Gonçalo Nuno Gomes Preto Ferreira Nunes

Organização social no peixe invasor *Australoheros facetus*: hormonas e comportamento

UNIVERSIDADE DO ALGARVE

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Mestrado em Biologia Marinha

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I declare to be the author of this work, which is original and unpublished.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

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11-KT	11-Ketotestosterone
11β-DH	11β-dehydrogenase
11β-HSD	11-hydroxysteroid dehydrogenase
11β-HSD1	Type 1 11-hydroxysteroid dehydrogenase
11β-HSD2.	Type 2 11-hydroxysteroid dehydrogenase
11β-OHT	11β-Hydroxytestosterone
A.Facetus.	*Australoheros facetus*
CBX.	Carbenoxolone
E2.	Estradiol
ETHOL.	Ethanol
GE.	Glycyrrhizic acid
IUCN.	International Union for Conservation of Nature
LEOA	Laboratório Experimental em Organismos Aquáticos
MR.	Mineralocorticoid receptor
NAD	Nicotinamide adenine dinucleotide
NADP(H).	Nicotinamide adenine dinucleotide Phosohate
RIA	Radioimunoassay
T	Testosterone
Vtg	Vitellogenin

Resumo

Espécies invasoras são um problema que tem vindo a ganhar reconhecimento nas últimas décadas e que afetam a maioria dos ecossistemas na Terra. São espécies que não são nativas da localização onde se encontram presentes e que são transferidas e introduzidas por ação humana tanto intencionalmente como acidentalmente, ao longo de barreiras biogeográficas. Estas espécies afetam a estrutura de espécies locais, a integridade das redes tróficas e funções críticas para o ecossistema sendo uma ameaça para diversidade biológica local, podendo também apresentar um impacto na economia local.

Portugal, devido à sua integração na Península Ibérica, uma região ladeada na sua totalidade pelo Oceano Atlântico, com exceção de uma comparavelmente pequena fronteira terreste, constituída pela cordilheira dos Pirenéus, faz com que tenha uma ictiofauna endémica muito característica, mas da qual vinte e três espécies são introduzidas. A ictiofauna portuguesa é composta por trinta e quatro espécies das quais 36% são endémicas e 23,5% são não-endémicas. Dentro destas espécies, podemos integrar a espécie alvo deste estudo, *Australoheros facetus*, conhecido localmente como Chanchito, um ciclídeo da família *Cichlidae*, originário da América do Sul, em particular da região do Paraná, no Paraguai e das bacias hidrográficas do Uruguai, inclusive, em afluentes costeiros.

A presença em Portugal da espécie *A. facetus,* deve-se essencialmente introduções antropogénicas, por ser uma espécie com elevado valor ornamental. É uma espécie que se pode encontrar com maior frequência em zonas ribeirinhas a sul do país, ao longo do rio Guadiana e afluentes, no reservatório da barragem do Alqueva e na ribeira do Odelouca.

O Chanchito é uma espécie que em Portugal não é considerada uma espécie invasora, estando descrita no Decreto-Lei. (nº 565/1999), como sendo uma espécie não endémica. Apesar desta avaliação, é uma espécie que deve despertar a atenção de uma avaliação mais cuidadosa, pois os seus impactos nas espécies endémicas e ecossistemas ainda são desconhecidos.

Este estudo pretende estudar a utilidade e aplicabilidade de diferentes tratamentos como método de controlo populacional em Chanchito, como forma de mitigar e conter o impacto que esta espécie poderá ter localmente. Para tal fim, é necessário entender os mecanismos envolvidos na reprodução desta espécie, em particular, o sistema endócrino

e a influencia que a estrutura e integridade social poderá implicar a nível fisiológica e comportamental, entre outras vertentes envolvidas no crescimento populacional desta mesma espécie.

Australoheros facetus, é uma espécie social, na qual se pode observar a formação de uma hierarquia dentro de uma população. Dentro desta hierarquia podem-se estabelecer dois tipos de fenótipos: os indivíduos dominantes e os indivíduos submissos. Este estabelecimento do estatuto social ocorre devido a interações agonísticas entre os indivíduos, em que, o individuo que vence essa interação é tido como dominante e o perdedor com submisso. Consoante o estatuto do individuo, este pode demostrar diferentes tipos de comportamento, com por exemplo, comportamentos agressivos como forma de ascender ou manter o estatuto na hierarquia ou reprodutores e de corte por parte de indivíduos dominantes ou comportamentos de fuga como forma de reduzir a exposição a interações agonísticas ou situações de maior stress em indivíduos submissos.

O contexto social encontra-se fortemente correlacionado com o nível de hormonas circulantes, sendo que este espectro social acarreta diferentes padrões endócrinos, como por exemplo, elevados níveis de estradiol (E2) ou testosterona (T) numa fêmea ou macho dominante, respetivamente, ou elevados níveis de cortisol em indivíduos submissos.

O trabalho desenvolvido nesta dissertação pretende descrever o impacto que substâncias como o Estradiol e a Carbenoxolona poderão ter nos níveis endócrinos e quais os efeitos que poderão ter na manipulação do comportamento em machos.

A Carbenoxolona (CBX) é um inibidor de 11β-hidroxi esteroide desidrogenase que é responsável pela conversão de testosterona em 11-Ketotestosterona (11-KT). Em teleósteos, 11-Ketotestosterona é um androgénio capaz de modular a frequência com que comportamentos agressivos, de corte e de guarda de postura ocorrem. Com a Carbenoxolona pretende-se inibir a produção de 11-KT e por sua vez, reduzir a frequência dos comportamentos de corte sem afetar as suas capacidades de estabelecer e manter o estatuto social de dominante.

Para a realização deste trabalho, recorreu-se ao stock de peixes desta espécie existente nas instalações do CCMAR. Os peixes foram amostrados, marcados e separados por seis grupos pequenos de 4 a 6 indivíduos do mesmo tamanho, com um individuo de tamanho superior em cada tanque, o individuo focal, para forçar a dominância do mesmo.

Os indivíduos focais foram submetidos a um período de pré-tratamento de 5 dias onde foram realizadas observações com o intuito de estudo comportamental. Após este primeiro período de observação os indivíduos focais foram mais uma vez amostrados e seguidamente injetados com soluções de estradiol, Carbenoxolona e controlo (Sham). Em seguimento ao tratamento, estes indivíduos foram novamente observados por um período mais longo de 7 dias, a fase pós-tratamento, para observar se os tratamentos teriam impacto sobre o comportamento do animal. No final do período de observações, estes indivíduos foram novamente amostrados e seguidamente eutanasiados para dissecação e identificação das gonadas.

As amostras de sangue recolhidas em cada amostragem foram utilizadas para estabelecer a existência de variações nos perfis hormonais, incluindo o Estradiol, a Testosterona, e a 11-Ketotestosterona.

Este procedimento foi repetido mais 3 vezes totalizando 4 séries, permitindo a realização de vários testes e obtenção de replicados.

O trabalho desenvolvido nesta dissertação demonstrou que a administração de Carbenoxolona em machos dominantes provocou uma descida considerável nos níveis de 11-Ketotestosterona e Testosterona, apesar do baixo número de replicados, não tendo demostrado diferenças significativas nas concentrações de E2 e Cortisol após tratamento. Para além disso, não foi possível observar um impacto significativo da Carbenoxolona em nenhum grupo de comportamentos, apesar que, é possível observar uma discrepância negativa à ordem de 10, na duração relativa média dos comportamentos reprodutores. Este valor não é significativo devido a um erro padrão elevado que poderá se causado, por o N de estudo ser pequeno, apesar que esta hipótese estudada não deve ser descartada em estudos futuros com uma população de estudo maior. Com os resultados obtidos neste trabalho podemos concluir que a 11-Ketotesterona foi inibida pela Carbenoxolona seguindo o resultado esperado, não afetando o comportamento agonístico do individuo o que será importante para a manutenção do seu estatuto social não afetando o processo de escolha de parceiro. Por outro lado, os resultados da análise comportamental, apesar de não serem conclusivos, pode-se concluir que com uma análise mais exaustiva pode-se determinar que os comportamentos de corte são reduzidos afetando a capacidade de o individuo produzir descendência, resultado que vai de acordo com o objetivo do trabalho desenvolvido nesta tese. Os constrangimentos mais significativos prendem-se, no entanto, com a dificuldade em discriminar machos de fêmeas devido à inexistência de qualquer dimorfismo sexual nesta espécie. O conceito estudado neste trabalho baseia-se na castração química dos machos, pelo que a sua efetividade só é garantida se estes puderem ser identificados *à priori*. Assim, apesar dos resultados serem promissores, será necessário encontrar uma metodologia que permita a seleção de uma população 100%

masculina, que possa ser tratada e libertada no meio. Ao contrário de outras metodologias, o método estudado não parece ter inconvenientes relativamente ao seu impacto sobre o meio ambiente e nas espécies nativas, já que não envolve a introdução de agentes químicos tóxicos no ecossistema e é seletivo para os organismos em causa.

Palavras-chave: *Australoheros facetus*; comportamento; hormonas; castração

Abstract

Invasive species are alien species that are introduced in new locations through anthropogenic means affecting endemic fauna and flora and local ecosystem.

Australoheros facetus, is a cichlid originating from South America that has been introduced in the southern riverine areas of Portugal. Although not classified as an invasive species, its impact in the ecosystem and endemic species is unknown.

This work intends to study the effectiveness of Carbenoxolone as a method of population control, and as a castration agent in male *A. facetu*s, through behavioural and endocrinal studies of this species.

Carbenoxolone (CBX) is an inhibitor of 11β-HSD, which is vital for the production 11-ketotestoterone. This androgen modulates the frequency and intensity of aggressive and reproductive behaviours and has important roles in spermatogenesis. CBX is expected not to affect the aggressive behaviour thus allowing the fish to maintain its dominance status and form a couple with the dominant female, the only female in the population capable of reproduce and hindering reproductive behaviour and subsequently reproductive capabilities.

Groups of similar size individuals were created with one individual slightly larger to force dominance. Then behavioural observations were conducted following a ethogram for this species before and after conducting the treatment in the dominant male, by injection of CBX in treated fish and vehicle in Sham fish.

There was no significant difference in the E2 and Cortisol levels between Control test and CBX treated males although there was a significant decrease in 11-KT and T. There was no significant difference in the aggressive behaviours between treatment phases and between treatments although there was a negative tendency in reproductive states after the treatment with CBX.

Thereby CBX does not affect agonistic interactions, not affecting the dominance and the ability of forming a dominant couple but will reduce courtship behaviour affecting the generation of offspring.

Key words: *Australoheros facetus*; behaviour; hormones; castration

1. Introduction

1.1.Invasive species

The introduction of non-native species can bear a broad spectrum of ecological and economic consequences as these can become invasive species which are a growing threat at a global level (Lodge, 1993; Mack et al., 2000; Williamson & Fitter, 1996).

The nomenclature "invasive species" is still prone to misconceptions as there is some uncertainty on whether the following impacts are from a negative or positive nature and different categorisation, which led to a diversion of viewpoints. There is still a lack of consensus on a spatial perspective as some utilise political space and others, ecological barriers such biogeographic or climatic regions (Boonman-Berson, et al., 2014) and there is still ambiguity about if invasive species refers to a single individual or a population as the environmental barriers that a population has to overcome to become self-sustained in a new location are more significant to the ones that a single individual have to overcome (Blackburn et al., 2011).

This dissertation follows along with the definition introduced by the International Union for Conservation of Nature (IUCN) in which invasive species are alien species, non-native, introduced in new habitats, harming the environments where they are introduced. It is important not to confuse with alien species as not all alien species are problematic (Balakrishna, 2001; Shine, et al., 2000). Human hand introduction, either intentionally or accidentally, is the main responsible for establishing species deemed as alien species (Shine et al., 2000).

These species are widespread across all major taxonomic groups negatively impacting most ecosystems (Richardson & Pyšek, 2012; Stohlgren & Rejmánek, 2014).

Invasive species result from dispersal and introductions of species to new habitats through an anthropogenic activity which diminishes biological diversity and compromises the integrity of historical ecological assemblages (Mack et al., 2000; Pyšek et al., 2012; Vitousek, 1990). Not only there is a direct introduction but there are also other causes indirectly related to human activity as climate change, human-induced terrain modulations and modified biochemical cycles, which, in the end, degrade the quality of goods and services that come from natural systems and a more sparse biological future (Seebens et al., 2015; Simberloff, 2013; Strayer, et al., 2006).

Introduction of fish species has been a practice that had a rise in popularity in the middle of the 19th century in Europe where fish species were transferred internationally to fulfil the food demand and for other purposes such as recreative or sports fishing (Baduy, 2017). The combination of the development of new artificial reproductive techniques after the end of World War II played an essential role in a further increase of the practice (Elvira, 2001; Ribeiro, et al., 2009).

The establishment of a novel invasive species depends on several factors, some related to their acclimation to the physicochemical environment, other related to its ability to assume an ecological niche and reproduce, for which the initial introduction pressure (events and number of fish) is fundamental. This process is propagule pressure.

Propagule pressure is then by definition, measured by the number of individuals and introduction frequency required for a species to be introduced in a new habitat to be considered as self-sufficient (Colautti & MacIsaac, 2004). The increment of this type of pressure is usually seen as number of times a species is introduced by human hand, but one can foresee that on certain types of species, the propagule pressure can be due a combination of human and natural conditions, such as to torrential rains that raise the water level of rivers overlapping the riverine area, creating connections between fish assemblages allowing the propagation of non-native species (Copp, 2006; Fobert et al., 2013). Global climate change is often appointed as one of the main culprits to facilitate the invasion of alien species due to the broad range of variables that it modifies such as water temperature and frequency of floods, often provoking critical droughts changing the assemblage of natural communities (Rahel, et al., 2008; Rius et al., 2014).

The introduction of new species has its most significant impact on endemic species that are geographically restricted making them prone to the effects of stochastic events (Bonn, et al., 2002; Godinho, et al., 1997; Pires, et al., 1999). It also exposes the endemic species to diseases and parasites as also predation and competition affecting the structure and equilibrium of the ecosystem which in the end leads to decrease of the endemic species population (Baduy, 2017).

How species distribute themselves across a new environment is tightly correlated to the environmental conditions of the location of those species. Environmental conditions can vary in terms of pH, temperature, salinity, dissolved oxygen, among others. In teleosts, homeostasis is essential for that establishment to happen. Thereby, to cope with conditions, fishes are required to adapt to differences in physical and chemical conditions of the water. Such factors can play a crucial role in the distribution of species, as individuals have an optimal range in which life is possible. Outside those boundaries, specific processes can be affected. Usually, invasive species have a broader range in which they tolerate certain environmental variables, such as temperature, salinity, pH or even pollutants. This tolerance is allowed by the modification of certain morphological, behavioural and physiological features in order to maximise their fitness (Lynch & Gabriel, 1987), which can be described as the species plasticity.

On a populational level, plasticity is critical on how species respond to the modulation of environmental factors, which by definition is, the response of one phenotype to a broad spectrum of variations along a timeline during the lifetime of an individual (Forsman, 2015; Lema, 2014). The adaptation process, not only occurs due to plasticity, as genetic polymorphisms, allelic variations in the genetic code of particular loci, also have a determinant role in the response (Forsman, 2015).

Plasticity comprises itself into two different types: development plasticity and acclimatisation. The development plasticity consists of the irreversible phenotypical variations that are fixed in the closure of the maturation phase, occurring during the developmental phase. On the other hand, the fast and reversible modifications that happen in one sole individual are denominated as acclimatisation and include metabolic switches such as endocrinal, metabolic and behavioural (Beaman, et al., 2016; Forsman, 2015).

Invasive species generate a globally spread problem engaging all continents inclusive Europe, to where many novel animals and plants were brought from the newly discovered lands overseas. This included fish as well. The Mediterranean bioclimatic region has 17 non-native fish species within the 91 freshwater fish species that currently inhabit in its streams, rivers and lakes (Ferreira et al., 2007).

The Iberian Peninsula is mostly surrounded by the Atlantic Ocean and the Mediterranean Sea, having only one well-defined land boundary, the Pyrenees mountain range, which is the main reason for its evident biogeographic isolation and explaining why this landmass has a distinct freshwater ichthyofauna (Almeida, et al., 2013; Oliveira et al., 2012).

It is essential to refer that a significant part of the presence of alien species in this region, nowadays toping around 23 species, are tied to anthropogenic introductions, as most of them are species used for ornamental or recreational fishing purposes (Leunda, 2010; Ribeiro & Leunda, 2012).

1.2.*Australoheros facetus* an alien species of concern

Cichlids, from the family Cichlidae, are found abundantly in Africa and Central and South America and scarcely in Asia, but no native cichlid species exist in Europe. This distribution pattern, alongside with an evident relation between cichlid phylogeny and sequences of Mesozoic continental rifting has led to accepting the hypothesis such patter might have arisen from Gondwanan vicariance followed by a rapid and successful regional allopatric speciation. This conjecture is still firmly accepted, despite the divergence of cichlids in the Early Cretaceous 130 Million years ago (Ma.) supported by this model, pre-dates the oldest known cichlids fossils by nearly 90 Million years (Ma.) (Friedman et al., 2013).

Cichlids are regularly known for having a lineage with high rates of diversification in relation to other families of freshwater fishes (Day, et al., 2008; Hulsey, et al., 2010; Kocher, 2004; McMahan, et al., 2013; Seehausen & Schluter, 2004) bearing around at least 1300 known different species and with estimates approaching 1900 species (Kullander, 1998). As inferred by Wagner, et al., (2012) one of the most widespread conceptions to be accepted as an explanation of Cichlids' high diversity, relies upon the aptitude of exploring new environments coupled with a competitive behaviour that nourishes in conditions of high variability. Such competition provides the means to species to coexist as resources individuals acquire morphological and physiological adaptations that allow a specialised resource utilisation thereby diminishing the pressure associated with competition (Arbour & López-Fernández, 2016; Burress, 2015).

The work developed in this dissertation targets the species *Australoheros facetus*, most ordinarily identified as Chameleon cichlid (Figure 1.1), a cichlid that was first described by Leonard Jenyns in 1842 as *Cichlasoma facetum*, nomenclature by which was most known until Kullander revised it in 1983 (Baduy, 2017). During the past two centuries, it also had other denominations, such as *Chromys oblonga* (Castelnau, 1855), *Heros autochthon* (Günther, 1862), *Heros jenynsii* (Steindachner, 1869) and *Heros acaroides* (Hensel, 1870) (Říčan & Kullander, 2008).

Figure 1.1) Exemplar of Australoheros facetus.

