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Effect of social isolation on aggressive behaviour of fish

Master's thesis

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Declaration

I hereby declare that this thesis entitled “Effect of social isolation on aggressive behaviour of fish” is my own work and all the sources have been quoted and acknowledged by means of complete references.

In Prague (26th of April 2017)

.....

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Abstract

The effects of social isolation on the agonistic encounters of males of *Betta splendens* were investigated. The literature review of this thesis focused mainly on the mechanisms of aggressive behaviour modulation by various levels of isolation from social networks. Also the effects of exposition to different sexes in social environment prior to agonistic encounters with conspecifics were summarized. Methods from the stated researches were considered in this thesis' experiment design.

58 male individuals of were housed individually in transparent boxes. They were divided into three groups according to the level of social isolation: socialized with male individuals ("M-socialized" males), socialized with female individuals ("F-socialized" males and fully isolated males ("Isolates"). The experiment took place two times with different durations to compare the effects of long-termed (6 weeks) and short-termed (3 weeks) isolation. Video-recording of every subject's reaction to mirror was carried out after both experiments. Despite having hypothesised, that isolation will result in increased aggressiveness, no significant ($p>0.05$) effect of treatment was noted after evaluating duration of two aggressive behaviours – opercular flaring and biting. Also there was no effect of isolation on the display readiness and the difference of latency to display between treatments was not significant ($p>0.05$) in isolates. Results were not in accordance with Lorenz's (1950) hypothesis that males elevate their aggressive reaction to their opponents after a period of social isolation, and results are discussed.

Abstrakt

Tato práce měla za úkol zkoumat vliv sociální izolace na agresi u samců Bojovnice pestré. Literární rešerše se soustředila na popis mechanismů, které zapříčiňují změny v agresi u izolovaných jedinců. Shrnutí bylo i dosavadní poznání o účincích rozdílných sociálních prostředí na agresivitu. Metody ze zmíněných prací pak sloužily jako podklad pro formulaci vlastního experimentu.

58 samců bylo umístěno jednotlivě do průhledných boxů. Rozdělení samců do skupin proběhlo podle úrovně sociální izolace: 1) Socializovaní s jinými samci („M-socializovaní“ samci), 2) socializovaní se samicemi („F-socializovaní“ samci) a 3) zcela izolovaní samci („Izolovaní samci“). Experiment byl proveden dvakrát s různou délkou trvání pro porovnání dlouhodobých (6 týdnů) a krátkodobých (3 dny) účinků izolace. Videozáznam reakce každého jedince na vlastní odraz v zrcadle byl pořízen při obou experimentech. Stanovená hypotéza, kterou bylo předpovídáno, že izolace bude mít za následek zvýšenou agresivitu, nebyla potvrzena. Jako určující hodnoty byly v tomto případě zvoleny dvě chování- kousáním a zdvihání žaberního víčka, a sledováno bylo jejich trvání. Nebyl také zaznamenán žádný vliv izolace na latenci k projevu první agresivní reakce. Rozdíl v latenci nebyl statisticky významný ($p < 0.05$) ani při porovnávání účinků rozdílných délek pokusu u izolovaných samců. Výsledky nejsou v souladu s Lorenzovo (1950) hypotézou, že samci zvyšují agresivní reakci vůči soupeřům po určité době sociální izolace. Výsledky jsou diskutovány.

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List of the abbreviations and terms used in the thesis

F-socialized– males that were socialized with females

M-socialized – males that were socialized with males

ITIS – Integrated Taxonomic Information System

s – seconds

SE – standard error

ANOVA – analysis of variance

1 Introduction

The reason why individuals behave aggressively has been a puzzling topic for mankind for a long time now. The origins and causes of aggressiveness were widely investigated especially over the last century. One of the observed phenomena was the males' overt enhancement of agonistic response to conspecific males after certain period of social separation (Lorenz, 1966). Siamese fighting fish *Betta splendens* Regan, 1910 is a perfect model organism for examination of this effect, with its set of apparent and easily observed aggressive behaviours.

It is apparent from literature that the attention was given mainly to the comparison of *Betta splendens* socially isolated males with conspecific males that have been socialized with other males. But number of papers proved that the choice of sex that is creating social environment is essential for the outcomes of aggressive trials. One of our concerns was to find out, whether or not socialization of subjects with females would bring any difference to socialization with males, and more importantly isolated males.

We also spotted a slight discrepancy in results of two studies monitoring two highly similar measures of aggression - latency to display to models (Halperin et al., 1992) and latency to approach to the mirror (Hinkel, 1972) post isolation. While Halperin et al. 1992 found the latency is decreasing within the isolated individuals, Hinkel reported the exact opposite. The main difference in chosen methods of these two papers was the selected length of isolation of subjects. This was the reason we decided to compare the latency to display to the mirror in visually isolated individuals; within the very same individuals in two treatment, one lasting 3 days and other 6 weeks. It was expected that the readiness to display would decrease with the length of isolation.

The review of literature covers the basic information necessary to understand underlying mechanisms connected to this topic.

2 Literature review

2.1 Siamese fighting fish

2.1.1 Biological background

Siamese fighting fish *Betta splendens* Regan, 1910 belongs to suborder Anabantoidei, the labyrinth fish, whose representatives are found in Asia and Africa (Nelson, 1994). According to Ruber et al. (2006) the suborder might have arose due to drift vicariance during the break up of Gondwana or Early Tertiary dispersal from Africa to Asia or from Asia to Africa.

The taxonomy of *Betta splendens* is formulated by ITIS (2017):

„ Superclass **Osteichthyes**

Class **Actinopterygii**

Subclass **Neopterygii**

Infraclass **Teleostei**

Superorder **Acanthopterygii**

Order **Perciformes**

Suborder **Anabantoidei**

Family **Osphronemidae**

Subfamily **Macropodinae**

Genus ***Betta***

Species ***Betta splendens***”

Species is distributed in Asia’s Mekong basin (Rainboth, 1996), living in small water bodies like rice paddies, lagoons, canals, marshes and ponds to medium and large streams (Taki, 1978). Having to survive in such space limited reservoirs, the whole suborder of Anabantoidei developed a specialized accessory labyrinth organ placed in the branchial cavity which provides them with the opportunity to respire air (Huang et al., 2011).

Males grow up to 5- 6 centimetres while females are smaller (Hoffman and Novák, 1999). Wild form has short fins and males grow into iridescent green or blue colours with blue-red rays in fins. Females remain inconspicuous with brownish colours

and horizontal stripes (Goldstein, 2004). *Betta splendens* consume zooplankton, mosquitoes and other invertebrate larvae in nature (Rainboth, 1996).



Figure 1. The appearance of male (left) and female (right) *Betta splendens* bred for long fins. (Source: Jane Burton/naturepl.com)

Wild fish differ from artificially bred ones in appearance. Traditionally, Bettas have been selected for traits that are advantageous in fights, as males are highly aggressive towards each other and have been used for combating competitions. These animals are preferred to have strong, heavy bodies, hard scales and relatively small fins, as the latter two features are the targets for biting in combat (Monvises et al., 2009). Fish bred for ornamental purposes on the other hand are favoured for bright, iridescent colours, strong scale patterns and various shapes and lengths of fins, (Monvises et al., 2009). Example of breed selected for long fins could be seen in Figure 1.

2.1.2 Bubble nest construction

Males of *Betta splendens* are, among other things, unique for they are the ones who provide parental care and nesting behaviour have been developed in them. The nests are built from bubbles that males produce with their pharyngeal organ. The air bubble is simply inhaled, vested with mucus and expelled to the water surface. This is repeated until a formation of coherent unit of bubbles is made (Braddock

and Braddock, 1959). During mating, male needs to readily pick up the eggs from female and fetch them up into the bubble nest. It is then widely defended against other fish by the male (Brown and Clotfelter, 2012; Braddock and Braddock, 1959). If any egg falls to the bottom, the male must react and pick them up again. He also refills the nest with new bubbles if any burst. The fry stays in the nest for some time after hatching (Braddock and Braddock, 1959).

Bubble nest is an effective tool for parental care – it ensures necessary oxygenation to provide faster embryo development. It was found out by Kang and Lee (2010) that the contents of the nests are glycoprotein rich, but not much more is known about the chemical composition of the material. After finding antimicrobial substances in nests of threespine stickleback *Gasterosteus aculeatus* Linnaeus, 1758 (Little et al., 2008), or African foam nesting tree frog *Chiromantis xerampelina* Peters, 1854 (Cooper and Kennedy, 2010), Brown and Clotfelter (2012) examined this effect in *Betta splendens* as well. Female can influence the immunity in eggs via differential allocation of nutrients and immune-stimulating molecules (Badyaev et al., 2008). Males on the other hand could only do so by providing any beneficial properties to the nesting material. The experiment of Brown and Clotfelter (2012) compared fry survival in regular tank water and water with extract from the nest. They also tested the effect of the foamy nest material against three microbes- bacteria *Edwardsiella tarda* Sakazaki 1962, (non-pathogenic) *Escherichia coli* T. Escherich, 1885 and oomycete *Saprolegnia parasitica* Coker 1923. The results indicated that there are no antimicrobial properties that would help eliminate those organisms, on the contrary the nest offered a rather favourable environment for the spread of *Saprolegnia parasitica*. It was suggested that the level of male attendance to the nest is responsible for the good larval survival rates. Brown and Clotfelter (2012) explained increased germination of *S. parasitica* oospores as that the nest material either had favourable conditions for oocyte germination or that the nest itself contained oospores from before. Also possible cleansing properties of the very mouthing of eggs by male were mentioned as probable defence against infection, as well as removal of infected eggs. A non-sterile substrate of the nest may also serve as a source of horizontal transmission of helpful microorganisms that are to be used as a first food for the fry. Brown and Clotfelter (2012) also speculate about bubble nest

being a good environment to enhance sperm longevity and therefore even for invading microorganisms.

