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Cognitive plasticity in teleost fish

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ABSTRACT

Phenotypic plasticity is a mechanism that permits to obtain different phenotypes from a single genotype, and it is widespread in the animal kingdom due to its adaptive value. Researchers have mostly focussed on the plasticity of behavioural and morphological traits, yet evidence suggests that also cognitive traits, i.e., those traits involved in perceiving, storing, and elaborating information from the environment, might display phenotypic plasticity. Here I presented an original work aimed to investigate cognitive plasticity in the vertebrate group with greater brain neurogenesis, and therefore the greater theoretical potential for cognitive plasticity, the teleost fish. I exploited three chosen based on their biology to investigate plasticity in response to different factors, *Poecilia reticulata*, *Oryzias latipes* and *Danio rerio*.

I exposed the subjects to different treatments in which I manipulated biotic and/or abiotic factors of the environment and then I administered batteries of cognitive tasks to the subjects to understand whether and how their cognition was affected. The factors investigated across five studies were: 1) the level of habitat complexity or environmental enrichment; 2) the level of resource predictability in the environment; 3) the social environment in terms of group size and group complexity; 4) the season; 5) a form of human-generated pollution.

The results obtained from the first four studies provided sufficient evidence to support the presence of phenotypic plasticity in fish cognition. For instance, in guppies, high habitat complexity determined a phenotype with greater learning abilities. Moreover, being in a stable and smaller social group improved inhibitory control. Several evidences point towards the idea that this plasticity might be adaptive: for instance, in predictable environments fish showed greater learning abilities, which are arguably beneficial to adapt behaviour to predictable conditions; conversely, in unpredictable environments, the fish developed enhanced inhibitory control and cognitive flexibility, cognitive functions that permit to adapt behaviour to changing situations. In medaka, I found that photoperiod is used as a proxy of the season to determine increased inhibitory and learning abilities in winter and I detected seasonal changes in the production of hormones that might be an underlying mechanism for the observed cognitive plasticity.

In the last study of the thesis, I exposed groups of zebrafish subjects to microplastic pollution and then I tested them in learning and cognitive flexibility tasks. I found no evidence of cognitive plasticity when looking at these phenotypes. However, a physiological analysis of brain tissues in a subsample of subjects indicated significant alterations due to the pollutant. I therefore conclude that the possibility of non-adaptive plasticity in fish cognition in response to human-induced pollution cannot be completely ruled out.

In conclusion, this thesis increased the knowledge of cognitive phenotypic plasticity in teleost fish suggesting that it is widespread, can be adaptive and can be triggered by different environmental factors. Future studies of fish cognition, especially those interested in interindividual variation, should consider the role of cognitive plasticity.

RIASSUNTO

La plasticità fenotipica è un meccanismo che permette di ottenere diversi fenotipi da un unico genotipo ed è diffuso nel regno animale per il suo valore adattivo. I ricercatori si sono concentrati principalmente sulla plasticità dei tratti comportamentali e morfologici, ma i risultati ottenuti in letteratura suggeriscono che anche i tratti cognitivi, cioè quelli coinvolti nella percezione, memorizzazione ed elaborazione di informazioni dall'ambiente, potrebbero mostrare plasticità fenotipica. Qui ho presentato un lavoro originale volto a indagare la plasticità cognitiva nel gruppo dei vertebrati con maggiore neurogenesi cerebrale, e quindi maggiore potenziale teorico per la plasticità cognitiva, i pesci teleostei. Ho sfruttato tre specie scelte in base alla loro biologia per indagare la plasticità in risposta a diversi fattori, *Poecilia reticulata*, *Oryzias latipes* e *Danio rerio*.

Ho esposto i soggetti a diversi trattamenti in cui ho manipolato fattori biotici e/o abiotici dell'ambiente e poi li ho sottoposti a una serie di compiti cognitivi per capire se e come la loro cognizione è stata influenzata. I fattori indagati nei cinque studi sono stati: 1) il livello di complessità dell'habitat o l'arricchimento ambientale; 2) il livello di prevedibilità delle risorse nell'ambiente; 3) l'ambiente sociale in termini di dimensioni del gruppo e complessità del gruppo; 4) la stagionalità; 5) una forma di inquinamento antropico.

I risultati ottenuti dai primi quattro studi hanno fornito prove sufficienti per sostenere la presenza di plasticità fenotipica nella cognizione dei pesci. Per esempio, nei guppy, l'elevata complessità dell'habitat ha determinato un fenotipo con maggiori capacità di apprendimento. Inoltre, essere in un gruppo sociale stabile e più piccolo ha migliorato il controllo inibitorio.

Diverse prove suggeriscono che questa plasticità potrebbe essere adattiva: per esempio, in ambienti prevedibili i pesci hanno mostrato maggiori capacità di apprendimento, che sono probabilmente utili per adattare il comportamento a condizioni prevedibili; al contrario, in ambienti imprevedibili, i pesci hanno sviluppato un maggiore controllo

inibitorio e flessibilità cognitiva, funzioni cognitive che permettono di adattare il comportamento a situazioni mutevoli. In medaka, ho trovato che il fotoperiodo è usato come indicatore della stagionalità per determinare le capacità inibitorie e di apprendimento aumentate nella stagione invernale e ho individuato i cambiamenti stagionali nella produzione degli ormoni che potrebbero essere un meccanismo di fondo per la plasticità cognitiva osservata.

Nell'ultimo studio della tesi, ho esposto gruppi di zebrafish all'inquinamento dovuto a microplastiche e poi li ho testati in un compito di apprendimento e di flessibilità cognitiva. Non ho trovato alcuna evidenza di plasticità cognitiva in riferimento a questi fenotipi. Tuttavia, un'analisi fisiologica dei tessuti cerebrali in un sottocampione dei soggetti ha indicato le alterazioni significative dovuto all'inquinante. I risultati ottenuti suggeriscono quindi che la possibile plasticità non adattativa nella cognizione dei pesci in risposta all'inquinamento indotto dall'uomo non può essere completamente esclusa.

In conclusione, questa tesi ha aumentato la conoscenza sulla plasticità fenotipica cognitiva nei pesci teleostei suggerendo che è diffusa, può essere adattiva e può essere innescata da diversi fattori ambientali. I futuri studi sulla cognizione dei pesci, specialmente quelli interessati alla variazione interindividuale, dovrebbero considerare il ruolo della plasticità cognitiva.

1. Introduction

1.1 Phenotypic plasticity

1.1.1 General concepts

Phenotypic plasticity can be defined as the potential development of different morphological, physiological and behavioural phenotypes from a single genotype. For instance, a well-known case of phenotypic plasticity regards an aquatic crustacean, *Daphnia cucullata* (Brooks, 1965; Tollrian, 1990; Tollrian & Laforsch, 2006). In this species, exposure of an individual to an environment with high levels of turbulence or an environment with the presence of invertebrate predators such as *Cyclops sp.* and *Daphnia magna* usually determines the development of individuals with larger protective helmets (Figure 1a). Moreover, in insects, we find several examples of plastic traits such as in *Precis octavia,* a butterfly*,* that can change its morph due to the characteristics of the seasons to better adapt to the habitat (Figure 1b). Another well-studied example of plasticity involves *Spea multiplicata* tadpoles (Levis *et al.*, 2015; Levis *et al.*, 2018). In certain situations, tadpoles with greater head size had a carnivore diet instead of an omnivore or detritivore diet. This morph also has a short intestine, and bigger jaw muscles to best fit with its specific food resource, in this case, a carnivore diet (Figure 1c). Further examples of phenotypic

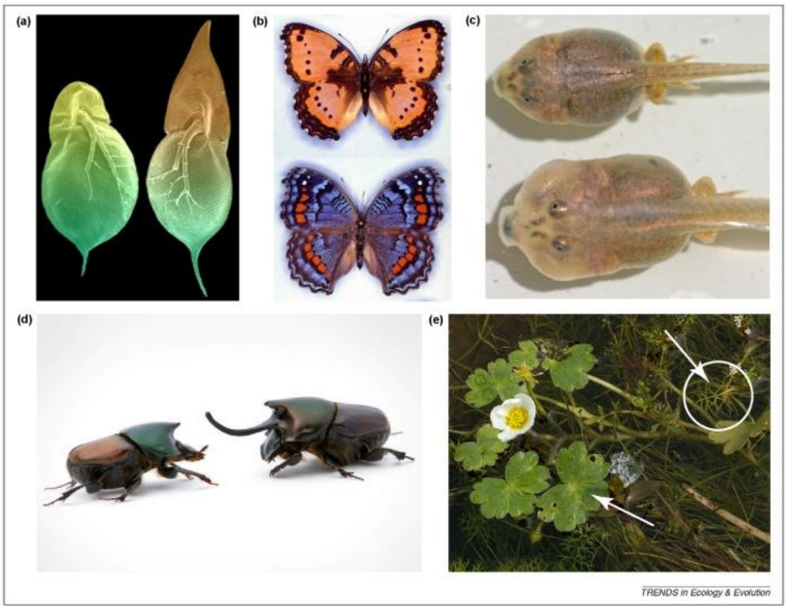

Figure 1*: Example of phenotypic plasticity both in plant and animals due to environmental chances. a) Normal (left) and predator-induced (right) morphs of water fleas, Daphnia cucullata (photo courtesy of Ralph Tollrian); (b) wet-season (top) and dry-season (bottom) gaudy commodore butterflies, Precis octavia (photo courtesy of Fred Nijhout); (c) omnivore (top) and carnivore-morph (bottom) spadefoot toad tadpoles, Spea multiplicata (photo by David Pfennig); (d) small-horned (left) and large-horned (right) dung beetles, Onthophagus nigriventris (photo by Alex Wild); (e) broad, aerial leaves and narrow, submerged leaves (circled) on the same water crowfoot plant, Ranunculus aquatilis (photo by John Crellin/FloralImages). Pfennig et al., 2010*

plasticity are illustrated in Figure 1. These are, however, only a small subsample of those available in the literature. In many decades of research in the field, phenotypic plasticity has been detected in virtually all organisms, including in plants and animals (Borges, 2005). Among animals, phenotypic plasticity is common in invertebrates (Androwski *et al.*, 2017) and vertebrates including mammals (Réale *et al.*, 2003), birds (Price *et al.*, 2003), fish (Meuthen *et al.*, 2018), amphibians (Middlemis Maher *et al.*, 2013) reptiles (Das *et al.*, 2020). Moreover, it has been shown that the environmental factors that may determine phenotypic plasticity are numerous as well. They include indeed, abiotic factors, such as temperature (Yampolsky *et al.*, 2014), the concentration of CO_2 (Cattano *et al.*, 2016) and O_2 (Storz *et al.*, 2010), pH (Mohamad *et al.*, 2021), and photoperiod (Lucon-Xiccato *et al.*, 2022). On the other hand, plasticity can be determined by a range of biotic factors such as availability and abundance of food resources (Newman, 1994), presence of predators (Brooks, 1965), presence of heterospecific competitors (Relyea, 2004) and social environment (Morand-Ferron & Quinn, 2011).

1.1.2 Adaptive plasticity

Many of the forms of plasticity described in the previous section are considered important determinants of individuals' fitness. The environmental conditions are not fixed. They may vary in space and in time, and this may determine different fitness optimums for individuals of the same species that live in different areas or at different moments. Plasticity can permit individuals to modify their phenotype to match the conditions to which they are exposed. This is called adaptive phenotypic plasticity and is well represented by the initial examples. For instance, individuals of *D. cucullata* that develop a helmet after experiencing cues of the presence of predators in the environment are less susceptible to predation. Similarly, tadpoles that develop the larger morph are usually observed when the pond begins to dry. Because of the larger morph, they can cope with the reduction of resources and time available for development by feeding on the other tadpoles in the pond.

An interesting model species for the study of plasticity in vertebrates is a teleost fish, the guppy, *P. reticulata*. The interest towards this species is related to its habitat, a set of small rivers in Trinidad and Tobago where the conditions change dramatically in a relatively small distance, as the river flows from the mountains to the sea. One of the factors involved is the presence of predators, which are abundant in the lower parts of the rivers. Studies in the guppies have revealed that the presence of predators may affect important life history traits such as reproduction, morphology and adult size. The risk of predation is one of the most important factors that lead to physiological, behavioural and morphological plasticity because the prey had to cope with the predator and has to implement appropriate solutions to avoid being prey. There are a lot of strategies that animals can adopt to avoid the risk of predation or to reduce the effect that predation can have on the population. Dzikowski and colleagues (2004) bring us an excellent example of predation connected with the inducible plastic reproduction in guppies. The researchers found that guppies reared in an environment with predators increased the number of offspring by reducing the inter-brood period and gave birth to larger size offspring respect to guppies reared in a condition without predators. This increased investment in reproduction is likely to shorten the life of a guppy, but under predation risk, the average life span is anyway reduced. Therefore, individuals can increase their fitness by boosting offspring production in early life. Another research on guppies and how predation can affect the morphology of the species was performed by Torres-Dowdall and colleagues (2012). They investigated plasticity in guppy from different populations experimentally reared in habitats with different levels of predation. Guppies that originated from habitats with either a high-predation and low predation ecotypes were exposed to an environment with predator cues and they found that guppy of both ecotypes showed the characteristic morph of high-predation ecotype, such as the fusiform shape of the head and the preference to stay more in the surface of the water column. This work suggests that the population from low-prediction risk when exposed to predator cues, can modify its behaviour and morphology to better escape the risk of predation. Therefore, these studies remark that adaptive phenotypic plasticity has a huge impact on the survival, reproduction and fitness of individuals (Doughty, 2004).

***Figure 2**: Morphological phenotypic plasticity in insects. (a, b) Discrete seasonal polyphenisms in Nemoria arizonaria caterpillars (fam. Geometridae). Summer brood feeds on oak leaves and resembles an oak twig. Spring brood feeds on and resembles oak catkins. Photos: E. Greene (Greene 1989). (c) Wet-season (left) and dry-season (right) Precis octavia (fam. Nymphalidae) butterflies, from Africa (McLeod 2007). Photos courtesy of F. Nijhout. (d) Many insects alter body color in response to rearing temperature: Romalea microptera grasshoppers (fam. Romaleidae) from south Florida reared at 35o C (top) and at 25o C (bottom). (e) Harlequin bugs, Murgantia histrionica (fam. Pentatomidae). Black and yellow individuals were reared at 22 and 30o C, respectively. (f) Nutrition strongly influences insect body size. Taeniopoda eques grasshoppers (fam. Romaleidae), from the Chihuahuan Desert in SE Arizona, showing plasticity in body size to nutrition. Males on left; females on right. Bottom four individuals from site that received ample rains and had lush vegetation; top four individuals from a site 15-km distant that received poor rains and had poor vegetation. In previous years, rain, vegetation, and grasshopper size patterns were reversed at these two sites (d-f: Whitman, unpubl.).*

However, one may ask why there is plasticity in so many traits. There is no simple solution to this question, but an important factor is the cost of plastic traits. In many of the examples reported, the phenotype that arises in a certain environment is not advantageous in other environments. For instance, a *D. cucullata* with the helmet has impairments in clutch size and consequently a reduced number of eggs (Riessen, 1984). Similarly, as mentioned, the guppies that increase the reproductive environments suffer from a reduction in other life history traits such as life span due to trade-offs (Dzikowski *et al.*, 2004). Therefore, if an individual develops one of these predator-related phenotypes in absence of predators, it will suffer the costs of plasticity without the advantage derived from a better response to the predators.

It seems reasonable that due to the costs and benefits of plasticity, it is critically important for the individual to be efficient in detecting the characteristics of the environment. This implies that individuals have to maintain different sensorial mechanisms that permit them to analyse the environment. With this regard, there are many forms of plasticity (Nijhout, 2003), based also on the fact that the variation of the environment can be discrete or continuous (Whitman & Agrawal, 2009). Speaking of plastic traits, we can observe a discrete response when the individual shift from one phenotype to another in response to a single encounter with a stimulus, such as a predator or a source of food. On the other hand, the continuous plasticity in response to variables such as temperature, illumination, etc. may determine a range of phenotypes that varies across a continuum.

1.1.3 Adaptive phenotypic plasticity and evolution

We expect that adaptive plasticity should be favoured by selection. It provides a rapid and flexible tool to increase individual fitness. Therefore, mutations that permit to obtain different phenotypes in different environments should accumulate in the population. A problem with this thinking is that an imaginary, extremely efficient plastic organism would be free from the need of adaptive evolution. Imagine an organism that responds immediately to environmental factors with the best possible phenotype. Selection on genes would not exist for this organism because its relative fitness would be always equal to 1. An interesting point to consider in this disquisition on evolution and plasticity is genetic mutations. Mutations on a specific trait are not necessarily involved to produce the trait plasticity, because the genotype related to such remains the same for all the various potential phenotypes. What is necessary is a gene that can turn on and turn off the plasticity process.

This conundrum has led many researchers to believe that plasticity is a problem for the theory of evolution. Consequently, variations in phenotype due to plasticity have long been ignored. However, some researchers have gradually started to propose integrations of plasticity in the concepts of genetic adaptive evolution, without a contrast between the two phenomena. For instance, Wilson and MacArthur in their most important work called "The theory of island biogeography" explicated that the modification has to be initially phenotypical, changing behavioural or morphological traits and only after this passage, thanks to natural selection, perhaps involving genetic assimilation' (Wilson & MacArthur, 1967). This point of view places plasticity even in a critically important role in genetic evolution. It implies indeed that traits that have initially evolved plastic mechanisms can produce the potential for genetic evolution.

Another point that gives relevance to evolution comes from a historical perspective. Baldwin and Morgan (Baldwin, 1896; Morgan, 1896), issued that plasticity has to be present in a population to prevent the risk of extinction because if the individual does not adapt itself to the changing environment, such as loss of available resources, that population could not survive to the environmental variations. In this contest, they inserted the importance of plasticity which can lead to the maintenance of genetic variation and speciation. There are several examples of this rescue-effect due to plasticity in the literature. For example, the study of Karkarey and colleagues (Karkarey *et al.*, 2017), in which they investigated how the fish *Cephalopholis argus* cope with changing structural environment with the massive loss of coral reefs. The researchers collected data about diet, foraging mode and foraging territory used. They discovered an increase in the size of the territory employed for foraging linked to the loss of structural complexity of the environment. Moreover, this fish when they are in a contest of high density of population, they used the ambush mode foraging and widely foraging in equal proportion. When fish are in a low-density structure population, they used more the widely foraging respect to the ambush foraging mode. Then, another finding was that the fish switched from an ambush foraging mode to a wider foraging mode. The results of this study show an adaptive plasticity in foraging behaviour that is likely performed by the animal with the ultimate scope to survive in a compromised situation.

Unfortunately, these theories, even when proposed at the beginning of the evolutionary research, have been often misunderstood by biologists, which tend to ‘believe’ in a central point of genes in evolution and see the theory of plasticity as adverse to that. For example, see the so-called Baldwin effect, is a term coined for a process of loss of plasticity in favour of genetically based variability, which actually has the opposite meaning compared to that of Baldwin’s original article (Scheiner, 2014).

In conclusion, it is nowadays clear that adaptive plasticity is a key biological phenomenon that is not against adaptive evolution: plasticity has evolved often due to adaptive evolution; plasticity may help to rescue populations in danger due to rapid environmental changes thereby maintaining also genetic variability that can be later used by adaptive evolution; and according to some scholars, new traits may arise mostly by phenotype plasticity and only later one became controlled by genetic producing evolutionary chances.

1.1.4 Non-adaptive phenotypic plasticity

Phenotypic plasticity can be adaptive when the individual in a changing environment or in response to variations of crucial factors has the capacity of modifying its characteristics to deal with the stressful condition in a favourable way (Whitman & Agrawal, 2009). This implies that adaptive plasticity has evolved due to selection (Grenier *et al.*, 2016). However, there is also another shape of plasticity, non-adaptive plasticity or maladaptive plasticity. This arises in various situations and with various consequences, but all related by the fact that is not a response evolved for a specific adaptive significance. For instance, some forms of non-adaptive plasticity arise in response to a new environment never experienced by the species. In such stressful situations, the individual can plastically change its trait because of inadequate responses. This maladaptive plasticity may reduce the fitness of the individual (Brady *et al.*, 2019). Alternatively, the trait may plastically lead to an instantaneous capacity to cope with the novel situation, but the phenotype shifts away from the optimum and develops a non-adaptive phenotype. This phenomenon is so marked that in the worst of cases, it can bring the population to extinction (Ghalambor *et al.*, 2007).

Nowadays, examples of maladaptive plasticity are unfortunately under our eyes due to climate change. A study of a migrating bird, *Luscinia megarhynchos*, showed us how individuals with long wings performed better the migration and there was a strong selection for birds with short wings than the long wings, suggesting the optimum morphology of the structure for the fly. But from 1994 to 2014 researchers noticed a strategy of this bird to change the size of the wings from long wings to shorter ones to minimize the cost to cope with the cost of reproduction in a hostile dryer habitat caused by global change (Remacha *et al.*, 2020). Due to global warming, lizards *Sceloporus tristichus* have been observed to plastically change their nest-building behaviour. To contrast the increasing temperature during oviposition, they shifted to a more superficial nest (Telemeco *et al.*, 2017). But this behaviour to adapt nesting to the temperature in the case of a situation of extreme hot can bring to a risk of a thermal shock for the eggs.

Maladaptive plasticity that compromises the fitness of the individual is influenced by 3 general factors: characteristics of the population, characteristics of the environment and the ecological and evolutionary feedback from the population and environment. Figure 3 represents the main 9 scenarios of the presence of maladaptive plasticity and the corresponding causes by Brady and co-workers (2019). The authors used a metaphor in which the target is the landscape fitness representative the optimum for the population, and the individuals of the population are represented by arrows. They divided into rows and columns considering respectively the trait in relation to fitness that can lead to maladaptation and then the causes that can influence the fitness landscape.

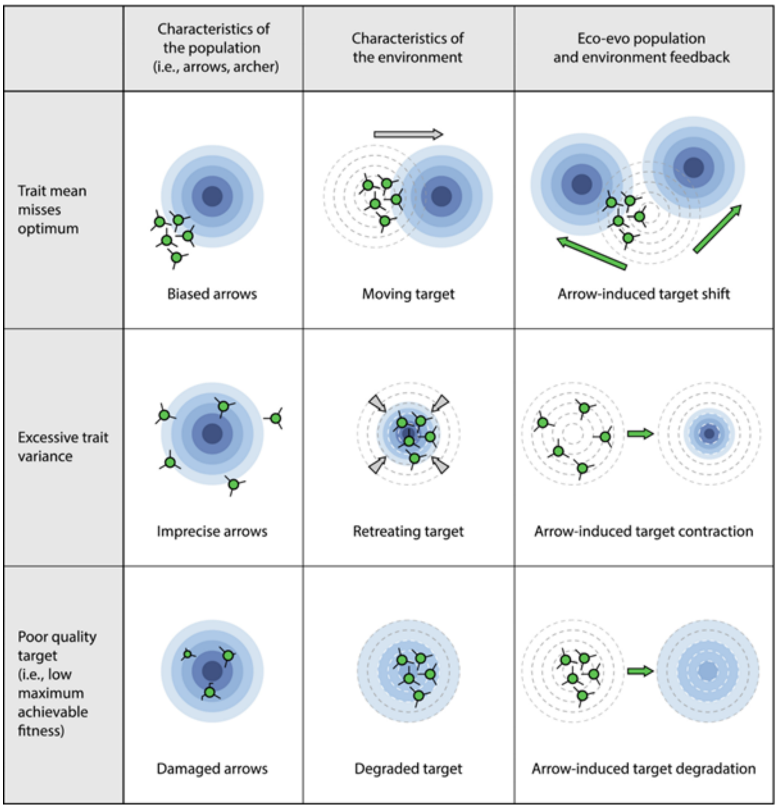

Figure 3: *Scenarios of maladaptation. Nine scenarios are illustrated using an archery metaphor of arrows and targets. In each scenario, arrows indicate representative individuals of the population while the target represents the fitness landscape. Rows indicate trait–fitness landscape scenarios that can generate maladaptation. Columns indicate various causes of the scenarios, involving either change in the focal population (left), change in the environment (middle), or eco-evolutionary/eco-plasticity feedbacks in which the focal population's evolution or dynamics alter the fitness landscape (Brady et al., 2019).*

In the box on the upper left part of the table, Brady and colleagues focused on changing characteristics of the population due to changing structure of the population (mutation, genetic drift and gene flow) in which the mean value of the population for a trait is shifted and missed the “optimum target” for the fitness. On the other hand, the shift can be provoked by maladaptive plasticity due to changing environment. These processes may help the phenotypic variation in a population that has temporary non-adaptive traits. Then, they highlighted a situation in which into the population there is an elevated phenotypic variance that, even if the population has a mean value of the trait perfectly adapted and in the optimum with intrinsic different characteristics, it deviated from the optimum. On the one hand, these situations can bring the population to have different phenotypes all adaptive to the environment and with a high probability closer to the optimum but with high competition between similar phenotypes. When there is a lack of phenotypic variance, it may lead to a maladaptive trait that can bring to the extinction of the population or a reduced fitness. In the third scenario is showed the problem of reduced fitness due to damage to the organism not dependent on a phenotype (pollution, high UV level). Then these phenotypes cannot always reach the optimum fitness due to this damage, reducing the number of individuals in the population. In these three scenarios described above, Brady and colleagues investigated the causes of maladaptive plasticity independent of the environment.

Moreover, the researchers explored how environment led to a non-adaptive trait. In the first scenario, is showed the risk of phenotypic maladaptive plasticity when the population has to cope with stochastic and unpredictable events such as an anthropic impact. In this case, the population may change its behaviour to survive to the new situation, but this change, which can have an advantageous result in optimal environmental conditions, can lead to a non-adaptive response with a consequent reduction of fitness. In another contest in which the changing environment with a loss of environment complexity, can lead to a reduction of the optimal fitness peak and a reduction of adaptive niches due to the development of maladaptive traits.

If the environment loses quality or is poor of resources, but the phenotype remains in the optimum fitness interval, the individual trying to fix their behaviour responses respect the situation, can have a reduced maximal fitness during the lifespan and can be also a situation of reduced dimension of population.

Over the characteristics of the population and of the habitat, the researchers explain the presence of maladaptive plasticity due to the ecological and evolutionary feedback responses given by the relation between phenotype and environment, dependent on each other. In this context, we can think about the coevolution of two species that live in the same habitat and that each species is part of the environment and can shift the peak of the optimum level of fitness for the other species due to the process of the evolution, such as the relation between a parasite to the host.

When we focused on the negative changing dimension of the population linked with the loss of several offspring and also reduced availability of resources, it may happen an event of competition between individuals of the population that may develop into the worst fitness due to maladaptation to the reduction of the dimension of population. Finally, the last scenario proposed is the opposite of the situation described before, in which is verified a higher density of the population due to scarcity of resources or absence of predators can have repercussions on the fitness due to the constant degradation of the habitat and a subsequent reduction of the population. This maladaptation to the high-density population condition can be transitory and prepare the basis for a new successive adaptation.

In conclusion, maladaptive plasticity can have different effects on a population linked to its characteristic, environment and feedback responses given the relation between these two factors. Moreover, non-adaptive traits may affect fitness, which can bring the population to a reduction of density, and extinction. On the other hand, since phenotypic plasticity may be transitory, maladaptive traits can lay the groundwork for a new adaptation.

1.1.5 Role of phenotypic plasticity in the mechanism of invasion by alien species

The consciousness about phenotypic plasticity can also help the researchers to fully understand some processes, for example, the invasion enacted by alien species. This mechanism is widespread and widely studied in plants, but it remains less investigated in animals. The power to have plastic traits enhances the invasion success into the early stage and preserves the risk of extinction thanks to the adaptation of a new optimal fitness peak related to the new environment. The defensive plastic behaviours may act as an advantage in the performance of the invasion and subsequently the success in the last part, the spread of the alien species

(Chapple *et al.*, 2012). The importance of to be plastic may avoid the extinction risk; moreover, the invader species can shape different phenotypes that can better adapt to the novel environment (Cordeschi *et al.*, 2022). An example that links the cognitive plasticity in learning with the success of invasion is given by Hazlett and colleagues (Hazlett *et al.*, 2002) in which they focused on an invader crustacean *Orconectes rusticus* and a native crustacean *O. virilis*. Then they subjected another pair of crayfish, (invasive: *Procambarus clarkii*, native: *Austropatmobius pallipes*) to the exposure of a novel odour and an alarm odour of a conspecific. They discovered that all four species can learn the association between the novel odour and the risk of predation, but interestingly the invasive species learned and memorized longer this information than the native species, suggesting higher cognitive plasticity.

Historically, the importance of the role of phenotypic plasticity in the process of invasion was not considered at all by researchers. Now, thanks to the growth of interest in this field, the literature is increasing. Also, in insects, we have suggestions for the mechanism of invasion that can be linked with behavioural plasticity. In Argentine ants, *Linepithema humile*, Sagata and Lester, observed that the propagule size of invasive species affects behavioural plasticity only in lab tests (Sagata & Lester, 2009). Specifically, they observed the invasion process of the invasive species and the native one *Monomorium antarcticum* both in the lab and in nature. They found out that the size of the propagule of invasive species in the lab was crucial for the success of the process, indeed only the larger group had an invasion success and replaced the native colony. Moreover, the behaviour of aggression was displayed more in larger propagules than in the smaller group of *L. humile*. On the other hand, in nature, the propagule size was not relevant for the resistance of *L. humile*, which coped with the situation also in the smaller group.

Ultimately, the likelihood of success of an invasion can increase due to the ability of alien species to be cognitively and behavioral plastic.

1.2 Cognitive plasticity

Cognition is composed of different processes that confer to an individual the capacity to perceive the stimuli in the environment, to learn a useful action in a specific moment and maintain it thanks to the capacity to memorize it. The study of cognitive plasticity is part of the field of phenotypic plasticity because the is the capacity of the individual that can adapt its cognitive system according to the environment. The capacity of an individual to be cognitively plastic can have effects on fitness because multiple reports indicate that individual cognition is related to survival and reproduction (Cauchard *et al.*, 2017; Cole *et al.*, 2012).

Cognitive plasticity is surely an added value to cope with the changing of the environment and to get important resources like food thanks to the perception of cues that are changed during the lifetime and re-elaboration of the previous just learnt response to a precise cue like a predation risk (Dzikowski *et al.*, 2004). In a work of Kacelnik and colleagues, it was tested the ability of localization of an acoustic cue by ferrets. The test consisted of problem-solving in which the ferrets previously trained to recognize a precise sound coming from spouts that produced water, had to choose from which one of twelve sounding spouts was working to earn the water and all subjects passed the test without errors (Kacelnik *et al.*, 2006). To test the capacity of plasticity to localize the right spout, researchers obturated the left ear of the ferrets and retested the animals in the task and they saw that the animals could find the origin of the sound even if they were deprived of an ear. This result is valid only for the individual that gets prior training, suggesting that having experience with a precise cue can help the animal to elaborate again the stimulus and to have a high performance in solving the situation.

