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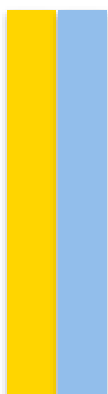

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Inês Caetano. **Behavioural and physiological assessment of socially isolated Zebrafish (*Danio rerio*)**

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MESTRADO EM CIÊNCIAS DO MAR - RECURSOS MARINHOS
RAMO DE BIOLOGIA E ECOLOGIAS MARINHAS

Behavioural and physiological assessment of socially isolated Zebrafish (*Danio rerio*)

Inês e Silva Caetano

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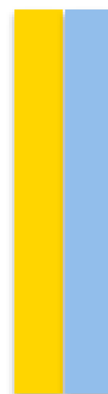

INÊS E SILVA CAETANO

Behavioural and physiological assessment of socially isolated Zebrafish (*Danio rerio*)

Tese de Mestrado em Biologia / Ecologias Marinhas do Mestrado em Ciências do Mar – Recursos Marinhos do Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto

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Inês e Silva Caetano

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“O mundo também existe para seres tu”

- Rui Louro Mendes

ABSTRACT

The zebrafish is a small sized aquatic vertebrate whose popularity as a model organism in research has been increasing rather quickly. The behavioural paradigms of zebrafish and mammals are highly similar, suggesting that many behaviours were conserved in terms of evolution across species, making *Danio rerio* a valid model organism. Ensuring welfare in laboratory conditions is key to having concise and precise results, with the least amount of bias possible. Zebrafish are highly social animals, and sometimes in a laboratory setting these fish need to be subject to certain periods of isolation, for example if you need to wait for genotyping results, in regeneration studies, during behavioural tasks taking several days, etc. Our aim with this project was to explore the possible impacts of social isolation that could happen in routine experiments in zebrafish, whilst understanding the impact it can have in further exposures to stressors. To achieve this, we exposed zebrafish to a 15-day isolation period, followed by the measurement of cortisol levels, and analysis of the oxidative stress levels for a deeper understanding of the impact of social isolation in zebrafish. We also conducted adequate behavioural tasks that are often used to assess anxious-like behaviours (white/black and novel tank test), and social behaviours (mirror biting test). In terms of cortisol levels, we did not find any significant difference, meaning that socially isolated zebrafish had cortisol values at control levels, and they also reacted normally to acute stress. In terms of behaviour, we only observed minor alterations but no difference between isolated and control animals. In terms of oxidative stress, for most analysis we did not report any differences as well. To sum up, zebrafish can be socially isolated for 15 consecutive days without altering their cortisol levels nor their stress response.

RESUMO

O peixe-zebra é um vertebrado aquático de pequena dimensão cuja popularidade como organismo modelo em investigação tem aumentado rapidamente. Os paradigmas comportamentais de peixe-zebra e mamíferos são muito semelhantes, sugerindo que vários comportamentos foram conservados a nível evolutivo, tornando o *Danio rerio* um organismo modelo válido. Portanto, é extremamente relevante garantir que esta espécie usada a nível mundial no ramo da investigação, seja mantida nas melhores condições laboratoriais possíveis. Garantir o bem-estar animal destes seres, é a chave para que os resultados obtidos, aquando da utilização dos mesmos, sejam mais concisos e precisos, com o menor nível de viés possível. Os peixes-zebra são considerados animais altamente sociáveis e, por vezes, em condições laboratoriais, estes animais são sujeitos a certos períodos de isolamento, como por exemplo, enquanto se aguardam resultados de genotipagem, em estudos de regeneração, durante ensaios comportamentais que demoram vários dias, etc. O nosso objetivo com este projeto é então explorar os possíveis impactos do isolamento social em peixes-zebra que podem ocorrer em procedimentos rotineiros, enquanto investigamos também o impacto que estes períodos podem ter em futuras exposições a *stressores*. De forma a realizar este projeto, expusemos vários peixes-zebra a um período de isolamento de 15 dias, seguido de quantificação dos seus níveis de cortisol e análise dos níveis de stress oxidativo para um estudo mais profundo acerca do possível impacto do isolamento social em peixe-zebra. Também realizámos vários testes comportamentais usados frequentemente para avaliar comportamentos típicos de ansiedade (o teste *"White/black*" e o *"novel tank"*) e comportamentos sociais (o teste de *"mirror biting"*). Relativamente aos níveis de cortisol, não foram encontradas quaisquer diferenças significativas, o que significa que os peixe-zebra que foram isolados dos seus conspecíficos tinham valores de cortisol semelhantes aos animais controlo e reagiram ainda de forma considerada normal ao stress agudo. Em termos comportamentais, apenas observamos pequenas alterações, mas sem diferenças entre animais isolados e controlo. Por fim, no que toca ao stress oxidativo, para a maioria das análises feitas, também não reportámos quaisquer diferenças. Em suma, os peixes-zebra podem ser isolados socialmente durante 15 dias consecutivos sem haver alterações nos seus níveis de cortisol nem na sua resposta ao stress.

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1. INTRODUCTION

Zebrafish, *Danio rerio*, has been increasingly used in research (Fig. 1). Thus, there is a need to consider the conditions of this species in captivity, keeping the fish capable of coping with the standard environment and procedures routinely performed in the animal facilities. In this sense, we need to comply with the animal welfare, and that the procedures are not going to interfere with the research outcomes.

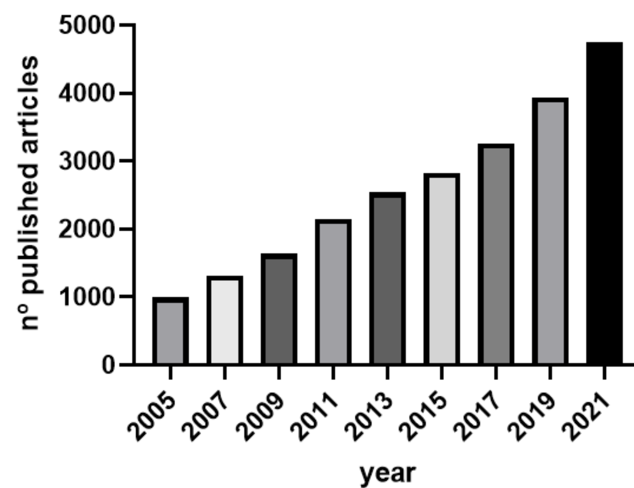

Fig. 1 - Number of publications mentioning zebrafish in the past 16 years (keyword "zebrafish" in Title/Abstract according to SCOPUS database).

1.1. *Zebrafish in nature*

Francis Hamilton was a Scottish physician that in 1822 discovered ten Danio species (Spence et al., 2008) in the Ganges river and its branches, and published the description of zebrafish as *Danio rerio* (Hamilton, 1822). The name "Danio" derives from the Bengali name "dhani" which translated to "of the rice field" (Talwar & Jhingran, 1991).
Zebrafish are usually distributed along the Ganges and Brahmaputra river basins in the North-Eastern India region, Bangladesh, and Nepal, but also in another places of Asia (Spence et al., 2006); (Talwar & Jhingran,1991); (Barman, 1991); (Spence et al., 2008) (Fig. 2). *Danio rerio* is one of more than 20 species that were reported to exist within the *Danio* genus and a part of the order Cypriniforms, and Cyprinid family (Miller & Gerlai, 2011), (McCluskey & Braasch, 2020).

Fig. 2 - *Danio rerio* distribution, adapted from the Natural Earth Data website (https://www.naturalearthdata.com/). Rows indicate where zebrafish is most predominant in nature.

Zebrafish is a diurnal species. It is considered a gnathostome (due to its jaw apparatus) and it has two paired fins – the pectoral and pelvic ones, and three single fins – the anal, dorsal, and caudal fin (McCluskey & Braasch, 2020). Zebrafish is described to be a small species (2-4 cm long as adults), to have a 'danionin notch' in the ventromedial margin of the dentary, and a unique colour pattern with darker and lighter horizontal stripes that can vary depending on the strains (Spence et al., 2008).

Fig. 3 - *Danio rerio* sexual dimorphism example. Copyright free for non-commercial use. Source: https://www.flickr.com/photos/8659392@N07/13896905021. The fish on top is a female and below is a male. Arrow points to the genital papilla of the female zebrafish.

Zebrafish live in social groups and can tolerate a wide range of temperatures, starting at winter temperatures that can reach 6ºC to extreme heat temperatures in the summer, surviving at 38ºC.

Their habitat preferences are usually slow-moving or still water bodies, margins of watercourses and canals, especially near rice-fields (Talwar & Jhingran, 1991); so, they prefer zones with vegetation, providing shades, and a silt substratum (Lopez-Olmeda & Sanchez-Vazquez, 2011). Although these animals are mostly seen in calm slow waters, (Daniels, 2002) also reports their presence within rivers and high-ground currents. The characteristics of zebrafish habitat allow these animals to flee and seek shelter from their predators, thus increasing their survival (Spence et al., 2008). The diet of *Danio rerio* is omnivorous and the majority of its feeding comes from ingestion of zooplankton and insects, although many other things have been found in their guts such as phytoplankton, algae, spores, etc (Spence et al., 2008). Zebrafish has sexual dimorphism, the males are usually slimmer, whilst the females are typically rounder, and the males also usually have longer fins. Females are often very well distinguished from males through the existence of the genital papilla (Fig. 3). Reproduction occurs most frequently within the first hour of the sunrise (Spence et al., 2006); zebrafish is a seasonal breeder in nature, breeding mainly during the monsoon season (Lopez-Olmeda & Sanchez-Vazquez, 2011). They do not have parental care, and larvae hatch at 72h post-fertilization (hpf), depending on the temperature (Miller & Gerlai, 2011).

1.2. *Zebrafish in Research*

Only in the late half of the 20th Century, around the 1970's, zebrafish were domesticated and introduced in laboratories (Sundin et al., 2019), but Creaser (1934) was already using it for developmental biology studies several years prior, and he is known as one of the pioneers of using *Danio rerio* in the molecular genetics field and embryology. In 2002, a review was published by Grunwald & Eisen (2002) summarizing how zebrafish started being introduced in the labs.

The scientist George Streisinger got a batch of *Danio rerio* from commercial suppliers and, throughout the years, made revolutionary discoveries, along with other researchers, leading us to the beginning of whole-genome sequencing of zebrafish at the beginning of the 21st Century (Grunwald & Eisen, 2002). As others, these animals have been kept in captivity for multiple generations, which could probably lead to the loss of some traits (Robison & Rowland, 2011). However, we have no certainties regarding the strain differences and how much intraspecies variation there is when comparing wild fish to captive zebrafish (Spence et al., 2008).

Not only laboratory strains are often divergent from the wild species, but *Danio rerio* can also differ between strains and within labs (Suurvali et al., 2020). Nevertheless, one of the most used strains are AB, which is often used in behavioural studies (Guo, 2004). These animals normally come from official breeding institutes that ensure compliance with high research demands for these animals (Delcourt et al., 2018).

At the National Center for Biotechnology Information there are more than 60 000 entries for zebrafish, and the Zebrafish Information Network (ZFIN) is a database available for all researchers with information regarding this species. Thus, this is the fish species with the most data available for research (Suurvali et al., 2020), and, on a global scale, more than 400 institutions use zebrafish in various fields of research daily (http://zfin.org) (Fig. 4).

Fig. 4 - *Danio rerio* in a laboratorial context example. Copyright and royalty-free from Getty Images website. Source: https://www.gettyimages.pt/detail/foto/fishing-zebrafish-from-tank-in-laboratory-imagem-royalty-free/551797967

Zebrafish has been used as an animal model in various fields of research such as biomedicine, molecular genetics, applied genetics, developmental biology, and neuroscience (Choi et al., 2021; Hruscha & Schmid, 2015; Schier & Talbot, 2005; Stewart et al., 2014; Veldman & Lin, 2008). These animals have also been proposed to replace the use of rodents in certain types of research (Graham et al., 2018), mainly because of their characteristics: small size, fast development, transparency during embryo stages and genetic resemblance to the human species (Lopez-Olmeda & Sanchez-Vazquez, 2011). In addition, compared to rodents, zebrafish demand less space per animal, have high reproductive rate and they are cheaper to sustain.

They reach sexual maturity after 3-4 months post-fertilization, and, when reproduced, females can hatch up to 200-300 embryos weekly. The embryos are transparent and have fast external development. This allows the observation of the embryos and larvae internal organs under stereoscopes, making it easy and accessible for genetic manipulation, embryological, and toxicological assessments (Delcourt et al., 2018). Since the cost of maintaining large quantities of zebrafish is low, screening studies with large pools can be done (Wiley et al., 2017), which is highly useful in toxicological and pharmacological research (Bambino & Chu, 2017) (MacRae & Peterson, 2015).

Another advantage of using zebrafish is that they have a rather long lifespan, living in a laboratory setting for an average of 42 months (Gerhard et al., 2002), allowing for researchers to do long-term studies.

The zebrafish genome sequencing was finished a couple of years ago in the UK Sanger Institute (Howe et al., 2013), making it a well described species for all types of studies. The behaviour of wild zebrafish is not well described, and this gap of knowledge is often attributed to the fact that zebrafish inhabit in turbid waters, making observations hard to analyse (Spence et al., 2008). Therefore, this makes the task of establishing optimal laboratory conditions for maximum animal welfare very hard, and also affects our perception of certain behaviours that can be relevant for assessing welfare since we may not notice them (Graham et al., 2018). In laboratories, zebrafish are usually kept in recirculating water systems, and housed in barren tanks with high animal densities', usually 5-8 fish per litre according with the literature and current guidelines (Aleström et al., 2020). The recirculation water systems have continuous filtering and aeration to ensure good water quality with stable parameters, such as temperature between 26-28.5ºC, pH between 6.8-7.5, and animals have a 14:10 light:dark cycle (Graham et al., 2018).

Zebrafish are social animals and form small groups called shoals (Suriyampola et al., 2016), and they can exhibit preferences regarding shoal size (Pritchard et al., 2001). Although it is difficult to register zebrafish behaviour in nature (Suriyampola et al., 2016), shoaling behaviour is easily observed and conserved in the laboratory settings. Thus, this behaviour allows the study of social interactions (Dreosti et al., 2015), social preferences (Engeszer et al., 2004), social learning (Lindeyer & Reader, 2010), social decision-making (de Polavieja & Orger, 2018), and social recognition (Madeira & Oliveira, 2017).

1.3. Social behaviour in zebrafish

Social behaviour is described as a complex behaviour, thus its underlying mechanisms and development are not fully known (Buske & Gerlai, 2011). Abnormal social behaviours are an underlying condition of many psychiatric and neurodevelopment conditions in humans such as depression (Hull et al., 2021), anxiety (Neumann et al., 2010), autism spectrum disorders (Barendse et al., 2018), but the mechanism that leads to these disorders is not thoroughly researched yet (Bartz & Hollander, 2006).

There are 24 000 known fish species that have tendency to aggregate in social groups at least in some stage of their life cycle.

These aggregations happen due to animals choosing to form intraspecific cohesive groups to obtain various kinds of individual benefits and it often occurs due to a series of extrinsic factors such as food, shelter or need for protection (Pitcher, 1998). Shoaling is a particular form of conspecific aggregation (Fig. 5). There are many definitions to what a shoal is but the one that is most accepted within the scientific community is that a shoal is an aggregate of fish that stay together due to social reasons (Pitcher & Parrish, 1993).