According to Braddock and Braddock (1959), the process of creation of the mucous bubble starts with expelling air from the labyrinth, followed by inhaling fresh air. Kang and Lee (2010) found after dissection of *Betta splendens* male's head that the pharyngeal organ is located right in the area through which inhaled air passes and it is covered with multiple papillae and crinkles in the epithelium. Interestingly, mucous goblet cells were found in both male and female epithelium. The pharyngeal organ was bigger in males than females thought and with higher number of mucous goblet cells. Braddock and Braddock (1959) subdued fish of both sexes to experiment in which they investigated the development of nesting behaviour and stated that the role of female's ability to build nests is still unclear.

It was reported that the size of the nest in various species of fish is significantly affected by the body size of nest holder, for example plainfin midshipman *Porichthys notatus* Girard, 1854 (DeMarini, 1988). However the importance of bubble nest size in overall reproductive success of Siamese fighting fish needs to be considered as Jaroensutasinee and Jaroensutasinee (2002) found out females showed no preference for males who built larger bubble nests. Also larger nests males were not more successful in male combats. Some level of nest size importance is however suggested by study of Bronstein (1981) who had been observing the behaviour of males with nests after presentation of opponents. After seeing a conspecific, males started further nest building and fixation, besides doing displays.

Clotfelter et al. (2006) found out that larger males did not build larger nests than their smaller conspecifics. However males, who displayed more frequently to female, did. The pair had then fewer eggs though, even when considering the body mass of female. On the other hand Harlioglu and Yonar (2008) found a positive but weak correlation between the size of constructed bubble nests and individual builder fish's size.

2.2 Aggression

The term aggression, despite being used in numerous studies on various topics, is perceived in several different ways by professional public. Therefore it could be a term describing several issues that are similar in appearance, but have distinctive genetic and neural control mechanisms, and also serve for different purposes (Ramirez and Andreu, 2003). In this thesis, the term was used in a context of agonistic behaviour and can be explained as behavioural patterns among conspecifics that have common functions of adaptations to situations that includes physical conflict (Scott, 1966). This comprises defensive, offensive and parental aggression (Nelson, 2005), while other authors like Ramirez (1981) or also include interspecific aggression into their definition.

2.2.1 Theory of honest signalling

During last forty years topics around the handicap theory and honest aggression were widely discussed amongst behavioural ecologists. Zahavi (1975) first talked about a selection for handicap, which proposes that some male's extensive and costly traits, for example antlers in moose *Alces alces* Linnaeus, 1758 (Solberg and Saether, 1993), are a measure of male quality to the females. Owning an extraordinarily conspicuous trait causes a lot of difficulties, one of them being easily noticeable by predator, which basically informs the female that the male is able to survive despite owning such a peculiarity. Mentioned traits thus serve as signals that pass information from transmitter to receiver, in this case male showing off to the female (Zahavi, 1975).

Signals evolved also for situations in which two individuals compete for one resource. Instead of performing exhaustive combat, the two could express their abilities or willingness to compete for the resource through display. This effect creates opportunities for a cheater that signals false abilities to their opponents though. Zahavi (1977) suggests signals must be honest to pay and that repetitive stereotypy of many displays comes from selection by receivers for a standard performance on the part of the transmitter. On the other hand, Krebs and Dawkins (1984) point to the presence of cheaters who manipulate when communicating, explaining that while cheaters evolve in the way of cheating, receivers also find better ways to detect dishonest signals. Grafen (1990) brought mathematical models to the discussion and stressed that the cost

of displaying at a level that is not respective to the individual is so high, that it does not pay to cheat, and supported Zahavi's (1977) theory. Later Johnstone and Grafen (1992) remarked that Zahavi's (1977) model could not be applied fully in real signalling systems, where perceptual error could be expected to some level. For example Számadó (2008) suggested that honest signalling is evolutionary stable only within some distance threshold. He adds that outside of this zone, there may be place for a mixture of honest and cheating displays that are evolutionary stable. To explain the existence of cheaters Dawkins and Guilford (1991) mention receiver cost in association with eliciting and evaluating honest signals that lead to certain level of dishonesty in populations. Such costs, along with the possible insufficiency of necessary signal correlates of quality, make truly honest signals less widespread, according to their comments.

2.2.2 Fish and aggression

2.2.2.1 Forming a hierarchy

Species that belongs to a group of animals that exhibit "exploitative competition" or "scramble competition" have the advantage of the resources being equally accessible to all individuals so they could take over them by simply being the first (Snaith and Chapman, 2008). Contest competition on the other hand is a concept, where aggression is a driving factor determining the winner of a mate, territory, food etc. and the resources are either won completely or not at all. Species from the second case then either build dominance hierarchies with various structures or defend territory when living in pairs or alone (Isbell, 1991).

Linear hierarchies are defined by one individual having over the other making a cascading chain of the ranks in the group. Despotic hierarchy contains one dominant individual and multiple subordinate members of the group, who are of the same rank (Chase, 1974).

Living in a group with a structure has demonstrable drawbacks for the subordinate members. It could be the dominant member deterring the subordinates from the food resources, such as documented for example in salmonids (Salmonidae) (Metcalf, 1986). Also subordinates exhibit increased levels of stress hormones - that

could be possibly caused by living in more or less constant fear from being intimidated by dominant members like in pumpkinseed sunfish *Lepomis gibbosus* (Linnaeus, 1758) (Erickson, 1967). Stress is often an originator of lowered growth rates within many species, as has been documented in brown trouts *Salmo trutta* Linnaeus, 1758 (Sloman et al., 2000). Fish were kept in pairs under laboratory conditions and allowed to establish social hierarchies, so that one became dominant and the other subordinate. Their standard metabolic rate was assessed. Subordinates resulted with significantly higher standard metabolic rate, increasing with the level of aggression that was received from dominant members of the group. Social stress therefore brings high metabolic disadvantage to the intimidated in this species (Sloman et al., 2000).

Magurran et al. (1991) emphasized that aggressiveness in some species could be affected by ecological history of specific populations. They visited eight locations with distinct populations of guppies in Trinidad and collected some individuals of each population for laboratory tests. It turned out that those that colonized sites with predators adapted through creating schools – aggregations of individuals that showed lessened intrasexual aggressiveness. On the contrary populations living in habitats with none or less of their predators were living more solitary lives and were significantly more aggressive to each other. Female aggressiveness was also tested on both wild-caught and laboratory-bred fish. Individual aggression in both groups increased with the group size and was not dependent on the size of the tank. Results confirmed the hypothesis – that there is a certain trade-off between staying in group (schooling) and resource defence.

The stability of group ranks is different across the taxa. There are species that maintain the hierarchical positions for a long time. Abbott et al. (1985) performed an experiment with rainbow trout. After establishing clear dominant-subordinate order in two differently sized trouts (5% difference), they separated these two fish for some time. That was done in order to feed up the subordinate individual to be bigger than the dominant. Although in some of the experiments the original subordinate became up to 50% bigger than the original dominant animal, the rank order did not change after reunion of these two. It became clear that in rainbow trouts rather prefer following their

previous experience than retest the opponent's abilities, probably because the chance of increasing weight or gaining stronger fighting abilities is low.

Oliveira and Almada (1996) studied the stability and structure of hierarchy in the cichlid African mouthbrooder *Oreochromis mossambicus* (Peters, 1852). Dominance structures appeared to be linear although the positions were changing from week to week. Chance of shifting between dominant and subordinate ranks was not too high, while shifts over other stages for one individual were more likely. Fish of this species showed that individuals are constantly challenged by their neighbours on rank order. Hierarchy did not come to stable point during the time this study was held.

2.2.2.2 Aggressive encounters in fish

Combats of conspecifics in animals are means of obtaining of resources, be it habitat, partner or food. Some of the species have developed intraspecific communication based on highly aggressive displays or combats to defend the territory (Alcock, 2001). However too much of effort put into aggression can be costly. Not only individual has to expend a lot of energy during performance of agonistic behaviour, but also loses time and is highly suspicious to predators. The sense of when is the point to stop is also essential, as the longer animal fights, the more costly it is going to be (Ichihashi et al., 2004).

As one could expect, the most indicative feature when speaking of advantage in fights is in most cases the size, or rather weight of the fighter. This is a case of African mouthbrooder as Turner (1994) suggests. He has put few male intruders into aquaria of resident males and let them fight. Series of displays occurred. In case when the intruder was smaller than resident male, it was quite easily beat-up. But the situation changed with the increase in intruder's size – they were able to win in 13 of 32 contests although not having the advantage of residency.

When size does not matter, it is more likely that other visual signals do. Red males of Green swordtail *Xiphophorus helleri* Heckel, 1848 were faster in latencies to show first sign of dominance, first attack and first approach than black males of similar size (Heuts and Nijman, 1998). Reddon and Hurd (2009) observed fights

of convict cichlids with two different common colour morphs – one wild type form with black bars and amelanistic form without the bars. Despite having size advantage in some cases, the amelanistic males were not able to increase their display rates and lost in significant amount of combats. Three spine stickleback is another example; in their case just a little red spot on their belly is indicative to conspecific males and also to females when mating period occurs. Interesting fact is that when a fight is held under blue light, the vivid red-bellied males lost their advantage, because the presented red colour was masked to black by the light. All males were of the same size. Red spot on the belly probably then serves as a threatening symbol more than it would be signalling any elevated fighting abilities of their owner as suggests Baube (1997) who held this study.

Green swordtail has also one trait that can be advantageous to the males besides colouration and that is a caudal fin elongation called sword. These swords have no practical use in the combat but serve as a kind of advantage; males with longer swords can defeat opponents sooner, and also have significantly more mating opportunities as it is also a trait that female is taking notice of. Males with shorter swords gave up earlier in fights with long-sworded males, at least when comparing the length of the sword with the body size (Prenter et al., 2008).

Those fish, which have the primacy of establishing a territory at a set place also have an advantage in fighting. It is well documented for example in Pearl cichlid fish *Geophagus brasiliensis* Kner 1865, and the effect in this species increases with environmental enrichment as Nijman and Heuts (2011) found out. It is energetically very costly to build up and defend the boundaries so timely establishment of territory is important.

