Tamara Priscila Faleiro Pereira Leite

DOES SALINIZATION OF FRESHWATER ALTER FISH BEHAVIOUR?

Dissertation in MsC in Ecology, supervised by Paulo Branco PhD and Professor Cristina Canhoto PhD,
presented to the Department of Life Sciences, Faculty of Science and Technology, University of Coimbra

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Universidade de Coimbra

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Faculty of Science and Technology
University of Coimbra

2018

“La natura è la fonte di tutta la vera conoscenza. Ha la sua logica, le sue leggi, non ha alcun effetto senza causa né invenzione senza necessità.”

Leonardo da Vinci

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Abstract

Freshwater ecosystems provide humankind with freshwater and food, and so, population tends to change landscapes around these valuable, yet very vulnerable, systems. The intensification of human activities surrounding rivers and streams is continuously threatening freshwater biodiversity and water quality, and further consequences include anthropogenic salinization. This major threat is mainly caused by irrigation, road salt addition and industrial discharges, and is known to have, in some cases, lethal effects on intolerant aquatic organisms. High salinity concentrations impair fish osmoregulation, growth, reproduction, overall fitness, and some studies indicate that it might also affect their behavioural responses.

In order to determine the effects of increasing salinity on freshwater fish boldness behaviour and cerebral lateralization we performed an experiment in mesocosms where Iberian barbels were exposed to three levels of salinity (0.9, 9 and 19 mS/cm) . Boldness was determined by the time that each individual took to emerge completely from a chamber. Lateralization was determined by performing a standard detour test where fish swam across a narrow channel; at the end of the channel were faced with a barrier being obliged to avoid the obstacle either turning left or right. Data analysis was interpreted considering the population trend.

The acute punctual stress caused by the tested salinity was not significant to significantly alter barbels' behaviour, but tendencies in high salinity levels were noticed, with more individual variation on the shy-bold gradient and with the population having a more bimodal distribution in terms of lateralization.

Broader investigations are crucial to better understand larger scale impacts of secondary salinization, and possible interactions between salinity and other environmental factors threatening rivers and streams.

Keywords: salinity, behaviour, boldness, cerebral lateralization, stressors.

Resumo

Os ecossistemas de água doce são fontes de água potável e alimentação para o ser humano, e por isso, a população tende a alterar paisagens ao redor destes valiosos, mas muito vulneráveis sistemas. A intensificação das atividades humanas adjacentes a rios está continuamente a ameaçar a qualidade da água e a biodiversidade destes sistemas aquáticos, e além disso, promove a salinização antropogénica. As principais causas deste grande problema são a irrigação, os sais de estrada e as descargas industriais, e, em alguns casos, pode provocar efeitos letais em vários organismos intolerantes. Concentrações elevadas de salinidade prejudicam a osmorregulação, o desenvolvimento, e o *fitness* dos peixes, e alguns estudos indicam que podem também afetar as suas respostas comportamentais.

Para determinar os efeitos do aumento de salinidade no carácter exploratório e na lateralização cerebral dos peixes, foram conduzidos ensaios comportamentais em mesocosmos com barbos-do-norte, que foram previamente expostos a três níveis de salinidade (0.9, 9 e 19 mS/cm). O caráter exploratório foi determinado através do registo de tempo que cada indivíduo demorou a emergir completamente do interior de uma caixa. A lateralização foi determinada através de um simples teste de desvio onde cada peixe percorreu um corredor estreito, e onde no final encontrou uma barreira que os faria decidir evitar o obstáculo pela direita ou pela esquerda. A análise de dados foi interpretada tendo em conta a tendência populacional. O stresse pontual e agudo provocado pelas salinidades testadas foi insuficiente para causar variação significativa no comportamento dos barbos, no entanto foram notadas ligeiras tendências nos maiores níveis de salinidade, com maior variação individual no gradiente de caráter exploratório, e com maior distribuição bimodal da população em termos de lateralização.

Investigações complementares são cruciais para melhor entender os impactos da salinização secundária em maior escala, e possíveis interações entre a salinidade e outros fatores ambientais que também ameaçam os cursos de água.

Palavras-chave: salinidade, comportamento, comportamento exploratório, lateralização cerebral, fatores de stresse.

Chapter I. General Introduction

Freshwater ecosystems are an essential good and magnets for human settlement, as the population develops, and we alter natural landscapes around running waters (Green et al., 2015). Freshwater represents only 0.01 % of the world's water and 0.8 % of the planet surface, and yet, nearly the entire world population is serviced by freshwater sources, in which we depend on for domestic, industrial and agricultural uses, energy, transportation, and leisure activities (Green et al., 2015; Malmqvist and Rundle, 2002). Rivers, lakes and wetlands have high biological value, because of their rich and unique biota, high diversity of fish and other vertebrates, and even greater diversity of invertebrates and algae (Janse et al., 2015; Malmqvist and Rundle, 2002).

These systems host over 10 000 fish species, which correspond to 40 % of the global fish diversity (Lundberg et al., 2000), and deliver important ecosystem services like freshwater availability, water purification, climate regulation, food and recreational values (Ely, 2005). Being some of the most endangered systems on the planet, it is crucial to recognise their value and their vulnerability (Dudgeon et al., 2006). Declines in biodiversity are far greater in freshwater than in terrestrial and marine ecosystems (Sala et al., 2000), and one of the reasons for these damages are human activities around the world. Biodiversity is put at risk and the systems are compromised by population growth, economic development, and poor environmental management (Green et al., 2015). The main problems are land-use, hydrological disturbances, pollution, climate change, overexploitation and exotic species (Dudgeon et al., 2006; Ely, 2005; Sala et al., 2000). There is a small number of water bodies that have not been altered in any way by humans, and impacting them will translate into vital ecological and potential financial losses that may well be irreversible (Dudgeon et al., 2006), since they shelter freshwater fish species (Cohen, 1970), which have great economic and nutritional value, regulate food web dynamics and act as links between ecosystems.

Over 70% of the 228 endemic fish species of the Mediterranean region are threatened with extinction or are already extinct (Smith and Darwall, 2006), and on a larger scale, according to a recent study (Collen et al., 2014), the global population of freshwater fish has declined by 76 % since the 1970s. In Spain, Portugal and Morocco, more than 20 % of the endemic species are declared Vulnerable (IUCN, 2008) and fish species are affected at least by 3 of the 6 main threats to their habitat -pollution, water extraction, agriculture, reservoirs, invasive species, and overfishing (Hermoso and Clavero, 2011). However, despite this distressing numbers, only 4.3 % of Mediterranean region freshwaters are protected.