This species belongs to Actinopterygii (ray-finned fishes) > Perciformes (Perch-likes) > Cichlidae (Cichlids) > Cichlasomatinae (FishBase, 2019). Its genus is Australoheros, named after the Latin word “australis” which means southern and “heros” as this genus is the most southerner genus of cichlasomatine cichlids and which is part of the tribe Heroini (Říčan & Kullander, 2008).

The original distribution of the Chameleon cichlid ranged across South America, specifically in Paraná, Paraguay and Uruguay basins, including coastal effluents (Bruno, et al., 2011; Říčan & Kullander, 2006, 2008).

The first records of this species were produced by Leonard Jenyns in 1842 in the “The zoology of the voyage of the H. M. S. Beagle Under the Command of Captain Fitzroy, R.N., during the Years 1832 to 1836”, where he described this species as *Chromis facetus* after Charles Darwin collected a specimen during his journey on board H.M.S. Beagle (Ringuelet, et al., 1967).

As a freshwater species, *A. facetus* can inhabit creeks, rivers, swamps and lakes with a particular emphasis in small pools and under rocks and branches or roots of aquatic or riparian vegetation which provide better shelter (Říčan & Kullander, 2008; Ruiz, et al., 1992).

The Chameleon cichlid originates from a subtropical climate where air temperatures vary between 25ºC and 30ºC (Riel & Baensch, 1991), although studies have proven that it can withstand water temperatures from 4.5ºC to 39ºC, depending on the temperature to which they are acclimated (Baduy, 2017).

It is also a species with tolerance for a reasonably high level of salinity for a freshwater fish, up to ~15ppt in brackish water where the chameleon cichlid is also found (Baduy et al., 2016; Gómez & Naya, 2007; Perazzo et al., 2011; Pereira, et al., 2011).

It is a species where the maximum length is approximately 19.3 cm SL (Andrade & Braga, 2005), although the most common length is around 18.0 cm (Kullander, 2003). The maximum reported age is of 10 years (Stawikowski & Werner, 1998).*Australoheros facetus* has in total 15 to 17 dorsal spines, 9 to 11 Dorsal soft rays, 5 to 7 anal spines and 7 to 9 anal soft rays. This species stands out from its congeners by having a lower jaw longer than the upper jaw, the shortest dorsal scale cover and least scaled dorsal and anal fins (Říčan & Kullander, 2008).

Regarding its feeding habits, it feeds on organic detritus and material that originates from plants, preying on small aquatic animals, including small fish, following an opportunistic diurnal feeder regime (Říčan & Kullander, 2008).

When considering the life cycle, *A. facetus* is an open substrate spawner, spawning at open environment, laying the eggs in a specific substrate, with well-described mating behaviour. Both male and female demonstrate parental care upon spawning by taking care of the eggs and fry (Axelrod, 1993) guarding the larvae and juveniles for the next 1 to 3 weeks (Říčan & Kullander, 2008). It usually spawns when temperatures rise above 28ºC to 30ºC depositing the eggs on hard surfaces such as rocks and pieces of wood (Říčan & Kullander, 2008) although it has been observed that populations outside the original range may spawn at lower temperatures of 24 ºC which can be explained by the acclimatization procedure that this species can go through as shown by Baduy, (2017).

The chameleon cichlid is a species with a well-defined social hierarchy among individuals of the same population. The establishment of this social hierarchy can occur in less than 24 hours and develops through a process of aggressive behaviours to define which is the most dominant individual. Such behaviour is mostly observed in males, engaging in aggressive behaviour over the other individuals in order to establish territory and access to females. In this species, only the dominant couple is allowed to reproduce within a territory, although the extension of this territory may vary according to fish sizes or population density (Baduy, 2017; Baduy, et al., 2017).

Some different physical pattern arise after the hierarchy is established according to the social status of the individuals. The dominant males and females show light colouration with darker vertical stripes along the body, while the rest of the individuals show no apparent skin pattern, except the ones that occupy the lowest levels of the hierarchy, which have a darker colouration throughout the entire body and even darker vertical stripes (Baduy, 2017; Baduy et al., 2017). However, the submissive individuals have a defining characteristic, which is a dark-eyed colouration.

From this information, one can extrapolate that vision plays a vital role in defining an individual place in the hierarchy alongside with body size, serving as a signalling method to the individuals, as also to reduce the contact with other individuals that might expose to harmful interactions (Alonso, et al., 2011; O'Connor, et al., 1999).

The current known distribution in Portugal goes across the Guadiana river and connected streams (Collares-Pereira, et al., 2000; Elvira & Almodóvar, 2001; Hermoso, et al., 2008; Ribeiro, et al., 2009), and recorded in the Alqueva reservoir in 2003 (Ribeiro et al., 2006) as also, in the Odelouca stream in 1997 (Pires et al., 2004; Pires, et al., 2010). In Portugal, according to Decreto-Lei. (n^{o} 565/1999), *Australoheros facetus* has been defined as an introduced species, and there are reports of its presence that date back to 1943 in Vouga River (Helling, 1943) which falls far from its northernmost current location (~300 km) Baduy, (2017).

Although its legal status as a non-native species may suggest a benign presence, there is no information regarding its impact, or lack of, on the populations of native species or in the riverine ecosystems.

1.3.The importance of behaviour on cichlids

The competition within animals is, in most cases, derivative from the necessity of obtaining food and establishing a territory. This process occurs when the benefits from this type of interactions overcome the energy required to obtain it. This type of interaction usually comes to form through agonistic interactions in which, in multiple aggressive interactions between two individuals, one limits the behaviour of the subordinate individual favouring themselves, gaining recognition as the "winner", thereby dominant (Vervaecke & Stevens, 2019). In a situation in which competition is intraspecific, a social hierarchy might arise with different degrees of dominance between each (Colléter & Brown, 2011; Dewsbury, 1982; Morse, 1974; Ramallo et al., 2015).

Within fish populations, some characteristics are thought to have more weight in defining the social degree of an individual, such as the body size, age, quality and availability of food, nutritional state and the overall condition of the group in which they fit (Maruska, 2014).

When establishing its status in the hierarchy, the behavioural pattern of these individuals can be increasingly aggressive, which several times ends up in severe physical damage or even death (Neat, et al., 1998). In other cases, some species develop specific characteristics that reduce their exposure to harmful interactions from others. Typically, the individuals that reveal a more submissive behaviour develop morphological, physiological and behavioural responses in order to mitigate any energetic costs that might result from the interaction (Keller-Costa, et al., 2016; Miyai et al., 2011; O'Connell, et al., 2013).

So generally, it is possible to observe two main types of phenotypes that might be associated with hierarchic interactions as identified by Maruska, (2014). There are the dominant individuals and the non-dominant, submissive individuals, both sitting in different ends of the spectrum when refereeing to physiological and behavioural characteristics associated with the reproductive potential.

Through previous studies, it is known that urine also plays a part in the resolution of conflicts and partner choice as also observed by Hubbard, et al., (2014), Keller-Costa et al. (2014) and Martinovic-Weigelt et al. (2012) in cichlids species such as *O. mossambicus*, *O. niloticus* and *Pimephales promelas*. Communication through urine is a method that is used by many species of fishes, including cichlids, as a way of releasing pheromones into the environment (Appelt & Sorensen, 2007; Keller-Costa et al., 2014; Yambe et al., 2006). This process has a significant increase during courtship and agonistic interactions between rival individuals, in particular, in the case of male African Cichlids (Barata et al., 2008; 2007; Maruska & Fernald, 2012). In the case of South American Cichlids, the release of urine as a means of communication has not been yet subjected to studies (Hubbard, et al., 2017).

Also, fishes can use biliary and intestinal fluids as olfactive cues to interpret and identify an individual social status. This process can be explained by the high olfactory sensibly that fishes have to biliary acids. In the case of *O. mossambicus*, the concentration and intensity of the biliary fluid are positively correlated with how greater the social status of the individual is (Hubbard et al., 2017).

1.4.The importance of circulating hormones in cichlids

The hierarchic status and the respective variation are some of the factors that take part in the endocrine profiles in cichlids, as varying patterns of circulating androgens arise through the exposure of an individual to different social contexts (e.g., Antunes & Oliveira, 2009; Dijkstra, et al., 2012; Oliveira, 2009; 2001; Sessa, et al., 2013). In the specific case of *Australoheros facetus*, 17β-Estradiol (E2) plays a vital role in females, and 11-ketotestoterone (11KT) seems relevant for both sexes. Although generally, in teleosts, 11-KT has been cited to be one of the androgens present in higher concentrations in males (Borg, 1994; Kime, 1993) with more significant concentrations in males than in females (e.g. Lokman et al., 2002).

The high social status can be related to a high concentration of 11KT independently of the individual's sex while in the opposite, low social status is connected to high concentrations of cortisol. High levels of this last hormone are usually correlated to situations of high stress felt by subordinate individuals when interacting with individuals of higher status affecting their reproductive capacities (Alonso et al., 2011; White, et al., 2002). Cortisol is a corticosteroid that is considered the major stress hormone in fishes is released when the hypothalamus–pituitary–interrenal (HPI) axis is activated. The expression of this hormone proportional to the stressful conditions to which the individual is exposed (Panagiotaki & Malandrakis, 2019). High concentrations of this hormone are normally observed in in subordinate fish (Bender, et al., 2008; Fox, White, et al., 1997).

Recent studies have revealed that 11-KT is not responsible for directly influencing the social status or aggressive behaviour but changing the frequency and intensity in which courtship and aggressive territorial behaviours occur (Almeida, et al., 2014; Hau & Goymann, 2015; Maruska, 2015; Wingfield, et al., 1990). On the other hand, 11KT is crucial in conflict resolution among individuals of the same species (O’Connell et al., 2013; Oliveira, 2004; Schulz et al., 2010). Both these factors, together with Testosterone (T), play roles in gonad maturation and gamete development.

11KT has a higher efficiency in the stimulus of secondary sexual characteristics, spermatogenesis and reproductive behaviours than T (reviewed by Borg, 1994). The synthesis of 11-KT has been identified in the testis and is strongly connected to the gonadosomatic index in males (Fostier, et al., 1984; Idler, et al., 1971; Kime & Manning, 1982; Sangalang & Freeman, 1974; Scott, et al., 1980).

T is an androgen that is secreted in males by the interstitial cells, Leydig cells, of the testes. This is induced by the release of luteinizing hormone by the anterior pituitary. In females is secreted by the adrenal cortex and ovaries, although in smaller quantities than in males. In mammals it is responsible for the maturation of the external reproductive organs (penis, scrotum), glands (prostate, seminal vesicles, bulbourethral glands) and ducts (epididymis, vas deferens, ejaculatory ducts) of the male reproductive tract (Furman, 2018). It is an androgen that has been shown by Baduy et al. (2017) to have a positive correlation with 11-KT as it is a precursor of the production of this last androgen.

The biosynthetic pathway that leads to the production of 11-KT involves different stages in which T is converted by 11β-hydroxylation to 1lβ-OH-T and finally to 11-ketotestosterone (Idler & Macnab, 1967; Idler, et al., 1976) through the action of 11-hydroxysteroid dehydrogenase (Kusakabe, et al., 2003). In mammals, it was possible to identify two different types of 11-hydroxysteroid dehydrogenase known as Type 1 and Type 2 (Stewart & Krozowski, 1997).

The Type 1 11β-HSD is a low-affinity oxidoreductase that is NADP(H)-dependent, expressed in the liver, adipose tissue, brain, lung, and other glucocorticoid target tissues. This type has been proven to act as reversible oxidoreductase *in vitro* as it can also work as a reductase in organs and tissues that have intact cells in an *in vivo* environment. Within the 11β-HSD type 1 expressing cells, this reaction can create glucocorticoids from inactive glucocorticoids (Honda, et al, 2008). Seckl & Walker, (2001) have shown that this type of 11β-HSD amplifies the action of glucocorticoids in local tissues.

On the other hand, the Type 2 11β-HSD have the function of deactivating glucocorticoids in mineralocorticoids, working as a very efficient dehydrogenase in the target tissues. This type of enzyme has an impact on kidney nephrons, colon, sweat glands, which are aldosterone target tissues and, in the placenta (Bambino & Hsueh, 1981). This enzyme can be responsible for metabolising bioactive glucocorticoids (cortisol and corticosterone) to inert 11-keto forms (cortisone and 11-dehydrocorticosterone) (Ozaki et al., 2006). This Type 2 11β-HSD is a high-affinity unidirectional oxidase that is also NAD-dependent. Teleosts have homologs with similarities to the Type 2 11β-HSD. This affirmation has been supported through studies done in rainbow trout (Kusakabe et al., 2003), tilapia and Japanese eel (Jiang et al., 2003).

The Japanese eel homolog metabolised cortisol to cortisone (Jiang et al., 2003) while the 11β-HSD2 in the rainbow trout also converted 11β-OHT to 11-KT (Kusakabe et al., 2003). Although the production of 11-KT via 11β-HSD is a vital process within the endocrinal system in teleosts, it has only been studied in the rainbow trout (Kusakabe et al., 2003).

E2 is an estrogen produced by the ovaries that as the function of mediating vitellogenesis and oogenesis in non-mammalian vertebrates and regulate gonadotrophins through by feeding back to hypothalamus and pituitary (Lubzens, et al., 2010). E2 is a product of the ovary, although there are many other tissues capable of producing it and using it in a paracrine or intracrine way (Nelson & Bulun, 2001) and is very active both in mammals as well in fishes (Sumpter, 1995).

Aromatase is a cytochrome P450 enzyme expressed in the female ovary and male testis that has the role of converting androgens into estrogens. Estrone, E2, and estriol are three estrogens that are synthesized through an aromatization step that causes each one of the estrogens to have a phenyl ring. Aromatization of estrogens is irreversible reaction that converts androstenedione in estrone, T in E2 and 16α-hydroxytestosterone to estriol (Wood & Cupp, 2018).

This estrogen is responsible for the expression of vitellogenin (Vtg) in the liver, which is a precursor of yolk proteins (Allner, et al., 1999; Arukwe, et al., 2000; Jobling, et al., 1996; Lim, et al., 1991; Sumpter & Jobling, 1995).

The liver of oviparous females, commonly produces Vtg, a complex phospholipoglycoprotein that is cleaved into yolk proteins, including lipovitellin and phosvitin once absorbed by the growing oocytes (Mommsen & Walsh, 1988; Tyler, et al., 2000). Not also is Vtg generally observed in females as it is also present in males but in a silent form due to the low levels of circulating E2 in males. Even though it is a silent gene, it can be expressed through the introduction of exogenous estrogens (Flouriot, et al., 1997; Wahli, et al., 1998). This type of treatment in males can also provoke the impairment of gonads function, including low sperm quantity, several cellular alterations and decrease of gonadosomatic index (Bieniarz et al., 1996; Christiansen, et al., 1998; Gronen et al., 1999; Jobling et al., 1996; Kinnberg, et al., 2000; Orlando, et al., 1999; Shioda & Wakabayashi, 2000), all phenotypic effects indicative of feminization (Filby, et al., 2007).

The greatest impact that the estrogen E2 has on behaviour is the capability of reducing the aggressive behaviours of dominant males exposed to it (Bell, 2001; Colman, et al., 2009; Filby, et al., 2012; Majewski, et al., 2002). E2 has also been described for causing the feminization of male individuals or, in more severe cases, the sex reversal of a fish population (Falahatkar, et al., 2014; Wang et al., 2008).

1.5.Methods of population control

Invasive species are becoming a growing concern putting in harm native species, populations and even ecosystems; hence, it is essential to develop new methods of population control.

Many studies are taking place to reach this objective by evaluating the impact of some types of destructive substances. Direct fishing is a standard method used for aquatic species. Many of these methods have proved to be effective in controlling the spread of some species, but in most cases, with the downside of affecting non-target species (Dalu, et al., 2015; Ribeiro, et al., 2015; Tate, et al., 2003).

Methods that rely on the use of physical barriers have increased safety although being less productive and consistent (Kates, et al., 2012; Noatch & Suski, 2012; Zielinski et al., 2014). These barrier methods also display certain flaws, because, besides blocking the dispersal of the target species, they also do not permit the displacement of non-target species to a particular location that can be crucial for a specific life stage of that species. For example, these methods can block the access of a non-target species to a location that could be optimal for reproduction and spawning (Baduy, 2017).

Some other methods based on the use of chemicals can induce a physical response on the target individual. Through an in-depth knowledge of specific mechanisms that take place in the life cycle of a species, such as chemical communication, hormonal cycles or life partner choices, it is possible to develop technics that can attract these species from the target location such as traps (Aquiloni & Gherardi, 2010; Johnson, et al., 2009; Sorensen & Stacey, 2004).

Johnson et al. (2013) studied the efficiency of the use of chemical compounds as a mean of population control in sea lamprey (*Petromyzon marinus*), an invasive species that has become a parasite in the upper Laurentian Great Lakes becoming one of the primary causes for the mortality of large- and medium-sized fish (Bergstedt & Schneider, 1988; Kitchell, 1990). The male mating pheromone, 3kPZS can be used to attract ovulated female sea lampreys upstream to the location where it was used (Siefkes, et al., 2005) or into traps (Johnson et al., 2009; Luehring, et al., 2011).

Understanding the way how certain species reproduce cam be a crucial tool to develop new technics of population control. This tool can be used to find new methods to inhibit the reproduction of a target individual directly.

Castration can be a reliable as a method of population control as it is used to impair reproduction. This method can be done through different ways: Physical castration chemical castration and eventually, genetical castration.

Physical castration is an effective way to inhibit reproduction, although it is an invasive method that relies on the removal of specific reproductive organs. This methodology demands tremendous experience because if the surgery is not conducted correctly, organs can regenerate and defeat the purpose of the study. It is a process that demands a more extended period of recovery and increases the risk of infections that can be harmful to the target individual, as also, propagate within an entire population, making it challenging to eradicate. The practical application of this method can be time-consuming when the objective is to control the reproduction within a population.

On the other hand, chemical castration relies on the application of a chemical substance that inhibits the release of circulating hormones that required for gonadal development and reproduction.

This can be done at different levels of the endocrine axis controlling sexual maturation and reproduction. Some available methods are agonistic substances of Gonadotropin-releasing hormone (GnRH), acting on upper levels of the axis to produce action downstream. The hypothalamus produces GnRH that acts as a stimulant for the production of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) in the anterior pituitary (Wierman, 2019). These act in the gonads to control steroidogenesis and the levels of sex steroids that then have an action stimulating the testicular development and spermatogenesis (Huhtaniemi, 2015) or stimulating folliculogenesis and oocyte maturation and release (Messinis, et al., 2014).