Fig. 5 - *Danio rerio* sexual shoaling example. Copyright free for non-commercial use. Source: https://www.istockphoto.com/pt/foto/zebra-fish-brachydanio-rerio-gm1250930417364917100?phrase=zebrafish

This should not be confused with schooling, which is a behaviour that described coordinated swimming movements (Delcourt & Poncin, 2012). Zebrafish maintain a reasonably high degree of shoaling throughout their lives, albeit particular biases for shoaling conspecifics seem to be learned (Kalueff & Stewart, 2012).This behaviour is thought to be highly conserved and a part of an evolutionary pathway. The characteristics of these groups of fish have been researched regarding ontogenesis (Fukuda et al., 2010), the consequences of external stressors (Brierley & Cox, 2010), and behavioural organization (Krause et al., 2000). The main advantage of shoaling is that it can protect animals from predation since it can confuse predators. In addition, a shoaling strategy provides more food resources to the group, and it can also potentiate the reproduction efficiency, since there is more access to mates (Buske & Gerlai, 2011).

However, fish inside the shoal may have to compete for resources or have less efficient in foraging behaviours when in tight groups (Miller & Gerlai, 2007). The size of zebrafish groups depends on water current and a series of other abiotic factors (Graham et al., 2018).

Other social behaviours such as social recognition are prevalent in zebrafish, and it is often characterized as the individuals' ability to recognize other individuals, and to learn to adapt their behaviours in future encounters according to past interactions. This is a vital feature of social skills in individuals, since it is necessary for territorial protection, formation of dominant hierarchies and breeding. Social recognition does not always need individual identification due to the fact that individuals classify and recognize other animals through social parameters like species, group member, family, sex, fertile state and hierarchical standing (Madeira & Oliveira, 2017). An individuals' tendency to dwell near conspecifics is known as social preference. This type of social behaviour in zebrafish is evaluated by monitoring how an individual reacts or engages with social stimulus. In addition, agonistic behaviours are also social behaviours displayed frequently by zebrafish, and they are often defined as a collection of broad, sophisticated, and species-specific actions that contributes to hierarchy's establishment. Although aggressive behaviours like chasing and biting are features of agonistic behaviour, building hierarchies does not depend on aggressive activity (Schneider, 2011). One example to study these social/ aggressive behaviours is the mirror test, which consists of presenting a mirror to the animal as stimulus. Zebrafish, just like other fish species, can display boldness through butting or biting a mirror. They also trace their own reflections while they swim in a quick back-and-forth movement. This test also shows researchers how individuals would interact with a conspecific (Kalueff & Stewart, 2012), without presenting another fish in the same tank, avoiding injuries during potential fights (Moretz et al., 2007). During the first minute of the mirror biting test, zebrafish baseline behaviour is typically characterised by periods of freezing. The animals will then gradually begin to investigate the tank, moving closer to the mirror. Most mirror biting behaviours will often happen between the third and fourth minute of the conventional six-minute test, depending on the size of the tank (Pham et al., 2012).

The characteristics of fish (sex, size, pigmentation, and stripe pattern) can have an impact in zebrafish social behaviour (Gumm et al., 2009; Rosenthal & Ryan, 2005; Ruhl & McRobert, 2005; Spence & Smith, 2007), which can also be affected by fear and/or anxiety. Therefore, the handling practices, housing conditions, chronic or acute isolation have to be carefully considered as they can impact the outcomes of research using zebrafish (Mahabir & Gerlai, 2017).

To minimize experimental errors or avoid the decrease in statistical power, it is extremely important to control the various factors that we know can influence zebrafish behaviour; this will also increase replicability within the scientific community (Mahabir & Gerlai, 2017).

1.4. Social isolation in zebrafish

The housing of a single zebrafish can be problematic because they are deprived of exhibiting their social behaviours. It has been described in the literature that social isolation can often bring consequences in terms of psychosocial stress, which can negatively impact the welfare of the isolated individuals (Holt-Lunstad & Steptoe, 2022; Kanitz et al., 2004). This is particularly serious in laboratory settings, where often the only environmental enrichment is the presence of conspecifics in their home tanks, allowing the establishment of a social hierarchy. Individuals obtain knowledge regards their social environment solely by direct engagement or indirect observations of conspecifics (McGregor & Peake, 2000). Toth et al. (2011) reported that social isolation in rats led to increased aggressiveness and a fragmentation of their behaviour, whilst other studies in rats showed that dopamine and serotonin neurotransmission levels were altered (dos Santos et al., 2010). Isolation was also tested in *Drosophila melanogaster* by Wahne et al. (2021), who concluded that it reduced sleep and affected the normal metabolism of these animals. Another study conducted in fruit flies reported that social isolation decreased their lifespan (Ruan & Wu, 2008). Social isolation has also been proven to decrease survival time in mice with liver cancer (Liu & Wang, 2005), and, when it occurs in an early life-stage, it can disrupt brain development and lead to behavioural abnormalities in later stages (Lapiz et al., 2003). In bees, isolation has been proven to decrease growth rates of sensorial and learning parts of the brain, leading to big impairments in these animals lives (Maleszka et al., 2009).

Some studies report that isolated zebrafish decreased shoaling activities, locomotor activity, anxiety and stress responses (Kerr, 1962; Parker et al., 2012). Isolated zebrafish before and/or during testing had reduced cortisol levels (Giacomini et al., 2015), lower number of proliferating cells in sensory regions of the brain (Lindsey & Tropepe, 2014), less ability to recognize novelty, and lower serotonin levels (Saif et al., 2013) compared with group housed animals. Shams et al. (2015) showed that social isolation for a long period (90 days) can change serotonin levels in zebrafish, as well as dopamine and their metabolites, whilst decreasing anxiety-like behaviours.

Due to the existence of gaps in research and ambiguous literature, there is a need to re-evaluate the housing that is employed in the laboratories that use zebrafish as an animal model, and its effects on research.

Although there are many studies reporting effects of social isolation in various species, zebrafish is still often isolated during or before experiments to allow individual identification, to recover from procedures or because isolation is the aim of the study. Scientifically, social isolation allows the identification of relevant individual behaviours (Delcourt et al., 2018); for example, the detection of less sociable animals might lead to the development of a new behavioural strain for research into the origin and treatment of autism disorders (Meshalkina et al., 2018). Furthermore, when a painful procedure is performed, such as surgery, the animal has to stay alone to monitor animals' welfare and fitness, to ensure the lack of health complications (Delcourt et al., 2018), and to avoid the potential harmful interference of a conspecific during recovery. Fin clipping is often used for genotyping, and researchers often house the animal alone while waiting the genotyping results, as they need to identify that animal. In addition, some behavioural tasks require several testing days (e.g., memory tasks with a learning curve) and the animal must be identifiable throughout the days (Al-Imari & Gerlai, 2008), thus the fish is single housed during the experiment. Surely there are several methods to identify individual fish (Reed & Jennings, 2011), but they are quite challeging for a small fish. If possible, the observation of natural marking patterns to identify a zebrafish is the best method to the animal, as it discards the need to handle and mark fish; it often happens when fish are kept in groups of four/five animals and it is possible to distinguish animals from each other solely based on colour patterns. Furthermore, there is the Visible Implant Elastomer technique, in which an elastomer material that contains a pigment is injected beneath the skin and solidifies. One disadvantage of this technique is the fact that fish need to be anaesthetised during the whole procedure (Rácz et al., 2021). Freeze branding is also an option for groups that cohabit in the same tank, where a cold needle (0ºC) is held against the animals' skin to mark the fish, although it can cause tissue necrosis and anaesthesia and analgesis should be used (Hadow, 1972) The removal of specific scales or fin clipping are other techniques also described, but anaesthesia and analgesia for the pain is also needed and the removal of scales and fins can cause wounds and create a pathway for potential infectious agents (Delcourt et al., 2018)

Therefore, isolating each animal throughout an experiment is the easiest technique to overcome the difficulty of recognizing different individuals. However, the period of isolation should be minimal, should not interfere with the experiment, and social isolation should only be adopted when no other alternative is possible (Delcourt et al., 2018).
In spite social isolation is well explored in many species, the studies in zebrafish often use extreme social isolation (a long duration, privation of all type of social stimulus, etc) (Anneser et al., 2020; Shams et al., 2015; Shams et al., 2017) and rarely study the type of social isolation routinely performed in laboratories. It is crucial to ensure that these types of social isolation does not alter physiological or behavioural profiles of the animal, such as stress (Forsatkar et al., 2017) and social behaviours (Shams et al., 2017)

1.5. Stress and anxiety-like behaviours in zebrafish

The term "stress" has been defined in different ways throughout the years. Tachè & Selye (1985) proposed that stress is defined as "the body's nonspecific response to any demand", and it is considered to be an element of the adaptation process that improves the body's ability to do a variety of tasks. The stress reaction is normal, adaptive, and helpful since these actions promote an increase in effective responses to aversions/ threats. The term "stressor" was coined to describe the stimulus or triggering component of stress (Tachè & Selye, 1985). Other describes stress as being a very individualized experience that differs from person to person, types of tasks, and depends on individual sensitivity and resilience (Fink, 2016). In fact, "stress occurs when environmental demands exceed one's perception of the ability to cope" that can be different between subjects (Fink, 2016).

Three primary interconnected systems are activated in the biological response to stress. The stressor information is first received by the brain's sensory receptors which analyse and compare the stressful event to the organism's current condition and prior stress background. The stressful stimulus activates the Hypothalamic-pituitary-adrenal axis (HPA) (or hypothalamo–pituitary–interrenal axis in fish) system: hypothalamus releases corticotropin-releasing hormone which binds to the pituitary leading to the release of adrenocorticotropin that reaches the adrenal gland (or interrenal gland in fish), initiating the synthesis and release of cortisol (Fig. 6). At the same time, Autonomic Nervous System will readily initiate the production and flow of catecholamines, noradrenaline and adrenaline, via the Sympathomedullary system.

The catecholamines will provoke an increase In blood pressure values and cardiac output, channel blood from the skin and stomach into the skeletal muscle and cause the liver to excrete glucose into the bloodstream (Fink, 2016).

Nevertheless, the specific mechanisms of glucocorticoids' protective activity are still unknown. Glucocorticoids work in tandem with adrenaline to raise blood glucose levels, providing the energy boost that is frequently required to overcome stress by aiding fight or flight mode. Glucocorticoids modulate the synthesis of prostaglandins and inflammatory cytokines, making them strong inhibitors of the immune response and inflammation processes (Fink, 2016). Increased levels of glucocorticoids act not against the cause of stress, but rather against the body's normal stress reactions, preventing such reactions from overshooting and jeopardizing the equilibrium and homeostasis. Thus, high glucocorticoid values improve stress tolerance and adaptability in the body. (Munck et al., 1984).

To sum up, there is a primary reaction that involves the release of catecholamines and cortisol into the circulation, while a secondary response involved alterations in various metabolic and physiological/ biochemical markers, for example, blood glucose, lactate, osmotic pressure, glycogen, oxidative stress status. This last parameter can be altered by the increase of the reactive oxygen species (ROS), which leads to the disruption of the cellular metabolism and regulatory pathways (Carneiro, García-Mesa, Sampaio & Planas, 2021). Finally, the tertiary response involves alterations in the whole animal, such as growth, reproduction, disease resistance and behaviour that can be adaptative or compromise survival, depending on the stressor intensity and on the capacity of the animal to respond and cope with this stressor (Barton, 2002; Barton & Iwama, 1991; Pavlidis et al., 2015).

In zebrafish, net handling, air exposure and increase in water current have been shown to induce acute stress responses in these animals, with whole-body cortisol levels peaking 15-30 minutes post-stress exposure. These procedures have also been proved to affect the expression of various genes that contribute to the regulation of the HPI (Fuzzen et al., 2010; Ramsay et al., 2009). Other identified stressors are crowded tanks leading to high density (Ramsay et al., 2006), and restraining that can also affect the overall animal welfare (Assad et al., 2020).

Often, stress not only alter cortisol levels, but also alter the state of anxiety and fear (Fink, 2016).

According to Craske et al. (2009) using Barlow's approach, "anxiety is a future-oriented mood state associated with preparation for possible, upcoming negative events"; and fear is "an alarm response to present or imminent danger (real or perceived)". An anxiety-like behaviour is characterized as being a complex behaviour provoked by dangerous or potentially dangerous surroundings and/or stimuli (Kalueff et al., 2013). It can manifest itself in various ways through multiple behaviours such as reduced exploration, geotaxis, thigmotaxis, preference for a black background, rather than a white background (Facciol et al., 2017), etc. Indeed, a new surrounding and/or stimuli or a decrease in risk-taking behaviours may reduce the general activity of the animal (Oswald et al., 2012). Fish exhibit geotaxis when it displays preference or move in the direction of the bottom of the tank, and it usually occurs when a threat is imminent (Rosemberg et al., 2011). Thigmotaxis is another anxiety-like behaviour which is a propensity for remaining near the wall of the tank, avoiding the centre open sections (Blaser & Rosemberg, 2012). Freezing can also be considered an anxiety-like behaviour since it happens when the fish is in the bottom of the aquarium and stop moving (apart from their gills and eyes). In most cases, freezing is the outcome of severe stress/anxiety or an element of submissive behaviours. When freezing is induced by stress, opercular motions (respiratory/gill movements) have usually a highly frequency; this should not be confused with immobility due to the fact that this behaviour is usually not accompanied by increased opercular movements (Cachat et al., 2011). Body colour change is an overall alteration in the body's pigmentation which often results in darker or lighter appearance and can also be an indication of anxiety (Zhang et al., 2010). Lastly, erratic movements can also be characterized as anxiety-like behaviours. These movements are drastic alterations in direction or speed, as well as frequent sudden darting. Several darts (quick speeding bursts in which the direction of the movement also changes in an apparently odd manner between the rapid darts) are generally reported in adult zebrafish, and acute stressors (predator exposure, warning signal release) or a general baseline of an anxious/fearful state usually elicit this type of response (Egan et al., 2009); (Kalueff et al., 2013).

One of the most used tests to assess anxiety-like behaviour in zebrafish is an open tank diving test, usually called novel tank. It enables individuals to demonstrate a wide range of natural and spontaneous behaviours alone during a short observation time. In the beginning it is expected that the animals bottom dwelled, as the threats may come from the surface (Kalueff et al., 2013). Nevertheless, the wide variety of behaviours elicited by this test may be a drawback for high-throughput assessments, as it takes some time to analyse, and the intra-group behavioural variation has to be taken in consideration(Kalueff & Stewart, 2012).

Nevertheless, it provides more information about the behavioural profile of the animal, which can be an advantage for more complex studies.

The white/black box or tank test has also been used in zebrafish as a simple and efficient task to assess anxiety-like behaviours (Kalueff et al., 2013). Intra-group variability is lower, and the stimuli used in the test (lighting system, colours of the tank walls, etc) are a lot easier to control and change if needed. However, it does not replace the novel tank test since white avoidance and vertical exploratory activities do not share the same pathways (Kalueff & Stewart, 2012).

