α-cyprinol-sulphate (5α-cholestane-3,7,12,26,27-pentol-27-sulphate; CYP-S) and taurolithocholic acid (TLC) were of similar potency with thresholds of $10^{-9.51\ (\pm 0.20)}$ M and $10^{-9.61\ (\pm 0.17)}$ M, respectively, whereas taurochenodeoxycholic acid (TCD) was significantly less potent with a threshold of $10^{-8.55\ (\pm 0.09)}$ M (one-way ANOVA, $F_{2,27} = 13.308$, $P<0.001$).

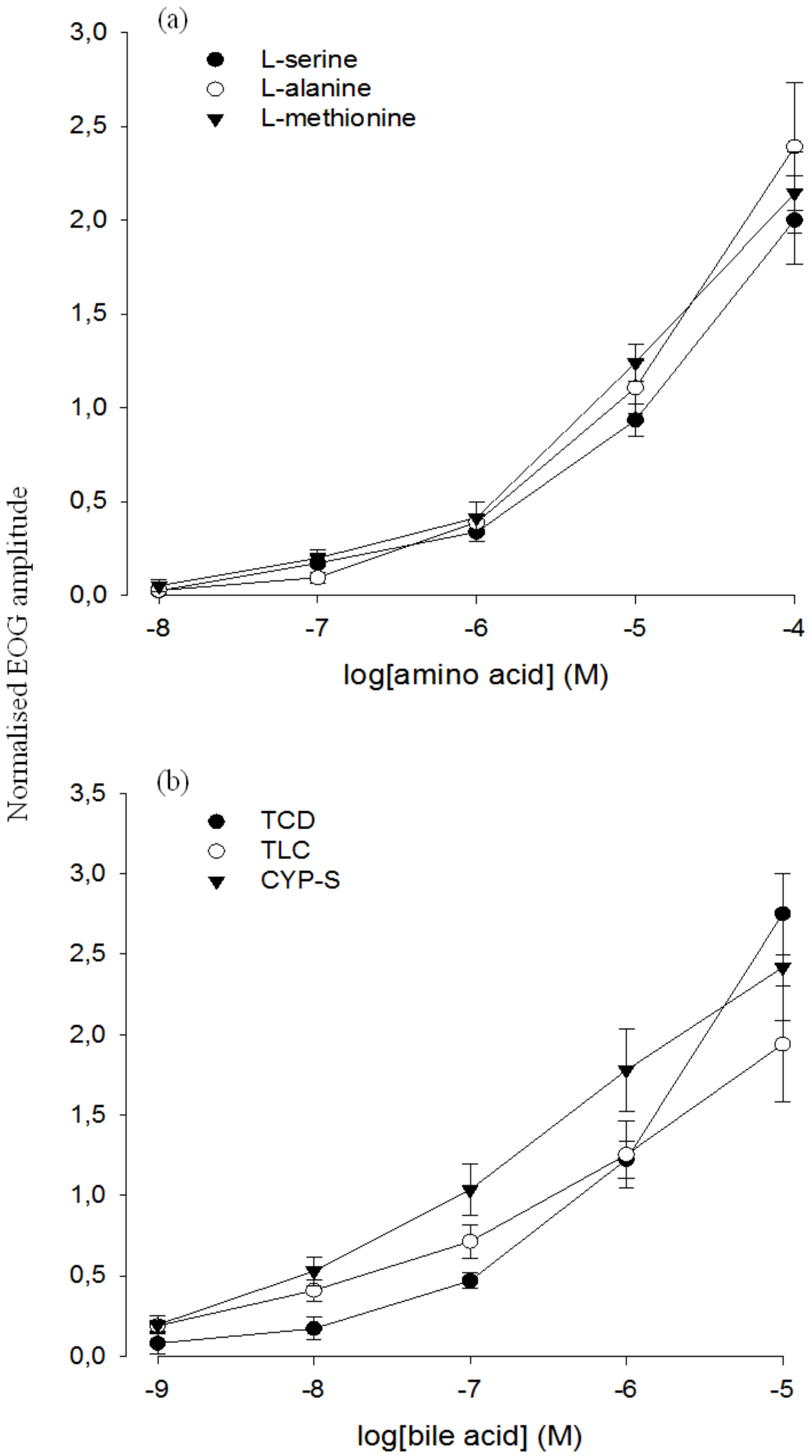

Figure.2. Semi-logarithmic plots of pooled normalized electro-olfactrogram (EOG) amplitude in response to different concentrations of (a) the amino acids L-serine (●, *n* = 11), L-alanine (○, *n* = 7) and L-methionine (▼, *n* = 7) and (b) the bile acids taurocehodeoxycholic acid (●, *n* = 9), taurolithocholic acid (○, *n* = 12) and 5α-cholestane-3,7,12,26,27-pentol-27-sulphate (▼, *n* = 7) in *Australoheros facetus*.

4. Discussion

The current study shows that conspecific intestinal and bile fluids are highly potent olfactory stimuli for *A. facetus*. Furthermore, the difference in potency of these fluids from males of different social status suggests that they may convey information of the social status of the donor. Given that bile fluid is a concentrated source of bile acids and that fishes in general have high olfactory sensitivity to bile acids, the higher potency of bile fluid from dominant males suggests that bile acids (or other odorants contained in the bile fluid) may contribute to the higher potency of intestinal fluid. In *A. nigrofasciata*, social stress (in this case cohabitation with dominant males) causes retention of bile fluid in the gall bladder (Earley et al., 2004); subordinate males may simply have less bile acids in their intestinal fluid. In the current study, however, intestinal fluid and bile fluid were roughly equipotent; bile fluid would have been diluted during its transit down the intestine, bile salts modified by bacterial action and the majority of bile acids resorbed (Hofmann, 1999), so it is likely that other odorants are also involved. For example, bile acids comprise *c*. 40% of the olfactory potency of intestinal fluid in the Senegalese sole *Solea senegalensis* Kaup 1858 (Velez et al., 2009). Whether the difference in potency is purely quantitative, or whether dominant males produce and release different odorants in their faeces remains unclear. Use of the faeces or intestinal fluid as a vehicle of chemical communication may be more widespread in fishes; for example, the olfactory potency of intestinal fluid, as well as urine, depends on reproductive status in another cichlid, *O. mossambicus* (Miranda et al., 2005) and *S. senegalensis* (Fatsini et al., 2017). While in the sea lamprey *Petromyzon marinus* L. 1758, bile salts have clearly defined pheromonal roles (Li et al., 2002; Sorensen et al., 2005), although not necessarily released in the faeces (Siefkes et al., 2003). Clearly, further studies are needed to isolate and identify the active components in the intestinal fluid and to establish their biological meaning, if any and role in chemical communication.

The current study also shows that *A. facetus* has olfactory sensitivity to amino acids and bile acids, in common with and similar to many other fishes (Hara, 1994). The thresholds of detection for the amino acids were similar to those of *O. mossambicus* (Kutsyna et al., 2016) although in the latter, L-methionine was significantly more potent than the other two (the sulphur-containing amino acids L-cysteine and L-methionine are usually the most potent olfactory amino acids in fishes). The thresholds of detection for the amino acids, however, were consistently (at least within the three tested) more potent (by at least one order of magnitude) in *A. facetus* than *O. mossambicus* (Huertas et al., 2010). The significance of this is unclear, as olfactory sensitivity to bile salts in teleosts, although widespread, is poorly understood (Buchinger et al., 2014). Nevertheless, it is consistent with bile salts comprising a proportion of the odour of intestinal fluid and a possible role in the signalling of dominance in *A. facetus*.

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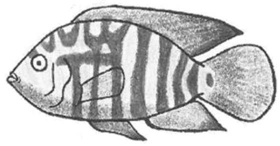
CHAPTER SEVEN

POTENTIAL INVASIVENESS OF *Australoheros facetus* (JENYNS, 1842) IN PORTUGAL ASSESSED BY FRESHWATER FISH INVASIVENESS SCREENING KIT (FISK) AND AQUATIC SPECIES INVASIVENESS SCREENING KIT (AS-ISK)



Potential invasiveness of A*ustraloheros facetus* (Jenyns, 1842) in Portugal assessed by Freshwater Fish Invasiveness Screening Kit (FISK) and Aquatic Species Invasiveness Screening Kit (AS-ISK)

Abstract

Invasive species are recognized as an important cause of biodiversity decrease. Unfortunately, legal regulations about prevention, control or eradication of invasive species are not always up-to-date, either due to failure in recognizing the problem, the lack of adequate scientific information or the long timings usually required to prepare the legislation. Pre-screening kits for potential invasive species are valuable tools for policy makers, since they provide information about *if* and *how* management measures should be taken. The freshwater Fish Invasiveness Scoring Kit (FISK) and the Aquatic Species Invasiveness Screening Kit (AS-ISK) have been suggested to be reliable tools to assess the potential risk of a species to become invasive. The present study applied the toolkits FISK v2 and AS-ISK to *Australoheros facetus* in Portuguese streams, in order to evaluate the possibility to classify this species as invasive in Portugal, in light of previous information and data presented in this thesis. The scores reached by the kits (FISK v2: 23; AS-ISK: 37) places *A. facetus* as a species with high potential of invasiveness and highlights the importance of frequent updates in both the field monitoring and the legal regulation and watch lists of invasive organisms.

Key-words: risk analysis, invasive species, freshwater fish, environmental monitoring, Iberian Peninsula

1. Introduction

Non-native species, when introduced in new environments (whether intentionally or not) can establish self-sustaining populations, become abundant, expand their distribution, and cause impacts to the environment, public health and economy. At this point, they acquire the status of invasive species (Casal, 2006; Clavero & Garcia-Berthou, 2005; Gallardo et al., 2016; IUCN, 2000; Mack et al., 2000). Monitoring populations of a non-native species that is likely to become an invader is a valuable step in management programs, since it is less costly than to try to eradicate a fully dispersed species (Epanchin-Niell & Hastings, 2010; Epanchin-Niell, 2017; Mack et al., 2000).

"What factors determine whether a species will become an invader or not?" was one of the three questions proposed by the program of the Scientific Committee on Problems of the Environment on biological invasions, in the 1980's (Drake, 1989). To identify such species, profiling can be used in a risk analysis, applying the knowledge on their biology and ecological features (Clavero, 2011; Vila-Gispert et al., 2005). In addition, it is important to clarify definitions of invasive species, suggesting some questions with focus in determining if *impact* and *success* are criteria to define invasive species, and if they are, specify which kind of impact and which measure of success were used (Heger et al., 2013).

The Weed Risk Assessment (WRA) for terrestrial plants (Pheloung et al., 1999) was first developed to assess if new plant taxa imported into Australia and New Zealand could become invasive through, mainly, analysing current status of the taxon in other parts of the world, its climate and environmental preferences and biological attributes. Adaptations of the WRA were the inspiration for the development of screening modules by the U.K. Department of Environment, Food and Rural Affairs (DEFRA) for the implementation of the European Regulation on the use of non-native species in aquaculture. One of those modules was the freshwater Fish Invasiveness Screening Kit (FISK) initially introduced in 2005 (Copp, 2013; Copp et al., 2008; Copp et al., 2005).

The FISK was revised from 2008 onwards, examining the potential relevance and application to warm temperate and tropical regions, as well in the original temperate zone, yielding FISK v2 (Copp, 2013; Lawson et al., 2013). The application of FISK v2 in the Iberian Peninsula (Almeida et al., 2013) is an easier protocol than the approach previously proposed by Clavero (2011), since the latter needs a detailed knowledge of the invasion pathways and the key biological features that determine invasion success.

These are troublesome to define or obtain, and are mainly focused in the early stages of invasion, while FISK can be built using abundance and distribution and environmental data. The calibration of FISK v2 for the Iberian Peninsula was performed through the analysis of 89 fish species by three assessors knowledgeable of the local fish fauna, and each one created a receiver operating characteristic (ROC) curve, used to assess the predictive ability of FISK to discriminate between invasive and non-invasive species (Almeida et al., 2013). From the ROC curves, the best FISK threshold was reached (20.25), distinguishing between "medium risk" species and "high risk *senso lato*" species.

In 2016, a new toolkit was published to incorporate the "sister" screening tools of FISK in only one toolkit [(freshwater invertebrates (FI-ISK), marine invertebrates (MI-ISK), marine fish (MFISK) and amphibians (Amph-ISK)], and to ensure that the protocols of the European Non-Native Species in Aquaculture Risk Assessment Scheme (ENSARS) are consistent with the minimum requirements for use with EU legislative instruments (Copp et al., 2016). This resulted in the Aquatic Species Invasiveness Screening Kit (AS-ISK), with changes that improve the clarity in the interpretation of questions and guidance and enhanced ecological applicability. In addition, in the AS-ISK toolkit, six questions on how the climate change models are likely to affect the invasiveness of the organisms assessed were added. Currently, few works have applied the new AS-ISK (but see Glamuzina et al., 2017; Tarkan et al., 2016; Tarkan et al., 2017), and none were applied in the aquatic ecosystems of the Iberian Peninsula. This leads to a lack of a precise threshold definition for this region to discriminate between high, medium or low risk of potential invasiveness.