Salinization of freshwater ecosystems

The intensification of human activities on surrounding freshwater ecosystems threatens water quality and biodiversity of these systems (Malmqvist and Rundle, 2002; Santos et al., 2015; Valle et al., 2015). Stream and land salinization constitute great environmental hazards all over the world (Bari and Smettem, 2006; Ghassemi et al., 1995). Salinity is defined as the total concentration of dissolved inorganic ions in water bodies or soil, being a component of all natural waters. Increasing the levels of those ions above standard levels is denominated salinization (Williams and Sherwood, 1994). Salinization modifies the chemical properties of soil and water by increasing ionic concentrations. This process is designated as “primary salinization” when originated from natural sources at rates independent from human activities, or it can be “secondary” when caused by anthropogenic influences (Cañedo-Argüelles et al., 2013). In the absence of human disturbances, natural salinization results from three main sources: weathering of the catchment, sea spray in coastal areas, and salts dissolved in rainwater as a result of evaporation of seawater (Williams, 2001). This is especially concerning in arid and semi-arid regions with seasonally hot dry climates since ions can be concentrated by evaporation and transpiration, whatever their source may be (Herbert et al., 2015).

Secondary salinization is mostly promoted by irrigation in agriculture (Clasen et al., 2018; Segurado et al., 2018). Because crops are not able to absorb the totality of the salts from the irrigation water and soil, salt content builds up in soil and water, intensifying salinization (Lerotholi et al., 2004). Subsequently, these salts are leached out through runoff and find their way to rivers and streams. Other important causes of water salinization are the use of road salts as de-icing agents, discharges from industrial activities or mining, activities that occur worldwide (Cañedo-Argüelles et al., 2013; Lerotholi et al., 2004; Williams, 2001). Therefore, the effects of anthropogenic salinization on aquatic systems are of great concern, as they will continuously be more intense and spread due to human water demand, and reduced rainfall promoted or amplified by anthropogenic climate changes (Vörösmarty et al., 2010). Precipitation has decreased 20 % in the past century, and will continue to decrease by 60 % in areas of the Iberian Peninsula. Additionally, as a consequence of temperature increase of approximately 5 °C, runoff in the Mediterranean basin is expected to drop as far as 40 % (Hermoso and Clavero, 2011). Forecasts point to an increase of evaporation in result of water temperature continuous increase and reduction of precipitation (Sereda et al., 2011). These human-caused climate changes will reduce catchment runoff (Nielsen and Brock, 2009), and river discharges (Alcocer and Sarma, 2002), which will result in a lower dilution capacity, therefore intensifying salt content of streams and rivers (Cañedo-Argüelles et al., 2013), putting at risk the ecosystem goods and services (Clunie et al., 2002; Ghassemi et al., 1995; Hart et al., 1991).

Further repercussions may also include water acidification (Lofgren, 2001) and translocation of toxic metals (Lewis, 1999; Norrström and Bergstedt, 2001), and several negative impacts on several organisms at different levels of the ecosystem organization, from initially increasing stress to eventually causing mortality (Eaton et al., 1999; James et al., 2003), feasibly affecting community structure. At an individual level, it affects osmoregulation, which has high metabolic costs, affecting the general fitness of organisms (Hart et al., 1991; James et al., 2003; Piscart et al., 2006). Animals regulate their internal osmotic concentrations according to their environment, but those that are poor regulators (stenohaline regulators) hardly adapt to wider than standard ranges of salinity, opposite to euryhaline regulators (Nielsen et al., 2003). Higher salt levels in water may result in lethal and sub-lethal consequences to aquatic species (Gillis, 2011; Hart et al., 1991; Kaushal et al., 2005). It disturbs growth, species distribution and abundance (Langhans et al., 2009; Rios-López, 2008), and their overall viability, thus being a selective pressure (Nielsen et al., 2003). Salinity contributes to metal accumulation in fish, as well when interacting with other factors such as pH, hardness and temperature, which may be harmful for human consumption of said fish (Canli and Atli, 2003). At a population/community/ecosystem level it affects the structure and functioning of the aquatic system (Kaushal et al., 2005) through influences on species relationships, such as competition or predation (Braukmann and Böhme, 2011; Judd et al., 2005; McEvoy and Goonan, 2003; Millán et al., 2011; Richburg et al., 2001). This stressor, when enough to be lethal, can change the community by adding salt-tolerant or removing salt-sensitive taxa that provide food or act as predators (Boulton and Brock, 1999; Nielsen et al., 2003). As species approach their tolerance thresholds, the effects of increased salinity become more severe across trophic chains, when intolerant species may disappear from the system, whereas tolerant species would be in competitive advantage (Hart et al., 1991; James et al., 2003). In addition, even at relatively low concentrations, there could also be several other impacts to the ecosystems, such as on: decomposition (Schafer et al., 2012), nitrogen fixation and nitrification of planktonic organisms (Groffman et al., 1995; Howarth et al., 1988), freshwater cyanobacteria activity (Baldwin et al., 2006; Elshahed et al., 2004; Hart et al., 1991), abundance and richness in algae (Herbst and Blinn, 1998), inhibition of sporulation in fungi (Byrne and Jones, 1975), diversity, richness and survivorship of invertebrates (Hart et al., 1991), and trophic complexity of food webs (Kaushal et al., 2005). These consequences can be perceived across all scales, from microhabitats in the water, individual streams and rivers, to whole landscapes (Naiman et al., 1992).

Impacts of salinization on freshwater fish

High salinity concentrations continue to take a toll on freshwater fish, as they drive loss of diversity or even extinction (e.g. carps, Koç 2008). Most freshwater fish are able to withstand salinities up to 7–13 g/L, which is their internal salt concentration, however, when external salinity exceeds this range, osmoregulatory mechanisms collapse, threatening fish's health (Bacher and Garnham, 1992; James et al., 2003). Salinity can alter regular metabolic rate of fish (Pistole et al., 2008), and osmotic stress will impact fish activity (Job, 1969), as they need to spend energy in ion regulation and osmoregulation (Cañedo-Argüelles et al., 2015). This assumption is logical when one considers that stress responses are energetically demanding while attempting to reach an equilibrium with the external environment, thus limiting the organism's available energy to other functions (Baitelman, 2013), such as growth (Iwama, 1998) and oxygen consumption (Febry and Lutz, 1987). In this case, fish ultimately have to increase foraging in order to maintain the energy level. Then, the direct impact of external salinity on fish development is an alarming issue (Boeuf and Payan, 2001).

As for disturbances on behaviour, some studies have shown that fish experiencing potentially harmful conditions, specifically sub-lethal levels of salinity, could display changes on behavioural responses, for example, on activity level, predator evasion and responses to stimuli (e.g. fathead minnows, Hoover et al., 2013). Overall, salinity impairs the general fish sensitivity to stress, and therefore, behaviour (Gül et al., 2004; Pottinger et al., 2002; Wenderlaar Bonga, 1997; Whiterod and Walker, 2006).

Effects of salinity on fish behaviour

Animal behaviour consists of responses to stimuli arising either from within the animal body or from the environment (Scott, 1958). It includes cognitive processes such as learning and decision making, that determine reproduction, foraging, interaction with predators and conspecifics (Shettleworth, 2001). Hence, when relating to the environment, it can be reduced to a trade-off decision between exploring, fleeing or freezing i.e. between foraging success or risk of predation (Gilliam and Fraser, 1987; Harcourt et al., 2009b; Riesch et al., 2009). Fish have to decide on the more productive foraging tactic, balancing the energy return and risk of predation associated. They can either prioritize low risk-low return tactics, to be safer, or high risk-high return tactics, to maximize fitness (Magnhagen and Staffan, 2005; Wilson et al., 1993).

