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# The role of dopamine for behaviour regulation in cooperatively breeding fish

**Mestrado em Biologia Evolutiva e do Desenvolvimento**

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# Índex

# Resumo

Este estudo tem como objectivo compreender o papel de um neurotransmissor, a dopamina, na regulação de comportamentos sociais e cooperativos. Para uma melhor compreensão de cooperação, é necessário compreender primeiramente o que é sociabilidade. Sociabilidade pode ser definida pela vivência de dois ou mais indivíduos como parte do mesmo grupo. Para a sociabilidade ocorrer, é ainda necessário, existir interacção entre os membros do grupo, tendo sempre em conta o contexto social em que a interacção ocorre, assim como o comportamento do indivíduo com o qual a interacção está a ocorrer. Após a percepção destes dois componentes cada membro do grupo terá que modelar o seu próprio comportamento para que a que a sua acção se enquadre ao que lhe é exigido pelo contexto social em que se encontra. O contexto social em que um animal se encontra é definido pelo conjunto de factores ambientais (eg. taxa de predação, recurso de alimento e a forma como os seus pares se estão a comportar). Apenas após a consideração dos aspectos referidos acima, é que os comportamentos cooperativos podem emergir.

Cooperação pode ser definida como uma acção que é realizada por um indivíduo A, que por sua vez beneficia um individuo B. Dentro de comportamentos cooperativos podemos definir vários tipos de interacções dependendo do receptor do benefício resultante dessa interacção. Esta troca de benefícios é designada de reciprocidade. A reciprocidade pode ser directa, quando dois indivíduos se entreajudam, ou indirecta quando numa interacção cooperativa o indivíduo beneficiado ajuda um terceiro indivíduo.

A investigação em cooperação e sociabilidade que tem sido desenvolvida têm-se focado maioritariamente na ecologia mas também nos aspectos evolutivos de como cooperação e sociabilidade poderá ter surgido e mantida ao longo do tempo. Contudo, pouco se conhece sobre os seus mecanismos regulatórios.

O presente estudo pretende desvendar o papel de um neurotransmissor na regulação de comportamentos cooperativos e sociais, a dopamina.

Dopamina é uma catecolamina que está envolvida em várias funções centrais de um organismo, tal como na locomoção, na cognição, na aprendizagem e no sistema mesolímbico de recompensa.

Em 2011 foi descrita a “Social decision-making network” que consiste num conjunto de núcleos cerebrais que estão envolvidos na regulação de sociabilidade, tal como o núcleo accumbens e a área pré-óptica, juntamente com o sistema mesolímbico de recompensa também está incluído na “Social decison-making network”. Admite-se desta forma, que um comportamento social para ser repetido, é porque despoletou alguma recompensa num “helper” (ajudante).

Tendo isto em conta o presente estudo tem como principal objectivo tentar uma melhor compreensão do papel da dopamina na regulação comportamentos sociais e cooperativos. Compreender de que forma a dopamina regula comportamentos cooperativos e sociais de ”helpers”.

Foi usado como objecto de estudo, o ciclídeo Neolamprologus pulcher, sendo que esta espécie vive em famílias com uma estrutura social robusta. Estas famílias são constituídas por um casal dominante e um conjunto de “helpers” que varia de um a trinta, em que apenas o par dominante se reproduz. Os ajudantes podem ser indivíduos sexualmente maturos ou não, o seu papel na família consiste em defesa do território contra predadores e intrusos,

manutenção do território e a ajudar a criar a ninhada dos dominantes. Os predadores podem ser predadores de ovos ou de adultos.

Para este efeito foram realizadas injecções intramusculares em indivíduos ajudantes, com agonistas e antagonistas específicos para receptores D1 e D2. Após a manipulação da actividade de cada um destes receptores realizou-se observações de forma a serem detectadas diferenças comportamentais (eg. número de comportamentos agressivos, submissos e de afilação).Para melhor compreensão da função dos receptores dopaminérgicos na regulação de comportamentos cooperativos e sociais em ajudantes, foram formadas 8 famílias com um casal dominante e dois ajudantes, um grande e um pequeno.

Começou-se por realizar um estudo de calibração para as dosagens das drogas em estudo, agonista de D1-like (SKF-38393), antagonista de D1-like (SCH-23390), agonista de D2-like (Quinpirole) e antagonista de D2-like (Metoclopramida).

Para a execução do estudo de calibração foram escolhidas 3 doses para cada uma das drogas, um dose alta, uma dose média e uma dose baixa, estas doses foram escolhidas tendo em conta estudos anteriores realizados noutros organismos. Como controlo foi injectada uma solução salina. Para este estudo foram criados dois grupos com 4 famílias cada, em que cada grupo apenas foi injectado com drogas para uma das classes de receptores, 4 famílias estiveram sujeitas ao tratamento para os receptores D1-like (8 ajudantes no total) e 4 famílias estiveram sujeitas ao tratamento para os receptores D2-like (8 ajudantes no total).

O desenho experimental consistiu em realizar várias sessões de observações de 15 minutos em diferentes tempos, uma observação antes da injecção, uma sessão de observação 15 minutos após a injecção, outra observação 30 minutos após a injecção e outra observação 60 minutos após a injecção.

Com este estudo foi possível concluir que os receptores D1-like e D2-like estão de facto a modular a agressividade, submissão e comportamento aflitivo dos ajudantes.

Após o estudo de calibração testou-se o papel dos receptores dopaminérgicos na regulação comportamental dos ajudantes, quando estes são sujeitos a diferentes contextos sociais. Para tal, foram usadas 10 famílias constituídas por um casal dominante e 2 ajudantes (um grande e um pequeno ajudante), a duas tarefas distintas mais uma tarefa de controlo. Estas tarefas consistiram em estimular certos comportamentos por parte dos ajudantes, tal como comportamentos de manutenção do território tal como escavar e limpar o abrigo e comportamentos de defesa contra intrusos. Para induzirmos comportamentos de ajuda na manutenção do território preenchemos o abrigo do casal com areia para induzir o comportamento de escavar por parte dos ajudantes para que estes desobstruíssem o abrigo. Para a indução de comportamentos de defesa do território foi apresentada à família um predador de ovos num tubo de apresentação, para induzir comportamentos agressivos contra o intruso por parte da família mas em especial por parte dos ajudantes.

Como controlo para as tarefas foi elaborada uma observação sem manipulação do ambiente antes e depois da injecção.

Semelhante ao que foi feito na primeira experiência foram realizados períodos de observação de 15min para cada tarefa, antes e depois da injecção.

Neste caso cada ajudante foi injectado intramuscular com uma dose de cada uma das drogas, agonista do D1-like (SKF-38393), antagonista de D1-like (SCH-23390), agonista de D2-like (Quinpirole), antagonista de D2-like (Metoclopramida), e controle (solução de 0.9% NaCl).

Esta experiência permitiu demonstrar que os receptores D2-like estão de facto a regular a agressividade, submissão e afilação em ajudantes de N.pulcher, no entanto foi observado que esta depende do contexto social a que o ajudante está sujeito. Isto porque não foi constatado um aumento no número de comportamentos agressivos em todas as tarefas apresentadas, apenas nas tarefas em que tal comportamento era contextualmente exigido (eg. Na presença de um intruso). É assim evidenciado que apesar da dopamina regular a sociabilidade de N.pulcher, especialmente os receptores D2-like, estes parecem estar a ter em conta o contexto social a que os ajudantes estão sujeitos.

Após a manipulação farmacológica dos receptores dopaminérgicos no cérebro dos ajudantes averiguou-se também de que forma a actividade dopaminérgica estava distribuída no cérebro de um ajudante. Para isso foram executadas microdissecções das macro-areas de cérebros de indivíduos ajudantes (que pertencem a uma família), e a indivíduos que não eram ajudantes (que se encontravam em tanques de agregação). Indivíduos de tanques de agregação também se encontravam dentro de uma estrutura hierárquica forte, mas no entanto esta não era uma família.

Dissecou-se as seguintes macro-areas: “forebrain”, tecto óptico, cerebelo, diencéfalo e tronco cerebral. Após as dissecções foram medidas as concentrações de dopamina e dos seus metabolitos (HVA e DOPAC), nas várias macro-areas em estudo.

Ao analisar a contracção total de dopamina e dos seus metabolitos no cérebro de ajudantes versus não-ajudantes, constatou-se a não existência de diferenças significativas entre estes. No entanto, quando analisadas as diferentes macro-areas separadamente, foi evidenciado que os ajudantes têm significativamente mais dopamina e HVA no “forebrain” comparativamente com os não-ajudantes. Considerando a concentração de DOPAC, observou-se ainda que os ajudantes têm significativamente mais DOPAC no diencéfalo e no tronco cerebral.

Estes resultados vão de encontro com estudos anteriores, que demonstram que áreas como o hipotálamo anterior estão envolvidas na regulação de comportamentos sociais, e que a elevada concentração de dopamina no “forebrain” está correlacionada com a percepção de uma recompensa. Os resultados deste estudo sugerem que os ajudantes de N.pulcher sentem uma recompensa por pertenceram a uma família. Sugerindo que, ao contrário do que se pensava anteriormente, ser um ajudante de uma família de N.pulcher é um caso de reciprocidade directa e não de um comportamento altruísta.

**Palavras-chave:** Sociabilidade, Cooperação, Neolamprologus pulcher, dopamina, receptores D1-like, receptores D2-like.

## Abstract

Cooperation is an evolutionary enigma that has intrigued biologists ever since Darwin. Much has been researched on the functional mechanisms of cooperation however; the physiological framework has only recently become a focus. Here we report on three experiments focussing the role of dopamine in social behaviour of a notorious cooperatively breeding teleost fish species. Dopamine is involved in the modulation of animals' reward system and social decision network, suggesting that it might be involved in sociability. We studied *Neolamprologus pulcher*, a cooperative cichlid fish from Lake Tanganyika, East Africa. These fish live in families with a dominant pair and a variable number of subordinates helping the dominant breeders in territory maintenance and defence, showing altruistic behaviour by engaging in alloparental care. We aimed at dopaminergic receptors D1 and D2, blocking or stimulating their activity with injections of agonists or antagonists (SKF-3893, SCH-23390, Quinpirole and Metoclopramide). Our data suggest that the two dopaminergic receptors have different regulatory roles for the social behaviour of these fish. The major focus seems to be on D2 receptor, which is influencing the aggressive, submissive and affiliative behaviour. Specifically, the D2 receptor is stimulated there was an increase of aggression, while when blocked it increases submission and affiliative behaviour. Interestingly, social context is the switch in which D2 influence is observe, helpers have into account the social context and they will not behave in discordance with the environment. Finally, when analysing the concentration of dopamine and its metabolites we found that helpers have a higher dopaminergic activity in the diencephalon and a higher concentration of dopamine the forebrain (e.g. Telecephalon). Higher levels of dopamine in helpers' telencephalon points out towards the direct existence of reward from living in a stabilized family. These data provide the first insight into the role of dopamine for the social behaviour of a cooperative fish species.



**Key-words:** Sociability, Cooperation, *Neolamprologus Pulcher*, Dopamine, D1-like receptors, D2-like receptors.

# General Introduction

## SOCIABILITY AND COOPERATION

Sociability is a term to define an interaction between two individuals that live in a group.[1] In order to live in groups, its members need to gain a genetically advantage, except in can of siblings.[1] In the particular case of siblings kin-selection is responsible for their group living, being a consequence of parental investment.[1] In non kin related groups they have several advantages for living in a group, for example it is known that in baboons, individuals that live in isolation do not survive for long due the large predation rate that exists in the environment.[2] Being social does not mean that the group members cooperate, in fact cooperation can be seen as a case of high sociability.

The term cooperation is applied to any interaction between individuals where the action of one individual benefits another [3]. A large spectrum of organisms engage in this positive trade-offs microorganisms (Lichens) as well as vertebrates (including mammals). For a long time cooperation has intrigued biologists: why should animals cooperate with each other? What is the advantage of cooperation?

In order to understand this cooperative behaviour we need look at the social structures behind it and analyse the benefits to each party involved, in particular the helper. "Helper" is the name given to the individual that benefits the other, in other words, the helper is the cooperator. An important point of analysis is whether the helper receives any pay-off from the receiver. This will allow differentiating between different ways of cooperation and biological interactions.

Cooperation interactions where the helper receives pay-off for his action can be divided in four categories, depending on the type of reward. First, there is the individual advantage, which occurs when cooperation is of advantage to the cooperator (helper). In this case, cooperation is motivated by a future benefit. For example, when an unmated male helps a mated male, in order to get his female mate when he dies[4].

Second, there is reciprocation, a type of cooperation where the helper cooperates only in order to receive the same treatment in the future from others in the population. This interaction it is often described as "reciprocal altruism"[5]. One example of this behaviour can be seen in vampire bats (*Desmodus rotundus*) which feed on mammalian blood. Sometimes, when some of the members of the population have been unsuccessful foraging, the more successful ones regurgitate to feed the unlucky ones[4].

At last there are instances where animals cooperate because they are Kin with the receiver, so in those cases the helper cooperates because that will increase his own fitness. Because his genes are in the receiver, the survival of the receiver is of interest to the

cooperator [4]. However, there are cases where the cooperator does not receive anything from this interaction. One of these cases would be a manipulated cooperator that helps without knowing. This is what happens in brood parasitism, when the parasitic specie lays his eggs on the host nest and leaves it there for the host to brood them. One example of that is the interaction between cowbirds and cuckoos, with the cowbirds parasiting the cuckoo's nest and the cowbirds infants mimicking the cuckoo's children[4].

Fourth, is cooperation without any pay-off to the cooperator.

## PHYSIOLOGY OF COOPERATION

For cooperation to be raised bonds are needed to be formed between group members.[6,7] These relationships are crucial for creating preferences where some individuals will be treated differently, where certain behaviours will perform exclusively towards individualized partners.[6]

Hormones and neurohormones play a very importance role in the bonding and in regulation of the exclusive individual specific behaviours performed, having both activational and organizational influence on general social behaviour.[6]

One example of the importance of hormones in modulation of social behaviours is the role of androgens. Androgens act as behavioural facilitators by modulating neural pathways of social behaviour, for instance androgens can regulate aggressive and sexual behaviour in male vertebrates.[6,8–11] Within a social network an individual androgen level will modulate perceptive, motivational and cognitive mechanisms, influencing future social behaviour efficiency. [10]

Hormones may modulate behavioural expression but they will not cause behaviour, behaviour is mainly driven by internal and environmental stimuli.[6]

Besides sex hormones, stress hormones, neuropetides and neurotransmitters can also modulate social behaviour.[6]

Neuropetides from vasoticin/oxytocin family can modulate social behaviours, in is known that species differences in Oxytocin receptors in the nucleus accumbens is associated with differences in matting systems.[12] In prairie voles it was shown by pharmacological manipulation of the oxytocin receptors induces the formation of partner preference.[13]

In cleaner wrasses (*Labroides dimidiatus*) it was found that arginine vasotocin (AVT) has a relevant role in reducing interspecific cleaning activities and modulates dishonesty, meaning that endogenous levels of AVT is directly modulating perceptive, motivational and cognitive mechanisms, affecting cleaning behaviours.[14]

Neurotransmitters and neuromodulators are also able to modulate social behaviour. In order to have flexible behaviours neuronal plasticity is needed, this plasticity can be achieved by chemical modulation.[6] Chemical synapses allow focal modulation of signal transmission, representing a modulation done by cell-cell signalling.[6]

In adult vervet monkeys (*Chlorocebus pygerythrus*) it was shown that by increasing central serotonergic activity with pharmacological stimulation resulted in the acquisition of high dominance status. [15]

In rats it was found that dopamine and serotonin are involved in in two different types of cost-benefit decision making. Dopamine was responsible for decisions concerning effort and reward delay, while serotonin was crucial for evaluation concerned with the reward delays.[16]

In teleost fish it was found that serotonin is neuromodulatory driver for social and cooperative behaviours, by pharmacological blockage of the serotonin-mediated response cleaner fish have decreased the number of cheats and increased aggression towards conspecifics.[17]

## DOPAMINERGIC SYSTEM

Dopamine is known to have a major importance in several central functions and behaviours, such as cognition, emotion, perception, motivation, reward, decision making and memory[18–20]. Dopamine is catecholaminergic neurotransmitter widely expressed in the brain [21]that is has been very well studied in mammals where it has been found that dopamine has four major pathways; the nigrostriatial, mesolimbic, mesocortical and tuberoinfular systems [22,23]

For the purpose of this thesis we will focus more on the role of dopamine in the mesolimbic reward system.