One of the species assessed by Almeida et al. (2013) to calibrate FISK v2 to the Iberian Peninsula was *Australoheros facetus*, which yielded a FISK score of 19.3 ± 5.8, (with mean certainty factor for all the 89 species of 0.83 ± 0.05) and was classified with medium risk to become invasive. Curiously, *A. facetus* was not present in the list of non-native/invasive species in the protocol proposed by Clavero (2011) for the Iberian Peninsula, and is currently classified as "harmless" by FishBase (www.fishbase.org, version 02/2017). This neotropical cichlid is native from South America, in the Paraná-Paraguay system and the Uruguay basin (Rícan & Kullander, 2006). It is a freshwater fish with bentho-pelagic habits, mostly diurnal and displaying opportunistic feeding behaviour, which spawns in hard substrate and exhibits bi-parental care of the eggs and fry (Baduy et al., 2017; Ribeiro et al., 2007; Ruiz et al., 1992).

As pointed out in Lawson et al. (2013), the FISK evaluation can highlight gaps in knowledge in non-native species. These gaps are especially critical for species classified as medium risk but can be suppressed with original research. The aim of the present work is re-applying the FISK v2 for *A. facetus* with the recent biological and ecological information for this species in Portugal, and to compare the new results with the data published in Almeida et al. (2013). In addition, the new AS-ISK toolkit will be applied as well. The new data from recent field and lab research should provide relevant information to the FISK and AS-ISK analysis for an update on the status of this species.

2. Material and Methods

The FISK v2 and AS-ISK toolkits and user guide were obtained from the CEFAS website (available at >http://www.cefas.co.uk<, accessed at 15th March 2017).

FISK v2 is composed of 49 questions, divided in two sections: (A) Biogeography and historical data of the non-native species, and (B) Biology and Ecology, both subdivided in several items. Each question can be answered using literature searches, with a possibility to respond “Don’t know” if the search returned no reliable results. In addition, the assessor is asked to give a degree of certainty that lead to that answer, which weights the given answers. “Medium risk” species have a FISK score within the interval of [1 to 19] and “high risk *senso lato*” species have a FISK score within the interval of [20 to 57]. The “high risk *senso lato*” species are subcategorized as “moderately high” (species with score [20 to 24]); “high” (species with score [25 to 29]), and “very high” (species with score [30 to 57]).

The AS-ISK toolkit is also composed of 49 questions that are part of the Basic Risk Assessment (BRA) module (some of them similar to those in FISK v2) and 6 additional questions that are part of the Climate Change Assessment (CCA) module. Like FISK v2, the assessor is asked to provide the certainty of each answer. In AS-ISK there is no option to respond "Don’t know" but there is the possibility of leaving up to 5 questions without an answer. As there is no available score threshold to distinguish between potentially invasive (high risk) and potentially non-invasive (medium to low risk) for the Iberian Peninsula, the same thresholds published in Almeida et al (2013) for FISK v2 were used in the present study.

The categories of the sectors affected in FISK v2 were “Aquaculture (A)”, “Environmental (E)”, “Nuisance (N)” and “combined (Cb)”. However, the “combined” category had no distinction of which sectors were combined. While for AS-ISK the sectors were categorized as “Commercial (C)”, “Environmental (E)” and “Nuisance (N)”, and the combinations among them were explicit (e.g. the code used for a combined “Environmental” and “Nuisance” were EN).

The questions were answered using the assessor’s expertise, scientific literature search, grey literature search (e.g. reports, working papers), online discussion forums and other "grey literature".

After completing the assessments, a report was generated with the scores for the species and corresponding certainty factor (APPENDIX II, FISK v2 in Table 1 and AS-ISK in Table 2).

3. Results

The result FISK v2 score for *A. facetus* was 23, classifying it as a “high risk” species, in the subcategory “moderately high risk”, according to the thresholds defined by Almeida et al. (2013) (Table 1). Four questions were answered with “Don’t know” due the scarcity of biological information. The most affected sector was “Environmental” with a score of 21.

The AS-ISK score for *A. facetus* was 27 for its basic risk assessment (BRA) and 10 for its climate change assessment (CCA). According to the threshold of 20, *A. facetus’* basic risk assessment (BRA) categorized it as “high risk” species and the most affected sector was “Nuisance” with a score of 22 (Table 1).

The change from “Aquaculture” in FISK v2 to “Commercial” in AS-ISK had some effects in the final output of the sectors affected, decreasing the impact in this sector. In the same way, there was a decrease in the impact in the “Environmental” sector; while for “Nuisance” the impact was much higher (Table 1).

The sub-section “Feeding guild” with 4 questions in FISK v2 was replaced by “Resource exploitation” with just 2 questions in AS-ISK. This replacement made the score in this sub-section increase from 1 to 7 (Table 1, Figure 1).

Factors increasing both scores included the fact that this species can be used as ornamental fish (domestication and cultivation, Figure 1), has already self-sustained populations outside its native range (invasive elsewhere, Figure 1), a climate match between its native range and the Iberian Peninsula, its wide range of temperature tolerance, certain degree of euryhalinity (undesirable and persistence traits; persistence and tolerance attributes, Figure 1).

See Appendix 1, Table 1 for the detailed questions and answers for FISK v2, and Table 2 for AS-ISK.

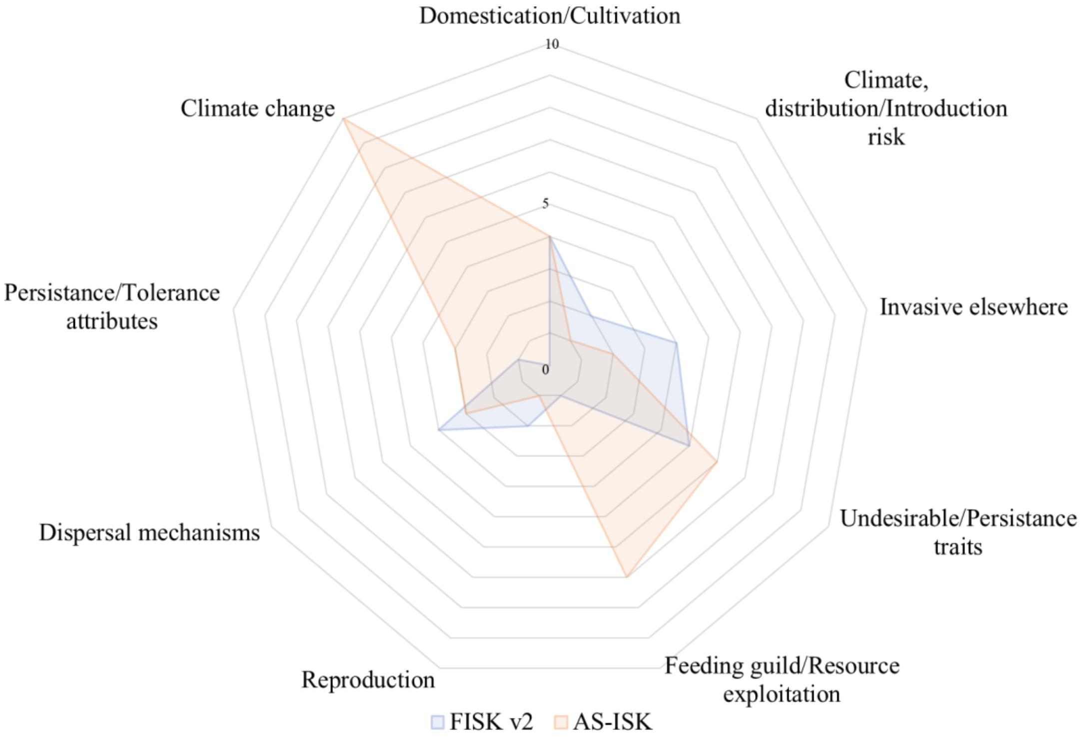


Figure 1. Scores achieved by *A. facetus* related to the using of the different approaches of FISK v2 and AS-ISK.

Table 1. Statistical summary of scoring of FISK v2 and AS-ISK for *A. facetus*.

Scores	FISK v2	AS-ISK
Total score	23	37
BRA score	23	27
CCA score	-	10
Outcome	**High**	**High**
Score partition		
A. Biogeography/Historical		
1. Domestication/Cultivation	4	4
2. FISK v2: Climate, distribution; AS-ISK: Climate, distribution and introduction risk	2	1
3. Invasive elsewhere	4	2
B. Biology/Ecology		
4. FISK v2: Undesirable traits; AS-ISK: Undesirable or persistence traits	5	6
5. FISK v2: Feeding guild; AS-ISK: Resource exploitation	1	7
6. Reproduction	2	1
7.Dispersal mechanisms	4	3
8. FISK v2: Persistence attributes; AS-ISK: Tolerance attributes	1	3
C. Climate change		
9. Climate change	-	10
Questions		
Answered	46	53
Not applicable	3	2
A. Biogeography/Historical		
1. Domestication/Cultivation	3	3
2. FISK v2: Climate, distribution; AS-ISK: Climate, distribution and introduction risk	5	4
3. Invasive elsewhere	5	4
B. Biology/Ecology		
4. FISK v2: Undesirable traits; AS-ISK: Undesirable or persistence traits	12	12
5. FISK v2: Feeding guild; AS-ISK: Resource exploitation	1	2
6. Reproduction	7	7
7.Dispersal mechanisms	8	9
8. FISK v2: Persistence attributes; AS-ISK: Tolerance attributes	5	6
C. Climate change		
9. Climate change	-	6
Sectors affected		
Aquaculture (FISK v2) / Commercial (AS-ISK)	18	8
Environmental	21	11
Nuisance	1	22
Thresholds		
Medium	19	19
High	20	20
Confidence	0.78	0.78

4. Dicussion

The freshwater Fish Invasiveness Screening Kit (FISK v2) and the Aquatic Species Invasiveness Screening Kit (AS-ISK), which establish the invasive potential of aquatic organisms, were applied and updated to include the novel information on the non-native cichlid *Australoheros facetus* in Portugal. This fish is already present in several streams of the Guadiana, Arade and Sado basins (Alexandre et al., 2012; Pires et al., 2010; Ribeiro et al., 2007), where it lives in localized but abundant groups. The FISK v2 analysis with our new data yielded a score for *A. facetus* of 23 - a significant increase from the previously described 19.3 (Almeida et al., 2013). This score is particularly relevant because it upgrades the species ranking to high invasiveness risk. Furthermore, the AS-ISK score was 27 for BRA, and increased to 37 when adding its CCA.

4.1. Reasons for increased invasive potential

Some features are described as key elements in the successful establishment of a non-native species: wide physiological tolerance, rapid sexual maturation, high fertility rates and parental care (García-Berthou, 2007; Ribeiro et al., 2008). It is worth noting that different life-history traits can affect the success of a new introduction differently depending on the stage of the invasion process, i.e. transport, establishment, spread and integration, since it is not just the abiotic factors in the invaded area that can act as filters but the biotic interactions with the native species matter as well (Moyle & Light, 1996; Ribeiro et al., 2008).

The increase in FISK v2 score places *A. facetus* with similar potential invasiveness to the pikeperch *Sander lucioperca* or the tench *Tinca tinca* (Almeida et al., 2013), in spite of these species presenting just few biological life-history traits in common (e.g. parental care in *A. facetus* and *S. lucioperca*, and extreme physiological tolerance in *A. facetus* and *T. tinca*) (Figure 2) (Ribeiro et al., 2008). The species most similar to *A. facetus*, in terms of traits such as trophic ecology, life-span, fecundity or abiotic tolerance is the black bullhead catfish *Ameirus melas* (Ribeiro et al., 2008), but interestingly, when *A. melas* was assessed by Almeida et al. (2013), it obtained a FISK v2 score of 32.7, much higher than that estimated for *A. facetus* at that time. Another surprising fact is that the cichlid *Astronotus ocellatus* has similar life-history traits with

A. facetus, and despite this, it is not established in Iberian Peninsula, although it has a high probability of establishment (Figure 2) (Range, 2013; Ribeiro et al., 2008).