2. AIMS OF THE STUDY

Like mentioned before, social isolation is often used in zebrafish research as a method for individual identification or when the fish is recovering from some procedure. However, the data available regarding the effects of short social isolation is rather scarce and contradictory. As referred above, some articles report that there are significant physiological differences, but others do not, depending on the methodology. There are some behavioural studies in this field, but oxidative stress assessment is rather scarce. Therefore, we intended to do an experiment that portrays the isolation conditions in which fish are maintained when they need to be separated for individual identification and evaluate the physiological and behavioural alterations of these fish. Thus, the main aim of this study is to understand if social isolation often performed in zebrafish facilities can influence their behaviour and physiological parameters and assess if socially isolated fish react as the grouped fish when exposed to an acute stressor. More specific aims are:

Task 1

- To establish and validate an acute stress protocol for adult zebrafish.
- To study the effects of social isolation on physiological parameters (cortisol, oxidative stress).
- To assess if social isolation alters the physiological response to an acute stressor.

Task 2

- To assess if socially isolated fish display any abnormal behaviours related with activity, anxiety, and social aggression (novel tank test, white/ black tank test, and mirror test).

3. MATERIALS AND METHODS

Ethical statement

All procedures were carried out under personal licenses approved by the Direção-Geral de Alimentacão e Veterinária, and a project license approved by the Animal Welfare and Ethics Review Body of the i3S. Thus, all procedures were performed according with the European Directive 2010/63/EU on the protection of animals used for scientific purposes, and its transposition to the Portuguese law, ''Decreto Lei'' 113/2013.

3.1. Animals and housing

Ninety-two adult mixed-sex AB zebrafish (*Danio rerio*) (seven to nine months old for the main study, weighing around 0.300 grams ± 0.100) bred in the Animal Facility of i3S were used. They were housed in a recirculating water system connected to a central unit of water purification and controlled conditions - temperature 27 ± 0.5 °C, pH of 7 ± 0.5, conductivity of 800-815 µS, in a facility with a 14:10h light:dark cycle. Adult zebrafish were housed in groups in 3.5L barren tanks (5-8 fish/ litre) before the experiment and were fed three times a day with a standardized commercial diet (Zebrafeed, SPAROS).

3.2. Task 1 – Physiological assessment

3.2.1. Establishment and validation of an acute stress protocol

To study the effects of social isolation on the stress response of zebrafish, i.e., on the cortisol levels, we had to make sure to use a validated stressor; thus, a valid acute stress protocol was established through a pilot test. The two protocols tested were based on Pavlidis, Theodoridi & Tsalafouta (2015).

Both protocols comprised two groups: the control, that was not exposed to an acute stressor and was only subjected to the normal routine of the facility; and the experimental group, that suffered an exposure to an acute stressor. A randomization between groups was done using an online randomization tool (https://www.random.org). In the end, to validate the efficacy of the acute stressor protocol, the experimental group should have a significantly higher cortisol levels than the control group.

We used surplus animals from the colony to maintain the number of zebrafish used to a minimum (Reduction). All animals were feed restrained 24h prior the experiment.

For the first protocol, we used 18-months old fish (mixed-sex), weight ranging from 0.29-0.47 g, 10 animals per group. First, both tanks were transferred to the procedure room for habituation 30 minutes prior to the test. For the acute stress group, the animals were transferred from their home tank (3.5L) to a new 3.5L tank with 2L of system water. Chasing was then initiated with a net, always following the fish as quickly as possible during five minutes. After chasing, fish were netted and exposed to air for 30 seconds, covering the net with a lid to prevent the fish from jumping. Then the fish was transferred to a 1-L tank to recover from these procedures. After 20 minutes, the fish were transferred to a new container with cold water at 1-4ºC and euthanised with the rapid cooling technique. Sex identification was done, and females had their eggs removed by squeezing the females' belly in an antero-posterior direction. A method of confirmation of euthanasia was done as well – decapitation. After this, the body of the fish were put inside the respective falcon tubes and frozen at -20ºC. For the control groups, individuals were simply transferred from their home tank to a new tank for euthanasia using rapid cooling, followed by decapitation. All the fish bodies were collected in falcons and stored at -20ºC until further analysis to quantify the cortisol (this methodology is described below).

For the second protocol, 12 (n= 6) 10 months old zebrafish weighing around 0.25-0.36g living in two tanks were used. Both tanks were transferred into the procedure room at the same time, but the control animals did not have a habituation period and were euthanised right away through rapid cooling, as described before; all these six fish were put in cold water at the same time. As before, sex identification was done, and females had their eggs removed through squeezing, followed by decapitation and storage of the bodies in their respective falcon tubes at -20ºC. The tank housing the acute stress group went through an approximate 30-minute habituation period. These animals were transferred from their home tank (3.5L) to a new tank with only 1L of system water. Chasing was then initiated as described before but only for 2.5 minutes. Then, the air exposure procedure was performed for 60 seconds. After this, the fish were transferred to a 1L tank with clean system water and remained there undisturbed for 20 minutes. Then, fish were again transferred to a new tank with cold water and euthanised using the rapid cooling technique. Sex identification was also done, and females had their eggs removed as described before. Decapitation was done and the animals' bodies placed in falcon tubes and stored at -20ºC to quantify cortisol later.

3.2.2. Effects of social isolation on zebrafish physiology

To assess if social isolation could affect responses to acute stressors, we used a tank with 20 mixed-sex zebrafish, around six months old. From these animals, 10 fish were transferred individually into new tanks with 1.1L, where they stayed for 15 consecutive days - the social isolation period. The isolation tanks were placed in a rack allowing them to visualize their conspecifics in the other tanks. Here, five fish were part of an isolated non-stressed group (INS), which means that they were not exposed to an acute stressor after an isolation period. The five remaining fish were exposed to an acute stressor after the 15-day isolation period (Isolated stressed - IS). For the control tank, 10 fish remained inside their home tanks: five of those fish were exposed to an acute stressor (Control stressed – CS) after the 15 days, whilst the other five did not go through this exposure to stress (Control non-stressed – CNS) and were euthanised right away. This was replicated with other batch of animals, thus, in the end, there were 10 animals per INS, IS, CS, and CNS group. There were 27 males and 13 females throughout the experiment, leaving us with a 2:1 ratio.

3.2.2.1. Acute stress protocol

The reasoning for the choice of the acute stressor protocol assessed in a pilot test is described in the results. The control group did not go through a habituation period, and once they entered the experimental room, they were immediately euthanised by rapid cooling (second protocol tested). Regarding the acute stress, the animals did a 20-minute habituation period, followed by placing them in a tank with 2L where they were chased with a net for five minutes. Then, the air exposure lasted 30 seconds. After the acute stress exposure, fish were left individually undisturbed for 20 minutes for recovery (first protocol tested). After recovery, all fish were euthanised through rapid cooling, sex identified, and females' eggs were removed as before. After decapitation, the bodies were collected in falcons with phosphate buffered saline (PBS, pH 7.4, Termofisher Scientific, Gibco™, USA) for cortisol assessment and the brains were dissected, placed in PCR Eppendorf tubes, and fast-frozen with liquid nitrogen for biochemical analysis. The tubes containing the animals' bodies were stored at -20ºC, and their brains were stored at -80ºC.

3.2.2.2. Cortisol assessment

The cortisol measurement method began by unfreezing the body samples and preparing the FastPrep tubes (2 mL) with 500 µL of PBS in each tube and named them with the respective falcons' labels. We transferred the bodies of the fish to these FastPrep tubes and cut their bodies with an ophthalmology scissor. The scissor was disinfected with alcohol and cleaned with PBS between tubes. Furthermore, five spheroid stainless steel beads (3 mm) were placed inside each tube for homogenization in the FastPrep-24™ Classic bead beating grinder and lysis system (MP Biomedicals; velocity 6 meters per second; time: 60 seconds; runs: 1). After homogenization, 750 µL of methanol were added to each tube, and all tubes were placed in a lab roller (24 rotations per minute; Mini Lab RollerTM Dual Format Rotator, Labnet) for further homogenization for 17 hours (overnight). Next day, the tubes were centrifuged for 10 minutes (10 000 xg) at 4ºC using an Eppendorf 5415R Refrigerated Centrifuge, followed by the transfer of the supernatant to a new Eppendorf tube. After all the tubes had their supernatant removed, the Eppendorf's were placed in an evaporator machine (Thermo Scientific™ Savant™ SPD121P Speed Vac) at 35ºC for four hours. After all the supernatant was evaporated, we added 500 µL of PBS to each tube and left them incubating in a refrigerator at 4ºC overnight. The following day, we added 500 µL of hexane to each tube and froze the tubes for 15 minutes (-20ºC). Furthermore, we discarded all the transparent top part and proceeded to assess cortisol levels according to the Salimetrics ® Cortisol Enzyme Immunoassay Kit (#1-3002), using spectrophotometry (Biotek® Synergy 2 microplate reader). In the end, 1 µL of the remaining samples were placed in the Nanodrop One (Thermofisher) to quantify the protein levels in each sample. Cortisol levels are expressed as pg/mg of protein for each animal.

3.2.2.3. Oxidative stress biomarkers and acetylcholinesterase assessment

Here we used the brains of the animals previously euthanised, like mentioned before, from the groups INS, IS, CS, and CNS (n= 5). Samples were homogenised in a Tissuelyser II (Qiagen, Hilden, Germany) in 500 µL cold buffer (0.32 mM sucrose, 20 mM HEPES, 1 mM MgCl2, and 0.5 mM phenylmethyl sulfonylfluoride (PMSF), pH 7.4) (Deng et al., 2009). After homogenization, protein was quantified spectrophotometrically (PowerWave XS2 /Take 3, Bio-Tek Instruments, USA) at 280 nm.

After this, aliquots were recovered for lipid peroxidation and protein carbonyls quantification and samples were centrifuged for 15 minutes at 10 000 xg at 4ºC (Prism R C2500-R - Labnet International, USA). Subsequently, supernatant was retrieved and transferred into new tubes that had been previously identified, and protein quantified as described earlier. This was then used for the following measurements.

Reactive Oxygen Species (ROS)

Twenty µL of sample were pipetted into each well on a 96-well plate followed by the addition of 100 µL of phosphate buffer with a 7.4 pH, and 8.3 µL of 2',7'-dichlorofluorescein diacetate (DCFH-DA) at 10 mg/mL. The ROS was assessed by reading DCF excitation at 485 nm and emission at 530 nm. Quantification was done using a DCF calibration curve (0-100 µM) (Deng et al., 2009).

Superoxide dismutase (SOD)

Using a 96-well plate, 10 µL of sample were pipetted into each well. Followed by this, 170 µL of potassium phosphate buffer at 50mM and pH 7.4 containing 0.6 mM hypoxanthine, 1 mM EDTA and 0.2 mM nitroblue tetrazolium (NBT) were also added to each well using a multichannel micropipette. The plate was then inserted in the plate reader and after two minutes, 20 µL of xanthine oxidase 40x diluted in PBS at 50 mM and 1 mM EDTA were added. Subsequently, the plate was read again for three more minutes at 560 nm to detect the photochemical reduction of nitroblue tetrazolium. Quantification was done using SOD standard curves (0-60 U/mL) (Durak et al., 1993).

Catalase (CAT)

On a 96-well plate 10 µL of sample were added to each well using a micropipette. This plate was read for two minutes at 240 nm and then, 90 µL of sodium buffer at 100 mM and pH 7.4 containing 20 mM H_2O_2 were also added to each well using a multichannel micropipette. The plate went in the reader again and stayed there for three more minutes for readings at 240 nm to detect the decomposition of hydrogen peroxide. Quantification was done using CAT standard curves (0-6 U/mL) (Greenwald, 2018).

Glutathione peroxidase (GPx)

Ten µL of sample were added into each well of a 96-well plate, followed by the addition of 200 µL of PBS at 100 mM and pH 7.0 containing 1 mM EDTA, 0.5 mM NaN_3, 0.120 mM NADPH, 2 U/mL glutathione reductase and 2 mM of reduced glutathione (GSH) to each well using a multichannel micropipette. The plate was then read at 340 nm wavelength for two minutes, and 10 µL of 1.5 mM H_2O_2 was added to each well. The plate was then inserted again and read for three more minutes. The activity of GPx was based on the extinction of NADPH (oxidation) at 340 nm using the extinction coefficient of 6.22 mM/ cm (Massarsky et al., 2017).

Glutathione Reductase (GR)

Ten µL of sample, 200 µL of 50 mM potassium phosphate buffer, 0.5 mM EDTA at a pH of 7.0 and 0.3 mM NAPDH were added to each well on a 96-well plate. The plate was put in the plate reader for two minutes, and 20 µL of GSSG at 10 mM were added to each well. The plate went to be read again for three more minutes at 340 nm to measure GR activity by the reduction of NADPH, using the extinction coefficient of 6.22 mM/ cm (Massarsky et al., 2017).

Glutathione S-transferase (GST)

In a 96-well plate, 10 µL of sample were added to each well, followed by the addition of 180 µL of potassium phosphate buffer at 100 mM, pH of 7.4 containing 1mM of 1-chloro-2,4-dinitrobenzene. The plate went to be read in the plate reader for two minutes at 340 nm. Fifty µL of GSH were then added at a 25 mM concentration to each well and then the plate was read again for three more minutes to estimate the activity of GST based on the formation of glutathione-dinitrobenzene using the extinction coefficient of 9.60 mM/cm (Habig & Jakoby, 1981).

Glutathione in a reduced form (GSH)

Ten µL of sample were added into each well of a 96-well plate using a micropipette. Followed by this, 190 µL of sodium phosphate buffer at 100 mM at pH 8.0 were added to each well using a multi-channel pipette and 20 µL of OPT (O-Phtaldehyde) at 1 mg/mL. Then, the plate went into incubation for 15 minutes at room temperature. Readings were performed at 320 nm and 420 nm for excitation and emission, respectively, on a Cary Eclipse spectrofluorimeter (Varian, USA), and quantifications were done using a calibration curve of GSH at 0-1000 µM (Misra & Niyogi, 2009).

Glutathione in an oxidized form (GSSG)

Using a 96-well plate, 10 µL of sample were pipetted into each well, followed by 4 µL of N-ethylmaleimide at 0.04 M. Then, after 30 minutes of incubation, 182 µL of NAOH at 0.1 M concentration was added. Followed by this, each well had 20 µL of OPT added at 1 mg/mL. Incubation was done for 15 minutes at room temperature. Readings were done as described for GSH, and quantification was done based on a calibration curve of GSSG with 0-1000 µM (Misra & Niyogi, 2009).

Thiobarbituric acid reactive substance (TBARS)

Ten µL of sample were pipetted into each well on a 96-well plate, followed by 70 µL of H_20, 50 µL of phosphate buffer at 50 mM at a 7.4 pH. Furthermore, 10 µL of butylated hydroxytoluene were added at a 1 mM concentration, and 75 µL of TBA 1.3% and 50 µL of trichloroacetic acid 50% were also pipetted into each well. The plate was incubated for 40 minutes at 60ºC and cooled in ice for 15 minutes. After cooling, 10 µL of SDS 20% was added. A calibration curve was done using 0-500 µM malondialdehyde for quantification, and the plate was read at 530 nm and 600 nm (Wallin et al., 1993).

Carbonyls

First, we pipetted 20 µL of sample into each plate well, followed by the addition of 20 µL of 10 mM dinitrophenylhydrazine. After this, we put the plate on a vortex and incubated the plate and samples for 10 minutes in a dark room at room temperature. Furthermore, we added 10 µL of NAOH 6M to the wells, followed by another 10-minute incubation. Samples were then read at 450 nm (Mesquita et al., 2014)

Lactate Dehydrogenase (LDH)

At the beginning, 10 µL of sample were pipetted into each well, followed by the addition of 200 µL of NADH at a 0.24 mM concentration. The reaction was initiated by the addition of 40 µL of sodium pyruvate at 10 mM to assess the oxidation of NADH to NAD+ with 6.22 mM/cm as the extinction coefficient. Readings were done for three minutes at 340 nm (Domingues et al., 2010).