If being plastic in a cognitive trait can be advantageous, on the other hand, this capacity is expected to be expensive metabolically (Aiello & Wheeler, 1995; Kotrschal *et al.*, 2013). Cognition has not have a direct connection to the structure and the size of the brain. On the other hand, there is evidence in which is shown that brain size can affect cognition in vertebrates in particular tasks. In the study of Buechel and colleagues (Buechel *et al.*, 2018) found in guppy, a teleost fish, that fish with a larger brain performed better in a reversal discrimination colour learning task but any significant differences were found during the previous colour learning task, suggesting that the brain size can influence the capacity to be more flexible in a task previously acquired, but not in a simple learning task in this species. This implies that a form of plasticity that increase flexibility may be associated with structural changes in the brain, and neural tissues are among the most expensive in terms of energetic requirements in the entire organism.

Another indicator to estimate the capability of cognition in a specific species or a subject is suggested in various studies and can be easily found by counting the number of neurons that compose the different structures of the brain, for example in mammals the neurons in the cerebral cortex and for the bird in the pallium described in Figure 4 (Herculano-Houzel, 2017). Moreover, in this paper, they showed that the number of neurons found in these structures is correlated with cognition and the capacity to solve a task. Specifically, a major number of neurons predict a good performance in a specific task of self-control between species. Again, if cognitive plasticity involves this trait, then its cost can be non-trivial.

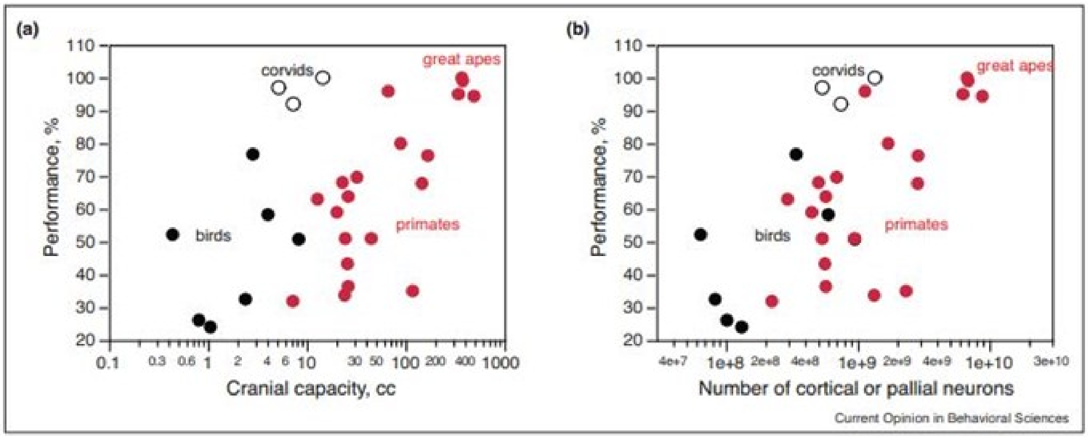

Figure 4: *Relationship between performance in a cognitive task and brain size or number of cortical neurons. Herculano-Houzel, 2017*

Focusing on teleost fish, we know that they have the great capacity to regenerate the neurons despite an injury of the brain and create new one also in adulthood (Zupanc, 2001), a process that in vertebrates like mammals is very limited. This makes this group an interesting starting point for the study of cognitive plasticity.

1.2.1 Natural environment and factors that determine cognitive plasticity

Given that cognition is the capacity to adsorb and remember crucial information from the habitat in which the individual lives, is commonplace to think about factors that can influence it. One of the most important factors that can influence cognitive plasticity is surely the complexity of the habitat, and environmental enrichment. As shown by Salvanes and colleagues, in *Salmo salar* there is evidence of higher expression of the proneuronal gene (NeuroD1) in fish that lived in enriched habitat than the fish maintained in a barren environment. Moreover, this enhanced expression caused an increase in learning ability in a spatial task (Salvanes *et al.*, 2013).

A result like the study described above is surely the research of Arechavala-Lopez and colleagues in which they maintained in two different groups of fish, *Sparus aurata*, each in an enriched condition and not enriched tanks without plants. Then they tested animals in an exploratory task and they found that fish treated with enriched tanks showed a higher explorative behaviour of the maze and had a greater capacity to solve the maze task than fish reared in barren tanks (Arechavala-Lopez *et al.*, 2020). Although not directly a cognitive measure, this effect could be due to changes in how subjects' brains perceived the environment.

Moreover, Kotrschal and Taborsky investigated how cognition can be influenced by factors such as food delivery. They involved individuals of *Simochromis pleurospilus*, cichlid fish, in learning task after a treatment which has a different quantity of food resources. They found that fish reared with an altered diet outperform fish with a constant amount of food resource in a learning task (Kotrschal & Taborsky, 2010a).

Seasonality is an important abiotic factor for individuals that have to change their behaviour after a change in temperature or photoperiod due to variable availability of resources such as food or home range. In rats, it was found an effect of photoperiod a diet in a task of object recognition. Specifically, rats maintained in a situation of long photoperiod (16 hours of light and 8 hours of dark) performed easily a NOR task independently from the diet (chow or fat diet). Rats with short photoperiod (8 hours of light and 16 hours of dark) showed impairment in NOR in both diets, suggesting that a short photoperiod may affect the recognition of familiar objects (McLean *et al.*, 2021).

Social interaction is crucial during the lifespan of an individual on survival. For example, in wild passerines great tits (*Parus major*) and blue tits (*Cyanistes caeruleus*) it was demonstrated how individuals of the larger social groups performed better and faster a foraging task. Moreover, they observed that if in the group there was an experienced individual, the group increased their problem-solving efficiency earning a larger amount of seeds (Morand-Ferron & Quinn, 2011).

This list of studies indicates that environmental factors that can determine cognitive plasticity are several. Moreover, this plasticity appears diffused in several major vertebrate lineages. However, the number of records is reduced compared to that present in the literature for other forms of adaptive plasticity. The gap is at least partly filled when considering indirect measures of cognitive abilities' plasticity, such as those presented in the following section.

1.2.2 Cognitive plasticity and neuronal plasticity

While the records of cognitive adaptive plasticity are not common in the literature, there are many studies of applied neuroscience that studied instead neuronal plasticity. Most of these studies are interested in models to understand and compensate for neurodegenerative diseases. They usually exploited simple environmental manipulations in the laboratory such as enrichment. Enrichment is described by Rosenzweig as the "combination of inanimate and social stimulation" (Rosenzweig & Bennett, 1996). Since the laboratory has standard and homogeneous conditions it is simple to implement rearing conditions that are enriched and present biotic and abiotic stimulations. One of the model organisms in the study of neuronal plasticity related to cognition is surely the rat. Several studies in the laboratory showed how enriched habitat can shape neural plasticity and cognition. Lores-Arnaiz and co-workers enrolled rats in a test of spatial cognition and working memory, in which the subject had to choose one of the 8 arms of the maze to gain the food (Lores-Arnaiz *et al.*, 2006). Before the spatial test, rats were divided and maintained in different conditions: the rats EE (with environmental enrichment) and rats SE (standard environment). The difference between the two conditions was the complexity of the cage of maintenance. In the group of EE rats, the cage was enriched with toys, different wooden walkways and walls provided with a net thanks to the rats can explore the cage entirely. In the other group (SE) the cage was empty without any enrichment, only the food and water were accessible. They found that the rats EE performed better in the spatial cognition test and reach the learning criterion faster than SE rats, suggesting a higher level of capacity in working memory. The cognitive change was also associated with measurable changes in neurons, i.e., neural plasticity.

Another interesting research was performed by Cao and colleagues, who manipulated the social environment. In this work, they investigate the role of social early isolation in mice related to environmental enrichment. They divided subjects into 4 groups: alone with environmental enrichment, provided with toys, food and shelters; alone without any enrichment; in groups with enrichment; in the group without enrichment. Then, mice were subjected to several spatial tasks and a social interaction test. The results of this study highlighted that both groups of mice treated with social isolation (SI) showed impairment with the spatial tasks and in working memory in the Y-maze. Concerning social interaction, the SI mice showed a decrease in socialization and an increase in anxious behaviour compared with the mice of the control treatment. Moreover, they found a neuronal plasticity impairment such as an increase in cellular apoptosis, and loss of synaptic protein that can lead to neuropathological disease. The present study indicated that living in an isolated habitat can have an impairment in cognition and this disease is implicated also in neuropathology related (Cao *et al.*, 2017).

Although these examples are not related to natural conditions and are therefore difficult to be associated with adaptive plasticity, they can still be useful to understand plasticity. Therefore, a comprehensive evaluation of cognitive plasticity should consider both the naturalistic treatments and the laboratory treatments designed for neuroscience research.

1.3 Cognitive abilities of teleost fish

Teleost fish are increasingly becoming central in the field of cognitive research (Bshary & Brown, 2014; Bshary & Triki, 2022; Laland, Krause & Brown, 2011; Oliveira, 2013; Rodríguez *et al.*, 2021). This is due to their advantageous characteristics in multiple disciplines, such as pharmacology, embryology, toxicology. For example, some species such as the zebrafish permitted to test new drugs on large samples of few-days old subjects, significantly speeding up the research (Caballero & Candiracci, 2018). In addition, it is possible to study the biological bases of fish cognition thanks to efficient mutagenesis techniques (Gerlai, 2015) and novel high-definition imaging techniques (Leung *et al.*, 2013). Findings in fish are then important for translational research because a high proportion of human genes related to cognitive diseases show extended homology with fish genes. Despite this interest in fish models, these translational studies do not tell us much about fish cognitive abilities. Perhaps because they are performed by experts in other fields such as genetic or because the advantages of the fish are mostly related to simple methodologies, translational studies only focus on a few, simple tasks.

One of the most studied abilities in teleost fish is habituation learning (Beppi *et al.*, 2021; Randlett *et al.*, 2019; Xu *et al.*, 2023). The capacity of learning is intrinsic in animals, even if some behaviours are inherited and innate, thinking a new-born mammal that nourishes from its mother's breast, or the pecking on the surface of the egg by a chick. Learning a task or an action can be advantageous in several situations, such as foraging, recognising the odour of the predator and also in mating, then is crucial for the fitness of the individual and its survival. Habituation learning is a simple form of learning common to most organisms. It is studied with an automatic setup that stimulates animals with disturbing agents, such as mechanic vibrations as described in a study on zebrafish larvae (Lucon-Xiccato, *et al.*, 2022). As habituation learning, researchers usually consider the rate of decrease in the response to stimulation. This simple method has permitted to discover of the underlying mechanism of learning on the association between stimulus and response from the individual and also the development of synaptic in relation to the stimulus.

Some studies on fish have focussed on simple forms of memory involved in remembering specific information (Baddeley, 2010). Memory is upstream the capacity to learn something. It is like a storage of information that the brain can stock and save until the next experience with this stimulus or information and ready-to-use. An explanation of how memory is involved in cognitive mechanisms is given by Gatto and colleagues which studied zebrafish and its capacity to remember specific objects after only one exposition. This kind of experiment is called NOR (novel object recognition) and they found out that larvae at 14 dpf, after the first exposition to an object, they preferred and explored more the familiar object than the new one, suggesting an innate capacity to recognize and store information about the environment from the stadium of larvae (Gatto *et al.*, 2022). Information acquired during life can remain in memory for a certain amount of time, as shown by Triki and Bshary in their work in which they focused on *Labroides dimidiatus*, a cleaner fish, and its capacity to remember for a period of eleven months a negative event like capture with nets, supporting the strong hypothesis that having memories about events that happened during the lifespan can help the individual to cope if the same event occurs again (Triki & Bshary, 2020).

Other paradigms used for translational research have permitted to describe other forms of learning, i.e., associative learning, in model fish such as the zebrafish. For example, *D. rerio* and *P. reticulata* can discriminate colour and associate one of them to the food, as described in a work in which researchers involved zebrafish in a discrimination colour learning in a T-maze in which each sleeve has a colour (red or blue in experiment 1 and green or purple in experiment 2). The fish had to choose between the 2 different colours presented and only one of them was rewarded with food. The result suggests that zebrafish can learn the association between colour and food (Colwill *et al.*, 2005). Another example of the ability to discriminate different stimuli is given by a study in guppy in which researchers tested them with a similar procedure used in prior studies in cognition, into a discrimination task in which fish had to choose between two stimuli with different quantity of food. They found that guppy can discriminate the food availability by considering the area occupied by the food and they preferred the larger quantity of two stimuli of food (Lucon-Xiccato *et al.*, 2015).

Considering these and other studies on translational research, the fish seems to possess a range of cognitive abilities and processes. However, these are far simpler than that observed in birds and mammals. This view has been recently changed by studies performed by researchers from other disciplines, such as behavioural ecology and comparative psychology. These researchers have attempted to develop more complex paradigms to investigate the presence of complex functions in fish.

Executive function is a set of mental processes that drive the decision of a subject, specifically when it has to cope with a situation in which instinct can betray and lead to a rushed and unfavourable choice. Executive functions are divided into 3 processes: inhibitory control, cognitive flexibility and working memory (Diamond, 2013). They are traditionally attributed to tetrapod cognition. However, researchers are identifying similar effects in fish. For instance, inhibitory control, or self-control, is the ability to block an action or a behaviour triggered by internal predisposition, such as an attack versus a prey by a predator, or by external stimulus, and that can be non-advantageous in that precise moment and implement later in a favourable condition. Recently, researched involved zebrafish in several tasks demonstrated the capacity of inhibitory control (Lucon-Xiccato & Bertolucci, 2020). When animals are exposed to a prey that they cannot reach, they progressively decrease of number of attempts to the prey throughout the experiment (Lucon-Xiccato *et al.*, 2020). Another species that has shown inhibitory control is *P. reticulata*, guppy (Lucon-Xiccato, Gatto, et al., 2017)

If learning is commonplace in animal behaviour, it is not so immediate thinking about the capacity to reverse the action just learn with the opposite stimulus. This is usually referred to as cognitive flexibility, one of the executive functions. Resuming studies cited above, which have been investigated after the learning discrimination also the capacity of cognitive flexibility in zebrafish, they confirmed the results just suggested by literature (Arthur & Levin, 2001), specifically fish can avoid the just learn choice in favour of the other stimulus that was not rewarded by food (Colwill *et al.*, 2005). Also, in the study of guppy, researchers investigated the ability to reverse their decision, but they reach the reversal learning criterion in more days than the learning task (about 6 days). Another confirmation of the presence of this capacity in teleost fish is given by Fuss and Witte that involved *Poecilia latipinna, Poecilia mexicana* and *P. reticulata* in a reversal learning task. Moreover, they found also differences in performance between sex and male of *P. latipinna* and *P. mexicana* learned faster the reversal discrimination in comparison to the conspecific females. In *P. reticulata* they found an inverse pattern, females learned faster the cognitive flexibility task, as suggested in the literature (Lucon-Xiccato & Bisazza, 2014). This experiment showed that cognitive flexibility can have differences between sex and also between species (Fuss & Witte, 2019).

Last, although less studied, it is worth mentioning that fish display also problem solving (Mair *et al.*, 2021), cooperation (Soares *et al.*, 2018), complex maze learning (Lucon-Xiccato & Bisazza, 2017), one trial learning of predators (Kelley & Magurran, 2003).

Overall, these records suggest that the cognitive repertoire of teleost fish is not significantly lower compared to that observed in tetrapods. This, along with the advantages identified by translational researchers, paves the way to the use of teleost fish for research on the evolution of plasticity.

1.4 Aim of the thesis

In this thesis, I try to investigate the presence of phenotypic plasticity in teleost fish cognition. In particular, I focused on three species selected for specific biological traits that can be associated to specific forms of plasticity, *P. reticulata* (Figure 5)*, O. latipes* (Figure 6) and *D. rerio* (Figure 7). The main goal of this work is to describe the presence of plasticity and to understand the mechanisms behind it and which factors influence the cognitive capacities of this species. Because the literature on fish cognitive plasticity is extremely reduced, I identified a set of factors that could be relevant based on the literature of other groups and I investigate their effects in fish. This resulted in five studies that are reported in the following chapters of the thesis.

In the first study, I considered the role of the habitat in which the individual lives and in particular a form of environmental enrichment. Indeed, environmental enrichment is one of the factors that determine reliable plasticity in mammals (Novkovic *et al.*, 2015). In my experiment, I raised newborn guppies in 2 different conditions: barren, empty laboratory tanks or tanks enriched with biotic and biotic stimuli. After this treatment, I assayed the subjects with a battery of cognitive experiments to measure if living in an enriched habitat can have an effect on capacity in inhibitory control, learning and cognitive flexibility as suggested in several studies (Ebbesson & Braithwaite, 2012; Pfennig, 2021).

Figure 5: *Poecilia reticultata. Credits: Wikipedia*

In the second study of this thesis, I was interested in investigating if living in a habitat with predictable or unpredictable food resources can modify the cognition of teleost fish. An extended literature connects this environmental factor to plasticity in many species (birds: van Overveld *et al.*, 2018 mammals: Ranc *et al.*, 2021 including fish but only for non-cognitive traits (Grand & Grant, 1994; Sloat & Reeves, 2014). I therefore hypothesise that this factor could also affect cognition. I raised guppies in a predictable condition, in which fish was fed at the same time of the day in the same foraging area and in an unpredictable condition, in which the food was randomly provided in the foraging area at different hours of the day. Then I tested them in a battery of cognitive tasks, in a colour discrimination learning task, a reversal learning task to measure cognitive flexibility, and in an inhibitory control task in foraging. I additionally performed behavioural tasks to address the presence of indirect effects of behaviour on cognition, such as open field to measure the components of explorative behaviour and social behaviour task to calculate social preference.

In the third study, I aimed to answer a question specific to the effects of the social environment in inhibitory control. Theory and comparative data indicate that animals exposed to a more social complex environment can deal with it only if they have sophisticated inhibitory capacities (Bond *et al.*, 2003; Byrne & Bates, 2007; Dunbar & Shultz, 2007; Jolly, 1966; Kamil, 2004). For instance, an individual has to repress aggressive behaviour when in presence of dominant individuals. I therefore raised guppies in different social environments including small groups, relatively large groups, stable groups, and unstable groups. I then assayed their inhibitory control capacities.

An interesting source of plasticity is seasonality because it involves plastic changes that are not fixed and can be reversed during the year following the season cycle. This has been shown to affect cognition in mammals (Lázaro *et al.*, 2018). To investigate seasonal plasticity in fish, I focussed on the medaka (*O. latipes*). This species lives in temperate environments and is therefore exposed to seasonal fluctuations. Moreover, it has been shown that it responds to seasons using photoperiod as a cue, which can be easily manipulated in the laboratory. I exposed groups of medaka

Figure 6: *Oryzias latipes. Credits: Wikipedia*

to a winter photoperiod or a summer photoperiod and then assayed in an inhibitory control task and discrimination learning task, plus behavioural tasks as control.

Figure 7: *Danio rerio. Credits: Wikipedia.*

Lastly, I used zebrafish to inquire about a very actual problem, the environmental pollution by common microplastics or biodegradable ones and if may change, after a long exposition, consequences in cognition. This would be a form of non-adaptive plasticity, useful as the first step to evaluate this problem in fish. After a treatment in which subjects were divided into two groups and fed each group with food mixed with these two kinds of microplastics, I measured learning and cognitive flexibility in the subjects.

2. Cognitive phenotypic plasticity: environmental enrichment affects learning but not executive functions in a teleost fish, *Poecilia reticulata*.

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> Simple Summary: Environmental enrichment is extremely important for an individual’s neural, cognitive and behavioral development. Emerging animal models, such as teleost fish, may con-tribute to our understanding of enrichment-driven cognitive plasticity. We studied the cognitive consequences of living in enriched conditions in *Poecilia reticulata*. In particular, we compared subjects raised alone in a barren aquarium versus subjects exposed to enriched aquaria (with conspecifics, natural substrate, plants, and live prey) in three different cognitive tasks to measure learning, inhibitory control, and cognitive flexibility. Our results showed that guppies from the enriched aquaria learned a color discrimination faster compared to the subject raised in barren conditions. However, in the two remaining cognitive tasks, we found no effect from the treatment, suggesting that enrichment does not affect inhibitory control and cognitive flexibility. This study reveals that enrichment-driven plasticity affects only specific cognitive abilities.

Abstract: Many aspects of animal cognition are plastically adjusted in response to the environment through individual experience. A remarkable example of this cognitive phenotypic plasticity is often observed when comparing individuals raised in a barren environment to individuals raised in an enriched environment. Evidence of enrichment-driven cognitive plasticity in teleost fish continues to grow, but it remains restricted to a few cognitive traits. The purpose of this study was to investigate how environmental enrichment affects multiple cognitive traits (learning, cognitive flexibility, and inhibitory control) in the guppy, *Poecilia reticulata*. To reach this goal, we exposed new-born guppies to different treatments: an enrichment environment with social companions, natural substrate, vegetation, and live prey or a barren environment with none of the above. After a month of treatment, we tested the subjects in a battery of three cognitive tasks. Guppies from the enriched environment learned a color discrimination faster compared to guppies from the environment with no enrichments. We observed no difference between guppies of the two treatments in the cognitive flexibility task, requiring selection of a previously unrewarded stimulus, nor in the inhibitory control task, requiring the inhibition of the attack response toward live prey. Overall, the results indicated that environmental enrichment had an influence on guppies' learning ability, but not on the remaining cognitive functions investigated.

Keywords: behavioral plasticity; cognitive control; fish cognition; habitat complexity; individual differences

Introduction

Individuals of the same species might experience different environmental conditions if they live in different habitats or if the conditions vary with time [1]. When a single phenotype or evolved differences between populations are not adequate to cope effectively with spatiotemporal fluctuations in environmental conditions, we expect selection to favor the evolution of adaptive plasticity mechanisms. Plasticity may allow an individual to develop a phenotype that confers fitness advantages in specific environmental conditions [2]. For example, studies in *Daphnia* spp. have demonstrated that individuals develop a defensive helmet when they detect predator cues in their environment [3,4]. Subsequent studies have provided compelling evidence of phenotypic plasticity for a range of morphological [5–7], physiological [8–10], and behavioral traits [6,11,12].

Recently, interest in the plasticity of cognitive traits has been growing. Cognitive abilities can substantially affect animals' fitness [13,14], and are involved in coping with changing environmental conditions [15,16]. Notably, cognitive abilities appear to be expensive to develop, as high metabolic requirements characterize the underlying neural tissues [17,18]. If an individual invests in the neural tissue necessary to achieve higher cognitive abilities, we expect the individual to compensate by reducing the investment in other tissues or functions. For example, guppies, *Poecilia reticulata*, artificially selected for increased brain size developed a shorter gut and demonstrated reduced investment in reproduction [18]. The trade-offs involving cognition make it possible to hypothesize the evolution of phenotypically plastic cognitive abilities that match the environment in which an individual develops and lives.

A well-studied example of cognitive plasticity is determined by a set of biotic and abiotic factors encompassing the presence of habitat complexities, social companions, and live prey, which are referenced collectively as environmental enrichment [19,20]. Environmental enrichment yields to brain changes and improved cognitive abilities in various species. In rodents, environmental enrichments, such as a large cage with social companions, toys, and hiding places, trigger brain plasticity, brain gene expression and, ultimately, cause an increase in learning and memory abilities [21]. In teleost fish, in which intense neurogenesis occurs throughout the life [22], research has been often linked environmental enrichment to cognitive abilities. For example, Salvanes and colleagues [20] found that juvenile *Salmo salar* reared in enriched environments, such as tanks with rocks and plants, display an increase in spatial learning abilities. Carbia and Brown [23] tested, with a spatial-learning task, individuals of Cocos frill-goby, *Bathygobius cocosensis* exposed to environments with or without rocks and found that fish from the enriched environment learned the task's solution faster. Strand and colleagues [24] showed that performance of juvenile cod, *Gadus morhua*, in learning to choose between prey via observational learning is influenced by environmental enrichment, consisting of rocks and seaweed.

Considering that research has described a number of different cognitive abilities in fish [25–27], it is surprising that evidence of environmental enrichment plasticity is limited to learning tasks. In this work, we aimed to investigate environmental enrichment effects on a set of three cognitive abilities in a teleost fish, the guppy *P. reticulata*. Before the cognitive testing, we raised newborn guppies in either barren aquaria or aquaria enriched with social companions, gravel bottoms, natural and artificial plants, and live prey. The three cognitive abilities investigated were learning [28], cognitive flexibility [29–31], and inhibitory control [32,33]. We measured learning in a canonical color discrimination task. Cognitive flexibility and inhibitory control are part of a family of cognitive functions termed executive functions [34]. They have a large impact on animal life because they activate, in conjunction with other functions, to execute complex behavior and solve many cognitive tasks. Cognitive flexibility allows an individual to change a certain behavior or action for one more adapted to the situation [35]. We measured it with a reversal-learning task, in which the fish had to learn to choose the color that was not rewarded in the learning task. Inhibitory control describes an animal's ability to inhibit a behavior or a response [34]. We used a procedure whereby the fish had to stop attacking a prey concealed behind a transparent barrier [32].

Based on prior studies, we expected that environmental enrichment would increase the subjects' learning performance, e.g., [20,23,24]. The presence of this effect on learning would also serve as a control to ensure that the enrichment treatment triggered cognitive plasticity. The absence of prior studies on cognitive flexibility and inhibitory control does not permit us to formulate a strong prediction on the outcome of these tests. However, molecular studies have showed increased neural plasticity due to enrichment in fish [36], and in humans, research has reported an association between neural plasticity and inhibitory control [37]. Therefore, our main expectation would be to observe increased inhibitory control and cognitive flexibility in response to enrichment in guppies.

Materials and Methods

Subjects

Overall, the study involved 39 juvenile guppies of a domestic strain (snake cobra green) collected on the day of their birth. We maintained the parents in standard 200-L aquaria equipped with biological filters and air pumps. Fluorescent lamps (30 W, 10,000 K) lit the aquaria with a 12 h:12 h light–dark cycle. We maintained the aquaria in a room with a controlled temperature (27 ± 1 °C) and fed the adult guppies twice per day with nauplii of Artemia salina and commercial flakes (Staple food Vipan, Sera, Heinsberg, Germany). The aquaria had a sex ratio of 50:50, and individuals could breed spontaneously. To minimize the risk of inbreeding, we routinely added 50 new guppies to the stock and relocated the guppies to different aquaria.

Environmental Enrichment Treatment

We performed the environmental enrichment treatment on 39 subjects. To begin the treatments, we collected these guppies from the maintenance aquaria the day of their birth. We intended to start the subjects' treatment as early as possible during the development, avoiding confounding effects due to uncontrolled experiences. As we kept the breeders in mixed-sex groups with free ability breed, we identified the new-born guppies by checking the maintenance aquaria daily. Upon collection, we randomly split the subjects into two groups for allocation to the two enrichment treatments (Figure 1a).

We assigned a group of 18 subjects to the treatment with enrichment. We housed these subjects in 6-L plastic aquaria (32 cm × 16 cm, h 14 cm; n = 18 aquaria). The aquaria' walls were covered with green plastic. The first environmental enrichment consisted of the presence of a social companion (i.e., one newborn guppy of the same age). Beside the presence of a social companion, we provided further environmental enrichments. We fed the subjects with live prey (*A. salina*), administered twice per day. The subjects had to chase and capture the prey by swimming in the aquarium and peaking at the substrate, a situation that is relatively similar to foraging for live invertebrates in nature. Therefore, the administration of live prey consisted of a form of behavioral enrichment for the subjects [38–40]. In addition, we provided gravel and plastic plants to simulate a natural environment [19,20].

We assigned the remaining 21 subjects to the treatment with no enrichment. We housed these subjects individually in barren plastic aquaria (21 cm × 15 cm × h 11 cm; n = 21 aquaria). The aquaria' walls were covered with green plastic. We fed these fish with dead *A. salina* twice per day. A. salina were killed with a freezing procedure, and were thawed before administration to the subjects. The frozen food ensured that the without-enrichment subjects had the same diet of the enriched-treatment subjects, but they were not exposed to the live prey.

For both experimental treatments, we changed water in the treatment aquaria every week. Each aquarium contained a small air stone to ensure water oxygenation. All remaining details of the treatment aquaria' condition resembled those of the maintenance aquaria (temperature ~27 °C, photoperiod 12 h:12 h). The treatments lasted 30 days, a period that is sufficient to determine cognitive plasticity in this species [41].

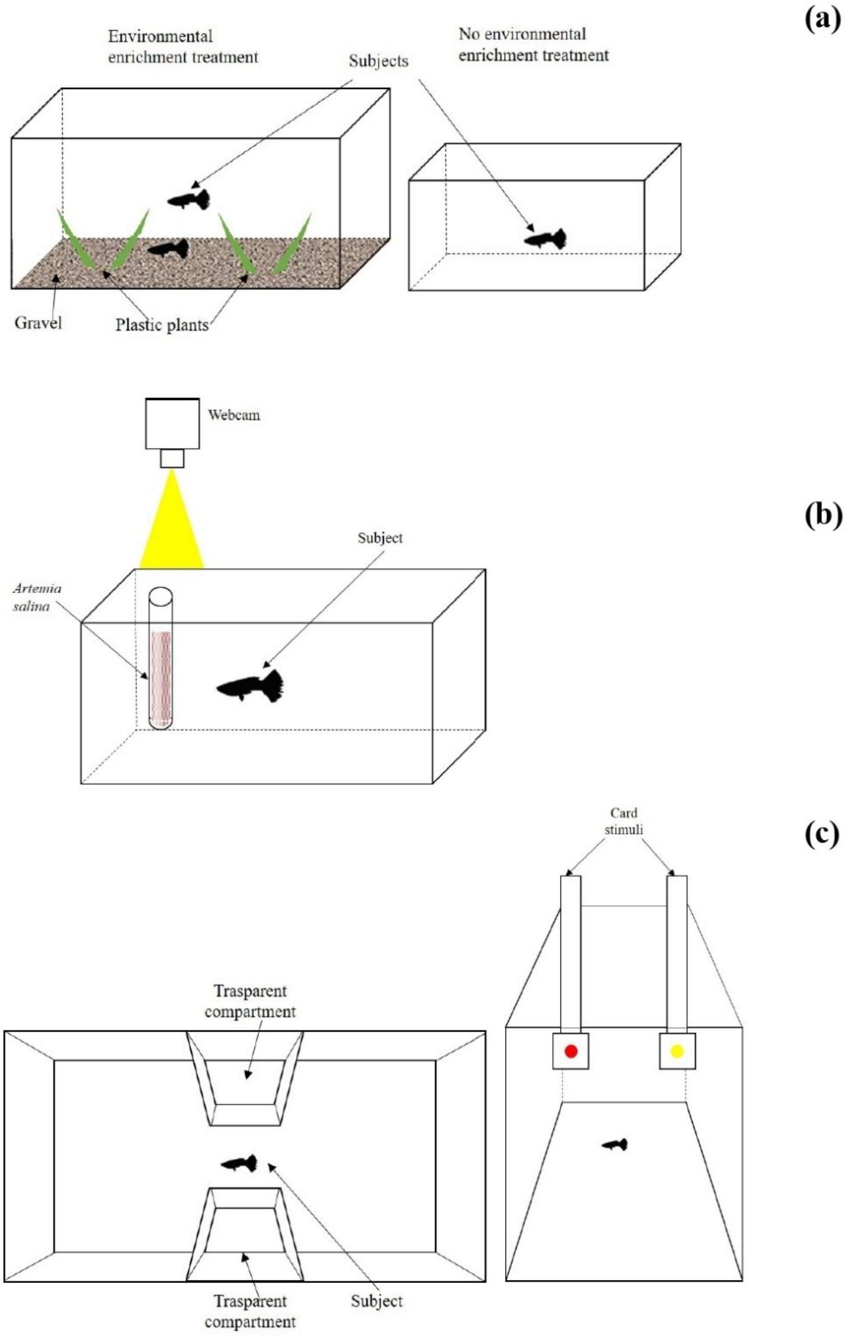

Figure 1. (**a**) Scheme of the aquaria used in the two treatments: the guppies of the enriched treatment (**left**) were housed in a 6-L aquarium with gravel bottom, plastic plants, and a conspecific, and they were fed with live *A. salina*; the guppies of the treatment with no enrichment were housed individually in a 2-L barren tank and fed with frozen *A. salina* (**right**). (**b**) Apparatus used in the inhibitory control task: the guppies were tested individually with a prey hidden inside a transparent tube. (**c**) Apparatus used in the learning task and in the cognitive flexibility task; the subjects were tested individually in the choice between two stimuli with different color, with a reward associated to the approach of the correct color.