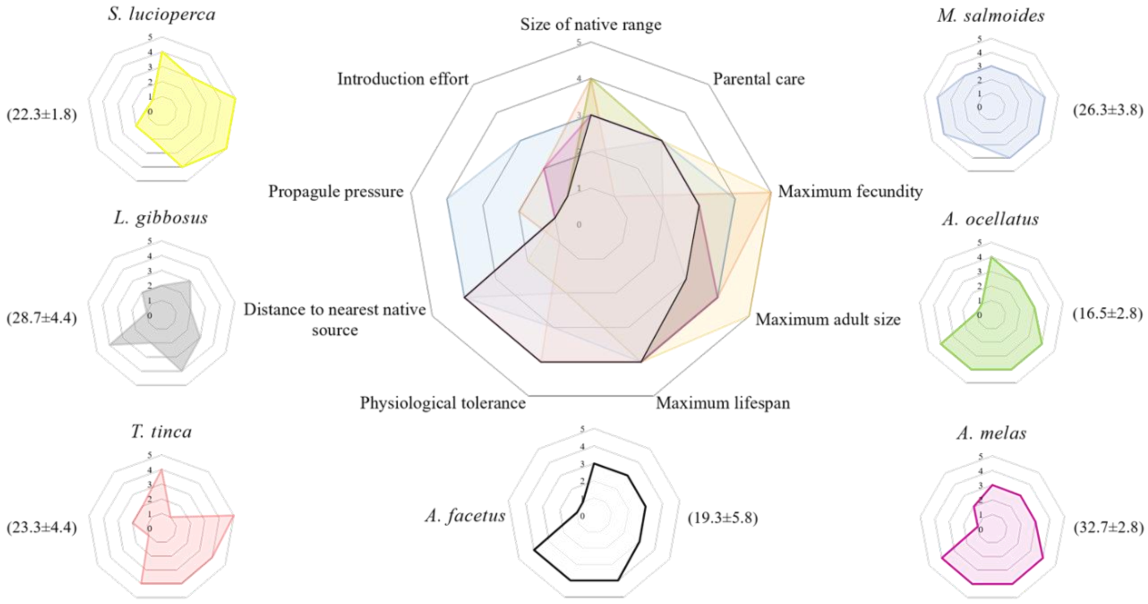


Figure 2. Diagram with some of the life-history traits and scores assessed by Ribeiro et al. (2008), showing some overlap among another non-native and invasive species and *A. facetus*. In parentheses the FISK v2 score from Almeida et al. (2013).

Although the possible direct impacts of *A. facetus* on native species, as would be the decline of abundance of native species abundance or the introduction of diseases, were not yet identified, there is circumstantial evidence that point to such deleterious effects, with recorded activities such as feeding on the eggs or larvae of other species or displacing other fish using a highly aggressive behaviour (Baduy et al., 2017; Ribeiro et al., 2007).

Although this neotropical cichlid seems to be restricted to a few basins in Southern Portugal, its local abundance seems to be increasing. The results described in chapter two of this thesis, despite being limited to a few sample points, showed high dominance of this species (abundance and biomass) in a fish fauna already threatened by human activities, poor in native and highly endemic species (Oliveira et al., 2012). During the hot and dry summers in Mediterranean regions (Gasith & Resh, 1999) such as is Southern Portugal, the fish fauna can be trapped in pools where the temperature increases rapidly and the dissolved oxygen decreases during the day, and even more at night in algae or plants are present; concomitantly, the resources diminish and thus the competition increases. On the other hand, the water temperature during winter can reach values at least as low as 6.6ºC (National Water Resources Information System – Portugal, available in http://snirh.pt/). The thermal amplitude in which *A. facetus* can live, as exemplified in this thesis, together with its generalist and opportunist feeding behaviour, could be a large advantage in Mediterranean streams (Baduy, Flávia et al., 2016; Ribeiro et al., 2007). In addition, we have shown that this species also presents salinity tolerance up to 15ppt, and possibly higher for smaller individuals (Baduy, F., Saraiva, J. L., et al., 2016; Baduy, F., Teixeira, D., et al., 2016). This suggests that *A. facetus* may use the upper and possibly the middle part of estuaries as bridges to reach new freshwater bodies. These appear to be examples of physiological plasticity that confers resilience to withstand abiotic variations. It remains to be seen if the species can thrive in such areas or if migrant individuals exposed to these conditions will maintain its fitness spreading throughout the invaded site.

Parental care and aggressive behaviour have been also discussed as key elements of successful invasions (Hudina et al., 2015; Juette et al., 2014; Marchetti et al., 2004). Aggression usually translates to dominance and brings advantage through priority access to limited resources, thus helping non-native species to acquire resources and enhance their competitiveness in a new environment. As described in chapter 5 and in Baduy et al. (2017), *A. facetus* is aggressive especially during the reproductive season. This may mean a competitive gain to sequester resources and quality habitats from native species, while its parental care allows its offspring to reach older stages, eventually to maturity, with less exposure to predation. Curiously, Ribeiro et al. (2008) established that low parental care is one of the characteristics that permits a non-native species to spread within Iberian Peninsula. Since most of native fishes do not present this kind of behaviour, the authors concluded that having features similar to the native fishes should enhance their spread. However, Grabowska and Przybylski (2014) found contrasting life-history traits among native and non-native fishes in Poland, with parental care (or some degree of) exhibited by the non-native fishes being the most distinctive feature. Therefore, *A. facetus* may in fact increase its competitive skills over the native fish fauna by differing in some biological attributes, thus taking advantage in face of a novel niche opportunity. In fact, two other successful non-native species, such as *Lepomis gibbosus* or *Micropterus salmoides*, both Centrarchids originally from North-America, present some degree of parental care, building nest sites and defending their young (Colgan & Brown, 1988). It is then likely that given the common ancestry and high endemism of Iberian species, in which these features were never developed, and constitutes a new dimension in terms of unexplored ecological niches.

Despite the amount of new information included in the analysis, it cannot be excluded that the discrepancy between the score presented here and that published by Almeida et al. (2013) could be due to the fact that in the present work the toolkit was applied by just one assessor. Is important to note that, as recommended by the Report to the European Commission about the project "Environmental Impacts of Alien Species in Aquaculture (IMPASSE)", a risk assessment should be carried out by more than one person, preferable by a multi-disciplinary team of recognised experts (Copp et al., 2008).

4.2. Evaluation of the toolkits

Both FISK v2 and AS-ISK have a friendly interface and are free, which can allow them to be a useful tool in management programs. And although FISK v2 has some ambiguous or repetitive questions they appear to be solved in AS-ISK. For example, very similar questions can lead the assessor to misunderstandings (e.g. Q1.02, Q 2.05 and Q 3.01 on the establishment of self-sustained populations outside their natural range– see annex). To deal with this problem, in AS-ISK the first two questions mentioned were removed and Q 3.01 was rewritten. In a general way, the questions and guidance in AS-ISK are clearer than in FISK v2. Moreover, questions about the taxon's presence in the surrounding area, possible pathways/vectors of introduction and socio-economic impacts, as well as the questions about predicted future climate change were included. Both questionnaires need an assessor with expertise in the field and a large degree of literature search. Nonetheless, the compilation of information and the creation of a species profile is easy. As observed by Glamuzina et al. (2017), both can provide similar risk rankings related to potential invasiveness (low, medium and high). Nonetheless, the AS-ISK score for *A. facetus* of 27 (BRA scores) place it as "high risk" *senso strictu* species (species with score [25 to 29]), while through FISK v2, *A. facetus* is categorized as "moderately high" (Almeida et al., 2013), which suggests the need for a calibration of AS-ISK for Iberian Peninsula.

4.3. The gaps in Portuguese legislation

As suggested in Almeida et al. (2013), the FISK assessment is a reliable tool to discriminate between invasive and non-invasive fish in Iberian Peninsula. The results presented here and in the remainder of this thesis highlight the importance of including *A. facetus* in the invasive species list in Portugal, with further monitoring and/or a more comprehensive assessment. Currently, *A. facetus'* status in Portugal is as *non-indigenous* (defined in Decreto-Lei nº565/99, that regulates the introduction into nature of non-indigenous species of flora and fauna), while in Spain *A. facetus* is recognized as an *exotic invasive* fish (Real Decreto 630/2013, that regulates the Spanish catalogue of exotic invasive species). Although Decreto-Lei nº565/99 is currently under revision, the information and novel guidelines proposed for alteration are, unfortunately, not available at present for public consultation. However, it is important to highlight that the Portuguese environmental legislation has some ambiguities that can lead to misunderstanding related to management of non-indigenous species. One of the major problems is that in the Decreto-Lei nº565/99 non-indigenous is defined:

> Article 1º
>
> Paragraph 3º: *Non-indigenous species listed in Annex I, which forms an integral part of this diploma, with the exception of those indicated as invasive, are considered for the purposes of this law, in each of the territories in which they are referred to, as indigenous species.*

As such, *A. facetus* is not currently legally recognized as an invasive species in Portugal and it is not clear what were the reasons behind the attribution of the status, as there was little published scientific information on the species at the time of the decree. This status opens the door for additional spreading, since from the sentence above, we can conclude that in Sado, Mira and Guadiana basins, where *A. facetus* is already referenced in the Decreto-Lei nº565/1999, there are no constrains to add further individuals in those basins. This gap, can enhance the species invasive potential, including in places designated to preserve the native biodiversity, such as the Guadiana Valley Natural Park (Decreto-Regulamentar, nº 28/1995) and the Guadiana Valley Special Protected Zone (Decreto-Lei, nº 384-B/1999) (Figure 3). Additionally, in 1980, Portugal signed the Convention on Wetlands (Ramsar Convention), assuming the obligations to promote the conservation of Wetlands and waterbirds, establishing Natural Reserves, and providing appropriate protection (Decreto, nº 101/1980). In 2012, the Vascão River, one of the collection sites for this thesis, and at present the largest river without artificial interruptions such as dams or reservoirs in Portugal was included as a Ramsar site (Ramsar, 2012).

Although the interpretation is that *A. facetus* cannot be introduced in river basins where the species was not yet recorded at the time of the decree, this also highlights the need for constant monitoring of the national rivers, as we now have evidence of *A. facetus* in locations not included in the mentioned annex. A direct consequence of the existing law would be that if the species is already in any location of the stream it can be translocated to any other pristine site of the same stream, or if exists in a given stream of a specific basin it can be introduced in any other stream of the same basin.

Despite the recent law (Decreto-Lei, nº 142/2008) that defines the introduction of non-indigenous species into the wild as very serious infringement or unlawful act punishable by a fine, (Article 43º, Paragraph 1º, item ‘t’), this same paragraph cites that this is only valid *if* it is predicted in its respective law (in this case, the Decreto-Lei nº565/1999). Nonetheless the law indicates general cautionary measures, and as ruled by the Environmental Policy in Portugal (Lei, nº 19/2014), the public action on the environment is subject to various principles, one of them is:

> Article 3º
>
> Item c) *Prevention and precaution, which require the adoption of anticipatory measures with a view to avoiding or minimizing, as a matter of priority, adverse impacts on the environment, whether natural or human, both in the face of immediate and specific hazards and in the face of future and uncertain risks, in the same way as they can establish, in the event of scientific uncertainty, that the burden of proof lies on the party alleging the absence of hazards or risks.*

In practice, there is a grey zone on what can and should be practiced in relation to this, and other, non-indigenous species, and most of the decisions are made by the services of ICNF, the Portuguese institute for Nature and Forest Conservancy, which is responsible for monitoring rivers, and carries out campaigns to eliminate non-indigenous fish, mostly in protected areas, as in the streams within the Guadiana Valley Natural Park.