There are certain species that do not fear to devote a lot of energy into their fights under a laboratory conditions. Although it has been previously described that fish of some species can evaluate and predict the outcome of a fight and therefore withdraw before the combat begins, sometimes the fish continue into the fight despite having such information (Maan et al., 2001). Neat et al. (1998) observed a continuous fight in redbelly Tilapia *Tilapia zillii* (Gervais, 1848) and found out that the loser of the fight

showed much higher concentrations of lactic acid in their muscles suggesting that the loser was defeated due to the subsequent fatigue. However this approach was questioned by Maan et al. (2001) with their study that experimented with South American cichlid fish *Nannacara anomala* Regan, 1905. They let the opponents to see each other before the conflict. After placing them into a less artificially designed tank that offered asymmetrical territory making, they obtained different results than the study that examined redbelly Tilapia. They assumed that the occurrence of predictors of conflict outcome and the following lack of response of the opponents is the result of unnatural designs that many experimenters before have used. Their possible explanation for previous studies' results was that fish in these test had no other option than to fight although they have already assessed the abilities of the opponent. This is called the "desperado effect".

In contrary to what have been stated in the beginning of this chapter, there are some cases when males that have a clear disadvantage (smaller body size for example) initiate the contests. Morrell et al. (2005) consider two explanations for this behaviour – it is a result of misperception of the likely losers that behold themselves as likely winners. When the resources are scarce and when the chances to win are close to half, they are predicted to be as aggressive as the other combatant. In case that the resources are relatively abundant and the chances to win are not so low, it may still pay off to be more aggressive than the opponent, as it is likely that the other male is going to consider the cost of such combat and leave for another more easily defendable source.

2.2.3 The influence of composition of social environment on aggressiveness across taxa

The social environment and its composition has an important role affecting individual's aggressive behaviour (Bends and Henkelmann, 1998; Lacava et al., 2011; Ruploh et al., 2013).

When testosterone levels are increased to levels at where they are no longer needed for reproduction, it is a result of reaction to various effects from social environment, as the challenge hypothesis suggests (Liening et al., 2012). Variations of sex ratio in populations are affecting aggressiveness for example in male blue-black

grassquits *Volatinia jacarina* (Linnaeus 1766). Lacava et al. (2011) exposed males of this species to either all-male, mixed, or one female company for one year. Monthly inspections of levels of testosterone in blood showed elevated values in all groups but the mixed one. All-male treatment had the earliest peak, steepest decline in testosterone levels and greatest number of aggressive encounters, one-female treatment showed on the contrary the latest first peaking of testosterone. The mixed group presented relatively continuous increases and decreases in the levels of this hormone (Lacava, 2011).

It seems that zebra finches *Taeniopygia guttata* (Vieillot, 1817) males' aggressiveness is affected by the composition of their social environment as well, but with different outcomes. Ruploh et al. (2013) kept zebra finches either in male-female pairs, male-male, or mix-compositional facilities during the adolescence. Opposite to blue-black grassquits, the males of male-female pairs resulted with highest levels of aggressiveness and excessive display over the other groups. Also, their attractiveness to females was the biggest. Males of the mixed treatment performed least of courtship behaviour, were least aggressive and attractive to females. Post-hoc trials revealed that the observed distinctiveness in aggressiveness and courtship intensity are stable over time, while the attractiveness to females may change (Ruploh et al., 2013).

Differences in aggressiveness in mammals that were kept in distinctive social environments can be observed as well, like the study with male house mice *Mus musculus domesticus* Schwartz and Schwartz, 1943 (Bends and Henkelmann, 1998). Subjects were kept as all-male groups of 6 individuals or 1 male+ 5 females groups. While more agonistic interactions were observed in subjects of the all-male treatment, novel intruder was attacked faster in the 1+5 group.

The influence of isolation on males' aggressive behaviour is discussed later in chapter "The effect of isolation on aggressiveness in non-fish taxa".

2.2.4 Audience effect in *Betta splendens*

It is essential to note that the agonistic fights within animals are almost never held at places that would be free of other conspecifics, and the information transmits

over a larger range than standard distance between individuals would be (Clotfelter and Paolino, 2003; Matos and McGregor, 2002). Absence of social vacuum creates space for contestant's behavioural alterations known as audience effect. It is defined as a phenomenon that appears when participant's behaviour is affected by characteristics or behaviour of the observer (Clotfelter and Paolino, 2003).

Betta splendens males are susceptible to be affected by both male and female observers during the contests (Doutrelant et al., 2001; Matos and McGregor, 2002). As Doutrelant et al. (2001) suggests when the observer is female, male increases behavioural display features that could be read by both sexes and simultaneously lowers the highly aggressive displays that are directed only towards males. On the other hand presence of male observer of similar size as the contestant's does not cause any significant difference in male's display. Doutrelant et al. (2001) explains that the presence of audience could be the reason why many conspicuous signals are adjusted to pass the information on both males and females.

Results of this study were homogenous with findings of Matos and McGregor (2002) who conducted similar experiment. Males attempted less biting and spent more time in the proximity of the rival during a fight with female audience in comparison to the situation with male audience. Matos and McGregor (2002) think this is caused by male's consideration of the trade-off between intimidating his opponent and not ejecting the female, who could be frightened by male's increased aggressiveness (described more in detail below). However their results differed from Doutrelant et al. (2001) in the case of combat with male bystander. Matos and McGregor (2002) found combatants with male observer more aggressive and the escalation of their reactions was also faster. Their possible explanation is that the distance between bystander and participants of the fight matters. In their case the gap was 7 cm, unlike in Doutrelant et al. (2001) that used 12.5 cm gap. As Bronstein (1983) proved, increasing the distance between tested fish reduces the amount of display.

Dzieweczynski and Walsh (2011) confirmed audience effect occurs not only for male-male interactions, but for pairs of Siamese fighting fish that perform courting behaviours. Especially the interactant-directed behaviour of subject was affected by

the presence of bystanders, audience-pointed behaviour was influenced only to some level. Interestingly, when presented with male opponent audience in structured environment during courting, males tend to increase and show off in larger intensities. Instead of trying to conceal the display behind the barriers, males tended to elevate those behaviours that could be read by both mate and rival, such as opercular flaring (Dzieweczynski et al., 2009).

Similarly also the audience may use the information gathered when observing aggressive displays of other conspecifics, or any other social interactions in which they take no part. Such phenomenon is called eavesdropping (Doutrelant and McGregor, 2001; Earley and Dugatkin, 2002). Oliveira et al. (1998) conducted an experiment in which bystander males were placed in aquaria separated with one-way see-through glass from the contestants. Their efforts were pointed towards testing whether audience is interested in observing other male's competition and eventually it was confirmed. Bystanders spent significant amount of time in the closeness of the two participants fighting, no matter the side or place where the fight was presented to them. Results were controlled for winner/loser effects (explained in paragraph below).

Clotfelter and Paolino (2003) performed two tests to map how male audience of two fighting males reacts and succeeds in future contests. It was revealed that bystanders to contests became dominant and more aggressive in display, chases and bites than "naïve" males which only had the chance to observe empty tank or two non-aggressive males previously. Naïve males were defeated in 80% of combats by bystanders. Although body size is truly important element in many fish species' aggressive encounters including Siamese fighting fish and Marble Cichlid *Astronotus ocellatus* Aggasiz 1831 (Jaroensutasinee and Jaroensutasinee, 2001; Beeching, 1992), body size difference of bystanders and naïve males evaluated by *post hoc* test was not significant in this case. Such interactions could affect and possibly form dominance hierarchy across taxa. Moreover this study observed so called "winner effect" - winning one combat may lead to series of further victories for one individual, in spite of having otherwise equivalent rivals (Dugatkin, 1997). Winner effect was registered within bystanders, which was explained as bystanders being primed for aggression by seeing

the two fighting animals. As well “loser effect” is analogically increased probability of losing a combat based on previous series of defeats and it appears to be more extensive than the winner effect and it could last from several hours to several days (Chase et al., 1994).

Observing of combatants could be advantageous also for nearby females. As the reliability of the signals transmitted by males during contests is really strong, female could use the information to assess the potential mate’s qualities (Doutrelant and McGregor, 2000). To prove this Doutrelant and McGregor (2000) conducted an experiment in which they monitored whether females of Siamese fighting fish are interested in presented male combat and if they then use the gained information in future. It appeared that the females which had seen the interaction preferred to visit the winner first and also displayed, spent more time near and looking at the winner. On the contrary, females that were not audience to a contest visited the loser first in more cases and did not prefer neither winner nor loser significantly in the other categories. Doutrelant and McGregor (2000) explain the naïve female visiting loser first as the attempt to avoid possible attack by an overly aggressive male that has (in her perspective) no obvious male opponent, who would elicit the aggressiveness.

Further studies showed that male’s behaviour connected with presence of audience is more complex than was previously believed. It appears that males are able to perceive the aftermaths of the presence of the audience and alter the behaviour to their benefits despite the fact that they are apparently going to lose present fight. In experiment of Herb et al. (2003), losers of fights had an opportunity to display either to naïve or eavesdropping females. As predicted, defeated males performed more gill cover erections and overall court behaviour towards naïve females who did not have the chance to see the male lose, whereas winners had no significant preference in females.

Peake et al. (2006) took a further look at the behaviour of combating males that is influenced by observers. They presented manipulated male combat to observing male; two males appeared to be interacting with each other to the bystander, while in fact they were displaying to the mirror. The interactions were arranged to increase

or decrease aggressive response of one male, while letting the other one be consistent in the display. Males were then presented to the bystander separately. Observers showed stronger response to male whose opponent's aggression was decreased and to the winners of controls, but they were not able to distinguish winners to losers when aggressiveness of one male was increased. Such result may imply the disability of observer to determine who is winner or loser, when the overall level of aggressiveness within the display is very high. As was previously stated by Smith (1982), male should withdraw from combat when realizing that his chances to win are scant, but this case shows males often continue to fight although they already know they shall lose. That might support the hypothesis called the "good loser" which says that males who lose while still performing well may experience less subsequent aggression from bystanders than when giving up combat after sensing that the opponent is stronger. This is caused by the previous bystander being confused by the high aggressiveness of both contestants (Peake and McGregor, 2004).