Other methods may act downstream, directly on steroidogenesis or influencing circulating steroid levels to impair gamete production and release and sexual behaviour.

CBX, a compound a derivative glycyrrhetinic acid (GE) which is an active mineralocorticoid compound of liquorice along with glycyrrhizic acid (Stewart, et al., 1990), It is believed to have the properties to be considered as a good chemical castration method.

This chemical compound has been proven to be a reasonably successful mean of treatment of gastric and duodenal ulcers throughout Europe (Loginov, et al., 1980).. However this use has been decreasing in popularity as CBX is a steroid congener which as undesired mineralocorticoid activity (Jewell, 2007).

CBX is also able to inhibit the production of 11β-hydroxysteroid dehydrogenase (11β-HSD), which many studies can support its importance of glucocorticoid conversion pathways in mammalian tissues (Jellinck, et al., 1993).

By inhibiting 11β-HSD, CBX can hinder the production of 11KT. As previously referred, 11-KT is involved in spermatogenesis and has a vital role on the frequency in which aggressive territorial and mate-guarding behaviours occur even though is not directly correlated with the expression of the hierarchic status or aggressive behaviour (Almeida et al., 2014; Hau & Goymann, 2015; Maruska, 2015; Wingfield et al., 1990). So, by administering CBX to a target individual, it is expected that this decrease their courtship behaviour but also result in impaired spermatogenesis, making them less capable of conceiving offspring.

E2 can also be administered as a method of population control because, even in highly dominant males it may induce a reduction of aggressive behaviour (Bell, 2001; Colman et al., 2009; Filby et al., 2012; Majewski et al., 2002) and feminization, making the target individual inapt to generate offspring.

Although genetical engineering is a powerful and emerging technology, with practical uses as a mean of inhibiting reproduction, it is still a taboo subject as the secondary effects that it bears are still not extensively studied. The introduction of genetically modified individuals to the wild it is still strictly controlled and, in most countries, illegal, which renders the applicability of this method as a mean of population control, unfeasible.

1.6.Work Objective

Australoheros facetus is an ornamental species that has been introduced in the southern riverine areas of Portugal. Although it is considered by law as only as an Alien species, the impact that it bears in the endemic species and ecosystem are still unknown. In order to mitigate the impact that this species might have, it is necessary to study new approaches to contain and impede this species from spreading.

In this dissertation, it is intended to study the concept and the respective applicability of CBX as a method of population control to be applied over male Chameleon cichlid individuals.

This work intends to study the impact that this compound can have in behaviours and endocrinal profiles and how can these affect the reproductive capability, not only at an individual level, as also in a populational level.

An observational approach to the behaviours allows them to be catalogued and accounted for, which in the end permits through a statistical approach to determine how they are modulated throughout different stages of this trial. This information combined with endocrine values retrieved at different stages can be used to determine how behaviours are impacted by the different endocrinal patterns.

Such information can be used to verify whether if the individual can maintain or not a dominant social status and still form a couple with the dominant female, the only female capable of generating an offspring, and can also provide information if the male is capable or not of displaying courtship behaviours, which are crucial for the success of producing offspring.

2. Materials and Methods

The work developed in this dissertation was comprised of different phases, each one requiring a certain degree of preparation to ensure the smooth running of the trial.

The first step was to gather the proper condition to distribute our population in smaller groups, thereby requiring smaller tanks that had to be prepared readapted to receive the different test groups ensure the best welfare conditions for our study population. This required forehanded preparation of an oxygenation and filtration system adapted to the physiological requirements for the target species.

Then the individuals had to be selected and distributed to ensure a homogeneous population throughout the different groups of the series and to select the proper focal individual slightly larger than the others to force the dominance which is essential for this study as it focusses on the social and endocrinal impacts of certain compounds in dominant male *A. facetus* individuals. This step required the sampling of a variety of variables such as size, weight and even the collection of blood samples to ensure a proper cataloguing of crucial data about test population that can be then used as a term of comparison. These are adequately identified using electronic tags preventing the loss of track of a certain individual.

After the groups are correctly organized, the observations before administering the test treatment can then be planned and initiated (Time zero or T0). This first stage of observations lasted for five days, where 5-minute observations were made every day in the morning and the afternoon and are vital to understanding where our focal individual sits on the group social hierarchy.

When the pre-treatment observations were concluded, the fishes had blood samples collected again and afterwards the treatments could then be prepared and administered (T1). Each of the treatments (Sham, E2 and CBX) were administered to the groups in the series pairwise granting a replicate per treatment in each series.

A new period of observations was conducted after the administration of the treatment lasting a more extended period of seven days. These observations allowed to observe the impacts that the administrated compounds cane have at a behavioural level and also ensuring enough time for these treatments to bear a differentiable effect.

At the end of the post-treatment observations, blood samples were collected using the same methodology used before (T2). Afterwards, the fishes were euthanized, and the focal individual dissected for direct observation of the gonads to determine the sex. If the non-focal fishers were only used once, they can then be reshuffled into new groups. If the groups are incomplete more fishes can be obtained from the main repository. If the non-focal individuals have been used more than once, new complete groups can be created.

The previous steps after the tanks preparation of the tanks were repeated in the same sequence for the other series.

When all the required series are completed, the blood samples can then be prepared and used in a RIA analysis to determine the levels level of the focal individual hormones at different stages of the trial (T0; T1; T2).

Finally, with behavioural data collected and the data relative to the hormone levels, it could then be compiled in an electronic support and be used for further analysis.

2.1.Tank assembly

To house the social groups used to establish hierarchy and evaluate the dominant and submissive behaviours it was required to prepare and adapt the tanks, before starting the procedure to provide the conditions necessary for the experimental setup.

For that, six fibreglass tanks were used, each with a volume of 260 litres as seen in Figure 2.1. These were opaque in three sides and featured a front window, made of glass. The same tanks were used throughout the experimental period, and several groups were placed consecutively in these tanks.

Figure 2.1) 260 litres tanks used in the experiment in LEOA, Universidade do Algarve.

Each tank was fitted with its biological filter, consisting in a ground platform that permitted the water circulation through the sandy bottom combined with a layer bioballs and corrugated pieces of a plastic tube that increased the surface area for the attachment of water purifying bacteria. The water circulation was created by running an air feed tube with a lead weight, and an air stone within a PVC tube that connected to the base aeration system and with a curve on the top end at the water surface. This tube system would serve as an airlift, in order to properly oxygenate the system and to recirculate water through the Bio Balls and pieces of corrugated plastic tube placed in the bottom.

The biological filter was completed by covering the previously referred assembly with a fine plastic net, followed a layer of nylon fibre which was later covered by a layer of a plastic net with larger holes. The biological filter was then finished with a layer of coarse sand and topped with a layer of gravel. This filter ensured the screen retained a significant part of circulating bacteria and detritus in the water. The filtering system combined with the capacity of allowing proper even oxygenation of the water and substrate avoided the growth and propagation of unwanted anaerobic bacteria that could reduce the quality of the water and health of the individuals in those tanks.

The temperature was kept within 20 and 22°C throughout the experiment without recurring to thermostats and through manual adjustment.

2.2.Creation of social groups

Four consecutive series of trials were performed, each with six different groups of fishes distributed in six previously prepared tanks.

The individuals used were selected from the stock of fish captured in the Vascão and Odelouca rivers, in previous years, and stocked at the facilities of CCMAR/University of Algarve. Four 2500 litres cylindrical tanks connected in closed circuit with a mechanical and biological filter, located in the exterior of LEOA (Laboratório Experimental para Organismos Aquáticos) one of the vivarium of aquatic organisms for the Faculty of Sciences of the University of Algarve, contained all the fishes used in this experiment (Figure 2.2).

Figure 2.2) Repository tanks of A. facetus in LEOA at Campus das Gambelas, Universidade do Algarve.

Initially, the fishes were collected from the previously mentioned tanks and stocked in smaller containers to select the individuals to be used. From here, these were anaesthetised with 2-Phenoxyethanol (Sigma-ALDRICH, ref. 77699) then weighed, measured, marked with pit tags (Trovan® TROVANUNIQUE™ ID-100A Microtransponder) and had blood samples collected for further steroid analysis.

Before the blood collection, povidone-iodine ointment (MEDA Pharma, Betadine®, ref. 9454728) was applied in the posterior part of the caudal peduncle, under the lateral line, where the needle was going to be inserted. The blood collection was done by using heparinized Terumo® U-100 syringes of 1ml with Terumo® Agani™ needles of 23Gx1/4" (0.6x32mm).

The blood was collected from the dorsal aorta or caudal vein that run under the spine (Figure 2.3.a) and then stored and refrigerated to be then taken to the laboratory to have the plasma separated from the cells through centrifugation and then frozen for further analysis.

Afterwards, the fish were injected with a pit-tag in the abdominal cavity using an implanter (Trovan® VetPlant) (Fighure 2.3.b) and the tag was checked with the scanner is to see if it was working correctly (Trovan® TROVANUNIQUE™ LID-573 Pocket Reader (USB)). This unique ID code was registered in a logbook with the number of the blood sample tube attached. The fish were transferred to a container with dechlorinated water and proper aeration system where they were able to recover from the anaesthesia after the sampling procedure.

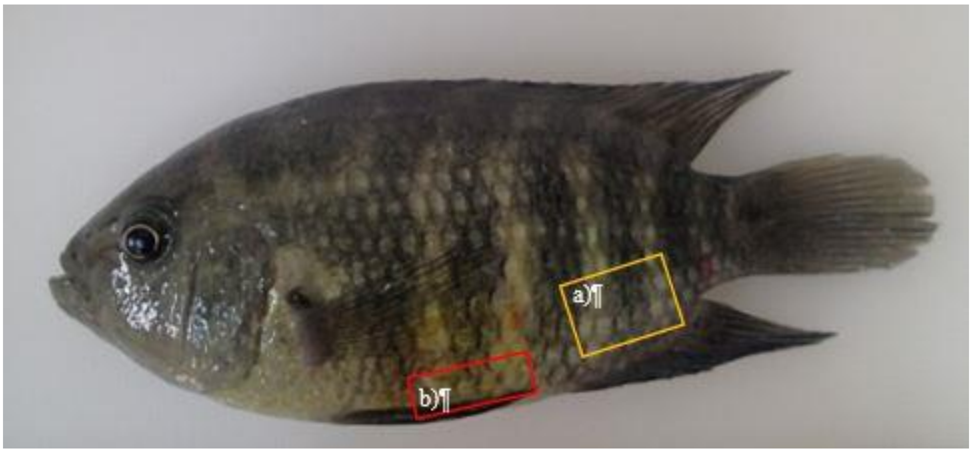


Figure 2.3) Fish sampling procedure. a.) Blood sample collection section; b.) Pit-tag injection placement.

This step was followed by the recording of the data on the experiment logbook and in digital support. This data was then used to determine which would be the most suitable group for a respective individual accordingly to their weight.

The weight and lengths of all fish were analysed to create six groups of 5-6 individuals of similar size, i.e. the variation in size in each tank should not be over 10%, even if there are significant differences between the average sizes of each tank. The same criteria were used for the weight variation throughout the different sets. Except for one individual per tank, that had a weight approximately 10-20% higher than the average of the rest of the group to force the dominance of that individual among the community formed within the corresponding tank.

After each trial, the non-focal fish were reshuffled into new social groups (except any injured fish) and the dominant fish was removed and sacrificed. Larger naïve fish obtained from the stock tanks selected to be the putative dominant male. All fish were placed in the group at the same time to allow to preclude former social interactions. Some fishes were taken from the repository tanks outside LEOA and sampled using the same methodology as former whenever it was required to complete the groups.

2.3.Observations

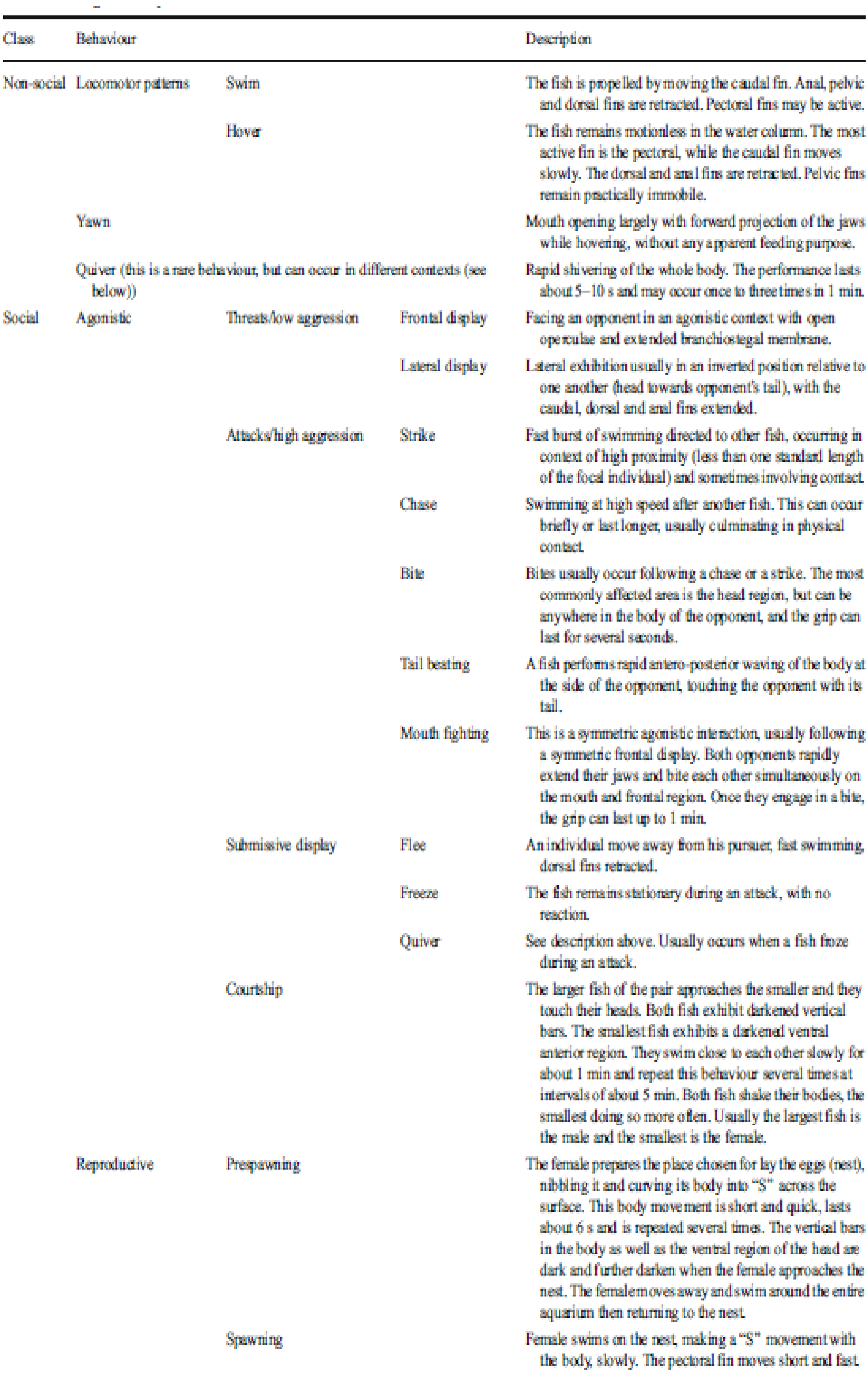
The behavioural analysis was conducted using the Software The Observer XT 8.0 from Noldus (Product key OB080-03880-DMAOANAA-R), which was used to register all the behaviours observed in the fishes assumed to be dominant.

The Observer XT is a software developed by Noldus as a presentation tool of observational data in animals by allowing the manual recording of events and its collection, management, analysis (Noldus, 1991). Not only can it be used in animal behavioural studies, but due to its reach and capacities, it can be used in all sorts of studies that involve the logging and compilation of observational data (Zimmerman, et al., 2009).

The Observer XT can be used to import videos and carry out observations offline as also, it can be used to do any observations while the software records the experiment directly through a USB camera device. The observational log can also be synchronised with the event registered in the video support (Zimmerman et al., 2009).

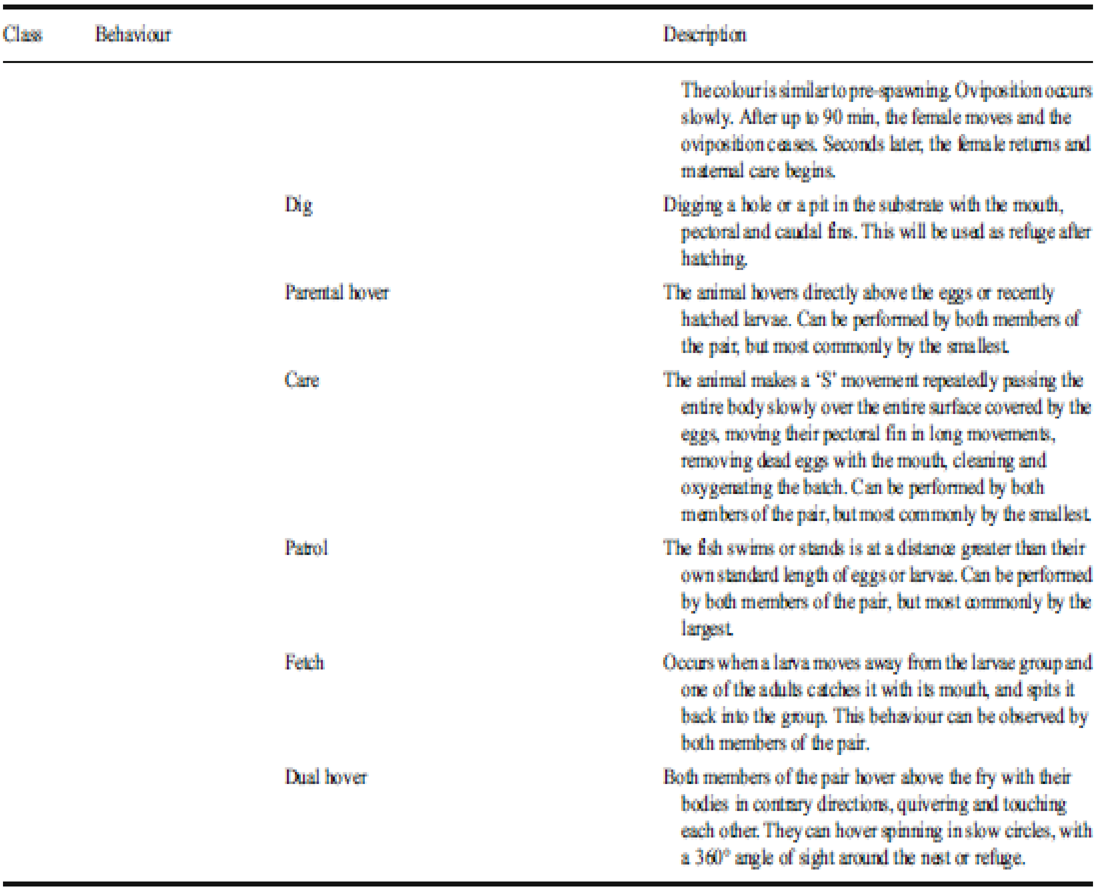
For the work developed in this dissertation, the software was set up for live direct observation with no video recording support with each observation limited to a time interval of 5 minutes and to do only single-individual observations.