Inhibitory Control

After 30 days of treatment [41], we subjected the guppies to the cognitive tasks. At this age, guppies are still juveniles as sexual maturation in this species occurs approximately at 3 months. We conducted the three cognitive tasks in the following sequential order: inhibitory control task, learning task, and cognitive flexibility task. This order reduced carryover effects across the tasks and exploited the contingency learned in the learning task as a starting point for the cognitive flexibility task (see below).

The inhibitory control task required the fish to inhibit the tendency to attack a live prey [32,33]. We conducted this task first, as it relied on a simple procedure and was less likely to cause carryover effects [42]. To perform the inhibitory control task, we initially housed each individual subject (n = 39) in a testing tank with green walls (4 L, 33 cm × 13 cm, h 15 cm; Figure 1b). Above the tank, we placed a transparent lid with a hole (⌀ 1.2 cm), through which we fed the fish, and on the day of testing, we presented the stimulus prey. We also installed a webcam 50 cm above the lid to record the trials.

The procedure of this task consisted in two phases: habituation phase and test phase. The habituation phase lasted 3 days during which we fed the guppies with dry food mixed with water, increasing to 2-, 4-, and 6-times per day. The food was provided from the hole in the lid of the testing aquarium. We used dry food rather than live prey to ensure that the food items did not spread in the apparatus. In this way, the subject became habituated to receive the food from that specific area of the aquarium. In the last day of the habituation phase, we checked that all the subjects consumed the delivered food.

After the habituation phase, the subject began the test phase, in which we exposed it to a transparent tube (10 cm, ⌀ 1.2 cm) with a solution of 4 mL of live *A. salina*. We inserted the tube from the hole in the lid, and we blocked it to suspend vertically in the water column (Figure 1b). In an earlier study, we determined that the number of brine shrimp in the tube was 470 ± 48 (mean ± SD; n = 10; [42]). We expected the guppies to try to reach the prey, given the initial habituation to feed in correspondence to the hole in the lid. However, guppies could not reach the prey because of the transparent tube, and we expected this to cause the inhibition of predation behavior. To score inhibition, we recorded the behavior of each fish for 20 min and used the recordings to count the number of attacks toward the prey. We scored an attack when the fish touched the tube's glass with its snout. Based on prior studies [32,33,42], we expected the fish to reduce the number of attacks over the testing time due to inhibition. There-fore, even if the test was continuous, we scored the data in 20-time bins of 1 min each. This allowed us to analyze the change in the number of attacks over the testing time. Fish with lower inhibitory control were expected to show a higher number of attacks and/or a smaller reduction in the number of attacks over time.

Learning

After the inhibition task, we randomly selected a subsample of 24 subjects (12 per treatment) to conduct the remaining tasks. This selection was necessary due to the logistic constraints associated with extensive training procedures. In the learning task, we trained the subjects to select a predetermined stimulus between two options, based on their color. We moved each subject to a larger glass tank (25 cm × 40 cm, h 25 cm) designed based on prior cognitive experiments in this species [43,44]. In this apparatus, we performed the learning and, thereafter, the reversal learning task (Figure 1c). This apparatus had an hourglass shape, with two main compartments, in which we presented the stimuli, and a central, narrow corridor. The corridor's walls were transparent plastic, to allow the fish to see the entire apparatus and the stimuli from each position. The external walls of the apparatus were covered with green plastic to simulate vegetation and avoid interference from external stimuli.

For the color discrimination learning task, we initially administered a 2-day habituation procedure to the subjects. During the first day of habituation, we presented to the subjects with a transparent card (4 cm × 4 cm) by means of a transparent support on the aquarium's wall. We inserted the card along one of the apparatus's short walls (Figure 1c). To avoid startling the subject and to give it the time to approach the card spontaneously, we waited to insert the card until the subject was in the opposite side of the apparatus. On the card, there was a drawn yellow or red circle (⌀ 1.8 cm), which served as the stimulus. The color of the circle assigned to each subject corresponded to the reinforced color used in the following phases of the learning experiment. We predetermined the assignments pseudo-randomly and counterbalanced them between subjects from the different treatments; we tested half of the guppies with the red stimulus and the other half with the yellow stimulus. A statistical test run after completing the learning experiment indicated no effect of color on learning performance (χ^2_1 = 0.501, p = 0.480). After presenting the card stimulus, we waited until the guppy approached it. Upon approach, we released a mix of crumbled dry food water using a Pasteur pipette. As the scope of the habituation phase was to train the fish to approach the card, we delivered small quantities of food to attract it even when the fish was at a distance. We repeated the procedure of inserting the card and delivering the food eight times, with an interval of 15 min between each trial. We alternated the card's presentation between the two short sides of the tank, and we varied the right-left position of the card. Only for this phase of habituation, we also housed 4 smaller guppies, randomly collected from the maintenance tanks, in the experimental apparatus [12,45]. This favored learning of the feeding schedule in the subject because, with the higher number of fish in the apparatus, it was more likely that one of them would notice the food and approach the card. Generally, the other fish rapidly followed and consumed the food. We removed these smaller guppies after this phase to avoid interference during the experiment.

On Day 2 of habituation, we subjected the guppies to 12 trials, in which we simultaneously introduced two stimuli with different colors, a card with a yellow circle and a card with a red circle. The two stimuli were placed as separate as possible on the tank's short wall (Figure 1c). The fish had to choose and approach the card with the color assigned in the prior phase (i.e., rewarded color). As in the previous phase, we inserted the cards when the subject was in the opposite half of the tank. Therefore, the subject had to swim through the corridor and position itself in the center of the apparatus when choosing between the two cards. To obtain a robust measure of choice, from this phase onward, we considered the subject’s approach to the card when it reached a distance smaller than 0.5 body lengths. When the subject approached the card with the rewarded color, we considered it a correct answer, and we provided the food reward. In the case of a correct choice, after rewarding the subject, we removed the card with the incorrect stimulus, and we let the subject to consume the food before removing the card with the correct stimulus and terminating the trials. If the subject chose the card with the incorrect color, we waited until the subject chose the right card, we removed the incorrect stimulus, and we provided the reward. This might re-quire multiple choices from the subject, but only until the maximum trial duration (15 min). After the subject consumed the reward, we terminated the trial as described above. In case of no choice (i.e., the fish did not approach any card), the maximum waiting time for a trial was 15 min. Then, we interrupted the trial and repeated it after an interval of 30 min.

After Day 2 of habituation, guppies started the test phase. We used only the data of this test phase to evaluate learning performance. The test phase consisted of a series of trials in which the fish could choose between the two stimuli. As described above, in each trial, we inserted both cards simultaneously in the aquaria during each trial, and we noted the choice of the fish. We administered food only when the subject chose the correct color (i.e., correct response) at first. If the subject chose the incorrect color, we removed both cards without delivering food. We administered 12 trials per day, but the number of test days varied according to each subject’s performance. We completed the testing when the subject reached a predetermined learning criterion of not more than seven errors in two consecutive days (<30% errors).

Cognitive Flexibility

We assessed cognitive flexibility with a reversal learning task. We presented the fish with the same color stimuli as in the prior task. However, to receive the food re-ward, the subject had to choose the card that was not associated with the reward during the prior learning task. Therefore, this task required the subjects to switch their learned preference between the two colors. The reversal learning task was administered to the 24 fish that completed the learning task, starting from day after each subject reached the learning criterion. One subject was discarded from the experiment because of an experimental error (final sample size = 23 subjects).

The reversal learning task took place immediately after each subject reached the criterion for the color discrimination learning task. We followed all the details of the procedure described for the test phase of the learning task. We administered 12 trials per day, until the subjects reached the criterion of maximum seven errors in two consecutive days (<30% errors). All other details of the procedure followed what was explained above.

Statistical Analysis

For the statistical analysis we used R Statistical software (available at: https://www.r-project.org/ (accessed on 21/12/2021). All of the data showed normal distributions. As we dealt with a small number of independent variables (maximum 2) and they were important for the scope of the study, we calculated significance of the terms from full models (i.e., we did not conduct model selection).

Initially, we analyzed the data of the inhibitory control task, which was composed by the number of attacks of each subject in each minute of the test. We applied Linear Mixed-Effects Models (LME; lme R function) to deal with the repeated measures. Beside the minute of testing, we fitted the treatment as fixed effect in the analysis; we also included subject ID as random effect to deal with the repeated measures. This analysis was expected to reveal differences in the overall number of attacks between the two treatments (main effect of treatment), as well as different trends in the reduction of attacks over time (interaction between minute of testing and treatment).

For the analysis of the learning task and the cognitive flexibility task, we adopted a similar approach. We analyzed the number of errors committed by each fish divided per each day of testing of the two learning experiments. As this latter data also included repeated measures, we used LME models fitted with the lmer R function to deal with differences in the number of training days between individuals. In these LMEs, we fitted the day of training (e.g., 1, 2, 3, …) and the treatment (enrichment versus no enrichment) as fixed effects; the subject ID was fitted as random effect. This analysis allowed us to detect differences in the overall number of errors to reach the learning criterion (main effect of treatment), as well as different learning trends across the two treatments (interaction between day of training and treatment).

Results

Inhibitory Control

Thirty-seven out of 39 guppies responded to the prey and attempted to capture them. The remaining two guppies, which were of the no-enrichment treatment, did not show interest for the stimulus prey and were removed from the dataset before the statistical analysis. Considering the 37 subjects that attempted to capture the prey, the overall number of attacks observed in the experiment was 166.24 ± 17.03 (mean ± standard deviation). The repeated measures LME indicated that the guppies decreased the number of attacks over testing time (minute of testing: $F_{1,735} = 38.040$; $p < 0.001$). Indeed, the number of attacks decreased from 29.46 ± 2.43 in the first minute of the experiment to 5.54 ± 1.06 in the last minute of the experiment.

The repeated measures LME did not detect significant differences in the number of attacks between the subjects of the enriched treatment and the subjects of the treatment without enrichments ($F_{1,735} = 0.13$; $p = 0.722$; Figure 2). In addition, there was no significant interaction between treatment and minute of the experiment ($F_{1,735} = 0.78$; $p = 0.378$).

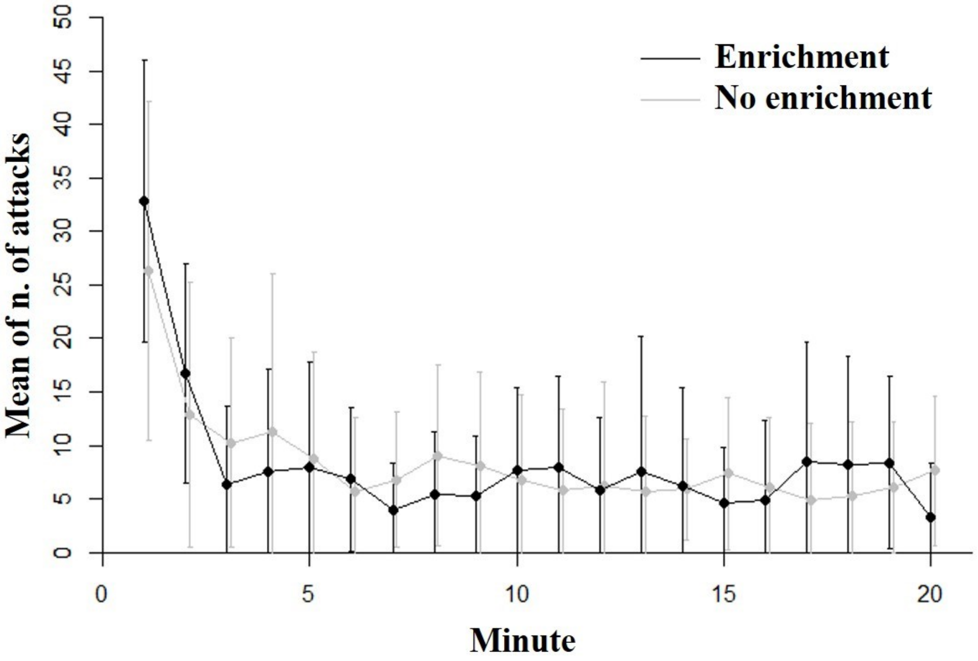

Figure 2. Results of the inhibitory control task. Number of attacks (mean _ SE) performed by the subjects of the two treatments in each minute of the test phase. The black line represents the treatment with environmental enrichment and the grey line the group without environmental enrichment.

Learning

In the learning experiment, all the guppies acquired the color discrimination and achieved the learning criterion. Considering all of the 24 subjects tested in the color discrimination learning task, the number of errors necessary to reach the criterion was 18.92 ± 4.30, corresponding to 4 days of training (4.00 ± 0.57). The repeated measures LME analysis on the number of errors showed a significant difference between treatments ($\chi^2_1 = 5.118$; $p = 0.024$; Figure 3a). The subjects of the treatment without environmental enrichment made more errors before reaching the learning criterion compared to the subjects of the treatment with environmental enrichment (Figure 3a).

In the repeated measures LME analysis, the day of training also had a significant effect ($\chi^2_1 = 27.905$, $p < 0.001$). More importantly, we found a significant interaction between treatment and day of training ($\chi^2_1 = 11.591$, $p < 0.001$). This indicated that fish from the enriched treatment displayed a quicker decrease in the number of errors across training days (Figure 3b,c), and therefore they had greater learning performance.

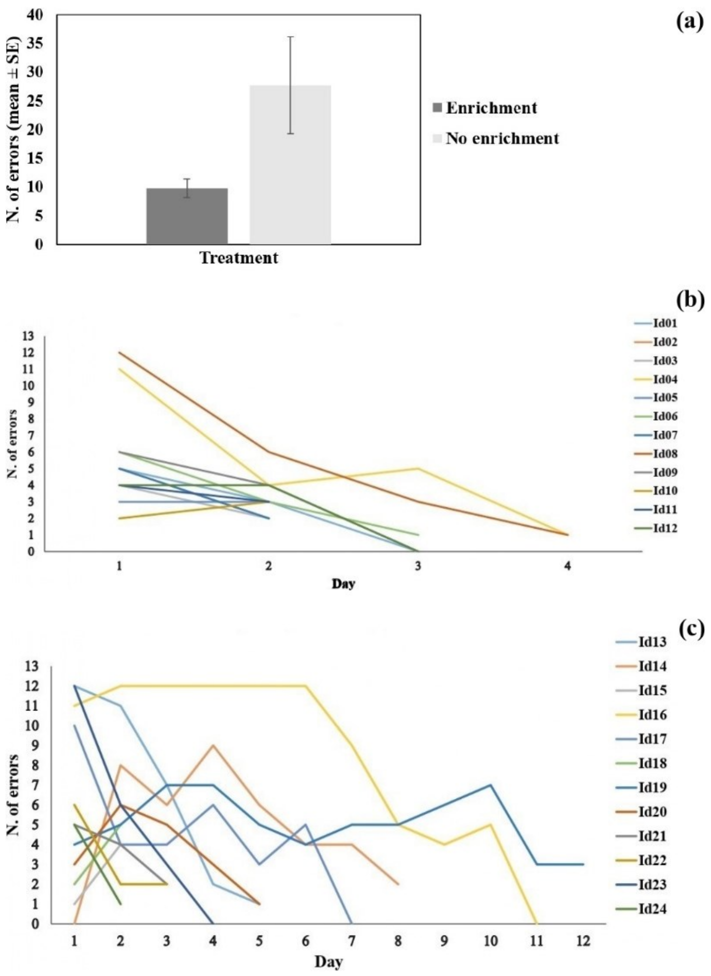

Figure 3. Result of discrimination learning task. (a) The number of errors to reach the learning criterion (mean ± SE) divided per treatment; the dark bar shows the subjects of the enriched treatment and the light bar the subjects without enrichment. (b) The number of errors made in each day of testing by the fish of the enriched treatment; each line corresponds to an experimental subject. (c) The number of errors made in each day of testing by the fish of the treatment without enrichment; each line corresponds to an experimental subject.

Cognitive Flexibility

In the reversal learning task, all of the 23 guppies tested achieved the learning criterion, with an average number of errors of 40.69 ± 7.43, corresponding to 7 days of training (6.95 ± 0.95). The repeated measures LMM showed no significant difference between treatments in the number of errors committed before reaching the learning criterion ($\chi^2_1 = 0.401$; $p = 0.527$; Figure 4a). The day of training had a significant effect on the number of errors ($\chi^2_1 = 100.637$; $p < 0.001$; Figure 4b,c), indicating an overall de-crease in the number of errors over testing days. Last, we did not find a significant interaction of treatment and day of training ($\chi^2_1 = 0.591$; $p = 0.442$; Figure 4b,c).

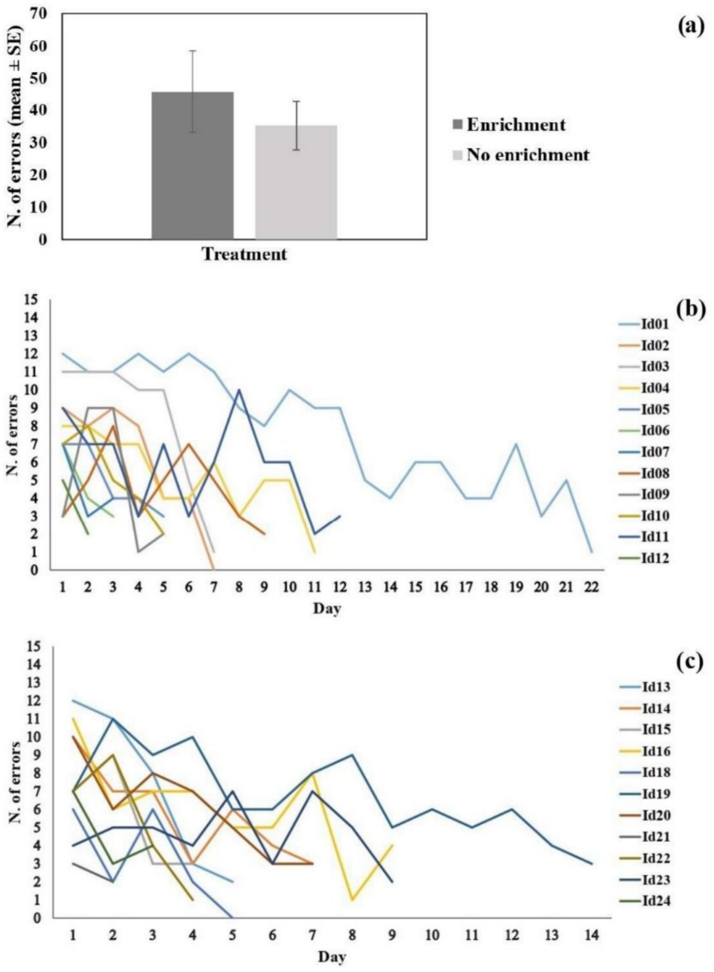

Figure 4. Result of the cognitive flexibility task. (a) The number of errors to reach the learning criterion (mean ± SE); the dark bar shows the subjects of the enriched treatment and the light bar the subjects without enrichment. (b) The number of errors committed in each day of testing by the fish from the enriched treatment; each line corresponds to an experimental subject. (c) The number of errors committed in each day of testing by the fish from the treatment without enrichment; each line corresponds to an experimental subject.

Discussion

A relatively extended literature has demonstrated that animals plastically alter their cognitive abilities in response to the level of environmental enrichment they experienced (e.g., [21,41]). In teleost fish, research has mostly reported this plasticity for learning capabilities (e.g., [23]); in this study, we investigated whether other cognitive abilities might be similarly plastic. Using the guppy as a study species, we assessed the effects of environmental enrichment on cognitive phenotypic plasticity, focusing on three different traits: learning, cognitive flexibility, and inhibitory control. We found an effect due to the environmental enrichment on the learning performance, but not in the other cognitive abilities investigated (i.e., cognitive flexibility and inhibitory control).

The effect of environmental enrichment on guppies' learning plasticity aligns with the results of prior studies in a range of species, including teleosts such as the striped knifejaw, *Oplegnathus fasciatus* [46]; the Atlantic salmon, *S. salar* [20], the cod, *G. morhua* [24]; and the Cocos frill-goby, *B. cocosensis* [23]. These teleost species were assayed for various forms of learning using social and spatial tasks. Considering that our task involved learning in a different setting (i.e., when discriminating between colors), a possible interpretation for the whole literature on teleosts might be the presence of an effect of enrichment on a single learning function that is recruited when learning in many situations. Clearly, at the current stage of research it is important to obtain more knowledge on different learning tasks to confirm this interpretation.

Several studies have investigated the molecular mechanisms of the enrichment-driven learning plasticity [47–49], and one of these studies involved a fish species [20]. Evidence indicated that Atlantic salmon, *S. salar*, with increased learning due to enrichment also displayed upregulation in the expression of the transcription factor NeuroD1 [20]. NeuroD1 is widely conserved in vertebrates, e.g., [50] and plays a critical role in nervous system development [50] and plasticity [51–53]. Therefore, future research should investigate NeuroD1 as a determinant of learning plasticity due to enrichment in guppies, as well as in other species. Among the alternative pathways, those involving hormones should not be ignored. Many hormones have widespread effects on learning, e.g., [53], and it is known that enrichment may alter hormonal production in fish, particularly in relation to stress hormones [54–56]. Indeed, without enrichment, guppies might suffer several forms of stress such as that due to absence of appropriate substrate [55]. It is worth considering that the mechanisms of the observed learning plasticity might involve neuroanatomical changes in guppies' brain. A recent study on this species found that individuals exposed to a spatially complex environment developed larger relative brain size and larger relative optic tectum size [36]. We also know that guppies with larger brain size display greater learning performance in discrimination tasks [18,57,58]. Therefore, brain anatomy changes might also have occurred in our guppies, although we cannot confirm this because we did not euthanize our fish for brain analysis.

More uncertainty remains on the ultimate causes of the observed plasticity in guppies' learning performance. At least two hypotheses deserve attention in future investigations. First, in nature, enhanced learning abilities might be advantageous when the environment is more complex. If this is true, then the effect on learning observed in the laboratory studies, such as the present one, is potentially due to an adaptive mechanism of plasticity. We can also speculate that, for fish exposed to a barren environment, low investment in a learning function, such as the color discrimination investigated in our study, would make available additional resources for the development of other traits [18]. As a second hypothesis, it is worth considering that the plasticity observed in the guppies of the present study might be due to a nonadaptive mechanism. For example, a complex environment might provide enhanced stimulation to the neural system, and this might cause a developmental improvement of learning [59]. In support of this idea, came the studies on fish bred in hatcheries without enrichments and released in nature for conservation purposes. The behavior of these fish often appears maladaptive compared to that of wild fish [60]. The same may apply if the effect observed on guppies' learning was due to stress hormones, as previously mentioned.

A question that remains unanswered due to our experimental design is whether one or some elements of enrichment, but not others, affected guppies' learning. Indeed, we simultaneously exposed the guppies of the enriched treatment to several stimuli (e.g., conspecifics and live prey). We did this in an attempt to obtain a larger and more robust effect, reducing the chances of type II errors. However, a study on lizards re-ported that social enrichment did not cause learning alterations [61]. Furthermore, in mice, a study revealed different effects on cognition according to the type of enrichment experienced by the subjects [62]. Similar variation in the effects of enrichment elements may occur also in guppies, and deserve investigations based on experiments that manipulate the elements one by one.

Regarding subjects' inhibitory control and cognitive flexibility, we did not find an effect of environmental enrichment. The number of attacks toward the unreachable prey and the number of errors in the reversal learning tasks were substantially equal in the fish of the two treatments. Because inhibitory control and cognitive flexibility are considered part of a family of functions with distinct characteristics (i.e., the executive functions [34]), one may argue that, for this reason, they have no plasticity. However, at least two published studies in guppies contrasted this hypothesis. These studies found that social environment affects inhibitory control and that predation risk affects cognitive flexibility [63,64]. Moreover, results in our recent study of another teleost fish, the medaka, found plasticity of executive functions [65]. Therefore, we can conclude that the two executive functions examined in our study are, at least, potentially plastic, which raises the question of why enrichment did not affect inhibitory control and cognitive flexibility in our study on guppies. Considering that we found a clear effect of our enrichment treatment on the learning ability of guppies, we can exclude methodological problems in our experiment (e.g., the treatment was not long enough, or not in a critical developmental period). Overall, the most likely explanation for the absence of plastic changes in guppies' inhibitory control and cognitive flexibility is that these specific executive functions do not respond to the specific environmental enrichment treatment that we performed.

At the current research stage, it is only possible to speculate about the evolutionary causes of the lack of plasticity observed for the two executive functions (i.e., inhibitory control and cognitive flexibility) in juvenile guppies. Arguably, cognitive flexibility and inhibitory control are not necessary to deal with the environmental factors that determine enrichment, at least in the guppies. This is expected to cause relaxed selection on the environment-driven plasticity of these traits. In other words, guppies' executive functions might not have the capability to change in relation to environmental enrichment. Before accepting this hypothesis, future research should investigate the effect of different enrichments on these and other executive functions in guppies to validate our findings. In this light, a comparison of studies in mice [66] and rats [41] reveals that these two rodents develop different cognitive flexibility responses in relation to the levels of environmental enrichment experienced. The same may occur in guppies, requiring further investigation with different enrichment treatments. We should not generalize the fact that we did not find plasticity of executive functions in guppies. Indeed, some aspects of executive functions vary substantially across vertebrate species [67], suggesting that there might be large interspecific variation in how executive functions respond to environmental enrichment. Possibly, other fish species will respond to environmental enrichment with marked plastic changes in executive functions.

Conclusions

Collectively, the findings of the present study indicate that enrichment does not equally affect all the cognitive functions of juvenile guppies, but rather causes plasticity on specific traits. Future research requires a greater effort to increase the number of species investigated and to assess subjects' performance in multiple cognitive tasks. This approach will help us to understand why enrichment only affects some cognitive functions and whether different species show different forms of plasticity.

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3. Adaptive phenotypic plasticity induces individual variability along a cognitive trade-off.

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Abstract

Animal species, including humans, display complex patterns of individual variability in cognition that are hardly explained. For instance, some individuals perform well in certain cognitive tasks but show difficulties in others. Here, we experimentally analysed the contribution of cognitive plasticity to such variability. Theory suggests that diametrically opposite cognitive phenotypes confer fitness advantages in environments with different conditions such as resource predictability. Therefore, if selection has generated cognitive plasticity mechanisms that permit individuals to match their cognitive phenotypes to the environment, this might produce complex cognitive variability. We found that guppies, *Poecilia reticulata*, exposed to an environment with high resource predictability (i.e., food available at the same time and in the same location) developed enhanced learning abilities. Conversely, guppies exposed to an environment with low resource predictability (i.e., food available at random time and location) developed enhanced cognitive flexibility and inhibitory control. These cognitive differences align along a trade-off between functions that favour acquisition of environmental regularities such as learning and functions that adjust behaviour to changing conditions (e.g., cognitive flexibility and inhibitory control). Therefore, adaptive cognitive plasticity in response to resource predictability, and potentially, similar factors, is a key determinant of complex patterns in cognitive individual differences.

Keywords: behavioural flexibility; cognitive ecology; cognitive plasticity; fish cognition; individual differences.

Introduction

That individuals display different cognitive abilities has been long acknowledged in human psychology (e.g., Gustafsson & Undheim, 1996; Humphreys, 1979), and evidence is now accumulating for a range of other animal taxa (e.g., mammals: Beran & Hopkins, 2018; birds: Langley *et al.*, 2020; teleost fish: Lucon-Xiccato & Bisazza, 2017; insects: Mery *et al.*, 2007). The pattern of this intraspecific cognitive variability is often quite complex, with individuals excelling at certain cognitive tasks but performing scarcely in others (e.g., Bebus *et al.*, 2016; Bensky & Bell, 2020; Ferrari, 2014; Kim *et al.*, 2018; Lucon-Xiccato & Dadda, 2017; Mazza *et al.*, 2018). Various hypotheses for this complex cognitive variability have been formulated and tested (e.g., covariation with personality traits: Carere & Locurto, 2012; energetic trade-offs: Kotrschal *et al.*, 2013), we currently do not have a clear explanation.

Intriguingly, the cognitive variability has been often observed for functions that fall into two categories: functions such as learning and memory that are advantageous in predictable environments, allowing to deal with consistent patterns and regularities (Carter *et al.*, 2014; Ehlinger, 1989; Mery *et al.*, 2007); and functions, like the executive functions named cognitive flexibility and inhibitory control (Bensky & Bell, 2020; Laschober *et al.*, 2021; Lucon-Xiccato *et al.*, 2020), that allow individuals to change their behaviour rapidly and are likely advantageous in ever changing situations (Diamond, 2013). We know that the level of resource predictability in the environment affects a large number of non-cognitive traits including foraging behaviour (Grand & Grant, 1994a; Sloat & Reeves, 2014; Stephens, 1993), aggressive behaviour (Goldberg *et al.*, 2001; Grand & Grant, 1994b), spatial behaviour (Cama *et al.*, 2012; Eide *et al.*, 2004; Lòpez-Lòpez *et al.*, 2014; Riotte-Lambert & Matthiopoulos, 2020), metabolism (Sloat & Reeves, 2014), stress (Gottlieb *et al.*, 2013), reproduction and life history traits (Webb & Marcotte, 1984; Zammuto & Millar, 1985). If environmental predictability also affects cognition, it might generate interindividual variability along a trade-off between cognitive functions advantageous in highly predictable versus unpredictable environments (Tellos-Ramos *et al.*, 2019). Considering that individuals of a species may be subjected to different levels of predictability across space and time (e.g., Eide *et al.*, 2004; Menge, 1972; Minckley *et al.*, 1999), a probable product of selection is a cognitive plasticity mechanism that permits individuals to match their cognitive phenotype to the levels of predictability experienced. This cognitive plasticity would be a critical contribution to the complexity of intraspecific variability in cognition.