For the study of cooperation and sociability the mesolimbic reward system has a major role, as it was described in 2011 by O'Connell and colleagues.[24] Social behaviour for being adaptive it must be rewarding in some way.[24] The dopaminergic system is responsible giving reward from a social interaction.[25]

Dopaminergic signalling is mediated by five distinct receptors that are organized in two clades: D1-like receptors that include the D1 and D5 receptors, D2-like receptors including the D2, D3 and D4 receptors. These two clades are distinguished by their interaction with the enzyme adenylyl cyclase (AC) , the D1-like receptors activate AC while the D2-like receptors inhibits it.[21]

It is known that these two clades of dopaminergic receptors have different affinities to dopamine, having the D2-like receptors close to 10-to-100-fold greater affinity

than the D1-like receptors[26,27]. Furthermore the D1-like receptors have a higher concentration postsynaptically, while the D2-like receptors can be found bout pre- and postsynaptically being mainly autoreceptors.[26]

The D2-like receptors can inhibit dopaminergic neuron firing, synthesis and release inducing a negative feed-back [27], while the D1-like receptors have a direct stimulation.

In 2011 O'Connell and colleagues have described the Social decision-making network (SDM).[24] In this study they took into account the Social Behaviour network described by Newmann in 1999,[28] but O'Connell added the mesolimbic reward system to the Social Behaviour network. O'Connel argued that a social behaviour should be some way rewarding in order for it to be continuous or repeated.[24] In the Social Behaviour network that was previously described it already included some brain nuclei that also belong to the mesolimbic reward system, such as the lateral septum (LS) and the bed nucleus from stria terminalis (BNST)/medial amygdala (meAMY).

With this network we can understand how crucial the dopaminergic system is for the regulation of social behaviour.

In cynomolgus monkeys it was shown the importance of the D2/D3 receptors availability for social rank formantion.[29,30]

In rats it has been shown by pharmacological manipulation that the blockage of the D2-like receptors increases aggression, while blockage of the D1-like receptors decreases aggression.[31] In praire voles was found that the D2-like receptors are responsible for pair bonding and partner preference.[32,33]

In teleost it was also shown the importance of the dopaminergic system in aggression [34–36]. In Artic charr it was shown that subordinate fish have lower dopaminergic activity which associated in recuduction of aggression.[36] In cleaner wrasses it was found that the D1 receptors are responsible for reward perception, perception of cost/benefits in an interaction with the clients, and in learning. [37,38] In cichlid fish (Aequidens pulcher) it was found that the administration of dopamine agonists and antagonist reduced aggression.[39]

## MODEL SYSTEM: NEOLAMPROLOGUS PULCHER

For better understanding the role of dopamine in social behaviour regulation we used the cooperative breeder specie Neolamprologus *pulcher*. This cichlid fish from the Lake Tanganyika lives in social groups inside of rocky habitats near the sublittoral zones of the lake (Poll 1974)[40]. These social families or groups usually consist of a pair of

dominant breeders and a variable number of subordinates of different size. On average the groups have between 5 to 6 helpers with ≥ 15mm standard length. However, the number of helpers can vary from 1 to 30 helpers for one family[41–43].

Helpers usually provide help with several chores such as brood care and defence, maintaining and improving the territory [42,44,45], both mature and immature helpers participate.

N. pulcher's behaviours and ecology it was studied for several years [41]and most of their behaviours have been described and catalogued (see Taborsky 1984)[44]. We can divide N. pulcher's behaviour in 6 categories: restrained aggression (fin spread, frontal approach, S-ben, head jolting); overt aggression (ramming, biting, mouth fighting); affiliative behaviour (bumping); submissive behaviour (tail quiver, hook display, zig-zag swimming); territory maintenance (digging, carrying, substrate cleaning); brood care (cleaning eggs, mouth-cleaning fry, fanning). [44]

Behaviours such as brood care and egg defence against predators are considered as altruistic behaviours, because helpers don't receive any direct reward from this behaviour.[46]

*Neolamprologus pulcher* has very complex social and cooperative interactions, for that reason our aim is to study the role of dopamine in the regulation of such complex interactions.

Our hypothesis is that *N.pulcher's* helpers might receive a reward from being part of a family, and that dopamine is regulating *N.pulcher's* interactions with the family members. For testing this hypothesis we performed pharmacological manipulations of the two clades of dopaminergic receptors, D1-like and D2-like, by injecting receptor specific agonist and antagonist of these two clades. Our interested is on the regulation of helping behaviour, interaction between helpers and dominants, for this reason our work is focus on helper's behaviour.

We predict that dopamine will in fact be regulating social interactions in N.pulcher, confirming dopamine's role in social decision as it was described by O'Connell. [24]

We will also measure the concentration of dopamine and tis metabolites to better understand where the behavioural regulation might take place, and where do helpers have a higher dopaminergic activity. With this analysis we also aim to study if helpers perceive a reward from belonging to a family.

Has it was described by O'Connell and Colleagues in 2011, we think that a social interaction should be somehow rewarding in order to be repeated, so we are expecting to find higher concentrations of dopamine in helper's forebrain which is a sign that they are perceiving a reward. [24,47]

# Chapter 1: The effect of different dosages of dopamine in the behavioural regulation of a cooperative breeding cichlid fish

## Introduction

Sociability and cooperation are a very complex set of behaviours, in which individuals need to have an integrative knowledge of the environment and social context in order to have an appropriate response to a given situation.[6] To behave appropriately an individual needs to collect relevant information, and process the acquired information in order to behave accordingly.[6] Nowadays, we have a very profound knowledge in behaviours and ecology of animals, but we still lack on knowledge about the mechanisms that are behind the integration of information.[6] For this reason the following chapter is going to focus on the role of the neurotransmitter dopamine.

Dopamine it is a catecholamonergic neurotransmitter known to have a major importance in several central functions and behaviours, such as locomotion, cognition, emotion, perception, motivation, reward, decision making and memory[18–20]. It is also known from studies in mammals that dopamine has four major pathways, the nigrostriatal, mesolimbic, mesocortical and tuberoinfular systems. [22,23] An abnormal dopaminergic signalling can originate a variety of brain disorders in humans, such as bipolar disorder, major depression and dyskinesia.[22,48–51]

Dopaminergic signalling is mediated by five distinct receptors that are organized in two clades: D1-like receptors which include the D1 and D5 receptors, and the D2-like receptors which include the D2, D3 and D4 receptors. The distinction between these two major categories is based on their interaction with the enzyme adenylyl cyclase (AC). The D1-like receptors activate AC whereas the D2-like receptors inhibit it.[21] It is known that there are affinity differences of dopamine for each receptor, the D2-like receptors have a higher affinity to dopamine than the D1-like receptor family.[26,27] Furthermore the D1-like receptors are in higher concentration postsynaptically, being thought that the $D1_A$ receptors have the higher influence in vertebrates [26]. On the other hand, the D2-like receptors can be found in both pre- and postsynaptically, being predominantly autoreceptors.[26] This means that the D2-like receptors are able to inhibit dopaminergic neuron firing, synsthesis and release, inducing a negative feedback[27], while the D1-like receptors have a direct stimulation. Thus it is expectable that D1-like receptors and the D2-like receptors to have opposite behavioural effects.[52] In teleost fish it has been shown in a cichlid fish (*Astotilapia brutoni*) that the D1 and D2 receptors are widely expressed in the telencephalon and diencephalon and some

mesencephalic structures.[24] These regions are known in across amniotes that involved the social behaviour regulation[28,53,54], suggesting that dopamine may play an important role in social behaviour in teleost fishes.[24]

Skuse and Gallager showed that the dopaminergic reward system is involved in the social brain[24], having a role in affiliation in the animal models[25]. In 2012, O'Connell and colleagues described the social decision-making network, as a combination of the social behaviour network[28] with the mesolimbic reward system, which they describe as network that governs stimulus evaluation and behaviour in social organisms.[24] Since our goal is to understand the underlying mechanisms in complex interactions such as sociability and cooperation we decided to use a cooperatively breeding cichlid fish, *Neolamprologus pulcher* as the model species for this study. *N.pulcher* is a cichlid fish endemic from Lake Tanganyika, one of the three big lakes in Africa known from its adaptive radiation. This fish live in rocky habitats in the sublittoral shores of the lake. They live in families with size-based hierarchy, consisting in a breeder pair and to 30 non reproductive helpers. Helpers are individuals that delay their reproductive period in order to stay in a family and help. Helpers perform alloparental care, shelter maintenance and engage territory defence against intruders.[42,55–58] Several studies have manipulated pharmacologically the dopaminergic activity in rats and fish brains[6,19,59–61], although *Neolamprologus pulcher* was never used for studying dopamine's role in N.pulcher's social behaviour. For this reason, and considering that receptors have distinct affinities and putative concentrations and distributions in N. pulchers brains, we deemed important to understand the role of different drug dosages. Thus, we performed a calibration study for this species in order to create a dosage/response curve. The aim of this study is to understand if dopamine and its receptors plays a role in *N.pulcher's* behaviour.

This study will be mainly focus on the role of the D1-like and D2-like receptors. It is known that these two receptors types have different roles and sometimes can produce antagonistic responses. It has been shown that the effect of D2-like antagonists increases aggression in rats whereas the administration of a D1-like antagonist decreases aggressive behaviours[31].In male prairie voles D2-like receptors are mediating partner preference [33].D1-like receptors are involved in recognition memory of familiarity and place of objects.[62]

In teleost fish it has been shown that the D1 receptor pathway has a greater role in reward associative learning than the D2 receptor pathway.[37] Moreover It was shown that dopaminergic blockage by administration of D1 and D2 antagonist that the dopaminergic system is involved in decision making in cooperative context.[38] For this reason, this study will make a broad observation of both major dopaminergic receptors' roles in the general

behaviour of *Neolamprologus pulcher*. We looked at performed aggression, submission, affiliative and maintenance behaviour. With this approach we want to create a stronger background for further experiments in *N.pulcher's* system. This calibration study will establish the effect produced by several drug dosages, providing strong evidence for following up experiments.

According to the literature mentioned before, we expected to find that dopamine is modulating behaviours such as aggression and/or affiliative behaviour.

## Materials and Methods

### Housing

We used second to fourth generation offspring's of wild caught *Neolamprologus pulcher* from Kasakalawe point near Mpulungo, Zambia. The fish were bred and housed at the Etologich Station Hasli, Institute of Ecology and Evolution, University of Bern.

We created 8 families with 4 members, organized in one couple and two helpers (one large and one small). All the fish had a minimum size difference of 5-10mm Standard Length (SL) between fish from different ranks. The families were kept in 50L tanks with two shelters and one refuge per tank. With a light: dark cycle of 13:11 at a room temperature of 27°C. All the fish were feed 6 days per week, with commercial cichlid food (tetra).

### Pharmacological manipulation

Our goal in this study is to see if dopamine has a role in the cooperative and social behaviour *of Neolamprologus pulcher*. In order to better understand the importance of this neurotransmitter we decided to pharmacologically modulate the receptor activity. For that we used SKF-38393 (1-phenyl-7,8-dihydroxy-2,3,4,5-tetrahydro-1H-3-benzazepine) hydrochloride as D1-like receptor agonist[19,21,37], and SCH-23390(7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine) hydrochloride as a D1-like receptor antagonist. For the D2-like receptor activity manipulation, we used Quinpirole hydrochloride (LY 171555), an D2-like receptor agonist, and Metoclopramide (4-Amino-5-chloro-N-(2-(diethylamino)ethyl)-2-methoxybenzamide), an D2-like receptor antagonist. These drugs will manipulate the activity of the dopaminergic receptors directly.[21]

For the purpose of this experiment we draw a dosage/time curve for each test drug. Tree dosages per drug were tested in accordance to previous work done with other species[19,37,38,63,64]

SKF-38393–0.5 µg/gbw, 2.5µg/gbw, 5.0µg/gbw;

SCH- 23390–0.1µg/gbw, 0.5µg/gbw, 1. 5µg/gbw;

Quinpirole – 0.5µg/gbw, 2.0µg/gbw, 3.0µg/gbw;

Metoclopramide - 0.5µg/gbw, 2.5µg/gbw, 5.0µg/gbw. . As control solution we used a saline solution (0.9% NaCl).

All the injections had a volume of 15µl per gram of body weight.

### Behavioural analysis

The number of aggressive behaviours performed by the focal helper were recorded, which included: overt aggression (i.e., with body contact such as biting, ramming, mouth-fighting) and restrained aggression (i.e. Fin spread and Opercula spreading). We also recorded the number of submissive behaviours (i.e. tail quiver) previous to aggression received by the focal helper, in order to make a ratio of submission performed per received aggression. Submission was usually towards a dominant (that may be any of the breeding pair or a larger helper) in order to stop the running attack and showing their subordinate position in hierarchy. Finally, we recorded affiliative behaviour, such as bumping (i.e. soft-touching the body of the recipient).

## Treatments

For this experiment we decided to divide the pharmacological manipulation in two treatments. 8 families were split into two equal groups (8 helpers per treatment). The first group of families was tested on the the D1-like receptors and was injected with three different dosages of SKF-38393, SCH-23390, plus the saline solution. The second group of families was tested on the D2-like receptors and was injected with three different dosages of Metoclopramide and Quinpirole, and the saline solution.

## Experimental protocol

During this experiment, all fishes were observed before and after the injection. Every behavioural observation period lasted for 15 minutes. Every observation was done using Observer 5.0 © (Noldus Information Technology). During the observation we noted each focal fish's behaviours, its social interactions and with whom it interacted. We had four different observational time points: before the injection, then at 15 min, 30 min and 60 min after injection.

At the beginning of the experiment we filled up the shelters with sand to stimulate digging behaviour, in order to see shelter maintenance behaviour by the helpers.

All tested fishes were measured, weighted, sexed and anesthetized with KoiMed© Sleep (0.15mL for a 300mL water anaesthesia recipient) before injection. The injections were done using 0.5mL insulin syringes (0.5mL M YJECTOR, Terumo Medical Corporation, Elkton, MD 21921, USA). The fishes were injected with 15µL/gbw [17].

After the injection the fishes were inserted in a recovery box with an air stone to recover from the anaesthesia. After the focal fishes were fully recovered from the anaesthesia, they were put back into their home tank, but kept inside of isolation net until the first behavioural measurement. For all the tested fishes we waited three to four days in between injections, depending on the stability of the test family. If one of the helpers was found evicted from the group or dead, that helper was replaced by another fish with the same size and sex. After the new helper had been accepted and the family had stabilized we proceeded with the experiment.

All assays were performed during the same time of the day (10am-20pm) to control for the normal circadian variation of the neurotransmitter and daily behaviour variation of the fish.

## Statistical analysis

All tests and plots were done using the software R (R Core Team, 2015 Vienna Austria) implemented in the user interface software RStudio© Version 0.98.1091 (2009-2014 RStudio, Inc).

We analysed the two treatments separately, since the fish was only exposed to a single treatment. Our experiment includes repeated measurements which we accounted for by defining fish identity as a random variable in our models. We started our analysis by subtracting the measurements before the injections as a baseline for all of the recorded behaviours. After this we log transform[ed] our data to fulfil the assumption of normality.. We

A

used package “lme4” for general linear mixed models (GLMM) analysis. In our models, we used the frequencies of performed behaviours such as aggressive, submissive, and affiliative or maintenance behaviour as dependent variable. As fixed factors, we used the different test drugs, such as SKF- 38393, SCH-23390, Quinpirole or Metoclopramide. For this we created subsets from the original treatment file, D1 treatment or D2 treatment. Our data was normalized by the log transformation, so we assumed in our model the normal distribution.

# Results

## a) D1 Treatment – Agonist

Analysing the results from the D1 treatment, the effect of D1 agonist in aggressive behaviour is increasing with the dosage (0,5ug/gbw p-value =0.514; 2,5 ug/gbw p-value=0,0654 0,5ug/gbw p-value <0,005 at 30 min; See Fig.1; See Supplementary information: S.I.Table 1). Indeed, aggression output is higher when animals were injected with 5ug/gbw, while the effect is observed 15min after injection (p-value=0,0668; See table 1; See Supplementary information: S.I.Table 1) but it is higher 30min after injection as it seems to decrease 60min after injection (p-value=0,0633; See Table 1). We also see a trend for the lower dosage (0,5ug/gbw) which seems to be consistent through the entire experiment (15min: p-value=0,0512; 30min: p-value=0,0514; 60min: p-value=0,0950; See Table 1; See Supplementary information: S.I.Table 1). On the other hand, when we look at the performed submission it seems that the only significant effect is an early stage (15min after injection: 0,5ug/gbw: p-value=0,2274; 2,5ug/gbw: p-value<0,05; 5ug/gbw: p-value<0,05; See Fig. 2; See Supplementary information S.I.Table 1). Regarding the performed affiliative behaviour no significant effect was found, except for a trend when using the 2,5ug/gbw dosage : p-value=0,0818; See Fig.3; See Supplementary Information: S.I.Table 1) or the higher (5ug/gbw: p-value=0,0814; See Supplementary Information: S.I.Table 1) dosage.