As number of studies examining audience effect and eavesdropping began increasing at the turn of millennium, some papers questioning the methods and effects of environment in such experiments also appeared.

Dzieweczynski et al. (2005) suggested that the audience effect might be context dependent and tested the audience effect with alterations in experimental conditions. It was found that both audience sex and presence of a nest had effect on the aggressive behaviour of interacting males. Also the context of the presentation of these two features had a great impact. There were notable differences in aggressiveness of contestants when interchanging contexts of fights – sex of the audience and presence of the nest of one of the subjects. For example males behaved more aggressively when none of them had a nest and were observed by male audience, than in the same case but with female audience. Results may be indicating that the interaction between audience and nest influenced contestant's behaviour, not audience's presence alone did. They have recommended further consideration of aspects of behaviour such as both external and internal factors, reproductive state and resource possession in experiments on audience effect.

Matos et al. (2003) raised questions about relevance of methods used to study the audience effect in previous papers, particularly the timing of the presentation of audience to contestants. The males that were pre-exposed to the audience before the beginning of the experiment exhibited higher levels of aggression, no matter if the audience was then present or not. When only one of the males had been pre-exposed to bystander, other (non-exposed) male tended to increase the aggressiveness to match the opponent. These results may imply that some of the previous experiments (Matos and McGregor, 2002; Doutrelant et al., 2001) may have been biased by untimely presentation of conspecific to the interacting fish.

2.2.5 Agonistic encounters in Siamese fighting fish

Due to its stereotyped and easily recognized aggressive behaviour, the Siamese fighting fish has been used as a model organism for many studies investigating agonistic behaviour in animals.

Over the second half of last century, multiple features and actions during the fight were observed by ethologists and later considered specific for this species. Some of those appear to be more consistent and indicative and now serve as the measures of aggressiveness in Siamese fighting fish males (Hinkel, 1972; Simpson, 1968; Halperin and Dunham, 1994).

In situation, where the selection on fecundity of the female is less intensive than sexual selection in a species, males are usually larger than females. In such mating systems, the size is a mean of assessment in female choice or could play role in male-male competition, which is the case of *Betta splendens*. Both process and outcome of a fight of two Siamese fighting fish is highly influenced by the size of the contestants. Jaroensutasinee and Jaroensutasinee (2001) proceeded an experiment with comparing smaller and larger contestants of wild type of *Betta splendens*. Large males were more successful in winning of contest, while the length of the fighting did not differ significantly among sizes. Larger males also attacked, chased and overall performed more of agonistic behaviour than smaller males. Interestingly none of this affected the female choice as they did not prefer larger males after presentation of two potential

mates with different sizes, which was not consistent with the results of Clotfelter et al. (2006) whose females accepted larger males better than smaller ones.

Karino and Someya (2007) studied the influence of sex, line and fight experience on the aggressiveness of Siamese fighting fish. After proceeding of a test on innate aggressiveness with a mirror image it was found that males invest more energy into the fight than females, also some line types were more aggressive than others. Those individuals also spent less time fighting and often lost the subsequent fights with other individuals. Winners of previous fights exhibited more aggressive behaviour and losers were less aggressive, which was the most notable and emphasized in lines that showed lesser innate aggressiveness.

Although long believed to be a privilege of mankind, brain lateralization has been found in other vertebrates and even invertebrates too (Frasnelli et al. 2012, Rogers, 2007, Leliveld et al. 2013). Lateralization is believed to be a helpful tool for example to enlarge number of tasks being done simultaneously (Rogers, 2007). Some consistency in lateralization during performance of aggressive behaviour in Siamese fighting fish has been documented by multiple studies. Cantalupo et al. (1996) let males interact with their own mirror image for 10 minutes and observed a consistency in their right or left eye preference during lateral display of the fish. Individuals showed preference of using one side for displaying both in frequency and duration of display. They also tested the same animals on the preference of the eye during performance of courtship behaviour to female two months later and the results were consistent. It was also confirmed by Takeuchi et al. (2010) who studied lateralization of aggressive behaviour with mirror image in twenty-five male fish, of which five preferred left eye for left display and eight used right eye during left displays. They also compared the side preference with morphological asymmetries and found that lateral difference in the craniovertebral angle corresponded with the side preference. Therefore they suggested that behavioural laterality is individually determined, in this case by external features.

Forsatkar et al. (2015) received different results. They found that *Betta splendens* males, who were holding nests at that time, preferred the use of the right eye, both before and after bubble nest building. Despite their prediction that

the preference would last and strengthen with aging of the eggs (and therefore their increased value) it did not last long after spawning, when later tested for laterality during aggressive display.

Castro et al. (2006) assessed the level of metabolic cost of display during fights with mirror image test. They found that level of oxygen consumption correlated positively with the spreading of dorsal fin and opercular flaring. Recording of interaction of two males showed that there was no significant difference between loser and winner of the fight in metabolic rate, but in the later night after fight winners exhibited increased oxygen consumption. These results support the hypothesis that fighting is costly and it does not pay to cheat by being overly aggressive when not being able to back it up by one's fighting abilities (Halperin et al., 1998).

The fact that Siamese fighting fish's aggressive response is modified with different coloration of the enemies is long known (Thompson and Sturm, 1965). After conducting series of presentations of differently coloured fish models to differently coloured subjects, they found that the fish responded the least to models that were resembling their own colour. On the other hand, stronger response was noticed to models that was opposite to their own. The strongest of all reactions was performed to the mirror, however since the perceived image is also much more complex and natural-like compared to models, they did not conclude that the response was influenced by the colour of the fish's image. Bando (1991) decided to test whether the fish is able to perceive and recognize shapes, particularly scale design and therefore potentially distinguish opponents from non-threat objects. Model with a sideways shape of Siamese fighting fish in the state of aggression was used to stimulate the individual. The reaction was strong when there was a fine distribution of natural-like colours and scale-like lines along the body of the model and was decreasing the more the scale pattern was disappearing. Bando (1991) then used models with circular shape with the same results – fish reacting more aggressively when the model had stronger scale patterns. According to the presented facts it is suggested, that this species is orientating not only with the colour recognition, but shape recognition is a great part of its visual perception.

2.2.5.1 Substances that affect aggressive behaviour of Siamese fighting fish

The series of behavioural patterns within a fight is of course stable in normal, undisturbed conditions. Aggressive response is different in environment which has been chemically or physically changed to not suit the species' requirements. Many of recent pharmaceuticals are designed to fit human need, but once released into any water environment by wastewater systems it often affects also other vertebrates that come in contact with the dissolved substance even in small concentrations. This is the case for example for European perch *Perca fluviatilis* Linnaeus, 1758 who was found affected by the content of oxazepam based tranquilizing drugs both in behaviour and storage of the drug in tissues (Brodin et al. 2013). The fact that Siamese fighting fish's behaviour could be affected by presence of artificial substances in water makes it a great biological indicator of water pollution. It is used extensively for assessment of various drugs or pollutive materials.

Mansur et al. (2012) exposed *Betta splendens* males to mercury chloride which is a substance widely polluting air, soil and water. In one test males underwent treatment with progressive dose of mercury chloride (0.04 mg) and were left to interact with their own mirror image, other group was exposed to acute dose of 0.2 mg. Both doses affected the animals in motor functions decreasing their mobility during aggressive display. The group with progressive dose differed in more aggressive to control group than males with acute dose. This fact may imply that the smaller progressive dose could affect the behaviour of this species on wider scale than acute dose.

Alternative resources are used in traditional medicine in different parts of the world, for example the extract of Rue *Ruta graveolens* L. used for reproductive control in men. Such substances can however cause modulation of reproductive traits in wildlife males and alter the behaviour, which is the case of *Betta splendens* as well, as Forsatkar et al. (2016) suggest. They found that injecting of Rue extract into tank water induces changes in fish aggressive and reproductive behaviour, also the willingness to explore new objects. Clotfelter and Rodriguez (2006) chose other reproductive behaviour modifying substances to test on *Betta splendens*,

phytoestrogens beta-sitosterol, equol and genistein along with the positive control 17 beta-estradiol. Genistein and equol significantly reduced manifestations of agonistic behaviour aimed towards mirror images of the males, proving that contamination with these phytoestrogens can alter the behaviour of aquatic wildlife.

2.2.5.2 Description of display and aggressive behaviour

The description of male combat and display behavioural patterns is overtaken from Simpsons' (1968) thorough summary: When male first beholds a conspecific, an instant quick approach towards the rival with erected fins and opercular gill covers could be observed. After that, male may swim away with "zig-zag" movements showing both sides of his body to the male behind him. The fish shows off switching from broadside to facing position. Facing position indices that the fish is ready to bite the enemy, opercular covers are erected, if already had not been before. Their lowering usually happens during displaying broadside. Flickers of the pelvic fin and beating of the tail also occurs while being broadside to the opponent. The fish is usually doing series of approaches and withdrawals to and from the rival, switching facing and broadside displaying once per visit. It is more probable that the facing fish will turn broadside if their opponent is also facing than if they were broadside displaying.

The opercular gill covers are erected during displays (see Figure 2), meaning the operculum is rising from its normal position and the branchiostegal membrane is blown up. As the end of this action is considered when the operculum is rapidly lowered, even though not fully closed. Gill flaring occurs synchronously with the fin spreading.

The threatening individual changes colour to the darker tones, especially in the head, ending with rich, intense colour of the fish. On the contrary, frightened individual goes paler and the vertical stripes on their body become visible sometimes.