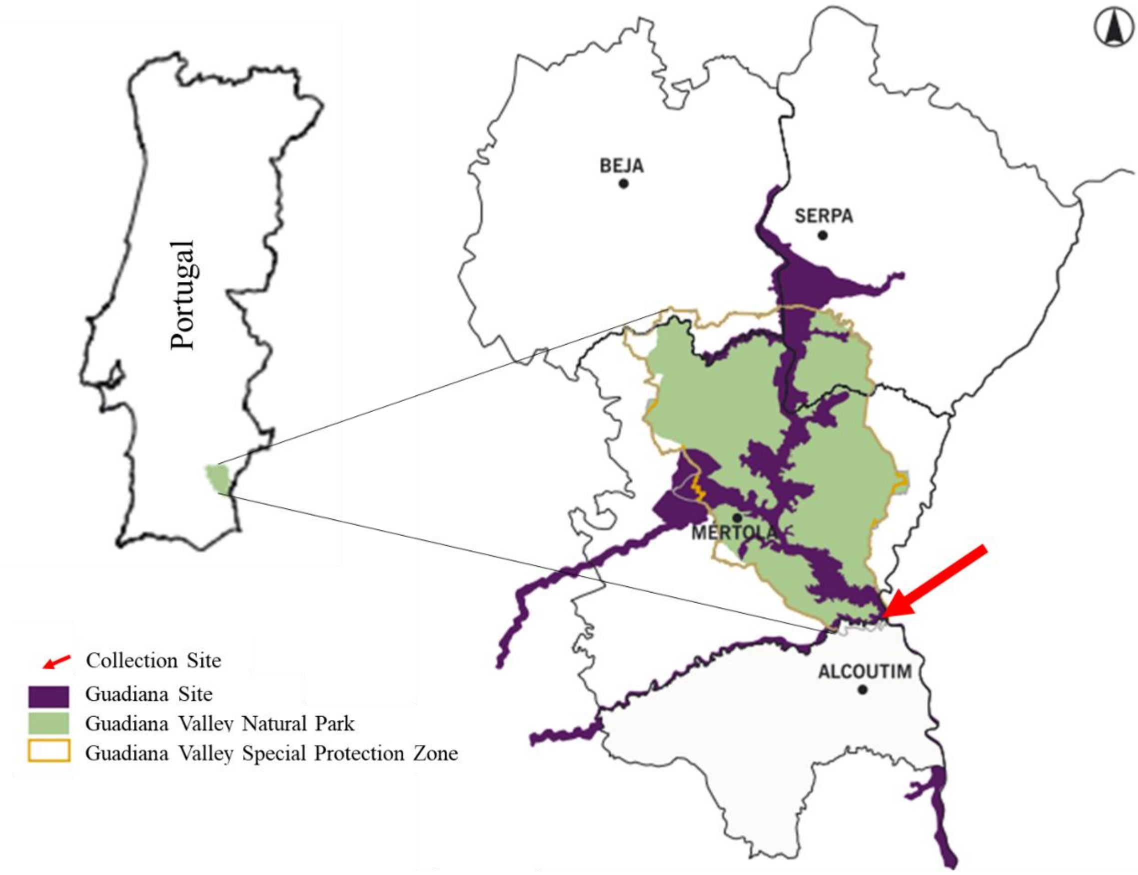


Figure 3. Location of the protected areas in Guadiana Valley. The red arrow indicates one of the collection sites used during this thesis, at Vascão River, in where, according to Decreto-Lei nº565/1999, *A. facetus* can be considered as an indigenous species. Adapted from Cardoso et al. (2008).

Additionally, Environmental Policy in Portugal (Lei nº 19/2014) states that the environmental policies are subordinated to the principle of “knowledge and Science”, and that the diagnosis and solutions for environmental problems must come from the convergence of social and scientific knowledge, and should take in account the involvement of the citizens during the elaboration and application of the environmental policy.

After the above, we hereby express the importance of communicating state-of-the-art knowledge that can help decision makers to prioritize *if* and *how* management measures should be addressed to potential invasive species, even if there is no available conclusive evidence of direct negative impacts (Hirsch et al., 2016). The present work can therefore be a key element in the management of freshwater basins where *A. facetus* is present.

Credits

The electronic tool kits are Crown Copyright (2011–2013). As such, FISK v2 and AS-ISK are freeware and may be freely distributed provided this notice is retained. FISK v2 was developed by G.H. Copp (Cefas) in collaboration with J. Hill, L. Lawson (both University of Florida), Scott Hardin (Florida Fish and Wildlife Conservation Commission) and L. Vilizzi (Muğla Sıtkı Koçman University and TÜBİTAK). The VBA for Excel and computational programming of FISK v2 was undertaken by Lorenzo Vilizzi, elaborated and expanded from the VisualBasic code in the original Weed Risk Assessment (WRA) tool kit of P.C. Pheloung, P.A. Williams & S.R. Halloy (1999) – these modifications are described in Lawson et al. (2013).

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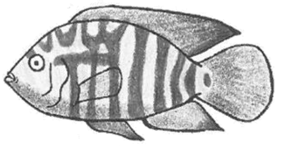
CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSION



1. General Discussion

One of the main goals achieved by this thesis is to show how important a careful collection of observational and/or experimental data about a non-native species is in order to provide a scientific basis for management, to predict future invasion hotspots, limiting the spread of populations and reducing the negative impacts on native biodiversity. In the process, we made important and novel observations about the basic biology of *Australoheros facetus* that provide new perspectives as to the potential for spreading of the species.

One of the great questions in Invasion Ecology, raised since the publication of “The Ecology of Invasions by Animals and Plants” by Elton (1958), is why some introduced species survive or not, and if they survived why some become invasive while others do not (Richardson, 2011; Williamson et al., 1986). To answer these questions there is an urgent need for studies focusing on physiology to address the potential invasiveness of non-native species. In fact, physiological tools can facilitate rapid assessments and signal to where direct efforts in efficient management programs in conservation (Lennox et al., 2015; McKenzie et al., 2016). An integrative approach can link physiology with invasion biology: for example, the endocrine analysis of stress and reproductive biology can pinpoint critical time windows to apply control measures; behaviour and metabolism can demonstrate interaction with abiotic tolerance limits underpinning the tolerance range for spreading. Overall, physiology can provide effective tools for controlling undesirable species (Lennox et al., 2015).

Cichlids can be found all over the world, despite their limited African or Neotropical origin. Members of this family are among the 20 most frequently introduced species in the Afrotropical, Neotropical, Australian and Oriental realms (Toussaint et al., 2016), and are also present in sites such as Iran (Esmaeili et al., 2016), Italy (Piazzini et al., 2010) and Japan (Sakano & Iguchi, 2009) in the Palearctic realm, and in California (Hovey & Swift, 2012) in the Nearctic realm. Some characteristics that make this family so widespread are related to their phenotypic plasticity, with ample physiological tolerance to environmental ranges (Chippari-Gomes et al., 2005; Febry & Lutz, 1987), although their aggressiveness and parental care could also enhance their abundances (Faunce & Lorenz, 2000). Because of these features they are usually traded as food or ornamental species, which can increase their propagule pressure in nature (Liew et al., 2013; Pullin, 1991; Sui et al., 2016).

In this context, this thesis aimed to study and analyse the mechanisms that allowed *Australoheros facetus*, to our knowledge the only cichlid found in the wild in Portugal, to adapt and spread, as well as to try to predict the potential occupation of new habitats in Portuguese waters. In addition, the current Portuguese legislation for exotic species was assessed. Using a standard risk assessment tool incorporating our new data, we suggest the re-classification of *A. facetus* as an invasive species in Portugal that should be closely monitored.

1.1. Increased presence of *A. facetus* in Portugal

The ichthyofauna of Iberian Peninsula is characterized by high degree of endemism and low number of native species (Filipe et al., 2010; Hermoso & Clavero, 2011; Magalhaes et al., 2002) and has been pressured by numerous interventions, such as construction of dams (Alexandre et al., 2012), increased pollution sources (De Miguel et al., 2016) and introduction of non-native species (Hermoso et al., 2012; Ilheu et al., 2014).

Regardless of the current distribution of *A. facetus* being still restricted to basins in Southern Portugal and some locations in Spain (Hermoso et al., 2011), it is important to note that this presence was not correlated with human-induced disturbance, as occurs with other non-native species, which could be evidence of its ability to adapt to different environments (Ilheu et al., 2014). In fact, there is little information to decide which are the preferred habitats. Our efforts to correlate the presence and/or abundance of *A. facetus* with environmental factors, biotic or abiotic, did not yield conclusive causal associations. What is certain is that in specific spots the species is highly abundant, and shows well-structured populations, with several breeding pairs surrounded by juveniles of different ages.

The estimates of relative abundance of *A. facetus* seem to be related to the season when the survey was conducted. The data reviewed in **Chapter two** shows that the discrepancies in the literature about the dominance of non-native species seem to be related to the pluviosity and consequent stream flow rate during sampling. Thus, we conclude that to fully assess the real presence and abundance of *A. facetus* (and other non-native species) in the Iberian Peninsula, surveys should be performed throughout the year, and preferably, in more than one year, to make sure that different hydrological conditions are covered. How this presence impacts or conditions the number or success of other native or non-native species is also to be determined. In this work, despite several attempts using different ecological indexes and methodologies, it was not possible to find correlations between the presence or number of *A. facetus* and the structure of the fish assemblage in each specific site. However, the presence of *A. facetus* in Southern Portugal is currently underestimated, especially at the Odelouca microbasin, where this species reaches a ponderal index above 95%. As *A. facetus* is characterized as a limnophilic species (Alexandre et al., 2012), and resilient to changes in temperature and dissolved oxygen in water (**Chapter three**), the formation of pools during the dry seasons can boost their abundance, even from few individuals as they have an laborious parental care (**Chapter five**), allowing their offspring to survive.

1.2. Environmental tolerance

Broad environmental tolerance is a common trait among successful invaders, given that abiotic parameters usually are the first filter that a species must transpose to establish itself in a new habitat (Kelley, 2014; Marchetti et al., 2004). This is especially true for fish that are directly affected by its surroundings. The African jewelfish *Hemichromis letourneuxi* for example, can tolerate progressive increasing salinities as high as 50ppt (Langston et al., 2010); and the oscar, *Astronotus ocellatus*, may support, at least for a short period, temperatures as extreme as 9ºC and 41ºC (Gutierre et al., 2016).

Different degrees of physiological plasticity can be the key to the increase of non-native species when the environment is disturbed, naturally or artificially. While low temperatures have been described as a barrier for some cichlids (Gutierre et al., 2016; Schofield et al., 2009), and the winter can be quite severe in Southern Portugal, the CTMin achieved by *A. facetus* during acute thermal stress can indicate resilience during cold fronts (**Chapter three**), or if isolated in river pools after a flood or placed in agricultural ponds. Regarding their response to long-term exposure, *A. facetus* showed a physiological and behavioural compromise, mainly observed in its reduced locomotor and feeding activities, as well through the stress markers assessed (especially plasmatic cortisol and *hsp70* gene expression). With a CTMax (LOE) as high as 36ºC, these fish will likely survive in isolated ponds, when rivers lose connectivity during the warm summer. If their tolerance to hypoxia matches, then they are in a position to be the sole winners when the rain comes again. These studies also permitted observations on the importance of temperature for *A. facetus* physiology and behaviour, as fish moved from lethargy at 7ºC to 12ºC to active swimming to feeding frenzy and social aggression, from 12ºC to 24ºC and then further with a period of 10 hours.

Another important environmental stressor assessed in this thesis was salinity (**Chapter four**). Although body size was not considered as a covariate in the response to the salinity trials, it seems that larger fish (~9 cm SL) were not able to cope with salinities above 15 ppt, while smaller fish (~5 cm SL) appeared to have little or no constraints at 15 ppt, and only impaired growth at 18 ppt. Despite the low survival at higher salinities, not all individuals showed abnormal physiological indicators, which may suggest some individual genetic variation that give the species the plasticity required to survive at hypertonic environments. Whether those more plastic *A. facetus* individuals, which tolerate salinity, would show such ability to reproduce in brackish water is unknown and would constitute important information.

Nonetheless, this important observation that *A. facetus* can live in brackish waters at least for a short period of time, potentially may allow them to use the upper and middle estuaries as bridges to reach other freshwater bodies. Given the flash floods which recurrently occur in the Mediterranean type streams (Gasith & Resh, 1999), and the likely salinization of estuaries predicted in the future climate scenarios (Cañedo-Argüelles et al., 2013), it is possible that some individuals may be dragged into more brackish environments. This will depend in part on the swimming ability of fish, another physio-morphological feature of the species that deserves attention.

1.3. Behaviour and chemical communication

Behavioural traits can be a key part in successful invasive episodes, but studies on this subject are scarce (exceptions include Blake et al., 2015; Holway & Suarez, 1999; Reisinger et al., 2016). High investment in reproductive strategies such as parental care, have been correlated with successful colonization of new habitats (Drake, 2007; Marchetti et al., 2004). In addition, aggressive abilities usually ensure the possession of territories and resources (Smith & Price, 1973). Those traits can allow the species to dominate and even exclude weaker competitors of another species through competitive and/or predatory interactions, especially in the establishment phase of an invasion (Hudina et al., 2014; Hudina et al., 2015; Pereira et al., 2014).

The behavioural repertoire shown by *A. facetus* (**Chapter five**) can reflect an advantageous weapon in Iberian watersheds, since the majority of the native fishes are cyprinids and typically show little or no kind of parental behaviour or aggressiveness (Hermoso et al., 2012; Marr et al., 2013; Ribeiro et al., 2008). However, to fully understand the interaction between behaviour and environment it is essential to look at the mechanisms underpinning behaviour. The endocrine profile described in chapter five is indicative of how the changing seasons can influence hormone levels, and concurrently aggression and reproduction. Joined with similar information on the molecular and physiological mechanisms underlying parental care, unfortunately not possible to study during the extent of this thesis, and confirmation with observations in the wild, it would mean the characterization of the reproductive repertoire of the species.

