

**Univerzita Karlova v Praze**  
**Přírodovědecká fakulta**

Studijní program: Biologie  
Studijní obor: Ekologie



**Bc. Lucie Kubická**

Behavioural differences between species and populations in the killifish genus  
*Nothobranchius*

Mezidruhové a mezipopulační rozdíly v chování halančků rodu *Nothobranchius*

Diplomová práce

Školitel: Doc. RNDr. Martin Reichard, Ph.D.

Praha, 2016

**Prohlášení:**

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze,

Podpis

## Poděkování

Ráda bych velmi poděkovala mému školiteli Martinu Reichardovi za veškerou pomoc, trpělivost a přátelský přístup a za užitečné rady týkající se mé diplomové práce. Podporou pro mě byli také Radim Blažek a Matej Poláčik, kteří mi pomáhali během pokusů. Také děkuji mému konzultantu Lukáši Kratochvílovi. V neposlední řadě chci vyjádřit veliké díky mé rodině a přátelům za veškerou podporu a trpělivost během celého mého studia.

Tato diplomová práce byla vypracována na Ústavu biologie obratlovců AV ČR, v.v.i. a byla podpořena projektem Grantové agentury ČR č. 506-11-112.

## Abstract

I used annual killifish *Nothobranchius orthonotus* to investigate two separate questions related to behavioural aspects of their life history. The first study focused on spatial cognitive ability of two *N. orthonotus* populations that originated from temporary pools that varied in their expected duration due to differences in annual precipitation patterns (dry and humid region). Test of cognition was based on the ability to learn to find food in a dichotomous test. It was assumed that learning ability differs between populations when tested in young and old age along with their difference in captive lifespan. Additionally, association between cognitive ability and other life history traits was tested. It was found that: (1) there was no difference in the ability to learn spatial task between populations; (2) both populations had similar lifespan with no cognitive decay in older age, and older fish from dry region population had committed even less errors in the learning task than they did as young fish; (3) fish were able to effectively learn the task but (4) solitary fish had lower learning ability than group-reared fish; (5) longer-living fish were quicker learners; (6) high resting metabolic rate was associated with more effective learning; (7) hesitant fish found the food reward in learning task in shorter time. The possible explanations of these findings are discussed. The second study focused on male-male aggression of *N. orthonotus* towards conspecific and heterospecific males of *N. furzeri* and *N. pienaarri*. The male colouration in *N. orthonotus* was assumed to serve as a cue for identification of opponents. The hypothesis was that male of *N. orthonotus* will show the highest level of aggression and interest towards conspecific male and to similarly coloured red male morph of *N. furzeri*. In contrast, the least aggressive behaviour was expected toward dissimilarly coloured yellow morph of *N. furzeri* and black male of *N. pienaarri*. The hypothesis was confirmed, showing that colouration plays a significant role in opponent recognition for *N. orthonotus* males, and several explanations are discussed.

**Key words:** *Nothobranchius*, *N. orthonotus*, killifish, personality, behaviour, cognitive ability, learning, male-male aggression, male colouration

## Abstrakt

V této diplomové práci jsem studovala dvě různá témata týkající se druhu halančička *Nothobranchius orthonotus*. První studie se věnovala prostorové kognitivní schopnosti dvou populací *N. orthonotus*, které pocházely z dočasných tůní lišících se v jejich předpokládané délce existence kvůli rozdílnému množství průměrných ročních srážek (suchá a vlhká oblast). Testování kognice bylo založeno na schopnosti naučit se nalézt potravu v dichotomickém testu. Předpokládalo se, že schopnost učení se bude lišit mezi populacemi v období dospělosti a stáří testovaných ryb. K dispozici byla data k různým znakům životní strategie, která se použila k testování jejich možných korelací s kognitivními schopnostmi. Bylo zjištěno, že (1) mezi populacemi nebyl rozdíl v prostorové kognitivní schopnosti; (2) délka života byla zhruba stejná u obou populací, přičemž starší ryby nevykazovaly zhoršení kognice, a starší ryby z populace ze suché oblasti dělaly méně chyb při testování než mladé ryby; (3) ryby byly schopné relativně rychlého učení, ale (4) ryby chované celý život izolovaně se učily pomaleji než ryby chované ve skupinách; (5) nejdéle žijící ryby byly rychlejší v učení; (6) čím vyšší byl klidový energetický výdej ryby, tím lépe se učila; (7) váhavé ryby nalézaly potravu v testu za kratší čas. V práci diskutuji možná vysvětlení těchto výsledků. Ve druhé studii se soustředím na samčí agresivitu druhu *N. orthonotus* vůči samcům vlastního druhu a dalších dvou druhů halančičků *N. furzeri* (se dvěma barevnými formami samců - červenou a žlutou) a *N. pienaarum*. Předpokládalo se, že červené zbarvení samců *N. orthonotus* slouží jako vodítko k identifikaci potencionálního soupeře. Hypotéza byla, že samci *N. orthonotus* budou projevovat nejvyšší agresivitu a zájem vůči samcům vlastního druhu, ale také vůči podobně zbarvené červené samčí formě druhu *N. furzeri*. Nejmenší agresivita byla naopak předpokládána vůči odlišně zbarveným samcům, tedy vůči žluté samčí formě *N. furzeri* a černě zbarveným samcům *N. pienaarum*. Tuto hypotézu se podařilo potvrdit a tím ukázat, že zbarvení samců *N. orthonotus* hraje důležitou roli v rozpoznávání ostatních samců a v míře použité agresivity a zájmu.

**Klíčová slova:** *Nothobranchius*, *N. orthonotus*, halančičci, personalita, chování, kognitivní schopnosti, učení, samčí agresivita, samčí zbarvení

# Obsah

<b>1 Introduction</b>	1
1.1 Cognitive abilities in fishes	1
1.2 Male-male aggression	5
1.3 African annual fishes	8
1.3.1 Introduction	8
1.3.2 Habitat, diet, distribution and phylogeny of the genus <i>Nothobranchius</i>	9
1.3.3 Sexual dimorphism and male colour polymorphism	13
1.3.4 Sex determination, sex ratio and reproductive behaviour	14
1.4 <i>Nothobranchius</i> species and their use as model organisms	15
1.5 Hypotheses and aim of the work	18
<b>2 Materials and methods</b>	20
2.1 Experiment 1 – learning ability	22
2.1.1 Details on the maintenance of experimental <i>Nothobranchius orthonotus</i>	22
2.1.2 Experimental methods	23
2.2 Experiment 2a – intraspecific and interspecific aggression of males	28
2.2.1 Experimental conditions	28
2.2.2 Experimental methods	29
2.3 Experiment 2b – interspecific aggression among males of <i>N. orthonotus</i> and two colour forms of <i>N. furzeri</i>	31
2.3.1 Experimental conditions	31
2.3.2 Experimental methods	32
2.4 Data analysis	32
2.4.1 Experiment 1 – learning ability	32
2.4.2 Experiments 2a a 2b - intraspecific and interspecific aggression of males	34
<b>3 Results</b>	36
3.1 Experiment 1 – learning ability	36
3.1.1 General results	36
3.1.2 Full dataset (all tested fish)	37
3.1.3 Learning and life history traits	41
3.2 Experiment 2a – intraspecific and interspecific aggression of males	46
3.3 Experiment 2b – interspecific aggression among males of <i>N. orthonotus</i> and two colour forms of <i>N. furzeri</i>	48
<b>4 Discussion</b>	51
4.1 Experiment 1 – learning ability	51
4.2 Experiments 2a and 2b – intraspecific and interspecific aggression of males	56
<b>5 Summary</b>	62
<b>6 References</b>	64

# 1 Introduction

## 1.1 Cognitive abilities in fishes

A broad definition of cognition includes all mechanisms that animals have for taking in information through the senses, retaining it, and using it to adjust behaviour to local conditions (Shettleworth, 2009). Major general cognitive traits include perception, learning, long-term memory and working memory, attention and decision making (Dukas, 1998). The importance of cognition is manifested in a large variety of behavioural traits that affect animal ecology and evolution, such as habitat selection, choice of food, predator avoidance, mate choice, social behaviour and behavioural shifts leading to adaptive radiations (Dukas, 1998). The phenotypic manifestation of cognitive abilities can be seen only indirectly by quantification of a change in behaviour. Thus, most animal cognition researchers have been interested in identifying and quantifying what cognitive ability(ies) cause observable behavioural changes (i.e. the mechanism of variation in behaviour) (Griffin *et al.*, 2015). In contrast, behavioural ecologists aim to determine the adaptive significance of a trait (i.e. the functional basis for variation in behaviour) (Griffin *et al.*, 2015). Specific experimental designs are being used so that they would be able to disentangle behavioural responses attributable to certain cognitive ability from other behavioural responses to different underlying cognitive mechanisms (Griffin *et al.*, 2015).

Throughout much of the 20<sup>th</sup> century, historical and social factors delayed extensive research on the evolutionary biology of animal cognition (Dukas, 2004). This was due to mostly independent development of ethology and animal psychology and their little communication (Richards, 1989). Important aspects of cognition have traditionally been in the domain of animal psychology which was studying learned behaviour in only few species in the laboratory conditions (Shettleworth, 2001). Conversely, ethology was at that time focusing mainly on innate behaviour of animals in the field (Shettleworth, 2001). Ethologists were using evolutionary thinking at work, whereas psychology in general was avoiding such thinking or was even opposed to it (Galef, 1998). Another reason for the little communication between these two fields was that ethology developed primarily by zoologists in Europe, whereas animal psychology developed mostly in North America (Shettleworth, 2001). However, at the end of the twentieth century, the old barriers began to fade rapidly thanks to integrative research on the genetics, neurobiology and evolutionary biology of cognition (Dukas, 1998; Shettleworth, 2009). There was the emergence of a number of subfields that are

integrating both psychological and biological approaches to mechanisms of animal information processing and decision making, such as cognitive ethology (Ristau, 1991), cognitive ecology (Real, 1993) and others. At the same time, research on animal behaviour in the field of behavioural ecology started to embrace studies of proximate cause (e.g. Krebs and Davies, 1997).

Since then, the research of cognitive abilities has been extensive and has showed many interesting new insights into various cognitive traits, their effects on fitness and evolution of cognitive traits. At first, cognitive ability was studied mainly in birds and mammals with only few studies investigating cognition in fish but by time this began to change (Odling-Smee and Braithwaite, 2003a). An example is research on learning ability which plays a pivotal role in the behavioural development of all vertebrates, including fish (Laland *et al.*, 2003). The articles concerning the role of learning in fish has grown from 70 published papers till 1992, to over 500 such papers till 2003, with majority being published shortly before that year, and this trend continues (Braithwaite, 2006; Laland *et al.*, 2003). This escalation in interest was caused by a big change in the way fish were perceived. The infamous image of fish having a 'three-second memory' and being able of only basic behaviours has been gone (or at least concerned to be obsolete) and that happened due to the growing evidence of quite complex cognitive abilities of fish (Laland *et al.*, 2003). For example, social intelligence of teleost fish is pursuing Machiavellian strategies of manipulation, punishment and reconciliation (Bshary *et al.*, 2002). Fish can also have stable cultural traditions (Warner 1988, 1990) and co-operate to control predators and catch food (Dugatkin 1997). The ability to recognize and remember individual conspecifics has been shown in shoaling species but they are also capable of monitoring the social prestige of others based on previous experience (Utne-Palm and Hart, 2000; Griffiths, 2003; Gómez-Laplaza, 2005) and even discriminate between different numbers of individuals in shoals (Agrillo *et al.*, 2007, 2008).

An interesting suggestion was that fish cognition is actually similar to cognition of primates, with employment of similar mechanisms to primates when they solve problems, and therefore in some cognitive domains, fishes can be favourably compared to nonhuman primates (Bshary *et al.*, 2002; Laland and Hoppitt, 2003). For example, some fish can use tools (Bshary *et al.*, 2002) and build complex nests either for egg laying or for shelter against predators (Paxton and Eschmeyer, 1998). Studies have shown impressive learning and long-term memory systems in some species and their importance in foraging activities of fish (Brown, 2001; Warburton, 2003). The environment that an animal inhabits largely affects development and evolution of morphology as well as its life-history characteristics and



behaviour (Brown and Braithwaite, 2005). Spatial complexity and spatial stability within a habitat has a major impact on speed of learning abilities of fish, the duration of memories, and the type of spatial cues they prefer to use for orientation (Odling-Smee and Braithwaite, 2003b). The environmental variability of the habitat in which fish are living seems to have effect also on the length of the memory window for prey (Warburton, 2003). The populations of the same species can differ in spatial learning and in the types of cues they choose for orientation according to the variation in the habitat complexity (Odling-Smee and Braithwaite, 2003b). For example in 4 species of intertidal gobies, local visual landmarks were used as reliable indicators only in stable, complex pond habitats (rock substrate) whereas body-centred method for orientation was used more in dynamic homogenous habitats (sandy sediment) (White and Brown, 2014).

On the other hand, natural selection will presumably favour higher cognitive abilities in environment that is rather unpredictable or changing (Brown *et al.*, 2003). Even a single change in resource availability in early life can permanently improve the learning abilities of animals because it signals the developing individual that it is inhabiting unpredictable environment (Kotrschal and Taborsky, 2010). The knowledge of the positive effects of environmental enrichment on behavioural flexibility can be used to improve post-release survival of captive-bred animals which are being intended to be released into the wild as a conservation strategy, like in the case of Atlantic salmon (*Salmo salar*) (Brown *et al.*, 2003; Salvanes *et al.*, 2013).

In general, it started to be assumed that learning and memory are suited accordingly to specific needs of a species habitat and way of life (Brown, 2003; Brown and Braithwaite, 2005). Not only habitat variability plays a role in learning but also predation pressure can shape learning rate, with lower learning rate in populations experiencing high predation pressure (Brydges *et al.*, 2008). Also, the variation in learning and memory ability in fish is very wide not only across the all known species but, in some cases, among the distinct populations and geographical variants of the same species as well (Odling-Smee and Braithwaite, 2003b). One of the most studied type of learning is spatial learning in which visual landmarks are used by fish and some studies even suggest that spatial abilities of fishes are comparable to those of humans (Sovrano *et al.*, 2002).

Generally, fish are the biggest extant vertebrate group inhabiting virtually every conceivable aquatic environment and thus there was sufficient amount of time for evolution of complex, plastic and diverse behaviour patterns that compete that of other vertebrates (Laland *et al.*, 2003). This new appreciation of fish as the taxon with wide behavioural and social

sophistication can help deeper understanding of the cognitive skills like perception, learning and memory.

Selection for increased cognitive ability is suspected to play an important role in evolution of larger brains (Striedter, 2006; Kotrschal *et al.*, 2013). It was shown that fish with larger brains had slower search time in the beginning but made fewer errors and learned faster in a spatial memory test (Kotrschal *et al.*, 2013, 2015). However, the size itself does not have to be the main reason for better cognitive ability as was discovered that numerous fine-scale structural differences in the brain are being linked to cognition (Toga and Thompson, 2005). Not only natural selection but also sexual selection can influence size of brain (Madden, 2001; Gonzalez-Voyer *et al.*, 2008). It was shown that sexual selection may be the driving force of evolution of cognitive ability in various species (Boogert *et al.*, 2011). Sexual selection acts on differences in reproductive success among individuals of the same sex that compete with each other (intrasexual selection) for the access to the opposite sex which is choosy (intersexual selection); hence this form of selection shapes mating system evolution (Darwin, 1871). Males are often under stronger sexual selection due to sex differences in search strategies and allocation of resources for gametes (Kokko *et al.*, 2006). In order to have selection on cognition, there must be heritable variation which affects individual' fitness (Dukas, 2004). The influence of cognition can be significant in the ability to find a mate, choose a mate or in success in fertilization, depending on what are the selective forces in a specific mating system (Smith *et al.*, 2015). It is predicted that selection for cognitive ability will be more pronounced in males as they are typically the sex with large variance in reproductive success and more strongly expressed traits like nuptial coloration, weapons or alternative mating behaviours (Smith *et al.*, 2015). There are empirical evidences of intersexual selection on cognition, with females preferring males with better cognitive skills, however, little is known on this in fish (Shohet and Watt, 2009; Boogert *et al.*, 2011). Relationship between learning accuracy and reproductive success was shown in males of rose bitterling (*Rhodeus ocellatus*) as well as heritability of that trait, implying that male cognitive traits may undergo intrasexual selection (Smith *et al.*, 2015).

Also, the social environment can shape fish cognitive ability in various directions, including effect on longer-term, evolutionary trajectories (Brown and Laland, 2003). In captivity, much less interactions are often available to fish and very little social stimulus can lead to a decrease in learning performance. It was shown that in some cases fish rely, in a significant way, on social learning through which they can acquire important knowledge, for example on antipredator behaviour, migration, orientation and foraging (Brown and Laland,

2003). For example, guppies proved to be able to learn the way to hidden food sources from knowledgeable conspecifics during an experiment (Laland and Williams, 1997). This ability was assumed to be generated by the shoaling social structure (Laland and Williams, 1997). It may be possible that by removing the chance to interact with other fish of its own species, learning ability would not have enough stimulus to develop to the same degree as if the fish was living in a group (Byrne and Bates, 2007). It was shown that high mortality rates of hatchery-reared fishes directly after their release can be decreased if the social learning processes are being used prior to their release into the wild (Brown and Laland, 2001). Despite those studies that demonstrate importance of social learning in fish, it is not currently known how much is this ability widespread among fish taxa. In my study of cognition, fish originated from two different social environments, with one half of fish being maintained in groups in larger tanks and the other half being isolated from any social interactions. Therefore, this gave me an opportunity to test whether these different social environments could have effect on the studied learning ability in *N. orthonotus*.

Taken together, fish have proved to be very flexible and pragmatic biological model system for studying cognitive abilities, their effects on fitness and evolution. However, further studies are needed for better and deeper understanding of how cognitive traits evolved, how they are associated with reproductive success and also what are the constraining trade-offs in their evolution (Smith *et al.*, 2015).

## **1.2 Male-male aggression**

Two key factors in Darwin's (1871) theory of sexual selection are female choice and male-male aggression. These two components often act simultaneously (in concert or in opposition) on the evolution of certain male traits (Benson and Basolo, 2006) but they can act separately as well (Hunt *et al.*, 2009). What is the purpose of male-male aggression? The outcome of agonistic behaviour (i.e. any kind of social behaviour related to fighting) can significantly influence distribution of resources and territories among individuals, and moreover, it determines in many cases who will breed within the population (Morris *et al.*, 1992). Therefore, winning in male-male contest can provide many benefits to male as he obtained better resources in various forms, such as superior breeding territories, and dominance. Also females often preferably mate with dominant males that are usually the winners of intrasexual competition (Andersson, 1996). For these reasons, male aggression may play important role in sexual selection of a species.

There are two types of competition; exploitative (occurs indirectly through common limiting resource) and interference competition (or direct competition) which occurs directly via aggression (Andersson, 1994). Aggressive encounters among conspecific males (i.e. within the species) have been well known and studied in evolutionary biology (Grether *et al.*, 2013). However, the aggression between heterospecific males (i.e. belonging to different species) is actually very common and its intensity can be the same as within species (Peiman and Robinson, 2010). The evolutionary consequences of interspecific interference competition have been much less studied even though it must have important ecological and evolutionary effects (Grether *et al.*, 2013). What is known about this phenomenon? It seems that interspecific competition can affect selection on encounter rates (i.e. habitat preferences and activity schedules), recognition of competitor and traits of fighting ability (i.e. weapons, body size) (Cody, 1969; Grether *et al.*, 2009; Laiolo, 2012). When two species live in sympatry long enough and they are competing for a limiting resource, selection may lead to convergence in traits involved in competitor recognition because of the benefits of recognizing a true competitor outside own species (Cody, 1969). Conversely, if there is no limiting resource that would create competition, the costs of accidental interspecific aggression may drive the selection towards divergence in traits for recognition (Grether *et al.*, 2009). Whether the outcome of sympatry will be divergence or convergence of agonistic signals and competitor recognition, depends on the intensity of interspecific exploitative competition (Grether *et al.*, 2009).

Besides two species competing for limiting resource, another way how interspecific aggression can arise is through their common ancestry as a byproduct of similar intraspecific competition (Schultz and Switzer, 2001; Tynkkynen *et al.*, 2004). Other possible alternative is arising of interspecific aggression as a byproduct of intraspecific competition in cases when speciation occurred in parapatry or sympatry, without allopatric phase (Grether *et al.*, 2013). Generally, species living in sympatry probably undergo selection which will eventually lead to change of the rate or intensity of aggressive encounters (Grether *et al.*, 2009) and cause sympatric populations to diverge from allopatric populations (Qvarnström *et al.*, 2012).

The way in which male-male competition is being expressed varies widely across animal kingdom and mating systems. Interference competition can include both apparent physical confrontation as well as non-tactile signalling contest (i.e. ritualized fight) through which information about opponent's strength is signalled (Moynihan, 1998). Decision of whether to initiate a contest or not, depends on the information which opponent gained before and during the encounter (Smith, 1982). In many confrontations, low-level agonistic displays are

sufficient for cessation of competition before the onset of physical fight (Enquist and Jakobsson, 1986). One of the important factors that can directly affect the outcome of aggressive encounter of two males is size (Andersson, 1994). Body size serves as a good predictor of physical strength and performance, with the larger individual usually winning (McCullough and Simmons, 2016). Size is also one of the best cues for estimation of the 'resource holding potential' (i.e. the measure of the individual's fighting ability) (Parker, 1974). Surprisingly, even when the favours are against the smaller subordinate males, they can still initiate the conflict more often than larger male (however, they still almost always lost) (Moretz, 2003). Aggressive decision can be affected by many traits as well as by who is a resource holder and who attacker, with the holder having greater expendable fitness budget and thus an advantage (Parker, 1974). When the asymmetries between the opponents are not significant enough to resolve the contest just by initial displays, the need for escalation arises and males physically attack each other (Leiser *et al.*, 2004).

Colour polymorphisms belong to one of the most striking examples of phenotypic diversity and this phenomenon has been used in evolutionary ecology to try to reveal the processes that underlie such remarkable diversity within species. Polymorphic species exhibit consistent genetic variation in colouration and they have provided insights into mechanisms responsible for individual variation, phenotypic variation and speciation (Gray and McKinnon, 2007; McKinnon and Pierotti, 2010). Colour polymorphism can be found among many animal species like fishes, frogs, lizards and birds (e.g. Hoffman and Blouin, 2000; Roulin, 2004). One of the possible mechanism maintaining this colour variation in the same species can be negative frequency-dependent selection (in which rare types are favoured by selection) (Sinervo and Lively, 1996). However, aggressive interactions among males can also act as a powerful selective force in the evolution of colour polymorphisms and its colour frequencies in the same species (Lehtonen *et al.*, 2015). One way how aggression can be maintaining particular colour morph is by fitness advantage of being more aggressive than other morphs (Dijkstra *et al.*, 2007). However, such competitive bias may have consequences not only in one species, but also in interactions with other species. Similarity in colour may give rise to more aggression even among sympatric species, where heterospecific intruders (belonging to different species) can be attacked as aggressively as conspecifics when their colouration is similar (Anderson and Grether, 2010). In nature, the aggression between species is actually very common and their intensity can be the same as within species (Peiman and Robinson, 2010; Grether *et al.*, 2013). Therefore, such biased levels of aggression can be strongly influencing patterns of species distribution and coexistence (Seehausen and Schluter, 2004).

Nevertheless, there have been only few studies considering this apparently important role of aggression and evolutionary consequences of heterospecific aggression (Grether *et al.*, 2013).

## 1.3 African annual fishes

### 1.3.1 Introduction

African annual fishes can be found in two clades within the family Nothobranchiidae (order Cyprinodontiformes) with Gondwanan distribution (Murphy and Collier, 1997). There is also the same ecological guild of related killifish (family Rivulidae) distributed in the Neotropics. They all share the same ability to live in a unique environment of isolated temporary pools filled mainly by rainwater (Watters, 2009). Most of the African annual fishes belong to the genus *Nothobranchius*, which currently contains over 70 described species (Froese and Pauly, 2016). This genus is distributed in woodland and grassland savannahs, ranging from the Nuba Mountains in Sudan to the northeastern part of South Africa (Wildekamp, 2004; Watters, 2009). The other closely related genera, *Pronothobranchius* and *Fundulosoma*, are more species poor and they inhabit Sahel region of West Africa (Valdesalici, 2013). The sister clade of African annual fishes consists of *Callopanchax* and *Fundulopanchax*, with the occurrence in West Africa, from which some species are known to be only facultatively annual (Reichard, 2015). However, most information on African annual fishes comes from *Nothobranchius* fishes as they started to play an important role as model organisms for several biological disciplines (Cellerino *et al.*, 2016). This chapter is focusing on this genus as it contains the *Nothobranchius orthonotus* (Peters), my study species (Figure 1.1).



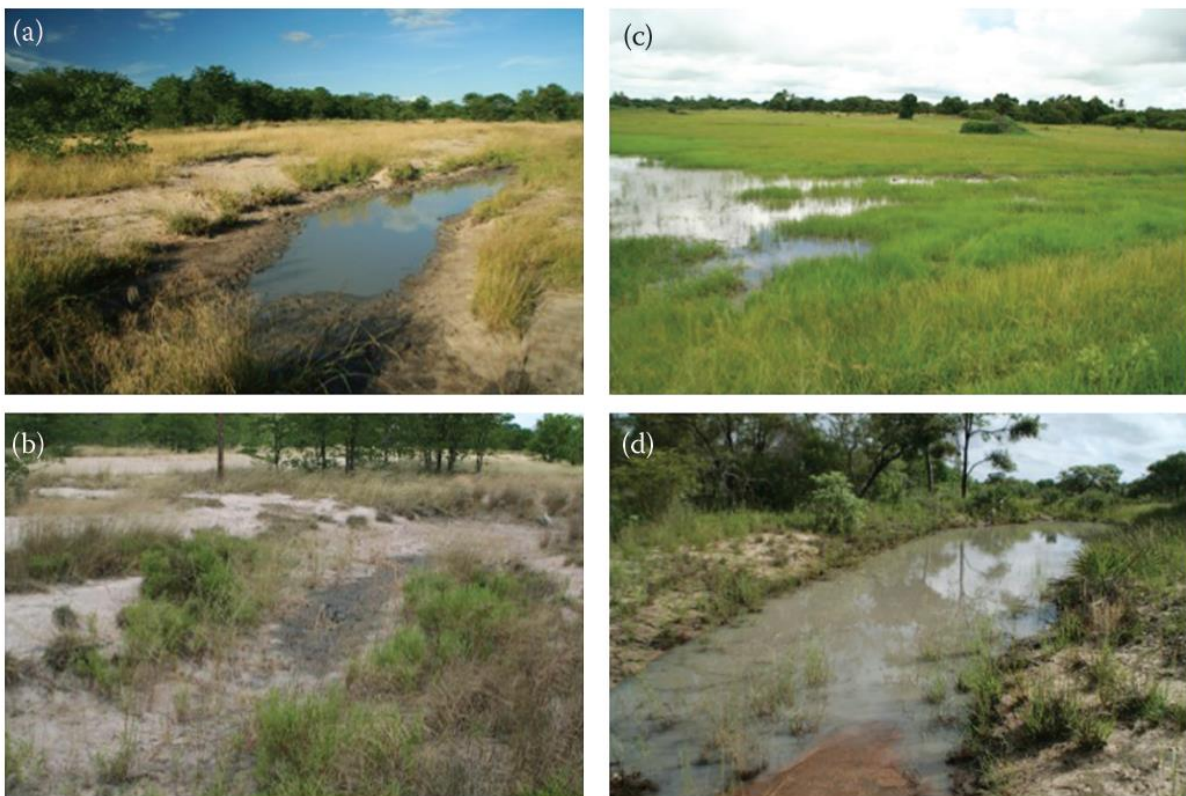
**Figure 1.1.** Photo of adult male of *Nothobranchius orthonotus*. (Photo by R. Žáček.)