Figure 2. Male of *Betta splendens* is visibly erecting the gill covers as a part of his display (Source: Farid Laid/www.seriouslyfish.com)

In the beginning of the display, when facing the rival, fish threat their opponent with biting attempts (called also biting threats). It means the mouth is open for a longer time than in biting itself, and not always directed towards the opponent. Biting occurs when operculum is lessened. Biting progresses into serious mouth fighting that could last for several minutes (see Figure 3). One fish bites and holds the others lip and they remain in such entanglement for some time, which could be fatal due to drowning.



Figure 3. *Betta splendens* male attempting to bite a male conspecific during a fight. (Source: Dwight Kuhn/dkphoto.photoshelter.com)

2.2.6 The effect of isolation on aggressiveness in non-fish taxa

The chance to interact with other conspecifics modulates behavioural responses widely across the species. In fact, those reactions are not consistent and the same in every taxa, but rather species specific. During past years, multiple studies were held in order to encode the importance of previous experience with conspecific in agonistic encounters, across the classes. It has been a field of study of Lorenz (1950; 1966) who was one of the first to mention that aggression may increase with the lack of performance of aggressive acts, sometimes referred to as “isolation syndrome” (Hatch et al., 1965). Below are presented cases that support this theory.

The literature has been for example largely speaking of the effects of isolation in house mice. For example Lagerspetz and Lagerspetz (1971) have been selecting mice males for aggressiveness for 19 generations. It seemed that animals from the aggressive strain that have underwent a treatment of two week long social isolation resulted with dramatically increased aggressive behaviour, compared to socially living males

of both aggressive and non-aggressive strain, who have showed no particular aggressiveness towards submissive opponent. Isolation induced aggression of mice males was also confirmed in Banerjee (1971), who reported that isolates kept their elevated aggressiveness up to fourth to sixth fighting session and then decreased it apparently after habituation to other conspecifics.

Stevenson and Rillich (2013) reported sharper aggression that escalated into several seconds running physical fights in isolated males of Mediterranean field cricket *Gryllus bimaculatus* De Geer 1773, in comparison to socially housed crickets, who performed only threat displays in majority of cases. Kuriwada (2015) moreover found that males isolated from their opponents also increase aggressiveness pointed to the females and therefore reduce their mating success.

In horses *Equus ferus caballus* Linnaeus 1758, the brief exposure in boxes prior to contact in the paddock could result in milder aggressive manifestations, even in mares, as Hartmann et al. (2009) found out.

2.2.7 The effect of isolation on aggressiveness in bony fish (Osteichthyes)

Earley et al. (2006) measured the aggressive behaviour and also levels of water-borne stress hormone cortisol in the tank during pre-fight and post-fight period in Convict cichlid fish. The isolates initiated significantly more intense and frequent reciprocal fights than the individuals previously kept in groups. Results moreover shown, that the isolated losers had significantly higher cortisol levels, than the group-housed losers in non-escalated contests.

Swordtail males are also susceptible to increased aggression after social isolation, which was proved by Franck et al. (1985). They performed two different aggression tests, one with live small and passive opponent and the other with mirror image. Four weeks long social isolation decreased the attack readiness in both experimental groups. Results suggest that external stimuli from other fish of the species are essential to maintain the levels of aggression.

Davis et al. (1974) investigated the effect of social isolation in Paradise fish *Macropodus opercularis* (Linnaeus, 1758), close relative to Bettas from family

Macropodinae. All of the individuals underwent different lengths of social isolation. They chose different experimental designs; first they measured the frequency of aggressive display that individual males and females targeted towards mirror image, second the display frequencies in interactions with live opponents. It turned out that increased aggressiveness was present in both sexes after isolation up to seven days, in both experimental groups.

On the other hand the situation may be different in species with mating systems in which both sexes are involved in parental care; Angelfish's *Pterophyllum scalare* (Lichtenstein, 1823) aggressive behaviour is apparently not increased post isolation. The species is living in pairs in serial monogamy (Cacho et al., 2007). Gomez-Laplaza and Morgan (1993) transferred juveniles into new aquaria with subsequent isolation. The level of aggressiveness decreased significantly after both group transfer and isolation, in comparison to previous measurements before transfers.

Cichlid fish *Pelvicachromis taeniatus* (Boulenger, 1901) is a species in which also both parents take care of the young and the mate choice is happening in both of the sexes (Hesse et al., 2016). Hesse et al. (2016) subjected both males and females to isolation treatment. Intrasexual aggressiveness decreased in both sexes within the isolated group. Above that the willingness to perform courtship behaviour was also weaker in socially isolated fish.

2.2.8 The effect of isolation on aggressiveness of *Betta splendens*

Methods of the studies examining the aggression in Siamese fighting fish males that have been socially isolated differ to a large extent and it is therefore difficult to compare the findings into integrated literature, though a lot of papers concluded similar results with just few tweaks in the experiments.

Zahavi's (1979) theory about handicap informs that there is a reason for using honest signals in communication channels. If false signals like exaggerative display not-corresponding to fish's abilities and state were rewarded, there would be no reason to "play fair" (for detailed explanation see the chapter Theory of honest signalling). Halperin et al. (1998) scheduled multiple live interactions after some period of social

isolation for males of *Betta splendens*. Group of male fish was primed (presented with a stimuli that could elicit a social response in receptive animal; by mirror in this case) and the aggressiveness was assessed. Primed isolates that were not stronger than their non-isolated opponents, resulted in fast escalation to tail beating and biting attempts – and usually lost. On the other hand, un-primed isolates were not hyper-aggressive from the beginning, did not cheat and did not lose as many fights as their primed conspecifics. Halperin et al. (1998) explain this as an effect of cheaters being exhausted from demanding display that does not correspond with their abilities and supported Zahavi's (1979) theory.

Hinkel (1972) recorded latency to approach to the mirror image, gill cover erection frequency and time spent having the opercula open in isolates after various lengths of social isolation; 15 minutes, 6 hours, 24 hours and 72 hours. The isolates became progressively more aggressive with increasing period of isolation. The latency to approach to the mirror was shorter than before isolation. Second experiment, in which Hinkel (1972) used conspecific model instead of mirror, did not elicit any major changes in the subject's aggressive behaviour.

Halperin et al. (1992) socially isolated male adults for up to 7 weeks and then evaluated the amount of aggressive behaviour with presenting series of models. The longer was the isolation, the stronger were the manifestations of aggressiveness to the last models presented, on the contrary aggression towards first presented model was decreasing with the growing length of isolation. In second part of experiment, isolates were exposed to visual stimuli that did not elicit the display, while controls had the chance to interact with male conspecifics once in two days for two minutes. The stimuli presented to isolates was below threshold level of starting a response, and was designed to prevent sensory deprivation. The isolates subsequently showed longer latencies to display, but higher display rates once it was elicited. Halperin and Dunham (1994) wanted to confirm findings from previous study (Halperin et al., 1992) with testing whether social overstimulation of males leads to waning of aggressiveness. Fish that were surrounded by conspecifics during the length of the experiment really showed reduced aggressiveness – performed less biting

of the opponents. Overly stimulated fish also never defeated the fish socialized group. To follow up with these findings Halperin et al. (1997) continued research with showing isolates brief glimpses of models or mirror that are below the threshold limit and therefore of subliminal value. Males became extremely hyper aggressive after being stimulated with these glimpses in subsequent aggressive actions. The models were presented in two different postures – facing, that indicates aggressive display and broadside which is used in multiple social interactions. Facing posture glimpse affected the fish to become more aggressive than the broadside posture glimpse; that indicates that their predictions were correct.

Charles (2003) followed up with Halperin's research having two experimental conditions; males that were physically isolated and socialized (three times a week for two minutes) and complete isolates. In this case, the social isolation did not alter the duration or frequency of aggressive behaviour after presentation of series of models or mirrors. Analogical experiment with live opponents did not bring any significant results.

Ichihashi et al. (2004) observed the development of aggressive behaviour in different rearing conditions with respect to isolation. They created four groups of juveniles; highly social group of individuals reared together, individually housed group of fish that had the sight into highly social group's tank, individually housed fish that only had the sight into each other's chamber within their group, and finally total isolates, that were reared separately in visual isolation. Interestingly after subsequently entering the fights with first (control) group, the total isolates won in significantly more cases than the other two groups. The measures of aggressive behaviour (butt-or-bite, chase, and gill-cover erect) were not different from the others during the fight though, but after termination of the combat indicated by loser being submissive, the total isolates continued to exhibit those aggressive manifestations even there were no apparent reasons to do so. Moreover it turned out that those continuous fights that appeared within fourth group are irreversible and last even after attempts for re-socialization. The second group of physically isolated fish also had increased aggression after sexual maturation. Ichitachi et al. (2004) suggest that winning of total isolates was caused

by not being able to stop at the right time rather than being extraordinarily aggressive during the combat. As an alternative explanation they propose that individuals from second and third group reduced their aggressive behaviour due to social overstimulation (Halperin and Dunham, 1994).

3 Aims of the thesis

The aim was to investigate the effect of social isolation on performance of agonistic behaviour in male individuals of *Betta splendens* Regan, 1910 targeted at male conspecifics. Also, a comparison of different social environments in the groups of socialized individuals was planned.

H1: The amount of aggressive response of isolated males will be higher than in socialized males.

H2: Latency to display will be quicker in short-term isolated males than in long-term isolated males (to compare Halperin et al., 1992 and Hinkel, 1972).

H3: The aggressiveness of males from the comparative groups (males socialized with other males × males socialized with females) will differ.

4 Materials and methods

4.1 Animals and experimental design

The research took place during September and November of 2016. Seventy- five Siamese fighting fish *Betta splendens* Regan, 1910 male individuals and fourteen females were obtained from a local ornamental fish dealer. Each fish was kept in 1000 ml transparent plastic box, with dechlorinated water that has been treated with a commercial conditioner for tap water Akvaregulátor (Hü-Ben, Czech Republic, year of manufacture: 2016) and vitamin solution for ornamental fish Fishtamin (Sera, Germany, year of manufacture: 2016).



Figure 4. The Setup of fish housing. Source: Author.