In our study, we tested the hypothesis that environmental predictability determines complex cognitive variability via cognitive plasticity. We manipulated temporal (Bassett & Buchanan-Smith, 2007) and spatial predictability (Grand & Grant, 1994a) of foraging resources in experimental populations of guppies, *Poecilia reticulata*, a teleost fish with heightened cognitive variability (e.g., Lucon-Xiccato *et al.*, 2020). The treatment exposed guppies to simulated environments with either a predictable food source available each day in the same place and at the same time or to an unpredictable food source available at pseudo random location and time. We then compared guppies exposed to the two treatments using assays for learning, cognitive flexibility and inhibitory control. Based on the aforementioned trade-off hypothesis (Tellos-Ramos *et al.*, 2019), we predicted a greater learning performance in guppies from the predictable treatment and a greater performance in the cognitive flexibility and inhibitory control tasks in guppies exposed to the unpredictable treatment. We additionally performed a set of behavioural tests because environmental predictability may affect fish behaviour (e.g., Sloat & Reeves, 2014; Goldberg *et al.*, 2001; Riotte-Lambert & Matthiopoulos, 2020) and this may indirectly determine cognitive changes (Savaşçı *et al.*, 2021; Trompf & Brown, 2014).

Materials and methods

Experimental manipulation of resource predictability

The experiments involved naïve, new-born guppies obtained from gravid females in our facility (electronic supplementary material, S1, section a). These subjects underwent exposure to environments with different levels of predictability for 20 consecutive days. We assigned a randomly chosen group of six individuals to each of 12 experimental aquaria (N = 72 guppies overall). Six experimental aquaria were assigned the predictable environment treatment and the remaining six experimental aquaria to the unpredictable environment treatment (N = 6 replicates).

The experimental aquaria were rectangular and contained four separate foraging areas, one in correspondence of each corner (electronic supplementary material, S1, Figure S1.1). We administered food to the guppies once per day in one feeding area of the experimental aquarium, with a different schedule for the two treatments. For half of the aquaria assigned to the predictable environment treatment, we provided the food at 11:00 h; for the remaining half to the aquaria of the predictable environment treatment, we provided the food at 15:00. Moreover, in the predictable environment treatment, the food was consistently administered in a predetermined foraging area per each aquarium. In the unpredictable environment treatment, we provided the food each day at a random time between 8:00 to 18:00 h and in a foraging area determined according to a pseudo-random scheme. Details of the treatment are provided in electronic supplementary material, S1, section b. At the end to the treatment, four subjects randomly collected from each experimental aquarium were used in the following assays (electronic supplementary material, S1, section c). As one subject showed signs of distress in the first assay, we interrupted the testing. Therefore, the sample used in the study was N = 47 guppies, 23 of the predictable environment treatment and 24 of the unpredictable environment treatment.

Learning assay

The learning assay was based on an established colour discrimination paradigm (Lucon-Xiccato *et al.*, 2022a; Montalbano *et al.*, 2022). Briefly, each subject was kept in an experimental apparatus consisting of two chambers connected by a central corridor (electronic supplementary material, S1, Figure S1.2). After a pre-test procedure (electronic supplementary material, S1, section d), the fish underwent 12 colour discrimination trials per day. In each trial, the experimenter inserted two stimulus cards in one of the two chambers of the apparatus. Each card had a circle coloured in either yellow or red. If the fish approached a predetermined correct colour, it received a food reward; otherwise, the experimenter removed the cards. The testing of each guppy continued until it reached a criterion of less than 30% errors. In each day of testing, we recorded the number of errors and the number of correct responses of each subject.

Cognitive flexibility assay

After the guppies reached the criterion of the learning task, we administered a reversal learning task to assess cognitive flexibility following previous studies in teleost fish (Lucon-Xiccato & Bisazza, 2014; Montalbano *et al.*, 2022). The apparatus and the procedure were the same as for the test phase of the learning task. However, the experimenter rewarded the choice of the previously unrewarded colour. The testing continued until each subject reached a criterion less than 30% errors, and in each day, we collected the number of errors and correct responses, as previously described.

Inhibitory control assay

Following a paradigm implemented in this species (Lucon-Xiccato & Bertolucci, 2019; Montalbano *et al.*, 2020), we assessed inhibitory control as the ability to withhold attempts to capture an unreachable prey behind a transparent barrier. Guppies underwent the assay individually in 4-L experimental aquaria (electronic supplementary material, S1, Figure S1.3). After a pre-test phase (electronic supplementary material, S1, section e), the experimenter inserted a glass tube with live *Artemia salina* in the apparatus and scored the guppies' behaviour for 20 min using a webcam. If a guppy touched the glass tube with the snout in the attempt to capture a prey, the experimenter recorded an error. Because of an issue with the webcam software, we did not retrieve the recordings of 4 subjects. Therefore, the sample size of this assay was 43 guppies, 22 of the predictable environment treatment and 21 of the unpredictable environment treatment.

Behavioural tests

First, we conducted a novel environment exploration test in an open-field arena (Brown *et al.*, 2007; Burns, 2008 Burns *et al.*, 2016). The guppies were observed individually in an unfamiliar, white, empty arena (electronic supplementary material, S1, Figure S1.4) for 20 min. During this period, using an automatic tracking system (electronic supplementary material, S1, section f), we measured the activity of the subject as distance moved and their boldness as time spent in the centre of the arena. Shier and more anxious individuals tend to avoid the centre of the apparatus where they perceive to be more exposed to potential predators (thigmotaxis behaviour; Blaser *et al.*, 2010; Kotrschal *et al.*, 2014).

Second, we conducted a social behaviour test (Cattelan *et al.*, 2019). The guppies were tested in the central comportment of a three-chamber apparatus (electronic supplementary material, S1, Figure S1.5). The two lateral chambers were divided from the central one by mean of a transparent partition. One lateral chamber contained a shoal of five guppies and the other was left empty. Using a camera on the ceiling, the experimenter recorded for 20 min the time spent by the subject close to the social stimulus (i.e., within 5 cm) and close to the empty lateral chamber. From this data, we calculated an index sociability of each subject. Further details of this procedure are reported in electronic supplementary material, S1, section g.

Results

Predictable environment enhanced learning

All the subjects tested (N = 47) reached the criterion in the colour discrimination learning task in a relatively short period of time (4.43 ± 2.58 days, mean ± standard deviation). The analysis on the number of errors across testing days found a significant decrease (Generalised Linear Mixed-effects Model, GLMM: $\chi^2_1 = 179.290$, $P < 0.001$). This suggested that the subjects progressively learned to choose the correct colour. Critically, the decrease in number of errors was steeper for the guppies of the predictable environment comparing to the guppies of the unpredictable environment

(GLMM: $\chi^2_1 = 18.769$, $P < 0.001$; Figure 1a). Therefore, learning was faster for the guppies of the predictable environment.

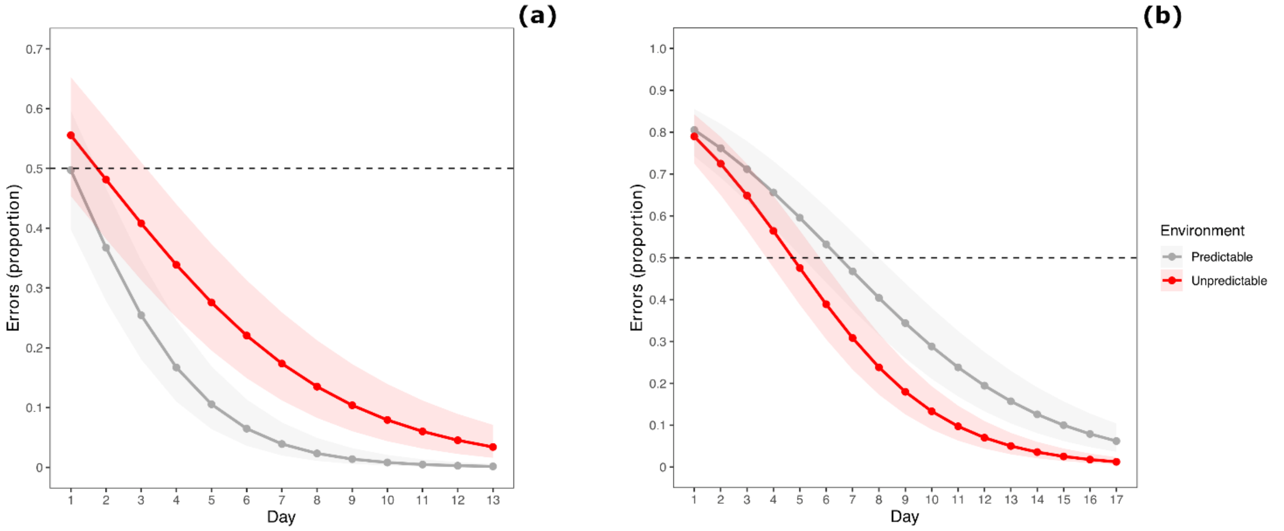

Figure 1. Results of the learning and the cognitive flexibility task. (a) Proportion of errors made by the subjects from the two treatments (predictable and unpredictable environment) when learning to discriminate between the two colour stimuli, divided per each day of the experiment. (a) Proportion of errors made by the subjects from the two treatments (predictable and unpredictable environment) when reversing the learned choice between the two colour stimuli, divided per each day of the experiment. In all the panels, points and shaded area represent mean and 95% confidence intervals estimated from the generalised linear mixed-effects model used in the analysis.

Unpredictable environment enhanced cognitive flexibility

All the subjects tested (N = 47) reached the criterion in the reversal learning assay. However, the number of days to the criterion in the reversal learning assay (8.36 ± 4.06 days) was approximately twice as that observed in the initial learning assay, suggesting a significant greater complexity of the cognitive flexibility assay for the fish (paired-samples t test: $t_{46} = 5.126$, $P < 0.001$).

The number of errors in the reversal learning assay significantly decreased across testing days (GLMM: $\chi^2_1 = 564.565$, $P < 0.001$), as expected due to subject's learning to handle the reversal learning task. Critically, the decrease in number of errors was steeper for the guppies of the unpredictable environment comparing to the guppies of the predictable environment (GLMM: $\chi^2_1 = 17.198$, $P < 0.001$; Figure 1b), suggesting greater cognitive flexibility in the guppies of the former treatment.

Unpredictable environment enhanced inhibitory control

27 out of 43 guppies attempted to capture the stimulus prey, on average within the third minute (±3.88, standard deviation) from the beginning of the test. The environment experienced by the subject did not affect whether (predictable environment: 16 out of 22 subjects; unpredictable environment: 11 out of 22 subjects; Fisher exact test: = 0.215) and when it approached the prey (two-samples t test: $t_{25} = 0.099$, $P = 0.922$; Figure 2a).

Overall, we observed 1661 attempts to capture the prey, with an average of 61.52 ± 63.39 (mean ± standard deviation) attempts per subject. The number of attempts was higher at the beginning of the experiment and then, decreased over testing time (GLMM: $\chi^2_1 = 111.241$, $P < 0.001$). Guppies from the predictable environment displayed a higher number of attempts (predictable environment: 74.69 ± 67.45; unpredictable environment: 42.36 ± 62.87; GLMM: $\chi^2_1 = 5.413$, $P = 0.020$; Figure 2b).

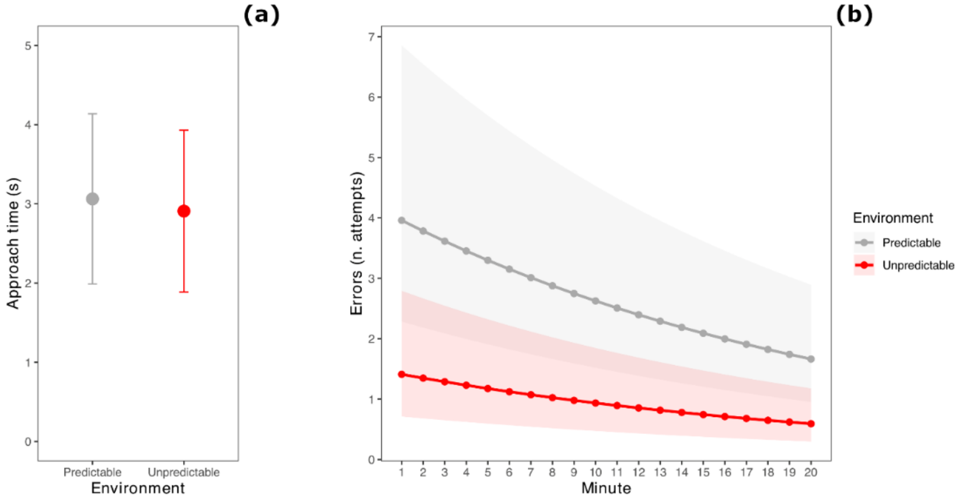

Figure 2. Results of the inhibitory control task. (a) Time taken by the subjects from the two treatments (predictable and unpredictable environment) to approach the stimulus prey; points and error bars represent means and standard errors, respectively. (b) Number of attempts to capture the prey performed by the subjects from the two treatments (predictable and unpredictable environment) in each minute of the test; points and shaded area represent mean and 95% confidence intervals estimated from the generalised linear mixed-effects model used in the analysis.

Level of predictability did not affect variance within experimental group

The performance variance observed within each of the two experimental groups of guppies was not significantly different in any of the cognitive tasks (Bartlett tests: number of errors in the learning assay: Bartlett's $K^2 = 0.273$, $P = 0.601$; number of errors in the cognitive flexibility assay: Bartlett's $K^2 = 0.033$, $P = 0.856$; number of number of attempts in the inhibitory control assay: Bartlett's $K^2 = 0.056$, $P = 0.813$).

Predictability affected guppies' activity but this did not explain cognitive plasticity

In the open-field test, the activity of the subjects decreased significantly across testing time (Linear Mixed-effects Model, LMM: $\chi^2_1 = 239.026$, $P < 0.001$). Moreover, guppies from the unpredictable environment showed greater activity (LMM: $\chi^2_1 = 4.547$, $P = 0.033$; Figure 3a). The time spent in the centre of the arena in the open-field test was significantly affected only by the experimental time (LMM: $\chi^2_1 = 7.055$, $P = 0.008$; Figure 3b). In the sociability test, none of the terms in the model, including the treatment, significantly explained the preference for the social stimulus (LMM: all P-values > 0.5; Figure 3c).

A regression analysis indicated that activity did not explain performance in the learning (linear regression: $t = -0.740$, $P = 0.463$, $R^2 = 0.012$), cognitive flexibility (linear regression: $t = -0.363$, $P = 0.718$, $R^2 = 0.003$), and inhibitory control task (linear regression: $t = 0.207$, $P = 0.838$, $R^2 = 0.002$). Therefore, the effects of environmental predictability in the cognitive tasks are not explained by the effect on behavioural activity detected in the open-field task.

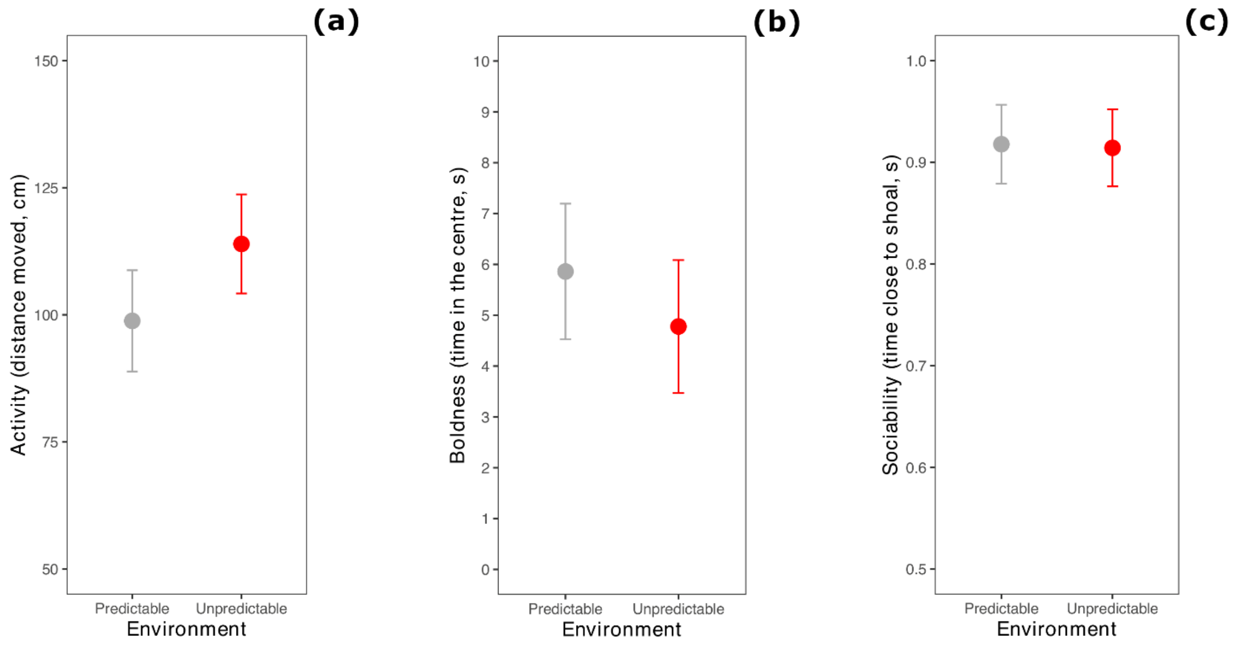

Figure 3. Results of the behavioural tests. (a) Activity measured as distance moved in the exploration test. (b) Boldness measured as time spent in the centre of the arena in the exploration test. (c) Sociability measured as time spent close to the social stimulus in the social behaviour test. In all the panels, points and error bars represent mean and 95% confidence intervals estimated from the linear mixed-effects models used in the analysis.

Discussion

Our study revealed that cognitive plasticity allows guppies to develop a cognitive phenotype that matches the level of resource predictability experienced in the environment, resulting in highly diversified individual variability. When food was predictably found in the same spatial location and at the same time of the day, guppies developed greater learning performance; when the location and timing of the food were unpredictable, guppies developed greater cognitive flexibility and greater inhibitory control.

We designed the study to experimentally investigate the effect of predictability and thus, we analysed populations of subjects exposed to very different levels of predictability. In nature, smaller fluctuations in levels of predictability experienced are likely to similarly determine plasticity-mediated cognitive variability between and within populations. Notably, the effect of predictability aligns with growing reports of cognitive plasticity in teleost fishes in response to other environmental factors (e.g., environment quality: Kotrschal & Taborsky, 2010; social environment: Lucon-Xiccato *et al.*, 2022b; enrichment: Montalbano *et al.*, 2022; predation risk: Villa Pouca *et al.*, 2021). All these forms of cognitive plasticity may interact in nature, thereby determining a broad spectrum of individual phenotypes. While cognitive plasticity has been studied also in other vertebrates, especially rodent models (e.g., Jankowsky *et al.*, 2005; Tang *et al.*, 2001), teleost's brain certainly displays a remarkable level of neural plasticity potential (reviewed in Ebbesson & Braithwaite, 2012), including extensive neurogenesis capacity in the adult (reviewed in Zupanc, 2006). Therefore, an interesting question for future research is whether cognitive plasticity determines individual differences in tetrapods with the same extent observed for teleost fish.

The plasticity due to levels of predictability has affected differently the three cognitive functions investigated in this study, aligning with the trade-off hypothesis proposed by Tellos-Ramos and colleagues (2019). This provides support to the idea that the observed cognitive plasticity is adaptive at least from two points of view. First, the trade-off hypothesis is based on the fact that enhancing functions such as learning and memory should be advantageous when the environmental conditions are predictable because they permit to rapidly and reliably exploit resources that are available consistently with the same spatial and/or temporal pattern. Conversely, executive functions such as cognitive flexibility and inhibitory control are involved in modifying individuals' behaviour (Diamond, 2013) and this should permit to rapidly adjust to resources that vary in space and time. Second, the function specificity of the observed cognitive plasticity further supports the idea of an adaptive mechanism that targets the functions more appropriate for each environmental condition. A more general, non-adaptive mechanism is indeed expected to determine wider and unidirectional changes (i.e., increase or decrease) in all the cognitive abilities of an individual, a scenario that markedly contrasts with what observed in our study. Function specificity has been also reported for other forms of cognitive plasticity: guppies raised in presence to biotic and abiotic stimuli developed greater learning ability compared to guppies raised in barren environments, but no differences were observed in inhibitory control and cognitive flexibility (Montalbano *et al.*, 2022). Many earlier investigations on plasticity and adaptive selection have focussed on general proxy of cognition, such as brain size (e.g., Burns *et al.*, 2009; Reyes *et al.*, 2022). Our findings suggest that a more precise approach that investigate cognition at the level of single functions is required to fully depict cognitive adaptation.

The unpredictable treatment also increased guppies' activity (but did not affect our measures of boldness and social behaviour). Variability in behavioural traits has been linked to cognitive variability (Carere & Locurto, 2011), including in our study species (Budaev & Zhuikov, 1998; Lucon-Xiccato & Dadda, 2017; Savaşçı *et al.*, 2021; Trompf & Brown, 2014). However, in our correlation analysis, the observed effect on activity did not emerge as a potential explanation for the differences observed in the cognitive tasks. Unpredictable environmental conditions are also known increase corticosteroids concentrations in all main vertebrate groups, including teleost fish (review by Wingfield & Ramenofsky, 1999) and these hormones may determine affect cognitive abilities in a range of tasks (Barreto *et al.*, 2006; Endo *et al.*, 1996; Saldanha *et al.*, 2000). Therefore, corticosteroids are proximate mechanisms worth investigating for the effect observed in guppies.

In conclusion, we demonstrated a form of cognitive plasticity driven by the levels of predictability of resources in the environment. The observed plasticity determined cognitive variability along a trade-off line between functions useful to learn and fixate a specific behaviour and functions that permit changes in behaviour. This highlights a previously unacknowledged role of cognitive plasticity in determining complex cognitive phenotypes. To fully understand cognitive variability in animals, research effort should be devoted to analyse other forms of cognitive plasticity and their interacting effects on individual's cognition. Altogether, multiple plasticity mechanisms might be responsible of very articulated individualities in cognitive abilities.

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Electronic supplementary materials S1

Supplementary methods

Experimental subjects

The guppies, Poecilia reticulata, used in the study belonged to a domestic strain named ‘cobra snake green’, maintained in our laboratory since 2012. Maintenance protocols followed typical laboratory standards. The guppies were kept in 400-L aquaria enriched with gravel bottom, aquatic plants, and water filters. Fluorescent lamps provided illumination with photoperiod 12: 12 h light: dark. By conditioning the facility, we kept the water in the aquaria at 27 ± 1 °C. Each aquarium contained approximatively 100 individuals with 50: 50 sex ratio. We fed the guppies twice per day with alternate live prey (*Artemia salina* nauplii) and commercial food flakes (Staple food Vipan, Sera, Heinsberg, Germany).

To obtain naïve experimental subjects, we removed juveniles from a set of maintenance aquaria containing several gravid females. Thereafter, we daily checked for new-born guppies in these aquaria. The new-born guppies collected were randomly assigned to the experimental treatments, with care to distribute equally fish from the same maintenance aquarium between the two conditions.

Details of the experimental treatments

The experimental aquaria (58.5 cm × 36.5 cm, h 30 cm) used for the predictability treatment were made of plastic to prevent the guppies to be exposed to external cues. Each aquarium housed six subjects. The bottom of the aquaria was provided with natural gravel. The water was constantly filtered and we performed a 50% water change each week. Other details, including temperature and the photoperiod, resembled those of the maintenance aquaria. At the four corners of the experimental aquaria, we built four separated chambers (8 × 8 cm) to delimit foraging areas for the subjects (Figure S1.1). Inside each foraging area, we placed a Petri dish (⌀ 6 cm). The guppies could access the foraging areas via an 8 × 8 cm hole.

To feed the guppies, the experimenter inserted a Pasteur plastic pipette in the predetermined feeding area, gently releasing a mixture of water and crumbled food flakes. The guppies were let to consume the food for 30 min, and then the experimenter removed the residual food from the Petri dish with a pipette. As a result of the Petri dish and the feeding areas, the food did not spread in the aquarium, ensuring localised foraging resources.

The predictability treatment lasted 20 days. Therefore, the subjects begun the assays before sexual maturation, which occurs around the age of three months in this species. Due to space constraint that limited the number of apparatuses in the laboratory, we assayed only four out of the six fish in each treatment aquarium.

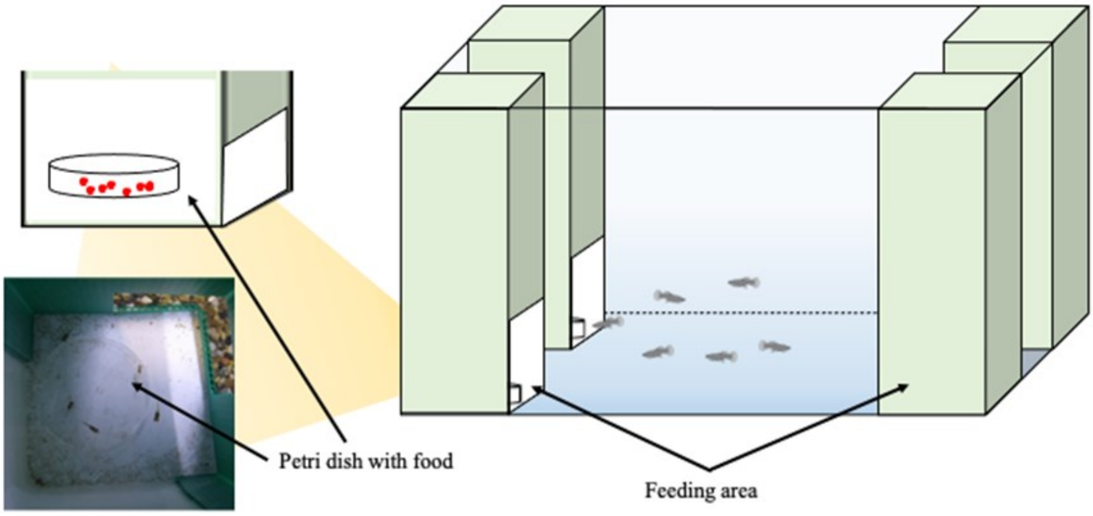

Figure S1.1. Aquarium used for the predictability treatment, details and lateral view.

Timeline of the assays

The order of presentation of the assays in the main text follows the logical order of the study. The assays were however performed with a different order, selected to avoid as much as possible carry over effects. In particular, we performed at first the assays that required shorter and fixed testing periods. The day after completing the treatment, we performed the exploration test and immediately after the sociability test. We then moved the subject into the aquaria for the inhibitory control assay, which we performed in the following four days. After the inhibitory control assay, the subjects were moved into the aquaria for the learning and the reversal learning assays. The length of these two assays was not fixed and varied according to the performance of each individual subject.

Apparatus and pre-test procedure of the learning assay

The experimental apparatus for the learning assay was built in a glass aquarium (25 cm × 40 cm, h 25 cm; Figure S1.2). The long walls of the aquarium were covered with a green plastic panel to resemble the colour of natural vegetation and the short walls of the aquarium with a white plastic panel to improve stimulus's visibility. The central corridor that divided the two main chambers was obtained with transparent plastics to allow the subject to see the stimuli from each position. The apparatus was enriched with natural gravel on the bottom and four social companions were housed in two lateral compartments located beside the central corridor (Figure S1.2). Illumination and temperature matched that of the maintenance and treatment condition. Outside the experimental trials, we also added an air stone in the apparatus. Water was refilled daily and the apparatus was cleaned when required but always outside the experimental trials.

The stimuli were white plastic cards (3 × 3cm) with a coloured circle (⌀ 1.8 cm) in the centre. To present the stimuli to the fish, we fixed each card to a transparent stick that could be attached to the short wall of the apparatus (Figure S1.2). The colour stimulus assigned to each subject was counterbalanced between the experimental groups.

The procedure of the learning task consisted of two phases: a pre-test phase and a test phase. During the first day of the pre-test phase, we moved the subject into the experimental apparatus and we waited until it resumed normal swimming behaviour. Thereafter, the experimenter gently inserted the card with the pre-assigned rewarded colour in correspondence of one of the short walls of the apparatus. We always presented the stimuli after ensuring that the subject was in the other chamber of the apparatus. The experimenter waited with the stimulus in the water until the fish approached it, and at that point, the experimenter administered a food reward consisting of nauplii of *A. salina* mixed with water. The food was released from a Pasteur pipette taking care to do not disturb the fish. As the approach, we considered when the subject swam at 0.5 body length or less from the stimulus, oriented toward the stimulus. If the subject did not approach the stimulus within 15 min, we considered the trial null and we repeated it after 10 min. We performed this procedure eight times, four in the morning and four in the afternoon. On the following day, we continued with the pre-test phase administering similar trials. However, in this second day, the experimenter simultaneously introduced both the stimuli in the apparatus, one on the left and one on the right of the chosen short wall. The left-right position of the stimuli was alternated according to a pseudo-random scheme. When the subject approached the correct stimulus, the experimenter released the food and removed the unrewarded stimulus. If the fish approached the unrewarded stimulus, the experimenter kept the stimuli in the apparatus until the subject performed a correct choice and obtained the food reward. If the fish did not perform the correct choice within 15 min, the experimenter removed the stimuli and repeated the trial after 10 min. In this second day of pre-test, we administered 12 trials (six in the morning and six in the afternoon).

The test phase, in which we scored the learning ability of the fish, commenced on the third day. The procedure of the test resembled that of the second day of the pre-test phase. The experimenter presented the two stimuli to the fish 12 times per day, alternating the side of the apparatus and the left-right position of the rewarded colour. The choice was scored as previously explained. However, if the subject chose the unrewarded card at first, the experimenter immediately removed both stimuli without administering the reward. Each day, the experimenter collected the number of correct choices and the number of wrong choices of each subject. When a subject made 17 correct choices out of 24 trials in two consecutive days, it was considered to pass the learning assay and the experiment was interrupted. The subjects that passed the criterion started the reversal learning assay for cognitive flexibility on the following day. The reversal learning assay was as the test phase of the learning phase, but the reward consistency was reverted between the two stimuli.

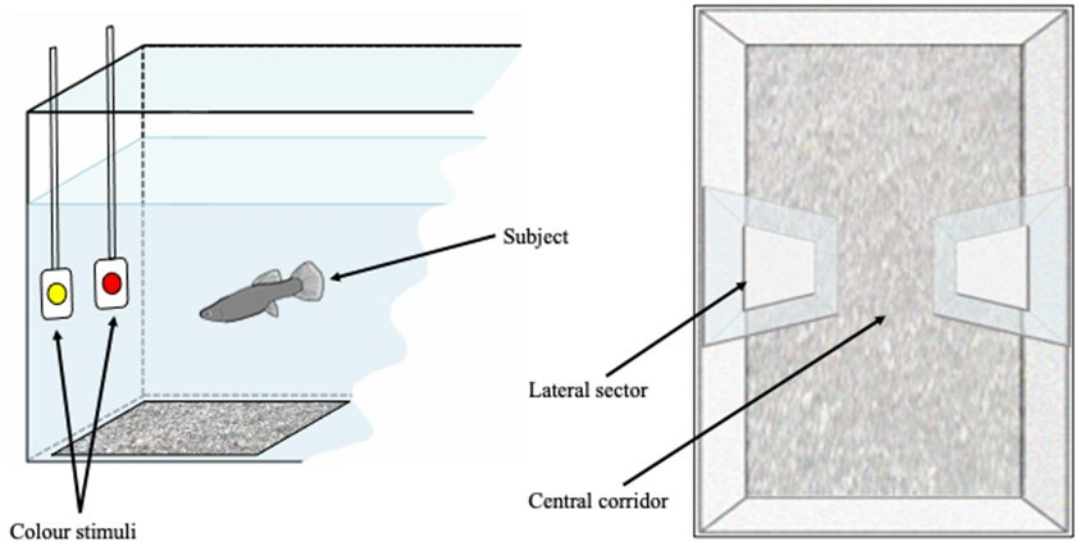

Figure S1.2. Experimental apparatus used for the learning and the cognitive flexibility assays, detail and top view.