Ultimately, behaviour can be influenced by this increasing stressor, and higher trophic levels, such as aquatic birds, mammals and reptiles, could suffer repercussions, when the community structure is affected by, for example, the possible extinction of intolerant species (Dudgeon et al., 2006; Fonseca et al., 2016).

Boldness

Personality includes the shy-bold continuum, identified in several species of: mammals (Lowe & Bradshaw 2001; Svartberg & Forkman 2002; Armitage & Van Vuren 2003), birds (Dingemanse et al., 2003), cephalopods (Mather and Anderson, 1993), and fish (Sneddon, 2003; Westerberg et al., 2004; Wilson et al., 1993). Individuals vary along this behavioural gradient from extreme shyness to extreme boldness (Magnhagen and Bunnefeld, 2009; Wilson et al., 1993), which corresponds to predisposition to take risks in unfamiliar environments (Coleman and Wilson, 1998; Riesch et al., 2009). Shyer individuals when in novel contexts retreat or become vigilant, to be safe. Bolder individuals, on the other hand, remain calm or become more curious and explore the surroundings, which puts them at higher risk. Individual variability of reactions can influence species fitness, anti-predator responses, social interactions and preferences (Harcourt et al., 2009a, 2009b; Réale et al., 2000). Boldness is associated with several behaviours, such as: predator inspection (Fraser et al., 2001), dispersion (Magnhagen and Staffan, 2005), and activity (Budaev, 1997a). Bold individuals are more likely to explore different areas, and forage more efficiently, even when facing predation threat (pumpkinseed sunfish, Coleman and Wilson, 1998; minnows, Murphy and Pitcher, 1997; Wilson et al., 1993). In some cases, due to discrepancy in predation exposure, prey animals often vary in their response to predators (Kamil and Balda, 1990; Magurran, 1990; Schjolden et al., 2005), thus affecting their abundance (Brown et al., 2005; Budaev, 1997a; Riesch et al., 2009). Studies reveal that smaller fish are more predated, and therefore, use a safer tactic and emerge later from shelter (i.e. shyer). In contrast, juveniles have faster metabolic rates and fewer body fat reserves than adults, thus are bound to risk their safety and start foraging (metabolic hypothesis, Krause et al., 1998), to maintain energy, increase food intake, and consequently growth rate (Brown and Braithwaite, 2004). Furthermore, boldness is correlated with leadership in schooling species (Harcourt et al., 2009a; Sih et al., 2004; Ward et al., 2004), then fish prefer to join groups of bolder individuals, because they are more likely to successfully find resources (Dyer et al., 2009; Magnhagen and Bunnefeld, 2009).

In conclusion, the shy-bold continuum is considered to be a fundamental gradient of behavioural variation (Frost et al., 2007), and disturbances, such as increasing salinity, on freshwater systems and fish populations could result in a cascade of behavioural and ecological consequences.

Cerebral Lateralization

Cerebral lateralization is defined as the asymmetry of the brain, when one hemisphere, or part of the brain, is structurally different from the other and/or performs different functions (Bisazza et al., 1998; Geschwind and Galaburda, 1985; Hines, 1987). Despite the initial belief that only humans were lateralized, evidence of brain lateralization is proven to exist among other mammals, birds and even lower vertebrates, for example, amphibians, reptiles and fish (Bisazza and Brown, 2011; Bradshaw and Nettleton, 1981; Cantalupo et al., 1995; Ward and Hopkins, 1993). In fish, cerebral lateralities are thought to benefit individuals when dealing with simultaneous stimuli and avoid conflict of responses that originate from the visual input of two laterally placed monocular eyes (Andrew et al., 1982; Bradshaw and Rogers, 1993; Rogers, 2002, 2000). The main factor determining lateralized responses of fish has proven to be the nature of the stimuli, for instance, females, predators or empty environment (Bisazza et al., 1997). Cerebral asymmetries may allow simultaneous processing and storing of information, for example, being vigilant for predators while foraging (Reddon & Hurd, 2008; Bisazza & Dadda, 2005; Brown et al., 2007). In fish, functional lateralization is mostly described through behavioural asymmetries (reviewed in Bisazza et al., 1998), such as: swimming counter-clockwise (Bisazza et al., 1998), and laterality in evasive responses (C-shape escape, Canfield & Rose, 1993; Cantalupo et al., 1995; Reebs, 2008) implied by presence of more scars in one side of the body (Reist et al., 1986).

Lateralization can occur at an individual or at a population level, depending on the tasks performed (Bisazza et al., 1998; Denenberg, 1981). A population is considered lateralized if more than half of the individuals are lateralized in the same direction. In this case, there must have been some selective advantages that sustained the maintenance of the directional bias (Bisazza et al., 2000). Predation pressure plays a determining role in strength and direction of lateralization of fish, promoting interspecific variation (Brown et al., 2007; Brown and Braithwaite, 2004). All gregarious species are highly lateralized (Bisazza et al., 2000) because coordination in shoals provides significant benefits (Bisazza and Dadda, 2005), and can be determining for fitness (Viscido et al., 2004) and survival in many natural scenarios such as feeding, predator interactions (Yasugi & Hori, 2012), and sexual interactions (Bisazza and Dadda, 2005; Rogers et al., 2004). More advantages of lateralization include better spatial orientation (Sovrano et al., 2005), faster reaction time, higher turning rates, and longer distance travelled in escape responses (Dadda et al., 2010). Despite the benefits of group lateralization, great variability is found in both the direction and strength of lateralization at the individual level (Reddon and Hurd, 2008; Vallortigara and Bisazza, 2002).

Lateralization in fish has been proven to be affected by some environmental factors, such as, elevated temperature and CO_2 (Domenici et al., 2014), and hypoxic conditions (Lucon-Xiccato et al., 2014), leading to believe that increasing salinity might also have similar effects on intolerant fish species.

Aim of this Study

The main focus of this thesis is the analysis of fish behaviour under the influence of high levels of salinity, more specifically, determining if salinity affects boldness behaviour or cerebral lateralization of fish. The stressor was tested on monitored fish, in mesocosms, in order to investigate its influence on behaviour and test the following hypotheses:

a) Increasing levels of salinity affect boldness behaviour of fish.

b) Increasing levels of salinity affect cerebral lateralization of fish.

Theoretically, it is known that salinity affects fish activity level and response to stimuli; so, it is expected that this stressor will alter the expression of some behaviours by promoting the adjustment of fish laterality (Lucon-Xiccato et al., 2014), and increasing shy behaviour or even, possibly stress fish to a point where they will take more risks and become bolder in an attempt to escape stressful conditions (Killen et al., 2013).

Chapter II. Does salinization of freshwater alter fish behaviour?