A small plant of *Taxiphyllum barbieri* Iwatsuki 1982 was put inside each box. Boxes were placed in shelves (see Figure 4) in stable conditions, with photoperiod 12:12 and temperature between 22-23°C. Fish were fed daily the same amount of micro granules for tropical fish Dajana Legend Tropical granules. The boxes were cleaned of silt

once a week and at least third of the original water replaced by fresh, aged water with appropriate amount of Sera fishtamin and Hü-Ben Akvaregulátor added. Each of the boxes had an opaque light brown cardboard division between them, in order not to let the fish to interact visually with neighbouring individuals (see Figures 5, 6 and 7).

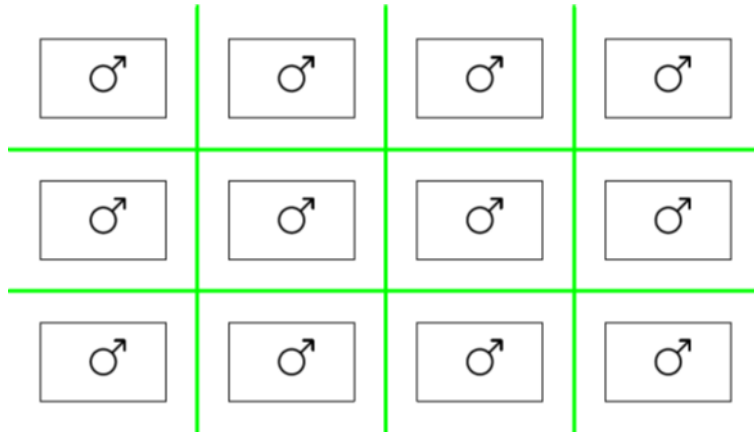


Figure 5. The layout of positioning of subjects socialized with male conspecifics in the shelf. Green lines represent solid partitions that were removed during socialization. Source: Author.

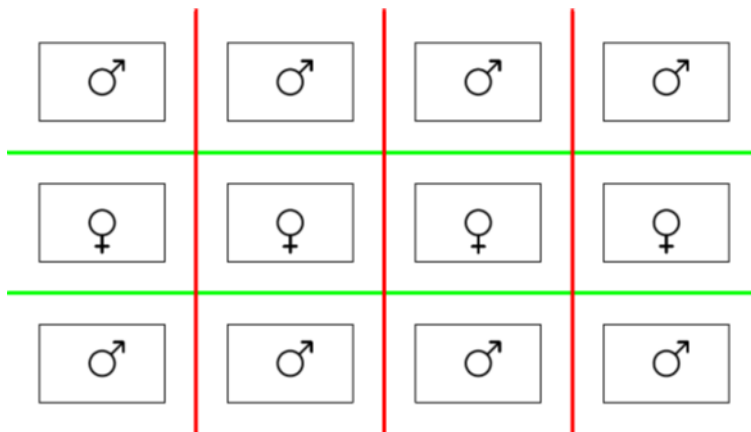


Figure 6. The layout of positioning of subjects socialized with female conspecifics in the shelf. Green lines represent solid partitions that were removed during socialization. Red lines stand for partitions that were not removed during socialization and that prevented visual contact with other males. Source: Author.

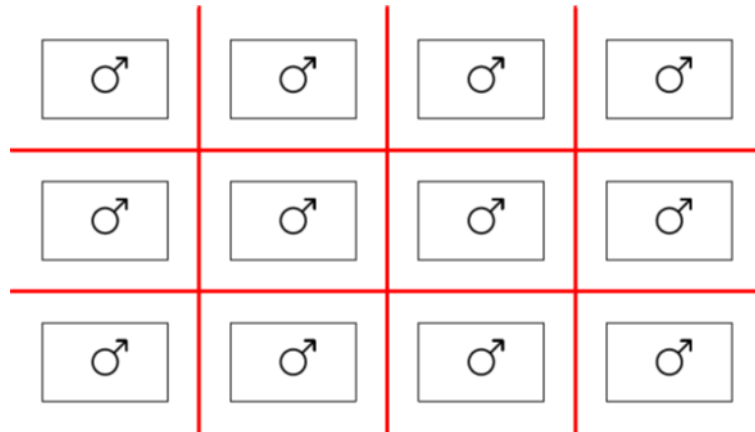


Figure 7. The layout of positioning of fully isolated subjects in the shelf. Green lines represent solid partitions that were removed during socialization. Red lines stand for partitions that were not removed during socialization and that prevented visual contact with other males. Source: Author.

In the beginning of the experiment, period of 12 days was set, during which fish could get used to new conditions. The long-termed (6 weeks) experiment followed. After that, transit period of seven days took place to prevent the effects of first experiment, so that the second, short-termed (3 days) could take place.

All the males were equally distributed into three groups of 25 individuals according to their colour phenotype (see appendices) as colour of the object that releases the stimulus may be important determinant of subject's behaviour in betta fish (Thomas and Sturm, 1965). Fish within one group were then randomly positioned next to each other. The three groups were made in order to differentiate the level of visual isolation: 1) males allowed to interact with conspecific males ("M-socialized" males); 2) males allowed to interact with conspecific females ("F-socialized" males), 3) male completely visually isolated from other individuals ("Isolated" males). The position of the groups in the shelves was randomized using Randomness and integrity services (2017) First two groups could interact 3 times a week for ten minutes during the long-termed experiment either with female or male conspecific, according to their group belonging and alternatively once a day for ten minutes within the short-termed experiment. The period of ten minutes was chosen so that every individual had a chance to interact with their neighbours and that the display was elicited.

Isolated males stayed divided by the opaque partitions for the whole length of the experiment. After three days of experimental treatment, fish were tested with mirror in 10l tank that was carefully cleansed after each testing with hot water and chlorine bleach Savo (Unilever, EU, year of manufacture: 2015) and then dechlorinated with water conditioner Akvaregulátor. The aggressiveness was evaluated by video recording of the behaviour targeted towards mirror image with DSRL cameras Canon EOS 500D (Canon Inc., made in Japan) and Nikon D5200 (Nikon Corporation, made in Thailand). The mirror was chosen to be a more accurate instrument than live opponent, as the live male does not control for all possible feedback effects (Hinkel, 1972). Four minutes of habituation period for the fish in experimental tanks with no distractions were chosen. The video recording of the behavioural response to the mirror took place for ten minutes. The beginning of the ten minute interval was considered as the moment when the mirror was presented to the fish, in the level of its sight. The insertion of mirror into the tank took place in situation where the fish was occurring in the middle third of the tank.

According to previously held studies (Simpson, 1968; Charles, 2003), the latency to first display to the mirror/rival, time spent opercular flaring and fin spreading were chosen to be the most indicative of *Betta splendens* aggressiveness. Opercular flaring and fin spreading are occurring mostly at the same time (Simpson, 1968), therefore for purposes of this study, gill flaring was chosen to be the indicative parameter. Also the occurrence and length of biting attempts was noted and evaluated, as it is easily recognizable aggressive behaviour that is widely used for determination of aggressiveness (Simpson, 1968; Halperin, 1998; Castro et al., 2006).

Conditions in short-termed and long-termed experiment were identical with the only difference in frequency of interactions of neighbouring socialized males: 3 times a week within long-termed experiment and once a day in short-termed experiment.

The purpose of holding the identical experiments with different lengths was to compare the reaction of the isolated fish, mainly the latency to fight, which was

believed to be shorter within isolates in general (Hinkel, 1972), but later contested by experiments of longer duration with different results (Halperin, 1992).

Ethical note: No animals were allowed to enter into physical combat or any other physical interaction. The housing and keeping method was chosen to suit the biological needs of the species and the facility resembled those used in similar researches.

4.2 Analysis

The measurements were gained using event-logging software BORIS v. 3.47 (Friard and Gamba, 2016) and evaluated using statistical software Statistica 10 (StatSoft Inc., 2011).

All animals that exhibited any unusual health or behavioural issues were excluded from the evaluation as well as individuals whose latency to display was longer than 3 minutes, with respect to that all those individuals met the conditions in both short-termed and long-termed experiment. Eventually 58 individuals were included into the analysis, with 20 in group 1, 19 in group 2 and 19 in group 3.

Before running the main analysis, Kolmogorov-Smirnov test for normality was used for each dataset. Analysis of variance – ANOVA was used for comparison of the length of observed behaviours between groups 1, 2 and 3, in particular for the length of opercular flaring. In cases where data were not normally distributed, which was latency and biting, non-parametric Kruskal-Wallis ANOVA test was used. For comparison of the changes in latency between treatments within one group, Wilcoxon test for comparison of two dependent variables was used in cases of datasets, which were not normally distributed. ANOVA repeated measures test was used when evaluating time spent opercular flaring, because its dataset was normally distributed. Many individuals did not perform biting behaviour at all.

5 Results

5.1 Latency to display

The data noting latency to display of the individuals were not normally distributed in both treatment durations (Kolmogorov-Smirnov test $d=0.19$, $p<0.05$, long-termed experiment and respectively $d=0.24$, $p<0.01$ for short-termed experiment).

In the **long-termed** (6 weeks) experiment **mean latency** to display \pm standard error was **37.74 s \pm 8.70 s** ranging from 4.87 s to 140.27 s in **M-socialized**; **25.14 s \pm 5.52 s** ranging from 2.43 s to 90.33 s in **F-socialized**, and **34.42 s \pm 8.65 s** with range from 2.93 s to 155.74 s in **Isolates**. In **short-termed** experiment (3 Days) the **mean latency** to display (\pm standard error) was **37.81 s \pm 10.32 s** in **M-socialized** ranging from 3.20 s – 161.90 s; **42.73 s \pm 12.05 s** with range 2.63 s – 166.07 s in **F-socialized** and **37.07 s \pm 8.81 s** ranging from 3.56 s to 138.02 s in **Isolates**.

Kruskal-Wallis test **long-termed** experiment ($H(2, N=58)=0.94$, $p=0.62$) confirmed that there were no significant differences between the latencies to display comparing the three experimental groups. Similarly, also the Kruskal-Wallis test revealed no significant differences in the case of **short-termed** experiment ($H(2, N=58)=0.07$, $p=0.97$).