When faced with such complex behaviours in social fish, it is unavoidable to ask whether there is communication and how individual recognition can be achieved to indicated who is who in the hierarchy, or between members of a reproductive pair, or their descendants. The olfactory system of *A. facetus* also shows the ability to discriminate between chemical odorants in the intestinal fluid from submissive or from dominant fish, thus indicating a chemical signalling system may be involved in the hierarchy formation and maintenance (**Chapter six**). Combining the findings on behavioural repertoire and hormonal profiles with knowledge of conspecific chemical communication could be the first step to develop management tools to control or eradicate invasive species, as recent techniques for controlling non-native species have focused more in species-specific effects, such as pheromone baits (Aquiloni & Gherardi, 2010; Witzgall et al., 2010) and traps (Johnson et al., 2009).

1.4. Risk Assessment

The results of the analyses of two risk assessment toolkits (**Chapter seven**) call for an update of the status of *A. facetus* in Portugal. Tools like these can help policy makers to direct efforts to control or mitigate impacts caused by non-native invasive species. This exercise also underlines the importance of gathering more information on the distribution and numbers of *A. facetus,* as shown in chapter two, and on the need to update the historical information on the occurrence of the species. Currently *A. facetus* is not considered an invasive species in Portugal, therefore the legislation that rules the management of this species is much too permissive. Little scientific information existed when the current status was determined, but surprisingly, instead of the *"when in doubt, protect"*, the approach followed was not the most conservative but rather a very lenient one: this non-native fish was considered as native within the basins where they already existed.

As observed with a quick on-line search, many hobby aquarists usually collect *A. facetus* in Portugal directly from ponds or streams, and not through sales in aquarium shops or commercial trade. This translocation of fish has the additional risk of facilitating the spread of diseases, given that in natural environments fishes can have a wide range of pathogens (Sampaio et al., 2015).

2. General Conclusions

The risk assessment analysis showed that *A. facetus* has a set of traits that may facilitate its spreading in Portugal, mainly in the Southern region where the winter is not as severe as in the North. In addition to human introduction, this species may also use the upper estuaries of Guadiana and Arade as bridges to reach other freshwater bodies. The potential for colonization of the upper and middle estuaries has yet to be established. However, it seems that *A. facetus* has no physiological constraints at salinities below 15 ppt and temperatures above 7 ºC, but it remains to be seen if it can reproduce in such conditions. In addition, parental care and aggressiveness allow this species to dominate over the local ichthyofauna, possibly displacing other fishes and guaranteeing high rates of recruitment.

It is important to keep in mind that scientific research should be the basis of environmental legislation (Figure 1). However, this is a dynamic system, where monitoring programs should provide the results from the conservation strategies in a way to refine or redirect efforts (McKenzie et al., 2016). It is also essential to maintain a good dialogue among researchers, decision-makers and population for the implementation of viable solutions.

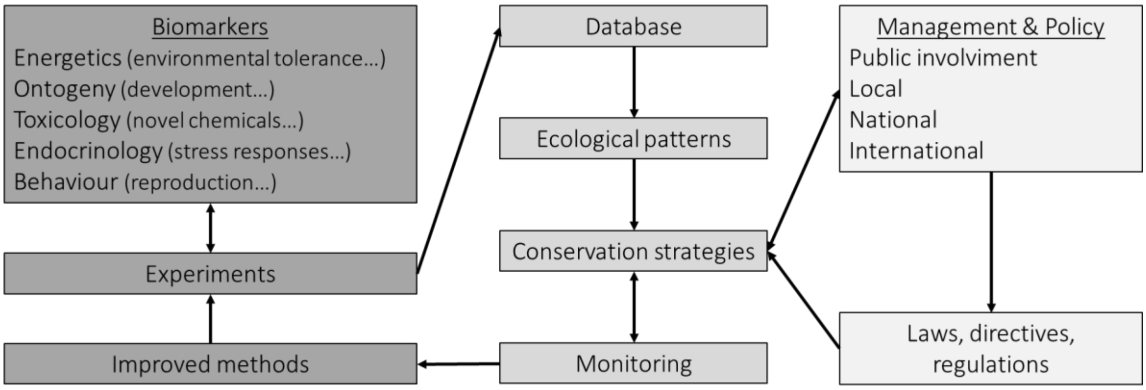


Figure 1. Diagram showing how dynamic the proposal of laws and regulation should be so that legislation is not outdated. It is important to note that this is a continuous feedback system. Diagram adapted from McKenzie et al. (2016).

3. Future Perspectives

The work presented in this thesis provided new insights about the ecophysiology of an invasive species and delivers an important biological basis for the design of species-specific management tools. However, it also gave rise to new questions, some of them summarized below:

A) How temperature and salinity interact on survival and reproduction? In the wild, especially is estuarine regions, salinity and temperature vary daily and seasonally. Manipulations of both these parameters will refine the knowledge produced in the present thesis and combined with metabolic and ecological studies better evaluate the fish's ability to acclimatize to these abiotic filters in nature.

B) What are the baseline levels of hormones in the wild and throughout the seasons? Knowledge on these issues is necessary to better understand the life-history of this species which does not show sexual dimorphism and display parental behaviour.

C) How do these populations relate to native ones and with other non-native species? Behavioural studies, especially in field, can answer about possible direct impacts of competition or predation on native species.

D) How phenotypic plasticity evolved in this species? Comparative physiology combined with genetics between native range and invaded range of *A. facetus* allow testing hypothesis about their evolution in the invaded area.

E) What is the mechanism behind mate choice and mate recognition? This species shows high social organization and chemical signalling through faeces, a communication channel that is rather unstudied. An array of questions about social competence may now be addressed in what might be a novel model species. An emergent field applied knowledge may be the development of chemical tools for management of invaded areas.

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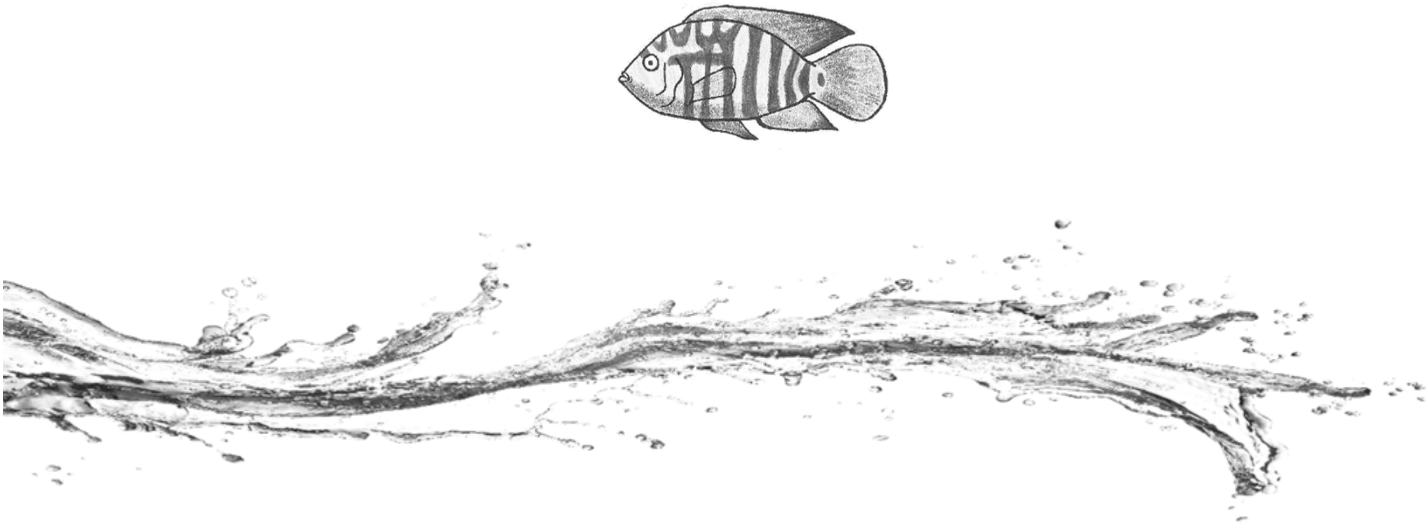
Appendix I



Appendix I

Table 1. Ethogram of *Australoheros facetus*.

Class	Behaviour			Description
Non-social	*a) Locomotor patterns*	i. Swim		The fish is propelled by moving the caudal fin. Anal, pelvic and dorsal fins are retracted. Pectoral fins may be active.
		ii. Hover		The fish remains motionless in the water column. The most active fin is the pectoral, while the caudal fin moves slowly. The dorsal and anal fins are retracted. Pelvic fins remain practically immobile.
	b) Yawn			Mouth opening largely with forward projection of the jaws while hovering, without any apparent feeding purpose.
	*c) Quiver**			Rapid shivering of the whole body. The performance lasts about 5-10 seconds and may occur once to three times in one minute. *This is a rare behaviour and can occur in different contexts (see below).
Social	*a) Agonistic*	i. Threats / Low aggression	Frontal display	Facing an opponent in an agonistic context with open opercula and extended branchiostegal membrane.
			Lateral display	Lateral exhibition usually in an inverted position relative to one another (head towards opponent's tail), with all fins extended.
		ii. Attacks / High Aggression	Strike	Fast burst of swimming directed to other fish, occurring in context of high proximity (less than one standard length of the focal individual) and sometimes involving contact.

(*Continued*)

Appendix I - Table 1 (*Continuation*)

Class	**Behaviour**			**Description**
Social	*a) Agonistic*		Chase	Swimming at high speed after another fish. This can occur briefly or last longer, usually culminating in physical contact.
			Bite	Bites usually occur following a chase or a strike. The most commonly affected area is the head region, but can be anywhere in the body of the opponent, and the grip can last for several seconds.
		ii. Attacks / High Aggression	Tail beating	A fish performs rapid antero-posterior waving of the body at the side of the opponent, touching the opponent with its tail.
			Mouth fighting	This is a symmetric agonistic interaction, usually following a symmetric frontal display. Both opponents rapidly extend their jaws and bite each other simultaneously on the mouth and frontal region. Once they engage in a bite, the grip can last up to one minute.
		iii. Submissive display	Flee	An individual move away from his pursuer, fast swimming, dorsal fins retracted.
			Freeze	The fish remains stationary during an attack, with no reaction.
			Quiver	See description above. Usually occurs when a fish froze during an attack.

(*Continued*)

Appendix I - Table 1 (*Continuation*)

Class	**Behaviour**		**Description**
Social	*b) Reproductive*	i. Courtship	The larger fish of the pair approaches the smaller and they touch their heads. Both fish exhibit darkened vertical bars. The smallest fish exhibits a darkened ventral anterior region. They swim close to each other slowly for about one minute and repeat this behaviour several times at intervals of about five minutes. Both fish shake their bodies, the smallest doing so more often. Usually the largest fish is the male and the smallest is the female.
		ii. Prespawning	The female prepares the place chosen for lay the eggs (nest), nibbling it and curving its body into "S" across the surface. This body movement is short and quick, lasts about six seconds and is repeated several times. The vertical bars in the body as well as the ventral region of the head are dark and further darkening when the female approaches the nest. The female moves away and swim around the entire aquarium then returning to the nest.
		iii. Spawning	Female swims on the nest, making a "S" movement with the body, slowly. The pectoral fin moves short and fast. The colour is similar to pre-spawning. Oviposition occurs slowly. After up to 90 minutes, the female moves and the oviposition ceases. Seconds later, the female returns and maternal care begins.

(*Continued*)

Appendix I - Table 1 (*Continuation*)

Class	**Behaviour**		**Description**
Social	*b) Reproductive*	iv. Dig	Digging a hole or a pit in the substrate with the mouth, pectoral and caudal fins. This will be used as refuge after hatching.
		v. Parental Hover	The animal hovers directly above the eggs or recently hatched larvae. Can be performed by both members of the pair, but most commonly by the smallest.
		vi. Care	The animal makes a "S" movement repeatedly passing the entire body slowly over the entire surface covered by the eggs, moving their pectoral fin in long movements, removing dead eggs with the mouth, cleaning and oxygenating the batch. Can be performed by both members of the pair, but most commonly by the smallest.
		vii. Patrol	The fish swims or stands is at a distance greater than their own standard length of eggs or larvae. Can be performed by both members of the pair, but most commonly by the largest.
		viii. Fetch	Occurs when a larva moves away from the larvae group and one of the adults catches it with its mouth, and spits it back into the group. This behaviour can be observed by both members of the pair.
		ix. Dual hover	Both members of the pair hover above the fry with their bodies in contrary directions, quivering and touching each other. They can hover spinning in slow circles, with a 360º angle of sight around the nest or refuge.

Table 2. Hormonal response of *Australoheros facetus* to group formation.

Asterisks mark significant differences at p<0.05. Initial: before group formation, Final: after group formation, F: females, NTF: non-territorial female, TF: territorial female, M: males, NTM: non-territorial male, TM: territorial male, SE: standard error. U-test value refers to Mann-Whitney U-statistic. t-test values refer to Student's t statistic. Asterisks show significant differences.