This chapter corresponds to the following paper in preparation:
Leite, T., Santos, J. M., Ferreira, M. T., Canhoto, C, Branco, P.
Does salinization of freshwater alter fish behaviour?
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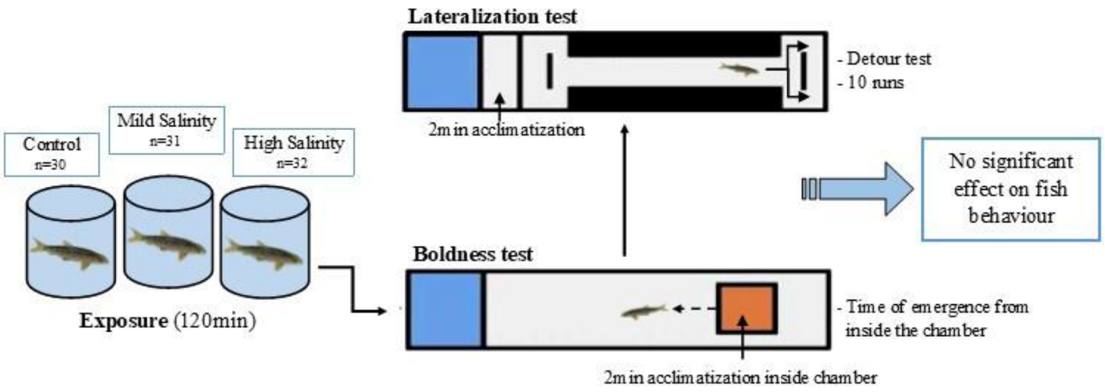
Abstract:

Stream salinization is a great environmental hazard being aggravated by anthropogenic disturbances. Harmful conditions, as increasing salinity in freshwater systems, may negatively affect river fish fauna and possibly influence fish behaviour, such as boldness and/or cerebral lateralization. The impact of salinity stress was evaluated by exposing Iberian barbels to three levels of salinity (0.9, 9 and 19 mS/cm) and by conducting boldness and lateralization experiments, regarding population trends. Results show that stress caused by salinity was not significant to evidently alter fish behaviour in any of the experiments, although some tendencies were observed in higher salinities, with greater individual variation on the shy-bold gradient, and with a more bimodal distribution of the population, in terms of lateralization.

Mediterranean fish species are especially resilient to various stressors, which may confer them additional resistance to salinity, and in this case, acute punctual exposure to increased salinity may not be detrimental for behaviour maintenance. More studies are necessary to evaluate further impacts of salinization on these systems, regarding other freshwater fish species and to test possible synergetic effects of different stressors in addition to salinity.

Graphical Abstract:



1. Introduction

Freshwater ecosystems are extremely vulnerable (Dudgeon et al., 2006), and the intensification of anthropogenic pressures is leading to water quality deterioration endangering many aquatic species (Malmqvist and Rundle, 2002; Santos et al., 2015; Valle et al., 2015). Stream and land salinization is a pressing environmental issue, especially in semi-arid and arid regions (Williams, 2001). This process can either be primary, when occurring naturally, or secondary when caused by humans (Cañedo-Argüelles et al., 2015, 2013). One of the main activities promoting secondary salinization is field irrigation in agriculture, since crops can only partially absorb the salts from the irrigation water (Lerotholi et al., 2004). Consequently, excess salts can be leached out through runoff and end up in rivers and streams. Other significant sources include the use of road salts as de-icing agents, industrial discharges or mining (Cañedo-Argüelles et al., 2013; Lerotholi et al., 2004). Most of these are point-source pressures that produce an acute, but temporary stress. Salinization damages on aquatic systems are a long-term concern, as they will continuously be more intense and spread due to human water demand and reduced rainfall promoted by anthropogenic climate change (Vörösmarty et al., 2010).

Anthropogenic salinization may cause great loss of freshwater biodiversity, putting at risk the ecosystem's goods and services (Clunie et al., 2002; Ghassemi et al., 1995; Hart et al., 1991). There might be toxic effects on water quality (Paul and Meyer, 2001) and the organisms (Eaton et al., 1999; James et al., 2003) through the acidification of streams (Lofgren, 2001) and translocation of toxic metals (Lewis, 1999; Norrström and Bergstedt, 2001). At an individual level, it affects osmoregulation, threatening long-term growth, reproduction, and overall viability of the species (Nielsen et al., 2003) because of the high metabolic costs (Cañedo-Argüelles et al., 2013; Piscart et al., 2006; Rind et al., 2017). At a community level, it affects the structure, functioning and sustainability of the aquatic system (Kaushal et al., 2005) through influences on species relationships, such as on competition and predation (Braukmann and Böhme, 2011; Judd et al., 2005; McEvoy and Goonan, 2003; Millán et al., 2011; Richburg et al., 2001). Hence, elevated salinity concentrations could have lethal effects on several species, possibly causing extinction (e.g. carps, Koç 2008), thus being a selective pressure.

Most freshwater fish species can tolerate salinities between 7 and 13 g/L, which is their internal salt concentration, however when external salinity exceeds this range, metabolism is compromised and osmoregulatory mechanisms fail (Bacher and Garnham, 1992; James et al., 2003; Pistole et al., 2008; Rind et al., 2017). This osmotic stress will impact fitness and development of fish (Boeuf and Payan, 2001), as more energy is spent on osmoregulation (Cañedo-Argüelles et al., 2015). Studies have shown that behavioural responses of fish can also be altered by sub-lethal levels of salinity, for example, overall activity, evasive responses, and ineffective responses to possible risk (e.g. fathead minnows, Hoover et al., 2013). Overall, fish experiencing potentially harmful conditions respond to stress by redirecting energy towards re-establishing homeostasis, and therefore, behaviour can be affected (Pottinger et al., 2002; Gül et al., 2004).

Behaviour, when relating to the environment, can be reduced to a trade-off decision between retreating or exploring the environment (Harcourt et al., 2009b; Riesch et al., 2009). Personality, includes the shy-bold gradient which describes the willingness of individuals to take risks in novel contexts (Magnhagen and Bunnefeld, 2009; Riesch et al., 2009; Wilson et al., 1993). Shy individuals retreat or become vigilant in unfamiliar situations, and bold individuals are more active and curious about the surrounding environment. Individual variation in personality affects how animals react to the physical environment, forage, reproduce and interact with prey, predators and conspecifics (Réale et al., 2000), therefore, it has repercussions for populations and can potentially influence social dynamics (Coleman and Wilson, 1998). Some situations promote bolder or shyer individuals, hence boldness can be associated with: predator inspection in school leaders, predator evasion (Fraser et al., 2001), dispersion (Magnhagen and Staffan, 2005), activity (Budaev, 1997b) and social preferences (Harcourt et al., 2009a, 2009b). Personality traits may be a strong driving force of evolution, as it could influence fitness of a given individual, and ultimately its survival (Frost et al., 2007; Toms et al., 2010). Concomitantly, cerebral lateralization is supposed to benefit individuals when receiving simultaneous stimuli and prevent conflict of responses (Bisazza et al., 1998; Bisazza and Brown, 2011; Halpern et al., 2005; Rogers, 2002). In fish, examples of functional lateralization are inferred from behaviour asymmetries (reviewed in Bisazza et al., 1998), such as: swimming counter-clockwise, and laterality in evasive responses (C-shape escape, Canfield and Rose, 1993; Cantalupo et al., 1995; Reebs, 2008), causing, for example, the presence of more scars in one side of the body (Reist et al., 1986). Interspecific variation in both strength and direction of laterality in fish may be caused by differential exposure to predation pressure (Brown et al., 2007; Brown and Braithwaite, 2004). At a population level, along with boldness, cerebral lateralization may determine survival in many natural scenarios such as feeding, predator interactions (Yasugi and Hori, 2012) and sexual interactions (Bisazza et al., 2000; Bisazza and Dadda, 2005), and plays a role on coordination among individuals living in social groups (Bisazza et al., 2000; Lucon-Xiccato et al., 2014). Strongly lateralized fish form more cohesive shoals, thus ensuring more efficient anti-predator responses during schooling manoeuvres (Bisazza and Dadda, 2005; Murphy and Pitcher, 1997), increasing fitness of said individuals (Pitcher and Parrish, 1993; Viscido et al., 2004).