Acetylcholinesterase (AcHE)

Ten µL of sample were pipetted into each well on a 96-well plate. 180 µL of 5,5′-dithiobis(2-nitrobenzoic acid) at 0.5 mM were also added, and the reaction began when 10 µL of acetylthiocholine iodide 20mM were added into each well. Readings were done for three minutes at 405 nm (Rodríguez-Fuentes et al., 2015).

3.3. Task 2 – Effects of social isolation on zebrafish behaviour

For the behavioural assessment we wanted to understand if there were any behavioural alterations induced by 15-days social isolation. In this task, we used 20 seven to nine-months old AB mixed-sex adult zebrafish - 10 were subjected to isolation for 15 days whilst the other 10 remained in their home tank, being the control group. One week prior to the isolation starting day, we separated the fish from the home tank (N=20) into three different tanks – one with ten fish, that would later be the control group and two tanks with five animals each. This was done to ensure that control groups did not suffer any kind of alteration due to the tank density reduction, having 3 weeks of habituation. To start the isolation period, the animals were individually placed in 1.1L tank in the same recirculating water system (Fig. 7). As before, the isolated animals could see their conspecifics. All animals had their tanks cleaned during the isolation period two times a week. Here we had 12 males and 8 females, which means we had a 3:2 male:female ratio.

Fig. 7- Illustration of how the animals were isolated in our facility.

After the 15-day isolation period, the animals were subjected to three behavioural tests in three consecutive days (one on each day): the white/black test, followed by the novel tank, and the mirror biting test in the last day. The tests were presented in a crescent order of potential stress induction. The order by which each animal was tested in each behavioural task was randomly determined using the online tool https://random.org.

The water used in the tests was from the same recirculating water system where the fish lived. The testing water was replaced between animals, and the apparatus used for each task was cleaned with a Virkon solution to disinfect and eliminate any residues or odours that could influence the next animal to be tested. In the end of all the tests, the animals were placed back into their original tank and kept for breeding.

3.3.1. White/black test

This test is based on the preference of adult zebrafish for darker backgrounds, and their avoidance for white ones (Facciol et al., 2019); an interference on this behavioural pattern may indicate alterations on anxiety levels. This test consisted of an acrylic tank with two equally divided zones: one black and the other white (floor and walls); a camera was placed above each tank to record the sessions. The tank was filled with 2L of clean system water. The water was exchanged between animals to ensure that no residues were affecting the following fish to be tested.

We used artificial lighting to ensure that no shadows appeared on video and each tank had their luminosity measured using the app “Smart luxmeter” (Smart tools®). We had two tanks being tested at the same time; thus, the apparatuses were on different parts of the room (Fig. 8).

Fig. 8- Illustration of how we conducted the white/black test in our facility. Two lamps were used to illuminate the room without causing shadows inside the apparatus.

The first apparatus had the black side with 32 lx, and the white side had 42 lx. The second apparatus had 48 lx in the black side of the tank, and the white side had an average of 60 lx. Each fish was placed individually in the white side of the tank and allowed to swim freely between both sides for six minutes. All animals had a habituation period of one hour in the procedures’ room before the start of the test. After the animals were exposed to the test, all returned to their home tank (control group) or to their isolation tank (isolation group). Here we measured several key behaviours such as time spent in the white and black compartment, attempts to enter the black side of the tank, latency to enter the black compartment and to re-enter in the white compartment, and the number of entries to the white side. We consider that the animal entered in one of the compartments when the whole body of the fish is inside of that part of the tank and stayed there for more than three video frames.

We also defined attempts to enter the black side of the tank as a moment where the fishes' bodies entered the black compartment for less than three video frames, and then turned back to the white part of the tank. The video analysis was conducted using the event logging software Ethowatcher (Universidade Federal de Santa Catarina, Santa Catarina, Brazil) (Crispim Junior et al., 2012).

3.3.2. Novel tank test

This test is the most used to test anxiety-like behaviours in zebrafish, consisting in placing the animal in a new environment; in the first minutes, the animal is expected to spend most of the time in the safest zone, the bottom of the tank (Cachat et al., 2010) Then, habituation is expected to occur, decreasing the responses to novelty overtime. One hour prior to the test, animals were transferred from the holding room to the experimental room. As a novel tank, we used a trapezoidal tank (3.5L tank, Tecniplast) with no lid and with a column of water 12 cm height. To start, the fish was netted and gently placed on the bottom of the tank. Then, the animal was allowed to explore the tank for six minutes, during which a camera placed on the side of the tank was recording the test (Fig. 9).

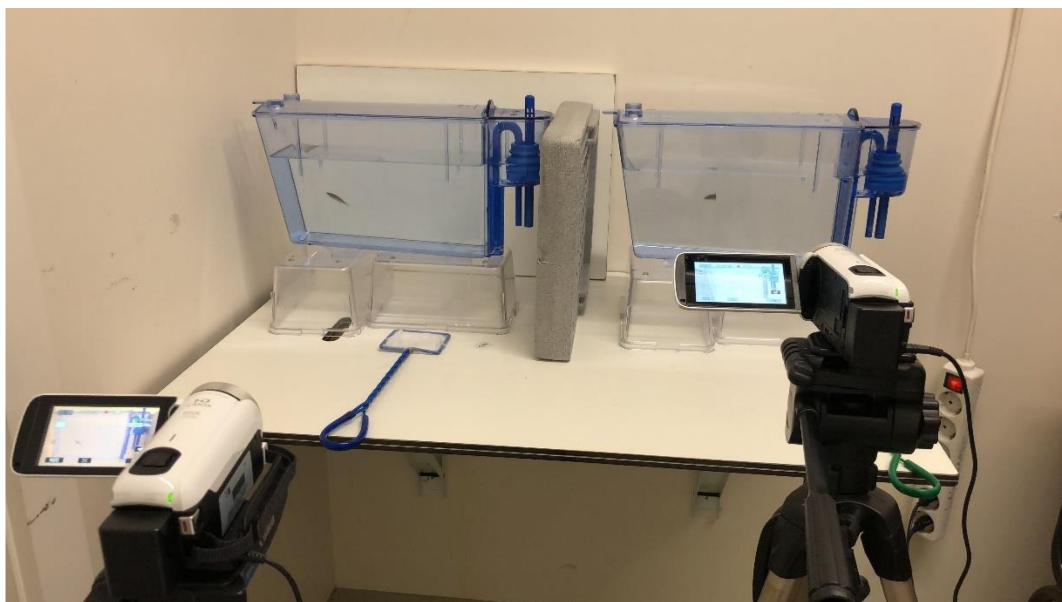

Fig. 9- Illustration of how we conducted the novel tank test in our facility.

After the recordings, control animals returned to their home tanks and isolation animals returned to their respective isolation tanks.

In this test we used the automated video-tracking software AnyMaze (Stoelting), where the tank was virtual divided horizontally into two equal zones (up and bottom). Here, we measured the total distance travelled by the fish inside the tank, the average speed, the maximum speed, the angular velocity (absolute turn angle/ time), the time and frequency of speeds above 0.07m/s, the freezing episodes and immobile episodes, and duration of immobility. Immobility is when the animal is standing still in one location but still perform other types of movements, such as moving their fins, for example. Freezing is a cessation of all movement in the body, except for breathing. These variables were also measured for each zone of the tank (upper and bottom part), except angular velocity. In addition, the number of entries in each zone, and the latency to enter the upper part of the tank was recorded. These variables were analysed considering the total duration of the test (six minutes). To study habituation to the novel tank, the number of entries, time spent, and distance swam in the bottom and upper part of the tank were also analysed each three minutes of interval.

3.3.3. Mirror biting test

A mirror image stimulation is often used to study zebrafish social/aggressive behaviour (Cachat et al., 2010). Here we assessed if social isolation could trigger abnormal agonistic behaviours, using mirror biting test behaviours as indicators of the animal behaviour towards a conspecific. All animals were transferred from the holding room to the experimental room one hour prior to the beginning of the test. For this behavioural task we had a custom mirror for the tank, and we also built in a 3D printing software (Autodesk Fusion 360) a custom piece to hold the mirror in place. The mirror was placed inside the tank at around 5cm from the filter. We used two cameras – one for a side and another for a top view.

We first set up a 3.5L trapezoidal tank (Tecniplast) with the mirror held by the 3D printed piece, and a 12cm water column. After this, we introduced the fish into the tank on the opposite side of the mirror and started recording for six minutes (Fig. 10). After the recording, all animals returned to their home tank, and the IS animals no longer remained in isolation.

Behaviours like time spent in the bottom portion of the tank, freezing frequency and duration, latency to the first approach, approach frequency and duration, latency to first mirror contact, mirror contact frequency and duration, frequency of mirror bitings, and number of aggressive tail beats were measured.

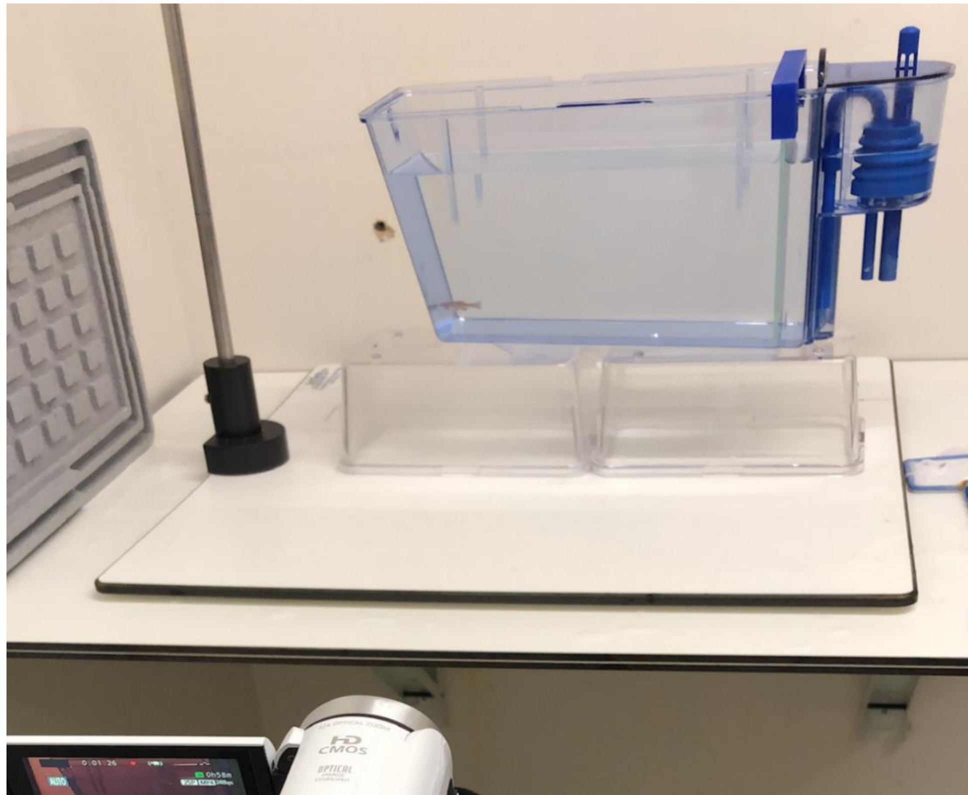

Fig. 10- Illustration of how we conducted the mirror biting test in our facility.

We defined approach as a moment where the whole body of the animal had crossed the approach line (at 2.5 cm from the mirror) and stayed inside the approach zone. Contacts were defined as the moment the animal crossed the contact line (at 0.5 cm from the mirror) and stayed inside the contact zone, where other behaviours like mirror biting and tail beats could occur. Mirror bitings were recorded when the mouth of the fish touched the mirror, often moving in contact with the mirror with an up and down or side-to-side movement; whilst tail beats occurred when the animals were in the contact zone, they turned around, beat their tails against the mirror, and then returned to the mirror, doing a 360º turn. AnyMaze Software was used to analyse the variables previously referred, except the mirror biting and aggressive tail beats events which were measured manually using the event coding software BORIS (Friard & Gamba, 2016).

3.4. Statistical Analysis

All data were analysed in terms of normal distribution (Shapiro-Wilk test) and homogeneity of variances between groups using the Levene's test. When normal distribution and homogeneity of variances were satisfied, we used parametric tests such as Students' t-test and the two-way analysis of variance (ANOVA) test for comparisons between two or more groups, respectively. When normality was not attained, we conducted a logarithmic transformation of the data.

If the data did not reach a normal distribution even after this transformation, we conducted a non-parametric Mann-Whitney test or a Kruskal-Wallis test for differences between two or more groups, respectively. Normally distributed data are expressed as mean± standard deviation and nonnormally distributed values as median [interquartile range]. Next, there is a more detailed description of the statistical tests used for each variable.

To assess differences between groups for cortisol levels, first we tested differences between sexes within each group using an independent Student's t-test, as not all the sexes were represented in all the groups. The sex did not contribute to any difference on cortisol; thus, this factor was not included in our model. Then, two-way ANOVA followed by the adequate post hoc test (Tukey's) was used; the group was the fixed factor, and the batch the random factor. The batch was the different weeks/ time of testing. For the establishment of the acute stress protocol, differences between groups regarding cortisol (stressed vs not stressed) were assessed with the independent Students' t-test.

For multiple comparison analysis of oxidative stress parameters between groups, we performed a two-way ANOVA followed by a Tukey post-hoc test with group and sex as fixed factor and batch as random factor.

For the white-black test statistical analysis we did a two-way ANOVA with group and sex as a fixed factor, and position of the apparatus in the room (two levels) as a random factor. We also did dependent t-tests to compare the time spent in the white and black compartments within each group. For one variable, latency to enter the black compartment, we performed a Mann-Whitney non-parametric test, due to the lack of normal distribution.

For the overall parameters of the novel tank, we performed a two-way ANOVA (with group and sex as fixed factors) or a Mann-Whitney nonparametric test to compare the variables between the two treatment groups, when normal distribution was not met. When comparing the parameters assessed between the top and bottom zone in each group, dependent Student's t-test was used. The same test was performed to compare time, distance, and entries in both compartments between the first three and the last three minutes of the test.

For the mirror biting test, group and sex were considered as fixed factors to the two-way ANOVA test for all the variables, except for the freezing frequency, in which we applied a Mann-Whitney test because the data did not have normal distribution.

Statistical significance was set at 0.05, considering two-tailed hypothesis. Data were analysed using SPSS 26.0 (IBM SPSS Statistics 26 Software; IBM Corp, Armonk, NY) for oxidative stress, cortisol, novel tank and mirror biting data and InVivoStat; UK (Clark et al., 2012) for the white-black test analysis. Graphical representations were created using GraphPad Prism 7 for Windows (Graphpad, Inc., San Diego, CA)

4. RESULTS

Detailed statistical results regarding comparisons between groups can be consulted in the annex I.

4.1. Task 1 – Physiological assessment

4.1.1. Establishment and validation of an acute stress protocol

To establish the acute stressor, we used two different protocols for validation by measuring whole-body cortisol levels. For the first protocol, there were statistically significant differences between control (1.84 pg/mg protein ± 0.48) and acutely stressed (5.61 pg/mg protein ± 0.78) group [t(18)=4.058, p<0.001]. For the second approach, we also found significant differences between control (0.53 pg/mg protein ± 0.13) and acutely stressed (6.32 pg/mg protein ± 2.01) group [t(10)=2.881, p=0.0163] (Fig. 11).