African annual killifishes are small fishes (3–15 cm total length), with short maximum natural lifespan (usually less than 12 months) because of typical annual desiccation of their pool habitats during the dry season (Cellerino *et al.*, 2016). The seasonality is largely affected by local climatic and geomorphological conditions. For example, differences in annual rainfall in southern Mozambique along the east-west axis are ranging from 1000 mm in a year (coastal areas) to only 300 mm annually (inland areas) (Reichard, 2015). These differences are influencing duration of each habitat existence and therefore also the time which the fish have to grow and reproduce. The *Nothobranchius* fish are adapted to regular annual desiccation of their habitat by developing some extreme life history traits to cope with such unique environment. After the pool is filled with water, they hatch from stored eggs in the substrate and they are able to grow very rapidly (Watters, 2009). Their sexual maturity is achieved just within few weeks and then they can reproduce daily with female fecundity being around 20 to 50 eggs per day (Haas, 1976a; Blažek *et al.*, 2013). For example, *Nothobranchius furzeri* (Jubb) has one of the shortest lifespans among all vertebrates with only 9 weeks survival in some populations (Valdesalici and Cellerino, 2003). Also the reproductive behaviour itself is simple and quick with egg spawning lasting around 1–3 seconds (Haas, 1976a). The eggs are laid in the sediment of the pool and they remain there after desiccation in diapause (Wildekamp, 2004). By diapause is meant developmental arrest accompanied by a reduction in metabolic rate (Podrabsky *et al.*, 2010) which is relatively rare trait among vertebrates but it is more common in plants and invertebrates (Murphy and Collier, 1997; Hrbek and Larson, 1999). During the deposition of gametes, they perform short courtship displays (rapid jerking movement of the pair close to the substrate) which helps to cover the eggs with sediment (Haas, 1976a). Hatching does not start immediately after the beginning of wet season but rather during the seasonal peak when the threat of sudden pool desiccation is the lowest (Polačik *et al.*, 2011).

### **1.3.2 Habitat, diet, distribution and phylogeny of the genus *Nothobranchius***

Despite the extensive geographic range, *Nothobranchius* distribution is rather mosaic due to the fact that not all temporary pools are suitable for these fish. This is caused by very strict requirements of a specific composition of the substrate (Quaternary vertisol soils) for the successful embryonic development of the eggs after the pool desiccation (Watters, 2009). These dark mud-rich substrates form impermeable layer after desiccation and thus enable

water to remain longer in a pool (Watters, 2009). Pools are typically situated within alluvia of streams and rivers but sometimes they are completely isolated from permanent waters, like in the dry part of *Nothobranchius*' range in Southwestern Mozambique (Figure 1.2). It is also possible to find *Nothobranchius* in remnant pools within the channels of temporary streams (Reichard *et al.*, 2009; Valdesalici *et al.*, 2012; Nagy, 2014) but so far it remains unclear whether these populations have the full life cycle within the stream pools or they consist of individuals flushed from nearby savannah pools during annual flooding (Reichard *et al.*, 2009).



**Figure 1.2.** Habitats of *Nothobranchius*. Photos of the same pool (with *N. furzeri* and *N. orthonotus*), in the dry part of the gradient in southern Mozambique, during wet (a) and dry (b) seasons. Photo (c), marsh in relatively humid coastal region of Central Mozambique (inhabited by *N. orthonotus* and *N. kryanovi*). (d) Remnant pool within the channel of temporary stream. (Taken from Reichard, 2015.)

The water quality can be very poor due to the organic input from large herbivores, mainly domestic cattle, which is urinating and defecating into the pools (Reichard *et al.*, 2009). In more humid areas, *Nothobranchius* species occur in less fragmented pools of inland marshes where they often live together with nonannual freshwater fishes (e.g. genus *Tilapia*, *Barbus*, *Clarias*), depending on duration of a connection to permanent waters (Watters, 2009; Nagy, 2014). Moreover, they may coexist syntopically with lungfish (*Protopterus* spp.) in isolated pools (Reichard *et al.*, 2009; Reichard, 2010; Valdesalici *et al.*, 2012). However, they co-



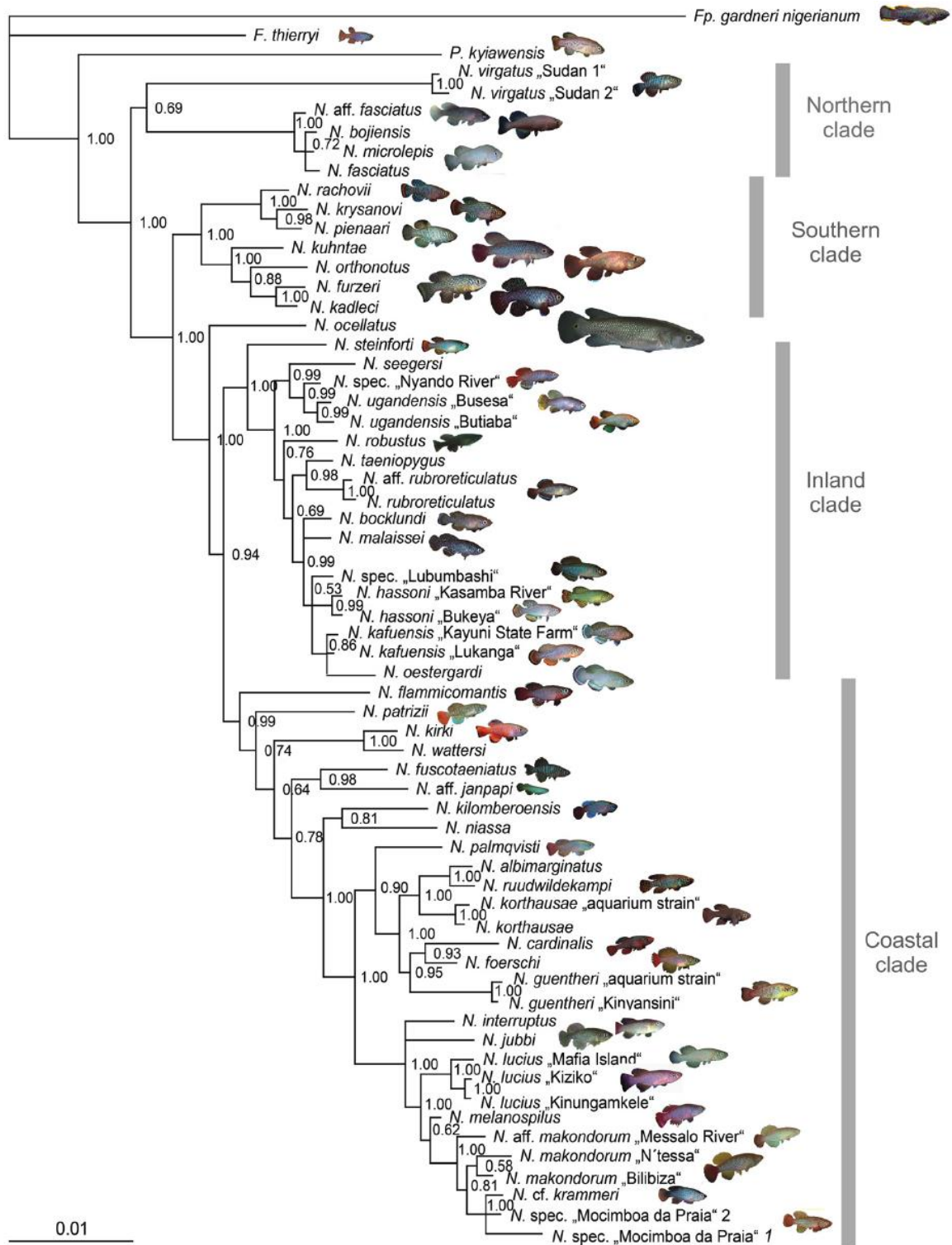
occur more commonly with various species of their own genus (Reichard, 2010). Most common is coexistence of two species (for example *N. orthonotus* with *N. furzeri*) though up to five species of *Nothobranchius* have been recorded in the same pool (Wildekamp, 2004; Reichard *et al.*, 2009).

*Nothobranchius* fishes feed largely on macroinvertebrates, containing both pelagic and benthic preys. However, diet of larger *Nothobranchius orthonotus* differ from other congeners as the fish sometimes feed also on small vertebrates such as tadpoles and even on lungfish juveniles (Polačik and Reichard, 2010). Food composition tends to be opportunistic and primarily depends on prey availability (Polačik and Reichard, 2010). When there are more *Nothobranchius* species in the same pool, their trophic niches become separated (Polačik *et al.*, 2014b). Predators feeding on *Nothobranchius* are most often birds (herons or kingfishers) but it is possible that are preyed upon by large belostomid hemipterans and lungfishes (Haas, 1976a; Reichard *et al.*, 2014). Males are more often targets of predators' attacks due to their conspicuous colouration, larger body and increased mobility associated with mate searching (Haas, 1976b; Polačik and Reichard, 2011).

The range of each *Nothobranchius* species can vary greatly in size but normally, it covers several river drainages. One of the most well-known range is the distribution of *N. orthonotus* that spans at least 1200 km north-south, from the northern part of South Africa to southern Malawi and part of Mozambique north of the Zambezi River (Reichard, 2015). The range of *N. furzeri* is also very well studied and it covers the area between the Save River in the north and the Lebombo ridge (South Africa) in the south (Reichard, 2015). These two species are often sympatric (Reichard *et al.*, 2009). Their range encompasses a relatively steep gradient of aridity and rainfall unpredictability which creates strong differences in the duration of habitats' existence for different populations (Polačik *et al.*, 2014a). Moreover, the duration of the wet season varies not only among regions and years but also among pools within a region, due to variation in their size and morphology (Reichard *et al.*, unpublished data). The most arid conditions are found inland while lowland areas closer to the Indian Ocean have relatively humid climate (Tozzini *et al.*, 2013).

There are four major phylogenetic clades within genus *Nothobranchius* and they are almost exclusively allopatric with minimum distribution overlap (Dorn *et al.*, 2014). There is the basal northern clade, the most researched southern clade distributed south of the Zambezi River (Cellerino *et al.*, 2016), the inland clade and the most species rich coastal clade inhabiting coastal basins between southern Kenya and northern Mozambique (Figure 1.3) (Dorn *et al.*, 2014). It is assumed that their range was strongly affected by the East African

Rift system which served as distribution boundary (Dorn *et al.*, 2014). The genus started to diverge coincidentally along with the aridification of East Africa and thus with the expansion of savannah habitats between 8 and 5 Mya (Dorn *et al.*, 2014). Current species are probably of Quaternary origin (Dorn *et al.*, 2014).



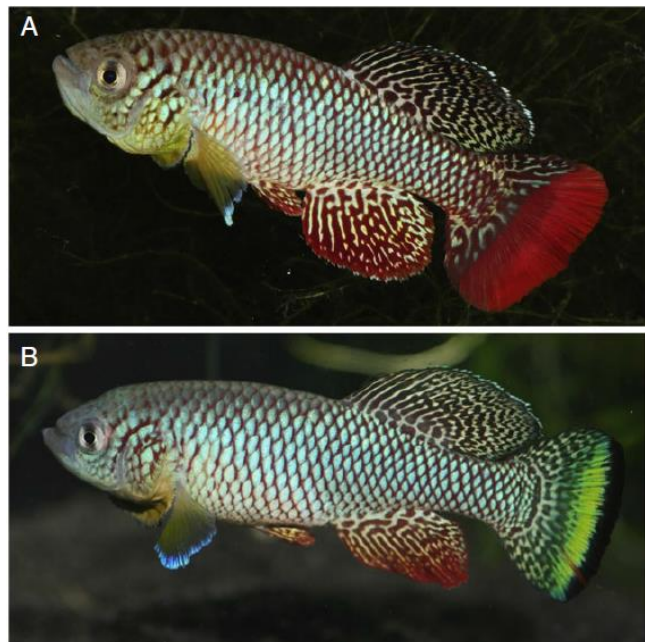
**Figure 1.3.** Phylogenetic tree of the annual killifish genus *Nothobranchius*. The tree is based on concatenation of partial coding sequences of six genes and fully partitioned dataset. Results of MrBayes run with posterior probabilities shown in the nodes. (Taken from Dorn *et al.*, 2014.)

### 1.3.3 Sexual dimorphism and male colour polymorphism

All *Nothobranchius* are highly sexually dimorphic mostly in coloration, but also in body size for some species (though negligibly in *N. orthonotus*). The sexes also have different lifespan in the wild (Reichard *et al.*, 2014). Males are robust and colourful, with two or more colour forms in some species (e.g. *N. furzeri*), while females are dull with translucent fins and pale brown body (Wildekamp, 2004; Reichard *et al.*, 2009). Two colour forms are also reported in *N. orthonotus* but the red form is very abundant throughout the whole species' range with only very few yellow (sometimes referred to as blue) males ever being captured (Wildekamp, 2004). *N. orthonotus* often co-occurs with *N. furzeri*, which has two male colour forms, yellow and red, with colours mostly distinctive on the caudal fin (Reichard *et al.*, 2009) (Figure 1.4). Both colour morphs of

*N. furzeri* are common which is important for my study of interspecific male-male aggression. The syntopic occurrence of two colour morphs in this species is quite common but pure one male morph populations exist as well (Reichard *et al.*, 2009). The red colour morph can be found as the only colour in the centre of *N. furzeri* distribution, while yellow morphs are conversely more abundant at its the periphery (Reichard *et al.*, 2009). Sexual selection is thought to be the driving force of the male colouration which is species-specific (Haas, 1976b; Wildekamp, 2004)

and in *N. furzeri* it is controlled by one major locus with yellow dominant over red (Valenzano *et al.*, 2009). The red colour morph can be found as the only colour of males in the centre of the *N. furzeri* distribution, while yellow morphs are conversely more abundant at the periphery (Reichard *et al.*, 2009). So far, despite the insights, it has remained poorly understood how this polymorphism evolved and how it is maintained (Dorn *et al.*, 2011). The male colour forms in *N. korthausae* species were studied in a greater detail but only very limited reproductive barriers were detected between them (Reichard and Polačik, 2010). These colour morphs were allopatric. For sympatric coexistence of colour morphs, one



**Figure 1.4.** Adult (A) red and (B) yellow coloured males of *N. furzeri*. (Taken from Cellerino *et al.*, 2016.)

possible mechanism that can play an important role in the evolution and stable coexistence of colour polymorphisms and morph frequencies is male-male aggressive interactions (Lehtonen *et al.*, 2015). For example, species colour polymorphism in cichlids (genus *Amphilophus*) was maintained by heterospecific aggression which was expressed more intensively towards similarly coloured males (Pauers *et al.*, 2008).

#### **1.3.4 Sex determination, sex ratio and reproductive behaviour**

Sex determination was studied in *N. furzeri* and *N. guentheri* and in both species it is strictly genetic (Ewulonu *et al.*, 1985; Valenzano *et al.*, 2009). Males were in both cases the heterogametic sex and therefore this could be widespread in *Nothobranchius* (Valenzano *et al.*, 2009). There should be balanced primary sex-ratio but this is not what was found in the wild, instead, the adult sex-ratio is often female biased (Reichard *et al.*, 2009). In captivity, no sex bias was observed and thus the most plausible explanation seems to be high male mortality caused by higher male predation also associated with male competition for mates (Reichard *et al.*, 2014). There is no strong territorial behaviour in *Nothobranchius* species but males establish a hierarchy based on the size (Haas, 1976a, b; Polačik and Reichard, 2009). Males compete with other males through ritualized aggressive contests for gaining access to mates (Haas, 1976a). Such contests often include mutual lateral displays with stretched unpaired fins and they are commonly resolved by one fish retreating. Threatening by extension of opercular and branchiostegal membranes is also common (Hass, 1976b). If these threats do not settle the contest, one fish usually attacks and bites the other male. Defeated opponent folds the dorsal fin and starts to reduce the intensity of colouration (Haas, 1976b). Male competition can be intense, leading to serious injuries to subordinate males, but the level of aggression differs in each species, with *Nothobranchius orthonotus* being considered as more aggressive species than *N. furzeri* or *N. rachovii* (Polačik and Reichard, 2011). Such disputes are often over occupancy in spawning arenas that are used for laying the eggs (Haas, 1976a). Eventually, dominant males are controlling these specific parts of the habitat that are preferred by females for spawning and where they tend to spawn more often (Polačik and Reichard, 2009). There is no female aggression in the genus *Nothobranchius*, probably because females are not active in courtship displays and attracting the mate; they simply decide whether to follow a male to spawn (Haas, 1976b).

## 1.4 *Nothobranchius* species and their use as model organisms

*Nothobranchius* fishes have been popular by hobbyists for their colouration long before their potential as model organisms in various biological disciplines was revealed (Genade *et al.*, 2005) and thanks to the popularity as aquarium fishes, detailed knowledge of their systematics, distribution and habitat was already available for scientists (Wildekamp, 2004). *Nothobranchius* started to be more intensively studied after the year 2003 due to the description of the extremely short lifespan of *Nothobranchius furzeri* in captivity (Valdesalici and Cellerino, 2003), which identified this species to be the shortest living vertebrate in laboratory conditions. Such short lifespan is the result of *Nothobranchius*' specific life history strategy to adapt to annual desiccation of their habitat and it is the reason why they have emerged as a model taxon.

Life history characteristics such as age at first reproductive event, reproductive lifespan or number of offspring, evolved through natural selection so that they would maximize fitness in the given environment (Stearns, 1992). Life history theory seeks to explain how this evolutionary force shape organisms to their optimum survival and reproduction (Stearns, 1992). The concept of trade-offs is essential in the theory as it states that all life history traits cannot be maximized simultaneously due to the limited amount of energy that animal disposes. Therefore, allocation into only some traits must be made with inevitable lost in other traits (Stearns, 1992). Each life history trait has its reaction norm which is described as an interval between extremes of phenotypic plasticity in genotype expression across a range of environments (Griffiths *et al.*, 2000). Since the habitat of *Nothobranchius* tends to be very unpredictable, the responses to environmental fluctuations show high phenotypic plasticity. For example, sexual maturation can be achieved in optimal conditions just within 18 days, but when there are suboptimal conditions, it can be delayed until the age of 5 weeks (Graf *et al.*, 2010). The flexibility of allocation between growth and reproduction is high as the fish can exhibit full compensatory growth after improvement in conditions without longer-term cost to fecundity (Vrtílek and Reichard, 2015). However, *Nothobranchius*' rapid growth and reproduction are disproportionately high and, according to the life history theory, this allocation should come at the cost of self-maintenance (Stearns, 1992). Indeed, studies have shown a high incidence of various internal damage, neoplasia and also apparent age-dependent degenerations all generally associated with ageing (Di Cicco *et al.*, 2011; Ng'oma *et al.*, 2014). The ageing is manifested in many different ways. There is age-related macroscopic phenotype expressed by typical habitus of emaciation, spinal curvature, and

reduced colouration in males (Lucas-Sánchez *et al.*, 2011) that were described in other fish species as well (Gerhard *et al.*, 2002). At a behavioural level, there is significant reduction in spontaneous locomotor activity (Lucas-Sánchez *et al.*, 2011) as well as lower learning performance compared to younger subjects (Valenzano *et al.*, 2006b; Terzibasi *et al.*, 2008). Ageing can be seen in histological level through various age-related organismal decay, primarily lesions in liver, kidney and heart (Di Cicco *et al.*, 2011). Cellular ageing is investigated by measuring the accumulation of lipofuscin (i.e. a pigment that serves as a marker of ageing rate due to its accumulation with increasing age in many organisms), accumulation of cells which lost their ability to enter the cell cycle or cellular changes in brain (Cellerino *et al.*, 2016). Overall, *N. furzeri* suffer from rapid ageing process that seems to be similar to that of other vertebrates, including humans (Cellerino *et al.*, 2016). These rapid ageing phenotypes of *Nothobranchius* (mainly *N. furzeri*) brought them under the spotlight in ageing research (Terzibasi *et al.*, 2007, 2008; Cellerino *et al.*, 2016).

The genus *Nothobranchius* offers a great opportunity to study the effects of extrinsic mortality on evolution of ageing because of their strictly nonoverlapping generations which experience different timing of sudden habitat desiccation (Tozzini *et al.*, 2013). By comparing lifespan and ageing across the gradient of life expectancy, strong differences were found between populations originating from arid areas and humid areas (Tozzini *et al.*, 2013; Blažek *et al.*, submitted). In captivity, populations of *N. furzeri* from arid areas had median lifespan (10% survivorship) of 17.5–29 weeks, whereas *N. orthonotus* from more humid region had a median lifespan 33 – 40 weeks (Tozzini *et al.*, 2013).

The use of this genus has not been restricted only to ageing research but it has been useful in many other scientific fields. Developmental biology use them for their specific embryogenesis (Levels *et al.*, 1986). *Nothobranchius* is also used as a model in quantitative genetics. The reason for that are intra-specific phenotypic differences among several species like colour morphs, behavioural traits or survival in captivity (Terzibasi *et al.*, 2008; Tozzini *et al.*, 2013) which enable to study genetic linkages in these traits and thus helping to reveal their evolutionary history (Cellerino *et al.*, 2016). The taxon is also well suited for ecological and evolutionary studies (Haas, 1976b; Reichard and Polačik, 2010). Mechanisms of speciation as well as sexual selection have been studied (Haas, 1976b; Polačik and Reichard, 2009).

Theory of sexual selection considers that females often preferably mate with dominant males (Andersson, 1994). Reason for choosing the successful male in intra-sexual competition is that the dominant male can provide direct benefits to female, such as superior breeding

territories, nesting sites, parental care or nuptial gifts (Andersson, 1994). *Nothobranchius* has been used for studying two key aspects of sexual selection, male-male competition (intrasexual selection) and female mate choice (intersexual selection) (Polačik and Reichard, 2009). Both selection pressures are assumed to be mutually reinforced (Polačik and Reichard, 2009). There is a lack of knowledge about mate choice in *N. furzeri* and little is known about female preference for male colour forms. Recently conducted pilot experiments did not find any consistent preference for male colour in female *N. furzeri* (Polačik and Reichard, unpublished observations). However, the brightness of male colour seems to play a role for females of *N. guentheri* who preferred to be associated with brighter males (Haas, 1976b). It is assumed that male colouration may be under sexual selection because it serves as a signal of dominance among males and can play role in female choice (Haas, 1976b). Male visibility to females ('sensory bias') may have the biggest role in sexual selection on bright male colouration (Cellerino *et al.*, 2016). Such showy sexual displays may be connected with increased mortality risk (Hunt *et al.*, 2004). The colour brightness is also affected by water turbidity in the habitat, the clearer the water is, the more colourful males are there (Cellerino *et al.*, 2016).

Cognition in *Nothobranchius* fish may play important role during life as their habitat is very unpredictable and variable. In such environments is expected to be beneficial to evolve reasonably good spatial learning, higher speed at which fish learn and better memory capabilities (Brown *et al.*, 2003). Better cognitive abilities allow to make more complex decisions and thus generating flexible behaviour which is crucial in changing environment.

There were two studies that tested cognitive ability of *N. furzeri* in relationship with ageing process, where they proved that temperature and resveratrol can delay cognitive decay connected with ageing (Valenzano *et al.*, 2006a, b). To test learning in *N. furzeri*, they used a modified protocol of active avoidance conditioning which was already used in zebrafish (Pradel *et al.*, 1999). The aim of this learning task was to learn fish to associate red light stimulus in one compartment in a tank with punishment (mechanical disturbance) if the fish stayed in that compartment. The trial was successful when the fish learned to avoid the negative stimulus by leaving the compartment after the light onset. Young fish showed effective learning ability with 73 % success rate after 50 consecutive trials (at 25 °C) (Valenzano *et al.*, 2006a). Older fish success was 43 % but when kept at 22 °C, they even outperform the younger fish having 85 % success rate (Valenzano *et al.*, 2006a). These studies gave the evidence that at least one species of *Nothobranchius* is capable of relatively fast learning which can be flexible depending on the environmental conditions.

Overall, *Nothobranchius* species have proved to be very valuable model taxon for several scientific fields thanks to its simple breeding in captivity, extremely short lifespan and unique life history adaptations.

## 1.5 Hypotheses and aim of the work

There have been several studies on effect of different habitat duration on life history traits in *Nothobranchius*, showing exceptional phenotypic plasticity and alternative life history strategies (Tozzini *et al.*, 2013; Polačik *et al.*, 2014a; Blažek *et al.*, submitted). However, nothing is known about how difference in habitat duration, and especially variation in life history traits associated with that difference, can affect cognitive ability in *Nothobranchius* species, and how cognitive ability is influenced by ageing process. Also, the evolution and maintenance of colour polymorphism in *Nothobranchius* remains poorly understood. According to studies on cichlids, one of the possible explanation of colour polymorphism within species as well as between species could be male-male aggression (Anderson and Grether, 2010; Lehtonen *et al.*, 2015). However, the role of aggression on colour polymorphism in genus *Nothobranchius* has not been studied so far.

In this master thesis, I conducted two separate studies. First, I tested cognitive ability of two populations of *Nothobranchius orthonotus* by testing their performance in a learning task. These two populations originated from regions which differ in mean annual precipitation (dry and wet region) and thus also in the existence of habitats. This major difference in habitat duration was assumed to have indirect effect on the cognitive ability via divergence in life history traits. Moreover, experimental fish were kept in two distinctly different social environments (singly-housed and group-housed fish) which could also have effect on cognitive ability. Second, I tested male-male conspecific and heterospecific aggression between *N. orthonotus*, and between *N. orthonotus* and two colour morphs of *N. furzeri* and *N. pienarii*.