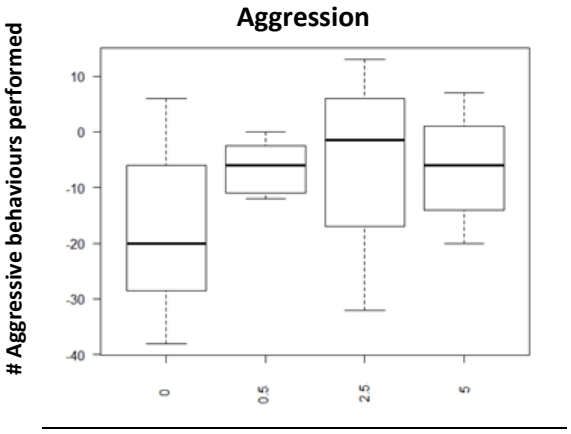


**Fig. 1: Performed aggression 30min after injection with D1-like agonist (SKF-38393); x axis: Dosages: 0- saline solution, 0,5ug/gbw, 2,5ug/gbw, 5ug/gbw; y axis: number of aggressive behaviours performed**

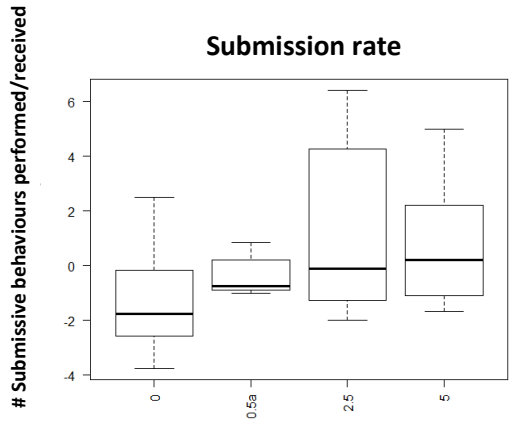


**Fig. 2: Ratio of Submission performed per received aggression at 15 min after injection with D1-like agonist (SKF 38393); x axis: Dosages: 0- saline solution, 0,5ug/gbw, 2,5ug/gbw, 5ug/gbw; y axis number of submissive behaviours performed per received aggression**

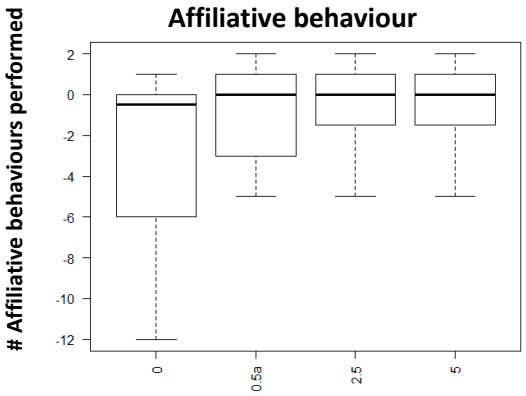


**Fig. 3 Performed affiliative behaviour 60min after injection with D1-like receptor agonist (SKF-38393); x axis: Dosages: 0- saline solution, 0,5ug/gbw, 2,5ug/gbw, 5ug/gbw; y axis - Number of performed affiliative behaviour**

## b) D1 Treatment – Antagonist

When we look to aggressive behaviour after the injection with D1-like antagonist, it seems that it is decreasing aggressiveness (See table 1); however we can only find a significant decrease of aggressiveness with the middle dosage (0,5ug/gbw; See table1; See supplementary Information: S.I.Table 2). This significant effect appears very early, 15min after the injection (p-value<0,05; See Fig.4; See supplementary Information: S.I.Table 2). This effect seems to decline with time but a trend is kept at 30min (p-value=0,567; See Table 1) and at 60min (p-value=0,0828; See supplementary Information: S.I.Table 2).

Looking at submissive behaviour, there seems to be an overall positive influence of SCH-23390. In this case, effective influence are seen in the lower (0,1ug/gbw) and middle (0,5ug/gbw) dosages (Figure 5). Also, a significant effect is found 15min after the injection, but only with the lower dosage (p-value<0,05; See supplementary information: S.I.Table2 ). 30min after injection all the dosages have a significant effect on performed submission (0,1ug/gbw:

p-value<0,05; 0,5ug/gbw: p-value<0,05; 1,5ug/gbw: p-value<0,01). However, only the lower and middle dosage kept the effect until 60min after the injection (0,1ug/gbw: p-value<0,05; 0,5ug/gbw: p-value<0,05) It seems that the drug is increasing affiliative behaviour with the lower dosage (0,1ug/gbw), but only having a significant effect 15min after the injection (p-value<0,05; See Fig. 6). Moreover, it seems that the middle and higher dosage are decreasing it, but compared with the control (saline) this is not a significant reduction.

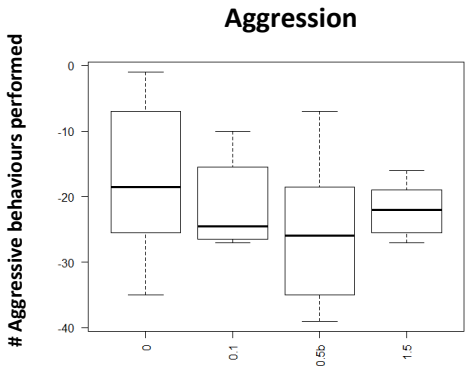


**Fig. 4 Performed aggression 15min after injection with D1-like antagonist (SCH-23390); x axis: Dosages: 0- Saline solution; 0,1ug/gbw; 0,5ug/gbw; 1,5ug/gbw; yaxis- number of performed aggressive behaviours**

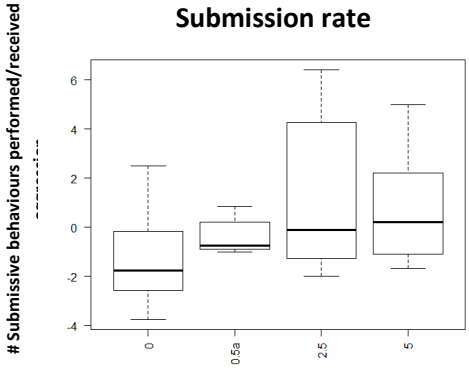


**Fig. 5 Ratio of performed submission per received aggression 15 min after injection with D1-like antagonist (SCH-23390); x axis: Dosages: 0- Saline solution; 0,1ug/gbw; 0,5ug/gbw; 1,5ug/gbw; y axis- number of performed aggressive behaviours**

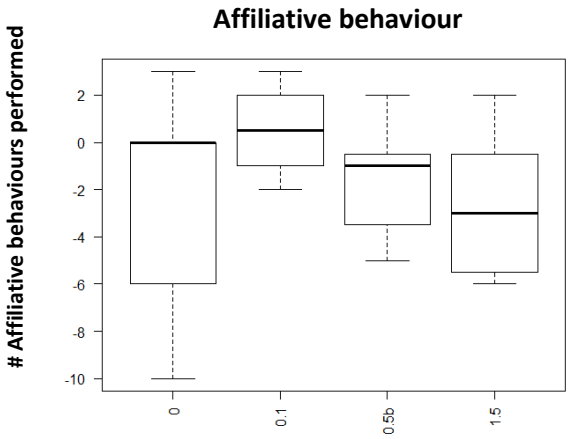


**Fig. 6 Performed affiliateve behaviour 15min after injection of D1-like antagonist (SCH-23390) ); x axis: Dosages: 0- Saline solution; 0,1ug/gbw; 0,5ug/gbw; 1,5ug/gbw; y axis- number of performed aggressive behaviours**

**Table 1 Summary chart results from D1-like receptor manipulation- ↑- increasing; ↓- decreasing; →- no effect. *- significant effect; .- trend.**

| | Performed aggression | Performed Submission | Performed Affiliative | Time |
|---|---|---|---|---|
| **D1** | | | | |
| **Agonist (SKF-38393) 0.5** | ↑. | ↑ | ↑ | 15min |
| (µg/gbw) | ↑. | ↑ | → | 30min |
| | ↑. | → | → | 60min |
| **Agonist (SKF-38393) 2.5** | ↑. | ↑* | ↑ | 15min |
| (µg/gbw) | ↑. | → | ↑ | 30min |
| | ↑ | → | ↑. | 60min |
| **Agonist (SKF-38393) 5** | ↑. | ↑* | → | 15min |
| (µg/gbw) | ↑* | → | → | 30min |
| | ↑. | → | ↑. | 60min |
| **Antagonist (SCH-23390) 0.1** | ↓ | ↑* | ↑* | 15min |
| (µg/gbw) | ↓ | ↑* | ↑ | 30min |
| | ↓ | ↑* | ↑ | 60min |
| **Antagonist (SCH-23390) 0.5** | ↓* | ↑* | ↑ | 15min |
| (µg/gbw) | ↓. | ↑* | → | 30min |
| | ↓. | ↑* | ↑ | 60min |
| **Antagonist (SCH-23390) 1.5** | ↓ | ↑ | ↓ | 15min |
| (µg/gbw) | ↓ | ↑* | → | 30min |
| | ↓ | ↑ | ↓ | 60min |

### c) D2 treatment - Agonist

No significant effects were found on aggressive and submissive behaviour by the D2 agonist treatment. However visual display of dosage response curve, concerning aggression shows an increase on aggression between the dosages 0,5 and 2ug/gbw, followed by a decrease with the dosage 3,5ug/gbw (See Fig.7; See Table 2; See Supplementary information: S.I.Table 3). For submissive behaviour there was a decrease between the 0,5ug/gbw and 2ug/gbw dosages (See Fig.8; See Table 2; See supplementary information: S.I.Table 3), and then an increase on preformed submission on 3,5ug/gbw dosage (See Table 2; See supplementary information: S.I.Table 3).

In terms of affiliative behaviour, the D2-like agonist seems to have an overall effect in increase it; however, its effect seems to decrease as the dosage increase (Fig .9). A significant effect was solely found when using the lower (0,5ug/gbw) dosage 15min after the injection (p-value<0,05; See supplementary information: S.I.Table 3). However, a trend was found with the middle (2ug/gbw: p-value=0,0871; See supplementary information: S.I.Table 3) and higher dosage (3,5ug/gbw: p-value=0,0871; See Supplementary information: S.I.T3) as well.

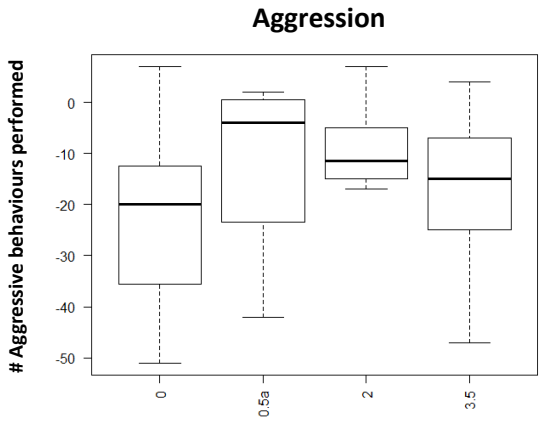


**Fig. 7 Performed aggression 15min after injection with D2-like agonist (Quinpirole); x axis: Dosage: 0- Saline Solution; 0,5ug/gbw; 2ug/gbw;3.5ug/gbw; yaxis- number of aggressive behaviour**

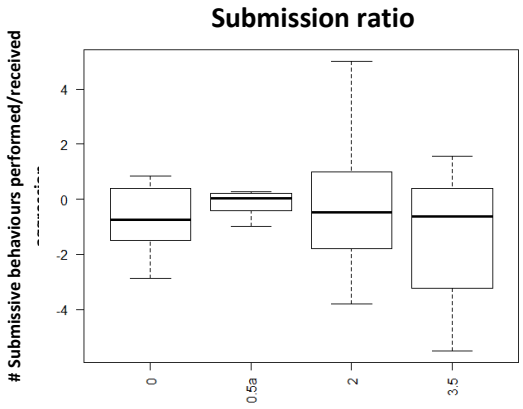


**Fig. 8 Ratio of performed submission per received aggression 15min after injection with D2-like agonist (Quinpirole); x axis: Dosage: 0- Saline Solution; 0,5ug/gbw; 2ug/gbw;3.5ug/gbw; yaxis- number of submissive behaviour performed per receive aggression**

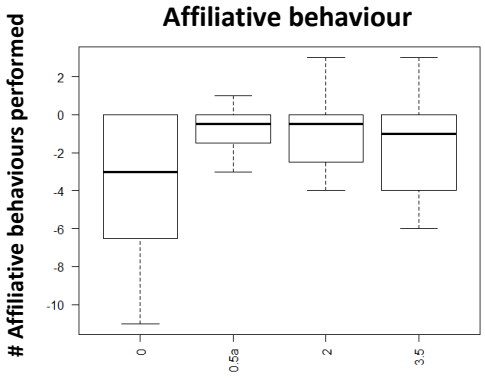


**Fig. 9 Performed affiliative behaviour 15min after injection with D2-like agonist (Quinpirole); x axis: Dosage: 0- Saline Solution; 0,5ug/gbw; 2ug/gbw;3.5ug/gbw; yaxis- number of affiliativebehaviour performed**

## d) D2 antagonist

Regarding the influence of the D2 antagonist, we were unable to find significant differences in aggression (Fig.10; Table 2; See supplementary information: S.I.Table 4) or submission (Fig.11; Table 2; See supplementary information: S.I.Table 4). However, affiliative behaviour had a similar effect than the one observed when injected with the agonist (Quinpirole): with the lower (0,5ug/gbw: p-value<0,05; See Supplementary information: S.I.Table 4) and middle dosage (2,5ug/gbw: p-value<0,05) which shows more consistent and lasting effect (kept for 60min)(See Table 2).

In general blocking the D2-like receptors seems to have an effect on affiliative behaviour (increasing it) when compared with the control (Fig.12). This effect is kept for 60 min with the lower and middle dosages (0,5ug/gbw; 2,5ug/gbw; See Table 2).

We did not find any effect in maintenance behaviour, such as digging, in either treatment; we could not test because of the lack of maintenance behaviours.

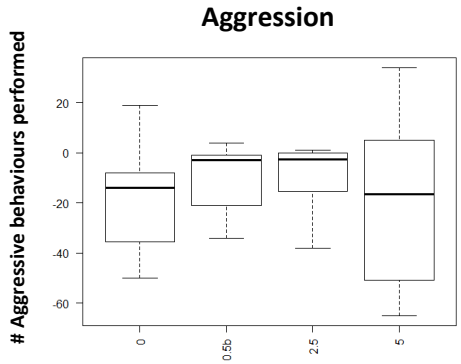


**Fig. 10 Performed aggression 15min after injection with D2-like antagonis (Metoclopramide); x axis: Dosage: 0- saline solution; 0,5ug/gbw; 2,5ug/gbw/5ug/gbw; yaxis - number of performed aggressive behaviours**

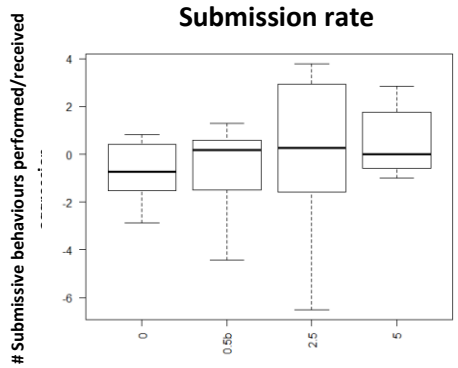


**Fig. 11 Rate of performed submissive behaviours per received aggression 15min after injection with D2-like antagonist (Metoclopramide); x axis: Dosages: 0- saline solution; 0,5ug/gbw; 2,5ug/gbw/5ug/gbw; yaxis - number of performed submissive behaviours per received aggression**

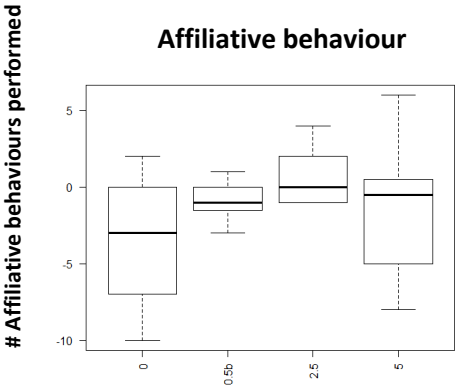


**Fig. 12 Performed affiliative behaviour 60min after injection with D2-like antagonist (Metoclopramide); x axis: Dosage: 0- saline solution; 0,5ug/gbw; 2,5ug/gbw/5ug/gbw; yaxis - number of performed affiliative behaviours**

**Table 2: Summary chart results from D2-like receptor manipulation- ↑- increasing; ↓- decreasing; →- no effect. *- significant effect; .- trend.**

| | Performed Aggression | Performed Submission | Performed Affiliative | Time |
|---|---|---|---|---|
| **D2** | | | | |
| **Agonist (Quinpirole) 0.5** | ↑ | → | ↑* | 15min |
| (µg/gbw) | ↑ | ↓ | ↑ | 30min |
| | ↑ | → | ↑ | 60min |
| **Agonist (Quinpirole) 2** | ↑. | → | ↑. | 15min |
| (µg/gbw) | ↑ | → | ↑ | 30min |
| | ↑ | ↑ | ↑ | 60min |
| **Agonist (Quinpirole) 3.5** | ↑ | → | ↑. | 15min |
| (µg/gbw) | ↑ | → | ↑ | 30min |
| | → | → | ↑ | 60min |
| **Antagonist (Metoclopramide) 0.5** | ↑ | ↑ | ↑* | 15min |
| (µg/gbw) | ↑ | ↓ | ↑* | 30min |
| | ↑ | ↓ | ↑. | 60min |
| **Antagonist (Metoclopramide) 2.5** | ↑ | ↑ | ↑* | 15min |
| (µg/gbw) | ↑ | → | ↑* | 30min |
| | ↑ | → | ↑* | 60min |
| **Antagonist (Metoclopramide) 5** | → | ↑ | ↑. | 15min |
| (µg/gbw) | → | → | ↑* | 30min |
| | → | ↑ | ↑ | 60min |

## Discussion

Building on the key influence of dopamine on animals' decision-making processes we aimed to find out more on its general effect in the context of cooperation. Research on fish is limited to few species [37,38] but none on the notorious cooperative breeder N pulcher. We first aim to draw a dosage/response curve for all the test drugs, agonist (SKF-38393) and antagonistic (SCH-23390) drug of D1-like receptors, and agonist (Quinpirole) and antagonist (Metoclopramide) of the D2-like receptor. This would allow us to know how the drugs are affecting fish behaviour, namely on aggression, submission, affiliative and maintenance behaviours, depending on the dosage. Overall we found that the two families of dopamine receptors have very distinct roles in behavioural regulation. In a constant environment, we saw that the D1-like receptors are modulating aggression and submission. The D2-like receptors seem to be modulating affiliative behaviour; however, pharmacological manipulation revealed that both agonist and antagonist produced increased behavioural performance.