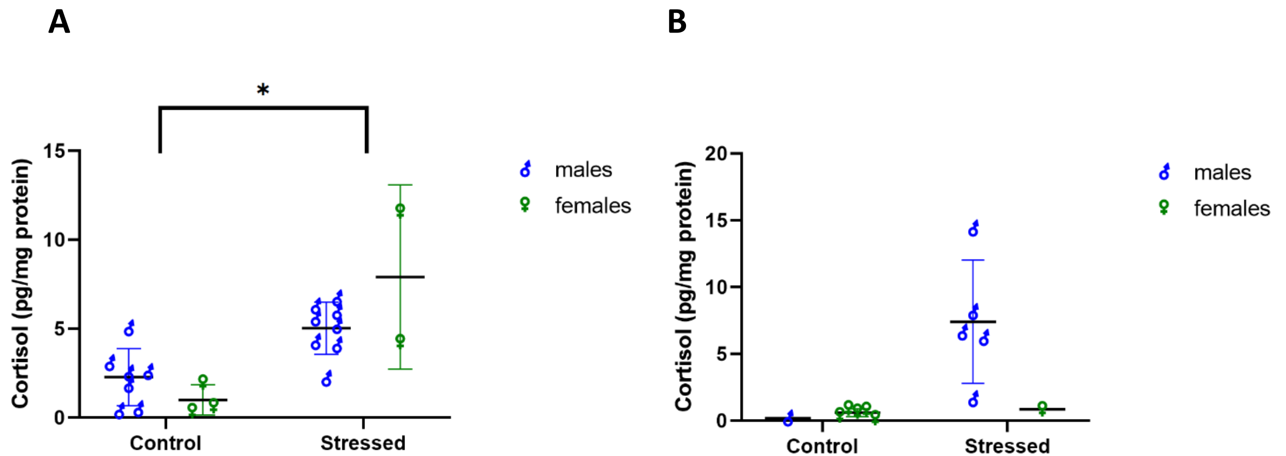

Fig. 11- Levels of cortisol (pg/ mg of total animal protein) measured in the whole-body of adult zebrafish from different groups: Control (WB CTRL, animals not subjected to an acute stress) and Stressed (WB STRESSED, animals exposed to one of the protocols of acute stress tested). A) cortisol levels of the animals subjected to the first protocol; B) cortisol levels of the animals subjected to the second protocol. Raw data is presented here as mean± standard deviation; each point represents an animal. *p< 0.001 for comparisons between control and stressed groups in the first protocol (A) (n= 10); *p=0.0163 for comparisons between control and stressed groups in the second protocol (B) (n= 6). Both data were analysed with an independent Students' t-test. In the protocol (A) there were n=7 males and n=3 females for the control group and n=8 males and

n=2 females for the stressed group; in the second protocol there were n=1 males and n=5 females for the control group and n=5 males plus n=1 females for the stressed group.

Thus, for the control group, we choose to use in the main experiment the protocol with the lowest cortisol levels, thus closer to the baseline- second protocol- where animals were placed in the experimental room and immediately euthanized. As both protocols induced stress to the animals, we choose the first acute stress protocol that use a shorter period of air exposure, minimizing zebrafish suffering.

4.2. Effects of social isolation on zebrafish physiology

4.2.1. Cortisol levels assessment

Here, in terms of cortisol levels there were no differences between sexes within each group, so this factor was not considered in the main analysis ($p>0.05$). Our results showed that control animals and isolated animals that were not exposed to acute stress had significantly lower values of cortisol than the ones that were exposed to our stress protocol. Control Stressed group (9.93 pg/mg protein ± 8.43) displayed significantly higher cortisol levels compared with Control Non-Stressed group (1.42 pg/mg protein ± 0.87) ($p< 0,001$), and with Isolated Non-Stressed group (4.16 pg/mg protein ± 4,97) ($p= 0.006$). In addition, Isolated Stressed group (8.96 pg/mg protein ± 4.97) also had higher cortisol levels compared with Isolated Non-Stressed group ($p= 0.009$) and with Control non-stressed group ($p< 0.001$) (Fig. 12).

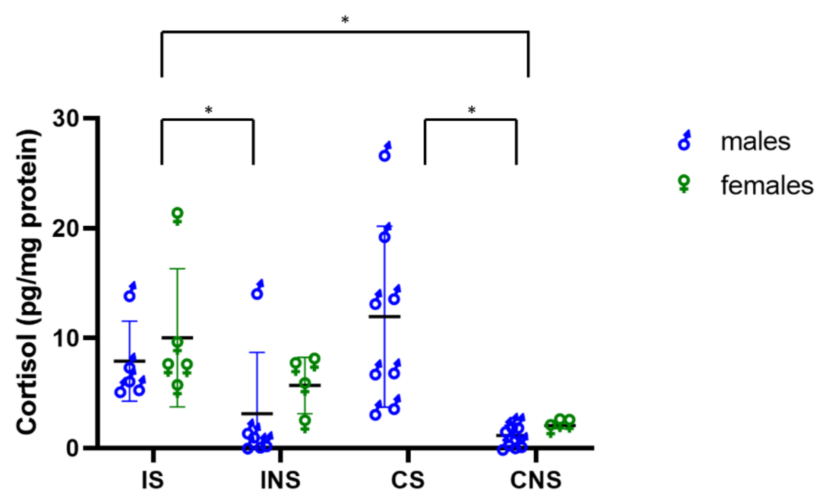

Fig 12- Levels of cortisol (pg/mg of total animal protein) measured in the whole-body of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation), isolated non-stressed (INS, isolated for 15 days), control stressed (CS, exposed to acute stress) and control non-stressed (CNS). The raw data is presented here as median and

5th to 95th data percentiles; each point represents an animal; n= 10. *p<0.001 for comparisons between CNS and CS and between CNS and IS; *p= 0.02 for comparisons between INS and IS; *p= 0.006 for comparisons between INS and CS; data were analysed with one-way ANOVA followed by Tukey's post hoc test in log-transformed data. CNS group: Females: n=3, Males: n=7; CS group: Females: n=0, males: n=8; INS: females: n=4; males: n=8; IS: females: n=5, males n=5.

No other significant differences were detected between groups regarding this variable ($p > 0.05$).

4.2.2. Reactive Oxygen Species (ROS) quantification

For the Reactive Oxygen Species assessment, the two-way ANOVA test detected no significant differences between groups, sexes, or any interaction between these two factors ($p < 0.05$) (Fig. 13).

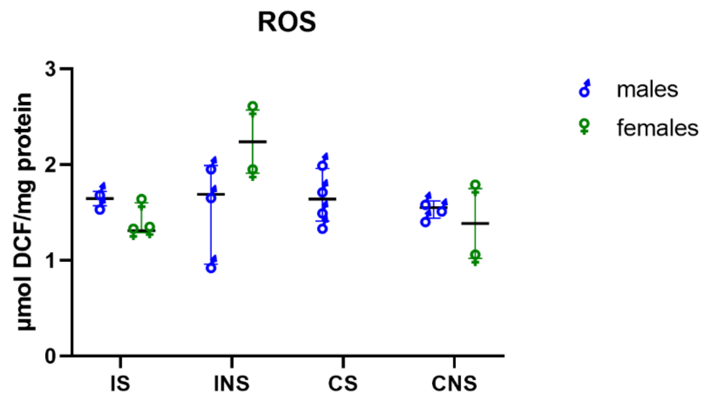

Fig 13- Levels of Reactive Oxygen Species (µmol DCF/mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation), isolated non-stressed (INS, isolated for 15 days), control stressed (CS, exposed to acute stress) and control non-stressed (CNS). The raw data is presented here as mean± standard deviation; each point represents an animal; CNS group: Females: n=2, Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

4.2.3. Superoxide dismutase (SOD) activity

This analysis showed that the activity of SOD was similar in all the groups tested (Fig. 14), thus there were no differences between the levels of this factor, nor between the levels of the factor sex.

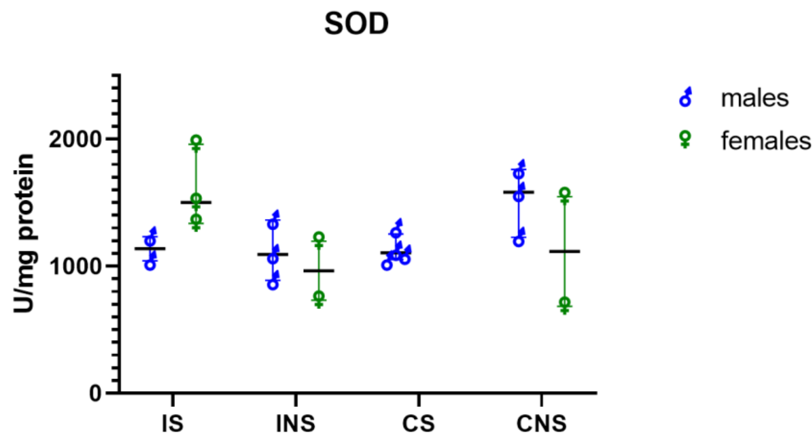

Fig 14- Activity of Superoxide Dismutase (U/mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation), isolated non-stressed (INS, isolated for 15 days), control stressed (CS, exposed to acute stress) and control non-stressed (CNS). The raw data is presented here as mean± standard deviation; each point represents an animal; CNS group: Females: n=2, Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

Also, factor sex and group did not have any interaction regarding SOD activity.

4.2.4. Catalase (CAT) activity

For the assessment of catalase activity, we used the same statistical tests as above, which showed no differences between the levels of the factors (group or sex), and no interaction between these factors was found (Fig. 15).

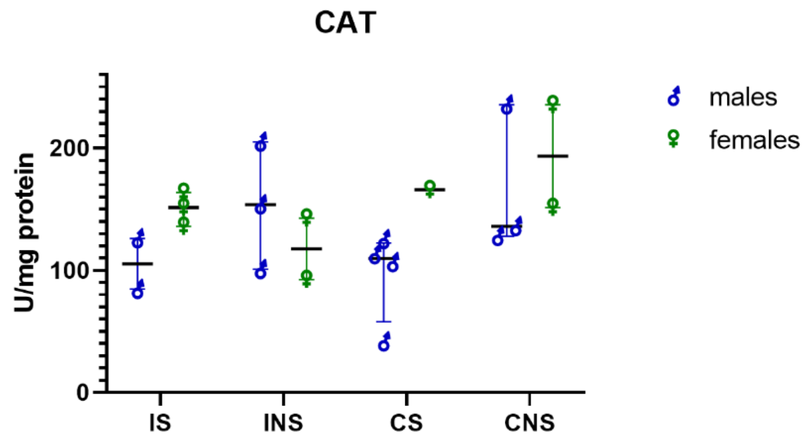

Fig 15- Activity of catalase (U/mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation), isolated non-stressed (INS, isolated for 15 days), control stressed (CS, exposed to acute stress) and control non-stressed (CNS). The raw data is presented here as mean± standard deviation; each point represents an animal; CNS group: Females: n=2, Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

4.2.5. Glutathione peroxidase (GPx) activity

No statistical difference was found between groups nor between sexes for the glutathione peroxidase activity (Fig. 16).

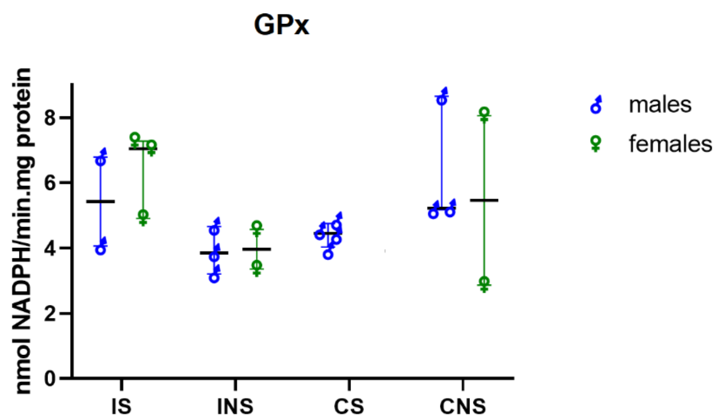

Fig 16- Activity of glutathione peroxidase (nmol NADPH/min.mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation), isolated non-stressed (INS, isolated for 15 days), control stressed (CS, exposed to acute stress) and control non-stressed (CNS). The raw data is presented here as mean± standard deviation; each point represents an animal; CNS group: Females: n=2,

Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

Also, there was no interaction between group and sex factor.

4.2.6. Glutathione Reductase (GR) activity

The activity of glutathione reductase was not different between sex, and no interaction was detected between the factor sex and group. However, there were differences between groups (p= 0.028). The IS group (10.36± 3.13 nmol NADPH/min.mg protein; p= 0.039); the INS group (9.65± 4.076 nmol NADPH/min.mg protein; p= 0.021); and the CS group (7.17± 1.21 nmol NADPH/min.mg protein; p= 0.009). revealed a lower GR activity when compared with the CNS group (27.90± 7.87 nmol NADPH/min.mg protein).

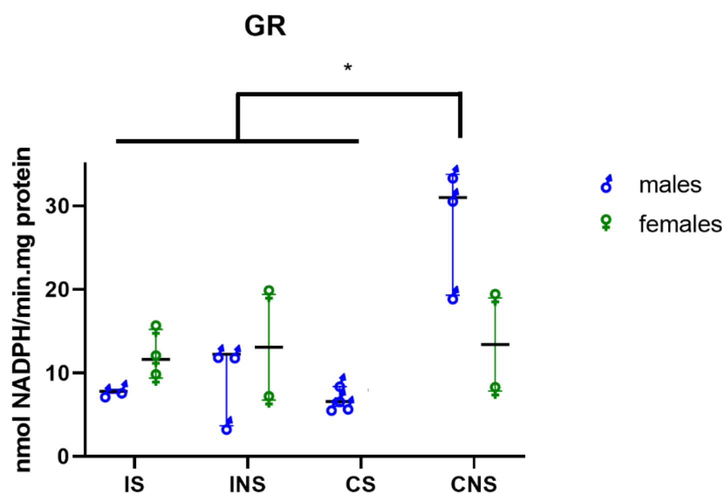

Fig 17- Activity of the glutathione reductase (nmol NADPH/min.mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation), isolated non-stressed (INS, isolated for 15 days), control stressed (CS, exposed to acute stress) and control non-stressed (CNS). The raw data is presented here as mean± standard deviation; each point represents an animal; n= 5. * p<0.05 for comparisons between CNS and all the other groups using two-way ANOVA followed by Tukey's post hoc test. CNS group: Females: n=2, Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

4.2.7. Glutathione S-transferase (GST) activity

The enzymatic activity of the Glutathione S-transferase was not influenced by the group tested nor by the sex of the animals (Fig 18).