The Observer XT was updated with all the relevant and known behaviours and behavioural groups of the species *A. facetus* as described by Baduy, (2017) without including modifiers.



Class	Behaviour			Description
Non-social	Locomotor patterns	Swim		The fish is propelled by moving the caudal fin. Anal, pelvic and dorsal fins are retracted. Pectoral fins may be active.
		Hover		The fish remains motionless in the water column. The most active fin is the pectoral, while the caudal fin moves slowly. The dorsal and anal fins are retracted. Pelvic fins remain practically immobile.
	Yawn			Mouth opening largely with forward projection of the jaws while hovering, without any apparent feeding purpose.
	Quiver (this is a rare behaviour, but can occur in different contexts (see below))			Rapid shivering of the whole body. The performance lasts about 5–10 s and may occur once to three times in 1 min.
Social	Agonistic	Threats/low aggression	Frontal display	Facing an opponent in an agonistic context with open operculae and extended branchiostegal membrane.
			Lateral display	Lateral exhibition usually in an inverted position relative to one another (head towards opponent's tail), with the caudal, dorsal and anal fins extended.
		Attacks/high aggression	Strike	Fast burst of swimming directed to other fish, occurring in context of high proximity (less than one standard length of the focal individual) and sometimes involving contact.
			Chase	Swimming at high speed after another fish. This can occur briefly or last longer, usually culminating in physical contact.
			Bite	Bites usually occur following a chase or a strike. The most commonly affected area is the head region, but can be anywhere in the body of the opponent, and the grip can last for several seconds.
			Tail beating	A fish performs rapid antero-posterior waving of the body at the side of the opponent, touching the opponent with its tail.
			Mouth fighting	This is a symmetric agonistic interaction, usually following a symmetric frontal display. Both opponents rapidly extend their jaws and bite each other simultaneously on the mouth and frontal region. Once they engage in a bite, the grip can last up to 1 min.
		Submissive display	Flee	An individual move away from his pursuer, fast swimming, dorsal fins retracted.
			Freeze	The fish remains stationary during an attack, with no reaction.
			Quiver	See description above. Usually occurs when a fish froze during an attack.
		Courtship		The larger fish of the pair approaches the smaller and they touch their heads. Both fish exhibit darkened vertical bars. The smallest fish exhibits a darkened ventral anterior region. They swim close to each other slowly for about 1 min and repeat this behaviour several times at intervals of about 5 min. Both fish shake their bodies, the smallest doing so more often. Usually the largest fish is the male and the smallest is the female.
	Reproductive	Prespawning		The female prepares the place chosen for lay the eggs (nest), nibbling it and curving its body into "S" across the surface. This body movement is short and quick, lasts about 6 s and is repeated several times. The vertical bars in the body as well as the ventral region of the head are dark and further darken when the female approaches the nest. The female moves away and swim around the entire aquarium then returning to the nest.
		Spawning		Female swims on the nest, making a "S" movement with the body, slowly. The pectoral fin moves short and fast.

Figure 2.4) Ethogram from A. Facetus retrieved from Baduy et al. (2017).



Class	Behaviour	Description
		The colour is similar to pre-spawning. Oviposition occurs slowly. After up to 90 min, the female moves and the oviposition ceases. Seconds later, the female returns and maternal care begins.
	Dig	Digging a hole or a pit in the substrate with the mouth, pectoral and caudal fins. This will be used as refuge after hatching.
	Parental hover	The animal hovers directly above the eggs or recently hatched larvae. Can be performed by both members of the pair, but most commonly by the smallest.
	Care	The animal makes a 'S' movement repeatedly passing the entire body slowly over the entire surface covered by the eggs, moving their pectoral fin in long movements, removing dead eggs with the mouth, cleaning and oxygenating the batch. Can be performed by both members of the pair, but most commonly by the smallest.
	Patrol	The fish swims or stands is at a distance greater than their own standard length of eggs or larvae. Can be performed by both members of the pair, but most commonly by the largest.
	Fetch	Occurs when a larva moves away from the larvae group and one of the adults catches it with its mouth, and spits it back into the group. This behaviour can be observed by both members of the pair.
	Dual hover	Both members of the pair hover above the fry with their bodies in contrary directions, quivering and touching each other. They can hover spinning in slow circles, with a 360° angle of sight around the nest or refuge.

Figure 2.5) Ethogram from A. Facetus retrieved from Baduy et al. (2017) (Continuation).

All the behaviour groups were considered as mutually exclusive as none of the behaviours included did occur at the same time as others and none was regarded as an exhaustively active behaviour group because no behaviour taken in the observation was continuously engaged. Non-social behaviours were discarded due to not be relevant for the objective of this work.

Most behaviours were logged as point events, which are events of short unquantifiable time span (Martin & Bateson, 2007), while Front and Lateral display, Mouth fighting, Freeze and Quiver were logged as state events as they have a quantifiable duration (Martin & Bateson, 2007).

2.3.1. Conducting the observations

The observations for each series were divided into a pre-treatment phase and post-treatment phase. The pre-treatment phase extended for five days, which allowed to standardise the behaviour of a particular individual and gather enough information to determine its place in the hierarchy. The post-treatment phase endured for seven days to allow the observation of a possible reaction curve of the individual’s behaviour to the treatment to which was subjected.

There were two observations per day for each tank, one during the morning and another in the middle of the afternoon.

For five minutes, any behaviour that the focal individual made was immediately logged using The Observer XT 8.0. This is a fast-paced process, so the person who is conducting the observations will require to conduct a few test observations before the trial to become used to the software.

While the observations took place, the person observing had to take some time and sat in front of each tank for the fishes in the tanks to get used to the observer, ensuring that their behaviour is not affected by the presence of the observer. In every observation, the observer used a white lab coat to grant that the observer is recognised and, due to the white colour being less intrusive and soothing ensuring that the individuals did not saw the observer as a possible threat.

2.4.Experimental inhibition of male features by chemical castration

The treatments were administered after five days of observations without any treatment. In each set, with six different focus individuals, two were injected with 17β-E2 (E2), two with the drug CBX and two only with the vehicle solution, which was coconut oil+Ringers/4%ETOH. The las ones served as a control and to verify if the treatment procedure per se, could affect or not the individuals' behaviour and levels of circulating hormones.

2.4.1. CBX injection preparation

It was first necessary to prepare a Ringer solution for freshwater teleosts to prepare the solution of CBX to be injected. The ringer was prepared accordingly to Hoar & Hickman, (1975).

The quantities of the components of the ringer were adjusted accordingly with the quantity of ringer necessary for the entire experiment and the pH adjusted to 7.6.

The CBX solution was then prepared previously to every treatment as CBX has a short shelf life when in solution form. The quantities prepared were adjusted to the weight of the individuals to be treated plus a margin in case if there were any problems with the injections. In a preliminary test it was injected 10mg of CBX per 100g of fish biomass as used in different studies conducted in other species (Derelanko & Long, 1981; Henman, 1970; Okabe, et al., 1976), but this test demonstrated that *A. facetus* could not cope with such concentration revealing problems in controlling the air volume in the swim bladder. This treatment caused the accumulation of excessive gas making it difficult for the fish to keep buoyancy, leading to death, most likely by exhaustion, although other toxicological effects cannot be excluded.

Thus, for the actual trials, 2.5mg of CBX disodium salt (SIGMA-ALDRICH, ref. C 4790) were used for every 100g of fish biomass to be injected.

As the total quantity of CBX solution injected per fish was 1ml for 100g of biomass, the previously referred proportion of CBX had to be dissolved in a solution composed of ringer and 4% of Ethanol 100% (to be identical to the vehicle used for E2). This solution was injected in a proportion of 200µl per 100g of biomass. The solution of CBX, Ringer and 4% of Ethanol 100% was then mixed in a proportion of 1:4 of coconut oil, meaning that was injected 800µl per 100g of biomass. The nominal CBX dose was 25 ug/g fish.

2.4.2. E2 injection Preparation

E2 solution was prepared by dissolving it with Ethanol 100%, diluting in Ringers to the appropriate concentration and was injected in conjunction with coconut oil as a vehicle.

The fish exemplars in this study that had this treatment administered were injected with E2 in a concentration of 1.5mg per 100g of fish biomass.

Estradiol-17β (Sigma) was used in a concentration of 15µg per gram of biomass to prepare the E2 solution. For every 100g of biomass to be injected, 1.5mg of E2 was dissolved in 400µl of Ethanol 100%. These values were then proportionally scaled up or down accordingly to the quantity of fish of biomass to be injected plus an extra if a problem would arise. E2 in a solution with ethanol 100% does not have a reduced shelf-life so, higher quantities of solution were prepared for further necessary treatments.

The previously referred solution was mixed with Ringers and then this solution was added to coconut oil in a proportion of 2:3 just before the treatment. Thereby, for each 100g of fish biomass to be injected, 200µl were E2 in Ringers plus ETHO4% solution and the other 300µl, coconut oil.

In the end, 1ml of the final solution was injected per 100g of biomass and the nominal E2 dose was 15 ug/g fish.

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2.4.3. Sham Injection preparation

Two focal individuals in each series were subjected to the injection procedure using just the vehicle substance, Ringers/4% ETOH and coconut oil, without recurring to both CBX and E2. This step allowed to test the effects of the procedure by itself and to serve as control where no treatment was applied.

As in the case of E2 and CBX, the individuals were injected with a total solution of 1ml per 100g grams of biomass, which was the same proportion used in the sham test, although these were only injected with the vehicle substance.

2.4.4. Treatment administration procedure

To treatment administer the treatment procedure the individuals were anesthetised using phenoxyethanol and by collecting blood samples in the same methodology as referred before. The treatments were administered using Terumo® U-100 syringes of 1ml with Terumo® Agani™ needles of 23Gx1/4" (0.6x32mm) via intraperitoneal injection in which the target substance was slowly administered in the abdomen region, posterior to the lateral fins. Having in account the length of the needles used (32mm), spacers of 20mm of Styrofoam were used to avoid any damage to the internal organs while administering the treatment.

In the end, the fish was placed in the same recovery conditions created after any sampling procedure.

2.5.Euthanization of fishes and sex identification

At the end of a series, sex identification differentiation was done through the direct observation of gonads. As *A. facetus*, does not present evident sexual dimorphism, no visual characters can be used for sex identification (Baduy, 2017), and non-lethal methods such as radioimmunoassay (RIA) analysis have been proven not to have high accuracy, thereby fishes had to be euthanised and dissected to corroborate the results from the assay.

The fishes were placed in low concentrations of Phenoxyethanol (2ml/L) to allow blood samples to be collected.

Then blood samples using the same methodology as referred in the group creation section. This step was only applied for the focal individuals.

Afterwards, the individuals were euthanized with an overdose of Phenoxyethanol (>10ml/L) and were left for a more extended period in the anaesthetic to ensure that these were not alive at the time of the dissection to ensure that the fishes were euthanised in the most humane conditions as possible.

Fish were sacrificed by section of the spinal corf and the dissection followed a simple protocol in which an incision is made from the urogenital orifice to the posterior region of the head along the ventral region. During the dissection, the pit tag was removed and stored in ethanol 100%.

Finally, the resulting information was logged, and a picture of the gonads was taken as seen in Figure 2.4.

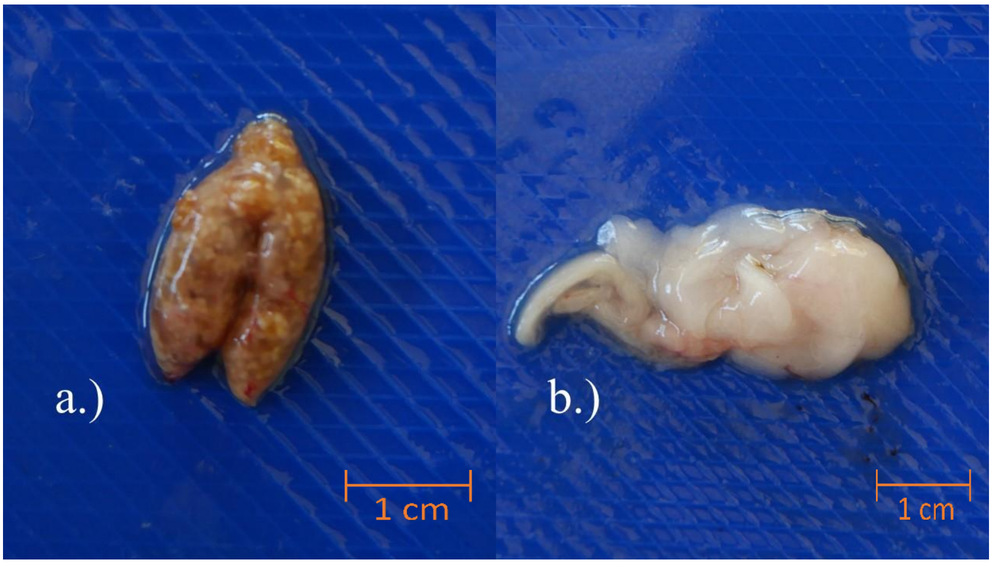

Figure 2.6) Gonads of a). Female and b.) Male of A. facetus.

2.6.Radioimmunoassay Analysis

As previously referred in this dissertation, the species *Australoheros facetus* does not show an evident sexual dimorphism (Baduy, 2017) and some further steps were required to verify the sex of each individual, in particular, the focal individuals.

Radioimmunoassays can be used to verify the levels of specific circulating hormones in the blood such as E2 which is a specific female hormone which that has an active role in the mediation of vitellogenesis and oogenesis in non-mammalian vertebrates. This process could also be used for cortisol, 11KT among others.

Radioimmunoassays employ the use of radioisotopes as detection labels (Deshpande, 2012). These labels are isotopic markers that allow easy detection of low quantities of antigens, antibodies, or antigen-antibody complexes (Cerda-Kipper, et al., 2018).

The basis on how Radioimmunoassays work is the competition for specific antibody sites for the hormone that is being evaluated between radio-labelled and unlabelled (our sample) antigens (Rao, 2005).The concentration of the analyte to be studied is scaled to the number of the antibodies binding sites available for interaction with the radiolabelled ligands (Maggio, 2018). Antibodies are mixed with labelled antigens in known concentrations that allowed that all antibodies binding sites to be saturated. In this step, the radiations levels at the beginning are close to 100% of maximal biding. Then the unlabelled antigens in plasma samples in unknown concentration are added to the mixture (Rao, 2005).

When the plasma is added, the labelled antigens start being removed from the binding sites as the hormone present in the serum start to compete for the same sites. The amount of hormone that will bind to these binding sites is proportionally to the concentration of hormones present in the sample. As the hormone being tested is not labelled, there will be a decrease in overall radioactivity as labelled antigens are washed away, thus being less than 100% maximal binding. The radioactivity decrease is proportional to the quantity of hormone present in the sample. The actual concentrations can be accurately determined by comparing to the decreasing binding percentages of a curve with known concentrations of unlabelled hormone. This method also involves a separation step, as during the incubation with antibody, labelled ligand and unlabelled ligand, two phases will be formed, one with the free ligand and another with the ligand bound to antibody. To separate these phases an absorbent media is added followed by centrifugation, and in this case activated charcoal was used as the absorbent.

2.6.1. Extraction of free steroids

The extraction process used in this work followed the methodology used by (Canario & Scott, 1989). This process was done to ensure that only the free steroids were left without any other component that might interfere with the RIA analysis.

The samples were defrosted and centrifuged in case of the existence of clots. These samples were then was transferred into glass extraction tubes and mixed with diethyl ether to extract proteins and centrifuged to separate both phases.

Afterwards, the samples were placed in dry ice to only solidify the water phase and the liquid phase decanted to glass vials placed in a warm dry bath to evaporate the ether. The last steps were repeated until only free steroids were left.

Finally, gelatine buffer was added according to the initial amount of the sample and frozen.

2.6.2. RIA Preparation

It is vital to refer that according to the different hormones to be analysed, different antibodies, labelled hormones and tracers were used. The evaluation of the analysis and crossed-reactions followed the procedures described by Rotllant et al. (2005) for Cortisol, by Kime & Manning (1982) for 11KT, by Mota, et al. (2014) for testosterone and E2 by Guerreiro, et al. (2002).

For the RIA, a standard curve was prepared to serve as a comparison term for result verification by using the unlabelled hormone at different known concentrations throughout twelve different solutions, with each one also having a replicate. This curve has also a Total sample, which represents the total number of counts possible, having only labelled hormone without any activated charcoal; the Blank sample which only contains labelled hormone and charcoal, is used to demonstrate what is the remaining activity resulting from unspecific binding and the Maximum which includes labelled hormone and the respective antibody but not unlabelled hormone, and it was used to determine which was the maximum of binding possible between the antibody and the tracer. The rest of the samples of the curve contain a series of different known concentration of the unlabelled hormone. Our unknown samples, the fish plasma samples, are treated as the curve samples.

The tubes were then filled with enough tracer (3H) in order to get 1500 counts per minute in the RIA counter for each 100 µl of solution. After overnight incubation in a refrigerator the solutions had the activated charcoal solution with dextran and buffer added except the total. The samples were finally transferred to scintillation vials with scintillation fluid and placed in a scintillation counter (Microbeta Trilux 1450- Wallac. Perkin Elmer) to start the counts. For E2, 17β Estradiol (Ref. E8875; Sigma) was used for the standard curve, then Antibody Estradiol-RDI-40 E2 and the Marker Estradiol, 17β-[2,4,6,7,16,17-3H(N)] (Ref. ARC- ART 1549).

For T, Testosterone - 4 Androsten-17β-ol-3 one (Refº T 1500; Sigma) was used for the standard curve, and then Testosterone antibody (Ref 20TR05; Fitzgerald) and the marker Testosterone-[1,2,6,7-3H(N)]-, 250µCi (9.25MBq) (Ref NET370250UC; Perkin Elmer).