Our D1-like receptor manipulation showed that independently of the dosage, there is an increase of the drug effect in N. pulcher's behaviour (i.e. aggression and submission) over time, more concretely, from the 15min to the 30min after injection. Overall, we begin to see a small decrease on the drug effect solely 60 min after the injection. The D1-like agonist is increased significantly the amount of aggressive behaviour, and submissive behaviour. Indeed, stimulating the D1-like receptors increased aggression with all the test dosages; however the higher dosage (5ug/gbw) was able to produce a significant increase of aggression, when compared to the control. Accounting for submissive behaviour, we also found that the D1-like receptor is generally increasing submission, although we found a significant difference with the middle (2,5ug/gbw) and higher dosage (5ug/gbw). Thus, the higher dosage of D1 agonist is having an effect both in aggression and submission.

As expected, the pharmacological blockage of the D1-like receptors was found to decrease aggression in N. pulcher in a stable environment, however only the middle dosage (0,5ug/gbw) revealed to have a significant effect. This effect on aggression could be seen 15min after the injection. When analysing submissive behaviour in relation to D1 blockage, we found a similar effect than the one observed in under effect of the D1-like agonist, but this time, it was the middle dosage (0,5ug/gbw) that produced an increase on submission thought the entire experiment.

In teleost fish, it has been shown that stimulating pharmacologically the activity of the D1 receptors in cleaner wrasses (Labroides dimidiatus) increases the number of inspection done to their clients. While blocking the D1 receptor leads to an increase of the tactile

stimulation of their clients. Showing that dopaminergic system has a role in intraspecific cooperation.[38]

Regarding the D2-like receptors manipulation we found that its stimulation leads to an increase on affiliative behaviour performed, from a general view. Only the lowest dosage of D2-like receptor agonist (0,5ug/gbw) produced a significant increase 15min after injection. We could not find any other relevant effect of D2-like receptor stimulation in a stable environment. For this reason we think that further investigation should be used the lowest dosage of D2-like receptor agonist.

Concerning the D2-like receptor antagonist we found the same general increase on affiliative behaviour, however we found a stronger effect, this because we found significant increases in all the test dosages. We found significant and lasting effect in both lower (0,5ug/gbw) and middle (2.5ug/gbw) dosages, however decided to use the lowest dosage because as it can be seen in table 2 this dosage seem to decrease submission, even though there was no significant effect. Our results in the D2-like receptors are in agreement with the results from Aragona and colleagues. In their study in prairie voles, they found that when D2-like receptors of the nucleus accumbens shell are activated pharmacologically, the males prefer to spend more time in contact with a familiar mate. [33]

In teleost fishes it has been shown that the blockage of the D2 receptors increases the amount of tactile stimulation done the cleaner wrasses to their clients, meaning that the dopaminergic system was modulating their perception.[38]

With these results we can conclude that as expected dopamine plays a role in the way the individuals from a family interact with each other by modulating aggressive behavior and affiliative behavior. Further studies are needed to understand how this works when the helpers are in different social contexts, where in the brain this is happening and whether there are differences in dopaminergic activity in the brain regions from the Social decision-making network.

# 2nd Chapter – The influence of the dopaminergic system to N. pulcher behavioural regulation: social Context manipulation

## Introduction

Cooperation can be considered as higher level of sociability. Cooperation can be defined as any behaviour that an individual does in order to benefit directly or indirectly another individual B. In some cases, cooperation can be based on direct or indirect reciprocity [65]. Reciprocity considers the benefit transaction that happens between individuals from a group. Direct reciprocity occurs when the action from the individual A directly benefits another individual B. Alternatively, indirect reciprocity takes place when the action from the individual A does not directly benefit the individual B but another individual C, which in turn will directly benefit individual B. For example: A helps C, and because C was helped before he will help B, creating a "helping chain". [3,5]

This "helping chain" can influence the social environment of the group. Of course the survival of helping always depends on the benefits/costs of helping itself and whether this behaviour is the best fitted strategy. This brings us to a new topic of game theory: the theoretical analysis how the two strategies of being cooperative or non-cooperative can co-exist in nature.

First of all, in order to understand what social environment/ context is, we need to take into account the animal ecology, its group composition, as well as their interactions within the group. When we talk about ecology, we should focus on the relationship between predator-prey interactions and resource availability. How does the focal animal act when facing a predator and/or an intruder invade his territory? Different social contexts have different behavioural demanding's; for instance, an individual behaves more aggressively when facing a predator or an intruder because the social context demands it. If the subject judges its social context incorrectly and performs misfit behaviours, that can bring loss of territory or even death of the subject. The concept of reciprocity can also be applied to subjects living in groups. Here, misfit behaviour can lead to expulsion from the group, bringing a consequent loss of recourses and vulnerability. [29,66,67]

In the previous chapter we started investigating the role of dopamine in the social behaviour of a highly social organism the *N.pulcher*. In this chapter we will continue to analyse it, also taking into account the social context that the helpers are in.

Dopamine it is a neurotransmitter that's very widely spread through the brain. [21] Dopamine takes action on the neural circuitry through a considerably slow modulation of the fast neurotransmission mediated by glutamate and GABA. [22]

There are two clades of dopaminergic receptors: the D1-like receptors and the D2-like receptors. These two clades were created to distinguish between the dopamine receptors that modulate adenylyl cyclase differently. In fact, while the D1-like receptors (D1 and D5) stimulate the production of adenylyl cyclase, the D2-like receptors inhibits it.[21,22]

It is known that the D1-like receptors and the D2-like receptors have different dopamine affinity; the D2-like receptors have 10-to-100fold higher affinity to dopamine than the D1-like receptors.[22,26]

The D2-like receptors activity can induce a negative feed-back that can inhibit dopamine neuron firing, synthesis and release[27] and then modulating D1-like receptors direct stimulating effect.

It is known that dopamine it is involved in sociability by taking part of the social-decision making network[28,68,69]. This network consist in a group of nuclei from different brain macro areas, such as the Nucleus accumbens, the prefrontal cortex, the medial nucleus of the amygdala and the ventral tegmental area, are connected with one another and regulate social behaviour. [24]

In 2016 Messias and colleagues have shown in cleaner wrasses (*Labroides dimidiatus*) that the modulation of dopaminergic activity modulates their learning ability, perception of reward and evaluation of cost/risks in a cooperative interaction with their clients.[37,38]

In the previous chapter we suggest that dopamine played a role in aggression, submission and affiliative behaviour of *N.pulcher*. This last category of behaviour is responsible for group cohesion. In fact affiliative behaviour is what creates the bonding between individuals and it is also responsible for keeping this bond.[33]

In this chapter we will continue to work with the same cichlid fish *N.pulcher* as model system to analyse the role of dopamine in sociability and cooperation.

As introduced in chapter one, the helpers perform several tasks in order to pay-to-stay in the breeder's territory, these talks include alloparental care, shelter maintenance and territory defence against predators and intruders. In addition to that, helpers will perform this large repertoire of cooperative behaviours according to their immediate social context. An example of this is how they will engage in more shelter maintenance when there are eggs in the territory.[56,70] There also seems to be a division of labour between helpers where, for instance, large helpers will defend the territory, while smaller helpers will engage in alloparental care and shelter maintenance. This makes sense as a smaller helper is less effective in territory defence when it comes to fighting bigger fishes and predators. Interestingly, a helper attacking an egg predator has been considered as an altruistic behaviour, because helpers are directly increasing breeders' fitness.[70,71] As a matter of fact, the helper will not receive any direct benefit from fighting off an egg predator, since it is not a threat to the helper itself, but only to the eggs that belong to the breeders. The question remains whether the helpers receive some form of reward through their "altruistic cooperation".

This chapter will explore the role of dopamine in two different social contexts: one were the helper should defend against an egg predator and one were the helper should engage in shelter maintenance behaviours. The analysis will also focus on the effect of the major types of dopamine receptors (D1 and D2), when pharmacologically modulated. We expect to see differences in the fish' drug response depending on the new social context that they are facing.

## Materials and Methods

### Housing

As in chapter 1, we used the second to fourth generation offspring of wild caught *Neolamprologus pulcher*, which were bred and housed at the Etologich Station Hasli, Institute of Ecology and Evolution, University of Bern. We thus established 10 families with four members: one breeding couple and two helpers (one large and one small). All the fish had a minimum size difference of 5-10mm Standard Length (SL) between them in order to easily establish an hierarchy. The families were kept in 50L tanks with two shelters and one refuge per tank. With a light: dark cycle of 13:11 at 27°C. All fishes were feed 6 days per week with commercial cichlid food (tetra).

### Pharmacological manipulations

In order to better understand the importance of dopamine for behavioural regulation in different social contexts, we have decided to keep the same approach used in the previous chapter 1. We used the same drugs used in our previous study, that were: SKF-38393 (1-phenyl-7,8-dihydroxy-2,3,4,5-tetrahydro-1H-3-benzazepine) hydrochloride as D1-like receptor agonist[19,21], and SCH-23390(7-Chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine) hydrochloride as a D1-like receptor antagonist. For manipulating the D2-like receptor activity we used Quinpirole hydrochloride (LY 171555), a D2-like receptor agonist, and Metoclopramide (4-Amino-5-chloro-N-(2-(diethylamino)ethyl)-2-methoxybenzamide), a D2-like receptor antagonist. . These drugs will act directly on the activity of the dopamine receptors, manipulating their activity[21]. As control we used a saline solution (0.9% NaCl). For this experiment, we used one dosage of each drug. We chose the dosages taking in account our previews results, as shown and discussed in the previous chapter: a) 5 µg/gbw of SKF-38393; B9 0.5µg/gbw of SCH-2390; c) 0.5 µg/gbw of Quinpirole and d) 0.5 µg/gbw of Metoclopramide. The dosages were also chosen taking into account the observed behavioural changes in aggression, submission, affiliative and/or maintenance behaviour. Additionally to that we took note for how long the effect lasted in order to have a sufficient time window for context manipulation and observation. All the injections had a volume of 15µl per gram of body weight (gbw).

### Behavioural analysis

The numbers of aggressive behaviours performed by the focal helper were recorded, which included: overt aggression (i.e. biting and ramming) and restrained aggression (i.e. Fin spread and Operculum opening display). We also recorded the number of submissive behaviours (i.e. tail quiver) previous to aggression received by the focal helper, in order to make a ratio of submission performed per received aggression. Submission was usually towards a dominant (that may be any of the breeding pair or a larger helper) in order to stop the running attack and showing their subordinate position in hierarchy. Finally, we recorded affiliative behaviour, such as bumping (i.e. soft-touching the body of the recipient).

## Experimental set-up

We used 10 families for this experiment, with a total of 20 focal helpers (10 small and 10 large helpers). We performed single intra-muscular, peripheral injections with agonists and antagonists of D1-like and D2-like receptors, plus the saline solution as a control (0.9%NaCl). We used a single dosage for each of the test drugs. Only one helper was injected per trial. Every focal helper had 3 days of break between trials in order to reduce the stress arising from capture and manipulation.

We performed then continuous live observations after the injection. For this, we used the software Observer 5.0© (Noldus Information Technology). Every observation period lasted for 15 min.

In this experiment consisted we directly manipulated the family environment to challenge the helpers' output behavioural response. Two distinct tasks were assigned to each family: a) the digging task, where the helpers were challenged to perform more shelter maintenance behaviour and b) the intruder simulation. In the first task, the shelters were previously filled up with sand before the observation. Then during the task we counted the number of digs (sand removal from the shelter) performed by the focal helper, plus any other interaction with the family members. Our second task consisted in challenging the helpers to defend against intruders. For the intruder task we decided to use the egg predator *Telmatochromis vittatus*. This species lives in sympatry with *N. pulcher*, and it is a natural egg predator of *N.pulcher*'s eggs.[46] During this task we observed the number of aggressive behaviours or aggressive displays performed towards the intruder by the focal helper, plus any other interaction occurring within the family. In the control situation we had fishes that did not face any kind of disturbance and registered every interaction within family members. The tasks were balanced in order to correct any sequential effects.

## Statistical Analysis

All tests and plots were done using the software R (R Core Team, 2015 Vienna Austria) implemented in the user interface software RStudio© Version 0.98.1091 (2009-2014 RStudio, Inc).Every focal fish was injected with all test drugs. This means that every fish was injected and observed 5 times. For this reason, in our analysis we included in the model repeated measures. We used general mixed models (GLMM) for our analysis. In our model we used the frequencies of aggression or submission (corrected for received aggression) or affiliative behaviour as dependent variables; as fixed factors we used treatment and we used fish ID as random factor. We assumed in our models the negative binomial distribution and our data was zero inflated.

# Results

## 1. Aggression

### 1.1. Control task

Higher levels of aggressive behaviour were observed in individuals injected with Quinpirole, when compared to control (p-value<0.05; See Table 3; See Fig. 13A). However, it seems that SKF-38393 (D1-like receptor agonist) and Metoclopramide (D2-like receptor antagonist) also influenced a tendencial increase in aggression (SKF-38393 p-value=0.065; Metoclopramide p-value=0.078; See Table3).

### 1.2. Digging task

During the digging task we did not found any significant effects .We saw that SKF-38393 (D1-like agonist) tend to increase performed aggression (p-value=0.092; See Table3; See Fig.13B). During this task no other drug produced an effect in aggression.

### 1.3. Intruder task

During the intruder task only the Quinpirole (D2-like receptor agonist) produces a significant increase on performed aggression (p-value<0.05; See Table3; See Fig.13C).

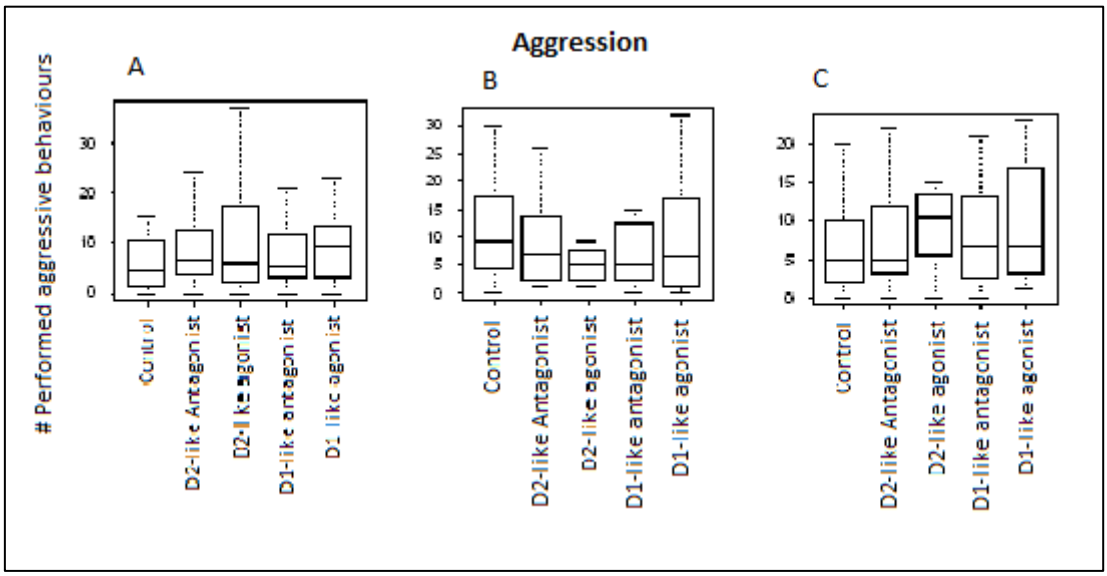


**Fig. 13 Number of performed aggressive behaviours observed. A- Control task; B- Digging task; C-Intruder task**

## 2. Submission

### 2.1. Control Task

The D2-like antagonist (Metoclopramide) tended to increase the performed submission during the control task (p-value=0.083; See Table3; See Fig.14A)

### 2.2. Digging task

No significant effects were found during the digging task. The D2-like antagonist (Metoclopramide) tended to increase the performed submission during the digging task, but it lack significance (p-value=0.057; See Table3; See Fig.14B).