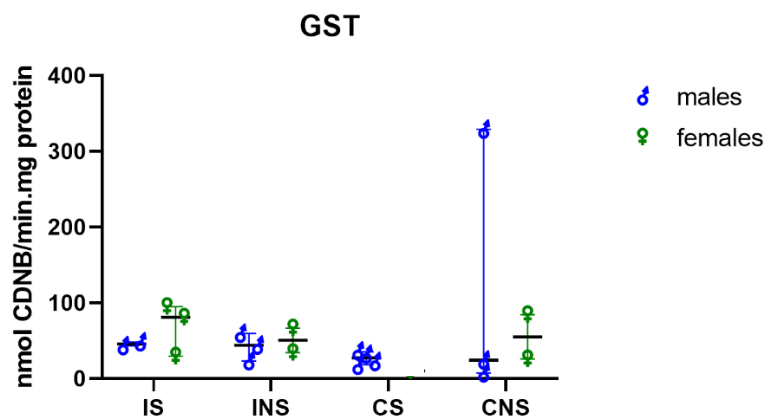

Fig 18- Activity of Glutathione S-transferase (nmol CDNB/min.mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation), isolated non-stressed (INS, isolated for 15 days), control stressed (CS, exposed to acute stress) and control non-stressed (CNS). The raw data is presented here as mean± standard deviation; each point represents an animal; n= 5. CNS group: Females: n=2, Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

In addition, these two factors had no interaction between them.

4.2.8. Glutathione in a reduced form (GSH) quantification

The quantities of glutathione in a reduced form were similar between groups (Fig. 19) and sexes.

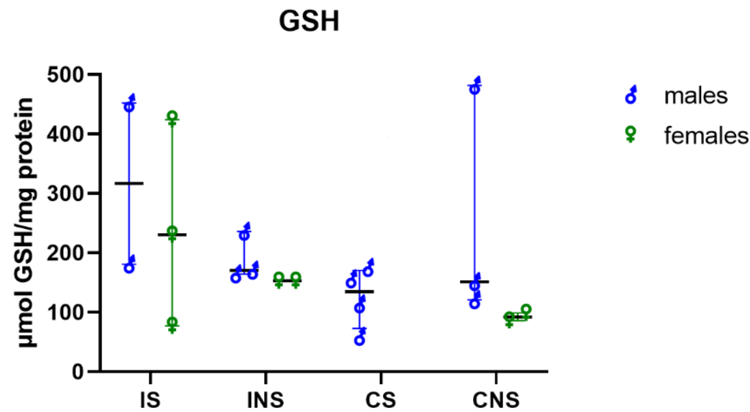

Fig 19- Levels of reduced glutathione (µmol GSH/mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation), isolated non-stressed (INS, isolated for 15 days), control stressed (CS, exposed to acute stress) and control non-stressed (CNS). The raw data is presented here as mean± standard deviation; each point represents an animal; CNS group: Females: n=2, Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

There was also no interaction between the factor group and the factor sex.

4.2.9. Glutathione in an oxidized form (GSSG) quantification

The values of GSSG observed were not different between sexes, nor between groups (Fig. 20).

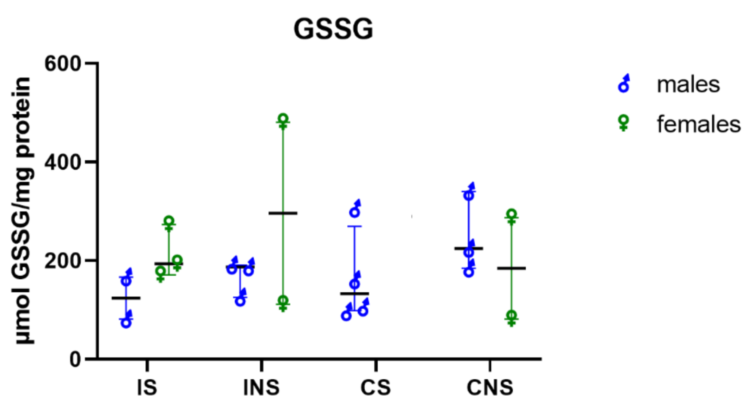

Fig 20- Levels of oxidised glutathione (μmol GSSG/mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation), isolated non-stressed (INS, isolated for 15 days), control stressed (CS, exposed to acute stress) and control non-stressed (CNS). The raw data is presented here as mean± standard deviation; each point represents an animal; CNS group: Females: n=2, Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

No interaction was detected between sex and group factors.

4.2.10. Thiobarbituric acid reactive substance (TBARS) qualification

There was no effect of group/ treatment or sex regarding the TBARS values (Fig. 21).

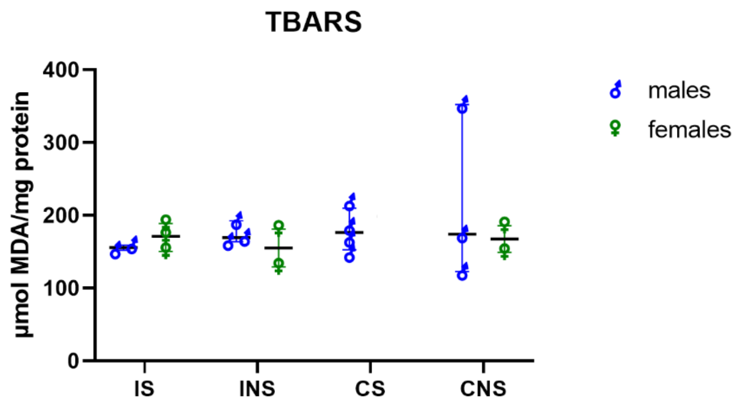

Fig 21- Levels of thiobarbituric acid reactive substances (μmol MDA/mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation), isolated non-stressed (INS, isolated for 15 days), control stressed (CS, exposed to acute stress) and control non-stressed (CNS). The raw data is presented here as mean± standard deviation; each point represents an animal; CNS group: Females: n=2, Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

Also, there was no interaction between these two factors (group*sex).

4.2.11 Carbonyls quantification

The levels of protein carbonyls were not significantly different between sexes or between groups (Fig. 22).

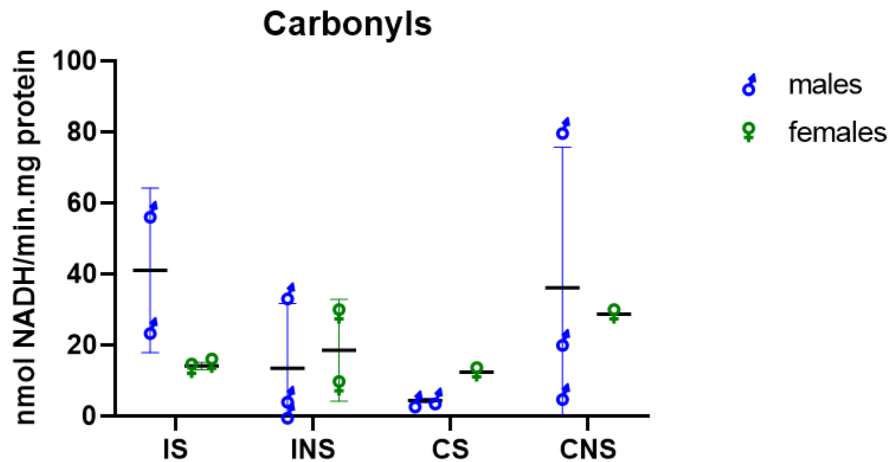

Fig 22- Levels of carbonyls (nmol NADH/min.mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation, n=4), isolated non-stressed (INS, isolated for 15 days, n=4), control stressed (CS, exposed to acute stress, n=3) and control non-stressed (CNS, n=4). The raw data is presented here as mean± standard deviation; each point represents an animal. CNS group: Females: n=2, Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

Also, group*sex interaction was not detected by our analysis.

4.2.12. Lactacte Dehydrogenase (LDH) activity

For the activity of the lactate dehydrogenase enzyme, we found that the factor sex and group did not influence the results obtained (Fig. 23), and that there was no interaction between these factors.

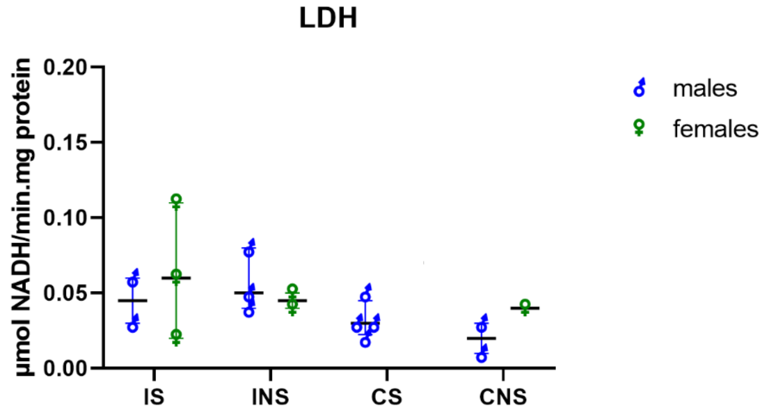

Fig 23- Activity of lactate Dehydrogenase (µmol NADH/min.mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation), isolated non-stressed (INS, isolated for 15 days), control stressed (CS, exposed to acute stress) and control non-stressed (CNS). The raw data is presented here as mean± standard deviation; each point represents an animal; n= 5, except in the CNS (n= 3). CNS group: Females: n=2, Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

4.2.13. Acetylcholinesterase (AChE) quantification

When comparing the different AChE levels between sexes or between groups (Fig. 24), no significant difference was detected, and no interaction was found between group and sex.

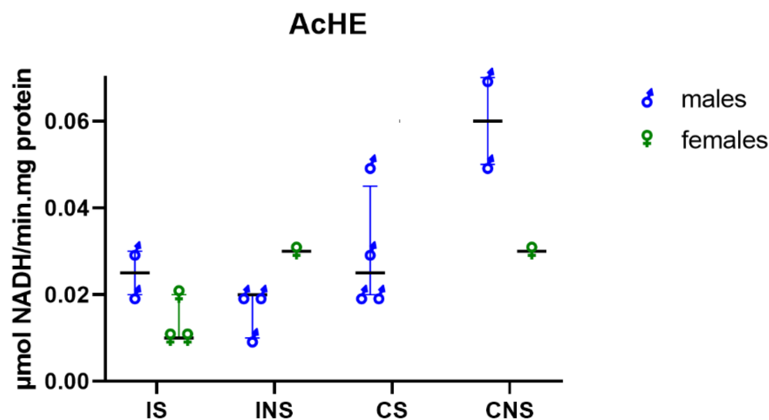

Fig 24- Levels of acetylcholinesterase (µmol TNB/min.mg of protein) measured in the brains of adult zebrafish from different groups: Isolated stressed (IS, exposed to acute stress after 15 days of isolation, n=5), isolated non-stressed (INS, isolated for 15 days, n=4), control stressed (CS, exposed to acute stress, n=5) and control non-stressed (CNS, n=3). The raw data is presented here as mean± standard deviation; each point represents an animal. CNS group: Females: n=2, Males: n=3; CS group: Females: n=0, males: n=4; INS: females: n=2; males: n=3; IS: females: n=3 males n=2.

4.3. Task 2 – Behavioural assessment

4.3.1. White/black test

For the white/black test, we used a custom-built white and black tank to assess anxiety-like behaviours in animals that were exposed to a 15-day isolation period *versus* animals that were housed with their conspecifics (control). There were no statistical differences detected between groups nor between sex regarding the number of entries in the white side, the time spent in each side of the apparatus (Fig. 25), the latency to enter in the black compartment, to re-enter in the white compartment, and the number of attempts to enter in the black compartment. Also, the random factor position of the apparatus did not influence the results obtained and no interaction was found between sex and group.

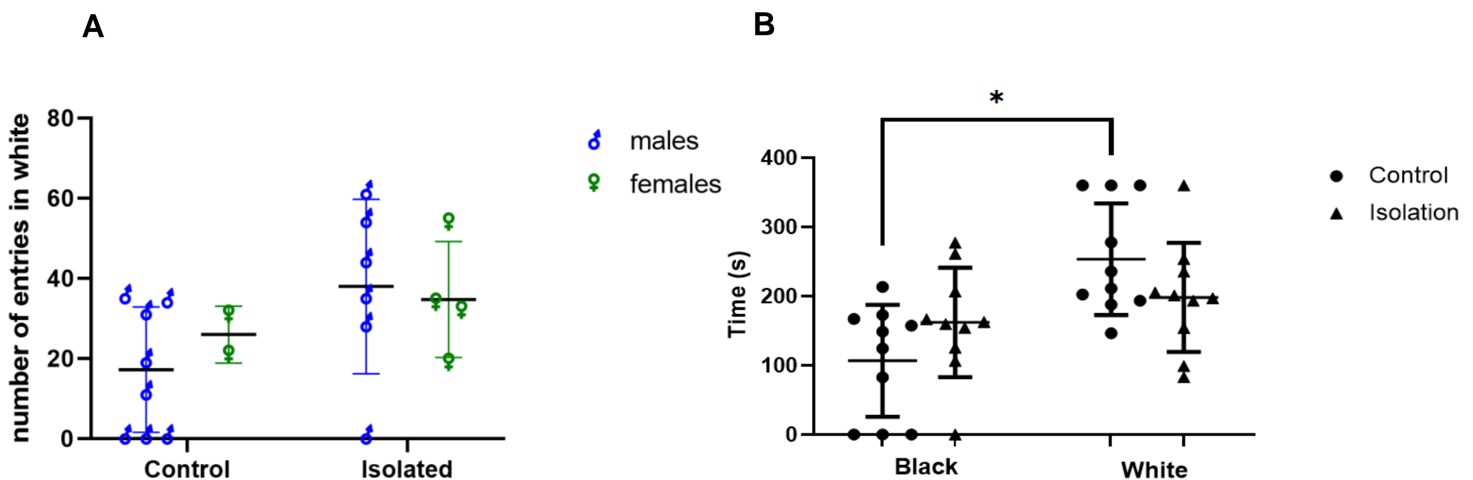

Fig 25- Number of entries in the white side (A), and time spent in each side of the apparatus (B) of the white-black test performed by different groups of adult zebrafish: Control (group of animals subjected to no treatment); and Isolation (group of animals isolated for 15 days and tested the day after the end of the 15-day isolation period). The raw data is presented here as mean± standard deviation; each point represents an animal; n=10; * p<0.05 for comparison between time spent by the control group in the black and white side of the apparatus using paired Students' t-test. (B). Control group: Males: n=8; Females: n=2; Isolated group: Males: n=6, females n=4.

When we compared the time spent in each side of the apparatus within groups, the control group spent more time in the white (253.51 seconds) than in the black (106.72 seconds) side of the tank (t(9) = 2.873; p= 0.018). The isolation group revealed no preference for any area.

4.3.2. Novel tank test

With the novel tank task, we wanted to compare activity, and anxious-like behaviour occurrences between individuals housed with their conspecifics and animals that were exposed to a 15-day isolation period. Regarding the total distance travelled by each animal, there were no differences between groups, but there was a significant interaction between the sex and group factor [F(1,16) = 6.095, p=0.025] and an influence of the sex factor [F(1,16) = 6.411, p=0.022].

Next, we checked the animals' average speed for the duration of the test, in which the factor group did not influence the results obtained, but again, we observed an interaction between the sex and group factors [F(1,16) = 7.208, p=0.016] and the influence of the sex factor [F(1,16) = 6.781, p=0.019]. Nevertheless, there were no differences regarding total distance or speed when comparing control and isolation animals within each sex. The maximum speed measured, and the angular velocity variables did not differ between groups, nor between sexes. We also analysed the time animals spent and distance swam at speeds above 0.07m/s, which may indicate erratic swimming; however, these variables were not different between groups, nor between sexes. Considering the overall analysis, we found that two control animals displayed one freezing episode each, and three animals from the isolation group displayed 9 different freezing episodes overall. For the immobile episodes, one animal from each group showcased this type of behaviour.