Ultimately, with high levels of salinity, fish are confronted with decisions, then behaviour and personality traits can be influenced by this increasing stressor as there could be disruption of behavioural expression (Hoover et al., 2013).

To our knowledge there are no studies that test the effects of increasing salinity specifically on boldness behaviour and lateralization of freshwater fish species, therefore our chief aim was to understand the effects of acute point-source anthropogenic salinization of rivers on freshwater fish by determining if salinity, as a stressor, can alter boldness behaviour and cerebral lateralization of fish. Theoretically, it is known that salinity affects fish activity level and response to stimuli (Hoover et al., 2013); so, it is expected that this stressor will alter the expression of some behaviours by promoting the adjustment of fish laterality, and increasing shy behaviours or even, possibly stress fish to a point where they will take more risks and become bolder in an attempt to escape stressful conditions.

2. Materials and Methods

2.1. Species selection

The Iberian barbel, *Luciobarbus bocagei* (Steindachner, 1864), is a freshwater fish from the Cyprinidae family. It is endemic to the Iberian Peninsula, and occurs in a wide range of lotic and lentic habitats and in almost all the river basins of northern and central Portugal from Lima to Sado drainages (Lobón-Cerviá and Fernández-Delgado, 1984). It has stable populations and is considered a non-threatened species by the IUCN Red List. Juveniles (9-13 cm Total Length, TL) were selected for this study as they may be more affected by salinity stress due to their higher surface/volume ratio. An eco-morphologic approach was followed, and the Iberian barbel was considered as representative of bottom oriented potamodromous medium-size cyprinids (Branco et al., 2013). This kind of approach has been considered suitable to study systems where several similar species exist, and when exhaustively studying all species present is logistically challenging (Boeuf and Payan, 2001). This species has the added advantage of being abundant in several systems which allows all fish to be captured in the same river stretch minimizing the probability of multi population capture and reducing the time taken for capture and concomitantly capture stress is maintained to a minimum. All of these contribute for a reduced experimental bias.

2.2. Fish capture and holding

Fish were captured from the Lizandro River (38°54’03.3” N, 9°21’42.2” W) in Carvalhal (Mafra, Portugal) in September and October 2017. Capture was achieved by using standard electrofishing protocols closely following the CEN standards (CEN, 2003). During the experiments, fish were maintained in tanks with freshwater at the School of Agriculture (University of Lisbon). Maintenance tanks (approx. 800 L) were kept under natural light and temperature, and with mechanical and biological filtration (Fluvial FX4) with a turnover of 1700 L/h. Fish were acclimated for at least 48 h and feeding stopped 24 h before the experiments took place. Fish were used only once and were never maintained for more than 5 days before they were returned alive to the same location from where they were captured. In total 93 barbels were used (TL: 11.34 ± 1.33 cm, mean ± SD).

2.3. Fish experiments

Fish experiments took place in a mesocosms system composed by 2 zinc channels (400 cm length x 40 cm width x 20 cm depth) (Fig. 1). Channels were fed by an *in situ* natural spring (pH = 8.06; Conductivity = 0.87 mS/cm; DO = 9 mg/L) and conducted to

3000 L central reservoir and evenly delivered to the channels. Each individual was tested for boldness, and then transported to the other channel to test for lateralization. Salinity levels of the mesocosms were maintained the same as the treatment being tested and water depth was maintained constant throughout treatments and replicates (10.06 ± 0.21 cm boldness and 7.49 ± 0.42 cm lateralization channel, mean ± SD).

Fish were randomly selected from the holding tank and transferred in small groups (5-8 individuals) to a treatment tank (40 L). Salinity levels were determined through conductivity measures. For the control group, fish were kept in freshwater with conductivity levels around 0.9 mS/cm, for 120 min previous to the experiments (Table 1) (Lucon-Xiccato et al., 2014). For the salinity groups, as there are studies that indicate behaviour impairment at salinities of 7.5 g/L (15 mS/cm) (common carp, Whiterod and Walker, 2006), we exposed the subjects to mild salinity (9 mS/cm) and high salinity (19 mS/cm) (Table 1) for 120 min previous to the experiments as well. To achieve the intended levels of salinity NaCl (common salt) was used (Cañedo-Argüelles et al., 2015).

Table 1. Mean ± SD of fish biometrics and water parameters collected from: **B**- boldness test channel; **L**- lateralization test channel. **n** = number of fish tested.

Treatment	**Total Length (cm)**	**Body Mass (g)**		**Conductivity (mS/cm)**	**Temperature (°C)**	**Depth (cm)**
Control (n = 30)	11.70 ± 1.29	13.55 ± 4.37	B	0.89 ± 0.02	19.54 ± 4.10	10.00 ± 0.17
			L	0.95 ± 0.06	19.34 ± 4.15	7.12 ± 0.54
Mild Salinity (n = 31)	11.03 ± 1.32	11.97 ± 4.58	B	9.44 ± 0.81	18.72 ± 2.61	10.27 ± 0.25
			L	9.51 ± 0.31	18.02 ± 2.97	7.70 ± 0.12
High Salinity (n = 32)	11.51 ± 1.32	13.50 ± 4.25	B	18.66 ± 3.29	16.72 ± 1.63	10.01 ± 0.17
			L	18.52 ± 1.37	16.41 ± 1.64	7.68 ± 0.12

Water parameters, such as conductivity, temperature and depth of the water column, were measured several times, both in the treatment tanks and in the mesocosms (Table 1), during the period of the assays to ensure similar conditions in every experiment (HANNA's multiparameter probe HI 9812-5).