Apparatus and pre-test procedure of the inhibitory control assay

The inhibitory control assay took place in 33 × 13 cm, h 15 cm, apparatuses (Figure S1.3). Each apparatus housed one subject. The apparatuses were built inside plastic aquaria with green walls. Each apparatus was provided with transparent lid with a hole (⌀ 1.2 cm) close to one of the short walls. An ELP 8MP Webcam was placed over the apparatus in proximity of the hole on the lid. Illumination and temperature were kept as described in the previous phases. Moreover, each apparatus contained an air stone that was removed before the experimental trials.

The experimental procedure was divided in a pre-test phase lasting three days and the test phase, which took place on the fourth day. During the pre-test phase, we fed the guppies with food flakes by inserting a Pasteur pipette into the hole of the lid. On the first day of the pre-test phase, we fed the fish twice; on the second day, four times; and on the third day, six times. To feed the fish, the experimenter first inserted the pipette in the water and then waited until the fish approached it. Only when the fish was close to the pipette, the experimenter released the food. This procedure motivated the subjects to rapidly investigate objects that entered the aquarium from the hole in the lid.

The day after completing the pre-test phase, the experimenter conducted the test phase in which we assayed the inhibitory control of the subject. To start the test, the experimenter introduced glass tube containing 4 mL of a solution of water and *A. salina* through the hole in the lid (Figure S1.3). In early experiments, we counted the number of prey contained in a tube under the microscope, finding that they were approximately 500 per tube. Because of the pre-test procedure, most of the subjects rapidly approached the tube. The tube was left in the water for 20 min. We scored the behaviour of the subjects from recordings obtained from the webcam. By playing back the recordings at reduced speed on a computer monitor, we counted the number of attempts (i.e., errors) made by subjects to capture the prey in each minute of the test. As an attempt, we considered each event in which the fish touched the glass of the tube with the snout. We additionally recorded in which minute after inserting the tube the subject performed the first capture attempt (approach time). Because of an issue with the recording software, we lost the recordings of five subjects.

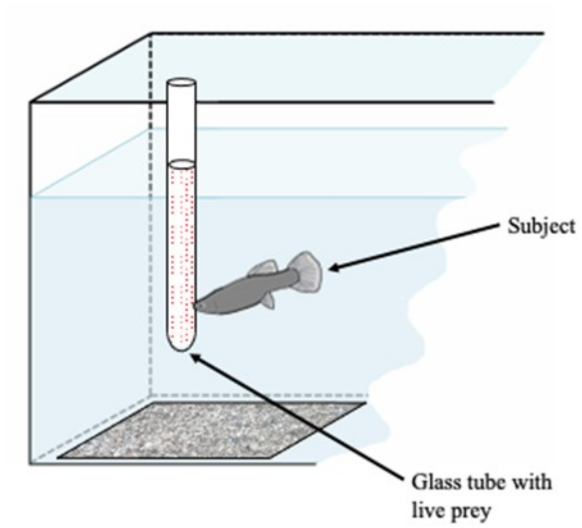

Figure S1.3. Detail of the apparatus used for the inhibitory control assay.

Apparatus and procedure of the exploration behaviour test

We measured exploration behaviour by exposing the subjects to an unfamiliar environment (open field arena; 20 × 20 cm, h 15 cm). The arena was made with white plastic panels and filled with 8 cm of water (Figure S1.4). Illumination was provided by a white LED strip placed 1 m above the arena. The temperature of the water was kept as in maintenance conditions (27 ± 1 °C). The arena was placed over a backlit IR LED table (λ > 980 nm). This, in conjunction with an infrared sensitive webcam (Monochrome GigE camera, Basler, Germany; resolution: 1280 × 1024), allowed us to track the behaviour of the subjects with an automatic system, i.e., a computer running Ethovision XT software (Noldus Information Technology).

To perform the test, the experimenter collected one subject and moved it in the centre of the arena. The subject was then left undisturbed for 20 min. The tracking software recorded the distance moved (i.e., activity) by the subject and time spent in the centre of the arena (i.e., boldness), at least 2 body lengths from the edges. The software was set to provide an output for each minute of the experiment separately. The subjects were tested sequentially, alternating between the two experimental treatments.

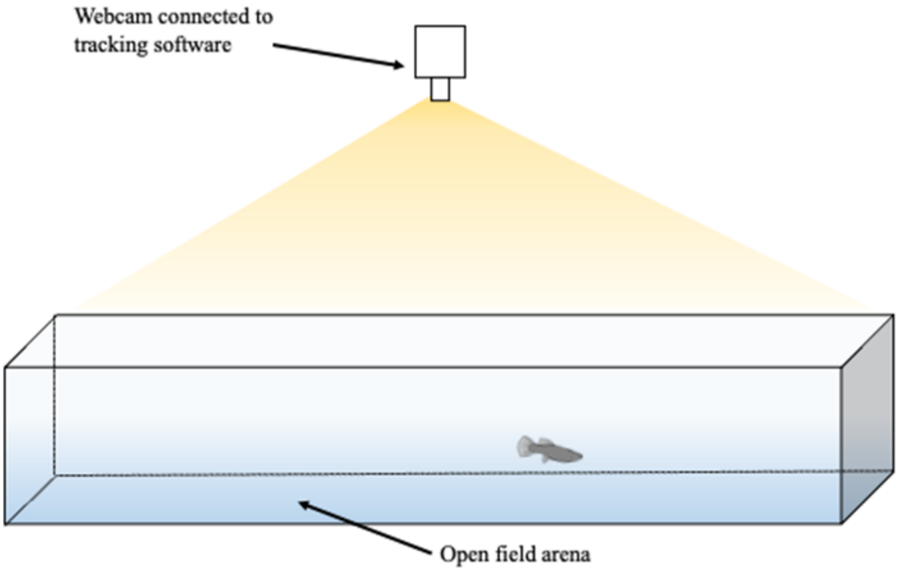

Figure S1.4. Diagram of the apparatus used in the exploration test.

Apparatus and procedure of the social behaviour test

In the social behaviour test, we investigated subjects' preference between a sector of the apparatus with a shoal of conspecifics and an empty sector. The experiment was conducted in a three-chamber apparatus (Figure S1.5) built inside a glass aquarium (60 × 40 cm, h 35 cm, with 15 cm of water). The walls and the bottom of the aquarium were covered with green plastic. The lateral chambers were separated from the central chamber with a wall made of transparent plastic. LED lamps placed over the lateral chamber ensured that they were highly visible for the subject in the central chamber. One of the lateral chambers was left empty during the experiment. The other chamber contained the social stimulus, i.e., a shoal of five guppies of approximately 3-4 weeks of age from the same population of the experimental subjects. The stimulus shoal was inserted in the apparatus at least one h before commencing the experiment for habituation. The left-right location of the stimulus shoal was alternated between subjects. A webcam was placed on the ceiling to record the central chamber of the apparatus.

The experimenter begun the test by introducing the subject in the central chamber and left it undisturbed for 20 min. The social behaviour was scored from the recordings played back on a computer monitor. An experimenter, blind with the respect to the identity of the fish in the recording, calculated the time spent close to the social stimulus (i.e., within 5 cm) and the time spent close to the empty chamber using the custom software CiclicTimer. These data were used to calculate an index of preference for the stimulus shoal as: time spent close to the shoal / (time spent close to the shoal + time spent close to the empty chamber). The index could have values ranging from 1 (high sociability) and 0 (low sociability).

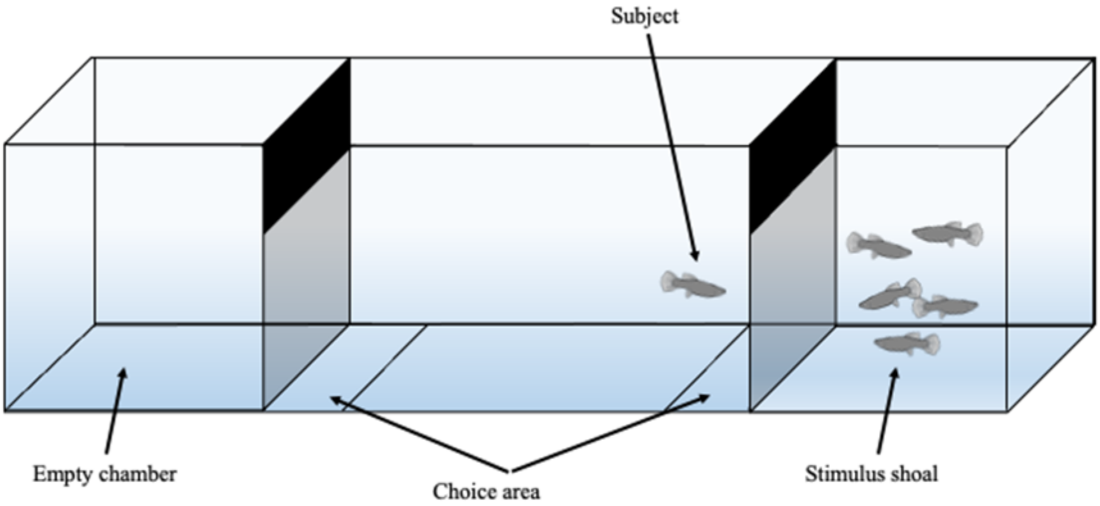

Figure S1.5. Diagram of the apparatus used for the social behaviour assay.

Statistical analysis

We analysed the data in R version 4.0.1 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). Significance level of the tests was set at P = 0.05. All statistical tests were two-tailed. Datasets were uploaded as electronic supplementary material S2.

The data of the learning and cognitive flexibility assays was analysed similarly. They consisted in multiple datapoints per each subject, one per each day of the experiment. The number of datapoints varied between subjects according to when they reached the criterion. Moreover, the data were not normally distributed because they consisted in a binary response variable, i.e., the number of errors and the number of correct responses recorded in each day. To handle data with this structure, we used Generalised Linear Mixed-effects Models (GLMMs) with binomial error distribution. The GLMMs were fitted with the glmer function of the lme4 R package and significance of the effects was tested with the Anova function of the car R package. All the GLMMs included subject ID as random effect to account for the repeated measurements. We initially fitted the models with predictability treatment, day of the experiment, and their interaction as fixed effects. We then dropped the non-significant terms before reporting the analysis in the main text. We also compared the number of days to the criterion between the learning and the cognitive flexibility assay using paired-samples t test.

For the inhibitory control assay, we first compared between the treatments the number of subjects that attempted to capture the prey at least once using Fisher's exact test. Then, we compared the approach time between experimental treatments using one-sample t test. The main data of the inhibitory control assay consisted in repeated counts of the attempts to capture the prey, one per each minute of the test. Data from the 16 subjects that did not attempt to capture the prey were removed before the analysis. We performed the analysis with a GLMM as described above for the learning and cognitive flexibility assays, but we used a Poisson error distribution given that the data were a count of events and we fitted minute of testing as the time variable. We further analysed the distribution of the cognitive data using Bartlett tests, looking for differences in variance between the experimental treatments.

The data of the behavioural experiments (exploration and sociability test) were analysed with a similar method. The depended variables were continuous measures: the distance moved, the time spent in the centre, and the preference for the social stimulus. They also had repeated measures structures because the data was collected for each minute of the experiment. We therefore used Linear Mixed-effects Models (LMMs), a version of GLMMs that is suitable for Gaussian distributed response variables. The models were fitted with predictability treatment and time of the experiment as fixed effects, and subject ID as random effect. Because one of the behavioural variables was affected by the treatment (activity as distance moved in the exploration test), we further analysed this variable to test whether it explained the effects observed in the cognitive assays. To do this, we performed three regression analysis between the activity (independent variable) and the measures of performance in the cognitive tasks (dependent variables).

Animal welfare note

The experiments complied with "Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes" and with Italian law "D. Lgs n. 26 4 marzo 2014 Attuazione della direttiva 2010/63/UE sulla protezione degli animali utilizzati a fini scientifici". The procedures were designed following the ASAB/ABS Guidelines for the Use of Animals in Research (https://doi.org/10.1016/j.anbehav.2019.11.002) and were approved by the ethical committee (OPBA) of University of Ferrara (permit TLX-2022-1).

4. Social environment affects inhibitory control via developmental plasticity in a fish

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Abstract

Living in a social group may impose cognitive demands, for example individual recognition, social memory, and the inhibition of behaviour when it is not adaptive. As the neural substrates for these cognitive skills are metabolically expensive, cognitive abilities may be positively related to the complexity of the social system. Where there is large spatio-temporal variation in the ecological conditions experienced and hence in the social system exhibited by species, selection may favour adaptive phenotypic plasticity of cognitive abilities involved in social tasks rather than evolved differences across populations. Here, we tested this hypothesis in a social living teleost fish, the guppy, *Poecilia reticulata*. We exposed new-born guppies to treatments that altered two parameters of social environmental complexity: group size (experiment 1) and group stability (experiment 2). Then, we assessed guppies' inhibitory control, the ability to withhold responding to a stimulus, a cognitive function that is critically involved in social interactions. In experiment 1, guppies reared alone showed higher levels of behavioural inhibition in a foraging task compared to guppies reared in pairs or in groups of 6 individuals. In addition, we found that individuals' variance in performance was smaller for fish raised as singletons. In experiment 2, guppies reared in a stable social group showed greater inhibition compared to guppies from groups subjected to frequent fission-fusion events. These results reveal phenotypic plasticity of inhibitory control in guppies, however, contrary to prediction, indicate greater inhibitory abilities developing in individuals exposed to 'simpler' social environments.

Keywords: executive functions; cognition; individual differences; *Poecilia reticulata*; social brain.

Introduction

The survival and reproduction of a social animal strongly depends on its ability to succeed in social interactions, which necessitates advanced cognitive skills to generate behavioural responses that are adapted to the social context (Fernald, 2017). Inhibitory control is one of the skills considered critically important to optimise social interactions (Amici *et al.*, 2008; Santos *et al.*, 1999), allowing the animal to withhold a behaviour when it is not adapted to the context (Diamond, 2013). For example, in several social species, subordinate individuals inhibit feeding or mating in presence of dominant individuals to avoid aggression (Byrne & Whiten, 1992; Estep *et al.*, 1988; Lindsay *et al.*, 1976).

As developing the neural substrates for cognition is energetically demanding (Kool & Botvinick, 2013; Kotrschal *et al.*, 2013), the evolution of cognitive skills involved in social tasks is expected to be strongly tied to levels of social complexity (the social brain hypothesis; Dunbar *et al.*, 1998). According to this hypothesis, inhibitory control should be enhanced in those species in which social relationships are, on average, more complex (Byrne & Bates, 2007; Bond *et al.*, 2003; Dumbar & Shultz, 2007; Kamil, 2004; Jolly, 1966). A comparative study on seven primates supported this prediction, finding that inhibitory control is enhanced in species with higher levels of fission-fusion dynamics (Amici *et al.*, 2008). Conversely, a subsequent study reported no effect of group size on inhibitory control in 23 primate species (MacLean *et al.*, 2014).

However, the social system experienced by members of the same species often varies widely as a result of spatio-temporal fluctuations in environmental conditions (Creel & Winnie, 2005; Crockett & Eisenberg, 1987; Edenbrow *et al.*, 2011; Foster *et al.*, 2012; Jo Safran *et al.*, 2004; Rushmore *et al.*, 2013; Seghers & Magurran, 1994). In addition, a growing literature suggests the presence of adaptive phenotypic plasticity in cognitive abilities in response to individuals' experiences during early life (Chivers *et al.*, 2016; Fond *et al.*, 2019; Kotrschal & Taborsky, 2010; Lucon-Xiccato *et al.*, 2016; Lucon-Xiccato *et al.*, 2020a; Salvanes *et al.*, 2013). An individuals' inhibitory control may therefore vary depending on the social environment experienced by an individual, perhaps especially early in life. Such adaptive phenotypic plasticity of inhibitory control would allow matching of the costs of the required neural substrates with the cognitive demands of the specific social environmental experienced by an individual when populations are exposed to changing socio-ecological conditions across generations. A study on a mammal, the spotted hyaenas *Crocuta Crocuta* (Johnson-Ulrich, *et al.*, 2020) and a study on a bird, the Australian magpie *Gymnorhina tibicen* (Asthon *et al.*, 2018) have provided some experimental support to the link between group size and inhibitory control at the intraspecific level. However, there has not yet been any investigation of the link between social complexity and inhibitory control within species in poikilothermic vertebrate taxa.

We tested the presence of a socially-mediated plasticity in inhibitory control in the guppy fish, *Poecilia reticulata*. In this species, several studies have reported refined inhibitory abilities (Lucon-Xiccato *et al.*, 2017; Santacà *et al.*, 2019) and remarkable intraspecific variability in cognition (reviewed in Lucon-Xiccato & Bisazza, 2017), including in inhibitory control (Macario *et al.*, 2021; Savaşçı *et al.*, 2021). Furthermore, variation in social environment has been consistently reported in natural guppy populations (Darden *et al.*, 2020; Edenbrow *et al.*, 2011; Magurran & Seghers, 1990, Seghers & Magurran, 1991; Seghers & Magurran, 1991). We analysed two parameters that are important determinants of social environment complexity, group size and group stability (Amici *et al.*, 2008; MacLean *et al.*, 2014). In our first experiment, we manipulated the group size experienced by guppies by maintaining subjects either alone, in pairs, or in groups of 6 individuals from their first day of life. In our second experiment, we manipulated group stability. We simulated fission-fusion events experienced by guppies in the wild (Croft *et al.*, 2003; Wilson *et al.*, 2014) and manipulated the exposure to these events by either keeping constant social groups or by regularly altering group membership. Following these social treatments, we measured subjects' inhibitory control. We expected that individuals' inhibitory control will be enhanced in experimental populations with larger social groups and the occurrence of frequent fission-fusion events (Amici *et al.*, 2008; MacLean *et al.*, 2014). Larger groups involve a greater number and diversity of social relationships, and hence maintaining a position in the hierarchy should require greater ability to inhibit behavioural responses. Populations with many fission-fusion events should be characterised by continuous changes in social networks and dominance hierarchies, requiring greater inhibition by the individuals to cope with an uncertain social environment.

Materials and methods

Subjects

We used guppies from a population of the snake cobra green strain, maintained in our laboratory since 2012. These guppies were chosen because they adapt quickly to training procedures involving interactions with human experimenters (Mair *et al.*, 2021; Montalbano *et al.*, 2020). The population's minimum size was approximately 1000 individuals. To reduce the chances of inbreeding, we moved guppies between multiple maintenance aquaria and we added new guppies once or twice per year. Mixed-sex groups of guppies were maintained in 200 L plastic aquaria, enriched with gravel and natural plants to simulate natural habitat. Aquaria were provided with air pumps, filters, and heaters (27°C ± 1°C) to ensure stable water conditions. Fluorescent lamps provided illumination with a light/dark cycle of 12h:12h. We fed the guppies twice per day with live *Artemia salina* and crumbled commercial flake food. In the current study, we used 144 guppies (72 in each experiment). They were collected in their first day of life as juvenile guppies begin to engage in social interactions approximately 72h after birth (Gorlick, 1976). This was done by isolating pregnant females and inspecting for the presence of offspring daily.

Social environment treatments

In experiment 1, we manipulated the size of the social group (1, 2, or 6 fish; figure 1a). Social group sizes used in the treatment have been reported in guppies' natural populations (Croft *et al.*, 2003). We randomly allocated 72 guppies to the 3 treatments: 6 groups of 6 subjects were maintained in 6-L aquaria; 12 groups of 2 subjects in 2-L aquaria; and 12 individual guppies in 1-L aquaria so that the density of individuals per water volume was equal in the 3 treatments. The treatment aquaria were enriched with gravel, natural and artificial plants, and an aerator. The treatments were maintained for 6 weeks and we changed 50% of the water from each aquarium with clean, conditioned and temperature matched water three times per week.

In experiment 2, we manipulated the stability of the social group (stable and unstable; figure 1b) using a resampling procedure to mimic a fission-fusion society, which is commonly reported in wild guppies (Auge *et al.*, 2016; Croft *et al.*, 2003; Wilson *et al.*, 2014). We randomly allocated 72 guppies to the 2 treatments. For the variable social group treatment, 6 groups of 6 subjects were maintained in 6 L aquaria as described for experiment 1. Three times per week, these 36 guppies were moved into a 20 L aquarium and immediately resampled to form 6 novel groups. For the stable social group treatment, 6 groups of 6 subjects were maintained in 6 L aquaria. Three times per week, each of these 6 groups was moved one at a time into a 20 L aquarium for 5 minutes and successively placed back into a new 6 L aquarium. This pseudo-resampling treatment controlled for the disruption of moving between aquaria, but maintained the membership of the groups throughout the treatment phase. The treatment phase lasted for 6 weeks and hence each group underwent 18 resampling/pseudo-resampling events. During the resampling/pseudo-resampling procedure, we performed a 50% water change in each aquarium.

Foraging inhibition task

After six weeks in the social treatments, each guppy was tested individually in a foraging inhibition task (figure 1c). Individual testing was necessary to assess individual differences in cognition. Guppies cannot be reliably sexed at this age and therefore our sample was comprised of mixed sex subadults. In the foraging inhibition task, we followed the paradigm developed for this species and other small teleost fish (Lucon-Xiccato *et al.*, 2020b; Lucon-Xiccato & Bertolucci, 2020). It relies on measuring the ability to inhibit foraging behaviour towards a visible prey that cannot be reached because of a transparent barrier. Individuals with higher inhibitory abilities are expected to attempt to attack the prey less often, after experiencing the transparent barrier. This paradigm has been shown to be successful in detecting significant individual differences and it correlates with other measures of inhibitory control (Lucon-Xiccato *et al.*, 2020b; Lucon-Xiccato *et al.*, 2020c; Montalbano *et al.*, 2020).

Initially, we moved each individual into an experimental aquarium filled with 4 L of water. By using multiple aquaria, we tested all guppies from an experiment at the same time. The experimental aquaria had green plastic walls and a transparent lid. Near the front of the lid (figure 1c), there was a circular opening (1.2 cm ∅) that allowed for feeding the subject and performing the experimental procedure. For 3 days (training phase), we fed the guppies with crumbled food mixed with water by inserting a Pasteur pipette through the opening. We took care to release the food only when the guppies showed interest toward the pipette. This habituated the guppies to receiving food in association with the opening in the lid. We performed this feeding procedure 2, 4, and

6 times on days 1, 2, and 3 of the training phase, respectively. All the fish promptly learned this feeding procedure.

On the day following the third day of training, we performed the foraging inhibition test. We inserted a transparent glass tube (length: 10 cm; Ø: 1.2 cm) into the experimental aquaria, through the opening in the lid. The tube contained 4 mL of a solution with live prey, *A. salina* (N = approximately 500). We video recorded the response of the subject for 10 minutes using a webcam (ELP 5-50 mm 8MP) placed 1 m above the experimental aquaria. From the video recordings played back on a computer monitor, we recorded the minute from the beginning of the experiment in which each subject attempted to attack the prey for the first time (latency). This variable was considered as a proxy of feeding/foraging motivation in the analyses assuming that more motivated individuals would approach the prey faster. We then scored the number of attacks performed by each subject for 10 consecutive minutes after the first approach to the live prey. This analysis allowed us to standardise the data for differences in motivation or other factors that could cause the subjects to initially approach the prey with different latency. In addition, the fact that the trial started from the first attack for each subject was useful for examining differences in inhibition rate between subjects and treatments: two subjects displaying a different number of attacks in a given minute implies that one of them was faster at inhibiting predatory behaviour after discovering that the prey was inaccessible. Attacks were counted when a guppy touched the glass tube with its snout. Because the fish could not capture the prey through the tube, they were expected to inhibit the attack behaviour. Individuals with higher inhibitory control were expected to perform fewer attacks. Prior experiments have highlighted that non-cognitive factors, such as neophilia/exploration and motivation did not affect this measure of performance (Lucon-Xiccato & Bertolucci, 2019). Fish that did not perform any attacks within 10 minutes were excluded from further analysis. The final sample size was as follows: experiment 1: 1 fish treatment $N = 11$, 2 fish treatment $N = 16$, 6 fish treatment $N = 27$; experiment 2: variable social group treatment $N = 20$, stable social group $N = 22$.

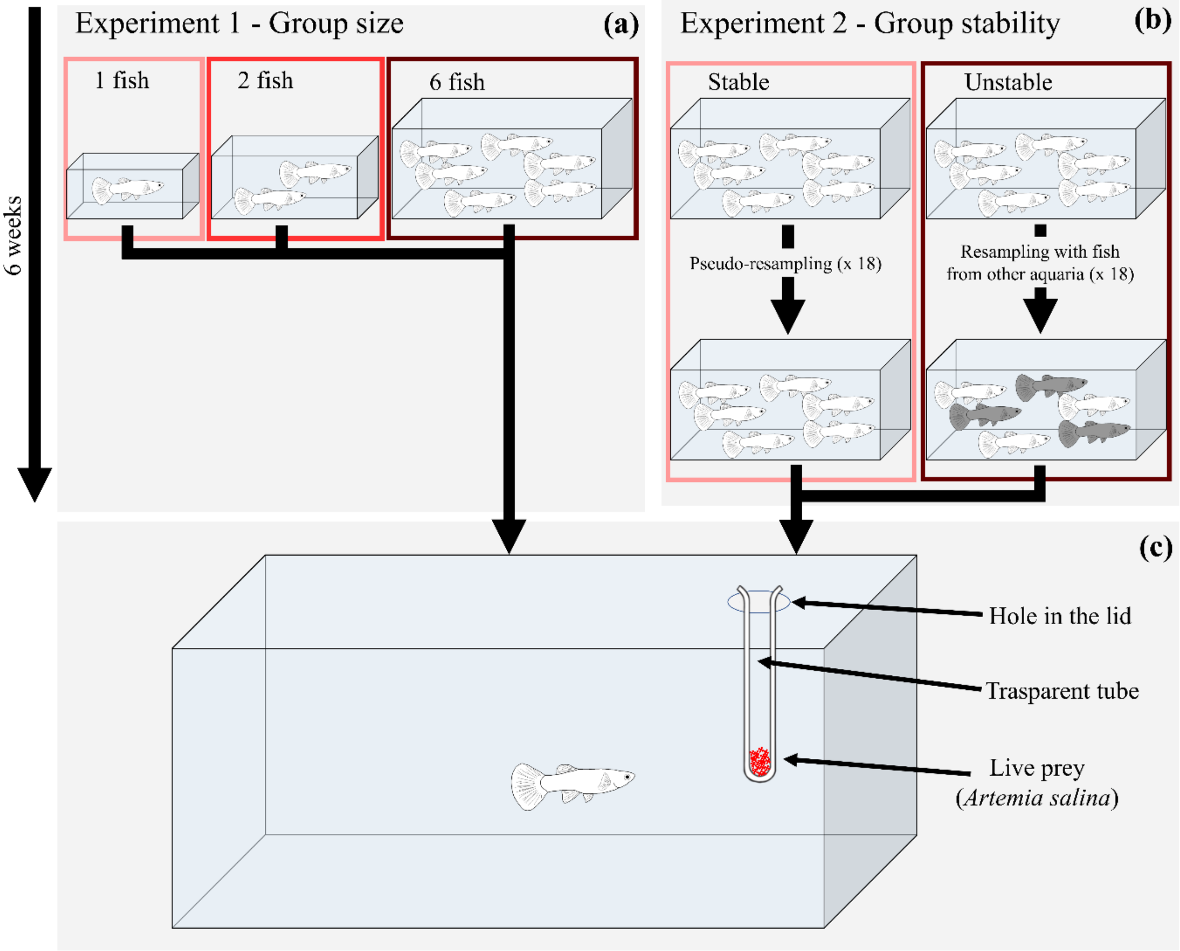

Figure 1. Overview of the experimental methods. (a) Scheme of experiment 1's treatment; (b) scheme of experiment 2's treatment; and (c) lateral view of the apparatus used for the inhibition task.

Statistical analysis

We used R Statistical software version 4.0.1 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). All statistical tests were two-tailed and significance was set at $\alpha = 0.05$ unless stated otherwise. First, we compared the measure of motivation (latency with which the fish firstly attempted to reach the prey) across the treatments of the two experiments. Given a non-normal distribution, we used non parametric tests (Wilcoxon test for experiment 1 and Kruskal-Wallis test for experiment 2). Then, we analysed the main dependent variable obtained for each fish, i.e., the number of attacks towards the stimulus prey in 10 1-min time bins. The use of time bins allows to compare inhibition rate across treatments (Lucon-Xiccato & Bertolucci, 2019; Lucon-Xiccato & Bertolucci, 2020). To account for the Poisson distribution and the repeated measures structure of this depended variable, we analysed it with generalised linear mixed-effects models with Poisson error distribution (GLMMs) fitted with the *glmer* function of the *lme4* R package (Bates *et al.*, 2007). We included social environment treatment and time (minutes from 1 to 10) as fixed effects, and subject ID as random effect in the models. The effect of time is difficult to model because its residual plotting suggested a non-linear effect on the dependent variable. This makes the data partially violating assumptions for covariance analysis (Ernst & Albers, 2017; Nimon, 2012). In addition, the experimental design based on recording the dependent variable in 1-minute bins makes the time variable *de facto* a non-continuous variable, which is also not ideal for covariance analysis. An alternative analysis approach would be fitting time as categorical factor; this approach is expected to achieve increased power in the comparison between the experimental groups in a specific experimental period but might suffer limits in considering the order of the time series. As each of the two aforementioned approaches has inferential advantages and disadvantages, we ran each model twice, first fitting time in 1-min bins as a categorical factor and then again fitting time as a continuous covariate. In experiment 1, the social environment treatment was treated as a fixed factor with 3 levels (social group size: 1 fish, 2 fish, or 6 fish). In experiment 2, the social environment treatment was treated as a fixed factor with 2 levels (social group stability: variable or stable). When significant interactions between time and social treatment were identified, we fit post-hoc models on each minute of the experiment separately using generalised linear models (GLMs) with Poisson error distribution and an α level corrected with the Bonferroni method. The post-hoc models were critical to assess differences in inhibition between the experimental groups. The number of attacks per minute was counted from the first attempt to attack the prey; therefore, differences in the number of attacks in a minute indicated that one group of subjects have a faster decrease in attack rate, which is indicative of greater inhibitory abilities. In experiment 1, to further investigate the differences between the 3 levels of the social environment treatment, we used Tukey post-hoc tests. Last, we compared variance of the overall inhibitory performance (sum of the 10 min bins) across the experimental groups of the two experiments using Bartlett test. We log transformed the data to account for the Poisson distribution. For Poisson data (range > 0) variance is expected to increase with sample size. In experiment 1, the sample size of the three experimental groups varied (range 11-27), which could artifactually lead to differences in variance. We therefore ran a simulation analysis that compared same-sized groups ($N = 11$) obtained from 10000 iterations of a resampling procedure for each experimental treatment group in experiment 1. For each simulated dataset, we ran a Bartlett test to compare the variances of the simulated groups and calculated an overall *P*-value supporting the presence of differences in group variances that are independent of sample size (P_{sim} = N non-significant P-values obtained in the 10000 simulations / 10000).