The frequency of entries, time spent (Fig 26), distance travelled, average speed and latency to enter in the upper part of the apparatus were analysed and no differences were detected between the factor levels (group or sex).

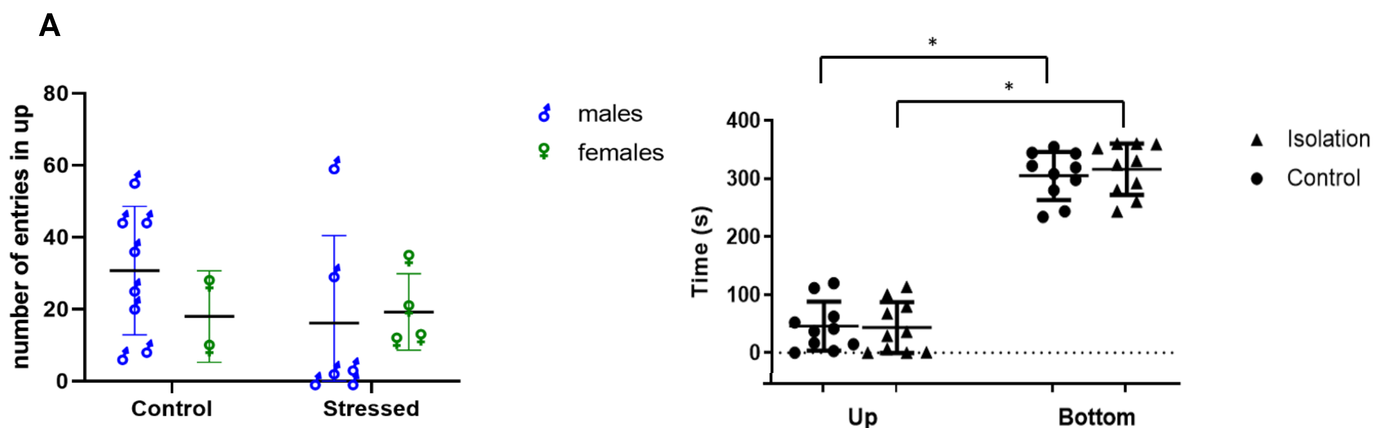

Fig 26- Number of entries in the bottom (A), and time spent (s) in the up and bottom zone (B) of the novel tank in each group of adult zebrafish: Control (group of animals subjected to no treatment); and Isolation (group of animals isolated for 15 days and tested in the novel tank two days after the end of the 15-day isolation period). The raw data is presented here as mean± standard deviation; each point represents an animal; n= 10; * $p<0.05$ for comparison of time spent between the up and bottom zone of the novel tank within each group, using paired Students' t-test (B). Control group: Males: n=8; Females: n=2; Isolated group: Males: n=6, females n=4.

Regarding the distance travelled and average speed in the bottom part of the tank, there were also no differences between groups or sexes, but a significant interaction between the group and sex of the animals was detected in both variables reported ($F(1,16) = 12.069$, $p=0.003$; $F(1,16) = 5.691$, $p=0.030$, respectively).

Both Control and Isolation group spent significantly more time in the bottom (41.91 seconds for control groups and 43.44 seconds for isolation group) than in the upper (304.59 seconds for control and 316.14 seconds for isolation group) zone of the novel tank ($t(9)= -10.278$, $p<0.001$ for Control group; $t(9)= -9.809$, $p< 0.001$ for isolation group) (Fig 26). Also, for the distance swam by the animals, we noticed that both the control and isolation group also swam bigger distances in the bottom part of the compartment (29.19 cm for the control group and 29.85 cm for the isolation group) than in the upper (5.29 cm for the control group and 4.38 cm for the isolation group) part of the apparatus ($t(9) = -9.115$, $p<0.001$ for control group; $t(9) = -7.716$, $p<0.001$).

Also, the values of the different variables were compared between the first three minutes and the last three minutes of the novel tank experiment to assess any process of habituation to the tank.

Therefore, the isolation group did not show any differences in any behaviour between the two time-points ($p>0.05$), while the control group showed differences between these time-points in the distance swam in the bottom compartment, and in the number of entries to the up zone. Thus, as the time of the experiment went by, from the third to the sixth minute of the novel tank test, the control animals swam less in the bottom zone (Fig 27A) ($t(9) = 2.587$, $p=0.029$), entered more often in the up compartment (Fig 27B) ($t(9) = -3.186$, $p=0.011$), and decreased the time spent at speed higher than 0.07 m/s ($t(9) = -4.118$, $p=0.003$).

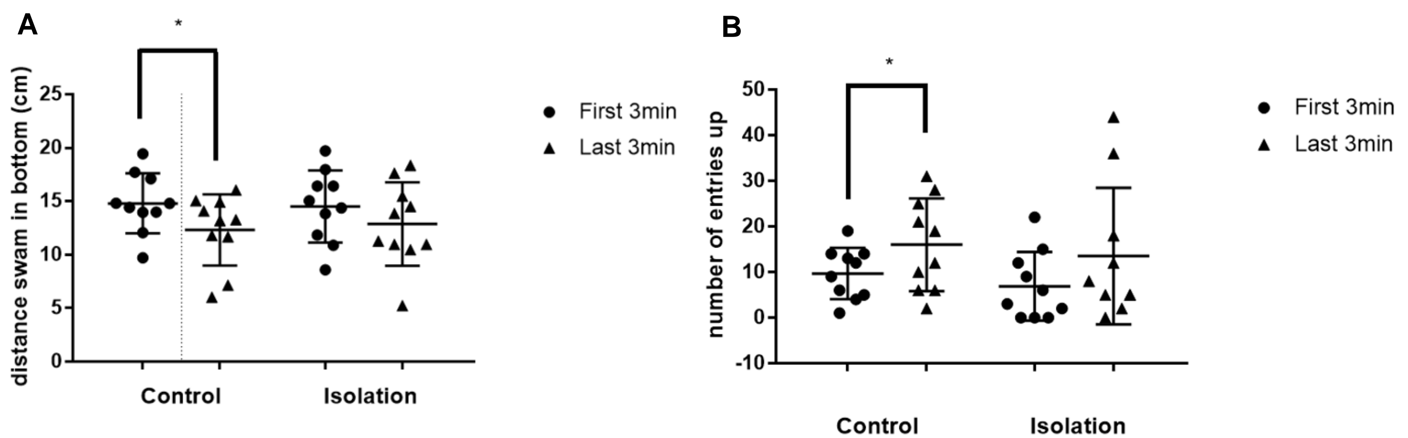

Fig 27- Distance swam in the bottom part of the tank (A); and number of entries in the upper part of the tank (B) in the first and in the last three minutes of the novel tank test by different groups of adult zebrafish: Control (group of animals subjected to no treatment); and Isolation (group of animals isolated for 15 days and tested in the novel tank two days after the end of the 15-.day isolation period). The raw data are presented as mean± standard deviation; n=10; * p<0.05 for comparisons between the first and the last three minutes of the novel tank test regarding distance swam in the bottom, and number of entries in the upper zone in the control group, using paired Students' t-test. Control group: Males: n=8; Females: n=2; Isolated group: Males: n=6, females n=4.

There were, although, no significant differences between groups in the first or in the last three minutes of the experiment (p>0.05).

4.3.3. Mirror biting test

For the mirror biting test, we wanted to check tendencies to exhibit anxiety-like and agonistic behaviours in isolated animals *versus* the control group. Control and isolation groups spent similar time in the bottom of the tank, showing also similar frequencies and durations of freezing behaviour. Furthermore, the latency for the animal to enter in the approach and contact zone of the tank, as well as the frequencies of entries in these zones and time spent there were not altered by the groups (Fig. 28) nor by the animals' sex.

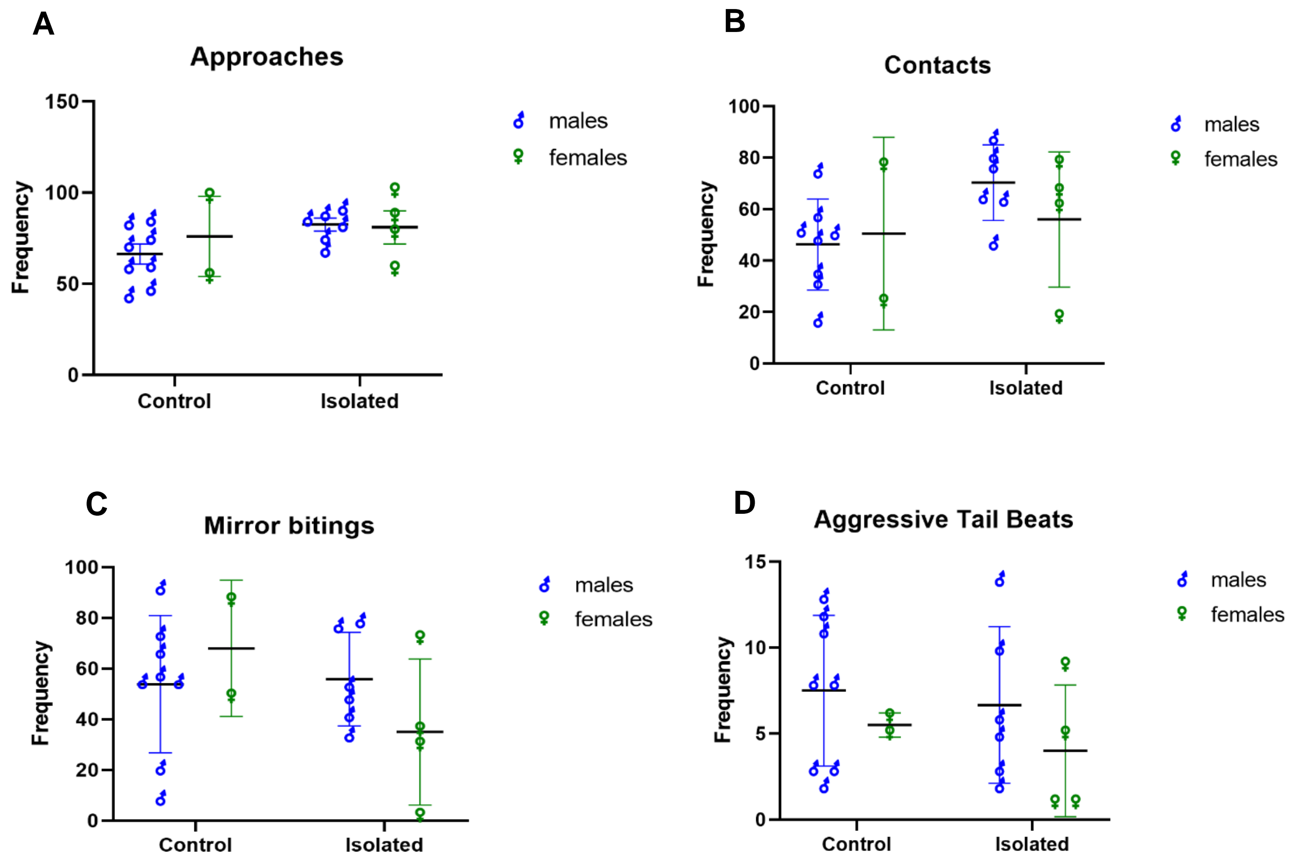

Fig 28- Frequencies of entering the approach (A), and contact (B) zone, and frequency of mirror bitings (C) and aggressive tail beats (D) in the mirror biting test using different groups of adult zebrafish: Control (group of animals subjected to no treatment); and Isolation (group of animals isolated for 15 days and tested in the mirror biting test three days after the end of the 15-day isolation period). The raw data is presented here as mean± standard deviation; each point represents an animal; n= 10. Control group: Males: n=8; Females: n=2; Isolated group: Males: n=6, females n=4.

There were also no differences between groups nor sexes regarding the frequency of mirror biting and aggressive tail beats.

5. DISCUSSION

In research, zebrafish is often housed alone for short periods, for example, when the animal needs to recover from a procedure, or when there is a need for individual identification. Examples of these are when genotyping is performed or when the animal is tested on behavioural tasks for several days. Several papers have been published regarding social isolation; however, few addressed the type of transient and not total social isolation that we often do in the laboratories. Thus, we intended to study if a 15-days social isolation, with visual contact, may alter some physiological parameters and behaviour. Our results indicate that this period did not have major impact on the parameters studied, as only minor alterations were detected: a decrease in the glutathione reductase activity, and a slight alteration on the behavioural profile in the white/black test, and on the habituation process in the novel tank.

To test if the 15-days isolation period caused alterations on how an animal respond to a stressful situation, an acute stress protocol had to be established. Both stress protocols tested induced a significantly increase in cortisol levels compared with the control animals. This was expected since acute stress spikes cortisol levels in zebrafish according to several studies (Gaikwad et al., 2011; Ramsay et al., 2009). For the first protocol we found that the control group had higher values and a more disperse data distribution than the control of the second protocol, where we euthanised the control animals immediately after being placed in the experimental room. In terms of the acutely stressed groups, data of stressed animals had a higher variability in the second than on the first protocol. Therefore, we found that the most optimal protocol would be to euthanise control animals immediately after being taken from the rack to obtain a better baseline for cortisol levels; and, for the acute stress protocol, to do 5 minutes of net-chasing in 2L of water, and 30 seconds of air exposure. Although both protocols induced stress validated by the cortisol levels, we decided to use the first protocol tested because it causes less harm to the animals (shorter air exposure), whilst still being an acute stress protocol.

According to our results, we concluded that 15-day isolation period did not affect cortisol levels in zebrafish when compared to the values from animals housed with their conspecifics, although some studies suggest that animals that went through isolation periods can have lower baseline cortisol levels than individuals housed with their conspecifics (Onarheim et al., 2022; Shams et al., 2017). The reason for this is that the

conditions and duration of these experiments conducted by other researchers included longer isolation periods (Forsatkar et al., 2017; Shams et al., 2018; Shams et al., 2017) or different types of social isolation. For example, Onarheim, Janczak & Nordgreen (2022) did a 5-day isolation period and found that isolated zebrafish displayed lower cortisol levels than socially housed individuals, but, during this isolation period, the animals were deprived of visual contact with their conspecifics. In our study, we wanted to mimic the social isolation that often happens in laboratories, so we focused on having the animals housed individually, but able to keep visualizing their conspecifics. We also observed that animals that went through this 15-day isolation period did not display a different response to acute stress. This is relevant since 15-day isolation periods for zebrafish are often used in laboratories, and our study showed that it will not affect stress studies, nor will it affect the physiological ability of the animal to respond to stress.

The potential effect of social isolation was also evaluated by other biochemical analysis, such as oxidative stress parameters and acetylcholinesterase, since those could be indicators that social isolation affect the animals' physiology on a deeper level. AChE levels and most of the oxidative stress parameters analysed were similar between groups. However, the glutathione reductase levels were significantly lower in the CS, IS and INS groups, which means that they suffered an inhibition when compared to our control/ baseline for GR values (CNS group). With this alteration and according to the GSH biosynthesis, it would be expected to also see significantly lower levels of GSSG and GSH, which was not the case. Zhao et al. (2009) stated that, under certain circumstances, the inhibition of GR may not influence the formation of ROS and enzymes involved in the GSH biosynthesis due to a mechanism of compensation, which can explain to a certain extent why a GR inhibition did not cause any disturbances on the rest of the GSH cycle and, consequently, did not influence the oxidative stress parameters.