2.3.1. Boldness test

Immediately after the end of the 120 min exposure period (no salinity, mild and high salinity), fish were tested for boldness. Fish were chosen randomly and gently poured from the holding container into the experimental chamber placed in the channel (25x25x30 cm) with a door (10x10 cm) and an opaque lid placed on top where they remained for a minimum of 2 min for acclimatization. After this time, the door was opened and the time the individuals took to emerge completely from the chamber was registered. If the fish had not emerged from the box after 6 min (360 s), the lid was

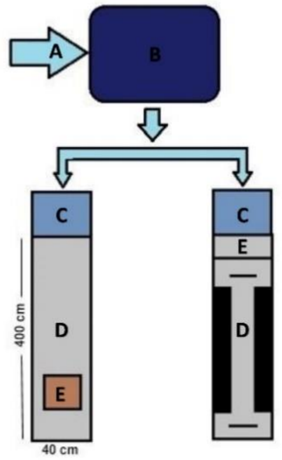


Fig. 1. Schematic overview of experimental system setup. **A** –water source inlet from a spring, **B** – central source tank with distributing pipe system, **C** – head containers, **D** – experimental area, **E** - acclimatization area. Photo in Appendix 1.

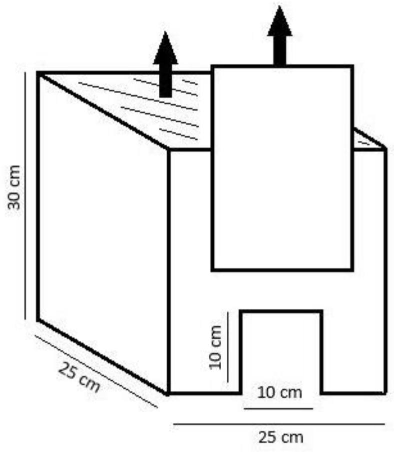


Fig. 2. Schematic representation of the boldness test chamber. Fish were placed inside the chamber with the lid on top. Door was opened after 2 min and fish were free to emerge. After 6 min the lid was removed. Time fish took to emerge from the chamber was recorded. Test was terminated after 8min.

removed, thus reducing the value of the refuge and encouraging fish to emerge from the box. If the fish still had not emerged after 8 min, we terminated the trial and gave the fish a maximum value of 480 s (Brown et al., 2005). Boldness was determined by the time fish took to emerge from the chamber (Fig. 2). The recorded time of each subject can be translated into a gradient of boldness (Brown et al., 2005). Only one fish was tested at a time.

2.3.2. Lateralization test

Individual behavioural lateralization was assessed by performing a standard detour test (Bisazza et al., 2000, 1997). Fish were transferred from the first channel into an acclimatization area in one end of the channel for a minimum of 2 min (E in Fig. 1), and after that into a double T-maze runway with a narrow channel with barriers at both ends (200 cm channel length x 40 cm total width x 10 cm channel width and 18 cm wide barriers placed 10 cm away from runway ends) (Fig. 3). Each fish was slowly encouraged to enter one end of the runway and swim towards the barrier using a fish net (encouragement stopped at channel mid length) and each individual performed 10 runs of the test (Domenici et al., 2014). The direction of turns in front of the barrier was recorded.

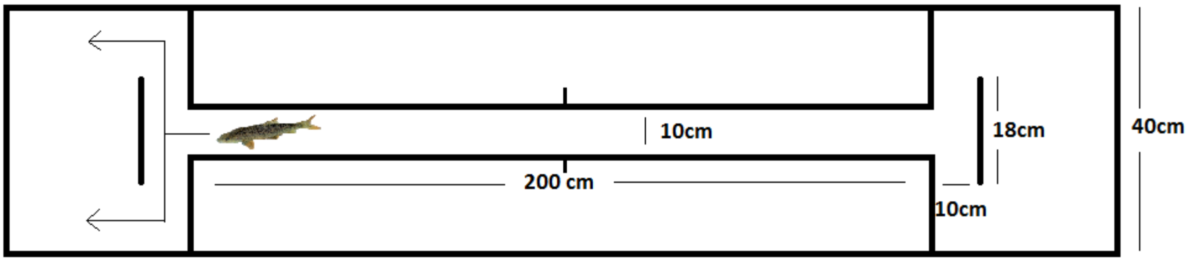


Fig. 3. Schematic representation of the double T-maze apparatus for the detour test. Fish could swim along the runway (alternately in opposite directions); left/right turning directions were recorded during the 10 runs of each fish.

Fish were measured for total length (cm) and weighted for body mass (g) only after testing each individual for both behaviours (Table 1), in order to decrease any effect on the experiments caused by stress induced by handling.

2.4. Statistical analysis

To determine differences in boldness between treatments, we performed a One-way ANOVA (Zar, 1999). If discrepancies were found to be significant, population's boldness was considered to be affected by salinity stress.

To make a comparison between fish and demonstrate their lateralization, for each individual a relative lateralization index, Lr *(1)*, and absolute lateralization, La *(2)*, (Bisazza et al., 1997) were calculated through the formulas:

Lr = [(turns to the right - turns to the left) / (turns to the right + turns to the left)]*100 *(1)* (Bisazza et al., 1997);

La = |Lr| *(2)* (Bisazza et al., 1997).

Fish were attributed values ranging from -100 (10 turns to the left) to 100 (10 turns to the right), and 0 to 100, respectively *(1, 2)*. To determine differences in laterality between treatments, a One-way ANOVA (Zar, 1999) was performed. If discrepancies were found to be significant, population's laterality was considered to be affected by salinity stress. The normality of the distribution of the control data was tested, and verified if it was maintained in the salinity treatments (Kolmogorov-Smirnov tests). Distribution of lateralization was analysed using t-tests against a theoretical normal distribution of Lr centred in 0.

All statistical procedures were conducted using STATISTICA 7 (StatSoft, 2004).

3. Results

Results (Fig. 4) show that in terms of boldness, in the control group, the distribution of emergence of fish along the boldness gradient was generally even. The highest frequency of emergence in the control group occurred in the 60 s right after the removal of the lid (33 %). In both salinity treatments, mild and high, the highest frequencies were recorded in the first 60 s of the experiment (35 %) and just after the removal of the lid of the container (47 %), respectively. Hence, originally the population has even number individuals along the gradient, ranging from extremely bold to extremely shy individuals. In the mild and high salinity groups, population laterality distributions were higher in the extremes - either extremely bold or extremely shy individuals. One-way ANOVA's assumptions were met and results show no significant differences between treatments, concerning boldness behaviour (Time of emergence: $F_{2, 93} = 1.275$, $p = 0.284$). Despite not finding any statistical differences in the data, it can be noticed a trend of decrease of the median when comparing the control with the salinity treatments (Fig. 4). Furthermore, the population seems to be more disperse along the boldness gradient with increasing salinity, when in the control group, the 25 %-75 % of the population is more focused in the intermediate levels of the axis (Fig. 4). Population dispersion, with increasing salinity, along the shy-bold gradient tends to be more skewed to the extremes, i.e. greater individual variation (extremely shy or extremely bold) than during the control (no salt added).