Animal welfare

Experiments were conducted in accordance with the ABS/ASAB 'Guidelines for the treatment of animals in behavioural research and teaching' (doi: 10.1016/j.anbehav.2019.11.002) and the law of the country in which they were performed (Italy, D.L. 4 Marzo 2014, n. 26). The Ethical Committee of University of Ferrara reviewed and approved the experimental procedures (protocol n. TLX 2-2018-PR).

Results

Experiment 1 – Group size

Analysis on the latency to attack the prey did not reveal differences between the two experimental treatments (*Kruskal-Wallis* $\chi^2_2 = 0.383$, $P = 0.826$; single fish treatment, mean ± standard deviation: 1.36 ± 1.21; 2 fish treatment: 1.38 ± 1.02; 6 fish treatment: 1.89 ± 2.49).

The GLMM on the number of attacks revealed a significant interaction between group size treatment and time ($\chi^2_{18} = 212.756$, $P < 0.0001$; figure 2). The interaction was significant also in the model fitted with time as continuous covariate ($\chi^2_2 = 249.796$, $P < 0.0001$). The main effect of time was also significant ($\chi^2_9 = 1979.135$, $P < 0.0001$) but the main effect of group size treatment was not ($\chi^2_2 = 2.121$, $P = 0.346$).

The significant interaction was further investigated by testing for the effect of treatment in each minute of the experiment separately with GLMs. The GLMs showed significant effect of group size treatment in minutes 1, 2, 3, 6, 7, 8, 9, and 10, but not in minutes 4 and 5 (table 1). Tukey's post-hoc tests on the GLMs with a significant effect of group size treatment indicated that the subjects from the single fish treatment exhibited lower number of attacks especially in the second half of the experiment (figure 2; table 1).

Analysis of individual variance in the number of attacks revealed a significant difference between group size treatments (*Bartlett's* $K^2{}_2 = 9.647$, $P = 0.008$; figure 3), mostly due to the single fish treatment exhibiting lower performance variance (0.35) compared to the 2-fish treatment (2.3) and the 6-fish treatment (2.52). The simulation analysis suggested that this effect was not due to the difference in sample size between groups ($P_{sim} = 0.012$).

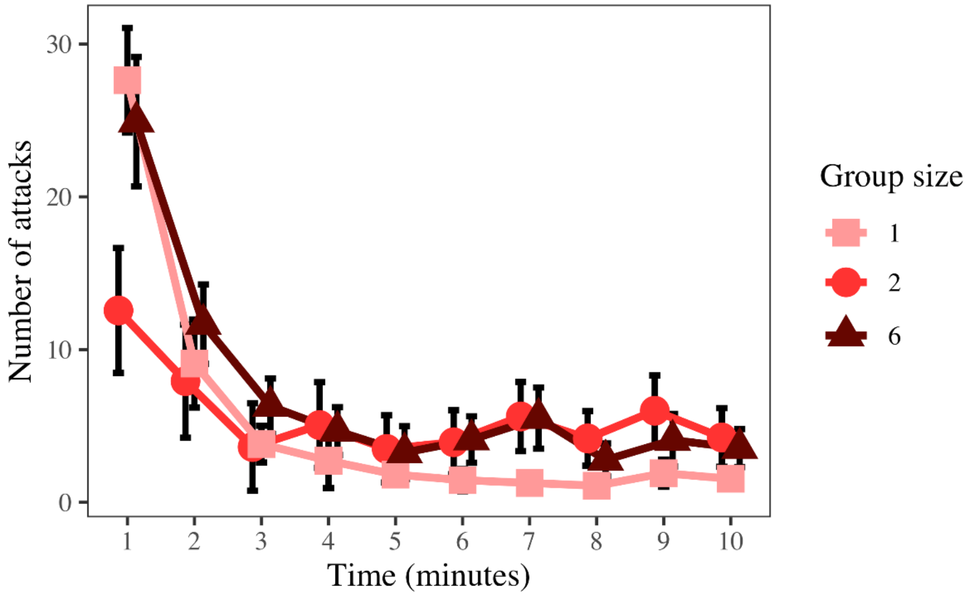

Figure 2. *Effects of group size on inhibitory control (experiment 1). Points represent mean number of attacks towards the prey and error bars represent SEM.*

Table 1. Post hoc analysis of the effect of group size treatment on inhibitory control (experiment 1)

Minute	GLM	Tuckey's post-hoc test
1	**$\chi^2_2 = 101.410$, $P < 0.0001$**	**Single vs. Pair: $P < 0.0001$** Single vs. 6 fish: $P = 0.290$ **Pair vs. 6 fish: $P < 0.0001$**
2	**$\chi^2_2 = 15.375$, $P = 0.0005$**	Single vs. Pair: $P = 0.564$ Single vs. 6 fish: $P = 0.075$ **Pair vs. 6 fish: $P < 0.001$**
3	**$\chi^2_2 = 18.423$, $P < 0.0001$**	Single vs. Pair: $P = 0.964$ **Single vs. 6 fish: $P = 0.010$** **Pair vs. 6 fish: $P < 0.001$**
4	$\chi^2_2 = 10.050$, $P = 0.006$	-
5	$\chi^2_2 = 7.798$, $P = 0.021$	-
6	**$\chi^2_2 = 20.464$, $P < 0.0001$**	**Single vs. Pair: $P = 0.001$** **Single vs. 6 fish: $P < 0.001$** Pair vs. 6 fish: $P = 0.958$
7	**$\chi^2_2 = 45.200$, $P < 0.0001$**	**Single vs. Pair: $P < 0.0001$** **Single vs. 6 fish: $P < 0.0001$** Pair vs. 6 fish: $P = 0.988$
8	**$\chi^2_2 = 24.464$, $P < 0.0001$**	**Single vs. Pair: $P < 0.0001$** **Single vs. 6 fish: $P < 0.0001$** Pair vs. 6 fish: $P = 0.988$
9	**$\chi^2_2 = 28.269$, $P < 0.0001$**	**Single vs. Pair: $P < 0.001$** **Single vs. 6 fish: $P = 0.036$** **Pair vs. 6 fish: $P = 0.007$**
10	**$\chi^2_2 = 17.292$, $P = 0.0002$**	**Single vs. Pair: $P < 0.001$** **Single vs. 6 fish: $P = 0.004$** Pair vs. 6 fish: $P = 0.489$

In the GLMs, a level for significance was corrected with the Bonferroni method (P=0.005). Bold indicates significant results.

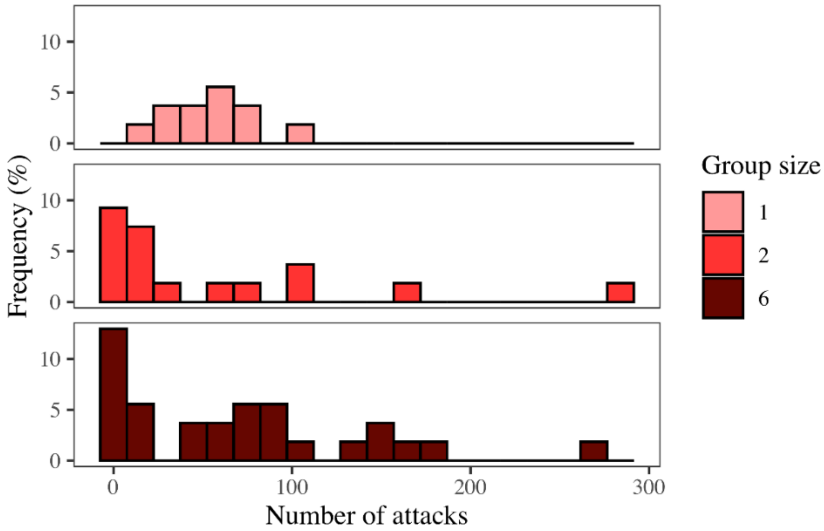

Figure 3. *Variability of individual differences in inhibitory control due to group size (experiment 1). Frequency distribution plot of number of attacks towards the prey performed by guppies throughout the test. (a) Single fish, (b) two fish and (c) six fish.*

Experiment 2 – Group stability

Analysis on the latency to attack the prey did not find differences between the two experimental treatments (*Wilcoxon* $W = 198$, $P = 0.535$; stable social group, mean ± standard deviation: 3.05 ± 2.95; unstable social group: 2.35 ± 2.45).

The GLMM on the number of attacks revealed a significant interaction between group stability treatment and time ($\chi^2_9 = 19.7499$, $P = 0.020$; figure 4). The interaction was marginally significant in the model fitted with time as covariate ($\chi^2_1 = 3.129$, $P = 0.077$). The main effect of time was also significant ($\chi^2_9 = 408.919$, $P < 0.0001$) but the main effect of group stability treatment was not ($\chi^2_1 = 1.945$, $P = 0.163$). The significant interaction was further investigated by testing for the effect of treatment in each minute of the experiment separately with GLMs. The GLMs showed that the subjects from the stable social group treatment performed a lower number of attacks in minutes 1 to 8 (table 2). This difference was not present in the last minutes of the task (9 and 10; table 2), suggesting an effect of habituation and/or learning.

Analysis of individuals' variance in the number of attacks indicated no significant difference between group stability treatments (*Bartlett's* $K^2_1 = 0.039$, $P = 0.843$; stable social group variance = 1.95; stable social group variance = 1.78).

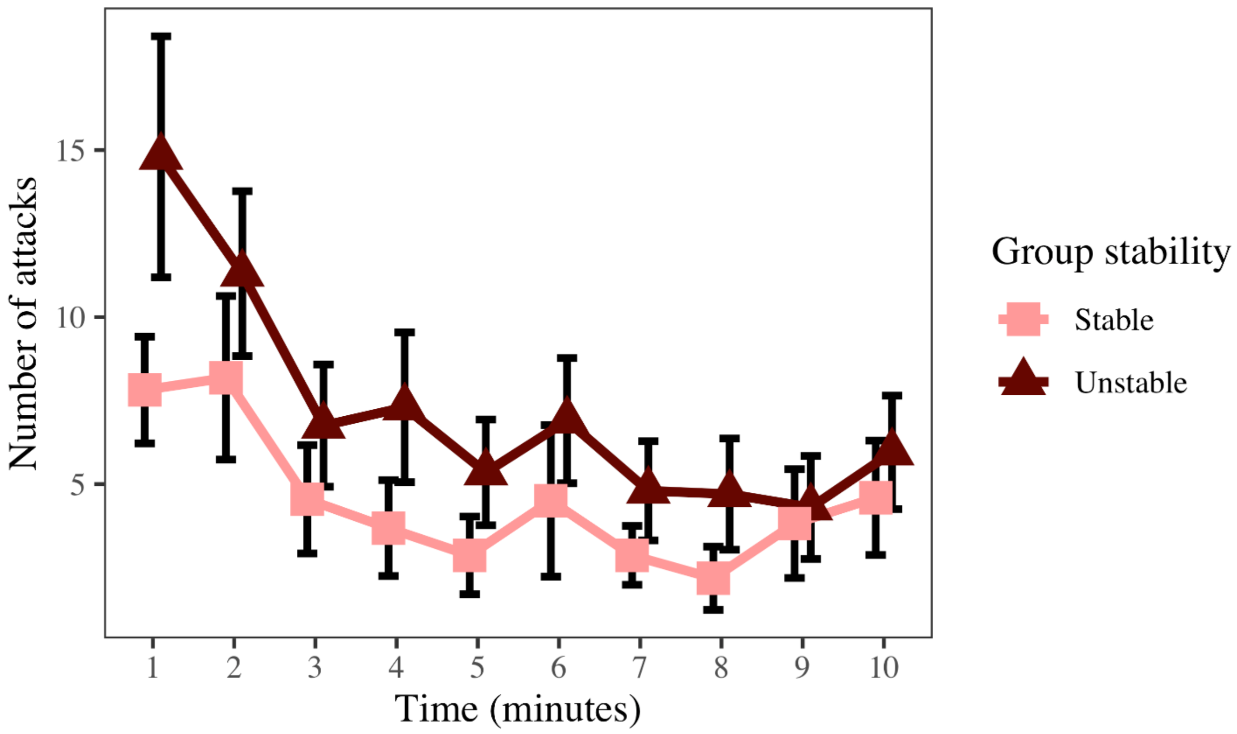

Figure 4. *Effects of group stability on inhibitory control (experiment 2). Points represent mean number of attacks towards the prey and error bars represent SEM.*

Table 2. Post-hoc analysis of the effect of group stability treatment on inhibitory control (experiment 2).

Minute	GLM
1	$\boldsymbol{\chi^2{}_1 = 46.131, P < 0.0001}$
2	$\boldsymbol{\chi^2{}_1 = 10.259, P = 0.001}$
3	$\boldsymbol{\chi^2{}_1 = 9.102, P = 0.003}$
4	$\boldsymbol{\chi^2{}_1 = 25.586, P < 0.0001}$
5	$\boldsymbol{\chi^2{}_1 = 16.099, P < 0.0001}$
6	$\boldsymbol{\chi^2{}_1 = 10.720, P = 0.001}$
7	$\boldsymbol{\chi^2{}_1 = 10.405, P = 0.001}$
8	$\boldsymbol{\chi^2{}_1 = 19.880, P < 0.0001}$
9	$\chi^2{}_1 = 0.600, P = 0.439$
10	$\chi^2{}_1 =3.689, P = 0.055$

In the GLMs, α level for significance was corrected with Bonferroni method (P = 0.005). Bold indicates significant results.

Discussion

The requirements for metabolically expensive cognitive functions due to group living (Kool & Botvinick, 2013; Kotrschal *et al.*, 2013) and the high levels of spatio-temporal variation in the social system experienced by many species (Creel & Winnie, 2005; Foster *et al.*, 2012; Rushmore *et al.*, 2013; Seghers & Magurran, 1994) suggest that social animals might exhibit adaptive phenotypic plasticity of cognitive abilities involved in social tasks (Asthon *et al.*, 2018; Johnson-Ulrich, *et al.*, 2020). Here, we reported evidence of such phenotypic plasticity for inhibitory control in a teleost fish, the guppy, *P. reticulata*. For half of the testing phase in experiment 1, the guppies reared alone exhibited a lower number of attacks towards the inaccessible prey. This effect indicates that guppies reared alone decreased the number of attacks after experiencing that the prey could not be reached more rapidly than did guppies reared in groups. Therefore, is possible to conclude that guppies reared alone exerted greater levels of behavioural inhibition. In experiment 2, the guppies reared in a stable social group consistently showed a reduced number of predation attempts, and therefore greater inhibitory control, compared to the guppies reared in groups with changing composition simulating a high turnover fission-fusion society, commonly observed in guppies from some natural populations (Croft *et al.*, 2003; Wilson *et al.*, 2014). Overall, these results suggest that simpler social environments characterised by small and stable social groups may favour the development of greater inhibitory control in guppies.

Interestingly, our results on the plastic response of the guppy to social experiences early in life do not fit with the logic of the social brain hypothesis which predicts that more complex social environments should be linked to greater cognitive abilities on evolutionary time scales (Dunbar, 1998). Empirical support for the social brain hypothesis has been mostly drawn from primates (e.g., Amici *et al.*, 2008; MacLean *et al.*, 2014), but it is also provided by studies in other mammals such as ungulates and carnivores (Dunbar & Shultz, 2007) and in some bird taxa (Ashton *et al.*, 2018; Emery *et al.*, 2007). However, evidence also indicates that this evolutionary hypothesis might not consistently apply across all taxa (e.g., Shultz & Dunbar, 2007). The specific ecological, life history, and social characteristics of a given species or taxon may affect the relationship between cognition and social system (Thornton & McAuliffe, 2015). Guppies show remarkable social sophistication, including tit-for-tat cooperation strategies, complex social networks and durable social relationships (Cattelan *et al.*, 2019; Croft *et al.*, 2006; Dugatkin, 1988), however, it is undeniable that the breadth and depth of their social behaviour repertoire is limited compared to that of higher vertebrates. For instance, highly structured primate societies with strong hierarchies and long-term relationships likely create a situation wherein an individuals' behaviours have long term consequences, providing selective pressures for heightened inhibitory control. Conversely, in guppy societies with high levels of fission-fusion events (Croft *et al.*, 2003; Wilson *et al.*, 2014), these consequences might be less relevant. High competitiveness during foraging (Bashey, 2008; Chuard *et al.*, 2018) allows a guppy to obtain more food leading to fitness benefits with limited socially mediated costs when other group members are absent or often changing. This might explain the finding that guppies raised in stable social groups developed higher inhibitory control compared to guppies raised in unstable social groups (experiment 2). Considering that aggression and competition increase with group size in guppies

(Magurran & Seghers, 1991), it also seems reasonable that guppies from the more complex social treatments may have developed reduced inhibitory control compared to guppies raised alone (experiment 1). However, this interpretation is difficult to reconcile with a recent report showing that in some circumstances foraging efficiency is improved when guppies are in larger social groups (Snijders *et al.*, 2021).

Before accepting an interpretation based on guppies' social ecology, two other explanations should be considered in future studies. First, the contradictory effects of social complexity on inhibitory control between guppies and primates may be due to differential effects of the social environment on developmental plasticity within species compared to interspecific variation on evolutionary timescales (but see Ashton *et al.*, 2018). Second, we cannot exclude that the domesticated nature of the guppies used in this study had a role in their response to the developmental social environment. For example, domesticated zebrafish, *Danio rerio*, rely less on social information than their wild counterparts (Zala *et al.*, 2012), and the fact that domesticated guppies shoal less than feral guppies (Swaney *et al.*, 2015) suggest differences in sociability might exist also between domesticated and wild guppies. To disentangle these possibilities, it will be necessary to gather more data on the social brain hypothesis in teleost fishes, as well as in other vertebrates. Further comparisons of wild and domestic guppy strains would also be informative.

Given the broad effects of social environment on the endocrine system (Hellmann *et al.*, 2015; Reddon *et al.*, 2015) and the effects of hormones on cognition (Eaton *et al.*, 2015), it is also worth considering hormonal underpinnings of the plasticity that we detected. In humans, substantial evidence indicates that stress hormones increase inhibitory control (reviewed in Shields *et al.*, 2016). Stress due to the lack of social companions might have caused the higher inhibition in the individuals raised alone in experiment 1. However, guppies are often found in small social groups (< 6 individuals) or alone in the wild (Croft *et al.*, 2006), suggesting that at least short-term isolation from social fellows is ecologically realistic, and unlikely to be highly stressful. Chouinard-Thuly *et al.* (2018) did not detect any effect of acute social isolation on the stress response in guppies. The stress mediated explanation does not seem to fit with the effects of group stability in experiment 2 because social fish prefer familiar conspecifics (Griffith & Magurran, 1999; Magurran *et al.*, 1994), and social disruption is likely to be more stressful than consistent group membership (Bhat & Magurran, 2006; Chivers *et al.*, 1995; Lucon-Xiccato & Griggio, 2017), yet the fish from the socially unstable treatment showed lower inhibitory control. It should be also noted that a recent study has suggested that early social experience has no effect on stress-induced cortisol levels in another species of fish (Antunes *et al.*, 2020). Collectively, the interpretation based on developmental organisation of the stress response seems an unlikely explanation for the results of this study, however, confirmation by examining the stress response in guppies following similar social manipulations would be worthwhile. Future studies should also investigate the effects on inhibitory control of other hormones. For example, manipulation of isotocin (the teleost fish homologue of the mammalian nonapeptide oxytocin) has been shown to alter the response to social disruption in the daffodil cichlid, *Neolamprologus pulcher*

(Hellmann *et al.*, 2015). The relationship between isotocin and inhibitory control is so far unknown, yet oxytocin has been related to inhibitory processes of hunger in rats (Stricker & Verbalis, 1987).

One or more non-hormonal mechanisms may be also involved in guppies' inhibitory control plasticity. For example, in fishes, the social environment can affect cerebral lateralisation (Biost *et al.*, 2013; Schaafsma & Groothuis, 2011), namely how the brain splits information processing between the two hemispheres. The degree of cerebral lateralisation affects inhibitory control performance in the zebrafish, *Danio rerio* (Lucon-Xiccato *et al.*, 2020c). Furthermore, both personality and brain size, which in fish, are affected by both group size (Castanheira *et al.*, 2019; Gonda *et al.*, 2009) and familiarity with group members (Galhardo *et al.*, 2012), have been related to inhibitory control (Buechel *et al.*, 2018; Lucon-Xiccato *et al.*, 2020b). These factors should be investigated along with physiological mechanisms to explain the effects observed in the present study.

Socially-driven plasticity of inhibitory control might have consequences for individual guppies and their fitness that go beyond social interactions. Core executive functions, to which inhibitory control belongs, are simple cognitive modules that are activated when an animal performs many activities (Diamond, 2013). For example, inhibition of a behaviour is expected to be advantageous anytime the animal must adapt to a changing situation (Chow *et al.*, 2020). Inhibitory control can help animals to deal with changes in levels of competition, including in the case of the invasion of an alien species (Hoare *et al.*, 2007). Literature also suggests that low inhibition and high persistency might help individuals to find the solution for a problem during foraging (Chow *et al.*, 2016). Other effects of inhibitory control can be visible during reproductive processes, such as mate choice, as suggested by two recent studies on sticklebacks, *Gasterosteus aculeatus* (Keagy *et al.*, 2019; Minter *et al.*, 2017). To understand these and other indirect consequences of socially-driven changes in executive functions it will be important to fully characterise their relationship with fitness.

Notably, our analysis indicated that the group size manipulation of experiment 1 not only affected average inhibitory control performance of guppies, but also its variability. Guppies reared alone displayed lower interindividual variance in performance compared to the other groups. Within-group variability was not affected by the manipulation of group stability performed in experiment 2. Changes in cognitive variance have recently been described in response to predation risk (Lucon-Xiccato *et al.*, 2020d). However, their causes and consequences are currently unclear. Undeniably, life in a group allows individuals to reach behavioural decisions based on collective mechanisms (Sumpter *et al.*, 2008; Ward *et al.*, 2008; Ward *et al.*, 2012). This may allow for the development of greater variability in cognitive performance as disadvantageous phenotypes can follow the group reducing the costs of poor decisions. Furthermore, it is possible that the social group favours the development of large differences between individuals in process similar to social niche partitioning (Montiglio *et al.*, 2013; von Merten *et al.*, 2017).

In conclusion, this study revealed phenotypic plasticity of inhibitory control in relation to the social environment experienced by developing guppies. Socially-driven phenotypic plasticity, as well as plasticity mediated by other factors (van Horik *et al.*, 2019), might therefore interact with genes (Langley *et al.*, 2020) in determining individual differences in inhibitory control in natural populations. Furthermore, the present study showed that the effect of social complexity on inhibitory control in the guppy is the opposite of what has been reported in mammals and birds (Asthon *et al.*, 2018; Johnson-Ulrich, *et al.*, 2020), suggesting that the relationship between sociality and cognition may not be straightforward and that the particulars of a species ecology, biology, or life history may have important effects which deserves attention in future research.

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5. Medaka as a model for seasonal plasticity: Photoperiod-mediated changes in behaviour, cognition, and hormones

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Abstract

Teleosts display the highest level of brain plasticity of all vertebrates. Yet we still know little about how seasonality affects fish behaviour and the underlying cognitive mechanisms since the common neurobehavioral fish models are native to tropical environments where seasonal variation is absent or reduced. The medaka, *Oryzias latipes*, which inhabits temperate zone habitats, represents a promising model in this context given its large phenotypic changes associated with seasonality and the possibility to induce seasonal plasticity by only manipulating photoperiod. Here, we report the first extended investigation of seasonal plasticity in medaka behaviour and cognition, as well as the potential underlying molecular mechanisms. We compared medaka exposed to summer photoperiod (16 h light: 8 h dark) with medaka exposed to winter photoperiod (8 h light: 16 h dark), and detected substantial differences. Medaka were more active and less social in summer photoperiod conditions, two effects that emerged in the second half of an open-field and a sociability test, respectively, and might be at least in part related to habituation to the testing apparatus. Moreover, the cognitive phenotype was significantly affected: in the early response to a social stimulus, brain functional lateralisation shifted between the two hemispheres under the two photoperiod conditions, and inhibitory and discrimination learning performance were reduced in summer conditions. Finally, the expression of genes encoding key pituitary hormones, *tshß* and *gh*, and of the *tshß* regulatory transcription factor *tef* in the brain was increased in summer photoperiod conditions. This work reveals remarkable behavioural and cognitive phenotypic plasticity in response to photoperiod in medaka, and suggests a potential regulatory role for the same hormones involved in seasonal plasticity of other vertebrates.

Keywords: fish brain; fish models; phenotypic plasticity; seasonality; stress research

Introduction

In the last decade, teleosts have gained importance as models for research on the brain, behaviour, and cognition, from both mechanistic and evolutionary perspectives (e.g., Bshary and Brown, 2014; Kotrschal *et al.*, 2013; Stewart *et al.*, 2014). A field in which fish models have made notable contributions is neurobehavioural plasticity research. The fish brain displays extensive plasticity in response to environmental factors (reviewed in Ebbesson and Braithwaite, 2012) and in contrast to the mammalian brain, its neurogenesis extends throughout life (Zupanc, 2006). Not surprisingly, plasticity in fish behaviour and cognitive function has been described for a range of factors such as predation risk (Herczeg *et al.*, 2016; Lucon-Xiccato *et al.*, 2020a; Thomson *et al.*, 2012), food availability (Kotrschal and Taborsky, 2010; Thomson *et al.*, 2012), social environment (Herczeg *et al.*, 2016; Lucon-Xiccato *et al.*, 2022), water flow (Bhat *et al.*, 2015), and lighting conditions (Chapman *et al.*, 2009; Ehlman *et al.*, 2015). Such plasticity may have contributed to the success of this group, which includes more than half of the extant vertebrate species (Ravi and Venkatesh, 2018).

Despite the widespread evidence for enhanced plasticity, behavioural and cognitive changes due to seasonality are poorly understood in fish. Only a few observations in the field of aquaculture and a few laboratory experiments have suggested that behaviour such as activity and foraging (Lucas and Batley, 1996; Smith *et al.*, 1993) and cognitive abilities such as spatial learning (Carbia and Brown, 2020) may vary across seasons. A potential reason for this gap in knowledge is that the fish species typically exploited as research models might not be appropriate to identify seasonal plasticity in behaviour and cognition. For example, the zebrafish, *Danio rerio*, the guppy, *Poecilia reticulata*, and African cichlids, which are among the most widely used models, are native to tropical habitats with very limited annual fluctuations in environmental conditions. Accordingly, they are not expected to display phenotypic plasticity in relation to seasons.

The medaka, *Oryzias latipes*, is particularly well suited for the study of seasonal plasticity. This fish species has recently gained importance as an animal model in several research fields, mostly due to the combination of powerful genetic tools it offers for the in-depth investigation of a range of biological mechanisms (Lin *et al.*, 2016; Takeda and Shimada, 2010; Wittbrodt *et al.*, 2002). The medaka is native to Eastern Asia, a temperate zone in which environmental conditions change significantly across the year. For example, the temperature to which medaka are exposed can vary between 4 and 40 °C (Hilgers and Schwarzer, 2019). Throughout this seasonal variation, medaka undergo distinct phenotypic changes related to the fact that reproduction occurs only during summer. For example, gonad maturation is triggered by exposure to summer conditions (Awaji and Hanyu, 1988; Koger *et al.*, 1999). For the purpose of studying seasonal plasticity in fish, the medaka offers a further advantage. Several studies have indicated that the behavioural and physiological changes which occur between seasons can be triggered by simply manipulating the duration of the photoperiod (Awaji and Hanyu, 1989; Fujisawa *et al.*, 2021; Ueda and Oishi, 1982; Urasaki, 1976). This marked response to photoperiod allows researchers to induce seasonal plasticity without the confounding effects of changes in temperature and food availability on metabolism and other physiological functions. Photoperiod-mediated plasticity of medaka behaviour and cognition may therefore provide an invaluable tool to study seasonal plasticity of vertebrates and its underlying mechanisms.

In this study, we have investigated behavioural, cognitive, and hormonal plasticity in response to photoperiod manipulation in the medaka. Subjects were exposed to one of two photoperiod treatments that mimic conditions experienced by medaka in its distribution range during summer (16 h light and 8 h dark) and winter (8 h light and 16 h dark). Several studies adopted this experimental design to examine physiological responses such as ovarian maturation, hepatic metabolism, and growth (Awaji and Hanyu, 1989; Davis *et al.*, 2002; Fujisawa *et al.*, 2021; Urasaki, 1976). The two photoperiods investigated have opposed biological meaning because medaka mate only in summer and reduce reproductive traits in winter (Awaji and Hanyu, 1988; Koger *et al.*, 1999). After the photoperiod treatments, we subjected medaka to a range of cognitive/behavioural and molecular analysis.

For the behavioural analysis, we initially focused on novel environment responses using an open-field test, which is considered by most researchers as a reliable measure of behavioural traits such as activity, exploration, anxiety, and boldness in fish (Burns, 2008; Lucon-Xiccato *et al.*, 2020b; Toms *et al.*, 2010; Szaszkiewicz *et al.*, 2021). We then assayed sociability using a shoaling apparatus, testing the preference of subjects to swim close to a shoal of conspecifics (Cattelan *et al.*, 2019; Mehlis *et al.*, 2015). Regarding cognition, we first explored the effects on brain functional lateralisation, the tendency showed by animals to process information differentially between the two brain hemispheres (Rogers and Andrew, 2002). Cognitive lateralisation often results in asymmetrical responses to stimuli, and we assayed the medaka in their lateralised response to a visual social stimulus (Lucon-Xiccato *et al.*, 2020d). We then studied medaka's inhibitory control, i.e. the cognitive function that allows withholding of behaviour, using a protocol in which the fish had to inhibit their tendency to feed on unreachable prey (Lucon-Xiccato *et al.*, 2020c; Montalbano *et al.*, 2020). Finally, we conducted two learning tests based on food conditioning: a colour discrimination learning task (Lucon-Xiccato *et al.*, 2019; Roy *et al.*, 2019) and a shape discrimination learning task (Lucon-Xiccato *et al.*, 2019). We expected to detect increased activity in the medaka from the summer photoperiod in the open-field test (Yokota and Oishi, 1992). Given the absence of established results on seasonal plasticity of the remaining behavioural and cognitive traits in teleost fish, we adopted an explorative approach.

Using qRTPCR, we then measured the mRNA expression in brain extracts of two key pituitary hormones, *tshß* (thyroid stimulating hormone *ß* subunit) and *gh* (growth hormone), as well as the transcription factor *tef* (thyrotroph embryonic factor), which is a key regulator of *tshß* gene transcription. Several studies in fish have shown that thyroid axis components, including TSH subunits, respond to environmental stimuli and that their levels change seasonally (Cowan *et al.*, 2017; Cole and Volkoff, 2020). Furthermore, *tshß* and *tef* are involved in the photoperiod sensing mechanism in mammals (Dardente *et al.*, 2010). In fish, GH levels also change with seasons and are correlated with feeding behaviour (Björnsson, 1997; Jørgensen and Johnsen, 2014).