### 2.3. Intruder task

The D2-like antagonist (Metoclopramide) produced a significant increase in performed submission (p-value<0.01; See Table3). Moreover, both D1-like receptor antagonist (SCH-23390) and agonist (SKF- 38393) showed a non-significant tendency to affect performed submission (p-value=0.06047and p-value=0.05907, respectively; See Table3; See Fig.14C).

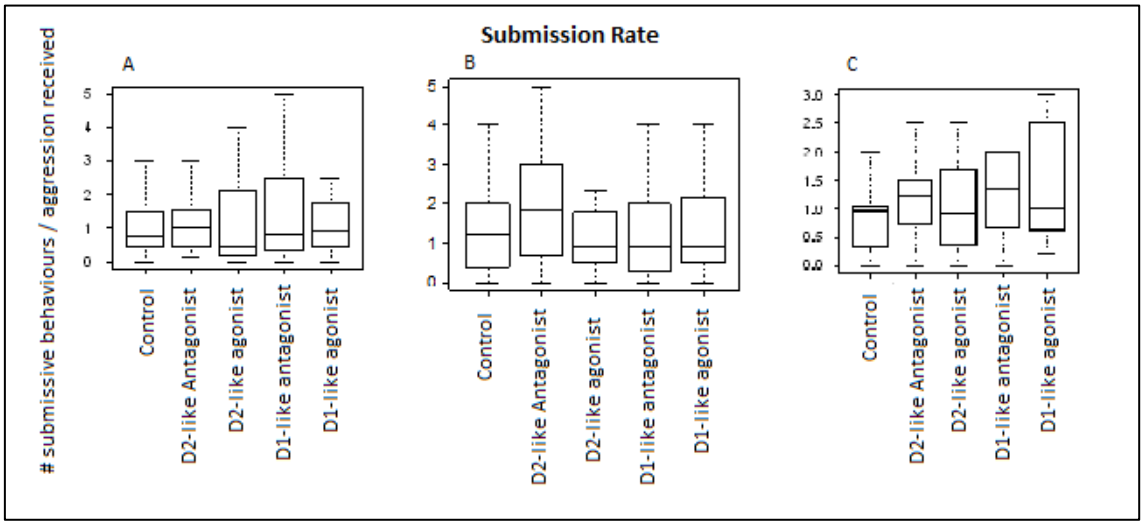


**Fig. 14 Number of performed submissive behaviours observed per received aggression. A- Control task; B- Digging task; C-Intruder task.**

### 3. Affiliative behaviour

#### 3.1 Control task

We found a significant increase on performed affiliative behaviour after the blockage of the D2-like receptors (p-value<0.05; See Table3; See Fig.15A).

#### 3.2. Digging task

During the digging task we could not find any drug effect on affiliative behaviour performed (See Fig.15B; See Table 3).

#### 3.3. Intruder task

No significant effects were found, however D1-like receptor antagonist (SCH-23390) showed a non-significant tendency to increase affiliative behaviour (p-value=0.096; See Table3; See Fig.15C).

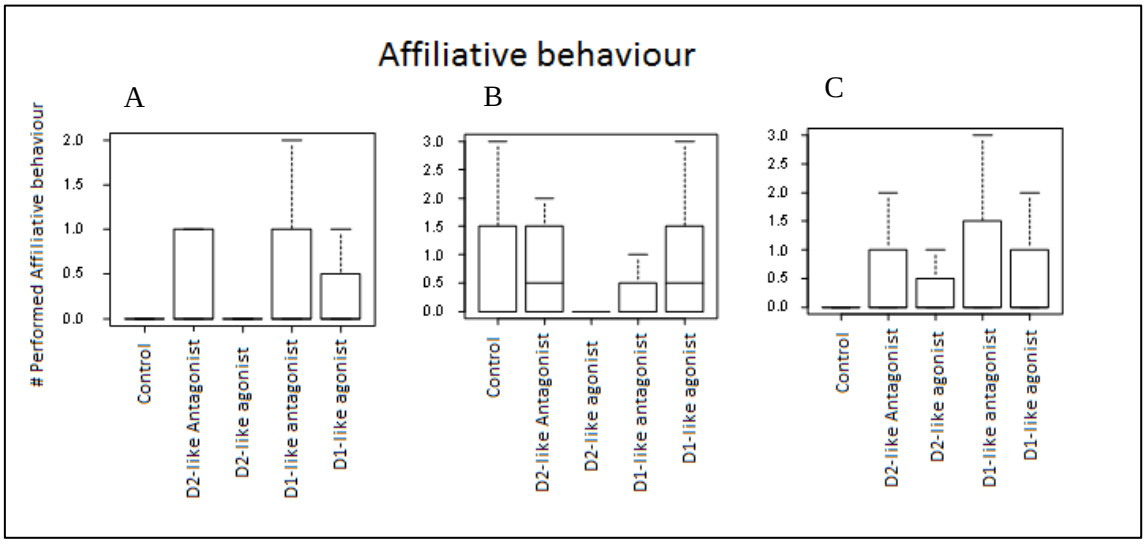


**Fig. 15 Number of performed Affiliative behaviour observed. A- Control task; B- Digging task; C-Intruder task.**

**Table 3 Statistical results from the pharmacological manipulation of D1-lile and D2-like receptors; . Trend, * p-value<0.05.**

| Drug | Behaviour | Task | Zero-Inflation | Estimate | SE | T-value | p-value |
|---|---|---|---|---|---|---|---|
| **D1-like agonist** | Aggression | Control | 0.031616 | 0.420 | 0.228 | 1.84 | 0.065 . |
| | | Digging | 0.052569 | 0.445 | 0.264 | 1.69 | 0.092 . |
| | | Intruder | 0.037622 | 0.2688 | 0.2134 | 1.26 | 0.208 |
| | Submission | Control | 1e-06 | 0.19844 | 0.20635 | 0.96 | 0.336 |
| | | Digging | 0.056104 | 0.14587 | 0.20833 | 0.70 | 0.484 |
| | | Intruder | 0.015478 | 0.37038 | 0.19621 | 1.89 | 0.05907 . |
| | Affiliative | Control | 0.30578 | -0.106 | 0.511 | -0.21 | 0.835 |
| | | Digging | 0.26409 | 0.479 | 0.495 | 0.97 | 0.33 |
| | | Intruder | 1.0003e-06 | 0.7727 | 0.6105 | 1.27 | 0.206 |
| **D1-like antagonist** | Aggression | Control | 0.031616 | 0.115 | 0.231 | 0.50 | 0.620 |
| | | Digging | 0.052569 | 0.164 | 0.259 | 0.63 | 0.528 |
| | | Intruder | 0.037622 | 0.1376 | 0.2186 | 0.63 | 0.529 |
| | Submission | Control | 1e-06 | 0.18007 | 0.20620 | 0.87 | 0.383 |
| | | Digging | 0.056104 | 0.22247 | 0.21574 | 1.03 | 0.302 |
| | | Intruder | 0.015478 | 0.39528 | 0.21055 | 1.88 | 0.06047 . |
| | Affiliative | Control | 0.30578 | 0.203 | 0.439 | 0.46 | 0.645 |
| | | Digging | 0.26409 | 0.224 | 0.451 | 0.50 | 0.62 |
| | | Intruder | 1.0003e-06 | 0.9470 | 0.5689 | 1.66 | 0.096 . |
| **D2-like agonist** | Aggression | Control | 0.031616 | 0.554 | 0.230 | 2.41 | 0.016 * |
| | | Digging | 0.052569 | 0.389 | 0.256 | 1.52 | 0.129 |
| | | Intruder | 0.037622 | 0.4700 | 0.2176 | 2.16 | 0.031 * |
| | Submission | Control | 1e-06 | 0.06071 | 0.21003 | 0.29 | 0.773 |
| | | Digging | 0.056104 | 0.25493 | 0.20452 | 1.25 | 0.213 |
| | | Intruder | 0.015478 | 0.17108 | 0.21050 | 0.81 | 0.41636 |
| | Affiliative | Control | 0.30578 | 0.352 | 0.590 | 0.60 | 0.551 |
| | | Digging | 0.26409 | 0.380 | 0.440 | 0.86 | 0.39 |
| | | Intruder | 1.0003e-06 | 0.0512 | 0.6341 | 0.08 | 0.936 |
| **D2-like antagonist** | Aggression | Control | 0.031616 | 0.418 | 0.237 | 1.76 | 0.078 . |
| | | Digging | 0.052569 | -0.127 | 0.256 | -0.50 | 0.620 |
| | | Intruder | 0.037622 | 0.0772 | 0.2215 | 0.35 | 0.727 |
| | Submission | Control | 1e-06 | 0.34716 | 0.20027 | 1.73 | 0.083 . |
| | | Digging | 0.056104 | 0.38659 | 0.20348 | 1.90 | 0.057 . |
| | | Intruder | 0.015478 | 0.57181 | 0.19665 | 2.91 | 0.00364 * |
| | Affiliative | Control | 0.30578 | 1.098 | 0.550 | 1.99 | 0.046 * |
| | | Digging | 0.26409 | 0.134 | 0.449 | 0.30 | 0.77 |
| | | Intruder | 1.0003e-06 | 0.7364 | 0.5792 | 1.27 | 0.204 |

## Discussion

We performed pharmacological manipulation (stimulation or blockage) of the D1-like and D2 like receptors of *N.pulcher* helpers in different social contexts. As expected, we found that the dopaminergic system is influencing social behaviour in accordance to social context in *N pulcher*. By stimulating the activity of the D2-like receptors we increased performed aggression (during the control task and intruder task), and this increase was observed in different social contexts. Contrarily, the blockage of the D2-like receptors produced a significant increase on performed submission and affiliation, also in different social context. Our results thus show that the activity and behavioural regulation by the D2-like receptors depends on the social context that helpers face. Against our initial predictions, D1 like receptor manipulations coupled with social context change did not amount to any significant effect. Our results show that the dopaminergic system is in fact regulating social behaviour, by modulation of social interaction between individuals. The D2-like receptors seem to have a higher importance in social behaviour regulation in *N.pulcher*.

However, there are first some requisites that they need to fulfil in order to interact in a group. They need to be able to acquire information from the environment they live in, as well as information from their conspecifics (i.e. whether they are in an aggressive status or not, or whether they are in the reproductive mood). After that they must integrate this information in order to perform a fitted behaviour. Misfit behaviour can be maladaptive and lead to expulsion from the group and/ or predation.[1,5,66]

In order understand this integration process; Newman in 1999 described the social behaviour network in mammals[28]. This network consists in several brain nuclei that are anatomically connected and are involved in social behaviour modulation. Later this network was extended to all vertebrates[53]. In 2011 O'Connell described the Social decision-making network, combining the social behaviour network with the mesolimbic reward system[24].

Our results show that the stimulation of D2-like receptors increases aggressive behaviour, which is similar to results observed in other model system. For instance, in 2000 Delville and colleagues have shown in mice that some of the nuclei from the social decision-making network are involved in modulation of aggression, particularly the activity of the D2-like receptors. The activation of D2-like receptors increases aggression in mice. [72,73] In teleost fish it has been shown that the dopaminergic system is also linked with aggression behaviour changes[34–36]

It has been shown that subordinate fish have higher dopaminergic activity in their hypothalamus.[74] In cichlid fish (*Aequidens pulcher*) it has been shown that administration of generalist dopamine agonists and antagonists reduced aggressive behaviours.[39]

Our results show the same pattern observed in mice and in another cichlid fish (Aequidens pulcher), and show the importance of the dopaminergic system in aggression regulation. This could be because the D2-like receptors are present in both pre- and postsynaptic neurons, and is responsible for dopamine reuptake, being able to create a negative feed-back that that may inhibit dopamine neuron firing, synthesis and release.[27,37] We think that the reason why we can see the D2-like pharmacological stimulation having an increase in aggression could be due to the difference in dopamine affinity that exists between the D1-like and D2-like receptors, where the D2-like receptors have a higher affinity to dopamine.[22,26] The D2-like receptors have this way a major role in the regulation of aggressive behaviours; however during the digging task (where maintenance behaviour was more demanding) we could not see any drug effect, while the intruder task showed the same effect as control task. This suggests that, as expected, the fish behave differently depending on the social context. So, independently of the drug manipulation, the information available from the environment has to be taken into account.

During the intruder task we saw that blocking the D2-like receptors induced a significant increase in submission performed. This corroborates evidence found in other teleost fish that submissive fish, which received aggressive from dominants, had lower concentration of dopamine in the brain then dominant fish.[35] Meaning that fish that are frequently attacked by dominants experience changes in the catecholamine levels when compared to dominants, however if subordinates do not receive aggression frequently there aren't any differences in catecholamine levels between dominants and subordinates.[34] Our results point out the importance of the D2-like receptors on the regulation of submissive behaviour, and goes on the same direction of it is known of dopaminergic activity in submissive fish. Subordinates have lower concentration of dopamine[34] and that the activation of the D2-like receptors can induce a negative feed-back that regulates the D1-like receptors activity.[27] Our results suggest that by blocking the D2-like receptors and blocking the negative feed-back we may also be enhancing D1-like receptors activity increasing the usage of dopamine leading to an increase of submissive behaviour. In fact our results from D1-like receptors stimulation we can see a trend that suggests that the D1-like receptors might be involved in the regulation of submissive behaviours, however this is just an hypothesis that should be tested by injection a mixture of D2-like antagonist and D1-like agonist We think that we only saw a significant effect of the D2-like receptors pharmacological manipulation in submission during the intruder task because it where the family members need to evict the intruder, increasing aggressive behaviours in all family members. Subordinates when receiving aggression from dominants need to perform submissive behaviours in order to "stop the attack" and show this submission.

The expression of affiliative behaviour in *N.pulcher* helps to maintain the bound created, group cohesion and stabilise the hierarchy within the individuals.[33,75] Our results for affiliative behaviour show that during the control task that blocking the D2-

receptors significantly increase this behaviour. Therefore, modulating the activity of the D2-like receptors changes how the fish keep their bound within the group. We could not find this effect during the digging and intruder tasks. We hypothesize that the mechanism regulating affiliative behaviour may be similar to the one described for submission regulation. However, we were only able to find a significant increase of affiliative behaviour after blocking the D2-like receptors, during the control set up, where no social manipulation was being done. Our results suggest that affiliative behaviour occurs in a stable social environment, that is why during the digging and intruder tasks we did found any effect in affiliative behaviour, because during these tasks helpers needed to engage other behaviours such as aggression and submission to fulfil the environmental demanding's.

Contrary to what was expected according to our calibration study, we could not find any effect of the D1-like receptors in *N.pulcher*'s behaviour. The major difference between this chapter and previous is the effect of social context, which provides a direct link between D2 receptor activity and the discrimination of social context. Moreover, this could be due to difference in dopamine affinity that exists between the D1-like and D2-like receptors. The D2-like receptors have a higher affinity to dopamine[22,26] than the D1. Thus the absence of results concerning the D1-like receptors in this experiment may due to the role of the D2-like receptor in environmental information integration.

With this experiment we showed that dopamine does, in fact, modulate sociability in *Neolamprologus pulcher*, modulating how individuals interact within their families through activation and inactivation of D2-like receptors. We suggest that the D2-like receptors are key receptors to modulate sociability. We also established that dopamine and its receptors are involved the discrimination of social context and modulate the fish behaviour differently according to it. In other words, dopaminergic activity enhances different behaviours depending on the information available.[66] These informed behaviours are advantageous to the fish, in order for it to properly fulfil the family needs.

Further laboratorial and field studies are needed to continue unravelling the role of dopamine in regulation of social and cooperative behaviours.