For cortisol, Cortisol-4-Pregnen-11b,17a,21-triol-3,20-dione (Ref H4001) was used in the standard curve, and then the Cortisol antibody (Ref. 20 CR50; Fitzgerald) and Hydrocortisone Marker (Cortisol, [1,2,6,7-3H(N)]-), [1,2,6,7-3H(N)]-, 250µCi (9.25MBq) (Ref. NET396250UC; Perkin Elmer).

For 11-KT, 11-Ketotestosterone- 4-Androsten-17β-ol-3,11-dione (Ref. K8250; Sigma) was used for the standard curve, and then the Antibody 11Kt- 4-Androsten-17β-ol-3,11 dione (Project #7398; Proteogenix) and the marker was produced in lab.

2.7.Statistical analysis

The data resulting from the observations was compiled with the software The Observer XT 8.0 by Noldus. After all the information was compiled, it was then exported to Microsoft Office Excel for descriptive statistical analysis.

2.7.1. Master file compilation

The extracted file was used to produce a master file containing all the information obtained during the practical work done in this dissertation, including also qualitative data such as Pit Tag code, standard length, total length, weight, sex, etc. The total duration of the state events was converted to a percentage relative to the duration of each observation. Information relative to the circulating levels of 11-KT, Testosterone, Cortisol and E2 at the 3 sampling points was also added.

The difference between hormone plasma concentrations at T2 *minus* T1 delivered the delta (Δ) values using the same methodology as (Saraiva, et al., 2017). The use of Δ provides a straightforward way to test variations in hormone levels since a unique value provides information both on the direction and the magnitude of the change.

All statistical tests were performed in the software IBM® SPSS® Statistics (Version 26.0.0.0).

2.7.2. Statistical Analysis in SPSS

The assumptions of normality of the data were verified prior to testing. When the data complied with assumptions of normality parametric statistics were used be used.

In case data did not comply with assumptions of normality, non-parametric statistics were used (see results for further information). For independent samples, Kruskal-Wallis tests were performed. For related samples (i.e. before and after treatment), Wilcoxon signed-rank tests were used.

In the data regarding males, the Estradiol treatment was excluded as there were only one usable male individual to be treated with E2.

3. Results

A total of 149 animals were used, divided in 4 sets of 6 experimental tanks (Figure 3.1). As it is impossible to discriminate males form females, the sex of focal individuals could only be assessed *a posteriori*. This resulted in 11 males, 11 females and 2 non-defined individuals. (Figure 3.2). As only males could be used for our experiment and the individuals treated to E2 were mostly females, E2 tests were discarded. With one CBX and one Sham treated male dying during the treatment and one Sham being discarded for not being in optimal conditions, the experimental population was reduced to 9 individuals. In each set of trials, two individuals were subjected to Sham treatment, two subjected to Estradiol treatment, and two be subjected to CBX treatment. Therefore, in a 24 total experimental population, eight fishes were treated with the same treatment.

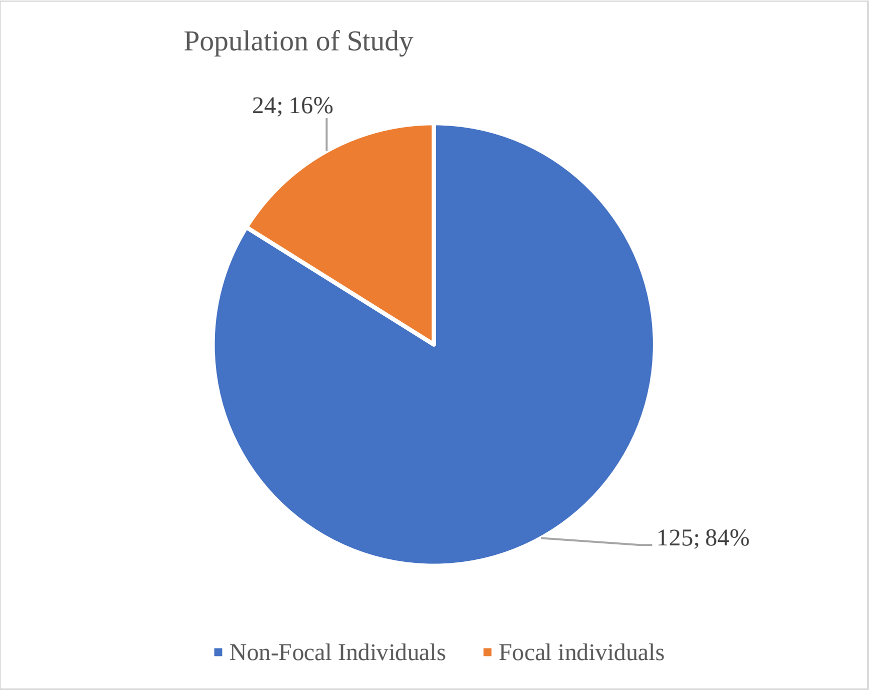

Figure 3.1) Pie Chart of the population of fishes used in the tests (Non-Focal individuals; N=125) (Focal individuals; N=24).

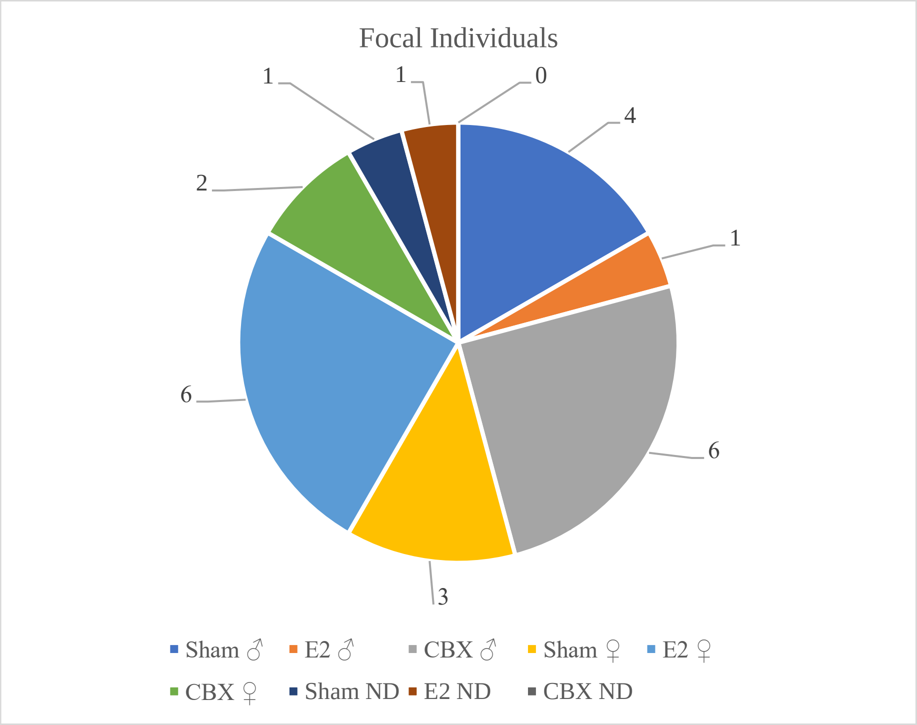

Figure 3.2) Pie chart of the distribution of the population of focal individuals according to the sex and the treatment that they were submitted to.

3.1.Hormone analysis

The mean hormone values before each treatment have similar values for all the tested hormones as observed by Baduy et al. (2017) in territorial male after the group formation with the exception of cortisol (96.61± 60.95ng/mL) that in the cited literature have slightly higher concentrations then what was obtained in this work.

In Figure 3.3 and 3.4 it is possible to observe a comparation of the mean level of tested hormones before(T1) and after (T2) the treatment for both tested treatments (Sham and CBX).

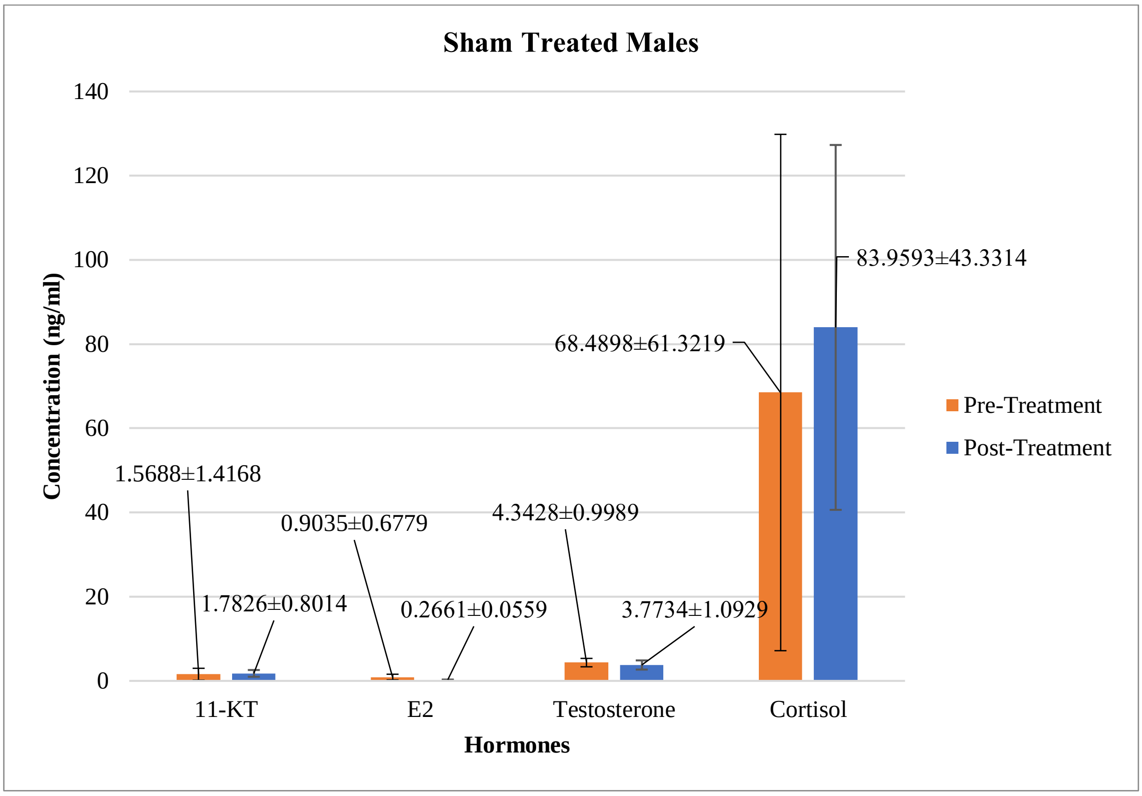

Figure 3.3) Bar chart of the mean hormone levels throughout the male tested population before and after the Sham treatment (N=4)(mean±SE).

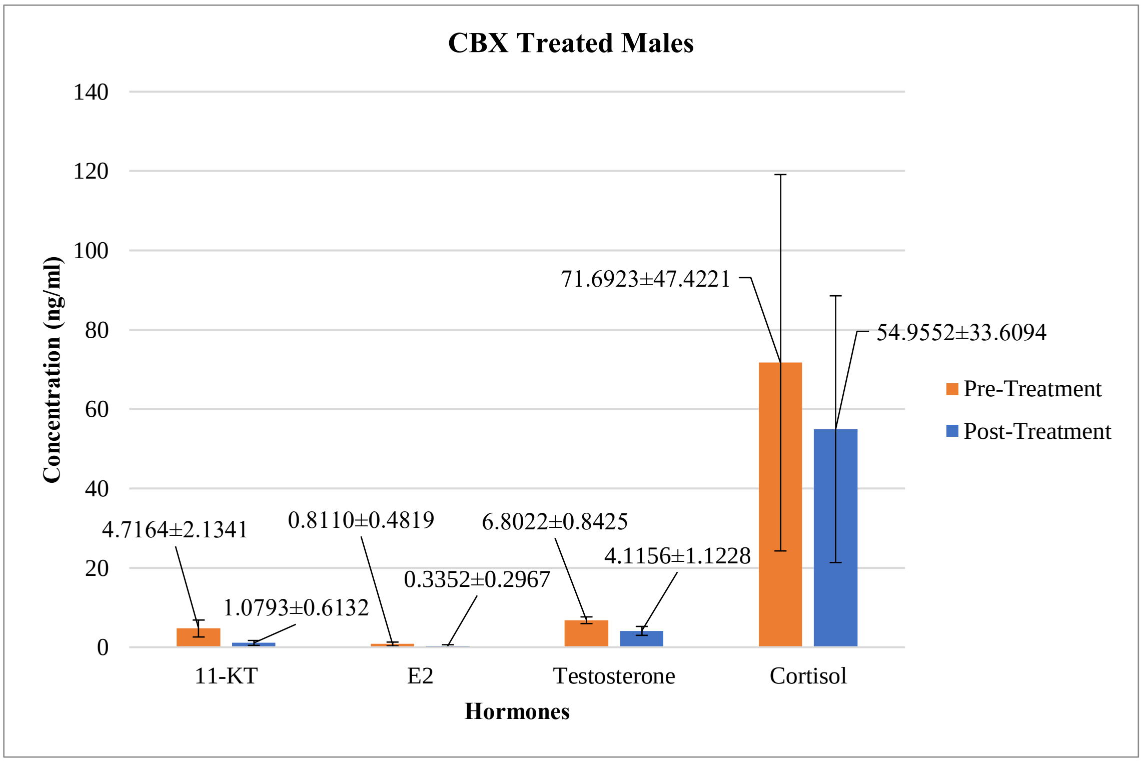

Figure 3.4) Bar chart of the mean hormone levels throughout the male tested population before and after the CBX treatment (N=5);(mean±SE).

Tests of normality applied to the Δ of Estradiol, Testosterone, 11-Ketotestosterone and Cortisol as dependent variables, treatment as fixed factor and Phase (pre-treatment and post-treatment) for male individuals did follow the assumptions of normality as the all the four test applied by SPSS under the fixed factor treatment in this test had a significantly higher than p=0.05 rejecting the null hypothesis.

Table 3.1)Multivariate General Linear Model Tests of Between-Subjects Effects for hormone Δ in males following CBX and Sham treatment[a. R Squared = ,579 (Adjusted R Squared = ,494); b. R Squared = ,073 (Adjusted R Squared = -,112); c. R Squared = ,725 (Adjusted R Squared = ,670); d. R Squared = ,120 (Adjusted R Squared = -,056);(Sham N=3; CBX N=4)]

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	Δ 11-KT	31,694	1	31,694	6,863	,047
	Δ E2	,101	1	,101	,396	,557
	Δ T	4,325	1	4,325	13,168	,015
	Δ Cortisol	1622,967	1	1622,967	,681	,447

The Multivariate General Linear Model for hormone Δ for males showed that CBX introduced significant differences after the administration of the treatment in 11KT (p=0.047) and in T after the treatment (p=0.015), and no differences were found after the treatment for Estradiol (p=0.557) or Cortisol (p=0.447) as it is possible to observe in Table 3.1.

In Figure 3.5 it is possible to observe that CBX significantly decreased the levels of 11-KT with a negative direction and higher magnitude when compared with Sham treatment. It is also possible to observe that T was also significantly impacted and although both treatments have the same negative direction, the CBX treatment revealed a greater magnitude.

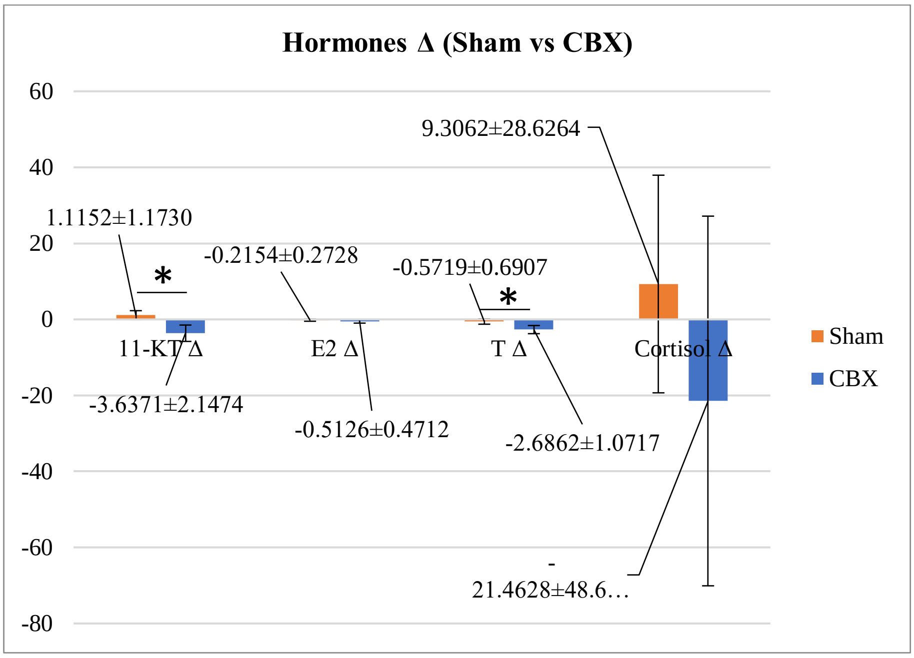

*Figure 3.5) Bar chart of the mean hormone Δ throughout the male tested population with Sham vs CBX (mean±SE)(*p<0.05).*

3.2.Behavioural analysis

3.2.1. Descriptive statistics

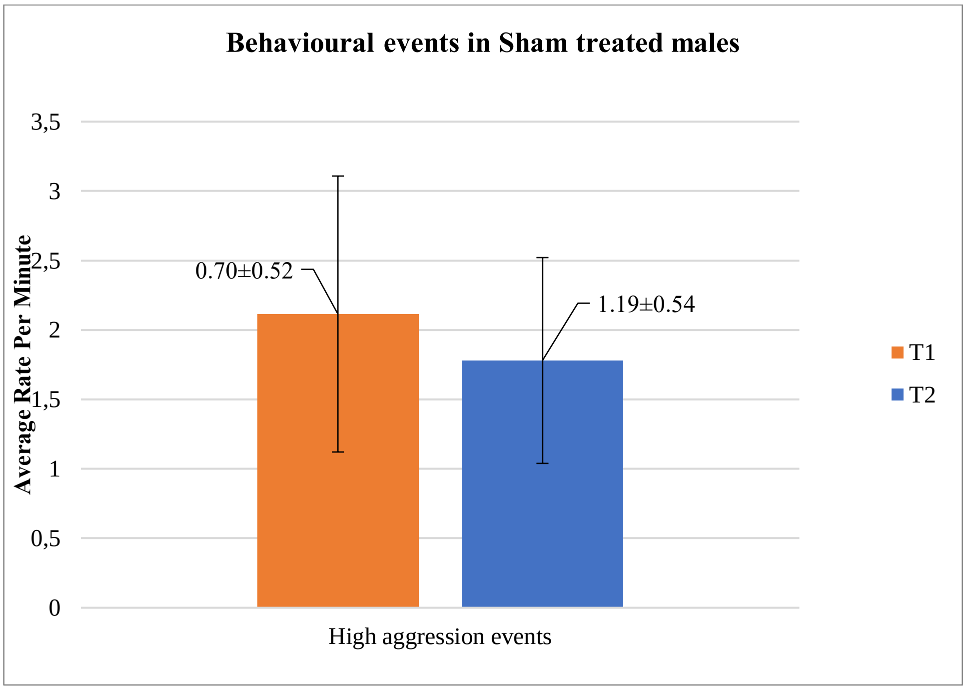

Figure 3.6) Bar chart of the mean Rate per Minute of Behavioural events for male tested population before and after the Sham treatment (N=5);(mean±SE).).