Regarding cerebral lateralization (Fig. 5), we confirmed that our data had a normal distribution of frequencies in relation with the lateralization index in each treatment (C: K-S ($d = 0.162$), $p > 0.2$; M: K-S ($d = 0.179$), $p > 0.2$; H: K-S ($d = 0.142$), $p > 0.2$). In none of the tested salinities fish turned more than 7 times to the right and the highest frequencies observed are on the left side of the axis (Fig. 5). Hence, we performed t-tests against a theoretical normal distribution of Lr centred in 0 and demonstrated that this population is slightly lateralized to the left, which is evident in every treatment (Control: M = -18.66, SD = 30.59 , t_{29} = -3.34, p = 0.002; Mild: M = -16.12, SD = 31.58 , t_{30} = -3.34, p = 0.007; High: M = -22.50, SD = 30.79 , t_{31} = -3.34, p = 0.0002). One-way ANOVA's assumptions were met and results show no significant differences between treatments, with respect to cerebral lateralization (Lr: $F_{2,\ 93}$=0.337, p=0.714; La: $F_{2,\ 93}$=0.227; p= 0.797). Nonetheless, some evidence of a bimodal distribution of frequencies exist in Figure 5.

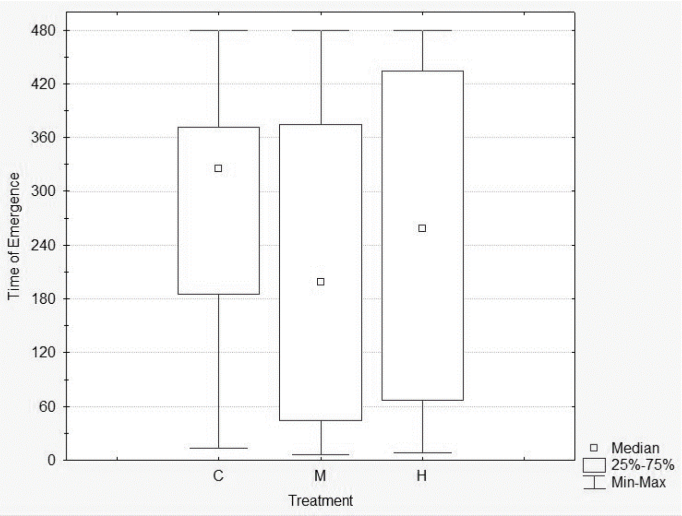


Fig. 4. Frequency distribution (%) of the Iberian barbel population's time of emergence (s) from the boldness chamber. **C**-control. **M**-mild salinity. **H**-high salinity. The lid was removed on second 361. No significant differences (One-way ANOVA) were found between salinity levels.

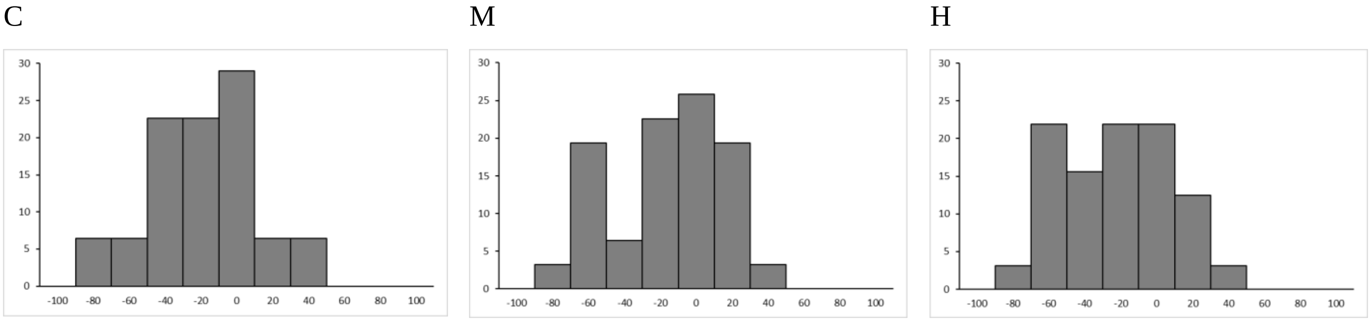


Fig. 5. Frequency distribution (%) of the Iberian barbel population's lateralization index values (Lr). A value of -100 represents a fish with 10 left turns in 10 trials; 0 value corresponds to a fish with 5 turns to the left and 5 turns to the right; 100 value corresponds to a fish with 10 turns to the right in 10 trials. **C**-control. **M**-mild salinity. **H**-high salinity. No significant differences (One-way ANOVA) were found between salinity levels.

4. Discussion

Anthropogenic salinization is a developing issue and raises concern because of the toxic salinity concentrations in freshwater ecosystems and its severe effects over river fish fauna (Dugan et al., 2017; Searle et al., 2016; Szöcs et al., 2014). Outcomes will be intensified by climate changes (Mantyka-Pringle et al., 2014), and these global variations will interact with increasing salinities, inducing physiochemical stress in freshwater ecosystems, by increasing ionic concentrations (Herbert et al., 2015). Harsh environments created by salinization might disturb fish's osmoregulation and metabolism in several ways, and therefore, their overall activity level, behavioural responses, and ultimately their survival (Boeuf and Payan, 2001; Gonzalez, 2012; Hasan et al., 2017; Rind et al., 2017). Our study tested a population of Iberian barbels in order to fill the gap related to direct effects of high levels of salinity on fish behaviour, particularly boldness and cerebral lateralization. This work can be viewed a starting point in the study of other effects related to salinity as a stressor.

Results show that acute and temporary salinity increase, up to 19 mS/cm (9.5 g/L), may not be translated into obvious effects on boldness or lateralization behaviour of this species, but as we observed tendencies of behavioural changes, with the increase of salinity, further studies are needed to support the hypotheses that fish diverge to the extremes of the shy-bold gradient and spread their lateralization, becoming a bimodal distribution of turning preferences. These divergent behaviour trends comply with similar studies on risk-taking behaviour of fish, where differential sensitivity to stressors was associated with different behavioural responses among individuals (*Dicentrarchus labrax* exposed to hypoxia, Killen et al., 2013). In terms of lateralization, the detected lateralization bias and bimodal distribution, has also been observed in recent studies, for exposures to hypoxia, elevated temperatures or CO_2 levels, and this behaviour impairment could be due to imbalances on hemisphere functioning and neurotransmission (Domenici et al., 2014; Lucon-Xiccato et al., 2014).

Since most freshwater fish species tolerate within the range of 7-13 g/L (James et al., 2003), and that there are evidences of behaviour modification at 7.5 g/L (erratic behaviour with mortality at 13 g/L, in common carps, Whiterod and Walker, 2006) and 8 g/L salinity (decrease in anti-predator responses in fathead minnows, Hoover et al., 2013), it is also likely that the osmotic threshold for this population of Iberian barbels (exposed to less than 0.5 g/L salinity in the Lizandro river) was not met during our experiment for the behavioural responses to be significantly affected, but slight behavioural variation at the population level started to be noticeable. So, it seems that besides different species having different tolerances to different stressors, there could also be observed individual differential susceptibility to those stressors (Killen et al., 2013; Segurado et al., 2011).