# 3rd Chapter – Dopamine Concentration in the macro-areas of the Brain

## Introduction

In cooperative interactions, some hierarchies may be created, in which one or more individuals that are dominant in relation to others.[75] These hierarchies are usually established through agonistic interactions between two individuals. At the end of these fights, one winner and one loser usually come out, with the winner becoming dominant over the loser. [1,76] The hierarchy is then established until the next fight that challenges the dominant to take its place in the hierarchy.[75,77] A hierarchy is also observed between several individuals within a group, which means that there will be one individual dominant towards the rest of the group with the rest of the individuals creating a stairway of dominance/submission.[75] Throughout nature we can find several examples of these hierarchies in vertebrates, such as chimpanzees.[77] The fact that these hierarchies are so widely spread may underline the importance of hierarchy is in social interactions in social groups.[1]

In a social interaction it is common for hierarchies to be created. Hierarchies provide guidelines for any interaction between two organisms, dictating how they should behave. In that way, an individual's position in the hierarchy should influence brain's dynamics, having consequences on neuronal activity. Indeed, it is possible to see differential brain activity according to the social status.[35] For instance, it is known that there are differences in the dopaminergic and serotonergic activity between dominant (winner) and submissive (loser) individuals.[47] For the purpose of this study we will focus solely on brain dopaminergic activity while being aware that there are also other neurotransmitters that play a role in these social interactions and stablishing the social status for instance, serotonin.[47,78]

Dopamine it is a catecholamine that it is synthetized from its precursor the amino acid Tyrosine is hydroxylated to L-DOPA which in turn is decarboxylated to dopamine (DA). DA is then enclosed inside of presynaptic vesicle for exocytosis to the synaptic cleft. DA then activates specific dopaminergic receptors in the postsynaptic neuron or in the presynaptic neuron for the re-uptake. Following that, DA undergoes another catalysis that degrades into two metabolites, 3,4-Dihydroxyphenyl-acetic acid (DOPAC) or 3-Methoxytyramine (3-MT). Both of these metabolites are then degraded into Homovanillic acid (HVA).[79–81]

Dopamine is a crucial neurotransmitter for several basic functions of the body, such as locomotion and learning.[21,37,82] It is known that dopamine is involved in aggressive behaviour in fish and mammals.[39,83–86] It was shown by Demski and colleagues in 1971 that stimulating the preoptic region in bluegill fish *(Lepomis macrochiru*) inhibited their aggressive behaviour and induced courtship. However, stimulating the surroundings of the lateral recess triggered aggression and feeding behaviour.[87] In 1991 Winberg and colleagues performed an experiment where they analysed the concentration of 5-HT and metabolite 5-HIAA, DA and its metabolite HVA, in order to evaluate if there were any dopaminergic and serotonergic differences in the brain macro areas between dominants and submissive individuals.[35] They found that dominant individuals had an increase of HVA in the telencephalon, meaning that dominants had increased their dopaminergic activity in this particular area without having any significant increase of DA. More recently, Teles and colleagues (2013) found that in zebrafish (*Danio renio*) winning a fight and becoming dominant triggered a social reward.[47] This reward could

be seen by the increase of dopaminergic activity in the telencephalon. They also found a negative correlation between the DOPAC concentration in the Diencephalon and aggressive behaviour, showing that this brain area is involved in modulation of submissive behaviour. Submissive fish also showed an increase of dopaminergic activity in the optic tectum. Similarly, in mammals several brain nuclei, such as anterior hypothalamus, nucleus accumbens, lateral recess, are important for the regulation of several social behaviours such as aggression, submission and pair bounding. [72,87–92] It is known that in prairie voles (*Microtus ochrogaster*) the DA in the nucleus accumbens is necessary for pair boing. Aragona and colleagues saw that administrating a DA antagonist in the nucleus accumbens disrupted couples.[90,91]

Examples stated above corroborate dopamine importance in modulating social encounters and sociability. Moreover, dopamine plays a role in the social decision-making network that consists of some of the nucleus and brain regions we introduced before that are connected to each other.[68] These nuclei and the mesolimbic reward system; together they create a network that modulates social behaviour.[28,68] Thus, considering the crucial influence of the dopaminergic system on social behaviours[33,93] and cooperative behaviour[37,38] we aimed to find out more regarding its general activity in a highly social cichlid fish such as *Neolamprologus pulcher*. As presented in the previous chapters, *N. pulcher* it is a cooperatively breeding fish, living in families with a very robust size-based hierarchy, where there are very explicit multi-individual dominant-submissive interactions. Every Helper is submissive to the breeders; but a large Helper will be dominant towards a smaller Helper. In other words, the Helpers can be submissive or dominant depending on whom they are interacting with. Our goal for the present study is to try to understand the role of dopamine as a modulator of sociability, how it is distributed across brain' more relevant areas, and where it has a higher activity. For that, we compared Helpers and non-Helpers: Helpers were defined as individuals that had been accepted in a family, while non-Helpers referred to the fish that were kept in sex-based aggregation tanks. All the fishes will be in well-structured hierarchy, the core difference is that Helpers belong to a family, meaning that they having several behaioural demanding's from being Helpers. While fishes belonging to sex-based tanks do not have the same demanding's and it is not expected from them several typical Helper behaviours, such territory maintenance. We expected to see a higher dopaminergic activity in the diencephalon and see less or no differences in the forebrain.

## Materials and Methods

### Housing and experimental design

All animals used were housed at the Etologische Station Hasli of the University of Bern, Switzerland, with a light:dark cycle of 13:11 at 27°C. 10 families with 4 members each were used, each with one couple and two Helpers (one large (50-40mm SL) and one small (40-30mm SL)), and they were kept in 50L tanks with two shelters per tank. The size difference between Helpers was always between 5-10mm Standard Length.

Control fish were housed in aggregation tanks divided by sex. The aggregation tanks had fish with several sizes. These fish did not belong to a family, but nevertheless they had a size-based hierarchy between individuals. All the fish were feed 6 days per week, with commercial cichlid food (tetra).

In the present study we wanted to locate differences in dopamine and dopamine metabolite concentrations in the brain, comparing fish with a Helper status with fish that did not belong to a family and therefore could not have a Helper status. So we wanted to test Dopamine concentration in the whole and macro areas, so that we could better understand if there were any differences between Helpers and non-Helpers. For that we created 10 families that all had one breeder pair and two Helpers: one small Helper (30-40mm) and one large Helper (40-50mm). There was always a minimum size difference of 5mm between the Helpers, in order to establish a stable hierarchy. The families were kept in 50L tanks.

The non-Helpers were of the same size as the Helpers, but they did not belong to any family and they were kept in 5 same sex aggregation tanks. Because of their size difference, the non-Helper fish were also in a stable size based hierarchy. We use 20 non-Helper fish (control fish) that we removed from the 5 aggregation tank, taking care to balance out the number of males and females.

### Sampling

The 40 fish used in this experiment were sacrificed with an overdose of MS-222, after which their spinal cord was sectioned. The brain was then macro dissected with naked eye into the following five brain areas: forebrain (olfactory blobs and telencephalon), optic tectum, diencephalon, cerebellum and brain stem. After the collection the brain tissue was immediately put into dry ice and stored at -80ºC, until analysis.

### Analysis of brain Dopamine and metabolites

The frozen macro areas were homogenized in 4% ice-cold perchloric acid containing 100ng/mL of 3,4-dihydroxybenzylamine (DHBA, the internal standard), using an ultrasound sonicater. After that we immediately put it in ice. Then, we centrifuged the solution at 10000rpm at 4ºC for 10 min. The supernatant was used for High performance liquid Chromatography with electrochemical detector (HPLC-EC), analyzing DA the it's metabolites DOPAC (3,4-dihyfrophenylaceticacid) and HVA ( 4-hydroxy-3-methoxy-benzeneacetic acid).[74]

The HPLC-EC consisted of a solvent delivery as system model 582 (ESA, Bedford,MA,USA), an autoinjector Midas type 830(Spark Holland Emmen, the Netherlands), a reverse phase collum (Reprosil-Pur C18-AQ 3µm, 100mm x 4mm collum, Dr. Maisch HPLC GmbH, Ammerbuch-

Entringen, Germany) kept at 40ºC, and an ESA 5200 Coulochem IIEC detector (ESA, Bedford,MA,USA) with two electrodes reducing and oxidizing potentials of -40mV and + 320mV. Before the analytical electrodes a guarding electrode with a potential of +450mV was employed to oxidase any contaminants.

The mobile phase was a solution of 75mM sodium phosphate, 1.4nM sodium octyl sulphate and 10µM EDTA in deionized water containing 7% acetonitrile brought to pH3.1 with phosphoric acid. The samples were quantified by comparison with a standard solution made in the lab with a known concentration of study monoamines. To correct for recovery we used DHBA as an internal standard using HPLC software Clarity ™ (DataApex Ltd., Prague, Czech Reepublic).

To normalize the brain monoamine levels, brain protein weight was determined with Qubit (Thermo Fisher) , using the company's protocol.

**Statistical analysis**

The statistical analysis was performed using RStudio© Version 0.98.1091 (2009-2014 RStudio, Inc). We used linear models for our analysis, having as dependent variables one of the test monoamines (DA, DOPAC or HVA, DA usage (DOPAC+HVA/DA); as fixed factors we first used treatment (control versus family). We performed linear models for whole brain concentrations for a preliminary analysis and then we decided to analyse every brain area separately. . We have tested for Size and sex differences, for including in the model if there were significant differences.

# Results

## Whole brain analysis

Overall, no significant differences in the whole brain concentrations of DA, DOPAC and HVA were found between helpers and non-helpers (See table 4). However, when analysing the DA turn-over (the [DOPAC]+[HVA]/[DA] ratio) we found a marginally significantly less DA turn-over in helpers (p-value=0.051; See table 4; See Fig.16).

**Table 4 Whole Brain analysis results from linear models**

| | F | DF | Estimate | SE | T-value | p-value |
|---|---|---|---|---|---|---|
| **DA** | 1.636 | 197 | 1.1818 | 0.9240 | 1.279 | 0.20240 |
| **DOPAC** | 0.03016 | 197 | -0.01191 | 0.06860 | -0.174 | 0.862 |
| **HVA** | 0.1414 | 197 | -0.007353 | 0.019554 | -0.376 | 0.707 |
| **DOPAC+HVA/DA** | 3.857 | 196 | -0.14956 | 0.07615 | -1.964 | 0.051. |

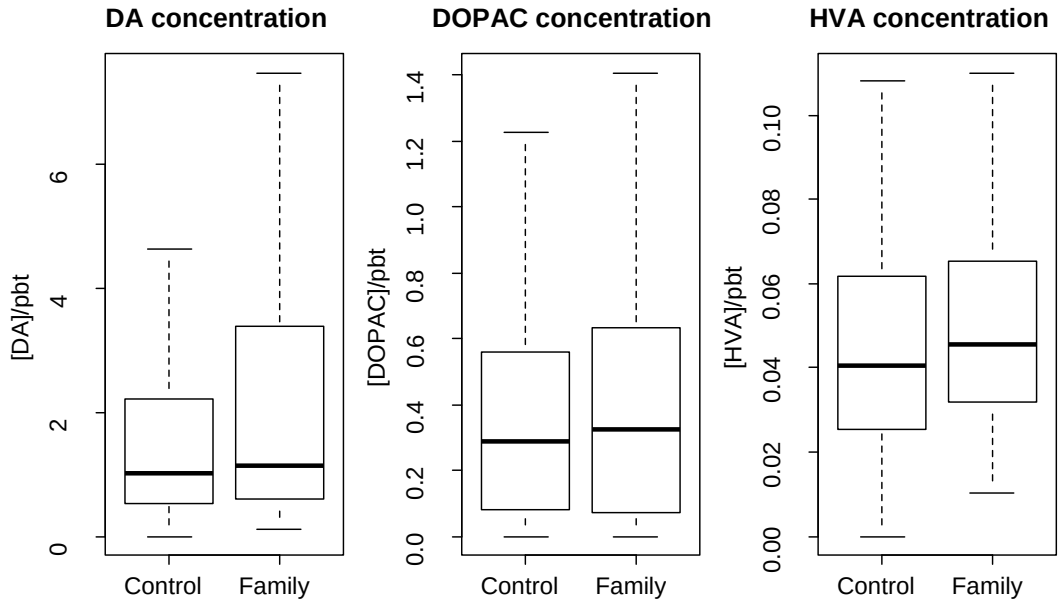


**Fig. 16 Whole brain comparison of DA, DOPAC and HVA concentration**

## Brain macro areas analysis

### *Dopamine concentration*

Regarding the concentration of dopamine (DA) across different macro areas, only in the forebrain helpers showed larger dopamine levels than control fish (non-helpers; $p<0.01$; Table5; See Fig.17). We did not find any differences in DA concentration in the other macro-areas (Table 5). We have included sex in the model concerning the cerebellum, because we found that there were significant differences between sexes. (See supplementary information: S.I.Table 5)

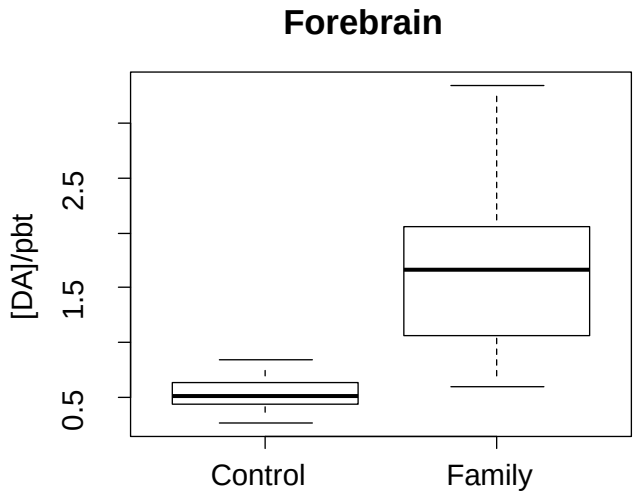


**Fig. 17 Dopamine concentration in the Forebain; yaxis- dopamine concentration ug per ug protein of brain tissue**

### *DOPAC Concentration*

For analysing the DOPAC concentration in the brain macro areas, we first tested for size and sex differences. We found sex differences in the cerebellum and size differences in the forebrain and brain stem (See supplementary information: S.I.Table5 and S.I.Table6). For further analysis we have included size or sex effects in models concerning the brain areas, such as the cerebellum and the brain stem.

Regarding the concentration of DOPAC in the different macro areas, we did not find any significant difference between Helpers and non-Helpers (see Table 5). In the Diencephalon there was a trend (p-value=0.07508; Table 5) for Helpers to have more DOPAC than non-helpers.

### *HVA Concentration*

We found Sex differences in the Cerebellum (See Supplementary information: S.I.Table 5). This result was taken into account in the model testing treatment differences in the cerebellum. The other brain areas did not show such effect.

There were no significant differences in the tested macro-areas with the exception of the Forebrain (Fig.18), where we found a significant increase of HVA in the family treatment (See Table 5).

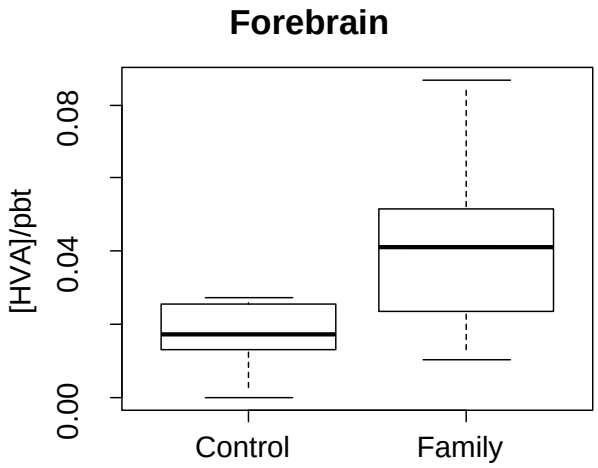


**Fig. 18 HVA concentration in the forebrain per protein of brain tissue; y axis: concentration of HVA per ug of proteinof brain tissue**

**Table 5 Dopamine and metabolites concentration (mean) in different brain areas in Helpers and non-Helpers and statistical results from linear models.**

| Brain area | Cathecolamines | Non-Helpers (ug/pbt) | Helpers (ug/pbt) | F | DF | Estimate | SE | T-value | p-value |
|---|---|---|---|---|---|---|---|---|---|
| **Forebrain** | DA | 0.89 | 1.25 | 7.759 | 38 | 0.9203 | 0.3304 | 2.786 | 0.00829* |
| | DOPAC# | 0.42 | 0.43 | 0.6506 | 36 | 0.24122 | 0.22261 | 1.084 | 0.2858 |
| | HVA | 0.02 | 0.03 | 9.134 | 38 | 0.017779 | 0.005883 | 3.022 | 0.00447* |
| **Diencephalon** | DA | 1.63 | 1.26 | 1.733 | 37 | -1.1239 | 0.8538 | -1.316 | 0.19614 |
| | DOPAC | 0.16 | 0.23 | 3.354 | 37 | 0.12769 | 0.06972 | 1.832 | 0.07508. |
| | HVA | 0.12 | 0.10 | 0.7537 | 37 | -0.08042 | 0.09263 | -0.868 | 0.391 |
| **Optic Tectum** | DA | 3.45 | 3.60 | 1.591 | 38 | 5.439 | 4.313 | 1.261 | 0.215 |
| | DOPAC | 0.26 | 0.19 | 1.791 | 38 | 0.15273 | 0.11414 | 1.338 | 0.1888 |
| | HVA | 0.09 | 0.09 | 0.8305 | 38 | 0.03018 | 0.03312 | 0.911 | 0.36788 |
| **Cerebellum** | DA+ | 1.04 | 1.11 | 2.208 | 36 | -0.11420 | 0.23317 | -0.49 | 0.6273 |
| | DOPAC+ | 1.03 | 0.54 | 10.05 | 36 | -0.2813 | 0.2000 | -1.406 | 0.168306 |
| | HVA# | 0.06 | 0.06 | 0.9461 | 36 | -0.017896 | 0.011288 | -1.585 | 0.122 |
| **Brain Stem** | DA | 4.25 | 4.47 | 0.7524 | 38 | 0.5169 | 0.5959 | 0.867 | 0.391 |
| | DOPAC# | 0.56 | 0.72 | 3.012 | 36 | 0.14986 | 0.12749 | 1.176 | 0.2475 |
| | HVA | 0.07 | 0.07 | 0.7537 | 37 | 0.004926 | 0.008267 | 0.596 | 0.555 |

### *Ratio of dopamine turn-over*

A significant difference was found regarding dopamine consumption in the Forebrain (p-value<0.001; Table 6) and Cerebellum (p-value<0.001; Table 6). We found a higher dopamine consumption in the Diencephalon (p-value<0.01;Table 6), but no differences in the Brain Stem (p-value=0.20014; Table 6) and in the Optic tectum (p-value=0.1329;Table 6).