Wilcoxon repeated measures test for evaluation of latencies between the two treatments in isolates revealed no significant ($p>0.05$) differences, nor in any of the socialized groups. **M-socialized** males resulted with $p=0.65$. Greatest tendency to decrease latency with long-termed experiment was present in the **F-socialized males**, however not significant ($p=0.23$). In last group of **Isolates** also slight but highly non-significant decrease of latency was marked ($p=0.97$). Figure 8 shows graphical comparison of the latency to display in all three groups of both treatments.

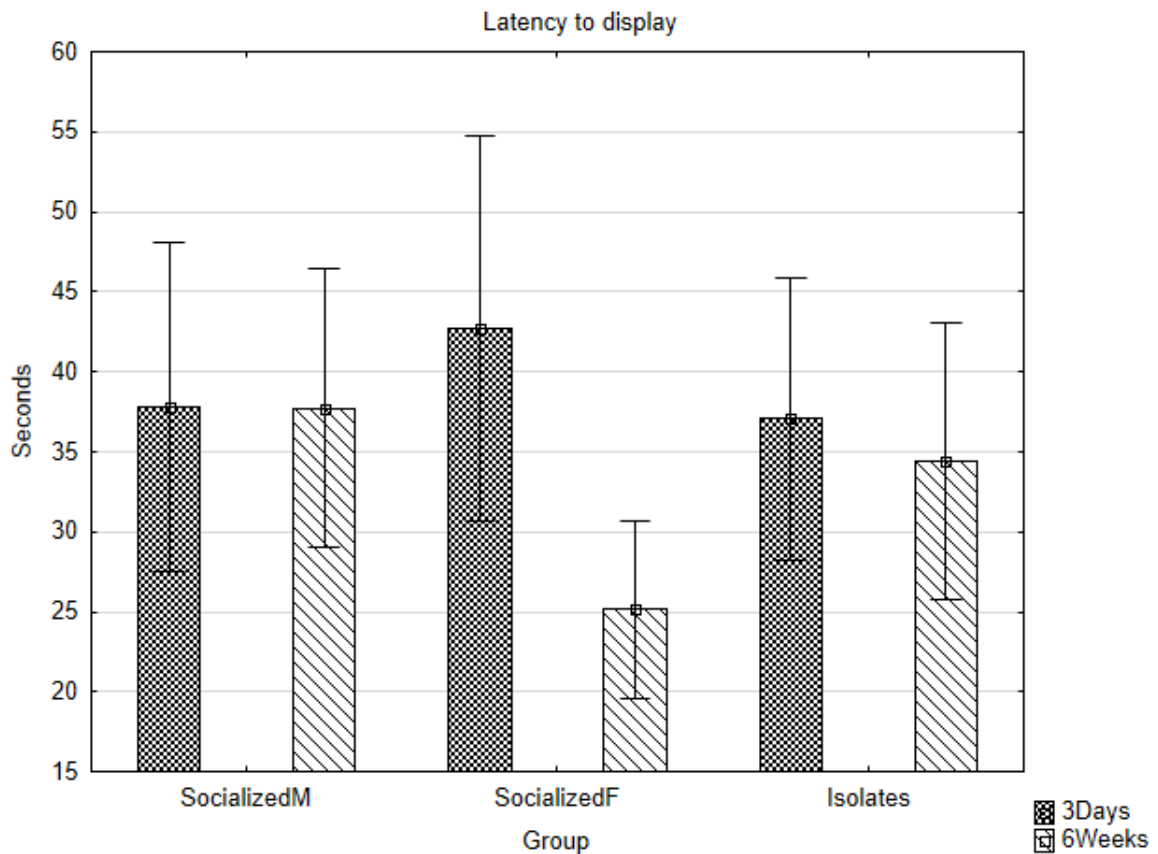


Figure 8. Visualization of the means with standard error plots of the latency to display, showing data gained from group of subjects socialized with male conspecifics (SocializedM), subjects socialized with female conspecifics (SocializedF) and fully isolated individuals (Isolates), in both 3 days long and 6 weeks long treatment. Source: Author.

5.2 Time spent opercular flaring

The normality of data distribution in the dataset of the length of opercular flaring was confirmed with Kolmogorov-Smirnov in the case of the long-termed experiment ($d=0.08$, $p>0.20$) and the short-termed experiment test ($d=0.07$, $p>0.20$).

In the **long-termed** (6 weeks) experiment, the **mean time spent opercular flaring** \pm SE was **285.09 s \pm 33.02 s** ranging from 48.89 s to 511.02 s in **M-socialized**; **300.55 s \pm 34.62 s** with range from 16.71 s to 515.32 s in **F-socialized** and **238,45 s \pm 27,91 s** ranging from 62.76 s to 461.48 s in **Isolates**. In the **short-termed** (3 days) experiment, the **mean time spent opercular flaring** \pm SE was **275.53 s \pm 33.19 s** ranging from 43.70 s to 517.73 s in **M-socialized**; **281.45 s \pm 33.66 s** varying from 14.31 s to 535.51 s in **F-socialized** and **289.34 s \pm 28.67 s** with range from 54.22 s to 528.43 s in **Isolates**.

Results of **ANOVA** test suggest that there is no significant difference in the time spent opercular flaring between groups of long-termed treatment ($p=0.37$), as well as in the short-termed treatment ($p=0.95$). Graphical comparison can be seen in Figure 9.

Repeated measure ANOVA showed no significant difference among the treatments (Wilks=0.91, $F(4, 108)=1.36$, $p=0.25$).

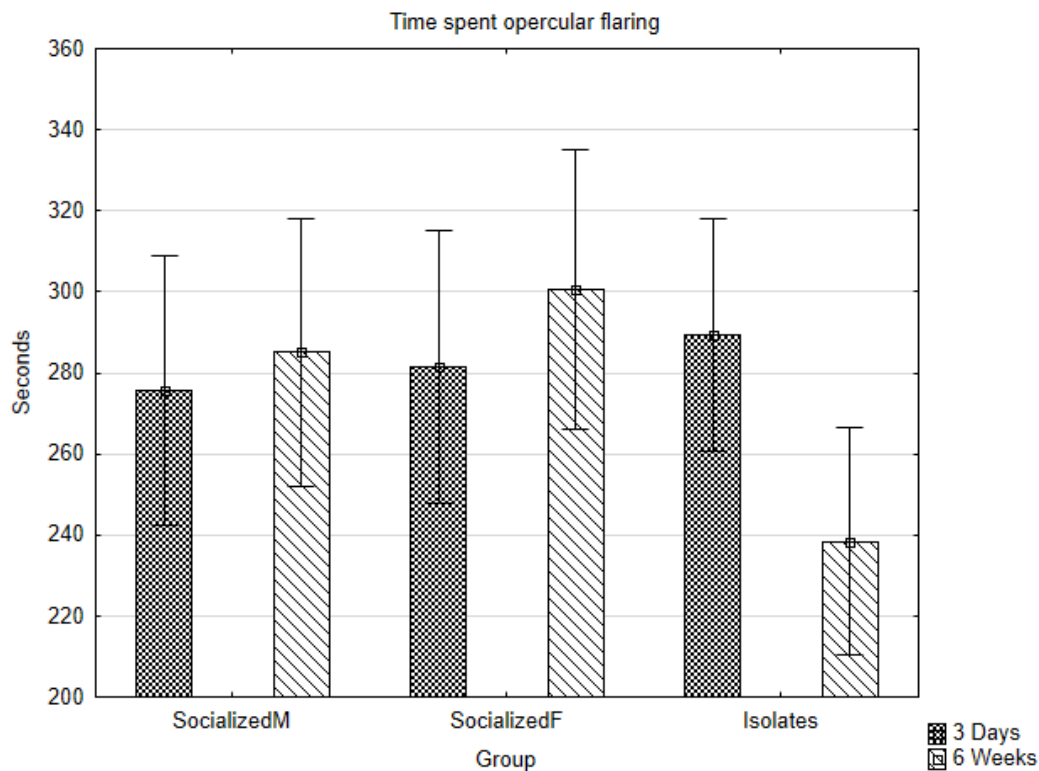


Figure 9. Mean±SE of differences in time spent opercular flaring in groups of subjects socialized with male conspecifics (SocializedM), subjects socialized with female conspecifics (SocializedF) and totally isolated individuals (Isolates) in both 3 days long and 6 weeks long treatment. Source: Author.

5.3 Time spent biting

Data distribution of values of time spent biting was not normal according to Kolmogorov-Smirnov test ($d=0.34$, $p<0.01$ in subjects of 6 weeks long experiment, and $d=0.30$, $p<0.01$ in subjects of 3 days long experiment).

In the **long-termed** (6 weeks) experiment, the **mean time spent biting ±SE** was **5.15 s ±2.95 s** with values ranging from 0 s to 57.96 s **M-socialized**; **9.31 s ±3.11 s** ranging

0 s – 43.52 s in **F-socialized**, and **6.55 s ± 3.54 s** with range from 0 s to 50.09 s in **Isolates**. **Short-termed** (3 days) experiment resulted with time spent biting **14.80 s ± 7.47 s** with range from 0 s to 142.36 s in **M-socialized**; **17.83 s ± 5.89 s** ranging from 0 s-92.38 s in **F-socialized** and **19.08 s ± 9.07 s** ranging from 0 s to 128.37 s in **Isolates**.

Kruskal-Wallis ANOVA test running showed no significant differences among the three groups in 6 weeks long experiment ($H(2, N=58) = 4.29$ $p=0.12$), as well as in the 3 days long experiment with values ($H(2, N=58) = 1.86$ $p=0.39$). Figure 10 shows results graphically.

Repeated measure Wilcoxon test showed significant drop in time spent biting after the long-termed experiment in **M-socialized** ($p=0.015$) males and **Isolates** ($p=0.038$) as well.