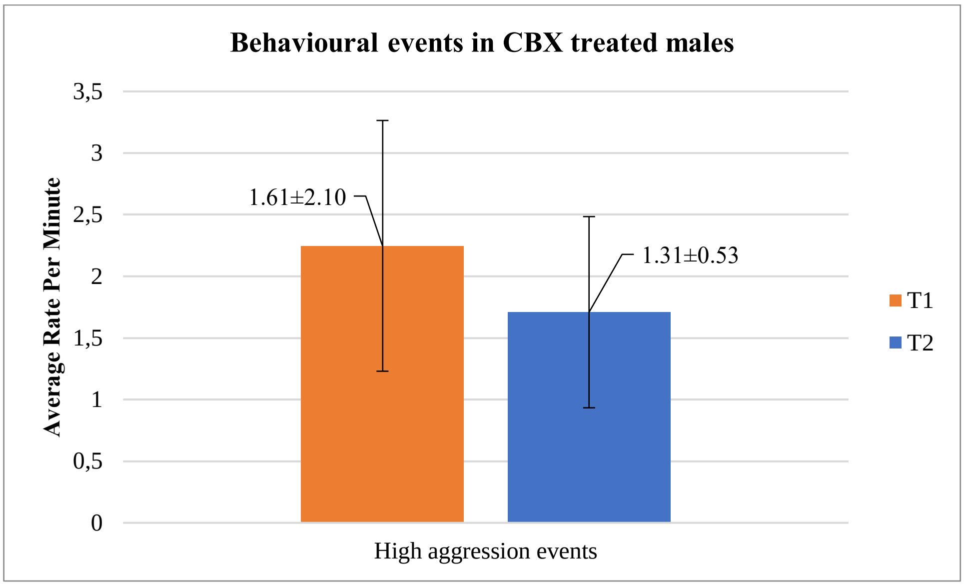

Figure 3.7) Bar chart of the mean Rate per Minute of Behavioural events for male tested population before and after the CBX treatment (N=5);(mean±SE).).

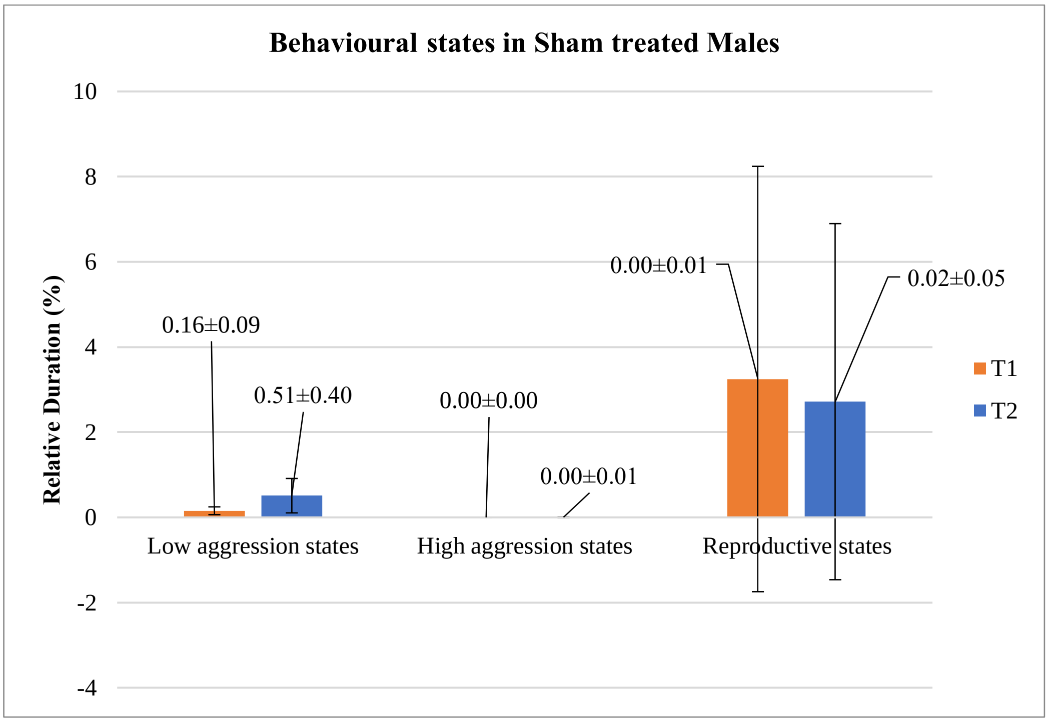

Figure 3.8) Bar chart of the mean Relative duration (%) for Behavioural States for male tested population before and after the Sham treatment (N=5);(mean±SE).).

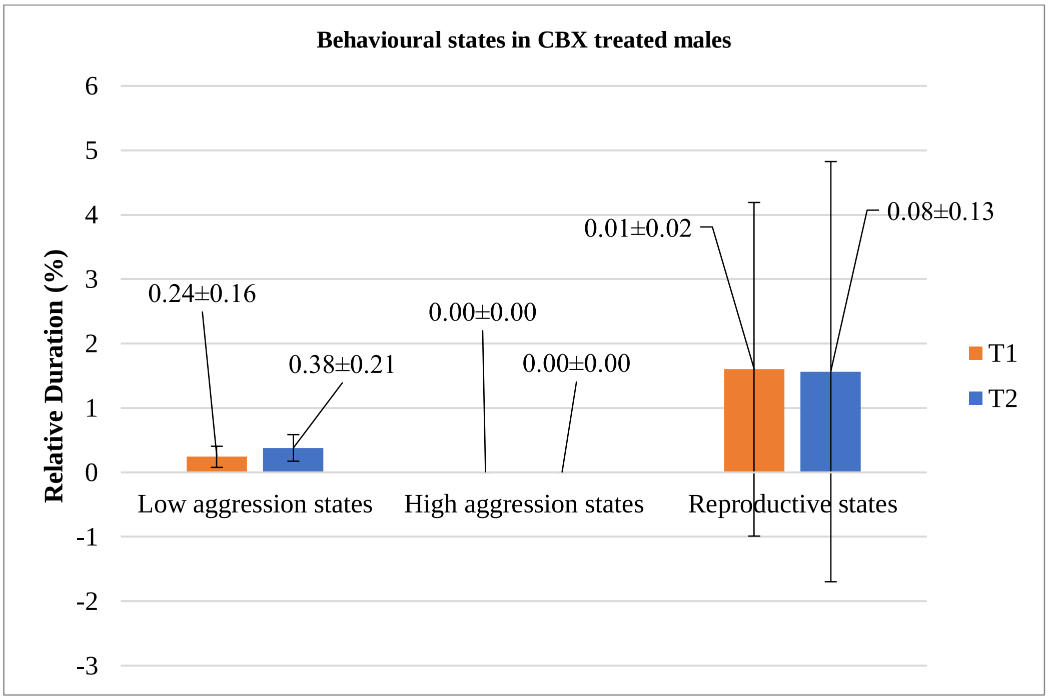

Figure 3.9) Bar chart of the mean Relative duration (%) for Behavioural States for male tested population before and after the CBX treatment (N=5);(mean±SE).).

Tests of normality applied to for group behaviours before and after the treatment for males treated with Sham or CBX individuals did not follow the assumptions of normality as the all the four test applied by SPSS under the fixed factor treatment in this test had a significantly lower to p=0.05 rejecting the null hypothesis.

Table 3.2) Nonparametric test Related-Samples Wilcoxon Signed Rank Test for behaviours in between both phases(pre-treatment and post-treatment) in tested male A. facetus submitted to Sham and Carbenoxolone treatment(p=0.05).

Treatment	Behaviours	Total N	Test Statistic	Standard Error	Standardized Test Statistic	Asymptotic Sig. (2-sided test)
Sham	Low-agression states	4	9,00	2,74	1,46	0,14
	High-agression events	4	4,00	2,74	-0,37	0,72
	High-agression states	4	1,00	0,50	1,00	0,32
	Reproductive states	4	6,00	2,74	0,37	0,72
CBX	Low-agression states	5	12,00	3,71	1,21	0,22
	High-agression events	5	4,00	3,71	-0,94	0,35
	High-agression states	5	0,00	0,00	-1,00	1,00
	Reproductive states	5	3,00	3,71	-1,21	0,22

The Kruskal-Wallis test for males treated with Sham or CBX did not reveal any significant difference for any group of behaviours between the pre-treatment phase and the post-treatment phase (Table 3.2). The Figures 3.6 and 3.8 demonstrate no differences in behaviours observed both for behavioural events and states respectively, for sham treated individuals. The same can be verified in Figures 3.7 and 3.9 for behavioural states and events, respectively for CBX treated individuals. With this information, CBX did not had any impact in any behavioural group, in specific, in the aggressive behaviour.

Comparation between treatments (Sham and CBX) at the same stage were also conducted with the comparation in the pre-treatment phase (T1) not showing any significant differences after a non-parametric test was applied, as observed in the Table 3.3. As no individual were not yet submitted to any kind of treatment it is expected not to see any differences, so no graphs for the T1 phase were required.

Table 3.3) ANOVA test for group behaviours Before the treatment (T1) for both treatments (Sham and CBX).

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	Sig.
Treatment	Low agression state	,038	1	,038	1,313	,290
	High agression events	,004	1	,004	,004	,953
	High agression states	,000	1	,000	.	.
	Reprodutive states	6,868	1	6,868	,239	,640

The behaviours observed for both treatments (Sham and CBX) were also compared in the post-treatment phase (T2) for both States and events as seen in Figures 3.10 and 3.11 respectively.

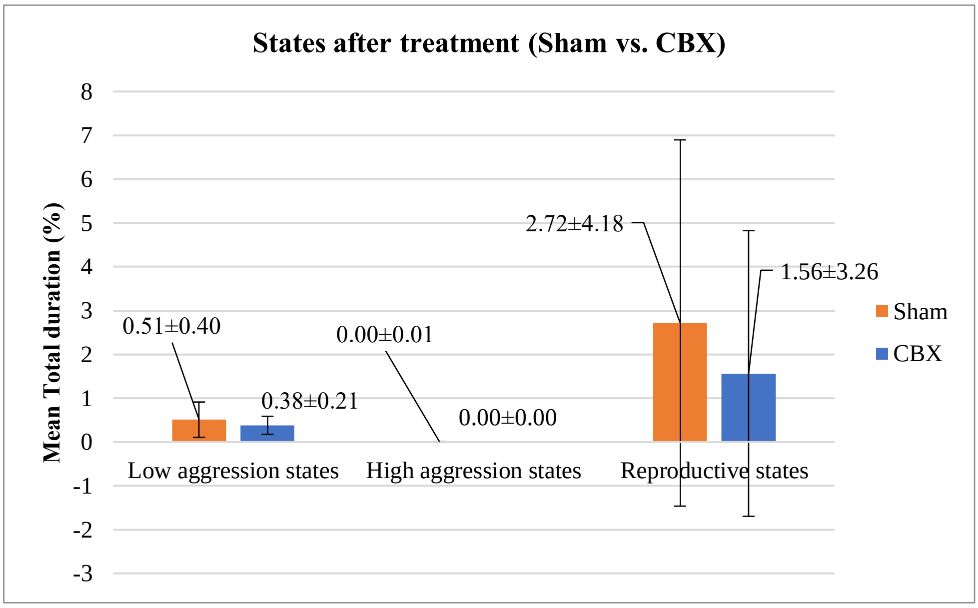

Figure 3.10) Behavioural States after treatment(T2) (Sham vs. CBX).

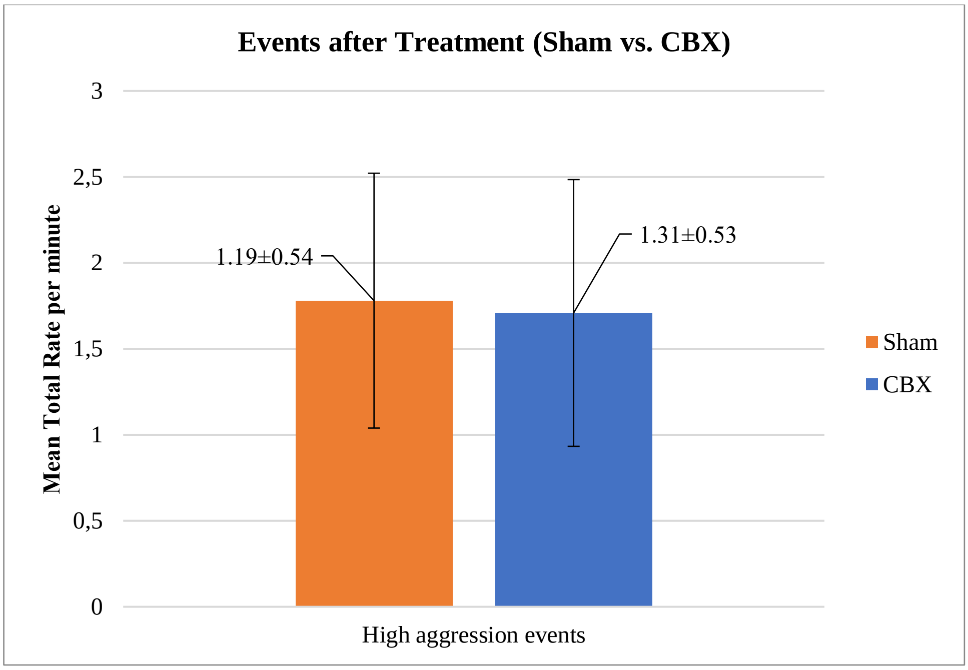

Figure 3.11) Behavioural Events after treatment (T2) (Sham vs. CBX).

The ANOVA applied to Behavioural States and Events between treatments in the post-treatment phase (T2) showed no differences, with all the analysed behaviours having a significance higher than p=0.05 not rejecting the null hypothesis. However, the Reproductive states had a p=0.084 hinting for a possible tendency that the CBX treated individuals engaged less in this type of behaviour than Sham treated individuals.

The correlation between each behaviour and the concentration of 11-KT for each treatment before and after the treatment (T1 and T2) were also represented in scatter plots as observed in the following figures.

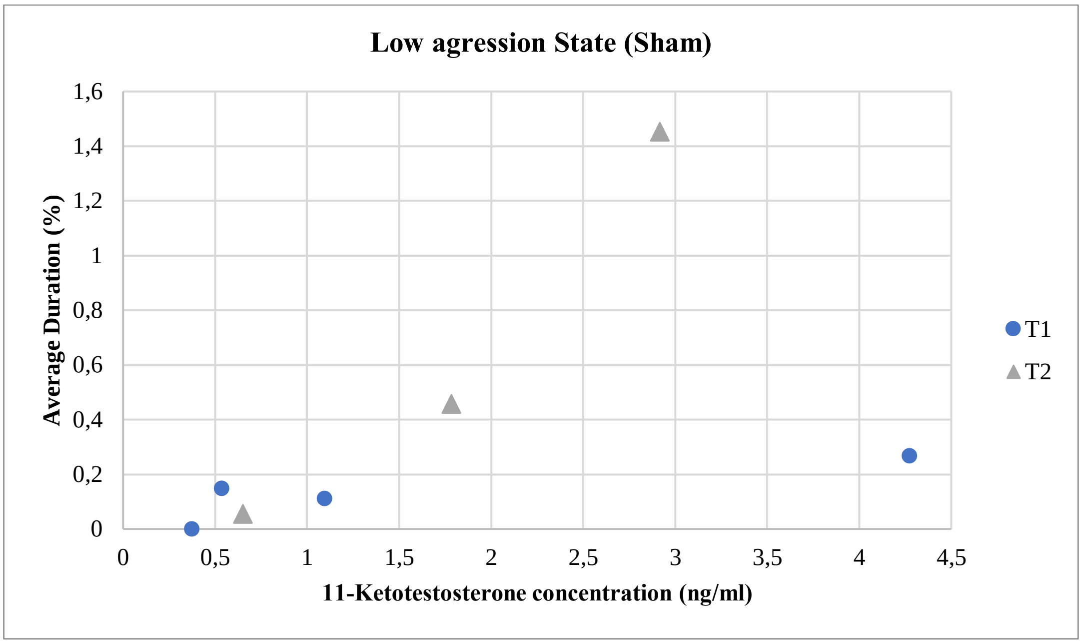

Figure 3.12) Scatter plot of the correlation of 11-KT levels with low aggression states before and after the treatment with Sham.