The aim of the first study of my master thesis was to measure the speed, accuracy and improvement of learning in *N. orthonotus* and, at the inter-population level, compare learning ability between two populations. At inter-individual level, my aim was to relate learning ability with other life history traits recorded for the same individuals, including their social environment and personality traits – boldness and overall activity.

The second study aimed to investigate differences in conspecific and heterospecific aggression, and estimate the possibility that heterospecific aggression is biased toward red



coloured heterospecific males and hence having the potential to contribute to existence of various colour morphs in the wild.

Several questions were addressed by these two studies:

- (i) Will the *N. orthonotus*' population from the dry region complete the learning task faster than population from humid area, despite making more mistakes (in the correspondence with pace-of-life syndrome)?
- (ii) Will the fish from dry region age faster and thus have lower learning ability in older age compared with the population from humid region?
- (iii) Does social environment of the fish play a role in learning ability? Do individuals from social groups perform better than singly-housed fish?
- (iv) Is there relationship between cognitive ability and other life history traits?
- (v) Are boldness and higher activity affecting cognitive ability in a way that the bold fish completes the learning task generally faster than shy fish, despite potentially making more mistakes?
- (vi) Assuming that *N. orthonotus* is most aggressive towards conspecific males, would they be comparably aggressive towards similar colour morph of *N. furzeri* (red) and less aggressive towards differently coloured males of *N. pienarii* (black) and yellow morph of *N. furzeri*?

## 2 Materials and methods

### Source populations and their collection

For both experiments, we used laboratory-reared descendants (F1–F3 generation) of 2 imported wild populations of *N. orthonotus*, which were collected from 2 sites with contrasting aridity in southern and central Mozambique (Table 2.1) (Blažek *et al.*, submitted) between 2010 and 2012. Steep cline of aridity in this area is generated by a decrease in precipitation with increasing distance from the coast and increasing latitude. Both populations from dry and wet region are genetically different from each other (Bartáková *et al.*, 2015) and they had enough time to evolve into different life history optima through time.

Males of *N. furzeri* and *N. pienaar*i were used as objects for aggressive behaviour of *N. orthonotus* in the experiments on intraspecific and interspecific male aggression. These two species were also imported between 2010 and 2012 (Table 2.1).

Collecting of fish was done by using a dip net with a triangular metal frame (45 x 45 cm, mesh size 5 mm) on a long (1.5 m) wooden pole. Number of hauls for each site was in the range of 15–40, depending on fish density. At some sites, a beach Seine net (length 2.7 m, depth 0.7 m, mesh size 4 mm) was used in addition or as an alternative to the dip net (Reichard *et al.*, 2014).

**Table 2.1** | Details on source populations and their collection sites

Species	Region	Collection code	River Basin	GPS coordinates	Aridity Index*	Noth. presence**	N founder males <sup>+</sup>	N founder females <sup>+</sup>
<i>N. orthonotus</i>	dry	MZCS NO002	Limpopo	S24.06347 E32.73220	0.3203	NO, NP, NF	6	22
<i>N. orthonotus</i>	wet	MZCS NO528	Pungwe	S19.69737 E34.78294	0.8115	NO, NP	16	26
<i>N. furzeri</i>	dry	MZCS NF222	Chefu	S21.87357 E32.80067	0.3082	NO, NP, NF	20	40
<i>N. pienaar</i> i	dry	MZCS NP505	Limpopo	S23.52978 E32.57793	0.2735	NO, NP, NF	10	22

\* data from CGIAR Consortium for Spatial Information: [www.cgiar-csi.org/Aridity](http://www.cgiar-csi.org/Aridity)

\*\* *Nothobranchius* presence: NO (*N. orthonotus*), NP (*N. pienaar*i), NF (*N. furzeri*)

<sup>+</sup> N founder males (females): the number of fish imported to the lab to produce F1 generation used in the experiments

### **Breeding conditions for *Nothobranchius* sp.**

Laboratory culture followed established protocols (Genade *et al.*, 2005; Blažek *et al.*, 2013; Polačik *et al.*, 2016). The eggs of parental generation were stored in an incubator (Pollab, Q-CELL 60-240) at 23.5 to 24.5°C for at least 16 weeks following standard methods (Blažek *et al.*, submitted), ensuring that all embryos developed via diapause. Embryos were hatched in 4 L tanks with well-oxygenated tap water (15–17 °C). Water temperature was gradually increased to 24–26 °C. Fish were raised in groups from the same cohort (hatching event) and population. From the age of 4 days, fish were housed either in a set of 20 L or 24 L glass aquaria, creating social groups (with maximum density 2 L/fish), or in small sized aquaria for only one individual fish (2 L volume). Individually kept fish were held in recirculation system (AquaMedic) in water with conductivity of 100-150  $\mu\text{S}\cdot\text{cm}^{-2}$ . Water temperature was measured continuously by HOBO loggers and was kept in all the tanks at  $26 \pm 2$  °C.

In the beginning, fish were fed ad libitum with brine shrimp nauplii 2 times a day and gradually weaned onto chopped frozen bloodworm (*Chironomus* sp. larvae) and/or *Tubifex* at an age of 10 days (*N. orthonotus*), 13 days (*N. furzeri*) and 22 days (*N. pienaari*). After another 7 to 10 days, combination of brine shrimp nauplii with the new food stopped completely and only bloodworms and *Tubifex* were used. Since then, fish were fed once a day ad libitum. Light conditions were kept at 14 h:10 h light:dark regime in aged tap water and 30–50 % of water was exchanged 1–3 times each week (Polačik *et al.*, 2016).

Since sexual maturation, fish were able to reproduce on a daily basis. Eggs for general maintenance of *Nothobranchius* cultures were obtained by breeding the fish in groups. Groups consisted of multiple spawning pairs in an aquarium. A glass jar filled with a 6 cm layer of boiled peat served as a spawning place inside the aquarium. Above the jar, there was at least 10 cm water to allow free movement of the fish. Peat was changed every two weeks, associated with egg collection. For more details on breeding, see the protocol by Polačik *et al.*, 2016.

The breeding of *N. furzeri* and *N. pienaari* was identical to breeding of *N. orthonotus*, except for higher fish densities (12 instead of 10 fish per tank, given their smaller body size).

## 2.1 Experiment 1 – learning ability

### 2.1.1 Details on the maintenance of experimental *Nothobranchius orthonotus*

Hatching of experimental fish (F1 generation) was done simultaneously on May 22, 2013 by watering the incubation substrate with dechlorinated tap water (16°C). During the hatching event, more than 500 juveniles hatched in each population. From the age of 4 days, fish were housed either in a set of 24 l glass aquaria, creating social groups of 5 males and 5 females, or transferred into individual tanks (2 L volume). Dead juveniles from social tanks were replaced with fish of the same age and housing density history from spare stock tanks. After reaching a size for individual marking in 6 weeks, fish from social tanks were briefly anaesthetized and a single Visible Implant Elastomer tag (Northwestern Marine Technology) was applied subcutaneously (Figure 2.1). Twelve aquaria were used for each population (social groups). Initial adult density of *N. orthonotus* was 10 fish per tank.

Tanks with group of fish were equipped with air driven sponge filters and 25–30 % of water was exchanged 2–3 times each week. Fish were kept under a 12:12 h light:dark regime in aged tap water (conductivity 550  $\mu\text{S}\cdot\text{cm}^2$ ). Water temperature was maintained stable at  $24 \pm 1$  °C. For the first 30 days, all fish were fed twice each



**Figure 2.1.** Photo of marked female (orange tag) and male (red tag) of *N. orthonotus*. (Photo by R. Žáček.)

day and once a day thereafter to satiation. Fish were initially fed with live *Artemia* sp. nauplii and their diet was gradually changed to larger food, frozen chopped bloodworm (*Chironomus* sp. larvae) and *Tubifex*, from the age of 10 days as the fish grew larger. All tanks received the same ration (approximately 15% of body mass of the fish in the tank) and fish were monitored daily. Any case of death was recorded and the fish removed.

### 2.1.2 Experimental methods

Cognitive abilities were tested according to modified protocol from the work of Kotrschal and Taborsky (2010), which was successfully used in some other species of freshwater fish (Mesquita *et al.*, 2015; Smith *et al.*, 2015; Roy and Bhat, 2016). It is based on the ability to learn to find the feed during dichotomous test. The first test was performed on young adult fish and it was completed between July 8 and July 26, 2013 with 80 fish being tested (fish were 7 to 9 weeks old). Half of the 40 fish were taken from individual aquaria and other half was from bred in groups. Second testing period, on older fish, was completed with the surviving fish from the first test (16 fish from individual aquaria, 12 fish housed in groups) and, additionally, using also other so far untested fish (1 fish from individual aquarium, 43 fish from groups). It was performed from January 23 to February 3, 2014 with 72 fish being tested (when they were 35 to 36 weeks old). One test lasted for 2 days. A set of 10 to 15 fish was tested during each 2 days period. Fish were tested individually; each in a separate aquarium. In total, 153 tests were made on 124 fish with 29 fish being retested in older age. One fish was showing very limited behavioural repertoire and died soon after experiment, and therefore it was deleted from the final dataset.

### Other functional assays of the study animals and their link to cognition data

Various data about two populations of *N. orthonotus* used in the tests were collected during their life (such as growth rate, lifespan, sexual maturity in females, metabolic rate, exploratory behaviour and boldness). Following measurements and tests were completed by other researches at the Institute of Vertebrate Biology in Brno (Radim Blažek, Caroline Methling, Radomil Řežucha).

**Growth rate** was calculated from the collected measurements of the fish size that had been obtained in regular intervals from the age of 6 weeks. Fish were measured every 2 weeks until the age of 14 weeks and once a month thereafter. In the procedure, fish were photographed from above in a gridded container with a thin layer of water. Measurements were made on digital photographs by program ImageJ. Fish were also measured earlier (before the age of 6 weeks), but no individual-based growth trajectories can be constructed due to the lack of individual marks. In individually housed fish, fish were measured every week initially and in the same interval as fish from social groups after 6 weeks.

The information about the **lifespan** is given in Table 2.2. The longest individual lifespan was 613 days in population NO528 (wet region) and 612 days in population NO2 (dry region).

**Table 2.2** | Details on lifespan in days for 2 populations of *N. orthonotus*

Population	Mean	Standard deviation	Median	Max
NO 528 (wet region)	309,6	121,13	301	613
NO 2 (dry region)	285,5	115,97	290,5	612

Max: Maximum lifespan

**Resting Metabolic Rate (RMR)** and **Maximum Oxygen Consumption (MO<sub>2</sub>max)** was recorded for 46 individuals from dry and wet population of *N. orthonotus*, using Loligo Systems Automated Aquatic Respirometry system. The protocol included 3 min chasing followed by 30 s air exposure (for estimation of MO<sub>2</sub>max). Fish were placed in 55 mL chamber submerged in a 10 L outer tank supplied with water from a reservoir, maintained at  $24 \pm 0.1^\circ\text{C}$  (Blažek *et al.*, submitted). Mass-specific oxygen consumption (MO<sub>2</sub>) was derived from the decrease of oxygen partial pressure in chamber during 5 min measuring period. Chambers were periodically flushed for 4 min followed by a closed 1 min wait period. Determination of MO<sub>2</sub>max was done for the first MO<sub>2</sub> measurement after the fish was placed in the chamber. Measurements of MO<sub>2</sub> during the following 24 hours were used to estimate RMR. Metabolic scope (MS) was quantified as a difference between RMR and MO<sub>2</sub>max (Blažek *et al.*, submitted). More details about the experimental method are in Blažek *et al.*, submitted.

A subset of observations (75 in total) were also tested on their exploratory behaviour and boldness as it could affect the performance in cognitive tests (Brown and Braithwaite, 2005). **Activity levels** was scored in a shallow opaque plastic aquarium (45 x 35 cm, water depth 2.5 cm). To standardize fish satiation and metabolic activity, the fish were fed *ad libitum* before and between trials. Each individually tested fish was transferred into the test tank and 1 min was given for acclimatization. After that, the movement was monitored using a camera mounted above the tank for 4 min. An external lamp was used to standardize light conditions (dim light). A ruler in the aquarium provided scale for software calibration. Videos of *N. orthonotus* and *N. pienaarri* were analysed by Tracker 4.82 using absolute distance travelled by individuals during 4 min (Blažek *et al.*, submitted). More details about the experimental method are in Blažek *et al.*, submitted.

**Boldness** was measured as time taken to leave a refuge in a novel environment, with similar design as in experiment of Brown and Braithwaite (2004). Experimental conditions (water quality, satiation and lighting) were the same as in exploratory behaviour analysis but fish were individually positioned in an opaque plastic container (8 cm length x 15 cm width x 16 cm height) with a sliding door, positioned by a side of the test aquarium (55 x 49 cm). The test aquarium, with water to a depth of 80 mm, had 3 sides covered by opaque foil to prevent any external disturbance. After 2 min time, the sliding door was lifted and the fish allowed to leave the container. The time taken to depart the refuge was measured (Time 1: tip of snout outside refuge, Time 2: entire body outside refuge) (Blažek *et al.*, submitted). Fish that did not leave the refuge within 10 min were given a maximum score of 10 min.

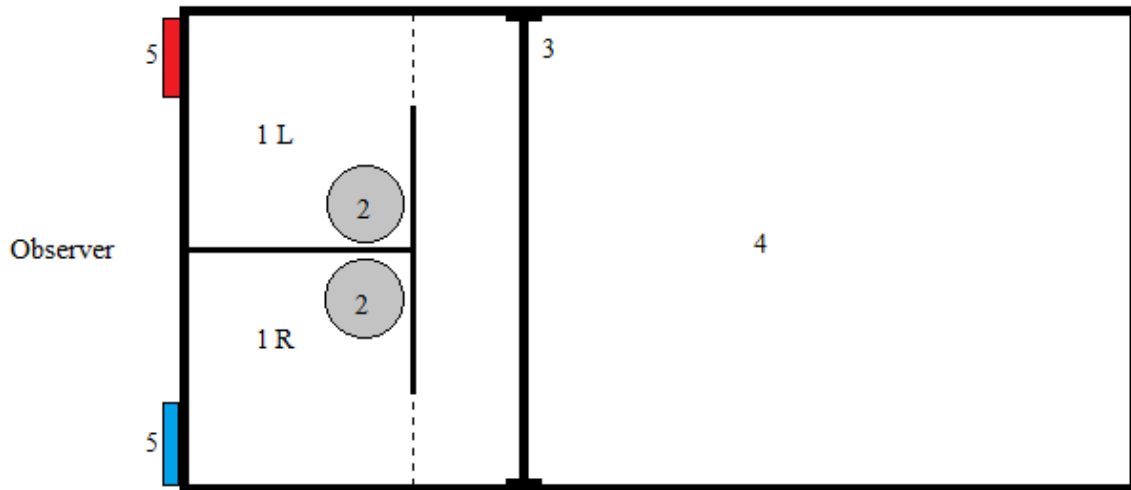
**Hesitancy** was a difference between Time 1 and Time 2 during boldness assay. It means that hesitancy measured the period between fish positioned tip of its snout outside the refuge and left the refuge with entire body.

These additional data collectively enabled more detailed analysis of the results of cognitive ability of individual fish and enabled to give more insight into the relation between life history adaptations of both populations and cognitive ability.

### **Experiment 1 – learning ability**

**Experimental set-up.** Test aquaria were 25 cm wide and 50 cm long, with water depth of 16 cm, separated into 2 parts by a removable PVC slate (3, Figure 2.2). In one part, there was a test apparatus consisting of 2 chambers (left, right, for an observer), made of black PVC, with entrance (dotted line, Figure 2.2). Inside each chamber was a glass bowl for the food, placed in the corner of the chamber so that fish could not see it before entering. There were red and blue coloured squares glued on the front aquarium glass, from outside (5, Figure 2.2). The coloured squares should serve as visual cues for the fish. The side of each colour was randomly predetermined. Every aquarium was visually separated from adjacent aquaria by an opaque cardboard paper to ensure that fish could not see each other.

Light conditions were approximately 12:12 hour light:dark and water was changed after the end of every test by aged tap water with temperature around 24 °C.



**Figure 2.2.** Test of cognitive performance. Schematic representation (top view) of the set-up used to test cognitive abilities of *N. orthonotus*. 1: chambers (L: left; R: right); 2: food bowl; 3: removable PVC slate; 4: space for fish between the tests; 5: blue or red squares; “Observer” refers to experimenter’s position during the test.

**Training phase.** Ten to 15 fish were placed in individual experimental aquaria the evening before the first day of the test so they could acclimatize to a new environment. Slate separating the compartments was not present so that fish could explore the whole area. The next day, slate was returned into aquarium. Feeding was done regularly 5 times in a day (with at least 2.5 hour time difference between any feedings) by inserting 3 thawed frozen bloodworms in the bowl inside the right or left chamber. The position of the food was based on predetermined random order. The chosen chamber for the food remained the same during the whole test (the same side position and colour). First 3 feedings were completed in training phase.

For each training, 3 bloodworms were placed in the bowl in one of the chambers separated by slate from the fish. Then the slate was slowly removed and the clock started. The clock was stopped when the fish arrived to the correct chamber and began to feed on the bloodworms, or once 600 s had elapsed. During this time I also recorded number of mistakes (i.e. entering the incorrect chamber). When a fish did not enter the correct chamber within 600 seconds, the time of the training was recorded as 600 s, and the testing area with the food was left accessible till the next training (Figure 2.3). Old uneaten food was removed before the next training. Training trials were repeated three times in a day.





**Figure 2.3.** Photo of one of the tested fish in experimental aquarium during training phase. Slate is removed from the aquarium and lies in front of it. The food can be seen in the right chamber.

**Test phase.** During the test trials I followed a similar procedure as in the training phase with two important differences. First, the slate, separating testing area with the rest of the aquarium, was placed back to the aquarium after 600 s of recording, regardless if the fish found the food or not. Second, drops of the water from the food were dripped 8 times into the water on several different places to disperse olfactory perception throughout the whole aquarium. This was done to prevent olfactory navigation to the food.

Tests were repeated 7 times, 2 tests in the first day (after training phase ended) and 5 tests in the second day.

**Repeated test with old fish.** The fish that were tested for their second time (after being tested the first series as young fish) had the same position and colour of the food chamber as before. This was done to examine whether the fish could be showing better performance in

once already known environment. The colour and position of the food chamber for the other fish was selected on basis of random, predetermined order.

## **2.2 Experiment 2a – intraspecific and interspecific aggression of males**

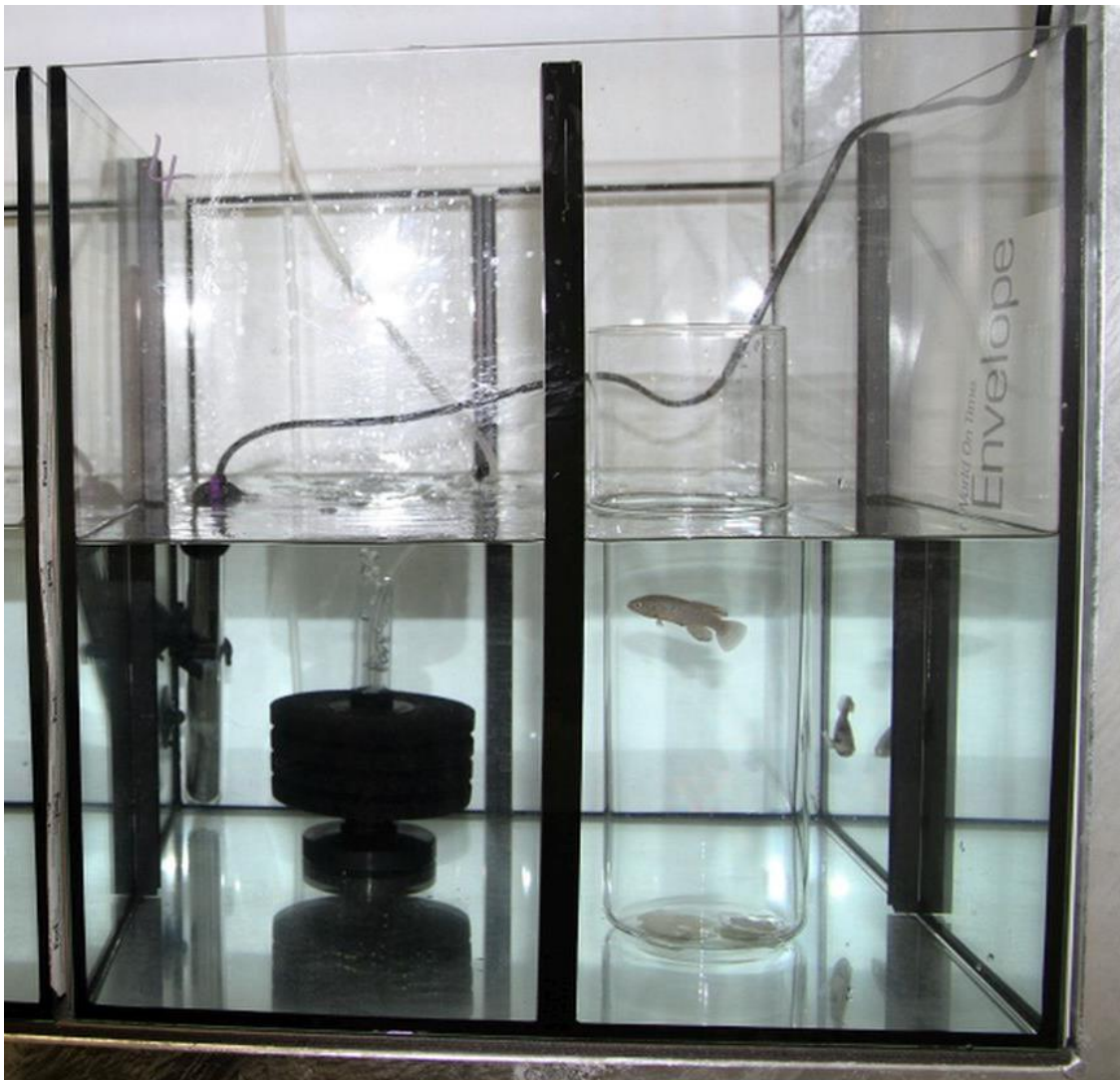
Male aggression of *N. orthonotus* (descendants of population from dry region, see Table 2.1) was tested against 4 different males (rivals), in the period from January 8 to January 11, 2016. In the test, the male was responding on one newly arrived male from the following set: male of their own species, red coloured male of *N. furzeri*, yellow coloured male of *N. furzeri*, and male of *N. pienaari*. The original population of the experimental (focal) males of *N. orthonotus* was living syntopically with a population of *N. furzeri* in which the red male colouration was predominant. Hatching of all experimental individuals of *N. orthonotus* (F4 generation) was done simultaneously on October 15, 2015. Hatching of *N. furzeri* and *N. pienaari* groups was done during the same day, August 28, 2015.

Breeding and maintenance of *Nothobranchius* species was the same as described above for the first experiment. Before the test, all fish lived in groups in aquaria with water volume of 20 or 24 L, except for a single male (NO17) who was separated due to his extremely aggressive behaviour. Fish density was 1 fish per 2 L of water. Experimental fish were moved to new individual aquaria (2 L volume, Aqua Medic recirculating system) for the testing period, to allow manipulation and individual recognition. In total, we tested 16 males of *N. orthonotus* in 4 tests.

### **2.2.1 Experimental conditions**

Experimental aquaria with size 40 x 40 x 40 cm were filled by aged tap water (water depth 20 cm) and equipped with air driven sponge filters. Heaters were adjusting temperature to  $24.5 \pm 0.5$  °C (the mean value: 24.8; standard deviation: 0.23). In the middle of the aquarium, near to front glass, one oval glass jar was inserted (2 L water volume) filled with tank water. The jar served as a temporary housing for a rival male (Figure 2.4). In the first half of the first 4 experiments (males NO1–NO4), glass jars with a rectangular shape were used, they were then replaced by the oval jars. There was no apparent change in fish response. Opaque papers were placed between aquaria to preclude fish to see adjacent males. Light conditions were approximately 12 h:12 h light:dark and water was exchanged after the end of the second test.

Fish were fed three times in a day by up to 10 bloodworms or until satiation. Feeding took place between the tests.



**Figure 2.4.** Photo of the experimental aquarium for the tests on male aggression. In the front, there is the oval glass jar with the rival male inside (*N. orthonotus*). Behind the jar, on the right, there is the focal *N. orthonotus* male. On the left, there are heater and sponge filter.

### 2.2.2 Experimental methods

In each test, 4 males of *N. orthonotus* were placed in the individual experimental aquaria. At least 1 hour was given to acclimatization to a new environment and then I started test one of the focal fish by putting first rival male in the glass jar. The order of *Nothobranchius* species in the glass jar was random (and predetermined, to ensure balanced design). After the rival male was put inside the jar, clock started measuring 420 s (7 mins). Male aggression was recorded during this time by recording 3 different categories of aggressive behaviour (see Table 2.3).

**Table 2.3|** Description of four behavioural categories recorded during experimental observations of male inter/intraspecific aggression

Behavioural category	Definition
<b>Frontal threat</b>	The male quickly swam towards the rival male or he was approaching him by jerking movement or he tried to bite.
<b>Opercular threat</b>	Male prominently extended the lateral projections of its opercular and branchiostegal membranes in close proximity to the rival male.
<b>Lateral threat</b>	The male was displayed laterally towards the rival male and spread his unpaired fins.
<b>Interest</b>	The male was observing or following the rival male from a close distance.

Male's reaction to rival male in the jar was frequently difficult to classify as one of the 3 forms of aggression because male was only observing and following the rival inside the jar in close proximity without clear displays of threatening. Moreover, males tend to stop or reduce their attacks after certain amount of time or unsuccessful attempts to bite because of the glass barrier that was prohibiting any closer encounter. Therefore, when focal male was observing or following the rival male from a close distance, this was recorded as an interest. As a consequence, fourth type of behaviour, "the interest", was measured. All these 4 reactions were recorded during 7 mins time by an observer (me) sitting in front of the aquarium. No quick movements were made during the test to avoid any startle response from fish. Shortly after the clock stopped, I removed the rival male used. The next rival male was inserted in the jar at least 20 mins after the end of the last test in order to provide enough resting time between the tests for the focal male. Focal males in other aquaria were tested in the meantime.