**Table 6 Dopamine turn-over (DOPAC+HVA/DA) statistical results from linear model; * p-value<0.05**

| Brain area | Estimate | SE | T-value | p-value |
|---|---|---|---|---|
| **Forebrain** | -0.40125 | 0.09749 | -4.116 | 2e-04* |
| **Diencephalon** | 0.37035 | 0.13113 | 2.824 | 0.00767* |
| **Optic Tectum** | 0.09169 | 0.05970 | 1.536 | 0.1329 |
| **Cerebellum** | -1.1505 | 0.3082 | -3.733 | 0.000651 * |
| **Brain Stem** | 0.10486 | 0.08042 | 1.304 | 0.20014 |

## Discussion

In this study, all the fish used for this experiment were living in well-established hierarchies, (even the fishes from the control group). However, our results showed that there are significant differences in the dopaminergic activity when the fishes are living in established hierarchies in a family-context compared to same-sex groups. Indeed, we found differences in the dopaminergic activity between Helpers and non-Helpers in several crucial brain areas, such as the Forebrain, the Diencephalon, the Cerebellum and Brain stem. Our results suggest that living in a family-context changes the dopaminergic activity of the brain.

In order to discuss these results, we need to take into consideration the behavioural profile of a Helper. A Helper belongs to a family that include a dominant breeding pair, that signal their dominance by performing aggressive behaviour to create a so-called hierarchy or family.[94,95] In that family, size difference between those more submissive (Helpers) may produce status variability between Helpers, i.e. a larger Helper may become dominant towards an smaller Helper. [95] Therefore, the Helper status is more than a standard dominant-submissive formation. Generally in families you have to create a multi-individual hierarchy, where some individuals switch between dominance and submission depending on whom they are interacting with. [94] This requires to family members in general and *N.pulcher* in particular, having a very precise system to perceive and integrate information, in order to know to whom they must behave submissively and to whom they need to show dominance.[5,56,74]

We first compared whole brain DA, DOPAC and HVA concentration (between treatments), but no significant differences were found. Interestingly, when analysing the ratio of DA usage, we found a decreasing trend suggesting that Helpers might be using less DA overall. When analysing sex and size differences we found that, in the cerebellum there were sex differences in the DA's and DOPAC's concentration in the brain, while HVA's concentration had a size effect. Also, while analysing size effects we also found size effect the DOPAC's concentration in the Forebrain and Brain Stem. These differences were found in both treatments (Helpers and Non-Helpers) (See supplementary information. S.I.Table 5; S.I.Table 6). We had these effects into account for our analysis by including this effect in the model used for these brain areas.

Our results concerning the Forebrain show that DA's and HVA's concentration is significantly higher in Helpers. We also found that Helpers use DA significantly less than non-Helpers. This decrease of dopaminergic activity seems somehow contradictory to the significant increase of HVA in the Helpers' forebrain. Although the concentration of HVA increased significantly in the Helpers' forebrain, its concentration is still very small in comparison to the DOPAC concentration. Interestingly, Helpers had significantly higher levels of DA in the Forebrain, but seemed to have lower levels of dopaminergic activity in the Forebrain. It is possible that in Helpers DA is accumulated in this area. It is known that dominant individuals have an increase of DA activity in the Telencephalon when compared to submissive individuals. This can also be seen in the concentration of HVA.[35] The increase of DA activity in Telencephalon is seen as a social reward that the dominants receive from reaching that status.[47]

In our study we also observed an increase of DA and HVA in Helpers' Forebrain, but not in usage of DA. These results might suggest that Helpers receive a social reward from being a

Helper, as fishes belonging to a family have more dopamine comparatively to fishes living alone. This pattern in Helpers' brains is similar to the one found in dominant Arctic char.[35]

Significant differences were also found on the Diencephalon between Helpers and non-Helpers. We saw a trend in DOPAC's concentration in the Helpers' diencephalon, were Helpers have more DOPAC however there is no significant difference when compared to the control (non-Helpers). These results go in the same direction than Teles et al., showing that the putative submission experienced by the Helpers may lead to a higher dopaminergic activity in the diencephalon.[47] Moreover, a positive correlation between submissive behaviour and DA concentration on the Diencephalon has been shown in zebra fish (*Danio renio*). The Diencephalon has several nuclei capable of modulating aggression, such as the pre-optic area and the lateral recess.[96] It has been shown in golden hamsters that the anterior hypothalamus and the nucleus accumbens are implicated in the regulation of aggression[92,97] and formation of pair bounding[91]. We did not found any significant difference in the concentration of DA or HVA in the Diencephalon. When analysing this ratio of DA usage, we saw that Helpers have a higher dopaminergic activity in the Diencephalon, meaning that Helpers have a higher dopaminergic activity in the Diencephalon than Non-Helpers. These results corroborate with the results from Teles et al., where submissive individuals (losers) also had an increase of dopaminergic activity in the diencephalon.[47]

We did not found any significant differences in DA, DOPAC or HVA's concentration in the Optic Tectum. It could be that generally, when it came to visual cues there was no difference between the two treatments. Indeed. Fishes from both treatments, lived in well-structured hierarchies, the core difference between the treatments was the absence or not of a family.

We did not find any significant differences on the Cerebellum concerning the concentration of the study cathecolamines (DA, DOPAC and HVA). Separately there was no difference between Helpers and non-Helper in the Cerebellum, however when analysing the ratio of DA's usage we can see that there is a significant difference, which means that the Helpers have less dopaminergic activity in the cerebellum when compared to the control. This difference may be due to a difference in the concentration of DOPAC that when separately analysed does not show us a significant decrease, but we can see from raw data that Helpers have less DOPAC in their cerebellum (See table 5). Also, it is known that the cerebellum is involved in learning[98] , such as spatial-learning.[99] The Cerebellum is also involved in integration of motor information[100] , taking this information into account it makes sense that we did not found any significant difference between Helpers and non-Helpers because their environments did not had any difference that could have an effect on their motor skills.

In conclusion, becoming a helper in a family seems to affect the general activity in the dopaminergic system of *N. pulcher*. It might be that group membership is rewarding, which could prevent helpers from dispersing. Further laboratorial and field studies are needed to understand the role of the DA system in this complex cooperative system.

# General Discussion and Final Remarks: The influence of environmental change to dopaminergic activity

Throughout this thesis we have been presenting our work on the role of dopamine for behavioural regulation in a cooperative breeding fish. After analysis the results of the experiments, we have a better knowledge on how dopamine is regulating social interactions, how it is involved in the integration of environmental information and which brain areas dopamine seems to take action on. First we discovered that the D2-like receptors have key role on the regulation of aggression, submission and affiliative behaviour in *N.pulcher*, and in social context information. Indeed, by changing N.pulcher's social environment while pharmacologically manipulating their dopaminergic activity the importance of D2-like receptors on N.pulcher's behaviour (Aggression, submission and affiliative e behaviour). We saw that depending on the social context of the focal helper the behavioural output could differ. This implies that is via putative changes in D2-like receptors that these animals are able to discriminate social environments and adjust behaviour according to the received social information.

In the second part of this thesis, we have shown that dopamine concentration in a helper's brain differs from a non-helper fish. The analysis showed no significant differences in the concentration of dopamine (DA), DOPAC and HVA in whole brain, between helpers and non-helpers. When looking at the concentration of DA, DOPAC and HVA in the brain macro areas, we found that the forebrain of the helper fish showed a significant higher DA concentration than non-helpers. Similarly, the Brain Stem and the diencephalon of the helpers showed a significant higher concentration of DOPAC. The analysis of the HVA concentration we found that helpers have a significantly higher concentration then non-helpers. In sum, these results lead us to think that there are two brain areas where the dopaminergic activity of N.pulcher is most relevant in terms of family living, the diencephalon and the forebrain. For example, in the diencephalon several brain nuclei are involved in social behaviours – such as the preoptic nucleus, the nucleus accumbens and the anterior hypothalamus[92,96,97] – which is coherent with the diencephalon having an important role in N.pulcher's sociability. As Teles and colleagues have showed in 2013 a higher dopaminergic activity in the telencephalon area can be correlated to the presence of a reward.[47] This could suggest that our results concerning dopaminergic activity in the forebrain (olfactory bulb and telencephalon) for helper fish indicate the presence of a reward. In that case, the N.pulcher helper fish would receive a reward from being in a family and fulfilling the requisite tasks of their hierarchy status. If that were to be true, the helping behaviour in Neolamprolus pulcher would be the result of direct reciprocity between helpers and breeders and not an altruistic behaviour from the helpers. To confirm this hypothesis, more studies would need to be done in the laboratory and in the field.

Finally, further experiments are needed to continue unveiling the importance of dopamine in social behaviours and cooperation, particularly in N pulcher. Further studies should be done to establish how the D1-like are and D2-like receptors are distributed in the brain, and what is the relation that these two clades of dopamine receptors have in social behaviour regulation In the future the study of dopaminergic activity in N.pulcher's brain should be conducted in order to understand which nuclei from the forebrain and diencephalon might be responsible for behavioural regulation.

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# Supplementary information: 1st Chapter

**S.I.Table 1 D1-like agonist results; . - Trend, * p-value<0.05, ** p-value<0.01**

| Drug | Behaviour | Time (min) | Dosage (mg/gbw) | Estimate | Std. Error | df | T Value | P-value |
|---|---|---|---|---|---|---|---|---|
| **SKF-38393** | Aggression | 15 | 0.5 | 0.7555 | 0.3653 | 21.0000 | 2.068 | 0.0512 |
| **D1-like agonist** | | | 2.5 | 0.6761 | 0.3653 | 21.0000 | 1.851 | 0.0784 |
| | | | 5 | 0.7062 | 0.3653 | 21.0000 | 1.933 | 0.0668 |
| | | 30 | 0.5 | 0.7220 | 0.3495 | 21.0000 | 2.066 | 0.0514 . |
| | | | 2.5 | 0.6796 | 0.3495 | 21.0000 | 1.944 | 0.0654 |
| | | | 5 | 0.7836 | 0.3495 | 21.0000 | 2.242 | 0.0359 * |
| | | 60 | 0.5 | 0.6415 | 0.3712 | 28.0000 | 1.728 | 0.0950 . |
| | | | 2.5 | 0.3493 | 0.3712 | 28.0000 | 0.941 | 0.3547 |
| | | | 5 | 0.7177 | 0.3712 | 28.0000 | 1.934 | 0.0633 |
| | Submission | 15 | 0.5 | 0.2946 | 0.2387 | 28.0000 | 1.234 | 0.2274 |
| | | | 2.5 | 0.5723 | 0.2387 | 28.0000 | 2.397 | 0.0234 * |
| | | | 5 | 0.5354 | 0.2387 | 28.0000 | 2.243 | 0.0330 |
| | | 30 | 0.5 | 0.3134 | 0.2131 | 28.0000 | 1.471 | 0.153 |
| | | | 2.5 | 0.2275 | 0.2131 | 28.0000 | 1.067 | 0.295 |
| | | | 5 | 0.1417 | 0.2131 | 28.0000 | 0.665 | 0.511 |
| | | 60 | 0.5 | 0.3118 | 0.2087 | 28.0000 | 1.494 | 0.146 |
| | | | 2.5 | 0.2650 | 0.2087 | 28.0000 | 1.270 | 0.215 |
| | | | 5 | 0.1709 | 0.2087 | 28.0000 | 0.819 | 0.420 |
| | Affiliative | 15 | 0.5 | 0.4338 | 0.2648 | 28.0000 | 1.638 | 0.113 |
| | | | 2.5 | 0.4329 | 0.2648 | 28.0000 | 1.635 | 0.113 |
| | | | 5 | 0.2899 | 0.2648 | 28.0000 | 1.095 | 0.283 |
| | | 30 | 0.5 | 0.3037 | 0.2469 | 21.0000 | 1.230 | 0.232 |
| | | | 2.5 | 0.3535 | 0.2469 | 21.0000 | 1.432 | 0.167 |
| | | | 5 | 0.3546 | 0.2469 | 21.0000 | 1.436 | 0.166 |
| | | 60 | 0.5 | 0.3969 | 0.2358 | 21.0000 | 1.683 | 0.1071 |
| | | | 2.5 | 0.4310 | 0.2358 | 21.0000 | 1.828 | 0.0818 . |
| | | | 5 | 0.4316 | 0.2358 | 21.0000 | 1.830 | 0.0814 |

**S.I.Table 2 D1-like antagonist results; . - Trend, * p-value<0.05, ** p-value<0.01**

| Drug | Behaviour | Time (min) | Dosage (mg/gbw) | Estimate | Std. Error | df | T Value | P-value |
|---|---|---|---|---|---|---|---|---|
| **SCH-23390** | Aggression | 15 | 0.1 | -0.2271 | 0.2540 | 21.2750 | -0.894 | 0.3814 |
| **D1-like antagonist** | | | 0.5 | -0.6080 | 0.2504 | 21.0330 | -2.428 | 0.0243 * |
| | | | 1.5 | -0.2688 | 0.2504 | 21.0330 | -1.073 | 0.2954 |
| | | 30 | 0.1 | -0.1614 | 0.2800 | 21.3320 | -0.577 | 0.5703 |
| | | | 0.5 | -0.5569 | 0.2762 | 21.0420 | -2.016 | 0.0567 . |
| | | | 1.5 | -0.3235 | 0.2762 | 21.0420 | -1.171 | 0.2546 |
| | | 60 | 0.1 | -0.3831 | 0.3024 | 21.4210 | -1.267 | 0.2187 |
| | | | 0.5 | -0.5435 | 0.2985 | 21.0800 | -1.821 | 0.0828 . |
| | | | 1.5 | -0.3285 | 0.2985 | 21.0800 | -1.101 | 0.2835 |
| | Submission | 15 | 0.1 | 0.5752 | 0.2380 | 28.0000 | 2.417 | 0.0224 * |
| | | | 0.5 | 0.2092 | 0.2380 | 28.0000 | 0.879 | 0.3869 |
| | | | 1.5 | 0.3563 | 0.2380 | 28.0000 | 1.497 | 0.1456 |
| | | 30 | 0.1 | 0.6592 | 0.2806 | 21.7520 | 2.350 | 0.02830 * |
| | | | 0.5 | 0.6582 | 0.2782 | 21.0810 | 2.366 | 0.02762 * |
| | | | 1.5 | 0.7958 | 0.2782 | 21.0810 | 2.861 | 0.00933 ** |
| | | 60 | 0.1 | 0.5129 | 0.2398 | 21.6810 | 2.139 | 0.0439 * |
| | | | 0.5 | 0.5249 | 0.2375 | 21.0630 | 2.210 | 0.0383 * |
| | | | 1.5 | 0.3422 | 0.2375 | 21.0630 | 1.441 | 0.1644 |
| | Affiliative | 15 | 0.1 | 0.5576 | 0.2505 | 21.8250 | 2.226 | 0.0366 * |
| | | | 0.5 | 0.3310 | 0.2487 | 21.0270 | 1.331 | 0.1975 |
| | | | 1.5 | 0.1790 | 0.2487 | 21.0270 | 0.720 | 0.4797 |
| | | 30 | 0.1 | 0.4107 | 0.3624 | 28.0000 | 1.133 | 0.267 |
| | | | 0.5 | 0.1818 | 0.3624 | 28.0000 | 0.502 | 0.620 |
| | | | 1.5 | -0.1040 | 0.3624 | 28.0000 | -0.287 | 0.776 |
| | | 60 | 0.1 | 0.27325 | 0.33401 | 28.00000 | 0.818 | 0.420 |
| | | | 0.5 | -0.06934 | 0.33401 | 28.00000 | -0.208 | 0.837 |
| | | | 1.5 | -0.10439 | 0.33401 | 28.00000 | -0.313 | 0.757 |