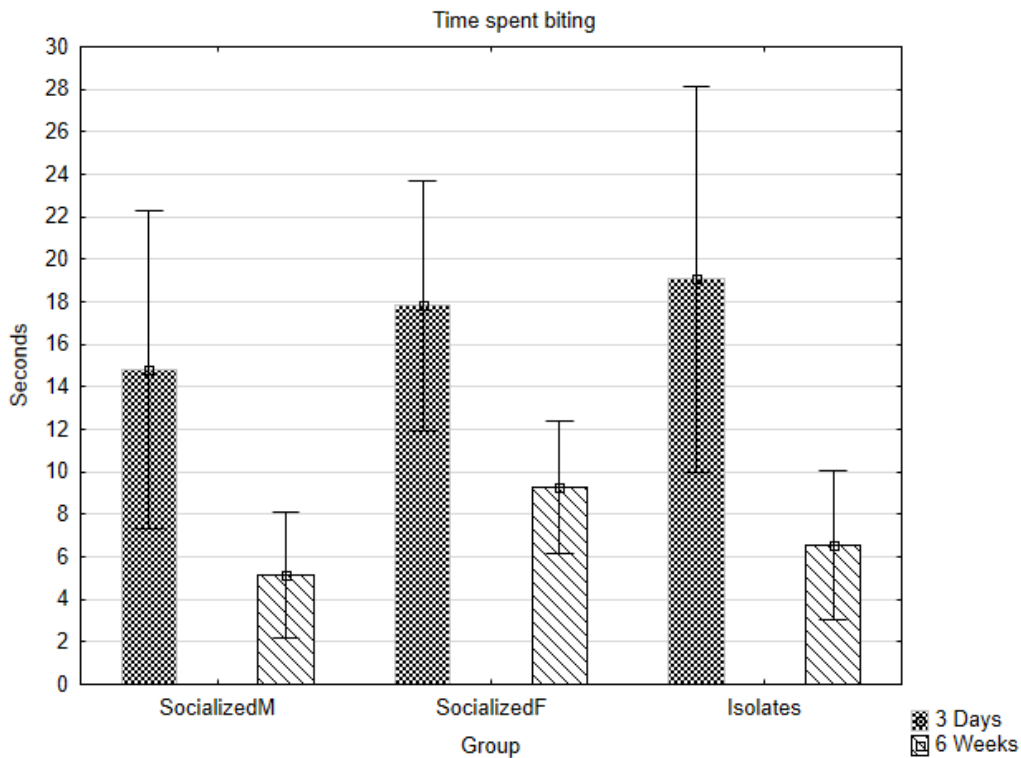


Figure 10. Mean ±SE of differences in time spent biting in groups of subjects socialized with male conspecifics (SocializedM), subjects socialized with female conspecifics (SocializedF) and totally isolated individuals (Isolates) in both 3 days long and 6 weeks long treatment. Source: Author.

6 Discussion

Presented experiment was assigned to reveal any differences in aggressiveness among *Betta splendens* males that were either socialized with other male conspecifics (“M-socialized males”), female conspecifics (“F-socialized males”) or completely visually and physically isolated individuals (“Isolates”). We hypothesised according to Halperin et al. (1992), Halperin and Dunham (1994), and Hinkel (1972) that isolated males will increase their aggressiveness compared to their visually-social conspecifics, and that the aggressiveness will grow with the length of the experiment (3 days vs. 6 weeks). That was supposed to be the result of absence of aggression releasing stimuli from other males. Stated predictions were not confirmed as it can be seen from the non-significant ($p > 0.05$) results of statistical comparison of two highly indicative aggressive behaviours – opercular flaring (and fin spreading) and biting, targeted towards mirror image. By contrast to Halperin et al. (1992), series of novel models was not used for this experiment’s evaluation. Rather than that, a mirror was supposed to elicit an aggressive response in subjects, as live opponent would do. In this part of experiment, the methods resembled Hinkel’s (1972) experiment, as he used the mirror image for evaluation too.

Increased aggressiveness in isolated *Betta splendens* males was not confirmed in a study by Charles (2003). She did a research in which she showed sequence of mirrors and models to visually-social males and visually-isolated males, for a period of 7-10 weeks. Social isolation had no effect on the frequency and duration of aggressive behaviour. Her possible explanation was that the results got biased during the process of data collection, as both visually-social and visually-isolated fish displayed high amount of behaviours that were expressing high fear. Ichihashi (2004), who did a comparison of aggressiveness between males reared in complete isolation and other males with some level of contact with conspecifics, found that the incidence of aggressive display (including gill cover erection) was not significantly different than in other males. However, they were able to assess the winners after physical combat with socially reared males, and found out that isolates not only won in significant amount of times, but also performed aggressive behaviour for unnecessary long period.

Unfortunately, a conclusion that isolated males perform more or less aggressive behaviour than socialized conspecifics cannot be made from results of this experiment, since data showed no significant difference, as well as in Charles' (2003) research. However excessive aggression post isolation in males of *Betta splendens* is expected, due to many paper's confirmations of this effect. There may be several conditions that could have influenced the results of this research, one of them being handling of the fish prior to the experimental evaluation. Fish had to be moved to the room of video-recording, and this could have resulted in frightening and biased behavioural response to the mirror image.

Another aim of this thesis was to document any changes in latency to display of the males, as it is stated to be changed post isolation period in *Betta splendens* males (Halperin and Dunham, 1994). Hinkel (1972) reported greater readiness to approach mirror images in isolated males, escalating with the length of experiments (duration of 15 minutes, 6 hours, 24 hours and 72 hours). On the other hand, Halperin et al. (1992) found that males isolated for 3 weeks eventually showed depressed display readiness. These findings were considered in this study. To unify and conclude integrated results, latency to display was chosen as a next measure in this experiment and it was evaluated after 6 weeks (long-termed experiment) and then after 3 days (short-termed experiment) in isolates. The hypothesis that short-term isolated males will result with quicker latencies and long-term isolated males with longer latencies to display was not confirmed by this study, as the results did not show any significant difference ($p > 0.05$) between the treatments. Again, it is assumed that the non-significant outcomes in latency were caused by possible disturbances that occurred during the experiment or its finalization with the video-recording. The greatest drop in the latency time was actually observed in the group of males socialized with females, when comparing treatments, however non-significant ($p > 0.05$). This effect could be attributed to the change of frequency of exposition to females (3 times a week in long-term experiment and each day in short-termed experiment). Possibly longer gaps between socialization with females may result in larger readiness to display to the opponent, but this cannot be concluded with such non-significant results.

One of the supposed outcomes of this study was also different level of aggressiveness between the F-socialized males and M-socialized males during the experiment. The papers that examine the influence of isolation on aggressiveness in males are comparing the groups of isolates with groups of males that have been kept in company of other males or presented with mirror images (Halperin, 1992; Halperin, 1998; Charles, 2003). But as far as one knows from the studies that tested the audience effect in agonistic encounters (Doutrelant et al., 2001; Matos and McGregor, 2002) and influence of the composition of social environment in previous housing (Lacava et al., 2011; Ruploh et al., 2013), gender that is chosen for socialization has a great impact on the tested subject in aggression tests. Therefore two variations of comparative groups were established; the F-socialized males and M-socialized males, to check for the possible differences in the outcome. Unfortunately none of the groups resulted with significantly different results that would represent contrast in aggression between those groups.

Unexpected results were observed in differences of time spent biting. The M-socialized males from the long-termed experiment resulted with significantly ($p=0.015$) less time spent biting, thus being less aggressive. Since the socialization occurred three times a week during the long-termed experiment and every day in short-termed experiment, result could be suggesting that the difference in aggressiveness may have been influenced by the very frequency of socialization. Such outcome could indicate that males are getting less aggressive when they have scarcer chances to perform aggressive behaviour, which is contradictory to Lorenz's (1966) hypothesis and also to findings of Halperin and Dunham (1994) who found that social overstimulation, in their case continual visual exposure, reduces subsequent aggressive behaviour. As a mean of evaluation, they also picked to assess biting, which was dramatically reduced in socially overstimulated fish.

Isolated males also showed significant ($p=0.038$) decrease in time spent biting in the long-termed of experiment compared to short-termed experiment, which could be possibly interpreted as that with the increasing length of isolation, males lower their subsequent aggressiveness. Similar trend was observed also when comparing the time

spent opercular flaring, however in this case the decrease in time spent performing of this aggressive behaviour was not significant. Again, statement that longer absence of aggression releasing stimuli results in lowering of aggressiveness is in contrast with Lorenz's (1966) hypothesis.

Tested hypotheses (H1: The amount of aggressive response of isolated males will be higher than in socialized males, H2: Latency to display will be quicker in short-term isolated males than in long-term isolated males and H3: The aggressiveness of males from the comparative groups) were thus not confirmed by this study.

7 Conclusion

Experiment testing differences in aggressiveness among *Betta splendens* isolated males, males socialized with other males, and males socialized with females was held. The aim was also to document the differences in latency to display in individuals after two periods of isolation of different length, as results from previously held studies were not uniform in the results (Hinkel, 1972; Halperin et al., 1992).

No significant difference ($p>0.05$) in time spent biting, and opercular flaring was found between the groups. That means that the isolates were not more nor less aggressive than the socialized individuals in this study. Also there was no significant difference ($p>0.05$) in aggression between the distinctly socialized groups (“F-socialized” and “M-socialized” males). The latency of isolates between treatments was not significantly different ($p>0.05$). Results were in both in consistency with some of the previously held studies and in contradiction to others. To conclude integrated results, it is suggested to repeat and continue the research. Methods comparison of this thesis and other studies was discussed.

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List of appendices

Appendix 1. Red-blue colour phenotype.

Appendix 2. Blue colour phenotype.

Appendix 3. Red colour phenotype.

Appendix 3. Yellow colour phenotype.

Appendices



Appendix 1. Red-blue colour phenotype. Source: Author.



Appendix 2. Blue colour phenotype. Source: Author.



Appendix 3. Red colour phenotype. Source: Author.



Appendix 3. Yellow colour phenotype. Source: Author.