Zebrafish have been utilised as a model for anxiety-like behaviours using the white/black tank test. Usually, animals often prefer to spend more time in the black than in the white compartment, thus, the major endpoint of this test is the spatiotemporal distribution of the fish between the two sides of the apparatus. In the white compartment, we also observed the frequency of entries and the latency to re-enter in this side to define the level of motivational conflict of the animals. It is described that zebrafish frequently make short (<1s) intrusions in the white compartment before swiftly retreating to the black side of the tank(Araujo et al., 2012); this behaviour is similar to rats' risk assessment behaviours. An approach-avoidance confluence is evident in this type of behavioural test since the animals' behaviour is influenced by the novelty of their surroundings.

Therefore, the degree of exploratory behaviour depends on fear impeding exploration, as novel situations can often generate concurrent feelings of apprehension or curiosity (Kalueff et al., 2013). Our white/black test results showed no significant differences between the isolated and control groups regarding all the variables tested. Contrarily to what would be expected, the control group spent more time in the white than in the black side, independently of the sex or position of the apparatus. This is probably due to the low luminosity inside the apparatus (mean of 45 lx), not causing an aversive reaction to the white side. The luminosity levels in the literature have high variations, for example from 77 to 875 lx (Facciol et al., 2019; Onarheim et al., 2022). Nevertheless, the isolated groups did not show this difference, having a different behavioural profile from the control group.

Another test used to assess anxiety was the novel tank test that was design to create a situation of novelty, which may lead to anxiety-like behaviours (Cachat et al., 2010; Rosemberg et al., 2011; Westerfield, 2000)). It is the animals' natural tendency to seek for asylum in an unknown surrounding through diving, freezing, and decreasing exploration, which serves as a basis for this test. An increase in exploration (e.g., higher activity, less freezing behaviours, and more entries to the upper half of the tank) often happen when the fish progressively adjust to its new surroundings (Cachat et al., 2010). In our novel tank test, we did not see any significant differences between groups when it comes to the number of entries, time and distance travelled in both the upper and bottom part of the apparatus, which means that isolated and control animals had similar behaviours. Indeed, both groups spent more time and had higher activity in the bottom part of the tank compared with the upper part. We did notice though, that as time went by, the control animals went through a habituation process; during the last three minutes of the test, they displayed more entries in the upper part of the tank, and less distance swam in the bottom of the tank compared with the first three minutes, indicating less activity in the bottom, and more activity in the up zone, where threats are more likely to occur. These indicators of habituation were not observed in the single-housed group. Nevertheless, these data has some variability within groups, which can be justified through animals' own boldness or shyness, i.e. inherent individual differences (Dahlbom et al., 2011; Wright et al., 2003). Other researchers, such as Shams et al. (2015), using the novel tank found that social isolation decreased thigmotaxis, and freezing episodes, i.e., a decrease on anxiety-like behaviours, which we did not observe in our study. This is probably because we used an isolation period 6 times shorter than the referred experiment (90-day isolation period).

Zebrafish can display boldness through butting, biting the mirror and by tracing their own reflection back and forth rapidly. The mirror biting reflects how the fish interact with a conspecific and is a key factor for studying social behaviours in this species (Kalueff et al., 2013). This test was conducted for 6 min, as after that, fish can start to display habituation to the mirror, with a steady decrease in mirror-biting activity as the novelty of the stimulus wanes (Pham et al., 2012). Although isolated for 15 days, isolation group behaved at control levels in all the variables studied in the mirror biting test.

The different behavioural profile of groups regarding the period spent in each side of the white/black tank test is not supported by other variables or tests (novel tank test), thus this could be a non-significant alteration, needing more tests to clarify this result; for example, using different luminosities in the apparatus. The process of habituation to a new environment may be delayed in the isolated animals, but, even for the control group, this process was not clearly displayed in all the variables tested for the purpose. Although different luminosities were used in the two behavioural apparatus used for the white/black test (15-20 lx of difference), the results are similar to what we observed in the novel tank test, since the control group spent more time in a location that should be more aversive, the white side, while simultaneously displaying habituation to a novel environment in the novel tank test, leading us to believe that these animals are bolder than the isolated animals. The social behaviour tested in this work seems to be intact after this period of isolation. Also, this treatment did not alter cortisol levels, nor oxidative stress levels; the only parameter altered did not influence the capacity of the organism to maintain the redox status. Therefore, there were no apparent signs of anxiety or stress in the animals housed alone for 15 days.

In conclusion, social isolation with conspecifics' visual contact for 15 days could be safely used regarding anxiety/ stress and social behaviour. However, other capabilities, such as learning and memory (Leser & Wagner, 2015) and other biochemical parameters, such as neurotransmitters levels (Shams et al., 2018; Shams et al., 2015) must be assessed to ensure that this social isolation protocol would not be an unwanted variable that could interfere with the research outcomes.

6. CONCLUSION

Social isolation is often performed in laboratories that use zebrafish for research purposes. The impact of these periods where the animals are restrained from interaction with their conspecifics needs to be further studied regarding animal welfare and post-isolation experimental outcomes.

Although there is some research on the impact of social isolation in zebrafish, the periods duration and conditions are very extreme and different from what is conducted in laboratories periodically. Usually, zebrafish need to be isolated for some days to two weeks depending on the purpose (genotyping, behavioural tests, regeneration studies, health condition evaluations etc). Other studies exposed zebrafish to long periods of time – ranging from one month to almost a year, or really short periods such as 24h in isolation. In our study, where we did a 15-day isolation period, and then the animals were submitted to a series of behavioural, physiological, and oxidative stress analysis, we did not find 15 days of social isolation to be harmful. Physiologically, cortisol levels stayed the same when compared to socially housed individuals, and the way the animals reacted to the exposure to an acute stressor was also similar to those who were housed with their conspecifics. Other physiological studies could be conducted, such as lactate or glucose levels quantification, which were not evaluated in our study. In terms of behaviour, we also did not see any significant changes in the animals' anxiety-like responses, meaning that the animals had similar reactions, whether they were housed alone, or with their conspecifics. When it came to oxidative stress analysis, we also did not find significant differences, although we think that it would be interesting to assess memory and neurotransmitter levels to further deepen the certainty that this isolation period can be done without causing harm to zebrafish.

To sum up, social isolation for 15-days, where the animals can visualize their conspecifics, does not seem to affect overall animal welfare nor the viability of experiments done after this period.

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Annex I: Description of the statistical analysis regarding the comparisons between groups of the cortisol levels assessment, oxidative stress parameters, and variables from the White/black, novel tank, and mirror biting test. * data were log-transformed to achieve a normal distribution

Protocol	Groups	Variables	Test	Statistical test value	Probability	Posthoc	Groups	Probability
Acute stress protocol I	Control vs Stressed	Cortisol levels	Independent t-test	t(18) = 4.058	<0.001			
Acute stress protocol II	Control vs Stressed	Cortisol levels	Independent t-test	t(10) = 2.881	0.016			
Cortisol assessment	IS vs INS vs CS vs CNS	Cortisol levels*	Two-Way ANOVA (group as fixed factor and batch as random factor)	F(1,6) = 5.838	0.133			
Oxidative stress	IS vs INS vs CS vs CNS	ROS	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 0.178	0.770			
	IS vs INS vs CS vs CNS	SOD	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 1.201	0.348			
	IS vs INS vs CS vs CNS	CAT	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 0.295	0.828			
	IS vs INS vs CS vs CNS	GPx	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 3.373	0.051			
	IS vs INS vs CS vs CNS	GR	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 4.181	0.028	Tukey	IS vs INS	0.986
						Tukey	IS vs CS	0.839
						Tukey	IS vs CNS	0.039

Protocol	Groups	Variables	Test	Statistical test value	Probability	Posthoc	Groups	Probability
						Tukey	INS vs CS	0.959
						Tukey	INS vs CNS	0.021
						Tukey	CS vs CNS	0.009
	IS vs INS vs CS vs CNS	GST	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 0.473	0.706			
	IS vs INS vs CS vs CNS	GSH	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 0.745	0.544			
	IS vs INS vs CS vs CNS	GSSG	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 0.291	0.831			
	IS vs INS vs CS vs CNS	TBARS	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 0.223	0.879			
	IS vs INS vs CS vs CNS	Carbonyls	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 0.223	0.391			
	IS vs INS vs CS vs CNS	LDH	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 0.830	0.505			
	IS vs INS vs CS vs CNS	AcHE	Two-Way ANOVA (group and sex as fixed factor)	F(3,13) = 2.421	0.127			
White/Black	Control vs Isolated	Time spent in white	Two-Way ANOVA (group and sex as fixed factor and position as random factors)	F(1,16)=1.08	0.315			

Protocol	Groups	Variables	Test	Statistical test value	Probability	Posthoc	Groups	Probability
	Control vs Isolated	Time spent in black	Two-Way ANOVA (group and sex as fixed factor and position as random factors)	F(1,16)=1.10	0.312			
	Control vs Isolated	Latency enter black*	Two-Way ANOVA (group and sex as fixed factor and position as random factors)	F(1,16)=1.15	0.301			
	Control vs Isolated	Entries black	Two-Way ANOVA (group and sex as fixed factor and position as random factors)	F(1,16)=3.22	0.093			
	Control vs Isolated	Entries white	Two-Way ANOVA (group and sex as fixed factor and position as random factors)	F(1,16)=3.31	0.089			
	Control vs Isolated	Re-enter white	Two-Way ANOVA (group and sex as fixed factor and position as random factors)	F(1,16) = 0.02	0.903			
	Control vs Isolated	Attempts black	Mann-Whitney	U=52.00	0.912			
Novel tank	Control vs Isolated	Total distance travelled	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.002	0.967			
	Control vs Isolated	Average speed	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.006	0.940			
	Control vs Isolated	Maximum speed	Two-Way ANOVA (group and sex as a fixed factor)	U=56.5	0.631			

Protocol	Groups	Variables	Test	Statistical test value	Probability	Posthoc	Groups	Probability
	Control vs Isolated	Absolute turn angle*	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 1.702	0.211			
	Control vs Isolated	Angular velocity	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.249	0.625			
	Control vs Isolated	Number activations	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.026	0.873			
	Control vs Isolated	Time activations	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.694	0.417			
	Control vs Isolated	Distance swam	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.015	0.903			
	Control vs Isolated	Entries upper	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.116	0.737			
	Control vs Isolated	Time upper	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 2.594	0.127			
	Control vs Isolated	Distance upper	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 1.366	0.260			
	Control vs Isolated	Average speed upper	Mann-Whitney	U=40	0.720			
	Control vs Isolated	Latency enter upper	Mann-Whitney	U=53	0.853			
	Control vs Isolated	Entries bottom	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.409	0.532			
	Control vs Isolated	Time bottom	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) =1.817	0.196			

Protocol	Groups	Variables	Test	Statistical test value	Probability	Posthoc	Groups	Probability
	Control vs Isolated	Distance bottom	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.696	0.416			
	Control vs Isolated	Average speed*	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.055	0.818			
	Control vs Isolated	Frequency freezing bottom	Mann-Whitney	U=57	0.631			
	Control vs Isolated	Duration freezing bottom	Mann-Whitney	U=58	0.579			
	Control vs Isolated	Total distance travelled 0-3 min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.017	0.898			
	Control vs Isolated	Total distance travelled 3-6min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.001	0.977			
	Control vs Isolated	Average Speed 0-3min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.834	0.375			
	Control vs Isolated	Average Speed 3-6min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.177	0.679			
	Control vs Isolated	Maximum speed 0-3 min	Mann-Whitney	U=40	0.796			
	Control vs Isolated	Maximum speed 3-6min	Mann-Whitney	U=44	0.684			
	Control vs Isolated	Absolute turn angle 0-3 min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.014	0.906			
	Control vs Isolated	Absolute turn angle 3-6min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.564	0.464			
	Control vs Isolated	Angular Velocity 0-3min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.017	0.897			

Protocol	Groups	Variables	Test	Statistical test value	Probability	Posthoc	Groups	Probability
	Control vs Isolated	Angular Velocity 3-6min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.374	0.550			
	Control vs Isolated	Number activations 0-3min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.052	0.986			
	Control vs Isolated	Number activations 3-6min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.194	0.665			
	Control vs Isolated	Time activations 0-3min	Mann-Whitney	U=49	0.971			
	Control vs Isolated	Time activations 3-6min	Mann-Whitney	U=54	0.796			
	Control vs Isolated	Distance activations 0-3min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.861	0.367			
	Control vs Isolated	Distance activations 3-6min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.446	0.514			
	Control vs Isolated	Entries upper 0-3min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.015	0.905			
	Control vs Isolated	Entries upper 3-6min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.163	0.692			
	Control vs Isolated	Time upper 0-3min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.145	0.709			
	Control vs Isolated	Time upper 3-6min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.122	0.731			
	Control vs Isolated	Distance upper 0-3min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.181	0.676			

Protocol	Groups	Variables	Test	Statistical test value	Probability	Posthoc	Groups	Probability
	Control vs Isolated	Distance upper 3-6min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.111	0.743			
	Control vs Isolated	Speed Upper 0-3min	Mann-Whitney	U=37	1.0			
	Control vs Isolated	Speed Upper 3-6min	Mann-Whitney	U=57.5	0.315			
	Control vs Isolated	Entries bottom 0-3min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.009	0.926			
	Control vs Isolated	Entries bottom 3-6min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.148	0.705			
	Control vs Isolated	Time bottom 0-3 min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.145	0.709			
	Control vs Isolated	Time bottom 3-6 min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.036	0.853			
	Control vs Isolated	Distance bottom 0-3min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.172	0.684			
	Control vs Isolated	Distance bottom 3-6min	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.029	0.867			
	Control vs Isolated	Speed bottom 0-3 min	Mann-Whitney	U=42	0.579			
	Control vs Isolated	Speed bottom 3-6min	Mann-Whitney	U=47.5	0.853			
Mirror biting test	Control vs Isolated	Time spent bottom	Two-Way ANOVA (group and sex as a fixed factor)	F(1.16)=0.783	0.389			
	Control vs Isolated	Latency approach	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16)=0.738	0.403			

Protocol	Groups	Variables	Test	Statistical test value	Probability	Posthoc	Groups	Probability
	Control vs Isolated	Number approaches	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 1.697	0.211			
	Control vs Isolated	Duration approaches	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16)=2.414	0.140			
	Control vs Isolated	Latency contact	Two-Way ANOVA (group and sex as a fixed factor)	F (1,16)=0.534	0.475			
	Control vs Isolated	Number contacts	Two-Way ANOVA (group and sex as a fixed factor)	F (1,16) = 2.044	0.172			
	Control vs Isolated	Contact duration	Two-Way ANOVA (group and sex as a fixed factor)	F (1.16)= 0.537	0.474			
	Control vs Isolated	Mirror biting frequency	Two-Way ANOVA (group and sex as a fixed factor)	F (1,16) =1.472	0.243			
	Control vs Isolated	Aggresive Tail Beats frequency	Two-Way ANOVA (group and sex as a fixed factor)	F(1,16) = 0.297	0.593			
	Control vs Isolated	Freezing frequency	Mann-Whitney	U=60	0.481			
	Control vs Isolated	Freezing duration	Mann-Whitney	U=60	0.481			