The Iberian barbel is a Mediterranean species, which by virtue of their environment are naturally affected by several pressures that translate into various stresses. Mediterranean rivers are known to be extremely impacted by anthropogenic activities (Hooke, 2006), and have harsh environmental settings that will be intensified by climate change, resulting in complex ecological impacts (Branco et al., 2016a). Therefore, it is possible that this population and other Mediterranean species have been selected to have an increased tolerance to various disturbances (Benton et al., 1994; Jones and Reynolds, 1997). Various cyprinids have high salinity tolerances, up to 13 g/L (*P. stymphalicus*, Bianco and Nordlie, 2008; *H. placitus*, Ostrand and Wilde, 2001). Particularly, some studies show that other *Luciobarbus* species have revealed higher resistance to stress when compared to other freshwater fish species (Kraiem et al., 1988; Tsigenopoulos et al., 2003), and the Iberian barbel was considered to have intermediate tolerance to human disturbances in a study in some Mediterranean rivers (Segurado et al. 2011). Furthermore, our study focused exclusively on Na^+ and Cl^-, while salinized waters are also dominated by other ions ($MgSO_4$ and $NaSO_4^-$), and it is possible that physiological and behavioural effects on fish could be more evident, and fish would be more responsive to other toxic combinations and concentrations of ions in the water (Hoover, 2017; Mount et al., 1997). Nonetheless, the population approach followed in this study is proved to be valid. The two studied behaviours seem to be deeply imprinted in the individual, and the population seems to have a stable representation of individual behaviours along each behavioural gradient. So much so, that even with high levels of salinity no significant differences arose at the population level. This constancy can nonetheless be an artefact of possible limitations of the methodology followed herein. For instance, the number of fish tested might have been insufficient to detect slight changes in populations with strong behavioural stability. A higher n would likely allow the observation of more differences in the dispersion of data, or (even though the distributions are normal in all treatments) the apparent trend to slight bimodal distribution in the lateralization index data for higher salinities could become more evident and statistically significantly different from a normal distribution. Additionally, exposure period (Domenici et al., 2014) or salinity levels (Kraiem et al., 1988) could have been more stringent, and thus, cause an alteration of barbel’s behaviour. Even so, the experiment was designed to simulate an acute, temporary, salinity increase that arises from the main causes of secondary salinization, for example, an industrial discharge or salt as a de-icing agent.

It is possible to further hypothesise that effects on behaviour could become more evident with multiple stressors in addition to increasing salinity, because in a natural setting salinity as a stressor, most likely, co-occurs with other stressors (e.g. flow reduction due to water abstraction, and nutrient enrichment due to land-use changes) that may interact synergistically, augmenting the effect of salinity acting in isolation (Piggott et al., 2015; Segurado et al., 2018). However, studies found no interaction between road salts and predatory stress (Daphnia, Hintz and Relyea, 2017) or, road salts and heavy metals (macroinvertebrates, Schuler and Relyea, 2018), for example. These specific stressors in addition to salinity only presented additive effects on species abundance and reproduction behaviour. Averse isolated stressors can also interact with the environment and each stressor's expression may change along natural gradients (Branco et al., 2016b; Segurado et al., 2018). Thus, using an approach applicable to a wide variety of environmental variables may be useful to further understand salinization effects on freshwater fish behaviour (Sokolova, 2013), because each species is likely to be affected differently by different kinds of pressures, as each might have a different level of tolerance. Some other stressors known to affect lateralization are suitable candidates to test interactions between different salinity levels. For instance, recent studies verified that other stressors were able to disrupt fish lateralization, enough to raise concern and suggest broad ecological implications (temperature and CO_2, Domenici et al., 2014; hypoxia, Lucon-Xiccato et al., 2014).

Broader investigations on the synergistic effect of different environmental factors would be essential to understand how isolated and interacting stressors can have distinct impacts on the community, the underlying physiological processes and ecological implications of the increasing worldwide secondary salinization. This knowledge would allow to evaluate greater scale implications on the freshwater systems as a whole, and implementation of more serious and effective conservation and management plans, preventing risky activities that are the main causes of this global problem (agriculture, irrigation, industrial discharges, and, in some countries, road salts). Using the Iberian barbel as a representative species of medium-size cyprinids might be useful to extrapolate the results to comparable species facing identical salinization scenarios. Moreover, some solutions to further contribute to our goal would be to test a larger population or an even higher level of salinity, perhaps illustrating sub-lethal conditions and observing possible shifts on population’s tendencies throughout the assays. Testing individual assessment of effects of stressor (same individuals in all treatments) and repeatability of behaviour (same individual tested for the same treatment through time) would be further valuable in disentangling the effects of salinity in fish behaviour. We encourage the expansion of the research to different freshwater fish species (with possibly lower tolerance to salinity) that would help to recognise their salinity thresholds and use them to implement effective conservation measures and appropriate ecological restoration actions for these sensible systems.

Chapter III. Final Remarks

Freshwater fish are great natural resources, and play a fundamental role in the ecosystem by regulating trophic dynamics, nutrient flux, sediment processes and acting as links between ecosystems. Humans take advantage of these resources in order to obtain food or income, not realizing that we are continuously destroying their habitat and jeopardizing river fauna sustainability. Human pressure is intensifying salinization in freshwater, and the triggered changes and their negative ecological impacts on these systems will endure and become more intense, at several scales, as population grows. Many of these changes will be irreversible, and therefore, it is urgent to understand and anticipate how ecosystems will react to future environmental changes caused by present and new stressors (e.g. climate change and habitat degradation, Friberg et al., 2011).

Although not statistically significant, the levels of salinity tested showed signs of affecting fish boldness and lateralization. The apparent tolerance to salinity shown by Iberian barbels (Kraiem et al., 1988), justifies the absence of extreme responses to salinity changes. The general results point to the need of larger-scale investigations, and because there is limited documentation on the adaptation of freshwater species to high salinity conditions, and not much is known about the underlying effects, specifically, on fish behaviour, we think future studies should consider:

a) Testing other fish species, to understand if the negative effects are evident throughout the taxa;
b) Testing even higher salinity levels (maybe >13 g/L), longer exposure periods, different ion composition, and acclimatization ability to better understand species salinity thresholds, in the context of Mediterranean rivers, and the repercussions on the ecosystems;

c) Relating mesocosms with field experiments, and complementing standard ecotoxicological studies with behavioural tests, considering interacting stressors and other levels of the trophic web.

This research would provide a more holistic and comprehensive assessment of the risks that freshwater systems are exposed to, and the costs of anthropogenic salinization to river fish fauna.

Our study is ecologically relevant, as it might offer a potentially useful new perspective on fish's behavioural response to stress caused by salinity. Additionally, the approaches we suggest can be used to predict how a community or population of organisms may respond to stressors (Friberg et al., 2011), since these long-term consequences can be perceived at all scales of the ecosystem, and their magnitude may be more intense and spread with the interaction with other global changes (Herbert et al., 2015; Vörösmarty et al., 2010). Hence, it's time, sooner rather than later, for effective conservation strategies and recovery policies. Management of the freshwater systems is necessary and essential, and measures based on good ecological understanding can prove to be determining for the survival of several freshwater species (Friberg et al., 2011; Malmqvist and Rundle, 2002).

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Appendix 1

Figure A. Mesocosms on campus.