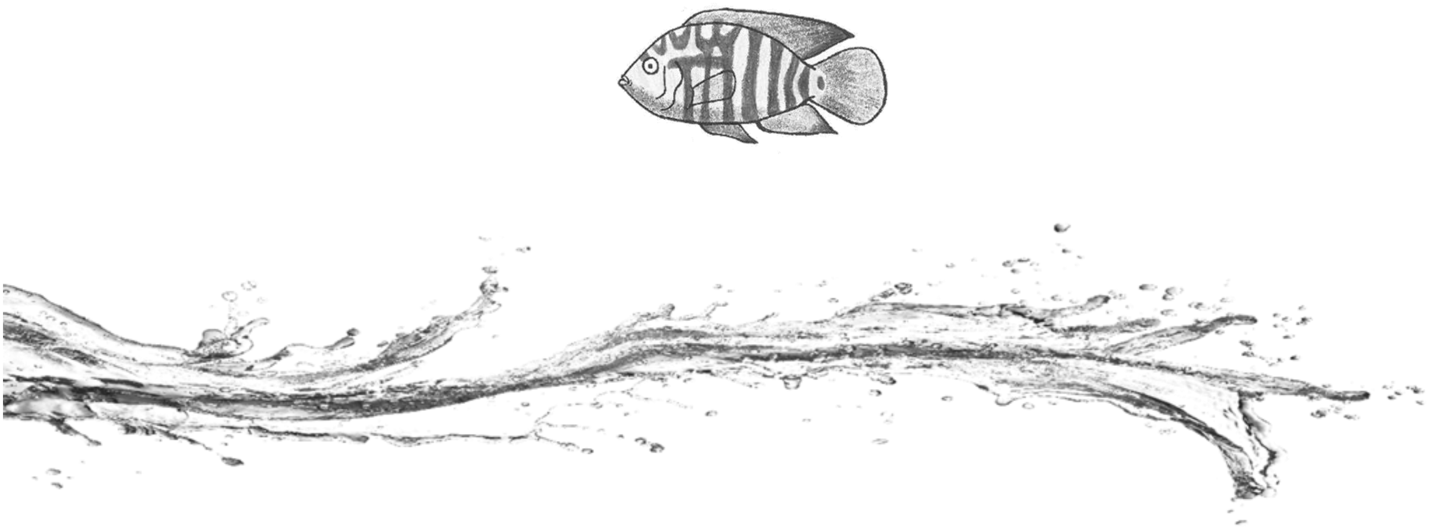
		F	NTF	TF	M	NTM	TM	F *x* M (test value, p)	NTF *x* TF (test value, p)	NTM *x* TM (test value, p)
E_2 (ng/mL)										
Initial	Mean	0.86	0.80	0.94					t=-0.65, 0.51	
	SE	0.15	0.20	0.23						
	n	32	19	13						
Final	Mean	1.31	0.98	1.72					t=-2.06, 0.04*	
	SE	0.23	0.25	0.41						
	n	32	18	14						
11KT (ng/mL)										
Initial	Mean	1.12	1.19	1.01	1.30	1.24	1.42	U=343.50, <0.01*	U=99.00, 0.35	U=141.50, 0.65
	SE	0.31	0.43	0.42	0.27	0.34	0.45			
	n	37	19	13	32	24	13			
Final	Mean	1.05	1.08	1.01	1.48	1.10	2.30	U=561.00, 0.29	U=118.00, 0.77	t=-2.25, 0.03*
	SE	0.26	0.34	0.40	0.29	0.29	0.62			
	n	41	18	14	32	29	13			

(*Continued*)

Appendix I - Table 2 (*Continuation*)

		F	NTF	TF	M	NTM	TM	F *x* M (test value, p)	NTF *x* TF (test value, p)	NTM *x* TM (test value, p)
Testosterone (ng/mL)										
Initial	Mean	4.45	4.56	4.31	5.42	4.13	7.99	U=415.00, 0.04*	t=-0.54, 0.59	t=-0.51, 0.61
	SE	1.16	1.75	1.37	1.13	0.53	3.19			
	n	32	19	13	36	24	12			
Final	Mean	3.28	2.39	4.43	2.58	2.48	2.82	U=605.50, 0.85	U=84.00, 0.11	U=114.00, 0.14
	SE	0.67	0.63	1.26	0.41	0.55	0.55			
	n	32	18	14	39	27	12			
Cortisol (ng/mL)										
Initial	Mean	335.14	333.50	336.78	249.26	241.19	257.33	U=450.00, 0.02*	t=-0.049, 0.96	t=-0.01, 0.98
	SE	31.65	42.53	46.87	29.27	32.57	48.64			
	n	31	17	14	42	29	13			
Final	Mean	246.01	324.52	167.50	130.11	163.30	96.61	t=-2.43, 0.01*	t=1.68, 0.10	t=2.71, <0.01*
	SE	40.49	53.30	60.95	36.68	40.81	60.95			
	n	30	17	13	42	29	13			

Appendix II



Appendix II

Report 1. FISK v2 protocol for *Australoheros facetus*

Risk Assessment area (RA): Portugal; threshold = 20.

Section:

- A) Biogeography
  - 1. Domestication / Cultivation
    - 1.01 (Cb). Is the species highly domesticated or widely cultivated for commercial, angling or ornamental purposes?
      Yes. Information on breeding and aquarium keeping of this species, can easily be found online, and it is known as the oldest Neotropical aquarium fish brought alive to Europe (Rícan & Kullander, 2006) (e.g. www.cichlidae.com/article.php?id=371).
      Certainty: *4*
    - 1.02 (Cb). Has the species established self-sustaining populations where introduced?
      Yes. There is scientific information available describing populations in introduced sites in Chile (Ruiz et al 1992) and Iberian Peninsula (Ribeiro et al 2007, Hermoso et al., 2011).
      Certainty: *4*
    - 1.03 (Cb). Does the species have invasive races/varieties/sub-species?
      Yes. This species come from a species complex, formerly named Cichlasoma facetum *(Rícan & Kullander, 2006). Although this term was not used, it is reasonable to assume this species as "invasive" from the results on impacts of* C. facetum *on autochthone fauna described by Ruiz et al. (1992). In addition, exotic populations in introduced sites may originally be from different races/varieties/sub-species of this complex.*
      Certainty: *3*
  - 2. Climate and Distribution
    - 2.01 (Cb). What is the level of matching between the species' reproductive tolerances and the climate of the RA area?
      3. Data collected from National System of Hydrological Resources, from Portugal and from Brazil, and presented in a recent study on temperature tolerance (Baduy et al., 2017b) and reproductive behaviour (Baduy et al, 2017a) in the RA area, show a high overlap between both regions. In addition, an approximation was done based in work presented by Peel et al., 2007. Both regions are classified as temperate with hot summers. The principal difference is that in the invaded area, the summer is dry, while in the native range there isn't a dry season (Peel et al., 2007).
      >http://snirh.apambiente.pt<
      >http://www.snirh.gov.br<
      Certainty: *4*
    - 2.02 (Cb). What is the quality of the climate match data?
      3. See Q 2.01
      Certainty: *4*
    - 2.03 (Cb). Does the species have self-sustaining populations in three or more (Köppen-Geiger) climate zones?
      Yes. There is online information about aquarium keeping in Germany and United States, despite no information about wild populations. Discarding these, wild populations outside its native range are found in Chile (Köppen-Geiger classification: Csb) (Ruiz et al 1992), Portugal and Spain

(Csa) (Ribeiro et al 2007; Hermoso et al., 2011), and in its native range (Cfa), in at least three climate zones.
Certainty: *3*

2.04 (Cb). Is the species native to, or has established self-sustaining populations in, regions with similar climates to the RA area?
Yes. Due to an approximation based in Peel et al. (2007), all regions are classified as temperate despite covering three different climate zones (see Q 2.03).
Certainty: *4*

2.05 (Cb). Does the species have a history of being introduced outside its natural range?
Yes. See Q 1.02.
Certainty: *4*

3. Invasive elsewhere

3.01 (Cb). Has the species established one or more self-sustaining populations beyond its native range?
Yes. See Q 1.02
Certainty: *4*

3.02 (N). In the species' introduced range, are there impacts to wild stocks of angling or commercial species?
No. There is no available information for that.
Certainty: *3*

3.03 (A). In the species' introduced range, are there impacts to aquaculture, aquarium or ornamental species?
No. See Q 3.02
Certainty: *3*

3.04 (E). In the species' introduced range, are there impacts to rivers, lakes or amenity values?
No. There is no available information for that.
Certainty: *3*

3.05 (Cb). Does the species have invasive congeners?
Yes. Although this term was not used, it is reasonable to assume this species as "invasive" from the results on impacts of C. facetum *on autochthone fauna (Ruiz et al. (1992). In addition, there are* Cichlasoma *spp described as invasive (e.g.* C. urophthalmus, *Adams & Wolfe 2007), although none* Australoheros *spp was yet described as invasive.*
Certainty: *3*

B) Biogeography/Ecology

4. Undesirable traits

4.01 (Cb). Is the species poisonous/venomous, or poses other risks to human health?
No. No such evidence was observed experimentally or in the literature.
Certainty: *4*

4.02 (Cb). Does the species out-compete with native species?
Yes. In the RA area, despite the paper about its feeding ecology had no conclusion about direct impacts (Ribeiro et al 2007), it is possible that A. facetus *predates on eggs or juveniles of other species. In addition, they can compete for territories, and its aggressive behaviour can displace other species (Baduy et al 2017).*
Certainty: *3*

4.03 (Cb). Is the species parasitic of other species?
No. No such evidence was observed experimentally or in the literature.
Certainty: *4*

4.04 (A). Is the species unpalatable to, or lacking, natural predators?

No. There are other non-native species in Southern Portugal observed eating juveniles of A. facetus *(e.g.* Micropterus salmoides*).*

Certainty: *3*

4.05 (Cb). Does the species prey on a native species previously subjected to low (or no) predation?

Yes. Although a paper about feeding ecology was inconclusive about direct impacts (Ribeiro et al 2007), it is possible that A. facetus *predates on eggs or juveniles of other species previously subjected to low (or no) predation.*

Certainty: *3*

4.06 (Cb). Does the species host, and/or is it a vector, for one or more recognised non-native infectious agents?

Don't know. There is no available information for that.

Certainty: *2*

4.07 (N). Does the species achieve a large ultimate body size (i.e. >15 cm total length) (more likely to be abandoned)?

Yes. The authors of the present study collected animals larger than 17cm of total length in Portugal.

Certainty: *4*

4.08 (E). Does the species have a wide salinity tolerance or is euryhaline at some stage of its life cycle?

Yes. During salinity tolerance trials, we observed normal behaviour and physiology within a range of 0-12ppt and despite some physiological cost at 15ppt, no mortality was observed at least for 30 days (Baduy et al 2016a; Baduy et al 2016b).

Certainty: *4*

4.09 (E). Is the species able to withstand being out of water for extended periods (e.g. minimum of one or more hours)?

Don't know. There is no available information for that.

Certainty: *2*

4.10 (E). Is the species tolerant of a range of water velocity conditions (e.g. versatile in habitat use)?

No. Cichlasoma facetum *subjected to velocities above 0.07 m/sec move in favour of the current and were passively dragged Gomez et al. (2003).*

Certainty: *4*

4.11 (E). Does feeding or other behaviours of the species reduce habitat quality for native species?

Yes. Its aggressiveness, territorial and reproductive behaviour can reduce quality habitat availability for other species through displacement (Baduy et al., 2017a).

Certainty: *4*

4.12 (Cb). Does the species require minimum population size to maintain a viable population?

No. Since this species have a high degree of parental care, can spawn several times during the reproductive season and has high fertility rates (Baduy et al., 2017a; Ruiz et al., 1992), it is reasonable to assume that it can maintain viable populations even when present in low numbers. In addition, in the RA area, during the rainy season or wet years, its population densities are usually low (Bernardo et al., 2003; Matono et al., 2012). However, it easily recovers to high abundances when the weather become dryer (See chapter 2 of the present thesis).