The rival male and the focal male in the aquarium were size-matched by the eye, so they would not differ significantly from each other as that could affect the aggressive behaviour. Owing to limited numbers of fish, tested focal *N. orthonotus* males were also used once (with one exception: male, with code NO2, was used twice as a rival male because no other size-matching male was available) during the testing period as rival males but not in the same day. Number of yellow coloured males of *N. furzeri* available for the tests was only 8. Therefore they had to be used twice in the test, with one exception, when one yellow male (code Y6) was used for the third time to replace too large male (code Y1) which would not match by its size with the particular tested male of *N. orthonotus*. The number of red coloured *N. furzeri* males was also 8 and all of them were used twice. Males of *N. piinaari* were 16 in total so each one was selected for the test just once. Also, every group had one spare male in the

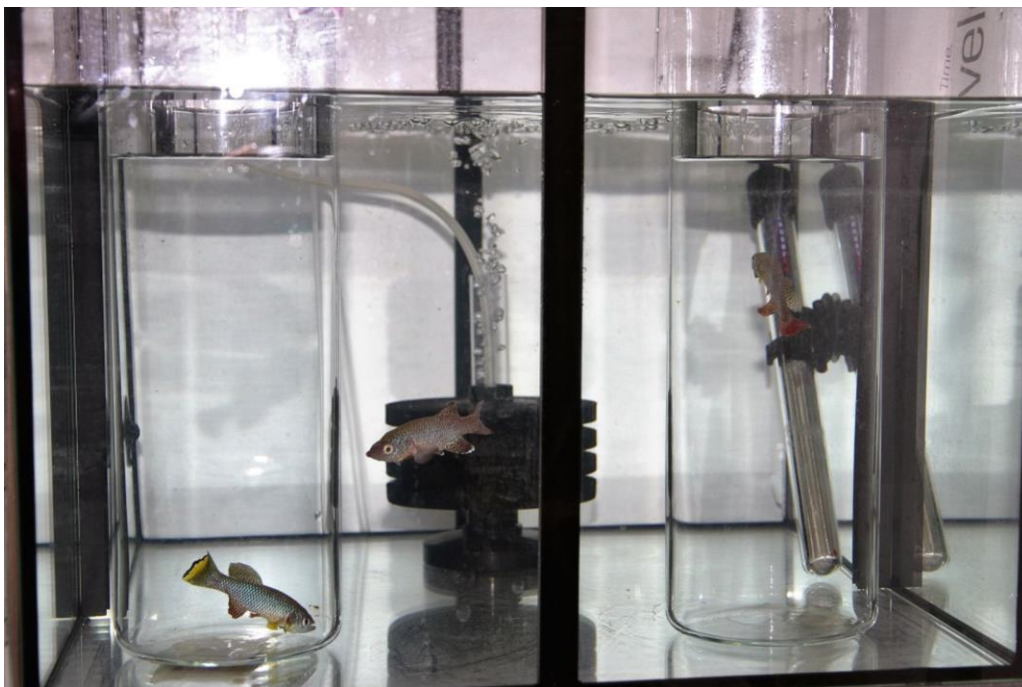
reserve if needed for the tests. One spare *N. pienaari* male was used because one rival male (NP16) fell from the net during handling and was excluded from further testing.

## **2.3 Experiment 2b – interspecific aggression among males of *N. orthonotus* and two colour forms of *N. furzeri***

In this experiment, male aggression of *N. orthonotus* was tested against two colour forms of *N. furzeri* simultaneously. It was performed from January 14 to January 16, 2016, with the same set of 16 males of *N. orthonotus* and 16 males of *N. furzeri* (8 red, 8 yellow) as in the previous test 2a.

### **2.3.1 Experimental conditions**

The experimental conditions were almost the same as in the previous test; the identical laboratory room and aquaria was used. However, I added one more oval glass jar into the aquarium. One jar was placed on the right side and the other one on the left side (Figure 2.5). The light in the experimental room was less bright than in the experiment 2a, to minimize as much as possible the stress for the fish from a new environment. Temperature was kept stable by heaters around 24.5 °C (the mean value: 24.8; standard deviation: 0.17). Water was changed after the end of the testing period.



**Figure 2.5.** Photo of the experimental aquarium with two males of *N. furzeri* in the glass jars. Inside the right jar, there is red coloured male. In the left jar, there is yellow coloured male.

### 2.3.2 Experimental methods

When the focal male was acclimatized in the experimental aquarium (minimum 1 hour), two different colour forms of males of *N. furzeri* (red form and yellow form) were placed into the glass jars, each colour form randomly to the left or right jar, and clock started measuring 420 s (7 mins). Three types of male aggressive behaviour were recorded: interest, jaw attack and threat by extension of supraopercular membrane (definitions in Table 2.3). Lateral threatening was no longer measured as it was not recorded almost at all during the previous experiment (more in Discussion).

In this test, we decided to use a slightly different recording method. Instead of just recording each type of aggressive behaviour separately and then summarize its amount in 7 mins time, I recorded behaviour of the tested male every 10 seconds during the test. Table, which was used for the records during the test, consisted of 3 rows: the left jar, no interest and the right jar, and each one was divided into 10 seconds intervals of 10 seconds (Table 2.4). In each interval, I marked one of the rows according to male’s behaviour. The “left jar” or “right jar” row, in given interval, was marked if the focal male was showing interest or aggressive behaviour towards the rival male in the certain jar. “No interest” row was marked when focal male did not perform any of the behaviour previously stated. After 7 mins time, males were returned to the individual aquaria and another male of *N. orthonotus* from the set was tested.

**Table 2.4 |** Sample table used in the experiment 2b for each focal male.

	code		size			date		temperature																																											
tested						time		aquarium																																											
in the jar			left:	right:		duration																																													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42									
left																																																			
no interest																																																			
right																																																			

## 2.4 Data analysis

### 2.4.1 Experiment 1 – learning ability

For each fish, we recorded the number of errors during test phase (ERR4\_7), the sum of time taken to locate food (TIME4\_7, sum of Time 4 to Time 7) and slope of learning line (SLOPE) that was a slope from a straight regression line fitted to the number of errors at Time

4, Time 5, Time 6 and Time 7. The slope took a negative value in most cases (fish improved in solving the task), but some individuals showed no improvement (slope = 0) and few individuals apparently got worse over time (positive slope).

Data on cognition were partitioned into two sets. The first set (set A) contained all tested fish (n = 152). For each of these response variables (ERR, TIME, SLOPE) we first fitted a full model with factors and covariates. Factors of the main interest were population identity (2 levels) and age (2 levels). For these factors, we also included their interaction, as we were primarily interested whether decline in cognition differs between populations. Additionally, we fitted the factors of Sex (2 levels), Social environment (2 levels) and Lifespan (age in days). Covariate Body size (continuous) and factor Colour (2 levels, colour of the visual mark) were not included in the model but were initially checked as a potentially confounding variables. Data inspection revealed no effect on the response variables.

As each response variable had different interpretation, three full models were fitted using General Linear Models (lm function) with Gaussian error distribution for SLOPE and Generalized Linear Model (glm function) with Gamma error distribution (strictly positive values) for TIME4\_7 and Poisson error distribution for ERR4\_7 (counts). Fitting ERR4\_7 using Poisson distribution resulted in overdispersion in the fitted values ( $\theta = 11.0$ ) and negative binomial model was fitted (resulting in no overdispersion;  $\theta = 0.99$ ). The full model was always inspected for overdispersion and then minimal adequate models were constructed using drop1 function. Drop1 function is based on log-likelihood tests. First, interaction was removed if non-significant and drop1 function was applied again. The best model was always checked for overdispersion. When the main effects (population, age and their interaction) were dropped from the minimal model, their (non) significance was taken from the full model. Some individuals (n = 28) were tested twice – as young and old fish. For this analysis, we assumed that the level of independence was negligible and we have not corrected for this pseudoreplication.

We compared individual performance over lifetime using paired tests. Given that data violated assumptions of Gaussian distribution, we used non-parametric Wilcoxon test. We compared all three measures for young and old age, paired across individuals.

A second dataset (set B) was a subset of fish that had additional covariates available (n = 75 fish). The covariates included Activity levels, Hesitancy, Boldness, Resting Metabolic Rate (RMR), Maximum Metabolic Rate (MO<sub>2</sub>max) and Metabolic Scope (MS). Prior to analysis, covariates were inspected for collinearity. Several covariates were highly correlated.

Therefore, some covariates were removed, retaining lifespan, activity level, boldness, hesitancy, Resting Metabolic rate, Maximum Metabolic rate and Metabolic scope. We then subjected these covariates to analysis of Variance Inflation and found that Metabolic scope was still highly collinear with the other two metabolic variables. Removing Metabolic scope from the set of covariates stabilised Global Variance Inflation Factor (GVIF) to  $<2$  which is well below generally accepted threshold ( $GVIF < 3 - 10$ ).

The same data distribution as for the full dataset were applied for the three particular response variables; ERR4\_7 was still overdispersed when using Poisson distribution and negative binomial distribution was used. We first constructed the full model without interaction (due to sample size limitation), with the exception of population by age interaction. We then sequentially removed non-significant terms until the best subset of predictors (based on Akaike Information Criterion, AIC). We report results from this set of best predictors (minimum adequate models).

Data were visualized using qqplot package in R environment. We illustrate the trends using raw data.

#### **2.4.2 Experiments 2a a 2b - intraspecific and interspecific aggression of males**

Data for each focal (experimental) male were tabulated with information on following factors: rival male species (*N. orthonotus*, *N. furzeri* red, *N. furzeri* yellow, *N. pienaari*), rank of the test (1, 2, 3, 4) and the counts of the three measures of aggression and interest. Exploratory analysis revealed that individual aggression types were relatively rare (zero inflated data). Therefore, a combined variable (named aggression) summed all individual aggressive behaviours into a single count. Interest was analysed separately as we were not certain whether this behaviour represented aggressive acts. We compared the counts of aggressive and interest behaviour separately, using Generalised Linear Mixed Models (GLMM) with Poisson error structure (counts) in lmer package. Response variables were (1) aggression and (2) interest. Fixed factors were rival male species and rank for the test. Given that each male was tested 4 times, we allowed for a random intercept for each male, adding a random effect of male identity (individual code of an experimental male; 1 | maleID). Models were fitted with and without the fixed effect “rank” and their fit compared using Akaike Information Criterion (AIC). Model outcomes were plotted using ggplot package in R environment.



Data for Experiment 2b were analysed in the same way as for Experiment 2a except that the fixed factor of Rank was not present because the design of the experiment was paired and not with sequential use of males. GLMMs with Poisson error structure were used to fit models with response variable Aggression (sum of Gill threat and Mouth treats) and Interest. Fixed factors were Rival male colour morph (red or yellow *N. furzeri*). The full model included also potentially confounding variable of side (left or right position of the glass jar in the experimental tank). However, this variable has never explained any significant part of variability and was always removed from the final models. In addition to GLMMs, we also compared the red and yellow males using paired t-test. The result of this analysis was qualitatively identical.

### 3 Results

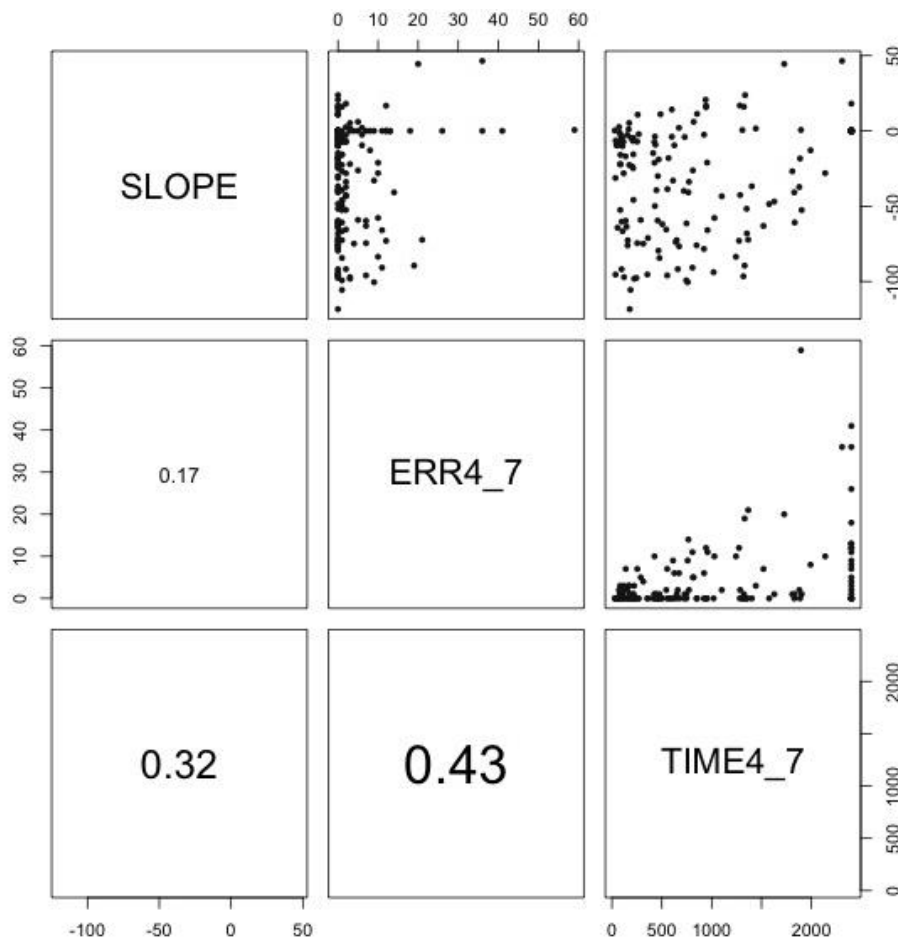
#### 3.1 Experiment 1 – learning ability

##### 3.1.1 General results

The three measures of cognition were correlated (Table 3.1) but not strongly collinear (Figure 3.1). Zero counts for the number of errors were not driven by fish undertaking no activity at all. There were 16 cases of fish not eating the reward food in the group of young fish and 6 cases in the group of old fish. The cases were balanced across trials.

**Table 3.1** | Correlation of three measures of cognition.

Correlated Data	Spearman's rank correlation 'rho'	
	'rho'	p-value
ERR4_7 and SLOPE	0.095	0.244
ERR4_7 and TIME4_7	0.361	< 0.001
SLOPE and TIME4_7	0.286	0.001



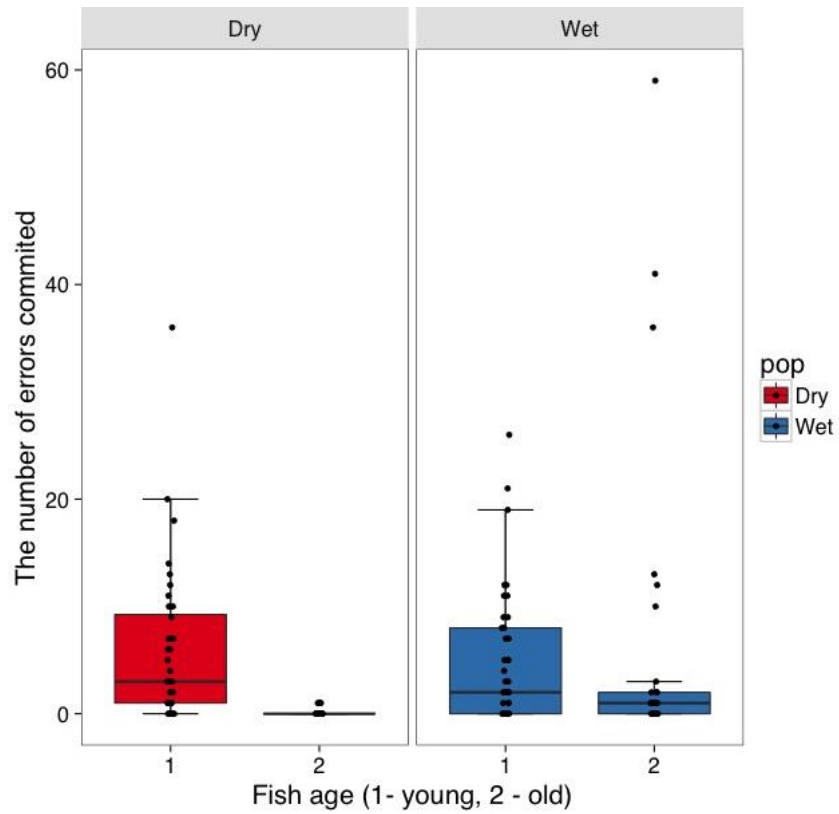
**Figure 3.1.** Correlation of three measures of cognition. Slope: slope of learning line; ERR4\_7: number of errors during test phase; TIME 4\_7: sum of time taken to locate food.

### 3.1.2 Full dataset (all tested fish)

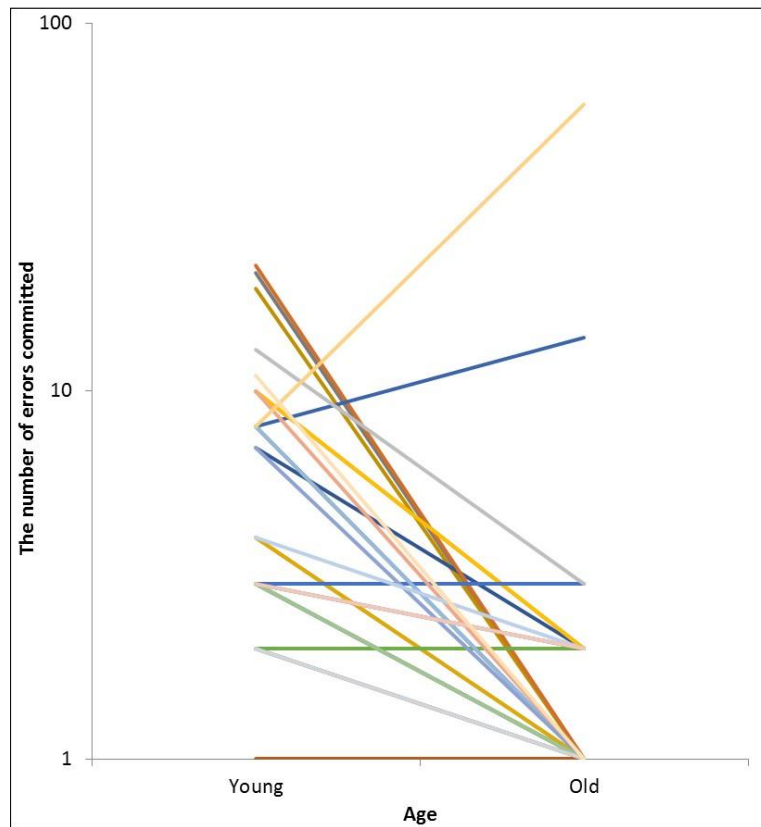
The number of errors committed by individual fish during testing phase (ERR4\_7) was much higher in young than in old fish ( $z = 4.68$ ,  $p < 0.001$ ) and this decrease with age was apparent in the population from dry region (NO002) ( $z = 5.85$ ,  $p < 0.001$ ; Figure 3.2). Overall, there was no difference between populations ( $z = 0.43$ ,  $p = 0.664$ ). The effect of age has been confirmed in paired test, when individual variability was controlled in a subset of fish tested as young and old (Wilcoxon test,  $p = 0.001$ ). Individual fish indeed improved their learning (Figure 3.3). Decrease in the number of errors was not driven by inactivity of old fish – they consumed the food reward more often than young fish (mean value, in percentage, of young fish eating the food: 71.9 %, and of old fish: 74.2 %). There was no difference in the number of errors committed by old fish between fish tested for the first time and fish tested for the second time (GLM with negative binomial error distribution,  $z = 0.62$ ,  $p = 0.534$ ) and no difference in their time taken to locate food (GLM with Gamma error distribution,  $t_{53} = 0.34$ ,  $p = 0.737$ ) or slope of the learning curve (LM:  $F_{1,15} = 1.03$ ,  $p = 0.313$ ). The same outcome was obtained for all fish or a subset with fish from group housing only.

Slope of learning line (SLOPE) did not vary with any of the factors (population:  $F_{1,148} = 0.93$ ,  $p = 0.34$ ; age:  $F_{1,148} = 1.11$ ,  $p = 0.30$ ; their interaction:  $F_{1,148} = 0.90$ ,  $p = 0.34$ ) (Figure 3.4). The overall mean value was -22.5 (s.e. 5.82) that was significantly different from zero ( $t_{148} = 3.78$ ,  $p = 0.002$ ). This indicates overall improvement in learning time over repeated trials. The paired analysis confirmed no difference between young and old fish (Wilcoxon test,  $p = 0.210$ ; Figure 3.5).

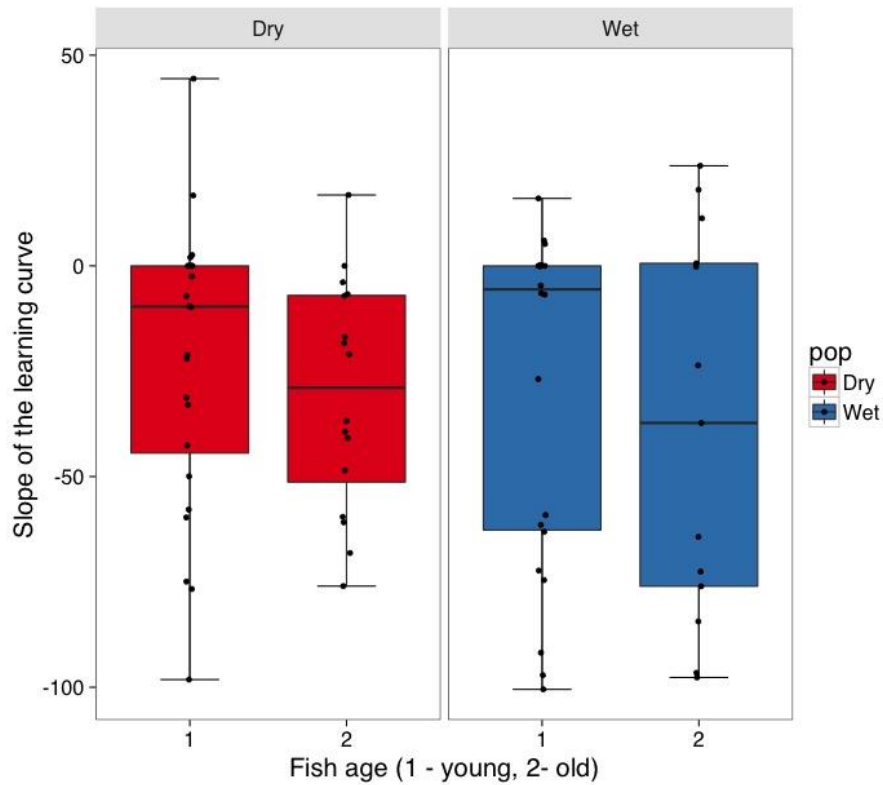
The cumulative time needed to consume the food during testing phase (TIME4\_7) varied between fish kept in social groups and fish kept individually (factor Group) (GLM with Gamma distributed errors:  $t_{151} = 2.44$ ,  $p = 0.016$ ). Solitary fish took longer to locate the food (Figure 3.6). There was no effect of population ( $t_{147} = 1.04$ ,  $p = 0.300$ ), age ( $t_{147} = 0.52$ ,  $p = 0.607$ ) or their interaction ( $t_{147} = 1.01$ ,  $p = 0.314$ ) (Figure 3.7). The paired analysis confirmed no difference between young and old fish (Wilcoxon test,  $p = 0.465$ ; Figure 3.8).



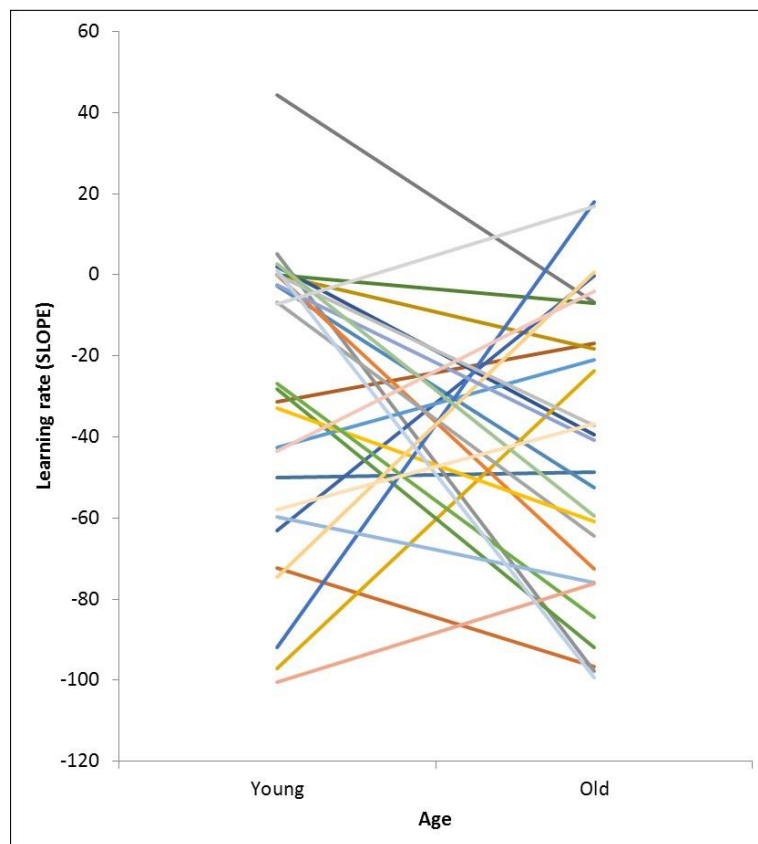
**Figure 3.2.** The number of errors committed in population from dry region (red) and from wet region (blue) with young and older fish groups. Apparent decrease in number of errors in older fish from dry region.