**S.I.Table 3 D2-like agonist results; . Trend, *p-value<0.05, ** p-value<0.01**

| Drug | Behaviour | Time (min) | Dosage (mg/gbw) | Estimate | Std. Error | df | T Value | P-value |
|---|---|---|---|---|---|---|---|---|
| **Quinpirole** | Aggression | 15 | 0.5 | 0.5795 | 0.3662 | 21.0000 | 1.582 | 0.1285 |
| **D2-like agonist** | | | 2 | 0.6660 | 0.3662 | 21.0000 | 1.819 | 0.0833 . |
| | | | 3.5 | 0.3921 | 0.3662 | 21.0000 | 1.071 | 0.2965 |
| | | 30 | 0.5 | 0.37686 | 0.37688 | 21.00000 | 1.000 | 0.329 |
| | | | 2 | 0.41232 | 0.37688 | 21.00000 | 1.094 | 0.286 |
| | | | 3.5 | -0.05535 | 0.37688 | 21.00000 | -0.147 | 0.885 |
| | | 60 | 0.5 | 0.6067 | 0.3709 | 21.0000 | 1.636 | 0.117 |
| | | | 2 | 0.5847 | 0.3709 | 21.0000 | 1.577 | 0.130 |
| | | | 3.5 | 0.2124 | 0.3709 | 21.0000 | 0.573 | 0.573 |
| | Submission | 15 | 0.5 | 0.18448 | 0.23444 | 21.00000 | 0.787 | 0.440 |
| | | | 2 | 0.02943 | 0.23444 | 21.00000 | 0.126 | 0.901 |
| | | | 3.5 | -0.29325 | 0.23444 | 21.00000 | -1.251 | 0.225 |
| | | 30 | 0.5 | -0.2248 | 0.2701 | 21.0000 | -0.832 | 0.415 |
| | | | 2 | -0.2057 | 0.2701 | 21.0000 | -0.762 | 0.455 |
| | | | 3.5 | -0.2790 | 0.2701 | 21.0000 | -1.033 | 0.313 |
| | | 60 | 0.5 | 0.01685 | 0.19269 | 21.00000 | 0.087 | 0.931 |
| | | | 2 | 0.05216 | 0.19269 | 21.00000 | 0.271 | 0.789 |
| | | | 3.5 | -0.21685 | 0.19269 | 21.00000 | -1.125 | 0.273 |
| | Affiliative | 15 | 0.5 | 0.5081 | 0.2210 | 21.0000 | 2.299 | 0.0319 * |
| | | | 2 | 0.3967 | 0.2210 | 21.0000 | 1.795 | 0.0871 . |
| | | | 3.5 | 0.3967 | 0.2210 | 21.0000 | 1.795 | 0.0871 . |
| | | 30 | 0.5 | 0.33429 | 0.25364 | 21.00000 | 1.318 | 0.202 |
| | | | 2 | -0.07766 | 0.25364 | 21.00000 | -0.306 | 0.762 |
| | | | 3.5 | 0.24081 | 0.25364 | 21.00000 | 0.949 | 0.353 |
| | | 60 | 0.5 | 0.2652 | 0.2524 | 21.0000 | 1.051 | 0.305 |
| | | | 2 | -0.1538 | 0.2524 | 21.0000 | -0.609 | 0.549 |
| | | | 3.5 | 0.1671 | 0.2524 | 21.0000 | 0.662 | 0.515 |

**S.I.Table 4 D2-like antagonis results; . Trend, * p-value<0.05, ** p-value<0.01**

| Drug | Behaviour | Time (min) | Dosage (mg/gbw) | Estimate | Std. Error | df | T Value | P-value |
|---|---|---|---|---|---|---|---|---|
| **Metaclopramide** | Aggression | 15 | 0.5 | 0.1906 | 0.3700 | 21.0000 | 0.515 | 0.612 |
| **D2-like antagonist** | | | 2.5 | 0.2658 | 0.3700 | 21.0000 | 0.718 | 0.480 |
| | | | 5 | -0.4296 | 0.3700 | 21.0000 | -1.161 | 0.259 |
| | | 30 | 0.5 | 0.2366 | 0.3715 | 21.0000 | 0.637 | 0.531 |
| | | | 2.5 | 0.2498 | 0.3715 | 21.0000 | 0.672 | 0.509 |
| | | | 5 | -0.4678 | 0.3715 | 21.0000 | -1.259 | 0.222 |
| | | 60 | 0.5 | 0.5643 | 0.4196 | 21.0000 | 1.345 | 0.193 |
| | | | 2.5 | 0.7096 | 0.4196 | 21.0000 | 1.691 | 0.106 |
| | | | 5 | 0.2195 | 0.4196 | 21.0000 | 0.523 | 0.606 |
| | Submission | 15 | 0.5 | -0.005906 | 0.210946 | 21.000000 | -0.028 | 0.978 |
| | | | 2.5 | -0.052606 | 0.210946 | 21.000000 | -0.249 | 0.805 |
| | | | 5 | 0.172807 | 0.210946 | 21.000000 | 0.819 | 0.422 |
| | | 30 | 0.5 | -0.32269 | 0.25502 | 28.00000 | -1.265 | 0.216 |
| | | | 2.5 | -0.22962 | 0.25502 | 28.00000 | -0.900 | 0.376 |
| | | | 5 | 0.08967 | 0.25502 | 28.00000 | 0.352 | 0.728 |
| | | 60 | 0.5 | -0.23356 | 0.14666 | 21.00000 | -1.593 | 0.126 |
| | | | 2.5 | 0.03103 | 0.14666 | 21.00000 | 0.212 | 0.834 |
| | | | 5 | 0.16943 | 0.14666 | 21.00000 | 1.155 | 0.261 |
| | Affiliative | 15 | 0.5 | 0.5560 | 0.2214 | 21.0000 | 2.512 | 0.0203 * |
| | | | 2.5 | 0.4934 | 0.2214 | 21.0000 | 2.229 | 0.0369 * |
| | | | 5 | 0.4307 | 0.2214 | 21.0000 | 1.946 | 0.0652 . |
| | | 30 | 0.5 | 0.7482 | 0.2548 | 28.0000 | 2.936 | 0.00657 ** |
| | | | 2.5 | 0.7640 | 0.2548 | 28.0000 | 2.998 | 0.00564 ** |
| | | | 5 | 0.5891 | 0.2548 | 28.0000 | 2.312 | 0.02836 * |
| | | 60 | 0.5 | 0.5297 | 0.2549 | 21.0000 | 2.078 | 0.0502 . |
| | | | 2.5 | 0.5768 | 0.2549 | 21.0000 | 2.263 | 0.0343 * |
| | | | 5 | 0.3441 | 0.2549 | 21.0000 | 1.350 | 0.1913 |

# Supplementary information: 3rd Chapter

**S.I.Table 5 DA, DOPAC and HVA concentration sex differences results; . Trend, * p-value<0.05, ** p-value<0.01**

| Treatment | Cathecolamines | Brain Area | F | DF | Estimate | SE | t value | p-value |
|---|---|---|---|---|---|---|---|---|
| **Helpers** | DA | Forebrain | 0.4893 | 18 | 0.3249 | 0.4646 | 0.699 | 0.493 |
| | | Diencephalon | 0.003114 | 17 | 0.01194 | 0.21394 | 0.056 | 0.956 |
| | | Optic Tectum | 0.6526 | 18 | -8.694 | 10.762 | -0.808 | 0.4297 |
| | | Cerebellum | 7.663 | 18 | 0.8713 | 0.3148 | 2.768 | 0.0127 * |
| | | Brainstem | 0.2245 | 18 | -0.6285 | 1.3266 | -0.474 | 0.641 |
| | DOPAC | Forebrain | 0.04254 | 18 | -0.07482 | 0.36276 | -0.206 | 0.83892 |
| | | Diencephalon | 0.9856 | 17 | -0.15559 | 0.15672 | -0.993 | 0.33474 |
| | | Optic Tectum | 1.085 | 18 | -0.2690 | 0.2582 | -1.042 | 0.31137 |
| | | Cerebellum | 0.002789 | 18 | 0.01374 | 0.26016 | 0.053 | 0.95846 |
| | | Brainstem | 2.919 | 18 | -0.29924 | 0.17516 | -1.708 | 0.105 |
| | HVA | Forebrain | 0.1337 | 18 | 0.004062 | 0.011110 | 0.366 | 0.719 |
| | | Diencephalon | 0.6259 | 17 | -0.01882 | 0.02379 | -0.791 | 0.439755 |
| | | Optic Tectum | 1.913 | 18 | -0.08524 | 0.06162 | -1.383 | 0.183494 |
| | | Cerebellum | 2.676 | 18 | 0.015854 | 0.009691 | 1.636 | 0.119 |
| | | Brainstem | 0.7507 | 18 | -0.012895 | 0.014884 | -0.866 | 0.398 |
| | DOPAC+HVA /DA | Forebrain | 0.1505 | 18 | 0.04961 | 0.12787 | 0.388 | 0.702572 |
| | | Diencephalon | 0.7487 | 17 | -0.2584 | 0.2986 | -0.865 | 0.399 |
| | | Optic Tectum | 0.1859 | 18 | 0.06403 | 0.14851 | 0.431 | 0.6715 |
| | | Cerebellum | 0.1391 | 18 | -0.09977 | 0.26753 | -0.373 | 0.71354 |
| | | Brainstem | 0.3805 | 18 | -0.12144 | 0.19685 | -0.617 | 0.54504 |
| **Non-helpers** | DA | Forebrain | 0.6256 | 18 | -0.4482 | 0.5667 | -0.791 | 0.4393 |
| | | Diencephalon | 0.6505 | 18 | -1.375 | 1.705 | -0.807 | 0.4305 |
| | | Optic Tectum | 0.03234 | 18 | 0.2411 | 1.3405 | 0.180 | 0.8593 |
| | | Cerebellum | 0.0773 | 18 | 0.08308 | 0.29882 | 0.278 | 0.784 |
| | | Brainstem | 0.3993 | 18 | -0.3822 | 0.6049 | -0.632 | 0.535 |
| | DOPAC | Forebrain | 0.04548 | 18 | 0.03010 | 0.14114 | 0.213 | 0.833518 |
| | | Diencephalon | 4.178 | 18 | 0.11842 | 0.05794 | 2.044 | 0.05588 . |
| | | Optic Tectum | 0.05596 | 18 | -0.02382 | 0.10070 | -0.237 | 0.816 |
| | | Cerebellum | 12.28 | 18 | 0.9221 | 0.2631 | 3.505 | 0.00253 * |
| | | Brainstem | 0.02246 | 18 | 0.01739 | 0.11607 | 0.150 | 0.883 |
| | HVA | Forebrain | 0.4127 | 18 | -0.00529 | 0.008231 | -0.642 | 0.528715 |
| | | Diencephalon | 0.6679 | 18 | -0.1511 | 0.1849 | -0.817 | 0.424 |
| | | Optic Tectum | 0.0004351 | 18 | 0.0009382 | 0.0449790 | 0.021 | 0.9836 |
| | | Cerebellum | 0.4761 | 18 | -0.01198 | 0.01736 | -0.690 | 0.499 |
| | | Brainstem | 2.992 | 18 | -0.019404 | 0.011218 | -1.73 | 0.101 |
| | DOPAC+HVA /DA | Forebrain | 2.827 | 18 | 0.2744 | 0.1632 | 1.681 | 0.11 |
| | | Diencephalon | 0.9738 | 17 | 0.10155 | 0.10291 | 0.987 | 0.33757 |
| | | Optic Tectum | 0.8146 | 18 | 0.02532 | 0.02805 | 0.903 | 0.379 |
| | | Cerebellum | 1.521 | 18 | 0.5072 | 0.4113 | 1.233 | 0.233364 |
| | | Brainstem | 0.8175 | 18 | 0.04019 | 0.04445 | 0.904 | 0.378 |

**S.I.Table 6 DA, DOPACC and HVA concentration size differences results; - Trend, * p-value<0.05, ** p-value<0.01**

| Treatment | Cathecolamines | Brain Area | F | DF | Estimate | SE | t value | p-value |
|---|---|---|---|---|---|---|---|---|
| **Helpers** | DA | Forebrain | 0.05658 | 18 | 0.08946 | 0.37607 | 0.238 | 0.815 |
| | | Diencephalon | 0.3085 | 17 | 0.09616 | 0.17313 | 0.555 | 0.585832 |
| | | Optic Tectum | 0.7025 | 18 | 7.207 | 8.598 | 0.838 | 0.413 |
| | | Cerebellum | 2.115 | 18 | 0.4136 | 0.2844 | 1.454 | 0.163122 |
| | | Brainstem | 0.0907 | 18 | 0.3208 | 1.0652 | 0.301 | 0.767 |
| | DOPAC | Forebrain | 1.003 | 18 | 0.2832 | 0.2828 | 1.002 | 0.330 |
| | | Diencephalon | 0.223 | 17 | 0.06175 | 0.13077 | 0.472 | 0.6428 |
| | | Optic Tectum | 0.2586 | 18 | -0.1074 | 0.2112 | -0.509 | 0.6173 |
| | | Cerebellum | 0.9976 | 18 | 0.2024 | 0.2026 | 0.999 | 0.3311 |
| | | Brainstem | 0.4528 | 18 | 0.1004 | 0.1492 | 0.673 | 0.51 |
| | HVA | Forebrain | 2.076 | 18 | 0.012171 | 0.008447 | 1.441 | 0.167 |
| | | Diencephalon | 0.2342 | 17 | -0.00950 | 0.019643 | -0.484 | 0.63463 |
| | | Optic Tectum | 0.001197 | 18 | -0.00179 | 0.051852 | -0.035 | 0.9728 |
| | | Cerebellum | 10.13 | 18 | 0.021156 | 0.006647 | 3.183 | 0.00515 * |
| | | Brainstem | 2.269 | 18 | 0.017250 | 0.011452 | 1.506 | 0.149 |
| | DOPAC+HVA/ DA | Forebrain | 2.524 | 18 | 0.15284 | 0.09620 | 1.589 | 0.12952 |
| | | Diencephalon | 0.1603 | 17 | -0.09927 | 0.24798 | -0.400 | 0.693910 |
| | | Optic Tectum | 2.959 | 18 | -0.19037 | 0.11067 | -1.72 | 0.10255 |
| | | Cerebellum | 0.9836 | 18 | 0.2075 | 0.2092 | 0.992 | 0.3345 |
| | | Brainstem | 0.7189 | 18 | 0.1323 | 0.1561 | 0.848 | 0.4076 |
| **Non-helpers** | DA | Forebrain | 0.7225 | 18 | -0.4707 | 0.5538 | -0.850 | 0.4065 |
| | | Diencephalon | 0.4202 | 18 | -1.090 | 1.681 | -0.648 | 0.525 |
| | | Optic Tectum | 0.01179 | 18 | 0.1427 | 1.3142 | 0.109 | 0.9148 |
| | | Cerebellum | 0.1218 | 18 | 0.1021 | 0.2924 | 0.349 | 0.731135 |
| | | Brainstem | 0.108 | 18 | -0.1963 | 0.5974 | -0.329 | 0.746 |
| | DOPAC | Forebrain | 0.02031 | 18 | 0.01972 | 0.13838 | 0.143 | 0.88825 |
| | | Diencephalon | 5.098 | 18 | 0.12560 | 0.05563 | 2.258 | 0.0366 * |
| | | Optic Tectum | 0.3481 | 18 | 0.05775 | 0.09788 | 0.590 | 0.5625 |
| | | Cerebellum | 0.3083 | 18 | -0.1841 | 0.3315 | -0.555 | 0.585577 |
| | | Brainstem | 4.748 | 18 | -0.22056 | 0.10123 | -2.179 | 0.0429 * |
| | HVA | Forebrain | 3.293 | 18 | -0.01361 | 0.007499 | -1.815 | 0.0863 |
| | | Diencephalon | 0.8116 | 18 | -0.1625 | 0.1804 | -0.901 | 0.380 |
| | | Optic Tectum | 0.02804 | 18 | 0.007373 | 0.044036 | 0.167 | 0.8689 |
| | | Cerebellum | 0.09555 | 18 | 0.005313 | 0.017189 | 0.309 | 0.76078 |
| | | Brainstem | 0.001784 | 18 | -0.00050 | 0.0118690 | -0.042 | 0.967 |
| | DOPAC+HVA/ DA | Forebrain | 0.6319 | 18 | 0.1344 | 0.1690 | 0.795 | 0.437 |
| | | Diencephalon | 0.008745 | 17 | 0.009782 | 0.104604 | 0.094 | 0.92659 |
| | | Optic Tectum | 0.004277 | 18 | -0.00184 | 0.028094 | -0.065 | 0.949 |
| | | Cerebellum | 3.682 | 18 | -0.7336 | 0.3823 | -1.919 | 0.071 . |
| | | Brainstem | 0.4062 | 18 | -0.02807 | 0.04404 | -0.637 | 0.532 |