Certainty: *3*

5. Feeding guild
    - 5.01 (E). If the species is mainly herbivorous or piscivorous/carnivorous (e.g. amphibia), then is its foraging likely to have an adverse impact in the RA area?
      Not applicable.
    - 5.02 (Cb). If the species is an omnivore (or a generalist predator), then is its foraging likely to have an adverse impact in the RA area?
      Yes. See Q 4.05
      Certainty: *3*
    - 5.03 (Cb). If the species is mainly planktivorous or detritivorous or algivorous, then is its foraging likely to have an adverse impact in the RA area?
      Not applicable.
    - 5.04 (Cb). If the species is mainly benthivorous, then is its foraging likely to have an adverse impact in the RA area?
      Not applicable.
6. Reproduction
    - 6.01 (Cb). Does the species exhibit parental care and/or is it known to reduce age-at-maturity in response to environment?
      Yes. This species has a striking bi-parental care (Baduy et al., 2017a). In addition, it was observed in captivity reproducing with ~5cm of standard length (with less than 1 year of age).
      Certainty: *4*
    - 6.02 (Cb). Does the species produce viable gametes?
      Yes. There is information about several self-populations in different areas, outside or within its native range (e.g. Ribeiro et al., 2007; Alexandre et al., 2012).
      Certainty: *4*
    - 6.03 (A). Is the species likely to hybridize with native species (or use males of native species to activate eggs) in the RA area?
      No. There are no records of congeners of this species or closely related species in the RA area that could hybridize with A. facetus.
      Certainty: 4
    - 6.04 (Cb). Is the species hermaphroditic?
      No. Although we observed a homosocial pair in experimental conditions (Baduy et al 2017a), we have no evidence of this possibility.
      Certainty: *3*
    - 6.05 (Cb). Is the species dependent on the presence of another species (or specific habitat features) to complete its life cycle?
      Yes. This species has adhesive eggs that are attached preferable to stones or another hard substrate. This seems to be the only habitat feature necessary to complete its life cycle.
      Certainty: *2*
    - 6.06 (A). Is the species highly fecund (>10,000 eggs/kg), iteropatric or has an extended spawning season relative to native species?
      Yes. Ruiz et al. (1992) counted 900 to 1034 eggs per female. Adding to this observation that the mean weight of reproductive fish observed in captivity was ~77g (Baduy et al., 2017a), it can be suggested a proportion of ~12000 eggs/kg.
      Certainty: *4*
    - 6.07 (Cb). What is the species' known minimum generation time (in years)?
      One year. We observed small fish (~ 5cm of TL) ~12 months old that hatched in captivity and followed throughout their life, breeding with viable eggs/offspring. There is no information available about wild populations.
      Certainty: *3*

7. Dispersal mechanisms
    - 7.01 (A). Are life stages likely to be dispersed unintentionally?
      Yes. It is likely that the juveniles could be dragged downstream (Gasith & Resh 1999; Baduy et al 2017b; Baduy et al 2016a; Baduy et al 2016b) and survive in brackish water since this species has a great environmental tolerance and flash floods are common in the RA area.
      Certainty: *3*
    - 7.02 (Cb). Are life stages likely to be dispersed intentionally by humans (and suitable habitats abundant nearby)?
      Yes. As an ornamental species, however very aggressive, is likely that people dispose unwanted individuals in rivers. Anecdotal reports of such behaviours are common.
      Certainty: *3*
    - 7.03 (A). Are life stages likely to be dispersed as a contaminant of commodities?
      No. There is no such evidence.
      Certainty: *3*
    - 7.04 (Cb). Does natural dispersal occur as a function of egg dispersal?
      No. A. facetus *lays adhesive eggs on the substrate and provides bi-parental care to the eggs and offspring (Ruiz et al 1992; Baduy et al 2017a).*
      Certainty: *4*
    - 7.05 (E). Does natural dispersal occur as a function of dispersal of larvae (along linear and/or 'stepping stone' habitats)?
      Yes. This species presents substrate spawning and bi-parental care of the clutch (Ruiz et al 1992; Baduy et al 2017a). However, due to the occurrence of winter floods in the RA area (Gasith & Resh, 1999), it is possible that the larvae are dragged downstream reaching new habitats. Adults of A. facetus *were no able to maintain its position in the water column in flow faster than 0.07m/sec (Gomez et al., 2003).*
      Certainty: *3*
    - 7.06 (E). Are juveniles or adults of the species known to migrate (spawning, smolting, feeding)?
      Yes. They are highly territorial and it is possible that breeding pairs should migrate, at a small scale, in order to find adequate sites for spawning (Baduy et al., 2017a).
      Certainty: *3*
    - 7.07 (Cb). Are eggs of the species known to be dispersed by other animals (externally)?
      No. There is no available information for that.
      Certainty: *3*
    - 7.08 (Cb). Is dispersal of the species density dependent?
      Yes. They are highly territorial and it is possible that breeding pairs should migrate, at a small scale, in order to find adequate sites for spawning (Baduy et al., 2017a).
      Certainty: *2*
8. Persistence attributes
    - 8.01 (Cb). Are any life stages likely to survive out of water transport?
      Don't know. There is no available information for that.
      Certainty: *2*
    - 8.02 (Cb). Does the species tolerate a wide range of water quality conditions, especially oxygen depletion and temperature extremes?
      Yes. This species has critical thermal maxima ranging from 36.5ºC to 39.1ºC and critical thermal minimum from 4.5ºC to 5.8ºC. The minimum value of dissolved oxygen during CTMax and Min trials was 4.6mg/L

(Baduy et al 2017b), and with high concentration of endosulfan (LC50=13.6ug/L; Crupkin et al., 2013).

Certainty: *4*

8.03 (A). Is the species readily susceptible to piscicides at the doses legally permitted for use in the risk assessment area?

Yes. Although, there is no available information for that.

Certainty: *2*

8.04 (A). Does the species tolerate or benefit from environmental disturbance?

Yes. This species has a wide environmental tolerance (Baduy et al 2017b), therefore it is reasonable to assume that it is tolerant to environmental disturbance. Furthermore, the RA area is known to have severe summer droughts (Gasith & Resh 1999) and this species was described in higher densities compared to native species in those conditions (Godinho et al., 1997).

Certainty: *3*

8.05 (Cb). Are there effective natural enemies of the species present in the risk assessment area?

Yes. It is unlikely that natural enemies exist among native species. However, among other exotic species, as M. salmoides*, it is possible that the eggs and juveniles could be predated.*

Certainty: *3*

Report 2. AS-ISK protocol for *Australoheros facetus*.

Sector codes (in parentheses): C = Commercial; E = Environmental; N = Nuisance. Responses are: Y = Yes; N = No; Nil = no change. Backward-compatibility (*BC*) of questions relative to FISK (v2) with corresponding *Q#* in parentheses: D = Different; N = New; NI = Near-identical; Sim = Similar. Certainty values range from 1 = Very uncertain to 4 = Very certain. RA: Risk Assessment area.

Risk Screening context:
Reason: The currently classification of this species in Portugal is as a non-native species, however not invasive. New data from a PhD carried out by Flávia Baduy, suggests the inclusion of this species in the "invasive species" list.
Taxonomy: It was formerly called as *Cichlasoma facetum*, and after genus review by Rivan and Kullander (2006). it was included in a new genus, *Australoheros*. Because of the confusion and new descriptions of species from the formerly called '*Cichlasoma*' *facetum* group, it is possible that the species found in Portugal came from different lineages, subspecies, or even species, from South America.
Native range: Coastal drainages from Southern Brazil, Argentina and Uruguay.
Introduced range: Southern Portugal.

Section:

A) Biogeography

1. Domestication / Cultivation

    1.01 (C) *NI(1.01).* Has the taxon been the subject of domestication (or cultivation) for at least 20 generations?
    Yes. Information on breeding and aquarium keeping of this species, can easily be found online, and it is known as the oldest Neotropical aquarium fish brought alive to Europe (Rícan & Kullander, 2006) (e.g. www.cichlidae.com/article.php?id=371).
    Certainty: *4*

    1.02 (C) *(D).* Is the taxon harvested in the wild and likely to be sold or used in its live form?
    Yes. Information on how to get some individuals in lakes and ponds in Lisbon or from some sites in Alentejo is available online. However, there is no information about official selling. (e.g. http://www.ciclideos.com/forum/ "Peixes que suportam o inverno em Portugal")
    Certainty: *2*

    1.03 (N) *NI(1.03).* Does the taxon have invasive races, varieties, sub-taxa or congeners?
    Yes. This species comes from a species complex, formerly named Cichlasoma facetum (Rícan & Kullander, 2006). Although this term was not used, it is reasonable to assume this species as "invasive" from the results on impacts of C. facetum on autochthone fauna described by Ruiz et al. (1992). In addition, exotic populations in introduced sites may originally be from different races/varieties/sub-species of this complex. Congener Cichlasoma urophthalmus, is known to have large impacts mainly in Florida (e.g. Porter-Whitaker et al., 2012).
    Certainty: *3*

2. Climate, distribution and introduction risk

    2.01 (E) *Sim(2.01).* How similar are the climatic conditions of the RA area and the taxon's native range?
    3. Data collected from National System of Hydrological Resources, from Portugal and from Brazil, and presented in recent studies in the RA area

(Baduy et al., 2017a, b), show a high overlap between both regions. In addition, an approximation was done based in work presented by Peel et al., 2007. Both regions are classified as temperate with hot summers. The principal difference is that in the invaded area, the summer is dry, while in the native range there isn't a dry season (Peel et al., 2007). >http://snirh.apambiente.pt < > http://www.snirh.gov.br/<
Certainty: *4*

2.02 (E) *NI(2.02).* What is the quality of the climate matching data?
3. See Q 2.01
Certainty: *4*

2.03 (CN) *D.* Is the taxon already present outside of captivity in the RA area?
Yes. Several studies describe this species in the wild ichthyofauna, mainly at Guadiana, Sado and Arade basins (e.g. Alexandre et al., 2012; Ribeiro e Collares-Pereira, 2010; Pires et al., 2010).
Certainty: *4*

2.04 (CN) *D.* How many potential vectors could the taxon use to enter in the RA area?
1. To enter in the RA area this species probably used just one vector: human (or as an ornamental species or for mosquito control) (Ribeiro et al., 2008)
Certainty: *3*

2.05 (CN) *D.* Is the taxon currently found in close proximity to, and likely to enter into, the RA area in the near future (e.g. unintentional and intentional introductions)?
Not applicable. The taxon is already present in the RA area.
Certainty: *4*

3. Invasive elsewhere

3.01 (N) *NI(3.01).* Has the taxon become naturalised (established viable populations) outside its native range?
Yes. There are records of this species in the wild in Chile at least since 1959 (Ruiz et al., 1992); in Portugal since 1940 (Ribeiro et al., 2007) and in Spain at least since 1980 (Doadrio, 2002).
Certainty: *4*

3.02 (C) *NI(3.02).* In the taxon's introduced range, are there known adverse impacts to wild stocks or commercial taxa?
No. There is no available information for that.
Certainty: *2*

3.03 (C) *NI(3.03).* In the taxon's introduced range, are there known adverse impacts to aquaculture?
No. There is no available information for that.
Certainty: *2*

3.04 (E) *Sim(3.04).* In the taxon's introduced range, are there known adverse impacts to ecosystem services?
No. There is no available information for that.
Certainty: *3*

3.05 (C) *D.* In the taxon's introduced range, are there known adverse socio-economic impacts?
No. There is no available information for that.
Certainty: *3*

4. Undesirable traits

4.01 (N) *NI(4.01).* Is it likely that the taxon will be poisonous or pose other risks to human health?
No. No such evidence was observed experimentally or in the literature.
Certainty: *4*

4.02 (N) *Sim(4.02).* Is it likely that the taxon will smother one or more native taxa (that are not threatened or protected)?

Yes. In the RA area, despite the paper about its feeding ecology had no conclusion about direct impacts (Ribeiro et al 2007), it is possible that A. facetus *predates on eggs or juveniles of other species previously subjected to low (or no) predation. In addition, they can compete for territories, and its aggressive behaviour can displace other species (Baduy et al 2017).*

Certainty: *3*

4.03 (N) *Sim(4.03).* Are there threatened or protected taxa that the non-native taxon would parasitize in the RA area? (obs.: in the guidance, it is used the sentence: “would become a *predator* or parasite (…)”)

Yes. As an omnivore species (Ribeiro et al., 2007; Ruiz et al., 1992), it is possible that A. facetus *predates on eggs or juveniles from other species as the threatened chub* Squalius pyrenaicus *and saramugo* Anaecypris hispanica.

Certainty: *3*

4.04 (N) *D.* Is the taxon adaptable in terms of climatic and other environmental conditions, thus enhancing its potential persistence if it has invaded or could invade the RA area?

Yes. Its wide environmental tolerance, especially to temperature but also to salinity changes (Baduy et al., 2017b; Baduy et al., 2016) could be one of the reasons for why this species adapted well to the differences between the dry summers of the Iberian Peninsula when compared to its native range (Peel et al., 2007).

Certainty: *3*

4.05 (E) *D.* Is the taxon likely to disrupt food-web structure/function in aquatic ecosystems it has or is likely to invade in the RA area?