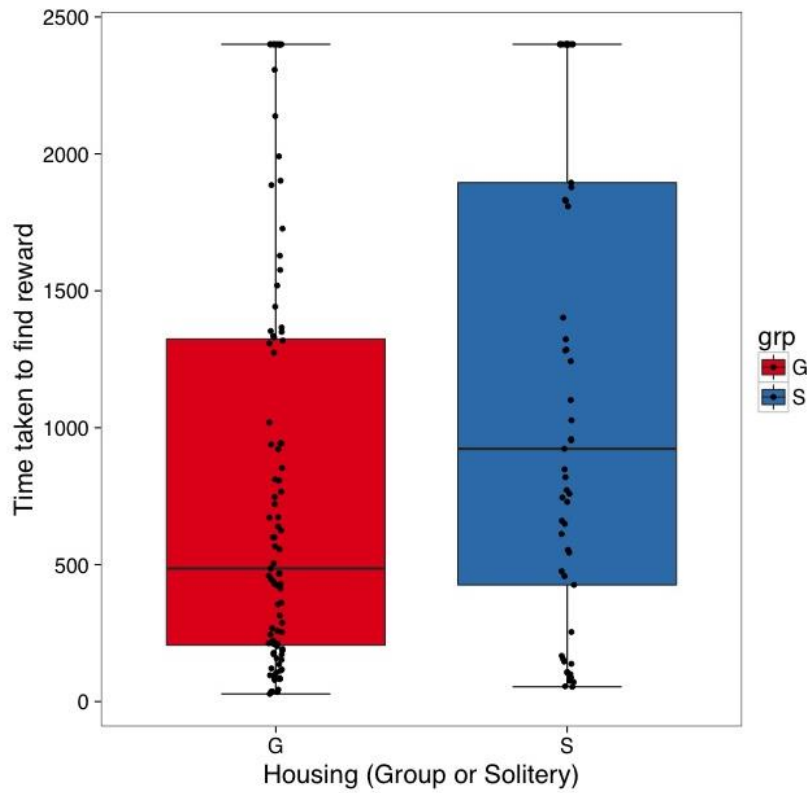
**Figure 3.3.** The number of errors committed in correlation with age of fish. Individual fish made fewer errors in their older age compared to young age. Each line corresponds to a single individual.



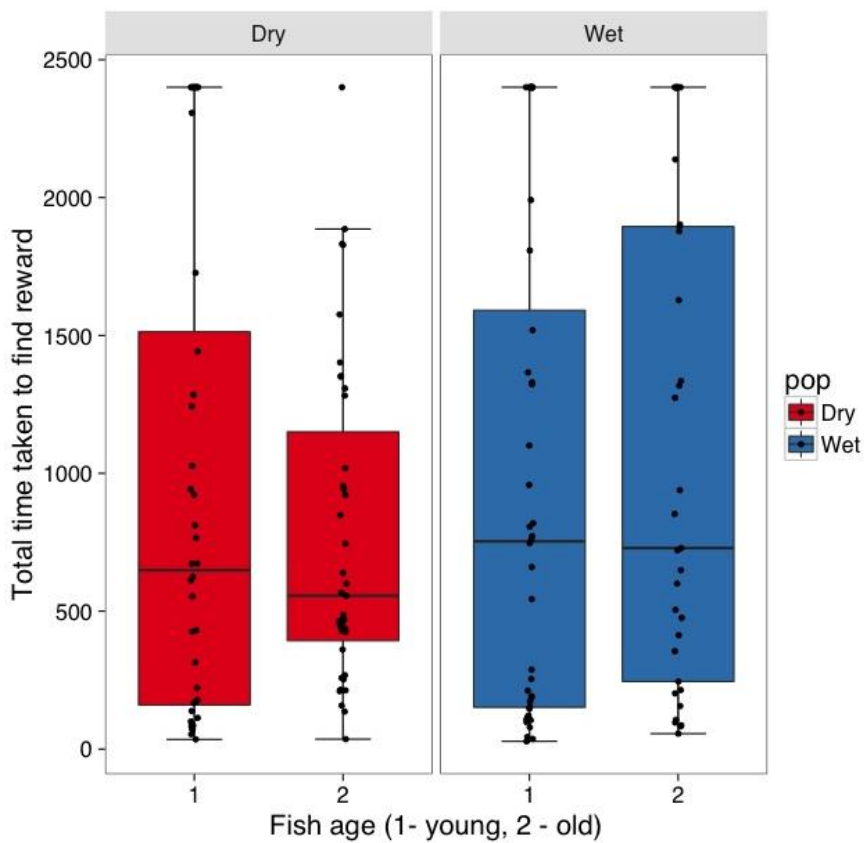
**Figure 3.4.** Slope of the learning curve in population from dry region (red) and from wet region (blue) with young and older fish groups. No apparent differences between populations or young and old fish.



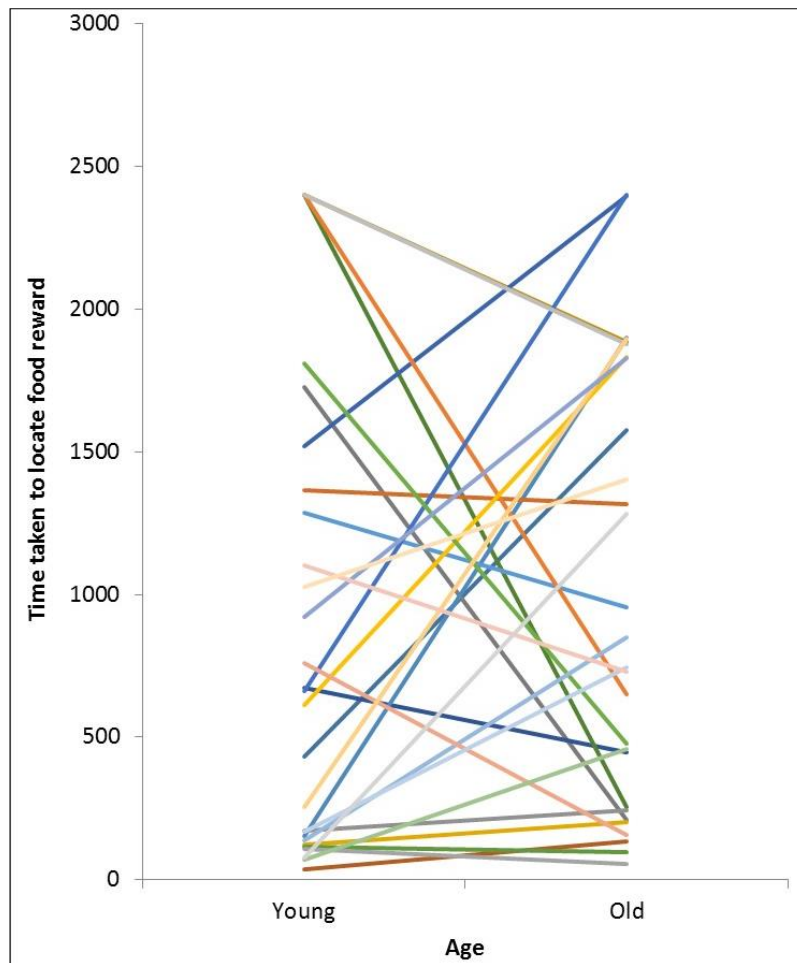
**Figure 3.5.** The speed of learning in correlation with age of fish. Young and older fish did not significantly differ in learning rate. Each line corresponds to a single individual.



**Figure 3.6.** The time needed to consume the food (TIME4\_7) dependent on the fish housing; in groups (red) or in isolation (blue). Fish kept in isolation in captivity took longer time to locate the food reward.



**Figure 3.7.** Total time needed to find the food by fish from dry (red) and humid (blue) region separated into young and older fish group. There was no significant effect.



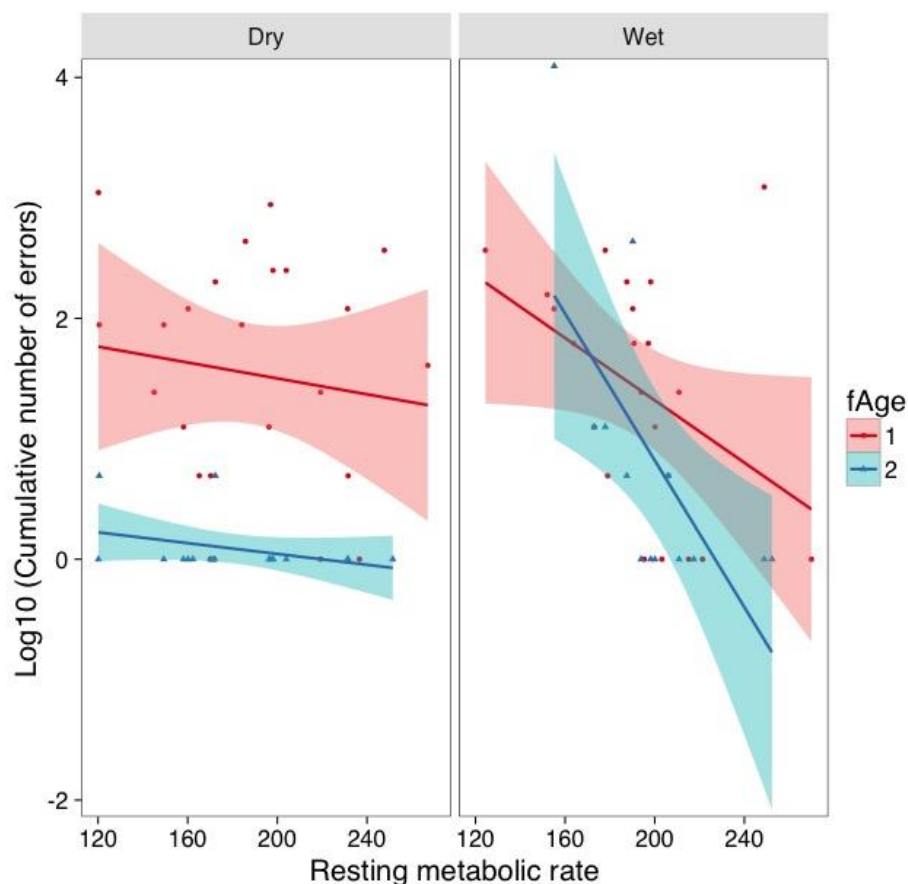
**Figure 3.8.** Time taken to locate food reward by young and old fish. Individual fish in young and older age did not differ in amount of time needed to locate food. Each line corresponds to a single individual.

### 3.1.3 Learning and life history traits

The number of errors committed by subject during testing phase (ERR4\_7) with additional covariates (for a reduced dataset,  $n = 75$  fish) matched that for the full dataset. The number of errors was much higher in young than in old fish ( $z = 4.80$ ,  $p < 0.001$ ) and this decrease with age was apparent in the population from dry region (NO002) (interaction:  $z = 4.04$ ,  $p < 0.001$ ). There was no difference between populations ( $z = 0.26$ ,  $p = 0.796$ ). Only Resting metabolic rate affected the number of errors committed by subject ( $z = 2.08$ ,  $p = 0.037$ ), with a decrease in the number of errors with increase in the RMR (Figure 3.9). The trend appeared much stronger in population from wet region but there was not enough observation to include interactions in the model.

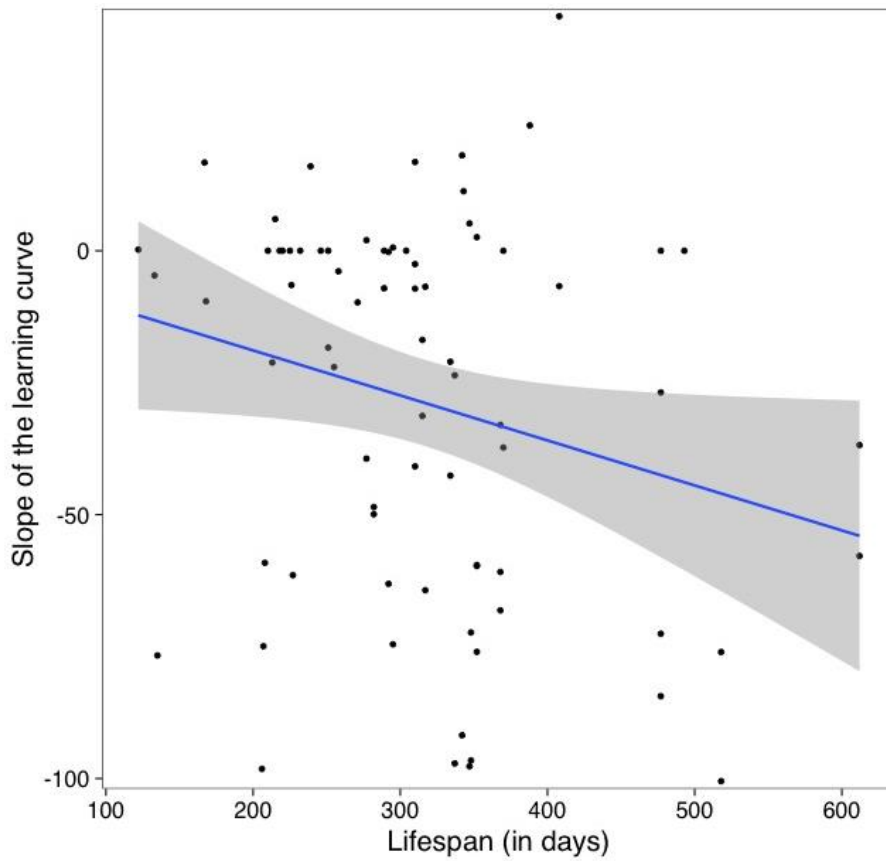
The values for slope of learning line (SLOPE) decreased with individual longevity ( $F_{1,72} = 4.46$ ,  $p = 0.038$ ; Figure 3.10) and tended to decrease with RMR ( $F_{1,72} = 3.87$ ,  $p = 0.053$ ) (Figure 3.11).

The best model for cumulative time needed to consume the food during testing phase (TIME4\_7) included the effects of social environment ( $t_{70} = 2.01$ ,  $p = 0.049$ , Figure 3.12), sex ( $t_{70} = 1.64$ ,  $p = 0.106$ , Figure 3.13) and hesitancy to leave the refuge ( $t_{70} = 1.53$ ,  $p = 0.131$ , Figure 3.14). There was no effect of RMR ( $t_{59} = 0.42$ ,  $p = 0.695$  in the full model), though the trend was comparable as for the other two cognition measures, especially in wet region population (Figure 3.15). Testing this association yielded significant effect of RMR by population interaction (interaction:  $t_{71} = 2.41$ ,  $p = 0.019$ ; RMR:  $t_{71} = 0.69$ ,  $p = 0.493$ ; population:  $t_{71} = 2.52$ ,  $p = 0.014$ ), though it must be noted that this specific model has been constructed only after the relationship between RMR and population identity was visualized graphically.

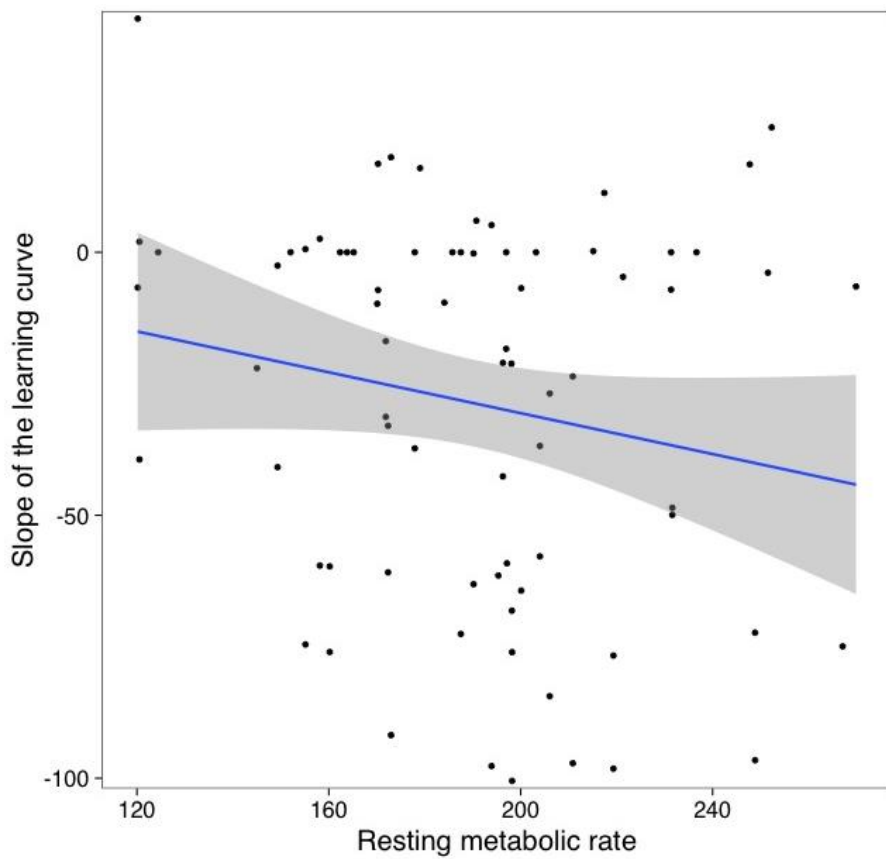


**Figure 3.9.** Fish with higher RMR made less errors, with the trend apparent in wet region population (right panel).

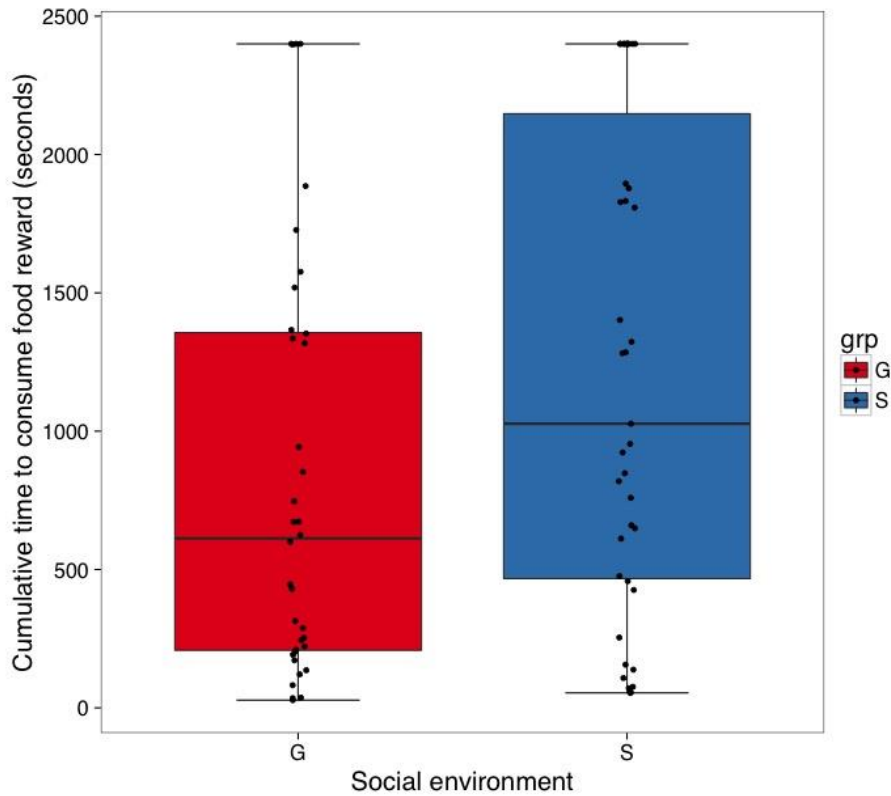




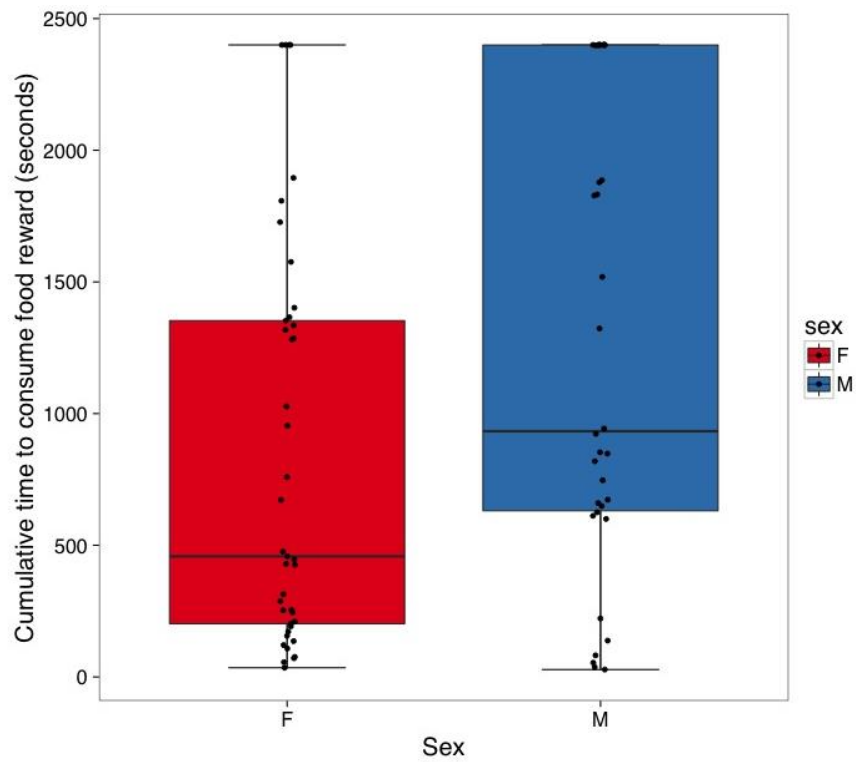
**Figure 3.10.** Slope of the learning line decreasing with lifespan.



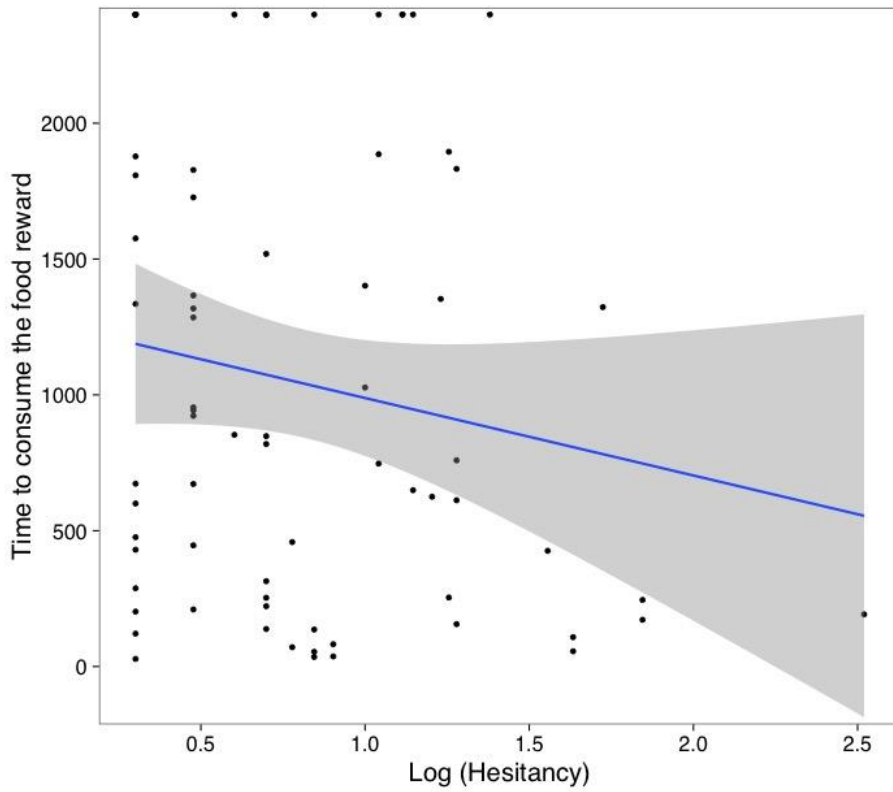
**Figure 3.11.** Decreasing slope of the learning line with increasing Resting metabolic rate.



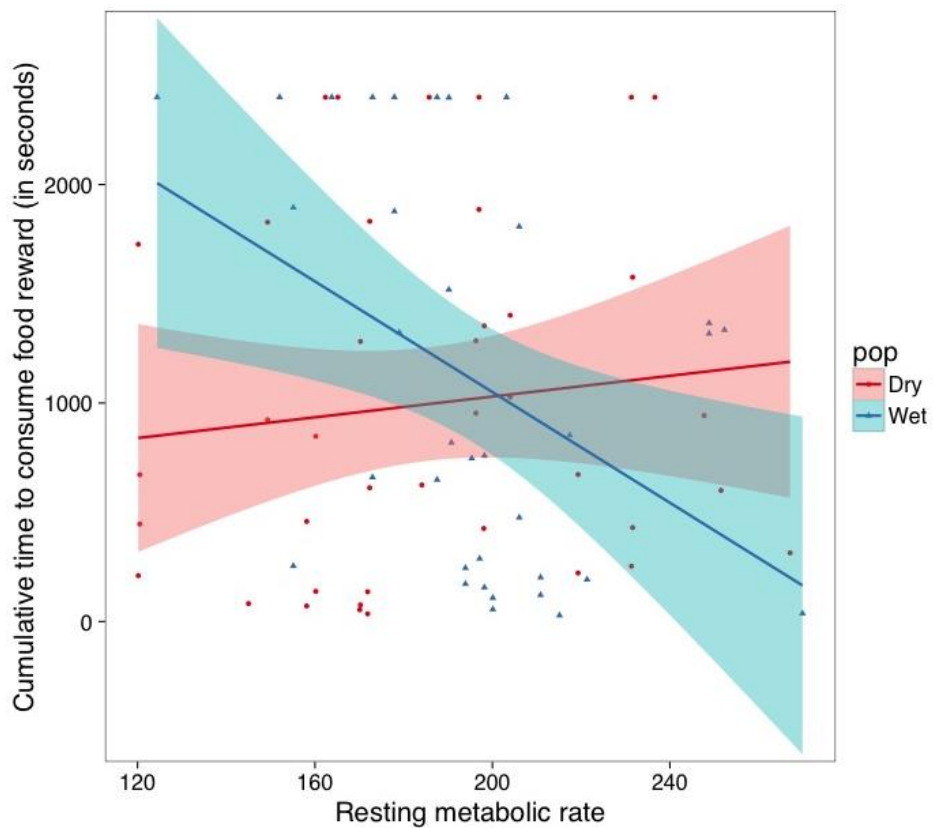
**Figure 3.12.** Cumulative time needed to consume the food during testing phase affected by social environment. (G) Fish housed in groups (red colour); (S) fish housed individually (blue).



**Figure 3.13.** Cumulative time needed to consume the food during testing phase affected by sex. (F) Females (red); (M) males (blue).