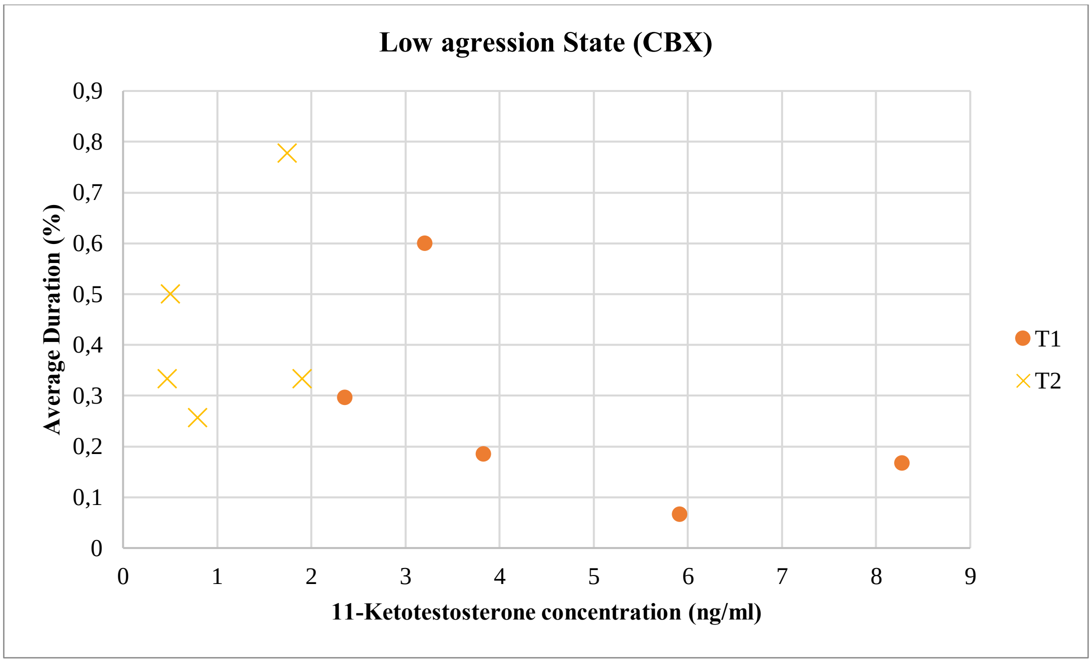

Figure 3.13) Scatter plot of the correlation of 11-KT levels with low aggression states before and after the treatment with CBX.

Low aggression states remain similar after the treatment with Sham (Figure 3.12) although before and after the treatment of CBX (Figure 3.13) the data seem to disperse with a visible distinction between the two phases. After the treatment with CBX the average duration of the low aggression states has slight increased.

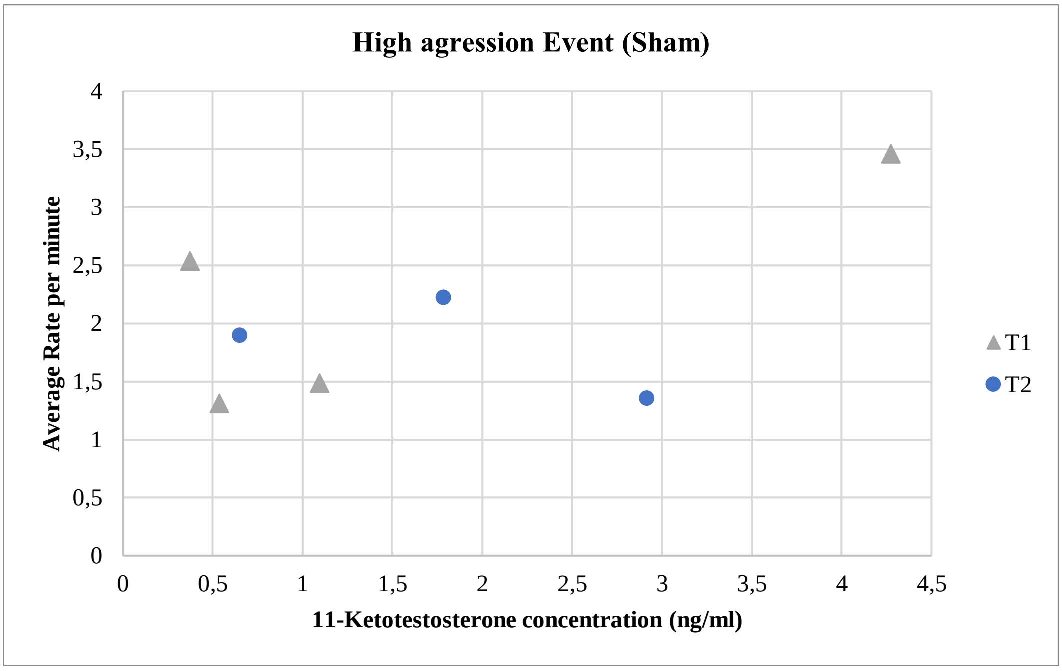

Figure 3.14) Scatter plot of the correlation of 11-KT levels with high aggression events before and after the treatment with Sham.

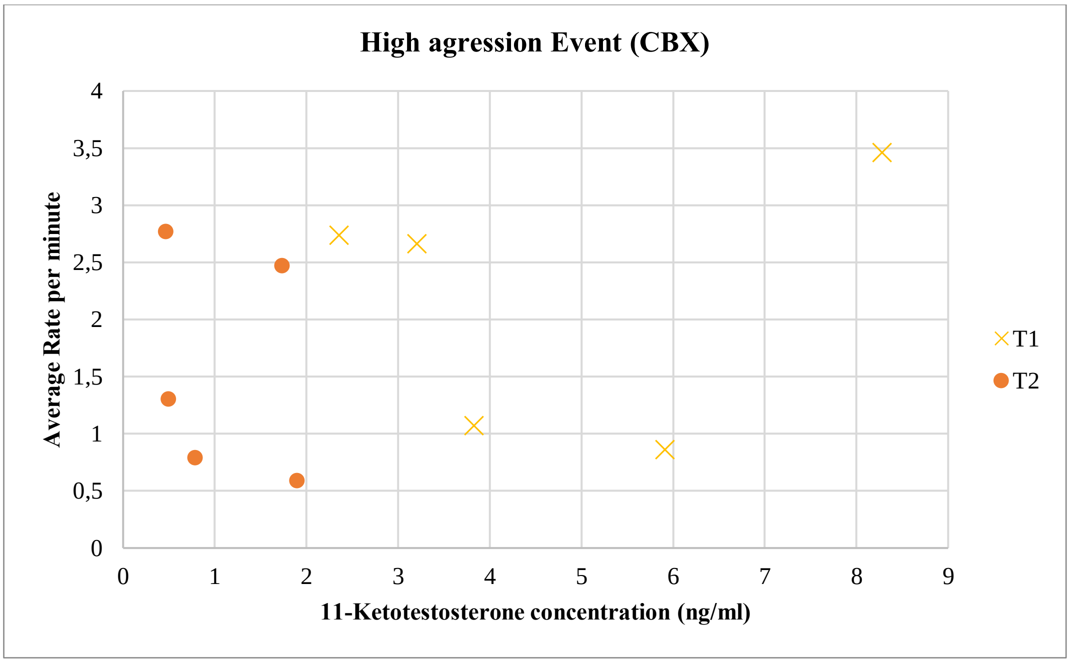

Figure 3.15) Scatter plot of the correlation of 11-KT levels with high aggression events before and after the treatment with CBX.

No visible difference is visible between phases on both treatments for High Aggression Events (Figures 3.14 and 3.15) as the data has a similar range in y axis.

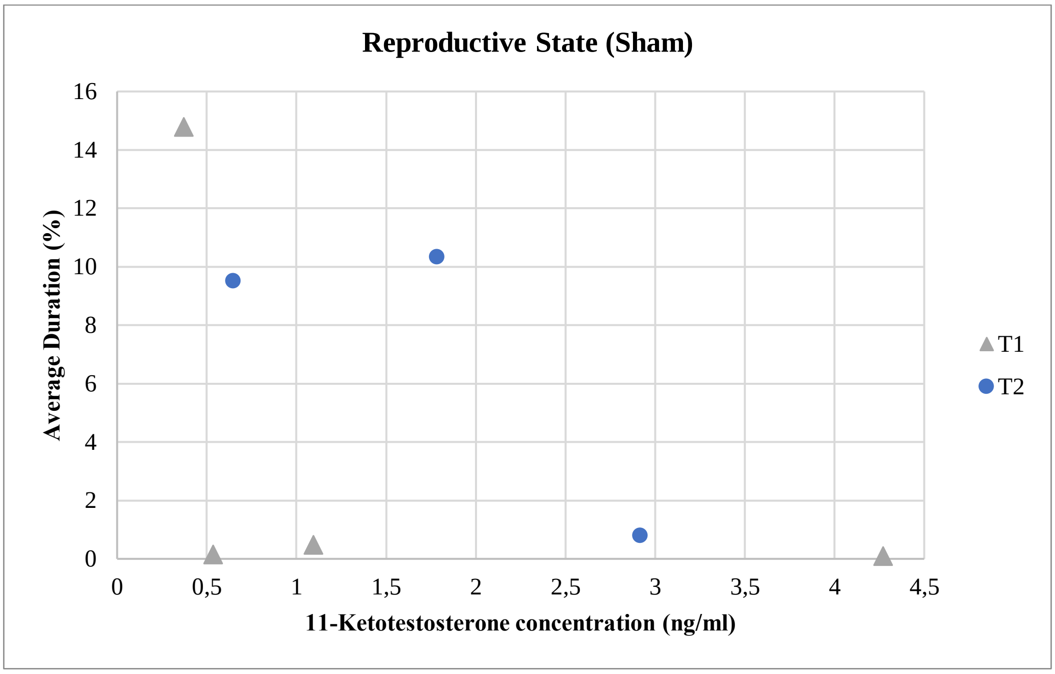

Figure 3.16) Scatter plot of the correlation of 11-KT levels with Reproductive States events before and after the treatment with Sham.

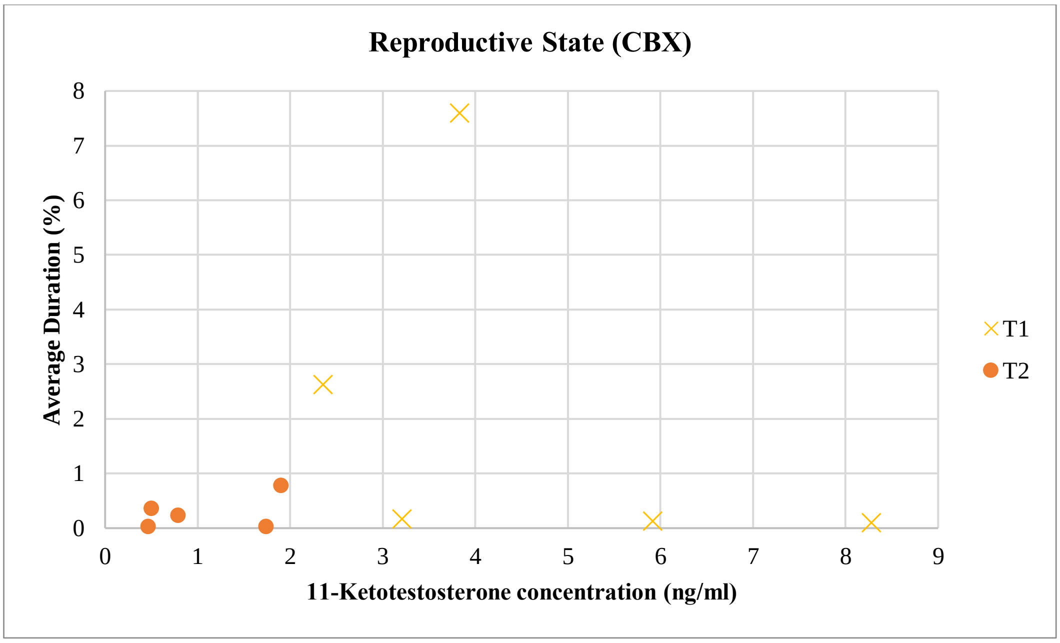

Figure 3.17) Scatter plot of the correlation of 11-KT levels with Reproductive States events before and after the treatment with CBX.

Before and after the treatment with Sham (Figure 3.16) it is not possible to identify any possible difference in the average duration of reproductive states, although after the treatment with CBX (Figure 3.17) longer reproductive behaviours can be observed.

4. Discussion

The focal objective of this work was to study the possible efficacy of hormonal treatments as a method of population control in *Australoheros facetus*. Our results show CBX to produce an impact in the circulating levels of hormones such as 11-KT and T, causing a significant decrease on concentration for both androgens, while for E2 and cortisol no changes were observed. This confirms that CBX is a valid method to impair synthesis of 11KT due to the inhibitive effect that it has on 11β-HSD (Kusakabe et al., 2003; Stewart et al., 1987), which is vital in the biosynthesis pathway of the production of this potent androgen(Idler & Macnab, 1967; Idler et al., 1976). CBX has shown no significant differences in any groups´ behaviours, including reproductive behaviours, although there seems to be a reduction in observed spawning behaviours. This difference opens the possibility for a more in-depth study over this matter with a longer period of observations and a greater target population.

The impacts of treatments over behaviour of dominant males

CBX appeared to have little or no effect in the duration or frequency of any type of agonistic behaviour, so it can be inferred that there was no impairment on the capabilities of the focal individual to reach and maintain a high social status within the population hierarchy, thereby not having any impediment of becoming dominant. This was confirmed by the behavioural observation between T1 and T2. High social status mean that the male individual has greater chances of forming a couple with a dominant female impeding other non-target individuals from forming a couple with this female.

It is known that in this species, only the dominant female is able to reproduce (Baduy et al., 2017) and that she is apparently selected by the dominant male(Enquist & Leimar, 1983; Parker, 1974), which prevents other couples to be formed in its territory. Thereby, only by affecting and impairing the capability of dominant male to reproduce and not its ability to become dominant, it is possible to create a dominant couple that is unable to reproduce. We observed a tendency to reduced reproductive states in males treated with CBX, when compared to males treat with vehicle alone. Although we have not observed changes between the percentage of times spent in specific courtship behaviours and that none of the males, either sham- or CBX-injected, showed spawning, the overall reduction in reproductive state (defined by the sum of all reproductive behaviours) in CBX-injected by dominant males, may considerably decrease the chances of the individual to be able to produce an offspring, as this types of behaviours are crucial to incite the female to engage. It is also likely that the CBX treatment, by reducing the levels of 11-KT, may reduce the male's fertility as this hormone is involved in spermatogenesis (O'Connell et al., 2013; Oliveira, 2004; Schulz et al., 2010) In addition, it is possible to speculate that if more time of observation was allowed, and if the CBX treatment was successful, the individual would not be able to take care and maintain the fry, due to lack of parental care, exposing them to outside risks decrease the possibility of surviving in the early life stages.

The impacts of treatments on circulating hormone levels

CBX is a compound derivative of glycyrrhizic acid, that can also act upon of 11β-dehydrogenase (11β-DH) that combined with 11-oxoreductase forms the enzymatic complex 11-hydroxysteroid dehydrogenase (11β-HSD) thereby also affecting 11β-HSD. 11-hydroxysteroid dehydrogenase is vital in the conversion of cortisol and cortisone (Kusakabe et al., 2003; Stewart et al., 1987). 11-KT is a crucial hormone that impacts the way how certain behaviours are expressed (Baduy, 2017). This hormone induces spermatogenesis and has important behavioural effects (O'Connell et al., 2013; Oliveira, 2004; Schulz et al., 2010). However studies revealed that 11KT could not be directly related to aggressive behaviour but connected to a modulation role in the frequency and intensity of aggressive territorial and mate-guarding behaviours (Almeida et al., 2014; Hau & Goymann, 2015; Maruska, 2015; Wingfield et al., 1990).

By inhibiting the action of 11-hydroxysteroid dehydrogenase, the conversion of 11β-OHT to 11-KT will be severely reduced after the administration of CBX, which is supported by our results.

In T, the non-parametric tests in males did reveal a significant difference from the control test (Sham) showing a negative pattern, meaning that there was an average decrease in testosterone from the day of the administration of CBX to the final day of observations. The androgen T is secreted in males by the interstitial cells, Leydig cells, of the testes. This step is induced by the release of luteinizing hormone by the anterior pituitary. In females is secreted by the adrenal cortex and ovaries, although in smaller quantities than in males (Furman, 2018). This androgen is important for 11KT as it takes part in the biosynthetic pathway that leads to its production. (Idler et al., 1976). Idler & Macnab (1967) conducted studies in *Salmo salar* which determined that testosterone is converted to 11β-hydroxylation to 1lβ-OH-testosterone and finally to 11-KT.

With the application of Carbenoxolone, it would be expected that 11β-HSD is inhibited, hindering the biosynthetic pathway previously referred and reducing the amount Of T converted to 11KT. The interruption of this pathway should disrupt all of the stages, causing an accumulation of T and possibly other by-products. The decrease of testosterone levels might therefore be explained by a negative feedback mechanism, eventually triggered by the saturation of T receptors, and acting on the synthesis or even clearance of testosterone when the concentration of testosterone or its by-products reach a certain plateau.

Cortisol, one of the most important stress hormones did not show any differences between individuals treated with CBX and Sham. This is a hormone that is modulated by social interactions and by the exposure or not to stressful conditions (Fox et al., 1997; Overli, et al., 1999; Pottinger & Pickering, 1992; Sloman, et al., 2001).

Studies conducted by Sapolsky, (1982, 1983) in anubis baboons (*Papio hamadryas anubis*) have shown that corticoid variations are not only connected with the social status of an individual but also with perceived predictability and control over certain challenges that emerge at different social levels (Ostner, et al., 2008; Sapolsky, 1992). The previous studies by Sapolsky, (1982, 1983) initially established that glucocorticoids are present in higher concentrations in low-ranking individuals than in with those in a higher ranking when in stable conditions. These subordinate individuals are exposed to higher costs has they are unable to predict and control certain experienced situations. The same is observed for the species *A. facetus* (Baduy et al., 2017).

However studies have shown that dominant individuals can also express higher levels of glucocorticoids than subordinate individuals when exposed a scenario of social instability causing the loss control and predictability in these situations (Sapolsky, 1993). In the present case, the only similar situation although not significant in our data, is in cortisol levels that slightly rise after the treatment. This can be explained by the population and interactions becoming unstable over the time, with the previously dominant males being temporarily removed from the tank, which decreases the predictability of possible interactions. Not knowing what type of interactions an individual might face can increase the stress exposure, thereby increasing the cortisol levels. Also, the manipulation of the individual to inject the treatment can introduce a new stress factor that can also explain the increase in cortisol levels.

As the agonistic interactions did not seem to be affected by the CBX treatment thereby not changing the exposure to stressful scenarios it is expected not to see a significant difference in cortisol levels, which this work confirms. Although a slight non-significant reduction of cortisol can be observed after the treatment with CBX which can be explained by decrease of interest of the focal individual in courting the female as mate choice requires expenditure of time and energy (Milinski & Bakker, 1992).

As previously referred, inhibiting the 11β-HSD will cause a negative feedback over the synthesis of 11-KT and testosterone derivatives that are crucial to this process.

Impacts of the hormones in behaviour

In this dissertation, one of the biggest objectives was to try to find a connection between the trial hormones and the modification of the frequency or amplitude of aggressive and reproductive behaviours. These behaviours can have a role in modulating how some individuals fit in the social hierarchy. In *A. facetus,* territorial males there is a relationship between sex steroids such as 11KT, and status after the formation of a hierarchy. Lower social status is usually related to higher levels of cortisol (Baduy, 2017).