No. From the data about its feeding ecology, as a generalist opportunistic fish, it is unlikely to occur a disruption in the food-web (Ribeiro et al., 2007; Ruiz et al., 1992).

Certainty: *3*

4.06 (E) *D.* Is the taxon likely to exert adverse impacts on ecosystem services in the RA area?

No. There is no available information for that.

Certainty: *3*

4.07 (N) *D.* Is it likely that the taxon will host, and/or act as a vector for, recognised pests and infectious agents that are endemic in the RA area?

No. There is no available information for that.

Certainty: *2*

4.08 (N) *D.* Is it likely that the taxon will host, and/or act as a vector for, recognised pests and infectious agents that are absent from (novel to) the RA area?

No. There is no available information for that.

Certainty: *3*

4.09 (N) *Sim (4.07).* Is it likely that the taxon will achieve a body size that will make it more likely to be released from captivity?

Yes. The authors of the present study collected animals larger than 17cm of total length in Portugal.

Certainty: *4*

4.10 (N) *NI(4.10).* Is the taxon capable of sustaining itself in a range of water velocity conditions (e.g. versatile in habitat use)?

No. Cichlasoma facetum *subjected to velocities above 0.07 m/sec move in favour of the current and were passively dragged Gomez et al. (2003).*

Certainty: *4*

4.11 (E) *Sim(4.11).* Is it likely that the taxon's mode of existence (e.g. excretion of by-products) or behaviours (e.g. feeding) will reduce habitat quality for native taxa?

Yes. Its aggressiveness, territorial and reproductive behaviour can reduce quality habitat availability for other species through displacement (Baduy et al., 2017a).

Certainty: *3*

4.12 (N) *NI(4.12).* Is the taxon likely to maintain a viable population even when present in low densities (or persisting in adverse conditions by way of a dormant form)?

Yes. Since this species have a high degree of parental care, can spawn several times during the reproductive season and has high fertility rates (Baduy et al., 2017a; Ruiz et al., 1992), it is reasonable to assume that it can maintain viable populations even when present in low numbers. In addition, in the RA area, during the rainy season or wet years, its population densities are usually low (Bernardo et al., 2003; Matono et al., 2012). However, it easily recovers to high abundances when the weather become dryer (See chapter 2 of the present thesis).

Certainty: *3*

5. Resource exploitation

5.01(E) *D.* Is the taxon likely to consume threatened or protected native taxa in RA area?

Yes. As an omnivore species (Ribeiro et al., 2007; Ruiz et al., 1992), it is possible that A. facetus predates on eggs or juveniles from other species as the threatened chub Squalius pyrenaicus *and the saramugo* Anaecypris hispanica.

Certainty: *3*

5.02 (N) *D.* Is the taxon likely to sequester food resources (including nutrients) to the detriment of native taxa in the RA area?

Yes. There is some overlap with native species (Ribeiro et al., 2007), although direct impacts are inconclusive. However, as Kodde and colleagues (2016) showed, the foraging success of the endangered native Southern Iberian chub Squalius pyrenaicus *is generally lower than that of the* A. facetus *when both species are present, especially when the temperature increases. It is therefore reasonable to assume that, in the presence of* A. facetus, S. pyrenaicus *could have a decrease in quantity and quality of its diet.*

Certainty: *3*

6. Reproduction

6.01 (N) *NI(6.01).* Is the taxon likely to exhibit parental care and/or to reduce age-at-maturity in response to environmental conditions?

Yes. This species has a striking bi-parental care (Baduy et al., 2017a). In addition, it was observed in captivity reproducing with ~5cm of standard length (with less than 1 year of age).

Certainty: *4*

6.02 (N) *NI(6.02).* Is the taxon likely to produce viable gametes or propagules (in the RA area)?

Yes. There is information about several self-populations in different areas, outside or within its native range (e.g. Ribeiro et al., 2007; Alexandre et al., 2012).

Certainty: *4*

6.03(N) *NI(6.03).* Is the taxon likely to hybridize naturally with native taxa?

No. There are no records of congeners of this species or closely related species in the RA area that could hybridize with A. facetus.

Certainty: *4*

6.04 (N) *NI(6.04).* Is the taxon likely to be hermaphroditic or to display asexual reproduction?
No. Although we observed homosocial pair during experiments (Baduy, 2017a), we have no evidence of this possibility.
Certainty: *3*

6.05 (N) *NI(6.05).* Is the taxon dependent on the presence of another taxon (or specific habitat features) to complete its life cycle?
Yes. This species has adhesive eggs that are attached preferable to stones or another hard substrate. This seems to be the only habitat feature necessary to complete its life cycle.
Certainty: *2*

6.06 (N) *Sim(6.06).* Is the taxon known (or likely) to produce a large number of propagules or offspring within a short time span (e.g. <1 year)?
Yes. Ruiz et al. (1992) counted 900 to 1034 eggs per female. This species can spawn at 15-day intervals during the reproductive season (Baduy et al 2017a).
Certainty: *4*

6.07 (N) *Sim(6.07).* How many time units (days, months, years) does the taxon require to reach the age-at-first-reproduction? [In the Justification field, indicate the relevant time unit being used.]
One year. We observed small fish (~ 5cm of TL) ~12 months old that hatched in captivity and followed throughout their life, breeding with viable eggs/offspring. There is no information available about wild populations.
Certainty: *3*

7. Dispersal mechanisms

7.01 (CN) *D.* How many potential internal pathways could the taxon use to disperse within the RA area (with suitable habitats nearby)?
>1. Winter floods are common in the RA area (Gasith & Resh, 1999), thus it is possible that the fish could be dragged downstream reaching new habitats since at water flows above 0.7m/sec A. facetus cannot maintain its position in the water column (Gomez et al., 2003). A second pathway is through aquarium trade. There is information available online about how to get individuals in lakes and ponds in urban or wild sites. However, there is no information about official selling. (http://www.ciclideos.com/forum/ "Peixes que suportam o inverno em Portugal")
Certainty: *3*

7.02 (EN) *D.* Will any of these pathways bring the taxon in close proximity to one or more protected areas (e.g. MCZ, MPA, SSSI)?
Yes. This species is already found at Guadiana Valley Natural Park.
Certainty: *4*

7.03 (N) *D.* Does the taxon have a means of actively attaching itself to hard substrata (e.g. ship hulls, pilings, buoys) such that it enhances the likelihood of dispersal?
No. There is no such evidence.
Certainty: *4*

7.04 (N) *NI(7.04).* Is natural dispersal of the taxon likely to occur as eggs (for animals) or as propagules (for plants: seeds, spores) in the RA area?
No. A. facetus *lays adhesive eggs on the substrate and provides bi-parental care to the eggs and offspring (Ruiz et al 1992; Baduy et al 2017a).*
Certainty: *4*

7.05 (N) *NI(7.05).* Is natural dispersal of the taxon likely to occur as larvae/juveniles (for animals) or as fragments/seedlings (for plants) in the RA area?

Yes. This species presents substrate spawning and bi-parental care of the clutch (Ruiz et al 1992; Baduy et al 2017a). However, due to the occurrence of winter floods in the RA area (Gasith & Resh, 1999), it is possible that the larvae are dragged downstream reaching new habitats. Adults of A. facetus *were no able to maintain its position in the water column in flow faster than 0.07m/sec (Gomez et al., 2003).*

Certainty: *3*

7.06 (N) *Sim(7.06).* Are older life stages of the taxon likely to migrate in the RA area for reproduction?

Yes. They are highly territorial and it is possible that breeding pairs should migrate, at a small scale, in order to find adequate sites for spawning (Baduy et al., 2017a).

Certainty: *3*

7.07 (N) *NI(7.07).* Are propagules or eggs of the taxon likely to be dispersed in the RA area by other animals?

No. There is no available information for that.

Certainty: *3*

7.08 (CN) *D.* Is dispersal of the taxon along any of the pathways mentioned in the previous seven questions (7.01–7.07; i.e. both unintentional or intentional) likely to be rapid?

Yes. The flash floods in the RA area can occur more than once a year.

Certainty: *3*

7.09 (N) *NI(7.08).* Is dispersal of the taxon density dependent?

Yes. They are highly territorial and it is possible that breeding pairs should migrate, at a small scale, in order to find adequate sites for spawning (Baduy et al., 2017a).

Certainty: *2*

8. Tolerance attributes

8.01 (N) *NI(4.09).* Is the taxon able to withstand being out of water for extended periods (e.g. minimum of one or more hours) at some stage of its life cycle?

No. There is no available information for that.

Certainty: *2*

8.02 (N) *NI(8.02).* Is the taxon tolerant of a wide range of water quality conditions relevant to that taxon? [In the Justification field, indicate the relevant water quality variable(s) being considered.]

Yes. This species has critical thermal maxima ranging from 36.5ºC to 39.1ºC and critical thermal minima from 4.5ºC to 5.8ºC. The minimum value of dissolved oxygen during CTMax and Min trials was 4.6mg/L (Baduy et al 2017b), and with high concentration of endosulfan (LC50=13.6ug/L; Crupkin et al., 2013).

Certainty: *4*

8.03 (N) *NI(8.03).* Can the taxon be controlled or eradicated in the wild with chemical, biological, or other agents/means?

Yes. Although, there is no available information for that.

Certainty: *2*

8.04 (N) *NI(8.04).* Is the taxon likely to tolerate or benefit from environmental/human disturbance?

Yes. This species has a wide environmental tolerance (Baduy et al 2017b), therefore it is reasonable to assume that it is tolerant to environmental disturbance. Furthermore, the RA area is known to have severe summer droughts (Gasith & Resh 1999) and this species was described in higher

densities compared to native species in those conditions (Godinho et al., 1997).
Certainty: *3*

8.05 (N) *NI(4.08).* Is the taxon able to tolerate salinity levels that are higher or lower than those found in its usual environment?
Yes. During salinity tolerance trials, we observed normal behaviour and physiology within a range of 0-12ppt and despite some physiological cost at 15ppt, no mortality was observed at least for 30 days (Baduy et al 2016a; Baduy et al 2016b).
Certainty: *4*

8.06 (N) *NI(8.05).* Are there effective natural enemies (predators) of the taxon present in the RA area?
Yes. It is unlikely that natural enemies exist among native species. However, among other exotic species, as M. salmoides*, it is possible that the eggs and juveniles could be predated.*
Certainty: *3*

9. Climate Change

9.01 (N) *N.* Under the predicted future climatic conditions, are the risks of entry into the RA area posed by the taxon likely to increase, decrease or not change?
Nil. The entry of A. facetus *into the RA area is mediated by human action, e.g. aquarium trade. It will probably not be affected by the predicted future climatic conditions.*
Certainty: *3*

9.02 (N) *N.* Under the predicted future climatic conditions, are the risks of establishment posed by the taxon likely to increase, decrease or not change?
Positive. This species is adapted to warmer waters, although can withstand temperatures as well (critical thermal maxima ranging from 36.5ºC to 39.1ºC and critical thermal minima from 4.5ºC to 5.8ºC; Baduy et al., 2017b). It is possible that an increase in water temperature could increase its abundance during winter and allow establishment in new areas.
Certainty: *2*

9.03 (N) *N.* Under the predicted future climatic conditions, are the risks of dispersal within the RA area posed by the taxon likely to increase, decrease or not change?
Positive. The forecasted increase in strength and frequency of floods can spur its dispersal by creating new pathways and connections between water bodies.
Certainty: *3*

9.04 (E) *N.* Under the predicted future climatic conditions, what is the likely magnitude of future potential impacts on biodiversity and/or ecological integrity/status?
Positive. This species seems to dominate the ichthyofauna during the hot dry season (together Lepomis gibbosus *in some sites, pers. obs), The forecasted climate change according to The International Panel on Climate Change (IPCC) is more frequent and extended periods of drought and water scarcity as well more episodes of flash-floods (Bates et al., 2008), so it is possible that the impacts on the endangered native fauna of Southern Portugal will increase.*
Certainty: *3*

9.05 (E) *N*. Under the predicted future climatic conditions, what is the likely magnitude of future potential impacts on ecosystem structure and/or function?

Positive. Due the observed dominance of A. facetus *during the hot dry season, a decrease in biodiversity of the native ichthyofauna could be expected, affecting the food web and disrupting the ecosystem structure and function.*

Certainty: *2*

9.06 (C) *N*. Under the predicted future climatic conditions, what is the likely magnitude of future potential impacts on ecosystem services/socio-economic factors?

Positive. If A. facetus *benefits from future climate changes, due to its ability to colonize new habitats (high parental care, high fecundity, high environmental tolerance, opportunistic feeding; Baduy et al 2017a,b; Ruiz et al, 1992, Ribeiro et al., 2007), it is possible that this species dominates the ichthyofauna, leading to a homogenization of the fish fauna, which can decrease the interest of people in fishing or diving.*

Certainty: *2*