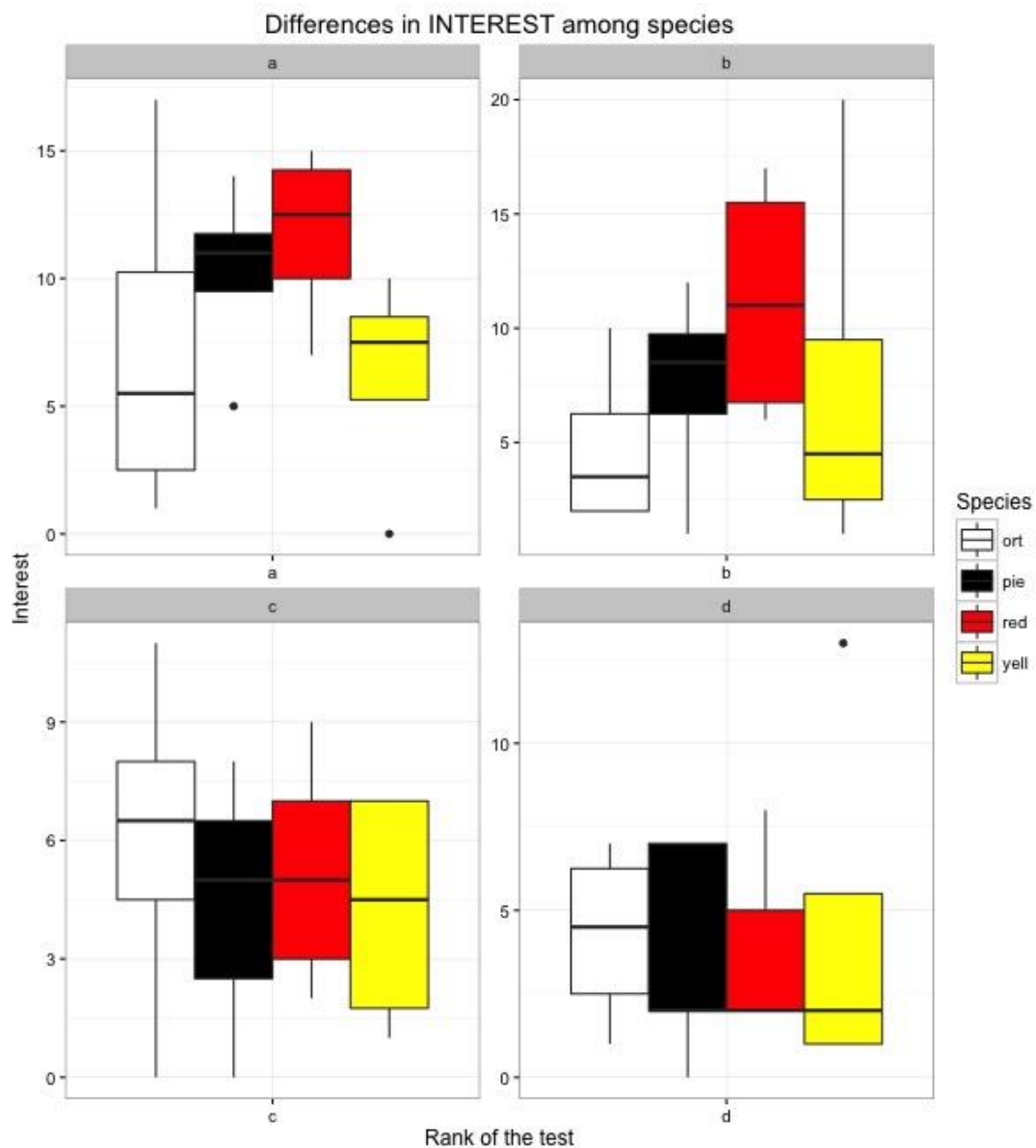
**Figure 3.14.** Cumulative time needed to consume the food during testing phase affected by hesitancy to leave the refuge. With increase in hesitancy, time for consumption of the food decreased.



**Figure 3.15.** Cumulative time to consume the food affected by Resting metabolic rate in population from wet region (blue) but not from dry region (red).

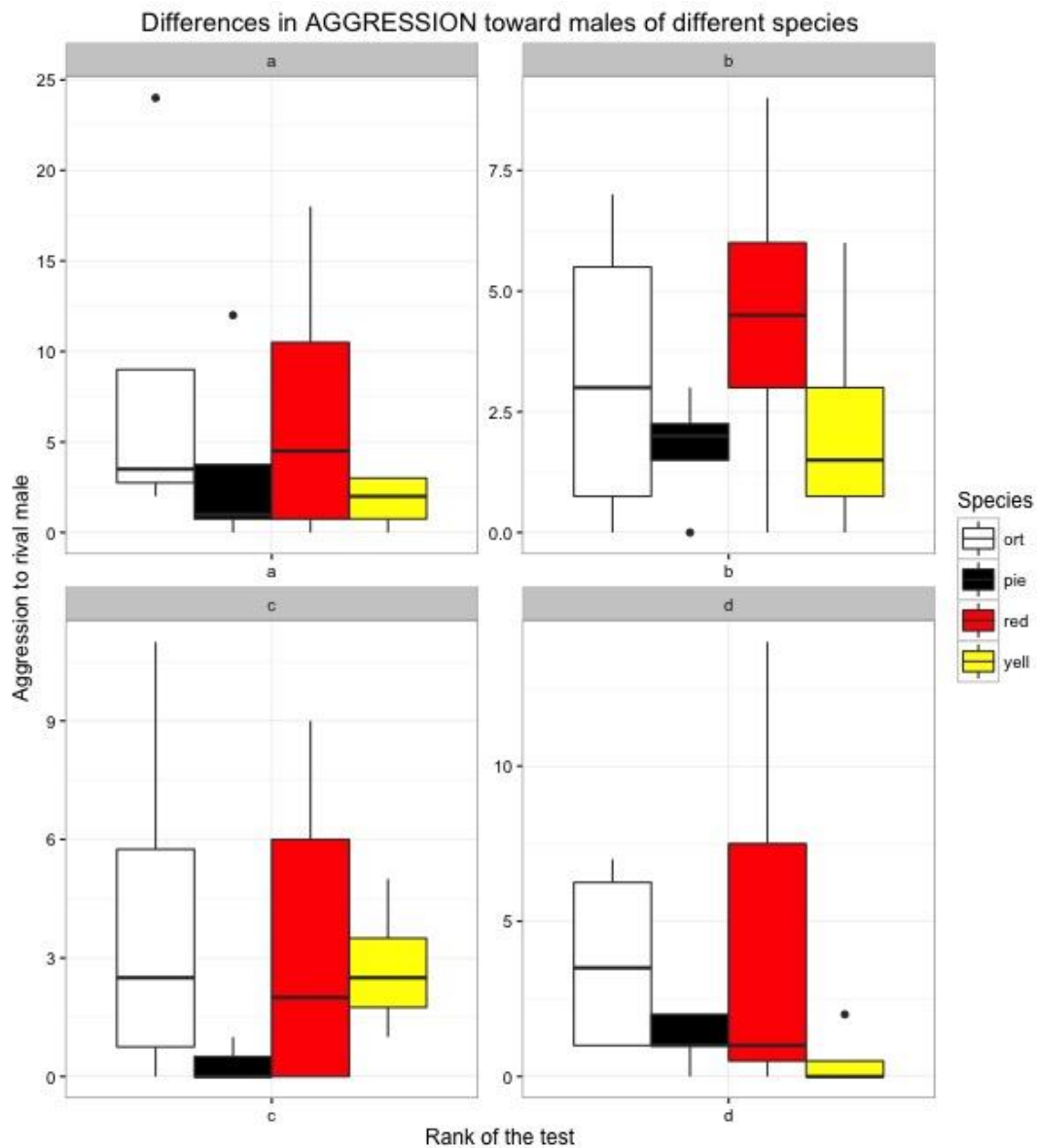
### 3.2 Experiment 2a – intraspecific and interspecific aggression of males

There was significant effect of male type on the interest rate by male *N. orthonotus* ( $F = 3.28$ ) and the rate of interest was modified by the rank of male presentation ( $F = 11.72$ ). Male *N. orthonotus* paid the strongest interest in red *N. furzeri* and the interest was generally higher and variability among rival male types larger when rival males were presented as the first or second in their sequence/rank (Figure 3.16).

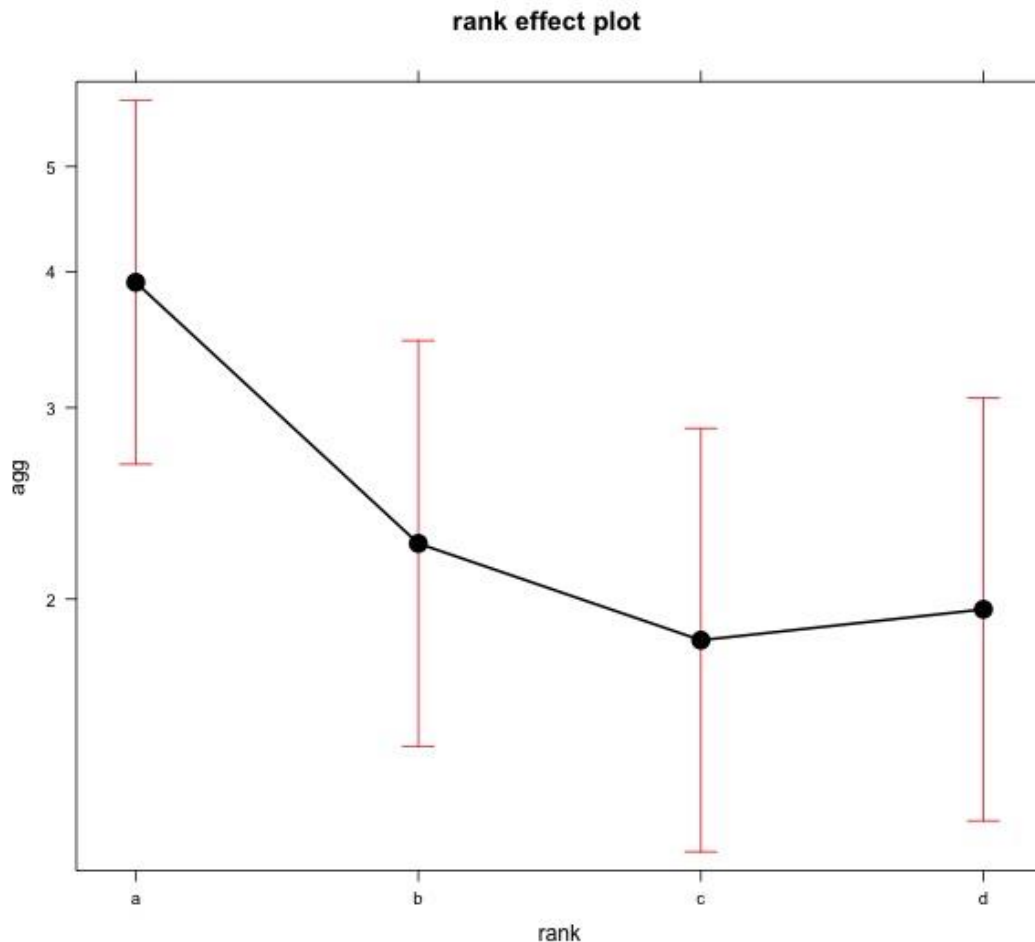


**Figure 3.16.** Difference in Interest among species. The Interest towards rival male affected by the rank in the experiment (a, b, c, d = 1, 2, 3, 4). Species: (ort) *N. orthonotus*; (pie) *N. pienaar*; (red) red *N. furzeri*; (yell) yellow *N. furzeri*.

Male *N. orthonotus* considerably differed in their aggression toward rival males ( $\chi^2 = 47.2$ , d.f. = 3,  $p < 0.001$ ). The highest aggression was directed toward conspecific males (*N. orthonotus*) and red *N. furzeri* and this was consistent across the sequence/rank of testing (Figure 3.17). Experimental male aggression gradually declined with further testing ( $\chi^2 = 20.2$ , d.f. = 3,  $p < 0.001$ ; Figure 3.18).



**Figure 3.17.** Difference in aggression to rival males of different species. Aggression affected by sequence/rank of testing. Species: (ort) *N. orthonotus*; (pie) *N. pienaar*; (red) red *N. furzeri*; (yell) yellow *N. furzeri*.



**Figure 3.18.** Aggression affected by rank of testing. Gradual decline in aggression with increasing rank of the testing sequence.

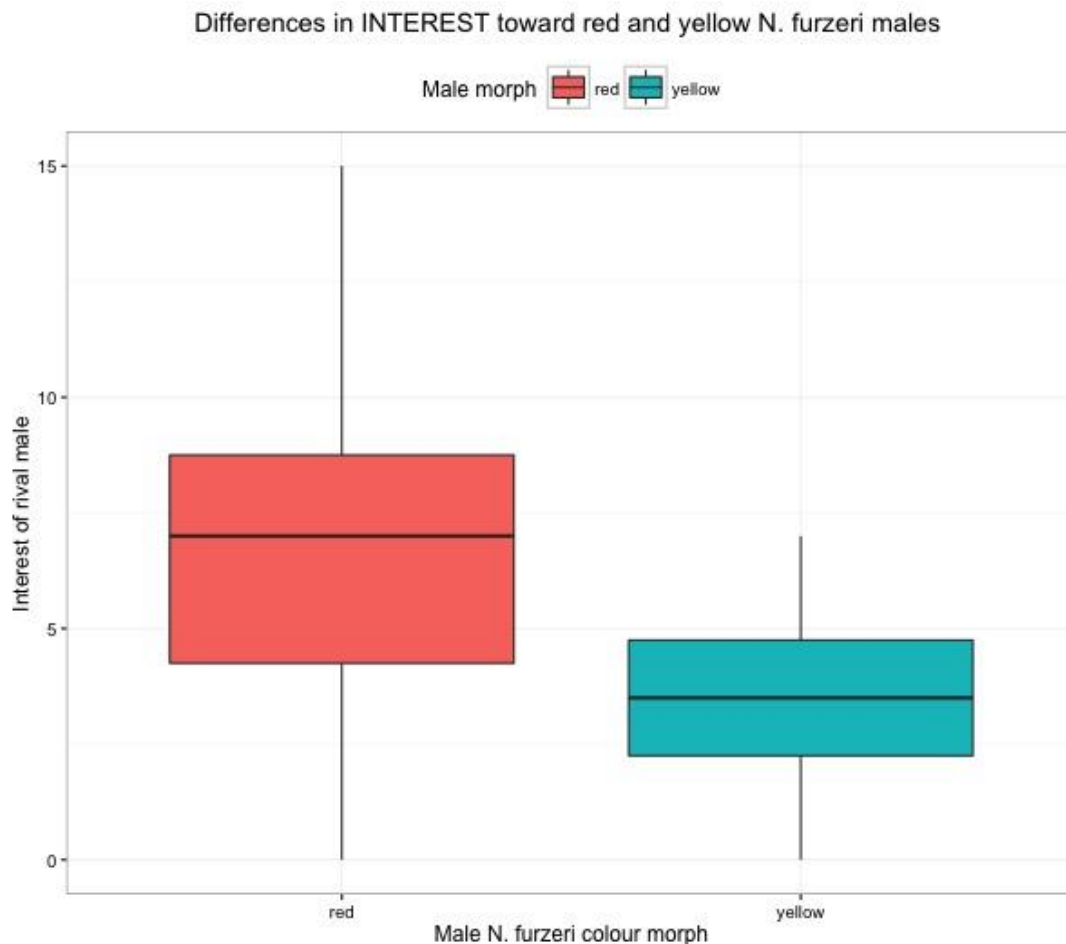
### 3.3 Experiment 2b – interspecific aggression among males of *N. orthonotus* and two colour forms of *N. furzeri*

There was strong positive relationship between male *N. orthonotus* aggression and his body size (TL) due to a single excessively aggressive male (ORT17) when Pearson correlation was used ( $r = 0.709$ ,  $p = 0.015$ ) but this trend ceased when non-parametric Spearman correlation was used ( $r_s = 0.101$ ,  $p = 0.767$ ). There was no difference in TL (body size) between red and yellow *N. furzeri* males (paired t-test:  $t = 0.16$ ,  $p = 0.88$ ).

Male *N. orthonotus* directed more interest on red *N. furzeri* than on yellow *N. furzeri* (GLMM:  $z = 3.35$ ,  $p = 0.001$ ). However, there was only a weak trend for *N. orthonotus* to address more aggressive attacks towards red *N. furzeri* than yellow *N. furzeri* (GLMM:  $z = 1.17$ ,  $p = 0.241$ ).

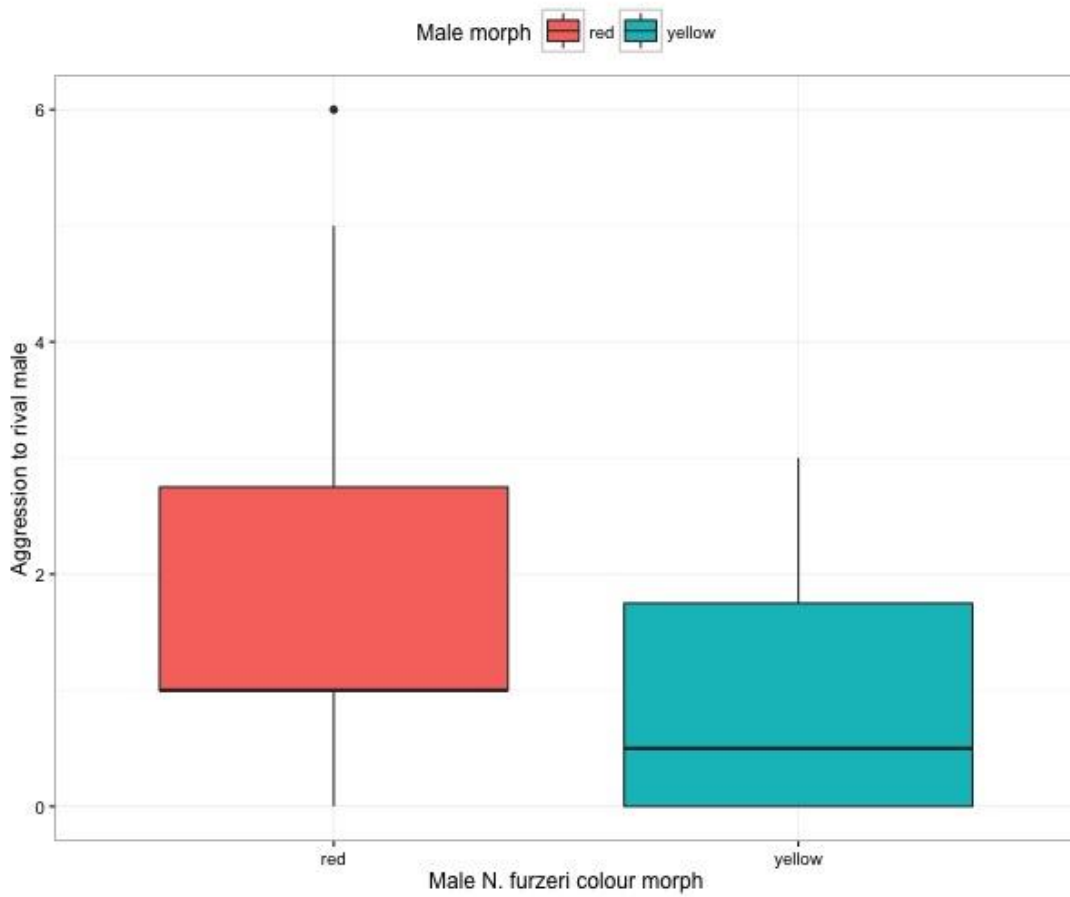
After removal of the excessively aggressive male ORT17, there was no qualitative change in the results. Male *N. orthonotus* still directed significantly more interest on red *N. furzeri* than on yellow *N. furzeri* (GLMM:  $z = 2.82$ ,  $p = 0.005$ ) (Figure 3.19). However, the trend for *N. orthonotus* to address more aggressive attacks towards red *N. furzeri* than yellow *N. furzeri* was stronger than in the full dataset (GLMM:  $z = 1.61$ ,  $p = 0.108$ ) (Figure 3.20).

The outcome was identical if paired t-test was used. Male *N. orthonotus* directed more interest on red *N. furzeri* than on yellow *N. furzeri* (paired t-test:  $t_{15} = 2.71$ ,  $p = 0.016$ ). There was no difference in aggression directed toward red and yellow males (paired t-test:  $t_{15} = 1.19$ ,  $p = 0.258$ ).



**Figure 3.19.** Differences in interest towards red and yellow *N. furzeri* males. Data after removal of the excessively aggressive male ORT17.

Differences in AGGRESSION toward red and yellow *N. furzeri* males



**Figure 3.20.** Differences in aggression towards red and yellow *N. furzeri* males. Data after removal of the excessively aggressive male ORT17.



## 4 Discussion

### 4.1 Experiment 1 – learning ability

In the first study of my master thesis, I tested cognitive ability of two populations of *N. orthonotus* that originated from temporary pools that varied in their expected duration. The results of my study suggest that (1) the two studied populations of *N. orthonotus* did not vary in the learning ability; (2) both populations had similar lifespan with no cognitive decay in older age, and older fish from dry region population had committed even less errors in the learning task than they did as young fish; (3) fish were able to effectively learn the task but (4) solitary fish had lower learning ability than group-reared fish; (5) there is a correlation between lifespan and learning rate, with long-living fish having faster speed of learning; (6) fish with high resting metabolic rate are more effective learners; (7) hesitant fish are able to find the food reward in shorter time than bolder fish.

It was predicted that *N. orthonotus*' population from dry region complete the learning task faster than population from humid region, despite perhaps making more mistakes. This hypothesis was based on the so-called “pace-of-life syndrome”, which suggests that rapid life history is associated with high metabolic rates and more active, aggressive and bolder behaviour (Réale *et al.*, 2010). The pace-of-life syndrome was successfully tested at the inter-population levels, showing for example that 4 populations of nestling stonechats (*Saxicola torquata*) from different environments adjusted their metabolic activity to the local conditions and even in common-garden conditions each population kept its own ‘pace of life’ along both physiological and life-history axes of variation (Wikelski *et al.*, 2003). In my study, the ‘pace of life’ of population from dry area was expected to be faster than in population from humid area due to the sooner habitat deterioration. Consequently, the overall more active and bolder behaviour of *N. orthonotus* from the arid area was assumed to lead to also faster completion of the learning task despite potentially making more errors as well. This would not mean that population from dry area has better cognitive ability. It would be just the consequence of more active and bolder behaviour. Nevertheless, the hypothesis was not proven as both populations performed equally well in the learning task with no difference in the number of mistakes. The possible reason for my observations could be that the difference between environmental conditions in humid and arid area was not large enough to cause a divergent selection pressure on the population-level life histories in dry and wet regions. Besides different duration length of the habitat, both populations still share similar environmental

conditions, predation risk and diet. Therefore, such similarity in niche prevents from generating alternative ecotypes and thus distinct inter-population pace-of-life syndromes. This finding is consistent with other aspects of the project on dry and wet region populations of four *Nothobranchius* species. The pairs of populations significantly differed in their lifespan and rate of functional ageing process in captivity (Blažek *et al.*, submitted) but short lifespan did not coevolve with pace-of-life syndrome (Blažek *et al.*, submitted), despite life history related to rapid growth and sexual maturation correlated with lifespan as predicted (Blažek *et al.*, submitted).

The other hypothesis was that fish from dry region will show lower learning ability in older age, in comparison with the population from humid region, due to their steeper ageing rate. This was not confirmed as the older fish from any population did not exhibit apparent decay in learning caused by ageing process. There was also no significant difference in lifespan between populations from dry and wet region, which is in contrast to other populations of *N. furzeri*, *N. pienaar* and *N. kadleci* originating from similarly varied areas (Tozzini *et al.*, 2013; Blažek *et al.*, submitted). Moreover, older fish from dry region population had committed fewer errors during the learning task than they did as young fish. Similar trend was seen in the population from humid region but it was not significant. Such result could be explained by several plausible factors. For example, the older fish from dry area may be overall less active and that could play a role in making fewer errors in total. However, this was shown as false because older fish consumed the food reward even slightly more often than young fish. Therefore, the older fish' appetite and thus the motivation to search for food reward were not reduced at all. It is possible that fewer errors made could mean that older fish from dry region indeed improved their learning ability in comparison to young fish. The explanation of such interpretation could be that the subset of older fish, which was tested for the second time, actually remembered the first experience to a certain extent. This was assumed to be highly unlikely and the dataset was not corrected for pseudoreplication. Direct test that directly compared performance of fish tested for the first or second time confirmed that this is not the explanation of the trend. The interval between the two testing periods was around 28 weeks (6 months) and that is a long period of time considering the fact that it is more than a half of their mean lifespan measured in captivity. Additionally, it seems that fish that live in a more enclosed environment, where food is abundant, do not need to remember the positions of specific food patches and thus spatial memory capacity may not be as important (Brydges *et al.*, 2008). Learning and memory are linked, however, the processes are distinct and memory has been studied far less than learning

ability (Shettleworth, 2009). Nevertheless, the fish showed to have at least two days memory length during the experiment. Besides the possible memory effect on the results, the experience from young age alone could have significant effect on further development of cognitive ability.

It is surprising that the ageing process did not seem to negatively influence the learning ability in older fish. The possible reason may be the overall slower onset of physiological decay linked to ageing than previously thought, resulting in rather gradual increasing of the fish mortality in populations. This probably affected the experiment on older fish in such way that the experimental fish were not showing enough pronounced ageing to cause significant decay in cognitive ability. In other studies on populations of *N. furzeri*, association was found between environment and ageing/lifespan, showing inter-population difference in lifespan and in beginning of physiological decay linked to ageing (Terzibasi *et al.*, 2008; Blažek *et al.*, submitted). In humid areas, where longer duration of habitat is generally expected, *N. furzeri* population aged in slower rate than its conspecific neighbour from arid area (Terzibasi *et al.*, 2008; Blažek *et al.*, submitted). Such association was not found in my studied populations of *N. orthonotus*, despite proven in the other three species of *Nothobranchius* within the project. However, dry-region population of *N. orthonotus* did display significantly faster increase in oxidative damage in heart, brain and liver, consistent with their more rapid functional deterioration. It seems that the link between life history evolution related to ageing and pace-of-life syndrome and cognitive abilities are not linked.

Overall, the slope of learning curve was negative, proving that fish were able to learn and improve during the testing period but no effect of either population origin or the age of fish was found. The found improvement of fish was not affected by olfactory cue navigation. To ensure that fish cannot use its senses to find the location of food, I dispersed the scent of the food throughout the whole aquarium using drops of the water from the food. Moreover, olfactory cue detection could not explain the improvement in the learning task; the fish would be either poor all the time or good all the time, depending on cue detection ability. Therefore, the fish proved to have good learning abilities by being able to learn the location of food after just 7 trials in two days. Demonstrated learning indicates that this ability is useful and beneficial for *N. orthonotus* in its natural environment which is considered to be very unpredictable. That is in accordance to the prediction that unpredictable and variable environmental conditions may favour selection on higher cognitive abilities due to need of more flexible behaviour (Brown *et al.*, 2003).