Based on these comparative data, we predicted that we would observe higher levels of expression of the genes tested in the fish from the summer photoperiod treatment compared to those of the winter photoperiod treatment.

Materials and methods

Experimental fish

For all our experiments, we used a wild-type strain of medaka (isogenic inbred 'iCab' strain) bred in the laboratory at the University of Ferrara. The iCab strain is derived from the Southern Japanese population and is a medaka inbred strain commonly used for basic research (Furutani-Seiki *et al.*, 2004; Loosli *et al.*, 2000; Lopez-Olmeda *et al.*, 2021; Spivakov *et al.*, 2014). All the subjects were adults at the time of testing (6-8 months). Before experiments, medaka were maintained under standard laboratory conditions: 200 L aquaria with air pumps and biological filters; temperature 28 ± 1 °C; feeding twice per day with live *Artemia salina* nauplii and dry food. The subjects were randomly selected among the stock population.

Photoperiod conditioning

To study the effect of seasonality on medaka, we exposed subjects to two treatments with different photoperiods (Awaji and Hanyu, 1989; Fujisawa *et al.*, 2021; Ueda and Oishi, 1982; Urasaki, 1976). Half of the medaka were exposed to a photoperiod consisting of 8 h of dark and 16 h of light (summer photoperiod) and half of the medaka were exposed to a 16 h of dark and 8 h of light period (winter photoperiod). These photoperiods were administered to groups of 10 subjects maintained in 100 L aquaria with biological filters. We exposed 6 groups of fish to the summer photoperiod ($N = 60$ medaka) and 6 groups of fish to the winter photoperiod ($N = 60$ medaka). Three groups per condition were used in the analysis of behaviour, and the remaining groups were used in the analysis of cognition and gene expression. We isolated the aquaria from the external environment by covering them with black plastic panels. We also placed a black plastic lid over each aquarium, containing a white light-emitting diodes (LED) strip (Superlight Technology Co. Ltd., Shenzhen, China) for illumination. The light was turned on between 6 a.m. and 10 p.m. for the summer photoperiod and from 10 a.m. to 6 p.m. for the winter photoperiod. Medaka were fed twice per day as under maintenance conditions. Each week, the aquaria were cleaned and half of the water was exchanged with fresh aquarium water. The photoperiod conditioning lasted for 30 days to ensure full acclimatization to the respective photoperiods (Koger *et al.*, 1999; Lopez-Olmeda *et al.*, 2021). After this period, the fish were tested in the following assays.

Analysis of behaviour

Behavioural test 1: Novel environment response

In this test, we measured the behaviour of the subjects when exposed to a novel, unfamiliar environment (open field). We tested 20 medaka from the summer photoperiod treatment and 20 medaka from the winter photoperiod treatment in the open-field test. After collection from the treatment aquaria, each subject was introduced into a test tank with white walls (40 × 40 cm, 15 cm height) filled with 8 cm of water (Figure 1a). The experimental tank was illuminated by a white LED strip and the temperature of the water was kept constant (28 ± 1 °C). The subject was then left undisturbed for 30 min. To allow recording of subjects' behaviour, the experimental tank was set on a backlit IR LED table ($\lambda > 980$ nm). An infrared sensitive webcam (Monochrome GigE camera, Basler, Germany; resolution: 1280 × 1024) was placed 1 m above the tank to record the experiment. Finally, a computer running Ethovision XT software (Noldus Information Technology, The Netherlands) was connected with the camera to calculate the dependent variables used in the analysis. As measure of activity, the software recorded distance moved and time spent moving at least 1 body length per second. Additionally, the software recorded a measure of anxiety called thigmotaxis and computed as time spent within 1 body length from the edge of the arena (Figure 1a).

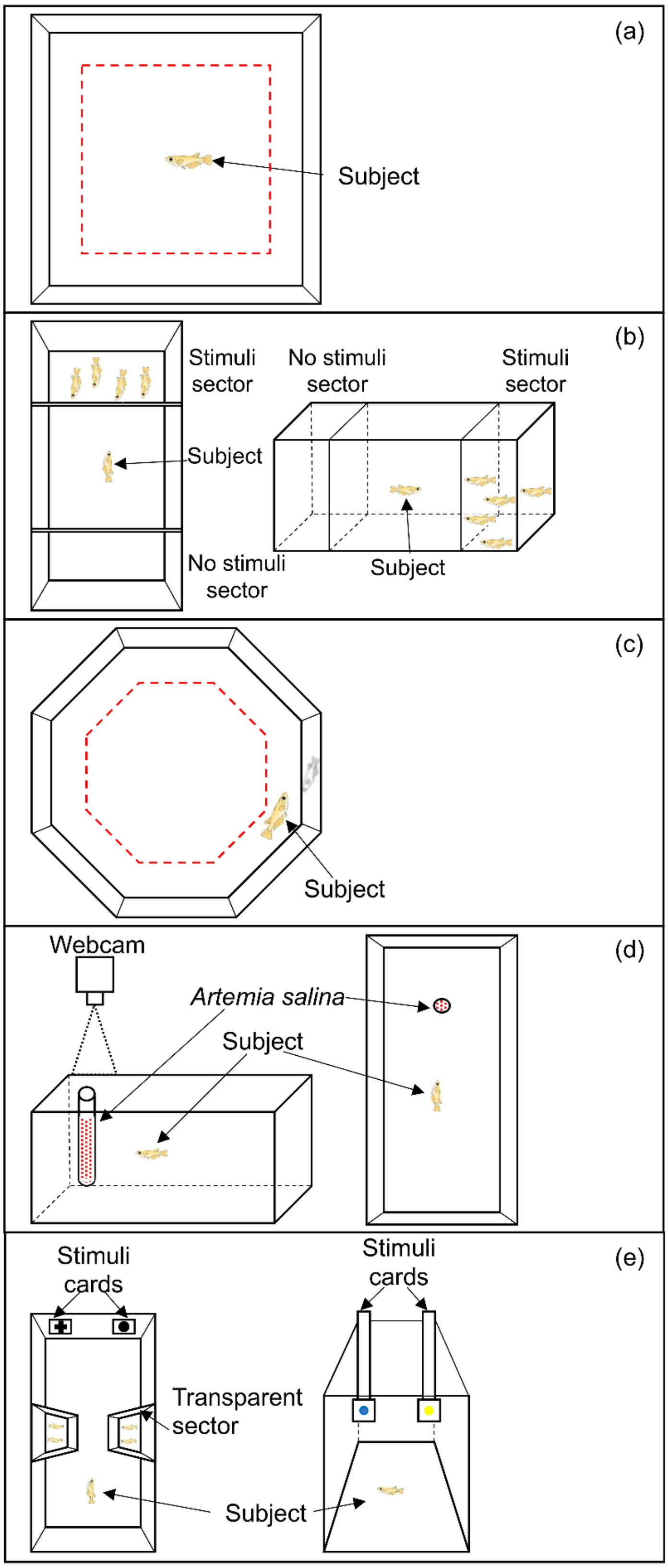

Figure 1. *Diagrams of our experimental set ups. (a) Open-field arena used to assess novel environment response; the dotted line represents the edge used to assess thigmotaxis. (b) Top (left) and side (right) view of the shoaling apparatus used to assess sociability. (c) Mirror apparatus used to assess brain functional lateralisation; the dotted line indicates the area in which subjects was considered to observe the mirror image. (d) Top (left) and side (right) view of the apparatus used to assess inhibitory control. (e) Top (left) and side (right) view of the apparatus used in the colour and the shape discrimination learning experiments.*

A potential confounding factor for the application of the open-field test, and more generally for tests that are based on habituation to novelty, is that the behaviour of most teleost fish studied is not constant over the course of the testing period (Bell and Peeke, 2012; Bruzzone *et al.*, 2020; Kotrschal *et al.*, 2014; Lucon-Xiccato *et al.*, 2014; Pazmino *et al.*, 2020; Stewart *et al.*, 2012; Wong *et al.*, 2010). This temporal variability has also been reported for medaka (Lucon-Xiccato *et al.*, 2020b; Matsunaga & Watanabe, 2010). For example, we previously demonstrated that medaka assayed with the open-field test exhibit a reduction in activity between 7 to 8 minutes after introduction into the arena (Lucon-Xiccato *et al.*, 2020b). The main explanation for this effect is that due to habituation, the novel environment becomes progressively familiar for the fish as the test continues. This change in the perception of the environment in turn alters fish behaviour (Bell and Peeke, 2012; Matsunaga & Watanabe, 2010). Not considering this temporal variation when comparing experimental groups is problematic because it might prevent to detect a behavioural difference. For example, Lucon-Xiccato and Dadda (2016) compared male and female guppies, in a test measuring the response to a novel object. On average, the fish apparently did not explore the object and there was no difference between the two sexes in the time spent close to the stimulus. However, when the temporal dimension was included in the analyses, it was found that fish actually explored the object in specific periods of the test. Males approached the stimulus at the beginning of the test and then reduced this exploration behaviour while females initially avoided the stimulus and only in the second half of the test, did they approach it. It was therefore important to consider the contribution of differences in temporal variation in behaviour in our study. The precise time window of the behavioural changes is difficult to predict *a priori* because it might vary according to several factors such as the age, sex, experience, and genotype of the subjects (Lucon-Xiccato and Dadda, 2016; Lucon-Xiccato *et al.*, 2020b; O'Neill *et al.*, 2018). Considering that frequently, differences were observed between the first and the second halves of the experiment, we clustered all the variables collected in the open-field test into two phases, corresponding to the first half of the experiment (minutes 1-10) and the second half of the experiment (minutes 11-20). Analysis of the dependent variable collected with this repeated measures structure allowed us to consider treatment effects that were dependent on experimental time.

Behavioural test 2: Sociability

In the sociability test, we presented the subjects with the choice to join a group of conspecifics or to stay alone, measuring their preference for the two options. The experimental tank was 20 × 60 cm (20 cm height) and filled with 10 cm of water (Figure 1b) at 28 ± 1 °C. It was divided into 3 sectors: a central sector (30 cm), hosting the subject medaka, and two lateral sectors (15 cm each) to present the experimental choices. One lateral sector hosted a group of 5 stimulus medaka, collected from standard maintenance tanks maintained in an intermediate photoperiod condition (12 h: 12 h). The second lateral sector was left empty, with no stimulus. Above each stimulus sector, we installed a pair of fluorescent lamps. The experimental procedure consisted of releasing an experimental subject (collected from the treatment aquaria) into the centre of the shoaling apparatus, and then leaving it there undisturbed, free to interact with its conspecifics. We recorded the experiment for 20 minutes with a camera placed above the apparatus. To measure the behavioural variables, we performed an offline manual analysis. An experimenter played back the recordings on a computer and by using the software BORIS (http://www.boris.unito.it) and calculated the time spent by the subjects within 5 cm from each of the stimulus sectors. As reported for the open-field test, previous studies indicate that the behaviour of the subjects in the sociability test might vary over the course of the experiment (Dadda *et al.*, 2007; Lucon-Xiccato *et al.*, 2017; Miller and Gerlai, 2007). This is again attributable to the effect of habituation: once introduced into the testing apparatus, the social response of the fish is usually stronger because the environment is unfamiliar and they try to find protection in the context of their social group (Hager and Helfman, 1991); as the fish progressively become familiar with the testing apparatus, this social attraction is expected to decrease. Critically, temporal variability in social response has been shown to influence the conclusions of such studies (Lucon-Xiccato *et al.*, 2017), requiring us to consider it in our experiment. We therefore assayed the sociability variable during two experimental phases (first and second half of the experiment), consistent with the design of our open-field test. In the experiment, we tested 20 medaka from the summer photoperiod treatment and 18 medaka from the winter photoperiod treatment.

Analysis of cognition

Cognitive test 1: Brain functional lateralisation

To assess brain lateralisation, we studied asymmetric responses of individual fish to being confronted with a social stimulus. The lateralisation test took place in an octagonal tank with mirror walls, exploiting the fish's social response to their mirror image (Figure 1c). Right hemisphere processing of the social visual information would cause the fish to observe the mirror image with the left eye and consequently to swim in a clockwise direction while trying to approach the mirror image. The opposite swimming direction would indicate left hemisphere processing of the stimulus. The mirrors of the apparatus were 17 × 15 cm each and were illuminated with a white LED strip placed 5 cm above. The apparatus contained 8 cm of water at 28 ± 1 °C. The experiment of each subject was recorded for 20 min with the Noldus set up described above. After testing, an experimenter scored the recordings to quantify the amount of time spent by each subject swimming in clockwise or anticlockwise directions within 1 body length of the mirrors using the software BORIS. As our lateralisation test is based on a social response, the behaviour of the fish was expected to vary over time as explained for the sociability test. This variation in lateralisation has already been reported in other fish species (Dadda *et al.*, 2010). To ensure that this potential temporal change in lateralisation was considered, we collected the data in the first (minutes 1-10) and the second (minutes 11-20) phase of the experiment separately, obtaining a data structure analogous to that of our behavioural tests. This data was used to calculate a lateralisation index for each phase of the experiment using the following formula: (time spent swimming clockwise – time spent swimming anticlockwise) / (time spent swimming clockwise + time spent swimming anticlockwise). The lateralisation index ranged between -1 to +1, with negative values indicating preference for left hemisphere information processing and positive values indicating preference for right hemisphere information processing. The sample size was 16 medaka of the summer photoperiod treatment and 14 medaka of the winter photoperiod treatment.

Cognitive test 2: Inhibitory control

In this test, we measured the ability of the subjects to withhold a particular behavioural response, namely their active feeding behaviour, in the event that they are not able to access their food. The inhibitory control experiment was performed in plastic tanks (33 × 13 cm, 15 cm height; Figure 1d) filled with 4 L of water kept at 28 ± 1 °C. The experimental tanks were provided with a plastic lid with a hole close to one of the short walls to permit feeding. White LED strips placed on the ceiling provided lighting with the treatment photoperiod of each subject. A back sector of the apparatus (5 cm) contained immature conspecifics as social companions.

Overall, the inhibitory control experiment lasted 4 days. The initial 3 days were part of a training phase; the inhibitory control testing took place on the fourth day. During the training phase, we fed the fish with dry food mixed with water for 3 days. The number of feeding events was increased across the 3 days: 2, 4 and 6 times per day, respectively. The food was delivered by means of a Pasteur pipette inserted into the hole of the lid. This protocol served to habituate the fish to receive food in correspondence with a specific area of the tank (i.e., under the hole in the lid).

After the training phase, the medaka underwent the testing phase. This consisted of a single trial that lasted 20 minutes. Instead of delivering food, we inserted a standard laboratory glass tube via the hole in the lid, with the tube suspended in the middle of the water column. Inside the tube, we placed 4 mL of a suspension of *Artemia salina* nauplii in water, which served as a prey stimulus for the medaka. With this procedure, subjects usually try to catch the prey inside the tube without success and slowly decrease their attack behaviour because the prey is not accessible. We recorded the behaviour of medaka with a webcam set 50 cm above the experimental tanks. The experimenter then played back the recording with reduced speed and counted the number of attacks directed towards the stimulus. Previous studies with this paradigm detected a substantial change in the number of attacks over the course of the testing time that indicated inhibitory control (Lucon-Xiccato *et al.*, 2022; Montalbano *et al.*, 2020). To consider this potential effect, which might differ between the experimental groups (e.g. Lucon-Xiccato *et al.*, 2022), we collected these inhibitory control data in the same two experimental phases described for our previous tests (minutes 1-10 and 11-20 of testing). The sample size for this test was as follows: $N = 21$ medaka from the summer photoperiod treatment; $N = 18$ medaka from the winter photoperiod treatment.

Cognitive test 3: Colour discrimination learning

In this experiment, we trained the fish to select a stimulus of a predetermined colour to obtain a food reward. We performed the task in glass tanks (25 × 40 cm, 25 cm height) filled with 20 cm of water (temperature: 28 ± 1 °C) and enriched with gravel bottoms (Figure 1e). Each tank hosted an individual subject for the entire experiment.

The tank was provided with two sectors in the centre of the long walls. They were made with transparent plastic (10 × 5 cm) and hosted two immature conspecifics as social companions. Due to these small sectors, the central sector of the tank was shaped like an hourglass, with a narrow central corridor and two main sectors in correspondence with the short walls of the tank. The walls of the tank were covered with green plastic. The apparatus was illuminated by white LED strips in order to expose the tested fish to a light cycle corresponding to their adapted photoperiod treatment (winter or summer). The stimuli to be discriminated were a yellow spot and a blue spot (Ø 1.6 cm). They were made of plastic and glued onto a 4 × 4 cm white plastic card, which provided a homogenous background. The card was presented to the subjects by mean of a transparent stick, which could be fixed to the short walls of the tanks to suspend the stimulus in the middle of the water column. Prior to the beginning of the experiment, we assigned a rewarded colour stimulus for each individual. Half of the subjects of each photoperiod treatment were assigned to the yellow stimulus and the other half to the blue stimulus.

The task procedure was composed of two consecutive phases: a training phase and a learning phase. The training phase lasted 2 days and consisted of a series of independent trials. On day 1 of the training phase, we performed 8 trials. In each trial of day 1 of the training phase, we inserted the rewarded colour stimulus into the tank, against one of the short walls of the tank. When the medaka approached the stimulus, we gently released a food reward close to the card with the help of a Pasteur pipette. The food reward was a suspension of *A. salina* nauplii in water. The short wall of the tank in which we presented the stimulus was alternated across the trials. On day 2 of the training phase, we performed 12 trials. In each trial on day 2, we introduced both stimuli (rewarded and unrewarded colour), at the two corners of the short wall of the tank. We administered food to the medaka only when they approached the rewarded colour.

After the 2 days training phase, the subjects underwent the testing phase. Only data from this testing phase were used to assess the learning performance. The testing phase was composed of a non-fixed number of days, which was determined based on the performance of each subject (i.e. upon achieving a learning criterion; see below). In each day of the testing phase, we administered 12 trials: in each trial, we presented both stimuli to the subject and we provided the food reward only if the subject approached the correct colour stimulus at first. If the subjects approached the incorrect stimulus at first, we removed the cards and did not deliver the reward food. As in previous studies on fish (Baratti *et al.*, 2021; Montalbano *et al.*, 2022), we imposed a learning criterion consisting of 17 correct choices out of 24 over 2 consecutive days. This corresponded to a choice accuracy of 70%, which is statistically significant ($X^2_1 = 4.167$, $P = 0.041$). When a subject achieved the learning criterion, the testing phase was terminated. The sample size for the colour discrimination learning task was 11 medaka of the summer photoperiod treatment and 12 medaka of the winter photoperiod treatment.

Cognitive test 4: Shape discrimination learning

Since we found an interactive effect of the colour assigned as rewarded in the colour discrimination test, we ran a second learning experiment that did not involve colour stimuli, i.e., shape discrimination. The protocol and the apparatus that we used for the shape discrimination learning task was the same as in colour discrimination learning, but using a different stimulus card. We used white cards with a black shape on the centre: either a cross (1.5 × 1.5 cm) or a circle (Ø 1.5 cm). We tested 22 medaka, $N = 11$ per each photoperiod treatment. We used the same criterion to define successful learning of the task: namely, 17 correct choices out of 24 over 2 consecutive days.

Analysis of brain gene expression

Gene expression was measured in 10 medaka brains sampled from each experimental group. Fish were always sampled 3 hours after lights on. After euthanasia, the whole brain of each subject was dissected under a stereomicroscope. We isolated total RNA using TRIzol™ reagent (ThermoFisher Scientific, Italy) following the manufacturer's instructions. We analysed the amount, quality, and composition of isolated RNA using BioSpec nano (Shimadzu Italia S.r.l., Italy). We then used DNase-treated RNA to perform cDNA synthesis in a final volume of 20 µl using the iScript™ cDNA synthesis kit (Bio-Rad Laboratories S.r.l., Italy). cDNA was PCR-amplified with the CFX Connect Real-Time PCR System using SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad Laboratories S.r.l., Italy). The thermal cycling conditions were as follows: 30 s of denaturation at 95 °C, followed by 40 cycles of a 15-s denaturation step at 95 °C and then by an annealing-elongation step for 30 s at 60 °C. After amplification, we performed a melting curve analysis to confirm amplicon specificity. All samples were analysed in triplicate. Gene-specific primers are indicated in Table 1. Finally, we calculated the relative expression levels of each sample by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001), using *S18b* as a housekeeping gene (Cuesta *et al.*, 2014).

Table 1. Primer sequences used in the RNA amplification.

Medaka Gene	Primer sequence
tshb; thyroid stimulating hormone, beta subunit	F- catccatatccaggccagag R-gcacaataggccaccaaagt
gh1; growth hormone1	F- ggaacaacgtcaactcagca R- tttgggtgagatttggttcc
tefa; thyrotroph embryonic factor a	F- tgggacaaaaccattcccta R- catcaggctcactggagaca
S18b; mrps18b; *mitochondrial ribosomal protein S18B*	F- tccccgagaaattccagcat R- ctcctccgttagctctccag

Statistical analysis

Data were analysed using R statistical analysis software. All the statistical tests were two-tailed because of the lack of specific predictions in some of the experiments. We selected different models for each experiment in order to fit the structure and the distribution of the dependent variable.

Behavioural tests

Considering the need to account for the well-documented temporal variability in behaviour in our tests (e.g. Kotrschal *et al.*, 2014; Lucon-Xiccato *et al.*, 2017), we collected two repeated behavioural measurements for each subject (in the first and second half of the test, respectively). This data structure prevented us from directly comparing the two treatments: an analysis of correlated samples without taking into account the correlation when computing the dispersion parameters would indeed represent pseudoreplication (Lazic, 2010). We therefore used models that included samples correlation. In particular, we fitted linear mixed-effects models (LMMs; *lmer* R function) with subject ID as random effect to deal with the repeated measurements. As independent variables, we fitted the photoperiod treatment (summer treatment versus winter photoperiod) and the experimental phase (i.e. first half versus second half of the test). In these models, we expected to detect a significant effect of experiment phase as evidence of the predicted behavioural change over time. The impact of the photoperiod treatment was expected to result in either a main effect of treatment or an interaction effect between treatment and experimental phase, which would indicate behavioural differences in a specific phase of the experiment. For this latter situation, we additionally ran post-hoc t-tests by analysing the two experimental phases separately, thereby allowing us to determine in which experimental phase the effect emerged.

Cognitive tests

In the lateralisation experiment, the dependent variable was similar in structure to those of the behavioural tests (two measurements in the two experimental phases). Therefore, we applied the analysis previously described (i.e. LMM with treatment and experimental phase as fixed effects, and subject ID as random effect).

For the inhibitory control experiment, the dependent variable was a count of events with repeated measure structure (i.e. one data point per each half of the experiment) to account for the temporal change typically observed in this test (Montalbano *et al.*, 2020). We therefore applied a generalised linear mixed-effects model (GLMM) with Poisson error structure. Besides the effect of photoperiod treatment, the model was fitted with the experimental phase and the subject ID (random effect) to handle the repeated measures. To account for the data distribution in the post-hoc test of this experiment, we used a general linear model with Poisson error structure.

For the two learning tests (colour and shape discrimination), the dependent variable was a single datapoint per subject (e.g. number of days to criterion in the learning experiments). Therefore, we used one-way ANOVA fitted with photoperiod treatment and stimulus assigned as rewarded in the learning experiments as fixed effects.

Brain gene expression

Data of the gene expression analysis consisted of a single observation for the same subject. We analysed these data with two samples t-test to compare the means of the subjects from the two photoperiod treatments. Data were log-transformed data before running the analysis because of a right-skewed distribution.

Ethical Note

Animal husbandry and experimental procedures were performed in accordance with European Legislation for the Protection of Animals used for Scientific Purposes (Directive 2010/63/EU) and National animal welfare standards (Tierschutzgesetz §11, Abs. 1, Nr. 1, AZ35-9185.64/BH, AZ 35-9185/G-179/13 for Germany; D.lgs. 26/2014, license 18/2017-TU, exp. procedures CB/01-2019 for Italy).

Results

Behavioural test 1: Novel environment response

For the first parameter of activity recorded (distance moved during the open-field test), we found a significant effect of photoperiod treatment modulated by experimental phase (LMM: $\chi^2_1 = 4.983$, $P = 0.026$). The effect of photoperiodic conditions was apparently related to the second half of the test, when medaka exposed to the summer photoperiod showed greater activity (Figure 2a). However, evidence from the post-hoc tests was not significant (first half: $t_{38} = 0.712$, $P = 0.481$; second half: $t_{38} = 1.267$, $P = 0.213$). In the initial LMM, the main effect of experimental phase was also significant ($\chi^2_1 = 7.561$, $P = 0.006$), but the main effect of the photoperiodic condition was not significant ($\chi^2_1 = 0.116$, $P = 0.734$).

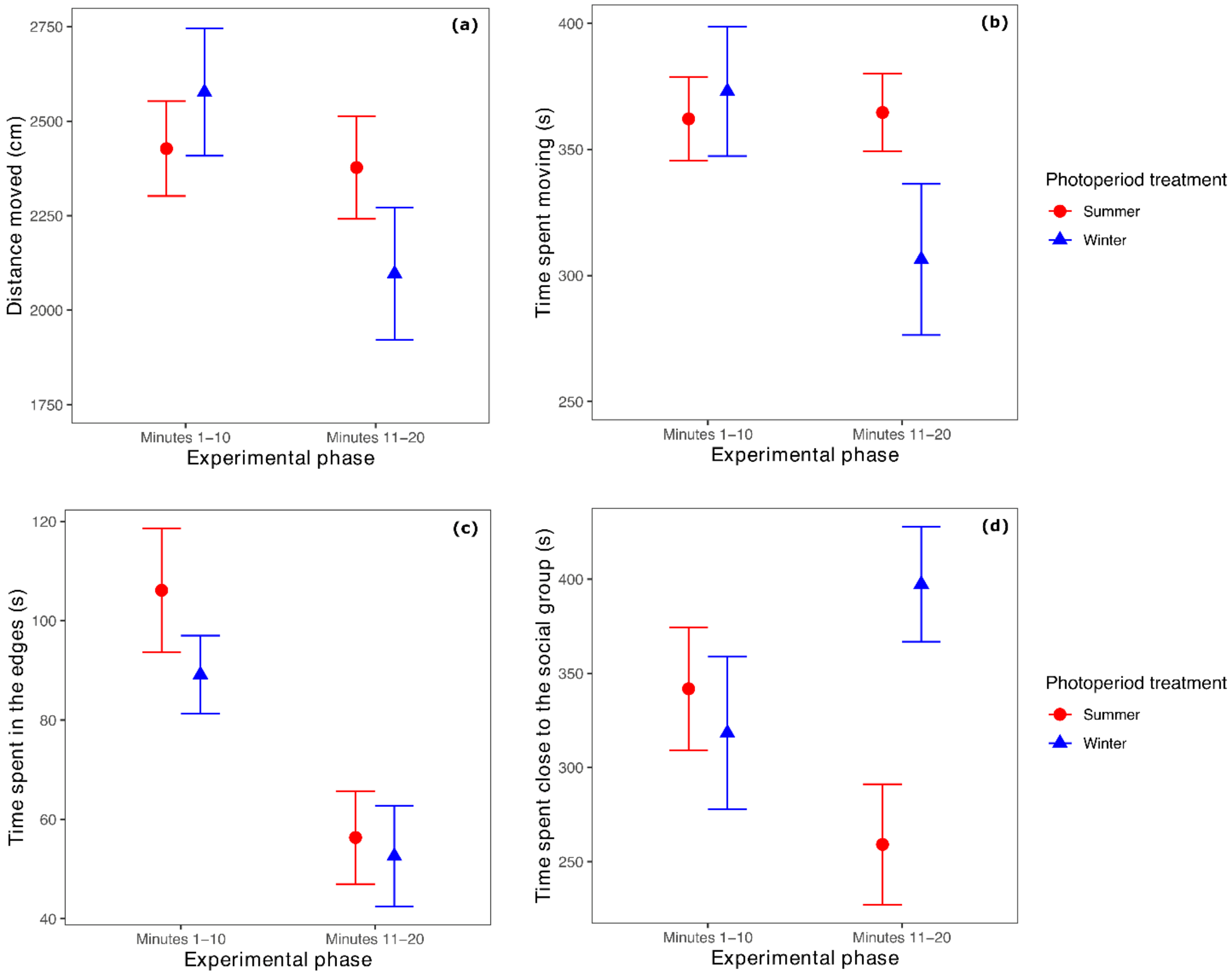

Figure 2. *Results of behavioural tests 1 and 2. (a) Distance moved in the open-field arena. (b) Time spent moving with 1 body length per second as threshold. (c) Time spent at the edges of the open-field arena. (d) Time spent close to the social group in the sociability test. Data points represent means and error bars represent standard errors.*

The second parameter of activity analysed, the time spent moving, revealed a pattern similar to that of distance moved. The LLM found a significant interaction between treatment and experimental phase ($\chi^2_1 = 6.022$, P = 0.014), a significant main effect of experimental phase ($\chi^2_1 = 5.177$, P = 0.023), and no significant main effect of treatment ($\chi^2_1 = 0.671$, P = 0.413). The post-hoc analysis to understand the significant interaction revealed that the two groups had similar activity in the first phase of the experiment ($t_{38} = 0.357$, P = 0.723); however, in the second phase of the experiment, the medaka from the winter photoperiod tended to spend less time moving ($t_{38} = 1.730$, $P = 0.092$; Figure 2b).

For the parameter "time spent along the edges of the open-field arena" (thigmotaxis), we found no significant difference between the medaka exposed to the two different photoperiodic conditions (LMM: treatment: $\chi^2_1 = 0.640$, $P = 0.424$; interaction treatment by experimental phase: $\chi^2_1 = 1.225$, $P = 0.268$; Figure 2c). The main effect of experimental phase was significant ($\chi^2_1 = 51.994$, $P < 0.001$).

Behavioural test 2: Sociability

Considering the entire testing time of the sociability test, the model of the time spent close to the conspecific shoal indicated that treatment marginally affected sociability (LMM, main effect of treatment: $\chi^2_1 = 2.724$, $P = 0.095$). The model term including the temporal variation (treatment by experimental phase interaction) revealed that the photoperiodic condition had a significant effect on sociability in relation to experimental phase ($\chi^2_1 = 5.588$, $P = 0.018$). At the beginning of the test, the medaka of both treatments showed similar attraction towards the stimulus shoal ($t_{35} = 0.453$, $P = 0.653$); thereafter, medaka exposed to the winter photoperiod spent more time close to the shoal compared to the medaka of the summer photoperiod treatment ($t_{35} = 3.092$, $P = 0.004$; Figure 2c). The main effect of experimental phase was not significant ($\chi^2_1 = 0.033$, $P = 0.856$).

Analysis of cognition

Cognitive test 1: Brain functional lateralisation

In the analysis of the lateralisation index, we found a significant effect of the photoperiodic conditions modulated by experimental phase (LMM: $\chi^2_1 = 10.203$, $P = 0.001$). In the first half of the test, medaka exposed to the summer photoperiod swam more often in a clockwise direction, which indicates left eye preference to observe the stimulus and therefore right hemisphere processing (post-hoc: $t_{32} = 2.699$, $P = 0.011$; Figure 3a); the opposite lateralisation pattern was exhibited by medaka exposed to winter photoperiod (Figure 3a). In the second half of the experiment, there was no difference between the lateralisation index of fish from the two photoperiod conditions ($t_{32} = 0.662$, $P = 0.513$). In the LMM, the main effect of experimental phase and the main effect of treatment were not significant ($\chi^2_1 = 1.527$, $P = 0.217$; $\chi^2_1 = 0.643$, $P = 0.423$, respectively).