In several cichlids, upon the perception of dominance, the levels of 11-KT rise in the dominant fish, which may be an indication that is vital in the resolution of social conflicts (Hirschenhauser, et al., 2004; O’Connell et al., 2013; Schulz et al., 2010). It is a hormone that is present in higher concentrations as higher the social status of an individual is. However, some studies have showed that 11KT is not directly connected to the expression of aggressive behaviour and hierarchical changes but have a moderation role, controlling and regulating the frequency and intensity of mate guarding and aggressive behaviours (Almeida et al., 2014; Hau & Goymann, 2015; Maruska, 2015; Wingfield et al., 1990). In our study, we have not evaluated the hormonal differences between submissive and dominant males. However, despite a reduction in 11-KT after CBX treatment, no differences were found in aggressive behaviour, thus in line with the studies mentioned above. This also adds to the usefulness of CBX as a population control method, since it may reduce the dominant male’s ability to reproduce, but not their ability to be dominant, thus effectively sequestering the reproductive females from the population.

Cortisol is a stress hormone that is present in higher concentrations in an individual when it is exposed to stressful (Fox et al., 1997; Overli et al., 1999; Pottinger & Pickering, 1992; Sloman et al., 2001). Subordinate individuals are usually exposed to situations of low predictability which does not allow an individual to know what interactions, positive or negative, can emerge, increasing the stress levels. In

In the Sham treatment it was not expected to observe any differences on the individual’s behaviour. This treatment worked solely as a control, to observe how behaviours and hormones would evolve in a normal scenario throughout the time. We have not observed any changes in cortisol before and after the treatment also in CBX treated fish, wish may indicate again that they have not lost their dominant status due to the treatment. In addition, this may also confirm that size is relevant to dominance, as previously shown by Baduy et al. (2017).

Methodological constraints

Despite the promising results, this approach shows several important constraints, one of them being our inability, at this point, to accurately discriminate males from females. In other well studied cichlids, such as most African species, and including the Mozambique tilapia (Barata et al., 2007; Oliveira & Almada, 1995), the males are well differentiated and form families with several females. Other less conspicuous species show differences in the urogenital papillae between males and females such as in *Oreochromis mossambicus* (Oliveira & Almada, 1995), thus still allowing for a clear separation between sexes. However, the individuals of *A. facetus* do not show any external secondary sex characters that allow to distinguish between sexes and effective sexing could only be performed *a posteriori*, when fish were euthanized.

Thus, the selection of the overall population used as focal individuals in this work reflected a 50-50 distribution, which may be a reasonable emulation of a real-world scenario, but which was not ideal for the work in hands as it is focused on methods of inhibiting the reproduction in male individuals.

Previously conducted studies, using Estradiol levels as a proxy were used to distinguish individuals of both sexes. Then a morphometric evaluation was conducted in order to observe if any morphometric characteristic existed that might be used to distinguish both males and females.

The RIA analysis for Estradiol did detect a steep variation pattern in E2 levels that allowed to establish a proxy threshold value that was used to differentiate both sexes. Although it was expected to see individuals with E2 and others without or minimal levels of this hormone, the pattern observed was close to a gradient. In these conditions a n arbitrary value was used to separate both sexes. The morphometric measurements used to identify any visual pattern were total length, fork length, predorsal length, head length, eye diameter, preorbital length, prepectoral length, prepelvic length, preanal length and max body depth measurements as described by FishBase (2010) as possible. These measurements were correlated with the E2 values using scatterplots and a regression analysis, but none of the measurements stood out as none of the coefficients of determination ($R2$) was strong enough to be able to correlate these two variables (data not shown).

With no non-intrusive methodology available that would allow a fast and easy way to distinguish both sexes, the test had to be conducted without previously identifying the sex of the focal individual. Furthermore, even if radioimmunoassays could have been used to identify the sex of an individual using the levels of Estradiol as a proxy, the introduction of the individual in groups and behavioural observations had to be done immediately after the blood collection to minimise the exposure of fishes to stressful scenarios as most as possible which can have an impact on how individuals behave and in the levels of circulating hormones, affecting the final results. As RIA analysis is a time-consuming process, there is no time to wait for the results after the first manipulation of the fishes.

In this study, the workload was divided into four sets of experiments in which six focal individuals distributed throughout six tanks. Each set is comprised of three pairs of individuals each one submitted to a different treatment. Carbenoxolone was our focal treatment as the Sham treatment was used as control and the Estradiol treatment as a positive control.

Not knowing the sex of an individual, excludes any possibility of filtering out female individuals, meaning that some of the conducted observations would not be used as this study focus itself primarily on males. This is the case of E2 tested individuals with just one male, one non-identified individual and six females. With just one male treated with E2 available it is not possible to draw any conclusion about the effects of this estrogen in males.

To overcome this major problem, and to allow this chemical castration method to be effectively, accurate methods for sex identification are necessary. Genotyping and the discovery of genetic sex markers would be a great advance, although its application may still be problematic, as the technology required can not be used in the field. However, it would allow to select a population of all male fish that could then be released upon castration. Another setback was that not all the individuals used in this test survived to the end of the trial after the administration of the treatment. This situation could be explained due to the variable response of an individual to the substance administered, as also to the method of treatment.

First, the E2 was the treatment that caused a higher number of fatalities. As E2 is a female hormone, it is expected that female chameleon cichlids already had an elevated concentration of this estrogen (Baduy et al., 2017). In a previous study it was shown that values in females were much higher than those in males. In fact, in such study E2 was virtually absent in males (Baduy et al., 2017), although, in a close species, *Cichlasoma dimerus*, considerable levels of E2 have been reported also in males (Alonso et al., 2011). As most of the individuals that were treated with E2 have after revealed to be females, injecting these individuals with this treatment would cause the levels of E2 to rise even further, probably reaching lethal concentration, although to our knowledge such situations have not been addressed or described in the literature. Another remote possibility is the feminization of males injected with E2. This has been shown to occur in other fish species (Jobling et al., 2002; Kidd et al., 2007), but generally with much longer exposure times and to higher E2 concentrations, and in most cases, when treatment was administered to juvenile fish. Thus, this was unlikely to have occurred in our population

Mortality was not only observed in E2 treated individuals as some carbenoxolone treated fishes also did survive until the end of the trial. In these fish, a clear change in swimming was noted almost immediately upon being placed in the tank after injection and demonstrated a drastic reduction in movements and a less responsive behaviour. By being less responsive, these individuals were more exposed to any threat that came from other individuals. In fact, these fish that were dominant before being removed from the tank were attacked by the hitherto subordinate fish. Previous preliminary observations have shown that CBX in larger concentrations can provoke partial paralysis and appear to alter the control over the swim bladder in *A. facetus*, causing buoyancy problems and even death, likely by exhaustion.

The treatment also might have caused some situations that could have aggravated the condition of the treated fishes. An intraperitoneal injection is an invasive procedure that requires the administration of the target substance in the abdominal cavity of the individual. Although spacers were used in this procedure, the exposed length of the needle might not have been small enough, for some individuals of a smaller size. This method might cause the internal organs to be punctured, and the treatment substance injected into it, with deleterious or even lethal effects

The preparation of the solution of CBX and E2 for injection was also cumbersome procedure because the solutions were difficult to mix with coconut oil. The vehicle has a high viscosity even when warm, leading to separation of the two phases of the final solution or to formation a thick gel clogging within the syringe, leading the user to exert excessive pressure that could provoke internal damage to the individual.

Summary and final considerations

In this work, no alteration was found in the average rate per minute of any type of behavioural events or in the average relative duration of any behavioural event after, and before the administration of CBX. CBX by inhibiting 11β-HSD can indirectly decrease of the production of 11-KT that can modulate the frequency and intensity of certain reproductive and agonistic behaviours. With a decrease in 11-KT it was expected for the individual to increase or at least maintain the frequency in which these types of behaviours occur.

In this work, aggressive behaviours, low-aggression and high-aggression, did not seemed to have been affected by the treatment, which is positive for the evaluation of CBX as method population control, as the individual can retain their social status as agonistic behaviours are vital to maintain dominance, thereby increasing the chances of forming a couple with the dominant male.

On the other types of behaviour, no significant differences were found, although in Reproductive states a non-significant trend was found, with a decrease in the average relative duration. This opens the door to more in-depth studies in which the use of a larger number of individuals would probably be required to confirm that CBX can produce a decrease in reproductive behaviours.

This decrease goes along of what it was desired to observe in this work, because as CBX does not affect the any aggressive interactions, required for maintaining social status, but can affect the reproductive capabilities, meaning that the individual will probably form a couple with the dominant female, the only female in the population that can actually reproduce, although it will not court with her, rendering the dominant couple unable to produce any offspring. This means that this population will not be able to grow decreasing the risk of expansion and the impact that this population can have in the endemic species and to the local ecosystem.

Our results present a proof of concept regarding the identification of a hormonal pathway through which we can disrupt the reproduction of an invasive species. This is the reason why this work focusses in the study of the behaviour and circulating hormones and the correlation between them in *A. facetus* to find which is the most appropriated mechanism to act in order to control the spread of this species.

This species has a social facet that can be modulated by hormones levels. By knowing how behaviour can affect the reproduction of this species, different endocrine modificators can be used to alter this species behaviours, instead of using a non-species-specific destructive method of population control.

The use of the chemical compound CBX has shown some positive results as a management technique as it can affect the normal formation of reproductive couples thus affecting the population capability of expanding. In this species, the generation of offspring is already limited to the dominant couple, thereby less individuals are needed to be chemically castrated, decreasing the workload required to start observing any result.

In this method, the effects are not immediately visible and require time for the treated individual to adapt and arise in the hierarchy of the population where they were introduced. The effectiveness also depends on the capability of an individual to become dominant, which requires the selection of only large-sized individuals to force the dominance.

It is still a cumbersome method to apply as male individuals have to be injected one-by-one and then released into the wild. Although the largest problem that this procedure faces, is the lack of fast and direct methods of sex determination in *A. facetus*.

The same problem revealed to be an impediment on the work developed in this dissertation as it only possible to determine the sex of the individual after the trials when fishes were euthanized, and a direct observation of the gonads was possible. This rendered many of the observation conducted on this test as unusable as this work focus in the effects of the treatments in males.

To solve this problem, a large test can be conducted where in depth observations of the morphology of the urogenital papilla to identify any visible differences followed by the sacrifice of the individuals to confirm the sex of the individual. This process requires the sacrifice of a large number of individuals and training by the person observing. Other possible solution can be preforming RIA analysis for E2 levels and sorting in advance way before preforming any trial or treatment allowing the individual to recover from the stress exposure of the handling process. This method is not infallible, although it can greatly increase the possibility of misidentification of the sex of the individuals and increase the success of any sex-dependent trial. With the possibility of rapidly genotyping many fish, the discovery and use of a sex marker could be a optimal solution to select and treat males to be released in the wild. Other alternatives, such as the production of fish genetic modified to be infertile, are of interest but collide with the regulations for environmental conservation.

These solutions can be applied in order to maximise the number of male test individual available for a further test to confirm the results obtained in this dissertation.

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6. Appendix

Table 6.1) Test population of focal individuals used across different treatments tested and survivability.

Set	Sex	Treatment	Survivability after treatment (Days)	Observations
Set 1	Male	CBX	0	Died from treatment in 29/05/19
Set 1	Female	CBX	7	Euthanised in 11/06/19 (Survived until the end of the treatment)
Set 1	Male	E2	7	Euthanised in 11/06/19 (Survived until the end of the treatment)
Set 1	Female	E2	7	Euthanised in 11/06/19 (Survived until the end of the treatment)
Set 1	Male	Sham	7	Euthanised in 11/06/19 (Survived until the end of the treatment)
Set 1	Female	Sham	7	Euthanised in 11/06/19 (Survived until the end of the treatment)
Set 2	Female	E2	2	Died in 27/06/19
Set 2	Female	E2	2	Died in 27/06/19
Set 2	Male	Sham	7	Euthanised in 03/07/19 (Survived until the end of the treatment)
Set 2	ND	Sham	No information	No information
Set 2	Male	CBX	7	Euthanised in 03/07/19 (Survived until the end of the treatment)
Set 2	Male	CBX	7	Euthanised in 03/07/19 (Survived until the end of the treatment)
Set 3	Female	Sham	0	Died from treatment 11/07/19
Set 3	Female	E2	4	Died 15/07/19
Set 3	Male	CBX	7	Euthanized 22/07/19 (Survived until the end of the treatment)
Set 3	Male	CBX	7	Euthanized 22/07/19 (Survived until the end of the treatment)
Set 3	Female	E2	7	Euthanized 22/07/19 (Survived until the end of the treatment)
Set 3	Male	Sham	7	Euthanized 22/07/19 (Survived until the end of the treatment)
Set 4	ND	E2	3	Died in 01/08/19
Set 4	Female	E2	4	Died in 02/08/19
Set 4	Female	CBX	7	Euthanised in 07/08/19 (Survived until the end of the treatment)
Set 4	Male	CBX	7	Euthanised in 07/08/19 (Survived until the end of the treatment)
Set 4	Female	Sham	7	Euthanised in 07/08/19 (Survived until the end of the treatment)
Set 4	Male	Sham	7	Euthanised in 07/08/19 (Survived until the end of the treatment)

Table 6.2) Mean values of behavioural data before the treatment with Sham in male individuals.

Series	Code	Treatment	Quiver (Mean Rate per Minute)	Frontal Display (Mean Total Duration (%))	Lateral Display (Mean Total Duration (%))	Strike (Mean Rate per Minute)	Chase (Mean Rate per Minute)	Bite (Mean Rate per Minute)	Tail Beating (Mean Rate per Minute)	Flee (Mean Rate per Minute)	Courtship (Mean Total Duration (%))	Dig (Mean Total Duration (%))	Parental Hover (Mean Total Duration (%))	Care (Mean Total Duration (%))	Patrol (Mean Total Duration (%))
1	D9C8	Sham	0,32	0,00	0,00	0,48	1,64	0,36	0,06	0,00	0,00	2,13	6,63	4,37	1,67
2	A87E	Sham	0,60	0,07	0,07	0,20	0,96	0,16	0,00	0,00	0,00	0,15	0,00	0,00	0,00
3	0CFE	Sham	0,84	0,00	0,11	0,38	1,02	0,09	0,00	0,04	0,04	0,44	0,00	0,00	0,00
4	B98B	Sham	1,36	0,00	0,27	0,28	2,06	1,12	0,00	0,02	0,00	0,10	0,00	0,00	0,00

Table 6.3) Mean values of behavioural data after the treatment with Sham in male individuals.

Series	Code	Treatment	Quiver (Mean Rate per Minute)	Frontal Display (Mean Total Duration (%))	Lateral Display (Mean Total Duration (%))	Strike (Mean Rate per Minute)	Chase (Mean Rate per Minute)	Bite (Mean Rate per Minute)	Tail Beating (Mean Rate per Minute)	Mouth fight (Mean Total Duration (%))	Flee (Mean Rate per Minute)	Courtship (Mean Total Duration (%))	Dig (Mean Total Duration (%))	Parental Hover (Mean Total Duration (%))	Care (Mean Total Duration (%))	Patrol (Mean Total Duration (%))	Dual Hover (Mean Total Duration (%))
1	D9C8	Sham	1,16	0,04	0,42	0,43	1,59	0,20	0,01	0,02	0,04	0,00	1,90	4,38	0,27	3,77	0,02
2	A87E	Sham	0,67	1,38	0,07	0,33	0,96	0,07	0,00	0,00	0,03	0,12	0,69	0,00	0,00	0,00	0,00
3	0CFE	Sham	0,57	0,06	0,00	0,57	1,13	0,20	0,00	0,00	0,00	0,81	0,58	7,81	0,33	0,00	0,00
4	B98B	Sham	1,36	0,00	0,40	0,29	1,21	0,66	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00	0,00

Table 6.4) Mean values of behavioural data before the treatment with CBX in male individuals.

Series	Code	Treatment	Quiver (Mean Rate per Minute)	Frontal Display (Mean Total Duration (%))	Lateral Display (Mean Total Duration (%))	Strike (Mean Rate per Minute)	Chase (Mean Rate per Minute)	Bite (Mean Rate per Minute)	Tail Beating (Mean Rate per Minute)	Flee (Mean Rate per Minute)	Freeze (Mean Total Duration (%))	Courtship (Mean Total Duration (%))	Dig (Mean Total Duration (%))	Parental Hover (Mean Total Duration (%))	Care (Mean Total Duration (%))	Patrol (Mean Total Duration (%))	Prespawning (Mean Total Duration (%))
2	235D	CBX	0,11	0,00	0,30	0,36	2,18	0,20	0,00	0,00	0,00	0,07	2,56	0,00	0,00	0,00	0,00
2	F9E0	CBX	0,29	0,04	0,15	0,20	0,67	0,18	0,02	0,20	0,04	0,07	0,11	6,96	0,19	0,26	0,00
3	44C2	CBX	0,42	0,00	0,07	0,16	0,62	0,08	0,00	0,84	0,00	0,00	0,13	0,00	0,00	0,00	0,00
3	896C	CBX	1,68	0,00	0,17	0,62	2,62	0,16	0,06	0,04	0,00	0,00	0,10	0,00	0,00	0,00	0,00
4	A789	CBX	6,60	0,10	0,50	0,34	1,28	1,02	0,02	0,04	0,03	0,00	0,03	0,00	0,00	0,00	0,13

Table 6.5) Mean values of behavioural data after the treatment with CBX in male individuals.

Series	Code	Treatment	Quiver (Mean Rate per Minute)	Frontal Display (Mean Total Duration (%))	Lateral Display (Mean Total Duration (%))	Strike (Mean Rate per Minute)	Chase (Mean Rate per Minute)	Bite (Mean Rate per Minute)	Tail Beating (Mean Rate per Minute)	Flee (Mean Rate per Minute)	Freeze (Mean Total Duration (%))	Courtship (Mean Total Duration (%))	Dig (Mean Total Duration (%))
2	235D	CBX	1,71	0,00	0,33	0,09	0,27	0,00	0,23	0,19	0,02	0,00	0,78
2	F9E0	CBX	1,38	0,10	0,15	0,31	0,32	0,11	0,05	0,49	0,15	0,08	0,15
3	44C2	CBX	0,55	0,00	0,33	0,35	1,83	0,58	0,00	0,40	0,00	0,00	0,03
3	896C	CBX	2,13	0,06	0,72	0,68	1,60	0,13	0,05	0,07	0,00	0,00	0,03
4	A789	CBX	1,44	0,05	0,45	0,23	0,61	0,46	0,00	0,09	0,36	0,05	0,31