The next hypothesis was that individuals housed in social groups in captivity will perform better than individually-housed fish. Indeed, the solitary fish took longer to find (locate and eat) food than fish reared in groups thus showing that the effect of social environment is strong enough to shape learning ability in *N. orthonotus*. Fish reared in groups has to compete for food and interact with other fish, which can all together lead to reduced shyness and stimulate cognitive ability. This is in contrast with single-reared fish which has no opportunity to interact with other fish or use any effort to get access to food. The detected correlation between social environment and learning ability is consistent with the general prediction that social environment can shape fish cognitive ability (Brown and Laland, 2003).

I found association between individual lifespan and the speed of learning. The quicker learner the fish was, the longer it lived. However, it is difficult to find appropriate explanation for this trend. Perhaps, longer lifespan could be associated with individuals of overall higher quality, demonstrated in several traits, including ability to learn. Nevertheless, it should be noted that this trend could be just an artefact of small sample size.

Various data about two populations of *N. orthonotus* used in the tests were collected during their life allowing me to study their possible effect on learning ability. An interesting correlation was found between the learning rate and resting metabolic rate (RMR). Fish from both populations were quicker learners and committed fewer errors in the learning task if they had higher RMR. Resting metabolic rate is whole body metabolism during a steady resting conditions and it is much more used as measurement of daily energy expenditure due to its easier accessibility than basal metabolic rate which must be measured only in very strict conditions to obtain total physiological equilibrium (Hulbert and Else, 2004). Why did fish with high RMR learnt faster and committed less errors? One possible interpretation can be that RMR of *N. orthonotus* is connected with another trait, significant to the cognitive ability, and that is relative brain size. There are studies that showed the important role of relative brain size in cognitive abilities within and between various animal taxa (e.g. Barrett and Henzi, 2005; Kotrschal *et al.*, 2013), and basal metabolic rate (BMR) proved to be correlating in significant way with brain size, at least in some terrestrial vertebrates (Martin, 1981; Isler and Van Schaik, 2006). The relative brain size can differ between individuals of the same species, for example, populations of marsh wren, *Cistothorus palustris*, had 30-40% difference in volumes of two specific areas of the brain involved in song due to different amount of songs learned in each population (Canady *et al.*, 1984). Additionally, the ratios of central nervous system metabolism to RMR in teleost fish was found to be similar to those described for other vertebrates (Mink *et al.*, 1981). RMR is considered as a good

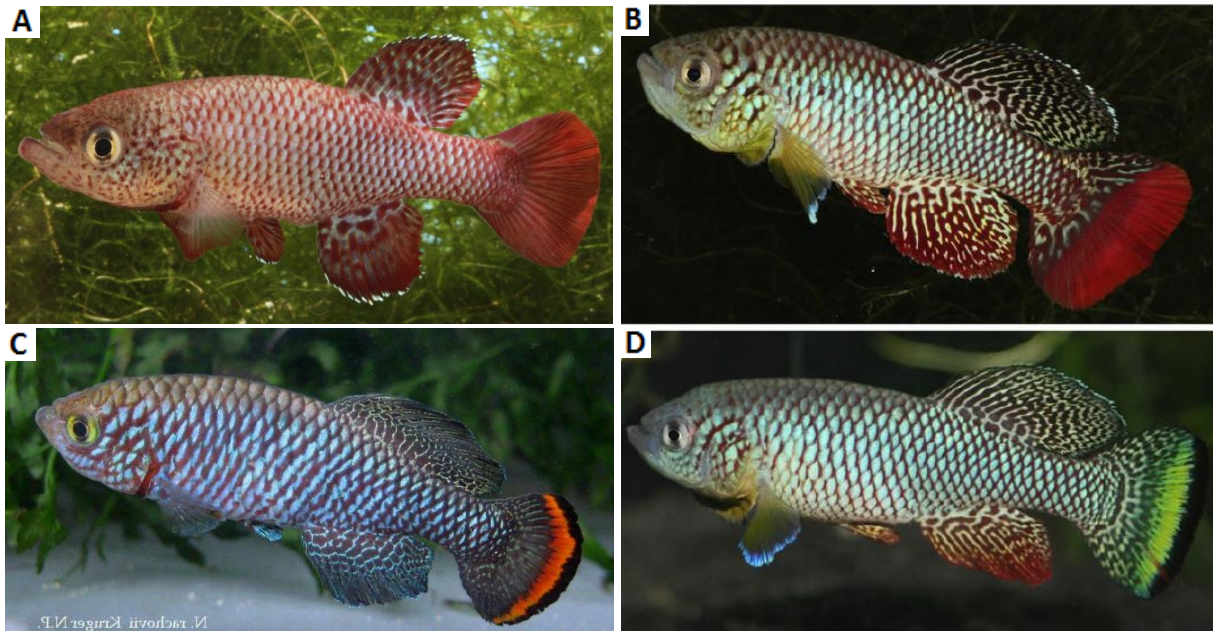
approximation of BMR and thus it is probable that increased RMR relative to body mass can serve also as the indicator of increased relative brain size. If that is true, tested *N. orthonotus* with high RMR showed higher cognitive ability due to larger brain size. In conclusion, these results suggest that the hypothesis about correlation between basal (or resting) metabolic rate and relative brain size is worth to pursue in further studies on *Nothobranchius*. In addition, the fish from wet region population that had high RMR also completed the learning task in shorter amount of time than fish with lower RMR. However, as we did not prove difference in lifespan or cognitive ability between the two populations, it remains unclear what caused this additional time difference between wet and dry region population and it requires further study with a specific hypothesis.

Another correlation appeared between the cumulative time needed to locate the food reward during the testing period and hesitancy to leave the refuge. Fish that took more time to leave the refuge after they initially emerged tended to spend less time to locate the food. This trend is in contrast to the hypothesis in my study which predicted that bolder or more active fish will show better performance in the learning task but at the expense of accuracy. In theory, bolder fish should begin exploring the maze sooner than shy fish and thus they would consequently discover the location of the foraging patch more quickly (Brown and Braithwaite, 2005). As a result, the bold fish may appear to be better at a cognitive task (Sih and Del Giudice, 2012). This theory was confirmed for example in the poeciliid species *Brachyraphis episcopi* where bold fish completed the maze faster than shy fish (Brown and Braithwaite, 2005). But this effect of boldness was also shown in other animals, such as ravens and chickadees (Stöwe and Kotrschal, 2007; Guillette *et al.*, 2010). High predation risk seems to increase the level of boldness in poeciliid fish and, on the other hand, shyness tends to increase with age (Brown *et al.*, 2005). In lizards, however, both 'bold' and 'shy' behavioural male types were more successful learners than intermediate males (Carazo *et al.*, 2014). Perhaps, it could be possible that shy individuals, when finally start to explore the area, tend to be more observant and attentive and thus spend less time by unfocused swimming which could ultimately lead to better efficiency of activity. Alternatively, recorded correlation could be connected with some other trait that we did not measure. Nevertheless, the explanation for our result that more hesitant, and thus shyer, fish spend less time locating the food remains unclear.

## 4.2 Experiments 2a and 2b – intraspecific and interspecific aggression of males

My hypothesis was that male aggression of *N. orthonotus* would be highest towards conspecific males but they would also show more aggression towards red morph of *N. furzeri* compared to yellow morph of NF due to more similar colouration between *N. orthonotus* and red *N. furzeri*. Lower aggressive behaviour was expected to be directed also to differently coloured males of *N. pienaarri* (black colouration) that were used as additional controls due to their sympatric occurrence with *N. orthonotus* and most distinct colouration. To test this hypothesis, two experiments were conducted. First experiment tested aggression of *N. orthonotus* towards 4 opponents (*N. orthonotus*, two coloured morphs of *N. furzeri*, and *N. pienaarri*), each tested singly. The second experiment focused solely on aggression of *N. orthonotus* towards two colour morphs of *N. furzeri*, which were presented simultaneously to the focal *N. orthonotus* male. Overall, the collected data supported the hypothesis; I showed that red coloured males of *N. furzeri* suffered the same amount of aggression as conspecific males (*N. orthonotus* males). Also, the interest was the highest towards red coloured males of *N. furzeri* and towards conspecific males. This considerable difference in expression of aggression remained consistent across the various sequences of testing (test rank order).

Why should *N. orthonotus*' males be more aggressive towards red *N. furzeri* than to yellow *N. furzeri* and *N. pienaarri*? We hypothesised that the difference in aggression stems from the close phenotypical resemblance between red *N. furzeri* and *N. orthonotus* (Figure 4.1A, B). Another two presented rivals were more divergent from *N. orthonotus* in their colouration, which was either yellow or black (Figure 4.1C, D). There are several studies that showed previously that interspecific aggression was significantly more intense if the species (and individuals) were phenotypically more similar to the aggressor (e.g. Seehausen and Schluter, 2004; Dijkstra *et al.*, 2007; Pauers *et al.*, 2008; Anderson and Grether, 2010). For example, the nuptial colouration in cichlids from Lake Victoria and Lake Malawi was found to be important for the recognition of competitors and even for the modulation of aggressive behaviour towards them (Seehausen and Schluter, 2004; Pauers *et al.*, 2008). In the study of Pauers *et al.* (2008) with species often co-occurring in the wild, males exhibited different types of aggressive behaviour depending on the opponent species. When the opponent was conspecific, he received more threats through lateral displays but when heterospecific,



**Figure 4.1.** Photographs of males of *N. orthonotus* (A), red *N. furzeri* (B), *N. pienaarri* (C) and yellow *N. furzeri* (D). (Photo A by R. Blažek; photos B and D taken from Cellerino *et al.*, 2016; photo C taken from nothos.webnode.cz.)

he received more bites (Pauers *et al.*, 2008). Lateral displays were seen as threats used by the individual to intimidate the opponent suggesting that he sees him as a rival and tries to communicate his superiority, rather than doing physical harm (Pauers *et al.*, 2008). Therefore, more lateral displays were interpreted as attempts to intimidate whereas the biting as attempt to chase opponent away (Pauers *et al.*, 2008). In my first experiment 2a, I recorded 3 different types of aggressive behaviour, aiming to reveal if there were any differences in their use toward individuals of the particular species. These 3 types were frontal threat that was characterized as attempts to bite the rival or as jerking movement towards him. The other two types were opercular threat, typical feature for this genus, and threatening by lateral display of the male's body. However, the latter proved to be, from all the types, the least discernible one and thus only few cases of lateral threatening were recorded during the experiment. This could have been caused by its problematic recognition or no actual occurrence. Frontal threat and opercular threat were performed more often but not in sufficient amount for each to be analysed separately. While one reason for a lower amount of aggressive behaviours of some focal males could be in higher level of stress during the experiment, time for acclimatization was more than sufficient when taking into account the ability of *Nothobranchius* fish to cope with stressful environment and its low demands on breeding and maintaining in captivity. Another reason could be intrinsically lower aggression level of some males or a failure to recognize the rival males in a jar as a real rival.

It should be noted that the light conditions inside the experimental room during the experiment 2a were probably not optimal as the light was brighter than in the room in which the fish were normally maintained. This was fixed before the experiment 2b started, but no distinct change in fish behaviour was found. Whatever the reason could be, the amount of each specific type of the aggressive behaviour was not numerous enough to conduct a separate test for that behaviour, and thus, we could not determine if there were differences in their use accordingly to rival male species identity. We combined all 3 individual aggressive behaviours into a single count to describe general aggression level and I was unable to test whether *N. orthonotus* is capable to distinguish between the conspecific male and red *N. furzeri* by displaying different threats. During the experiments I did not notice any significant evidence for this possibility but more males would have to be tested to enable me more relevant conclusion on this hypothesis.

However, the colour similarity is not the single possible explanation at hand for increased aggression of *N. orthonotus* towards red male of *N. furzeri*. It is also possible that the bright colouration of the red morph of *N. furzeri* could play its role in the resulting higher level of aggression. This possibility is based on studies which showed that orange and reddish colouration are often associated with aggressive signalling among various animal taxa. An example can be brightly coloured males of three-spined stickleback *Gasterosteus aculeatus*, in which higher intensity of red colouration is used among males as threat signal, reflecting the social dominance and it is being preferred in female choice (Candolin, 1999). This brings another aspect, often connected with these bright colouration, and that is dominance of such individuals among conspecific males (e.g. Pryke, 2009). Comparing the 3 studied species (*N. orthonotus*, *N. furzeri* and *N. pienaarri*), *N. orthonotus* is generally considered to be the most aggressive in wild populations (Wildekamp, 2004), with *N. furzeri* showing intermediate level of aggression and *N. pienaarri* showing the lowest aggression of all three species (Polačik and Reichard, 2011).

Colour morphs are often associated with different life histories and behavioural tactics (Sinervo *et al.*, 2000) and thus this could have effect on the ultimate level of aggression towards certain colour morph. If life histories of the morphs differ, the coexistence of colour polymorphism is then enabled by environmental variability and fluctuations of population factors (Sinervo *et al.*, 2000). However, the connection between differences in colouration and life history has not been documented so far in *Nothobranchius*.

One of the main predictors of the level of interspecific aggression is the magnitude of niche overlap (Peiman and Robinson, 2010). The more overlaps in resources occur between



species, the more they tend to exhibit aggressive behaviour to each other (Peiman and Robinson, 2010). It is known that several species of *Nothobranchius* commonly co-occur syntopically in the pool (Wildekamp, 2004) and this could indicate that niche overlap is the trigger of interspecific aggression. In the most intensively researched area in southern Mozambique, the co-occurrence of *N. furzeri* with *N. orthonotus* and *N. pienaarri* was studied and it was found that all species (in various combinations) inhabit the same pool relatively often (Reichard *et al.*, 2009). The abundance of either red or yellow morph of *N. furzeri* in Mozambique populations is variable with overall more common red phenotype (Reichard *et al.*, 2009). Generally, *N. orthonotus* is considered to be intermediate in its habitat requirements between *N. pienaarri*, a coastal plain species (Wildekamp, 2004), and *N. furzeri*, which inhabits more inland areas with turbid water (Reichard *et al.*, 2009). There is some evidence that *Nothobranchius* species, when living syntopically, exhibit differences in morphology (within the limits of low general variability in the genus) and in diet composition to decrease niche overlap (Polačik and Reichard, 2010; Polačik *et al.*, 2014b). Nevertheless, there is still considerable overlap in the diet and differentiation can be apparent only if the habitat is resource-abundant (Polačik and Reichard, 2010; Polačik *et al.*, 2014b). Even more, spatial aspect of *N. orthonotus* and *N. furzeri* co-occurrence in pools is stronger than that between *N. pienaarri* and the other two species (Reichard *et al.*, 2009) and they could possibly compete for the best spawning areas with the specific substrate that is needed for embryonic development. Although the male-male aggression is present, males do not defend a strict territory (Wildekamp, 2004). This may be the consequence of very small habitat that often covers area of only a few square metres (Polačik and Reichard, 2010). In such conditions, any spatial or habitat segregation is severely limited (Reichard *et al.*, 2009). Regardless to possible high and frequent niche overlap between *N. orthonotus* and *N. furzeri*, the stated hypothesis alone cannot sufficiently explain differences that I found in the aggression of *N. orthonotus* towards red and yellow morphs. However, one factor could play a role in the results and that is the detected higher abundance of red male morph of *N. furzeri*, which population lived syntopically in the same pool as the *N. orthonotus*' source population. The experimental males are descendants of this population (collected from the dry region) and thus the aggression bias could be influenced by overall higher amount of encounters with red morph. Although this seems unlikely to be the explanation of my findings, I cannot exclude the possibility that it affected the level of aggression. Other population of *N. orthonotus*, syntopic with *N. furzeri* population consisting predominantly of yellow morph, would have to

be tested to undoubtedly eliminate the effect. Unfortunately, such population was not available in the laboratory during the testing period.

Higher aggression of *N. orthonotus* towards similar red males of *N. furzeri* probably originates from species recognition errors in a competitive context that can ultimately lead to character displacement in agonistic signals and competitor recognition functions (Anderson and Grether, 2010). Agonistic character displacement is the process of phenotypic evolution caused by interference competition between sympatric species and it changes traits affecting the rate, intensity or outcome of interspecific aggression (Grether *et al.*, 2009). There is also likely recognition error in a mating context (these two errors is difficult to separate) supported by evidence showing that *N. orthonotus* was actively trying to mate with *N. furzeri* females (Polačik and Reichard, 2011). Their offspring successfully hatched but its survival was low (Polačik and Reichard, 2011). Interestingly, males of *N. furzeri* do not seem to be interested in females of *N. orthonotus* and when forced to mate, the eggs were not viable (Polačik and Reichard, 2011). This interspecific reproductive interference caused by indiscriminating males of *N. orthonotus* can be preventing interspecific divergence in traits used to recognize competitors (Drury *et al.*, 2015). Consequently, it can be one of the factors causing persistence of interspecific aggression (Drury *et al.*, 2015). This could be applied to *N. orthonotus* interspecific aggression as well. The occurrence of these errors in recognition is within zones of sympatry between closely related species that came into secondary contact (Anderson and Grether, 2010).

Male-male competition was studied in more detail among the haplochromine cichlids in Lakes Victoria and Malawi (Dijkstra and Groothuis, 2011). It is assumed that competition among cichlid males serves as a source of negative frequency-dependent selection which enables maintenance of rare colour morphs (Seehausen and Schluter, 2004). The resulting disruptive selection may facilitate diversification processes, including character displacement (Grether *et al.*, 2009), speciation and the syntopic co-occurrence (Mikami *et al.*, 2004; Pauers *et al.*, 2008). For example, the widespread cichlid genus *Pundamilia* in Lake Victoria contains sympatric closely related species that display nuptial colours at opposite ends of the spectrum and colour polymorphism within a single population is relatively common trait in this group (Dijkstra and Groothuis, 2011). One of its populations consists of two genetically determined incipient species or colour morphs (red and blue) (Seehausen *et al.*, 2008). They use colour as a cue in intrasexual communication and in female choice (Dijkstra *et al.*, 2005). Males direct more aggression towards similarly coloured males (Dijkstra *et al.*, 2008) but red males exhibit more aggressive behaviour and win more often against the blue males in dyadic contests

between the two morphs (Dijkstra *et al.*, 2005). Another study on species complex *Pundamilia* demonstrated that aggression bias varies between stages of speciation and that this bias alone does not have to be strong enough to stabilize the process of speciation (Dijkstra *et al.*, 2007). The cichlid fish example served as an illustration of far more widespread phenomenon of direct agonistic behaviours toward similarly coloured rivals occurring in fish, birds and insects (e.g. Pauers *et al.*, 2008; Alatalo *et al.*, 1994; Tynkkynen *et al.*, 2004; Anderson and Grether, 2010). Also, asymmetric dyadic dominance relationships are relatively common (e.g. Pryke and Griffith, 2006; Kingston *et al.*, 2003).

In conclusion, the results of two experiments show that *N. orthonotus* directs higher levels of aggression towards red morph of *N. furzeri* than to dissimilarly coloured males (yellow morph of *N. furzeri* and *N. pienaari*). Our findings suggest that males of *N. orthonotus* use the male colouration for identification of opponents, and the colour similarity of heterospecific red males of *N. furzeri* leads to increase in *N. orthonotus* aggressive behaviour towards them. The other factors which could contribute to the higher aggression towards red *N. furzeri* are considered to be the red colouration by itself and niche overlap, especially in our *N. orthonotus* population. Another possibly important factor may be different behavioural tactics and life history traits between the two colour morphs of *N. furzeri*, however, this was not studied and thus remains unknown. To test whether aggressive competition among heterospecific and conspecific males can serve as a diversification force in *Nothobranchius* species, more field and experimental investigations are needed, along with more research on dominance and female mate-choice.

## 5 Summary

In the study on cognitive ability of *N. orthonotus*, two populations that varied in their expected lifespan were tested on how their learning ability differs between them in young and old age and how learning is associated with other life history traits. The predictions on inter-population differences and temporal patterns were not confirmed but learning was associated with other life history traits. Predicted inter-population difference in learning ability, as part of the pace-of-life syndrome differences, was not met probably due to shared environmental conditions, predation risk and diet among both populations. Such niche similarity perhaps prevents divergent selection pressure on pace-of-life syndrome phenotypes between dry and wet regions. No cognitive decay was apparent in older age and older fish from dry region population committed even less errors in the learning task than they did as young fish. Such result showed that the onset of physiological decay linked to ageing was much slower than previously thought and not yet pronounced in tested older fish to cause a reduction in spatial cognitive abilities. However, both populations of *N. orthonotus* proved to be effective learners with at least two days memory length. Social environment proved to play an important role in stimulation of the development of cognitive ability as solitary fish were inferior to group-reared fish in time needed to complete each learning trial.

An interesting finding was that there is correlation between lifespan and learning rate, with long-living fish having faster speed of learning. One suggested explanation was that longer lifespan is perhaps associated with overall higher individual quality in several traits, including learning ability. However, artefact of small sample size could cause this trend as well. Another unexpected finding was the effect of high resting metabolic rate (RMR) on learning rate. Individuals that had higher RMR were showing increase in speed of learning. This correlation was thought to be probably caused by a link between RMR and relative brain size of an individual, with higher RMR indicating higher brain size relative to body size. Lastly, the effect of hesitancy (willingness to leave refuge after inspection started) was shown to be the opposite of what was predicted. Less hesitant fish did not find the food reward in shorter time. Instead, hesitant fish were quicker to locate the food in each trial. This result was difficult to explain and remains unclear, however, it could be possible that shy individuals, when finally start to explore the area, tend to be more observant and attentive and thus ultimately spend less time by unfocused swimming, which could ultimately lead to better efficiency of activity.

In my second study on *N. orthonotus*, I tested aggressive behaviour between conspecific and heterospecific males (*N. furzeri* and *N. pienaari*). The findings were consistent with the hypothesis that *N. orthonotus* will be most aggressive towards males of its own species and also towards red male morph of *N. furzeri* due to similar colouration. The lowest aggression was expressed toward yellow morph of *N. furzeri* and males of *N. pienaari* with black colouration. Also, the interest was the highest towards red coloured males of *N. furzeri* and towards conspecific males and it remained the same across the various sequences of testing. I offered several explanations for this trend and for each one I discussed their probability in my study. The most probable explanation was assumed to be the use of male colouration for identification of opponents in *N. orthonotus*, causing higher aggression towards similarly coloured heterospecific red males of *N. furzeri* and lower aggression towards dissimilarly coloured yellow *N. furzeri* and *N. pienaari*. Factors that could have affected results were magnitude of niche overlap, generally bright red colouration associated with aggressive signalling or possibly different life histories and behavioural tactics of *N. furzeri*' colour morphs but that has not been documented so far. Alternatively, it may be because the source population of used experimental *N. orthonotus* lived syntopically with *N. furzeri* and in that pool was higher abundance of red male morph. Overall, higher aggression of *N. orthonotus* towards similar red males of *N. furzeri* probably originates from species recognition errors in a competitive context and it may ultimately lead to character displacement in agonistic signals and competitor recognition functions.

## 6 References

- Agrillo, C., Dadda, M., and Bisazza, A. (2007). Quantity discrimination in female mosquitofish. *Animal cognition*, 10(1), 63-70.
- Agrillo, C., Dadda, M., Serena, G., and Bisazza, A. (2008). Do fish count? Spontaneous discrimination of quantity in female mosquitofish. *Animal cognition*, 11(3), 495-503.
- Alatalo, R. V., Gustafsson, L., and Lundberg, A. (1994). Male coloration and species recognition in sympatric flycatchers. *Proceedings of the Royal Society of London B: Biological Sciences*, 256(1346), 113-118.
- Anderson, C. N., and Grether, G. F. (2010). Interspecific aggression and character displacement of competitor recognition in Hetaerina damselflies. *Proceedings of the Royal Society of London B: Biological Sciences*, 277(1681), 549-555.
- Andersson, M. B. (1994). *Sexual selection*. Princeton University Press.
- Andersson, M., and Iwasa, Y. (1996). Sexual selection. *Trends in Ecology & Evolution*, 11(2), 53-58.
- Barrett, L., and Henzi, P. (2005). The social nature of primate cognition. *Proceedings of the Royal Society of London B: Biological Sciences*, 272(1575), 1865-1875.
- Bartáková, V., Reichard, M., Blažek, R., Polačik, M., & Bryja, J. (2015). Terrestrial fishes: rivers are barriers to gene flow in annual fishes from the African savanna. *Journal of Biogeography*, 42(10), 1832-1844.
- Benson, K. E., and Basolo, A. L. (2006). Male–male competition and the sword in male swordtails, *Xiphophorus helleri*. *Animal Behaviour*, 71(1), 129-134.
- Blažek, R., Polačik, M., and Reichard, M. (2013). Rapid growth, early maturation and short generation time in African annual fishes. *EvoDevo*, 4(1), 1.
- Blažek, R., Polačik M., Kačer P., Cellerino A., Řežucha R., Methling C., ... and Reichard M. (2016). Intra-specific variation in ageing and its life history consequences in annual fishes. Submitted to *Proceedings of the Royal Society B, London*.
- Boogert, N. J., Fawcett, T. W., and Lefebvre, L. (2011). Mate choice for cognitive traits: a review of the evidence in nonhuman vertebrates. *Behavioral Ecology*, 22(3), 447-459.
- Braithwaite, V. A. (2006). Cognitive ability in fish. *Fish physiology*, 24, 1.
- Brown, C. (2001). Familiarity with the test environment improves escape responses in the crimson spotted rainbowfish, *Melanotaenia duboulayi*. *Animal Cognition*, 4(2), 109-113.
- Brown, C. (2003). Habitat–predator association and avoidance in rainbowfish (*Melanotaenia* spp.). *Ecology of Freshwater Fish*, 12(2), 118-126.
- Brown, C., and Laland, K. (2001). Social learning and life skills training for hatchery reared fish. *Journal of Fish Biology*, 59(3), 471-493.
- Brown, C., and Laland, K. N. (2003). Social learning in fishes: a review. *Fish and fisheries*, 4(3), 280-288.
- Brown, C., Davidson, T., and Laland, K. (2003). Environmental enrichment and prior experience of live prey improve foraging behaviour in hatchery-reared Atlantic salmon. *Journal of Fish Biology*, 63(s1), 187-196.
- Brown, C., and Braithwaite, V. A. (2004). Size matters: a test of boldness in eight populations of the poeciliid *Brachyrhaphis episcopi*. *Animal Behaviour*, 68(6), 1325-1329.

- Brown, C., and Braithwaite, V. A. (2005). Effects of predation pressure on the cognitive ability of the poeciliid *Brachyrhaphis episcopi*. *Behavioral Ecology*, *16*(2), 482-487.
- Brydges, N. M., Heathcote, R. J., and Braithwaite, V. A. (2008). Habitat stability and predation pressure influence learning and memory in populations of three-spined sticklebacks. *Animal Behaviour*, *75*(3), 935-942.
- Bshary, R., Wickler, W., and Fricke, H. (2002). Fish cognition: a primate's eye view. *Animal cognition*, *5*(1), 1-13.
- Byrne, R. W., and Bates, L. A. (2007). Sociality, evolution and cognition. *Current biology*, *17*(16), R714-R723.
- Canady, R. A., Kroodsma, D. E., and Nottebohm, F. (1984). Population differences in complexity of a learned skill are correlated with the brain space involved. *Proceedings of the National Academy of Sciences*, *81*(19), 6232-6234.
- Carazo, P., Noble, D. W., Chandrasoma, D., and Whiting, M. J. (2014). Sex and boldness explain individual differences in spatial learning in a lizard. *Proceedings of the Royal Society of London B: Biological Sciences*, *281*(1782), 20133275.
- Cellerino, A., Valenzano, D. R., and Reichard, M. (2016). From the bush to the bench: the annual *Nothobranchius* fishes as a new model system in biology. *Biological Reviews*, *91*, 511–533.
- Cody, M. L. (1969). Convergent characteristics in sympatric species: a possible relation to interspecific competition and aggression. *Condor*, *71*(3), 223-239.
- Darwin, C. (1871). Sexual selection and the descent of man. *Murray, London, 1871*, 589.
- Di Cicco, E., Tozzini, E. T., Rossi, G., and Cellerino, A. (2011). The short-lived annual fish *Nothobranchius furzeri* shows a typical teleost aging process reinforced by high incidence of age-dependent neoplasias. *Experimental gerontology*, *46*(4), 249-256.
- Dijkstra, P. D., Seehausen, O., and Groothuis, T. G. (2005). Direct male-male competition can facilitate invasion of new colour types in Lake Victoria cichlids. *Behavioral Ecology and Sociobiology*, *58*(2), 136-143.
- Dijkstra, P. D., Seehausen, O., Pierotti, M. E., and Groothuis, T. G. G. (2007). Male-male competition and speciation: aggression bias towards differently coloured rivals varies between stages of speciation in a Lake Victoria cichlid species complex. *Journal of evolutionary biology*, *20*(2), 496-502.
- Dijkstra, P. D., Seehausen, O., Fraterman, R. E., and Groothuis, T. G. (2008). Learned aggression biases in males of Lake Victoria cichlid fish. *Animal Behaviour*, *76*(3), 649-655.
- Dijkstra, P. D., and Groothuis, T. G. (2011). Male-male competition as a force in evolutionary diversification: evidence in haplochromine cichlid fish. *International journal of evolutionary biology*, 2011.
- Dorn, A., Ng'oma, E., Janko, K., Reichwald, K., Polačik, M., Platzer, M., ... and Reichard, M. (2011). Phylogeny, genetic variability and colour polymorphism of an emerging animal model: the short-lived annual *Nothobranchius* fishes from southern Mozambique. *Molecular phylogenetics and evolution*, *61*(3), 739-749.
- Dorn, A., Musilová, Z., Platzer, M., Reichwald, K., and Cellerino, A. (2014). The strange case of East African annual fishes: aridification correlates with diversification for a savannah aquatic group?. *BMC evolutionary biology*, *14*(1), 210.

- Drury, J. P., Okamoto, K. W., Anderson, C. N., and Grether, G. F. (2015). Reproductive interference explains persistence of aggression between species. *Proceedings of the Royal Society of London B: Biological Sciences*, 282(1804), 20142256.
- Dukas, R. (1998). *Cognitive ecology: the evolutionary ecology of information processing and decision making* (Vol. 1). University of Chicago Press.
- Dukas, R. (2004). Evolutionary biology of animal cognition. *Annual review of ecology, evolution, and systematics*, 347-374.
- Enquist, M., and Jakobsson, S. (1986). Decision making and assessment in the fighting behaviour of *Nannacara anomala* (Cichlidae, Pisces). *Ethology*.
- Ewulonu, U. K., Haas, R., and Turner, B. J. (1985). A multiple sex chromosome system in the annual killifish, *Nothobranchius guentheri*. *Copeia*, 1985(2), 503-508.
- Froese, R. and Pauly, D. (eds) (2016). FishBase. World Wide Web electronic publication. Available at [www.fishbase.org](http://www.fishbase.org), accessed Mar 2016.
- Galef Jr, B. G. (1998). Edward Thorndike: Revolutionary psychologist, ambiguous biologist. *American Psychologist*, 53(10), 1128.
- Genade, T., Benedetti, M., Terzibasi, E., Roncaglia, P., Valenzano, D. R., Cattaneo, A., and Cellerino, A. (2005). Annual fishes of the genus *Nothobranchius* as a model system for aging research. *Aging cell*, 4(5), 223-233.
- Gerhard, G. S., Kauffman, E. J., Wang, X., Stewart, R., Moore, J. L., Kasales, C. J., ... and Cheng, K. C. (2002). Life spans and senescent phenotypes in two strains of Zebrafish (*Danio rerio*). *Experimental gerontology*, 37(8), 1055-1068.
- Gómez-Laplaza, L. M. (2005). The influence of social status on shoaling preferences in the freshwater angelfish (*Pterophyllum scalare*). *Behaviour*, 142(6), 827-844.
- Gonzalez-Voyer, A., Fitzpatrick, J. L., and Kolm, N. (2008). Sexual selection determines parental care patterns in cichlid fishes. *Evolution*, 62(8), 2015-2026.
- Graf, M., Cellerino, A., and Englert, C. (2010). Gender separation increases somatic growth in females but does not affect lifespan in *Nothobranchius furzeri*. *PLoS One*, 5(8), e11958.
- Gray, S. M., and McKinnon, J. S. (2007). Linking color polymorphism maintenance and speciation. *Trends in Ecology & Evolution*, 22(2), 71-79.
- Grether, G. F., Losin, N., Anderson, C. N., and Okamoto, K. (2009). The role of interspecific interference competition in character displacement and the evolution of competitor recognition. *Biological Reviews*, 84(4), 617-635.
- Grether, G. F., Anderson, C. N., Drury, J. P., Kirschel, A. N., Losin, N., Okamoto, K., and Peiman, K. S. (2013). The evolutionary consequences of interspecific aggression. *Annals of the New York Academy of Sciences*, 1289(1), 48-68.
- Griffin, A. S., Guillette, L. M., and Healy, S. D. (2015). Cognition and personality: an analysis of an emerging field. *Trends in ecology and evolution*, 30(4), 207-214.
- Griffiths, S. W. (2003). Learned recognition of conspecifics by fishes. *Fish and Fisheries*, 4(3), 256-268.
- Griffiths, A. J., Miller, J. H., Suzuki, D. T., Lewontin, R. C., and Gelbart, W. M. (2000). Norm of reaction and phenotypic distribution.
- Guillette, L. M., Reddon, A. R., Hoeschele, M., and Sturdy, C. B. (2010). Sometimes slower is better: slow-exploring birds are more sensitive to changes in a vocal discrimination task. *Proceedings of the Royal Society of London B: Biological Sciences*, rspb20101669.



- Haas, R. (1976a). Behavioral biology of the annual killifish, *Nothobranchius guentheri*. *Copeia*, 80-91.
- Haas, R. (1976b). Sexual selection in *Nothobranchius guentheri* (Pisces: cyprinodontidae). *Evolution*, 614-622.
- Hoffman, E. A., and Blouin, M. S. (2000). A review of colour and pattern polymorphisms in anurans. *Biological Journal of the Linnean Society*, 70(4), 633-665.
- Hrbek, T., and Larson, A. (1999). The evolution of diapause in the killifish family Rivulidae (Atherinomorpha, Cyprinodontiformes): a molecular phylogenetic and biogeographic perspective. *Evolution*, 1200-1216.
- Hulbert, A. J., and Else, P. L. (2004). Basal metabolic rate: history, composition, regulation, and usefulness. *Physiological and Biochemical Zoology*, 77(6), 869-876.
- Hunt, J., Brooks, R., Jennions, M. D., Smith, M. J., Bentsen, C. L., and Bussiere, L. F. (2004). High-quality male field crickets invest heavily in sexual display but die young. *Nature*, 432(7020), 1024-1027.
- Hunt, J., Breuker, C. J., Sadowski, J. A., and Moore, A. J. (2009). Male–male competition, female mate choice and their interaction: determining total sexual selection. *Journal of evolutionary biology*, 22(1), 13-26.
- Isler, K., and Van Schaik, C. P. (2006). Metabolic costs of brain size evolution. *Biology Letters*, 2(4), 557-560.
- Kingston, J. J., Rosenthal, G. G., and Ryan, M. J. (2003). The role of sexual selection in maintaining a colour polymorphism in the pygmy swordtail, *Xiphophorus pygmaeus*. *Animal Behaviour*, 65(4), 735-743.
- Kokko, H., Jennions, M. D., and Brooks, R. (2006). Unifying and testing models of sexual selection. *Annual Review of Ecology, Evolution, and Systematics*, 43-66.
- Kotrschal, A., and Taborsky, B. (2010). Environmental change enhances cognitive abilities in fish. *PLoS Biol*, 8(4), e1000351.
- Kotrschal, A., Rogell, B., Bundsen, A., Svensson, B., Zajitschek, S., Brännström, I., ... and Kolm, N. (2013). The benefit of evolving a larger brain: big-brained guppies perform better in a cognitive task. *Animal behaviour*, 86(4), e4-e6.
- Kotrschal, A., Corral-Lopez, A., Amcoff, M., and Kolm, N. (2015). A larger brain confers a benefit in a spatial mate search learning task in male guppies. *Behavioral Ecology*, 26(2), 527-532.
- Krebs, J. R., and Davies, N. B. (1997). The evolution of behavioural ecology. *Behavioural ecology*, 3-18.
- Laiolo, P. (2012). Interspecific interactions drive cultural co-evolution and acoustic convergence in syntopic species. *Journal of Animal Ecology*, 81(3), 594-604.
- Laland, K. N., and Williams, K. (1997). Shoaling generates social learning of foraging information in guppies. *Animal Behaviour*, 53(6), 1161-1169.
- Laland, K. N., and Hoppitt, W. (2003). Do animals have culture?. *Evolutionary Anthropology: Issues, News, and Reviews*, 12(3), 150-159.
- Laland, K. N., Brown, C., and Krause, J. (2003). Learning in fishes: from three-second memory to culture. *Fish and Fisheries*, 4(3), 199-202.

- Lehtonen, T. K., Sowersby, W., and Wong, B. B. (2015). Heterospecific aggression bias towards a rarer colour morph. *In Proc. R. Soc. B* (Vol. 282, No. 1815, p. 20151551). The Royal Society.
- Leiser, J. K., Gagliardi, J. L., and Itzkowitz, M. (2004). Does size matter? Assessment and fighting in small and large size-matched pairs of adult male convict cichlids. *Journal of Fish Biology*, *64*(5), 1339-1350.
- Levels, P. J., Gubbels, R. E. M. B., and Denucé, J. M. (1986). Oxygen consumption during embryonic development of the annual fish *Nothobranchius korthausae* with special reference to diapause. *Comparative Biochemistry and Physiology Part A: Physiology*, *84*(4), 767-770.
- Lucas-Sánchez, A., Almáida-Pagan, P. F., Madrid, J. A., De Costa, J., and Mendiola, P. (2011). Age-related changes in fatty acid profile and locomotor activity rhythms in *Nothobranchius korthausae*. *Experimental gerontology*, *46*(12), 970-978.
- Madden, J. (2001). Sex, bowers and brains. *Proceedings of the Royal Society of London B: Biological Sciences*, *268*(1469), 833-838.
- Martin, R. D. (1981). Relative brain size and basal metabolic rate in terrestrial vertebrates. *Nature*, *293*(5827), 57-60.
- McCullough, E. L., and Simmons, L. W. (2016). Selection on male physical performance during male–male competition and female choice. *Behavioral Ecology*, arw033.
- Mckinnon, J. S., and Pierotti, M. E. (2010). Colour polymorphism and correlated characters: genetic mechanisms and evolution. *Molecular Ecology*, *19*(23), 5101-5125.
- Mesquita, F. O., Borcato, F. L., and Huntingford, F. A. (2015). Cue-based and algorithmic learning in common carp: A possible link to stress coping style. *Behavioural processes*, *115*, 25-29.
- Mikami, O. K., Kohda, M., and Kawata, M. (2004). A new hypothesis for species coexistence: male–male repulsion promotes coexistence of competing species. *Population Ecology*, *46*(2), 213-217.
- Mink, J. W., Blumenshine, R. J., and Adams, D. B. (1981). Ratio of central nervous system to body metabolism in vertebrates: its constancy and functional basis. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *241*(3), R203-R212.
- Moretz, J. A. (2003). Aggression and RHP in the northern swordtail fish, *Xiphophorus cortezi*: the relationship between size and contest dynamics in male–male competition. *Ethology*, *109*(12), 995-1008.
- Morris, M. R., Batra, P., and Ryan, M. J. (1992). Male-male competition and access to females in the swordtail *Xiphophorus nigrensis*. *Copeia*, 980-986.
- Moynihan, M. (1998). *social regulation of competition and aggression in animals*. Smithsonian Institution Press.
- Murphy, W. J., and Collier, G. E. (1997). A molecular phylogeny for aplocheiloid fishes (Atherinomorpha, Cyprinodontiformes): the role of vicariance and the origins of annualism. *Molecular Biology and Evolution*, *14*(8), 790-799.
- Nagy, B. (2014). *Nothobranchius milvertzi*, a new species of killifish from the Lushiba Marsh in the Lake Mweru drainage, Zambia (Teleostei: Cyprinodontiformes: Nothobranchiidae). *Ichthyological Exploration of Freshwaters*, *24*, 347-360.

- Ng'oma, E., Reichwald, K., Dorn, A., Wittig, M., Balschun, T., Franke, A., ... and Cellerino, A. (2014). The age related markers lipofuscin and apoptosis show different genetic architecture by QTL mapping in short-lived *Nothobranchius* fish. *Aging (Albany NY)*, 6(6), 468.
- Odling-Smee, L., and Braithwaite, V. A. (2003a). The role of learning in fish orientation. *Fish and Fisheries*, 4(3), 235-246.
- Odling-Smee, L., and Braithwaite, V. A. (2003b). The influence of habitat stability on landmark use during spatial learning in the three-spined stickleback. *Animal Behaviour*, 65(4), 701-707.
- Parker, G. A. (1974). Assessment strategy and the evolution of fighting behaviour. *Journal of theoretical Biology*, 47(1), 223-243.
- Pauers, M. J., Kapfer, J. M., Fendos, C. E., and Berg, C. S. (2008). Aggressive biases towards similarly coloured males in Lake Malawi cichlid fishes. *Biology Letters*, 4(2), 156-159.
- Paxton, J.R., and Eschmeyer, W.N. (1998). *Encyclopedia of fishes*. Academic Press, San Diego, Calif.
- Peiman, K., and Robinson, B. (2010). Ecology and evolution of resource-related heterospecific aggression. *The Quarterly Review of Biology*, 85(2), 133-158.
- Podrabsky, J. E., Tingaud-Sequeira, A., and Cerdà, J. (2010). Metabolic dormancy and responses to environmental desiccation in fish embryos. In *Dormancy and resistance in harsh environments* (pp. 203-226). Springer Berlin Heidelberg.
- Polačik, M., and Reichard, M. (2009). Indirect fitness benefits are not related to male dominance in a killifish. *Behavioral Ecology and Sociobiology*, 63(10), 1427-1435.
- Polačik, M., and Reichard, M. (2010). Diet overlap among three sympatric African annual killifish species *Nothobranchius* spp. from Mozambique. *Journal of Fish Biology*, 77(3), 754-768.
- Polačik, M., and Reichard, M. (2011). Asymmetric reproductive isolation between two sympatric annual killifish with extremely short lifespans. *PLoS one*, 6(8), e22684.
- Polačik, M., Donner, M. T., and Reichard, M. (2011). Age structure of annual *Nothobranchius* fishes in Mozambique: is there a hatching synchrony?. *Journal of fish biology*, 78(3), 796-809.
- Polačik, M., Blažek, R., Řežucha, R., Vrtílek, M., Terzibasi Tozzini, E., and Reichard, M. (2014a). Alternative intrapopulation life-history strategies and their trade-offs in an African annual fish. *Journal of evolutionary biology*, 27(5), 854-865.
- Polačik, M., Harrod, C., Blažek, R., and Reichard, M. (2014b). Trophic niche partitioning in communities of African annual fish: evidence from stable isotopes. *Hydrobiologia*, 721(1), 99-106.
- Polačik, M., R. Blažek, and M. Reichard. 2016. Laboratory breeding of the short-lived annual killifish *Nothobranchius furzeri*. *Nat. Protoc.* doi: 10.1038/nprot.2016.080, in press.
- Pradel, G., Schachner, M., and Schmidt, R. (1999). Inhibition of memory consolidation by antibodies against cell adhesion molecules after active avoidance conditioning in zebrafish. *Journal of neurobiology*, 39(2), 197-206.
- Pryke, S. R. (2009). Is red an innate or learned signal of aggression and intimidation?. *Animal Behaviour*, 78(2), 393-398.

- Pryke, S. R., and Griffith, S. C. (2006). Red dominates black: agonistic signalling among head morphs in the colour polymorphic Gouldian finch. *Proceedings of the Royal Society of London B: Biological Sciences*, 273(1589), 949-957.
- Qvarnström, A., Vallin, N., and Rudh, A. (2012). The role of male contest competition over mates in speciation. *Current Zoology*, 58(3).
- Real, L. A. (1993). Toward a cognitive ecology. *Trends in Ecology and Evolution*, 8(11), 413-417.
- Réale, D., Garant, D., Humphries, M. M., Bergeron, P., Careau, V., and Montiglio, P. O. (2010). Personality and the emergence of the pace-of-life syndrome concept at the population level. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 365(1560), 4051-4063.
- Reichard, M. (2010). *Nothobranchius kadleci* (Cyprinodontiformes: Nothobranchiidae), a new species of annual killifish from central Mozambique. *Zootaxa*, 2332, 49-60.
- Reichard, M. (2015). The Evolutionary Ecology of African Annual Fishes. *Annual Fishes: Life History Strategy, Diversity, and Evolution*, 133.
- Reichard, M., Polačik, M., and Sedláček, O. (2009). Distribution, colour polymorphism and habitat use of the African killifish *Nothobranchius furzeri*, the vertebrate with the shortest life span. *Journal of Fish Biology*, 74(1), 198-212.
- Reichard, M., and Polačik, M. (2010). Reproductive isolating barriers between colour-differentiated populations of an African annual killifish, *Nothobranchius korthausae* (Cyprinodontiformes). *Biological Journal of the Linnean Society*, 100(1), 62-72.
- Reichard, M., Polačik, M., Blažek, R., and Vrtílek, M. (2014). Female bias in the adult sex ratio of African annual fishes: interspecific differences, seasonal trends and environmental predictors. *Evolutionary Ecology*, 28(6), 1105-1120.
- Richards, R. J. (1989). *Darwin and the emergence of evolutionary theories of mind and behavior*. University of Chicago Press.
- Ristau, C. A. (1991). Cognitive ethology: The minds of other animals.
- Roulin, A. (2004). The evolution, maintenance and adaptive function of genetic colour polymorphism in birds. *Biological Reviews*, 79(4), 815-848.
- Roy, T., and Bhat, A. (2016). Learning and Memory in Juvenile Zebrafish: What makes the Difference—Population or Rearing Environment?. *Ethology*, 122(4), 308-318.
- Salvanes, A. G. V., Moberg, O., Ebbesson, L. O., Nilsen, T. O., Jensen, K. H., and Braithwaite, V. A. (2013). Environmental enrichment promotes neural plasticity and cognitive ability in fish. *Proceedings of the Royal Society of London B: Biological Sciences*, 280(1767), 20131331.
- Seehausen, O., and Schluter, D. (2004). Male–male competition and nuptial–colour displacement as a diversifying force in Lake Victoria cichlid fishes. *Proceedings of the Royal Society of London B: Biological Sciences*, 271(1546), 1345-1353.
- Seehausen, O., Terai, Y., Magalhaes, I. S., Carleton, K. L., Mrosso, H. D., Miyagi, R., ... and Imai, H. (2008). Speciation through sensory drive in cichlid fish. *Nature*, 455(7213), 620-626.
- Shettleworth, S. J. (2001). Animal cognition and animal behaviour. *Animal behaviour*, 61(2), 277-286.
- Shettleworth, S. J. (2009). *Cognition, evolution, and behavior*. Oxford University Press.

- Shohet, A. J., and Watt, P. J. (2009). Female guppies *Poecilia reticulata* prefer males that can learn fast. *Journal of Fish Biology*, 75(6), 1323-1330.
- Schultz, J. K., and Switzer, P. V. (2001). Pursuit of heterospecific targets by territorial amberwing dragonflies (*Perithemis tenera* Say): a case of mistaken identity. *Journal of Insect Behavior*, 14(5), 607-620.
- Sih, A., and Del Giudice, M. (2012). Linking behavioural syndromes and cognition: a behavioural ecology perspective. *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 367(1603), 2762-2772.
- Sinervo, B., and Lively, C. M. (1996). The rock-paper-scissors game and the evolution of alternative male strategies. *Nature*, 380(6571), 240-243.
- Sinervo, B., Svensson, E., and Comendant, T. (2000). Density cycles and an offspring quantity and quality game driven by natural selection. *Nature*, 406(6799), 985-988.
- Smith, J. M. (1982). *Evolution and the Theory of Games*. Cambridge university press.
- Smith, C., Philips, A., and Reichard, M. (2015). Cognitive ability is heritable and predicts the success of an alternative mating tactic. *In Proc. R. Soc. B* (Vol. 282, No. 1809, p. 20151046). The Royal Society.
- Sovrano, V. A., Bisazza, A., and Vallortigara, G. (2002). Modularity and spatial reorientation in a simple mind: encoding of geometric and nongeometric properties of a spatial environment by fish. *Cognition*, 85(2), B51-B59.
- Stearns, S. C. (1992). *The evolution of life histories* (Vol. 249). Oxford: Oxford University Press.
- Stöwe, M., and Kotrschal, K. (2007). Behavioural phenotypes may determine whether social context facilitates or delays novel object exploration in ravens (*Corvus corax*). *Journal of Ornithology*, 148(2), 179-184.
- Striedter, G. F. (2006). Précis of principles of brain evolution. *Behavioral and Brain Sciences*, 29(01), 1-12.
- Terzibasi, E., Valenzano, D. R., and Cellerino, A. (2007). The short-lived fish *Nothobranchius furzeri* as a new model system for aging studies. *Experimental gerontology*, 42(1), 81-89.
- Terzibasi, E., Valenzano, D. R., Benedetti, M., Roncaglia, P., Cattaneo, A., Domenici, L., and Cellerino, A. (2008). Large differences in aging phenotype between strains of the short-lived annual fish *Nothobranchius furzeri*. *PLoS One*, 3(12), e3866.
- Toga, A. W., and Thompson, P. M. (2005). Genetics of brain structure and intelligence. *Annu. Rev. Neurosci.*, 28, 1-23.
- Tozzini, E. T., Dorn, A., Ng'oma, E., Polačik, M., Blažek, R., Reichwald, K., ... and Cellerino, A. (2013). Parallel evolution of senescence in annual fishes in response to extrinsic mortality. *BMC evolutionary biology*, 13(1), 77.
- Tynkkyne, K., Rantala, M. J., and Suhonen, J. (2004). Interspecific aggression and character displacement in the damselfly *Calopteryx splendens*. *Journal of evolutionary biology*, 17(4), 759-767.
- Utne-Palm, A. C., and Hart, P. J. (2000). The effects of familiarity on competitive interactions between threespined sticklebacks. *Oikos*, 91(2), 225-232.

- Valdesalici, S. (2013). *Pronothobranchius chirioi* n. sp. a new annual killifish species from the Niger River drainage, with redescrptions of *P. kiyawensis*, *P. gambiensis* and *P. seymouri* (Cyprinodontiformes: Nothobranchiidae). *Killi-Data Series*, 2013, 21-41.
- Valdesalici, S., and Cellerino, A. (2003). Extremely short lifespan in the annual fish *Nothobranchius furzeri*. *Proceedings of the Royal Society of London B: Biological Sciences*, 270(Suppl 2), S189-S191.
- Valdesalici, S., Bills, R., Dorn, A., Reichwald, K., and Cellerino, A. (2012). *Nothobranchius niassa* (Cyprinodontiformes: Nothobranchiidae), a new species of annual killifish from northern Mozambique. *Ichthyological Exploration of Freshwaters*, 23(1), 19.
- Valenzano, D. R., Terzibasi, E., Cattaneo, A., Domenici, L., and Cellerino, A. (2006a). Temperature affects longevity and age-related locomotor and cognitive decay in the short-lived fish *Nothobranchius furzeri*. *Aging cell*, 5(3), 275-278.
- Valenzano, D. R., Terzibasi, E., Genade, T., Cattaneo, A., Domenici, L., and Cellerino, A. (2006b). Resveratrol prolongs lifespan and retards the onset of age-related markers in a short-lived vertebrate. *Current Biology*, 16(3), 296-300.
- Valenzano, D. R., Kirschner, J., Kamber, R. A., Zhang, E., Weber, D., Cellerino, A., ... and Brunet, A. (2009). Mapping loci associated with tail color and sex determination in the short-lived fish *Nothobranchius furzeri*. *Genetics*, 183(4), 1385-1395.
- Vrtílek, M., and Reichard, M. (2015). Highly plastic resource allocation to growth and reproduction in females of an African annual fish. *Ecology of Freshwater Fish*, 24(4), 616-628.
- Warburton, K. (2003). Learning of foraging skills by fish. *Fish and Fisheries*, 4(3), 203-215.
- Warner, R. R. (1988). Traditionality of mating-site preferences in a coral reef fish. *Nature*, 335(6192), 719-721.
- Warner, R. R. (1990). Resource assessment versus tradition in mating-site determination. *American Naturalist*, 205-217.
- Watters, B. R. (2009). The ecology and distribution of *Nothobranchius* fishes. *Journal of the American Killifish Association*, 42(2), 37-76.
- White, G. E., and Brown, C. (2014). Cue choice and spatial learning ability are affected by habitat complexity in intertidal gobies. *Behavioral Ecology*, aru178.
- Wildekamp, R. H. (2004). *A World of Killies: Atlas of the Oviparous Cyprinodontiform Fishes of the World* (Volume 4). American Killifish Association, Elyria.

### Internet reference

<http://nothos.webnode.cz/fotogalerie/n-pienaari/#!>