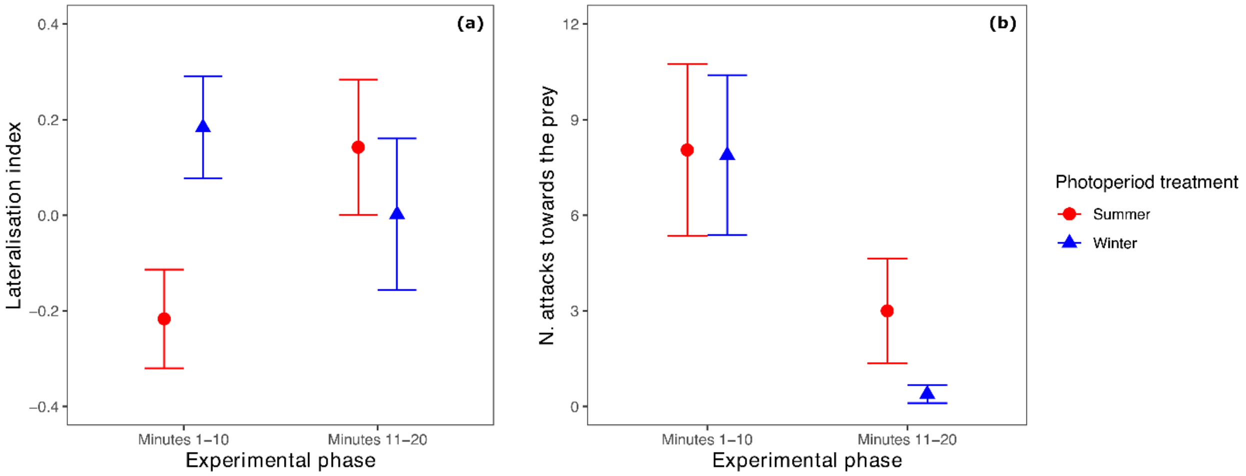

Figure 3. *Results of cognitive tests 1 and 2. (a) Cerebral lateralisation index indicating preference for processing social information with the right hemisphere (positive values) or left hemisphere (negative values). (b) Number of attacks in the inhibitory control task. Data points represent means and error bars represent standard errors.*

Cognitive test 2: Inhibitory control

The analysis of inhibitory control revealed a significant difference in the number of food "attacks" between the medaka from the two photoperiod treatments in relation to the experimental phase (GLMM: $\chi^2_1 = 23.684$, $P < 0.001$). This interaction was due to the fact that there was no difference between the two photoperiod conditions in the first half of the experiment (GLM: $\chi^2_1 = 0.031$, $P = 0.861$), but the main effect of photoperiod condition was significant in the second half of the experiment (GLM: $\chi^2_1 = 0.293$, $P < 0.001$). This suggests that the medaka exposed to the winter photoperiodic conditions displayed a reduced number of attacks in the second half of the test (Figure 3b) while in contrast, the medaka under summer photoperiodic conditions made continuous attempts to reach their prey, which is indicative of lower inhibition. In the initial GLMM model, the main effect of experimental phase was significant ($\chi^2_1 = 79.343$, $P < 0.001$) and the main effect of treatment was not significant ($\chi^2_1 = 0.293$, $P = 0.589$).

Cognitive test 3: Colour discrimination learning

All the subjects achieved the learning criterion in the colour discrimination task. In analysis of the number of days necessary to reach the learning criterion, we found a significant effect of treatment modulated by the rewarded colours associated with each subject) (ANOVA: $F_{1,19} = 4.389$, $P = 0.049$). Summer photoperiod treated medaka required more time to achieve the learning criterion, but only for those subjects which were assigned with blue as the rewarded colour (Figure 4a). The main effect of rewarded colour was significant ($F_{1,19}$ = 5.297, P = 0.033), and the main effect of photoperiodic condition was not significant ($F_{1,19}$ = 1.642, P = 0.209).

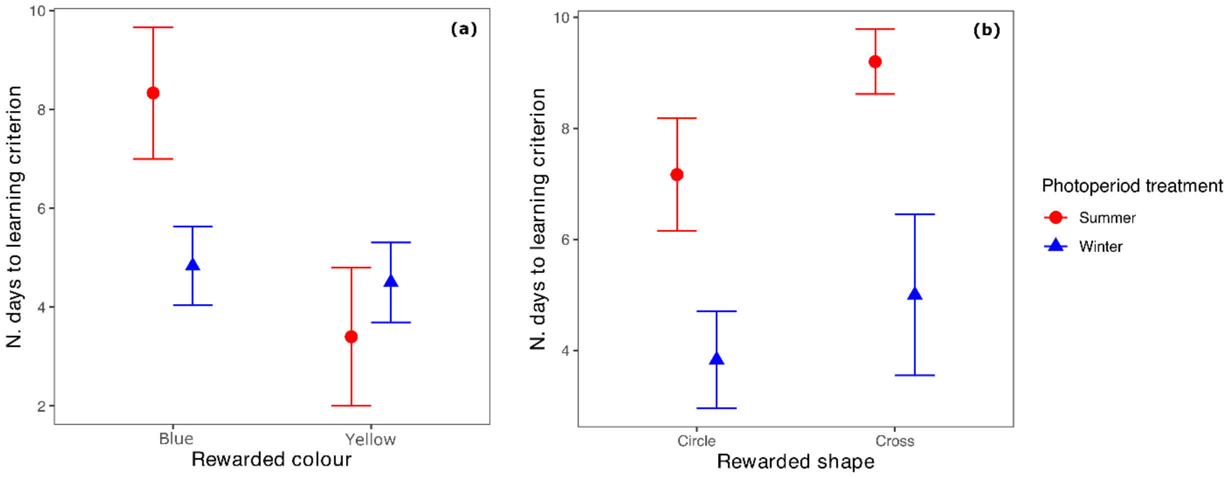

Figure 4. *Results of the discrimination learning tests (cognitive test 3 and 4). (a) Number of days required to achieve the learning criterion in the colour discrimination task, divided per rewarded colour assigned to the subjects. (b) Number of days required to achieve the learning criterion in the shape discrimination task, divided per rewarded shape assigned to the subjects. Data points represent means and error bars represent standard errors.*

Cognitive test 4: Shape discrimination learning

All the subjects achieved the learning criterion in the shape discrimination task. The analysis of the number of days required to reach the learning criterion in the shape discrimination experiment revealed a significant main effect of the photoperiodic conditions (ANOVA: $F_{1,18}$ = 13.423, P = 0.002), indicating slower learning rate in the medaka exposed to the summer photoperiod treatment (Figure 4b). The main effect of rewarded stimulus and the interaction involving rewarded stimulus and treatment were not significant ($F_{1,18}$ = 2.453, P = 0.135; $F_{1,18}$ = 2.453, P = 0.677, respectively).

Analysis of brain gene expression

In the brain gene expression analysis, we found significant seasonal differences of expression levels for all mRNAs investigated (t-test: *tshβ*: $t_{11} = 2.632$, $P = 0.023$; *tef*: $t_{16} = 2.380$, $P = 0.030$; *gh1*: $t_{16} = 3.465$, $P = 0.003$). In all cases, the expression was higher for the medaka brain under summer photoperiod conditions (Figure 5).

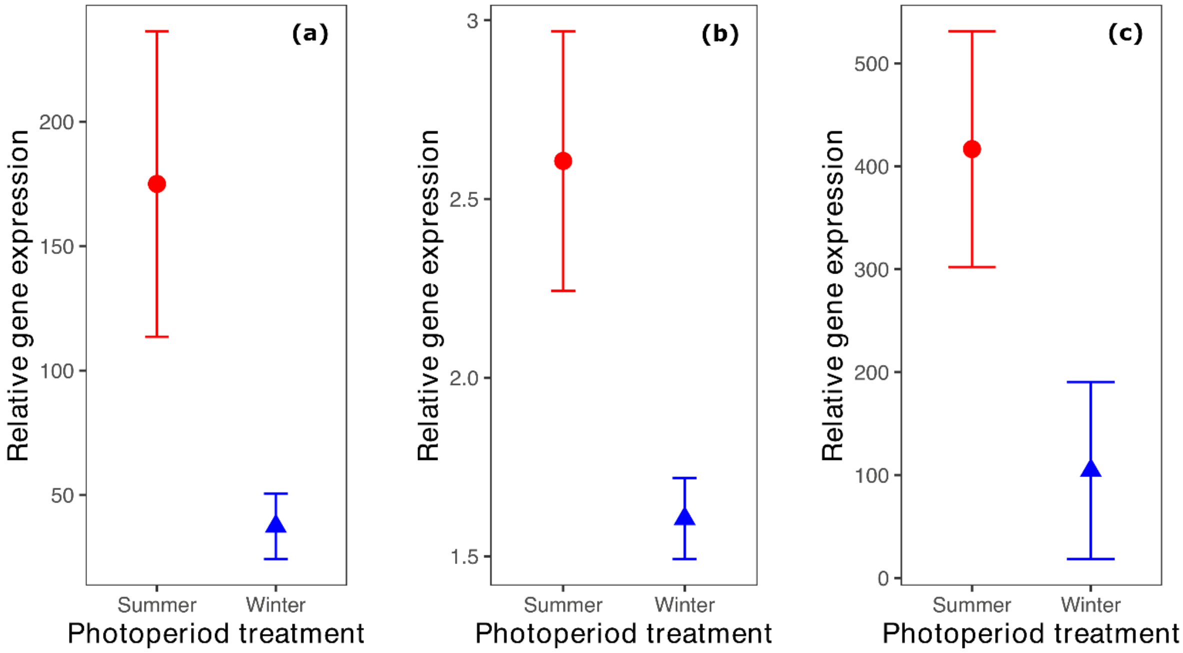

Figure 5. *Results of brain gene expression analysis. Relative expression of (a) tshβ, (b) tef, and (c) gh. Data points represent means and error bars represent standard errors.*

Discussion

In this study, we exposed medaka fish to two naturally relevant photoperiod conditions to investigate the effect of seasonal changes on behaviour and cognition. Specifically, we used a long photoperiod to simulate summer, during which reproduction occurs, and a short photoperiod for simulating winter. Our results show that the behavioural and cognitive phenotype of medaka plastically responds to the changes of photoperiod. Moreover, gene expression analysis reveals that the behavioural adaptations are accompanied by substantial changes in hormonal regulation between the different photoperiodic conditions.

The two behavioural tests conducted in our study were designed to analyse two key traits relevant for the fitness of the species and that are often adopted in applied research on fish models (e.g., Hong and Zha, 2019), namely the response to a novel environment and sociability. The novel environment test (open-field test) indicated a difference in activity in terms of the distance moved and the time spent moving by medaka exposed to summer photoperiod compared to those exposed to the winter photoperiod. In both cases, the effect was mediated by the experimental phase. Graphical inspection (Figure 2a and 2b) suggests that the effect was mostly due to medaka of the winter photoperiod showing reduced activity in the second half of the experiment, although the post-hoc tests failed to confirm this and only detected a marginal effect possibly due to lack of power. These parameters (distance moved and time spent moving) are mostly associated with activity although some studies associated it with forms of anxiety (Toms *et al.*, 2010). A third behavioural parameter that we measured, namely the spatial preference for the edges of the novel environment (thigmotaxis), did not significantly vary between fish from the different photoperiods. Since thigmotaxis has been clearly associated with anxiety (Schnörr *et al.*, 2012), we conclude that the downregulation of metabolic activity and other physiological traits due to the winter-like photoperiod were more likely involved in the altered swimming activity detected with the variables distance moved and time spent moving (Fujisawa *et al.*, 2021). In contrast to a previous study (Yokota and Oishi, 1992), in our experiments the temperature was kept constant. Therefore, the reduced activity is not the result of a lower water temperature during the winter period, and rather points towards the presence of a ‘winter’ behavioural phenotype induced by photoperiodic changes. It is worth noting that the temporal dimension of the difference in activity might indicate a role for habituation. Changes in activity in the open-field test are often associated with the fish becoming familiar with the novel environment and thereby reducing their exploration (e.g., O’Neill *et al.*, 2018; Pazmino *et al.*, 2020). From this perspective, it is possible that the winter photoperiod-treated medaka habituated faster to the open-field arena or reduced their exploration behaviour sooner.

Our second behavioural test indicated reduced sociability of medaka exposed to the summer photoperiod. Social aggregation in fish is associated with various activities such as defence from predators (Hager and Helfman, 1991) or improving foraging (Harpaz and Schneidman, 2020). Arguably, these activities assume a different relevance under different seasonal conditions (e.g., Harvey and Nakamoto, 2013), thereby representing a selective pressure for the evolution of behavioural plasticity in sociability (Johnsen and Hasler, 1977; Koizumi *et al.*, 2017). In the case of medaka, high sociability during the winter season might be due to an increased need for predator avoidance, for example to counterbalance the costs of reduced activity or changed ecological parameters such as the presence of shelter (Fukuda *et al.*, 2006; Fujisawa *et al.*, 2021). However, to the best of our knowledge, the fitness significance of social attraction has not been fully described in this species making it difficult to interpret the significance of the observed plasticity. Alternatively, aggression in medaka, a behavioural trait usually negatively related to sociability (Kasper *et al.*, 2019), has been associated with reproduction (Howard *et al.*, 1998; Yamashita *et al.*, 2020) and with other factors that vary with seasons such as food availability (Robb and Grant, 1998). Therefore, it is possible that the reduced sociability observed in the medaka adapted to the summer photoperiod is the consequence of an increase in aggression to cope with summer foraging and reproductive activities. Notably, the effect on sociability was observed during the second phase of the experiment. This is not unexpected because our protocol exploited the social attraction in response to a novel/unfamiliar environment (Lucon-Xiccato *et al.*, 2017). As experimental time proceeds, the relative impact of the testing environment changes for the subjects, shifting progressively from unfamiliar to familiar, and this in turns alters the social response of the fish. We therefore speculate that habituation might be involved in the observed difference in sociability between medaka from the winter and the summer photoperiods, as proposed for the open-field test. More generally, our study emphasises the importance of incorporating temporal measures when analysing medaka behaviour.

Our cognitive experiments revealed substantial phenotypic variation according to photoperiod. Lateralisation is the tendency to split information processing between the two brain hemispheres, thus representing a mode of functional brain asymmetry which is commonly observed in vertebrates (Bisazza *et al.*, 2000). We found that medaka exposed to the summer photoperiod preferentially used the left hemisphere for processing the image of a conspecific, at least in the first part of the experiment when the attraction towards the social stimulus was higher due to the unfamiliar testing environment. In the same time window, the medaka under winter photoperiod conditions showed the opposite hemisphere processing preference. Since we used a lateralisation test that was based on social interactions (Moscicki *et al.*, 2011), the first explanation for the observed lateralisation plasticity is that it is linked to the seasonal plasticity in sociability observed in our previous experiment. However, the results of our lateralisation test may be considered as a proxy for a more general lateralisation phenotype because previous studies have revealed consistency across diverse lateralisation tests (Dadda *et al.*, 2012). Therefore, the overall conclusion from our study is that photoperiod generates major changes in functional lateralisation of the brain. Seasonal plasticity of lateralisation potentially has several explanations. A shift towards right-hemisphere processing has often been observed as a result of high stress levels (Rogers, 2010), a finding which also holds true in fish (Lucon-Xiccato *et al.*, 2014). Medaka might therefore shift to right hemisphere control during winter to cope with more stressful environmental conditions. Lateralisation is also critical for foraging (Giljov *et al.*, 2009) and mating (Torres-Dowdall *et al.*, 2020); thus, the observed plasticity of lateralisation might be due to different occurrence of these activities between summer and winter. Alternatively, the plasticity of medaka lateralisation might be associated with seasonal variation in ecological parameters such as predation risk, vegetation abundance, or light exposure, which are all known to affect cerebral asymmetric functioning in anamniotes (Dadda & Bisazza, 2016; Dadda *et al.*, 2010; Lucon-Xiccato *et al.*, 2020e; Lucon-Xiccato *et al.*, 2020f).

The two remaining cognitive traits measured in this study (inhibition and learning) also showed variation across the photoperiod treatments. Specifically, exposure to summer photoperiod conditions was associated with lower inhibitory capacity. With our test, inhibition consisted of a reduction in attacks targeting unreachable prey over the course of the testing time (Lucon-Xiccato *et al.*, 2022). The medaka from the summer photoperiod tried to reach the prey throughout the entire test, whereas those from the winter photoperiod inhibited this response after the first ten minutes of the experiment. The effect on inhibitory control might also be related to the known link between lateralisation and this ability (Lucon-Xiccato *et al.*, 2020d). From an adaptive point of view, reduced inhibitory control might be advantageous during competition for food or mating. These activities mainly take place in the summer season for this species, thereby explaining the reduced inhibition observed in the subjects exposed to the summer photoperiod.

The final cognitive effect of the photoperiod that we observed was a reduced discrimination learning ability in medaka adapted to the summer photoperiod. The effect on discrimination learning also aligns well with our previous findings in this species (López-Olmeda *et al.*, 2021). In that previous study, it was revealed that medaka, on average, reduce their learning performance during foraging in summer months, an effect that is thought to be related to hormonal changes or shifting interest towards mating. In support of this interpretation, the effect was mostly observed in males. It is worth noting that one of our learning experiments was based on colour discrimination and these results are difficult to interpret. Although, on average, medaka which experienced the summer photoperiod treatment took longer to learn colour discrimination, this effect was mediated by the rewarded stimulus assigned to each subject. In particular, summer photoperiod-treated medaka apparently learned much faster to select the yellow stimulus versus the blue stimulus. Such an effect is indicative of a seasonal shift in spontaneous colour preference (Roy *et al.*, 2019), which is likely due to a reported change in the photoreceptor population in summer versus winter conditions (Shimmura *et al.*, 2017). This finding is relevant for methodological purposes as it suggests caution in designing and interpreting colour learning experiments in medaka. Indeed, the results may vary according to the season and the lighting conditions.

Our gene expression analysis supports previous studies revealing that photoperiod treatment alters hormone production in medaka. The expression of both *tshß* and *tefa* were upregulated in summer-adapted fish. It is known that in fish, the transcription factor TEF binds to D-box enhancer elements in the promoters of light-inducible genes (Vatine *et al.*, 2009; Mracek *et al.*, 2012). In mammals, TEF also binds to a D-box in the *tshß* promoter and upregulates gene expression under long day conditions (Dardente *et al.*, 2010). Further experiments will be required to evaluate whether seasonal variation of *tshß* expression is directly light-driven by TEF via its regulation of D-box elements. It is also important to consider a possible role for deep brain photoreceptors in determining the phenotypic shift observed in medaka. Non-visual opsins are widely expressed in the fish brain (Sato and Ohuchi, 2021; Foulkes *et al.*, 2016). Furthermore, the photoperiodic regulation of seasonal reproduction in

Japanese quail is mediated by deep brain photoreceptors expressing Opsin 5 that are located in the paraventricular organ and extend fibres to the pars tuberalis of the pituitary to translate lighting information into *tshβ* expression (Nakane *et al.*, 2010). Finally, melatonin, a key hormone involved in the photoperiodic signalling pathway, may also play a role in the seasonal control of *tsh* expression. Indeed, melatonin exposure has been shown to reduce *tsh* mRNA levels in medaka pituitary organ culture (Kawabata-Sakata *et al.*, 2020). GH is also a central mediator of seasonal changes in physiology and behaviour such as feeding, swimming, aggression, and anti-predator behaviour (Canosa *et al.*, 2007). Since previous investigations in zebrafish have shown that GH overexpression has significant effects on the brain with an improvement in long-term memory (Studzinski *et al.*, 2015), seasonal changes in the cognitive abilities of medaka could be due to altered expression of this pituitary hormone. Overall, these results reinforce the notion that seasonal changes in photoperiod alter the general hormonal phenotype of medaka. Hormonal modulation pathways have been described in vertebrates for both the behavioural (e.g., Killen *et al.*, 2021; Yamashita *et al.*, 2020) and cognitive traits (e.g., Riedstra *et al.*, 2013; Rogers, 1974) described in this study. Therefore, hormonal changes resulting from changes in photoperiod may potentially underlie the plasticity that we have observed in medaka behaviour and cognition. Further insights into hormonal control might derive from studies exposing the medaka to intermediate photoperiods (i.e. spring-like and autumn-like conditions), corresponding to the biological phase in which the shift between summer and winter phenotype is expected to occur (Awaji and Hanyu, 1989).

In conclusion, our study reveals widespread behavioural and cognitive alterations in response to two critical photoperiod lengths in medaka, as well as accompanying changes in genes linked with hormonal regulation. This indicates considerable plasticity in these traits in natural populations of medaka, potentially mediated by a light-hormone regulatory axis. Thereby, our work paves the way for investigations of plasticity under other photoperiod conditions and for the use of this species as a model to explore the origins of seasonal behavioural diseases.

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6. Does microplastic ingestion affect fish cognition?

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Abstract

Animal cognition displays remarkable plasticity in response to environmental conditions and consequently, is potentially susceptible to non-adaptive changes due to anthropogenic activities. A pervasive source of pollution for aquatic environments is represented by microplastics, which can be ingested by fish and other organisms potentially altering many traits, including cognition. In this study, we analysed the cognitive consequences of microplastic ingestion in a teleost fish, the zebrafish. We measured brain activity, learning ability with a visual discrimination task, and cognitive flexibility with a reversal learning task of zebrafish fed either a diet including microplastics obtained from a common synthetic polymer, polyethylene (PE), a diet including microplastics from a biodegradable polymer, polybutylene adipate terephthalate (PBAT), or a control diet without microplastic. We found that PE microplastics significantly reduced brain activity in the zebrafish. However, the three experimental groups showed no differences in learning a colour discrimination task and subsequently, in reversing the learned association. Moreover, no detectable changes due to microplastics were observed in interindividual cognitive variability. We conclude that microplastics derived from synthetic polymers did alter brain functioning at the molecular level, but these alterations did not result in detectable changes in the cognitive phenotype in the tasks investigated.

Keywords: cognitive plasticity; cognitive variance; conservation behaviour; *Danio rerio*; fish cognition; individual differences; aquatic pollution.

Introduction

For most vertebrate taxa, evidence suggests that cognitive traits, such as learning and memory capacities, varies substantially among individuals (reviewed in Lucon-Xiccato & Bisazza, 2017; Thornton & Lukas, 2012) and that this cognitive variance is a significant determinant of animals' fitness (Cauchard *et al.*, 2013; Huebner *et al.*, 2018; Smith *et al.*, 2015). While part of the cognitive variance is attributable to genes (e.g., Langley *et al.*, 2020; Smith *et al.*, 2015), a large proportion derives from cognitive plasticity driven by the environment experienced by the individuals. For example, tadpoles raised in presence of predators develop greater capacity to learn novel threats compared to tadpoles raised in environments with no predators (Ferrari, 2014; Lucon-Xiccato *et al.*, 2016), and fish and mammals raised in structurally complex habitats develop greater learning capacities (Montalbano *et al.*, 2022; Salvanes *et al.* 2013; Van Praag *et al.*, 2000).

The aforementioned cases depict forms of adaptive plasticity that have probably evolved due to selection because they confer fitness benefits to individuals in specific environments (Via *et al.*, 1995). However, considering the high plastic potential of cognition and the rate of environmental changes in the Anthropocene, the question arises on whether animals now display new forms of cognitive plasticity, including non-adaptive plasticity. A number of scholars have been asking this question recently, finding more than one confirmation. For instance, velvet geckos, *Amalosia lesueurii*, incubated under climate warming conditions develop slower learning (Dayananda & Webb, 2017), and zebra finches, *Taeniopygia guttata*, subjected to disturbance by traffic noise showed decreased cognitive performance in several tasks (Osbrink *et al.*, 2021). In aquatic species, evidence suggest various cognitive impairments due to hypoxia (Lucon-Xiccato *et al.*, 2014), which occurrence is increasing due to human pollution (Diaz & Rosemberg, 2008), ocean acidification (Domenici *et al.*, 2012; Ferrari *et al.*, 2012), and various materials spilled in the environment during human activities (Grassie *et al.*, 2013).

One of the most concerning forms of pollution is related to plastic waste. In particular, small plastic fragments (i.e., microplastics) take decades to fully degrade and are virtually ubiquitous in aquatic habitats (Li *et al.*, 2018; Li *et al.*, 2020; Lindeque *et al.*, 2020; Sharma *et al.*, 2021). The tiniest microplastics (i.e., < 1 mm) are similar in size to many marine organisms, such as fish eggs, larvae, and unicellular algae (Rowenczyk *et al.*, 2020) and can be ingested by larger organisms such as fish (Galafassi *et al.*, 2012; Parker *et al.*, 2021; Pinheiro *et al.*, 2017; Silva-Cavalcanti, 2017). Once ingested, microplastics may determine a range of impairments on gene expression, physiology, life history, and behaviour (e.g., Critchell, & Hoogenboom, 2018; Limonta *et al.*, 2019; Mallik *et al.*, 2021; Uy & Johnson, 2022). Negative effects have been also reported for microplastic derived from biodegradable materials (e.g., Kedzierski *et al.*, 2018). In the brain, microplastics determine the alteration of at least one neurotransmitter, the acetylcholine (reviewed in Xiong *et al.*, 2023). However, the effects of microplastic ingestion on aquatic species cognition are mostly unknown, with only one study directly investigating a cognitive output and finding an impairment in decision making of the European hermit crab, *Pagurus bernhardus* (Crump *et al.*, 2020).

In this study, we exploited the zebrafish, *Danio rerio*, an emerging model in research on microplastics (reviewed in Bhagat *et al.*, 2020) and in cognitive sciences (reviewed in Meshalkina *et al.*, 2017), to investigate the effects microplastic ingestion on cognition. We exposed experimental groups of zebrafish to a diet supplemented with microplastic based on either common commercial grade plastics widely used for flexible packaging, polyethylene (PE), or a common biodegradable polymer (PBAT, polybutylene adipate terephthalate). After the treatment, we investigated subjects' brain activity via genes expression analysis (Calvo & Schluessel, 2021) and we performed cognitive phenotyping with a colour discrimination and a reversal learning task (Montalbano *et al.*, 2022), two assays measuring learning and cognitive flexibility, respectively. Considering that prior studies revealed forms of plasticity that involved not only the average cognitive phenotype but also its variability (e.g., Domenici *et al.*, 2012; Lucon-Xiccato *et al.*, 2022b), we included this aspect of fish cognition in our analyses.

Materials and methods

Animal welfare note

The experiments of this project have been approved by the Ethical committee of University of Ferrara (OPBA) and by the Italian Ministry of Research (MIUR; protocol n. 446/2021-PR). Moreover, the behavioural observations were designed to avoid stress and discomfort to the subjects and followed the ASAB/ABS Guidelines for the Use of Animals in Research (https://doi.org/10.1016/j.anbehav.2019.11.002).

Subjects

The zebrafish used in the study were adult individuals of an outbreed wild-type line maintained in our facility at University of Ferrara. The stock consisted of approximately 500 individuals at the time of the experiments. The subjects were tested at approximately 6 months of age. Before the testing, the subjects were maintained in standard laboratory conditions (temperature: 27 ± 1 °C; photoperiod: 14-10 h light-dark) in groups of 12-15 individuals in 75 L glass aquaria. Twice per day, the zebrafish were fed with alternate live prey (*Artemia salina* nauplii) and commercial food (Staple food Vipan, Sera, Heinsberg, Germany).

Experimental treatments

The experimental treatments followed the procedure of an early study (Limonta *et al.*, 2019). We randomly selected 24 groups of four zebrafish (overall N = 96 subjects) each from different maintenance tanks. We placed each group in a 2 L glass aquarium provided with an aerator stone. The aquaria were maintained in a room with temperature of 27 ± 1 °C and 14: 10 h light: dark photoperiod. Each group of zebrafish was randomly assigned to one of three treatments, thereby obtaining 8 experimental replicates. The three treatments consisted of a diet as follow: 1) 0.01 g of food plus 100 µg PE microplastics per fish; 2) 0.01 g of food plus 100 µg PBAT microplastics per fish; 3) control diet of 0.01 g food per fish. As food for the treatments, we used commercial dry granules (Tetra, Melle, Germany). The fish were fed their experimental food once per day (h 09:00 - 11:00). After administering the food, the experimenter visually checked that the fish consumed it entirely. The experimenter also performed a complete water change and cleaned the aquaria to avoid presence of residuals. The treatment lasted 20 days.

We obtained the microplastics by milling commercially available polymer pellets. The PE pellets were purchased from DOW with trade name Dowlex 2629 as LLDPE type used for film and food packaging. The PBAT pellets were purchased from Ecoworld® Biodegradable Polymer and are commonly adopted to manufacture certified biodegradable and compostable shopping bags, food packages, mulch films, and garbage bags. The microplastics were prepared by using a Retch mill (AM 200) and then characterised by Retch vibratory sieve machine (AC 200) equipped with 9 sieves with square apertures ranging from 25 to 1000 microns. The plastic pellets of PE and PBAT were milled as received without adding any additive. The selected milled plastic powders were separated into nine fractions and evaluated by using EasySieve software (Retch) for calculating the average powder dimensions and their distributions. For the experiment, we selected as microplastics the powder fractions ranging from 50 to 400 µm with a mean dimension of the microplastics in the range 265-280 µm, a sample useful to demonstrate general effects of microplastics that can be found in nature.

Brain activity

We adopted a method based on measures of immediate early gene expression, which is increasingly used to investigate fish cognition (Calvo & Schluessel, 2021). At the end of the treatment, 36 randomly chosen zebrafish (control treatment: N = 12; PE: N = 12; PBAT: N = 12) were sacrificed for the brain activity analysis. We performed the euthanasia with MS-222 overdose. We extracted whole brain under a dissection microscope and we isolated total RNA from 12 zebrafish brain pools (each made of 3 brains; N = 4 pools per treatment) with TRIzol reagent (Thermo Fisher Scientific, USA). We measured concentration and purity using Biospec-nano (Shimatzu, Kyoto, Japan); genomic DNA was removed with a DNaseI (Sigma-Aldrich, USA) treatment performed according to the manufacturer's instructions. RNA (1 μg) was reversed transcribed using iScript™ cDNA Synthesis Kit (Biorad, Milan, Italy).

We assessed relative gene expression by real-time quantitative PCR using SsoAdvanced Universal SYBR Green Supermix (Biorad, Milan, Italy) on a CFX Connect Real-Time PCR Detection System (Biorad, Milan, Italy) instrument. Genes of interest (*c-fos* and *egr-1*) and housekeeping genes used to normalise gene expression (*ef1a* and *18S*) were analysed with the primer sequences listed in Table 1. We verified the efficiency of the primers sets by constructing standard curves and we used the dissociation curves to confirm the specificity of the amplicons. Last, we calculated the relative levels of expression with the $2^{-\Delta\Delta CT}$ method (where CT is the cycle number at which the signal reaches the threshold of detection) (Livak and Schmittgen, 2001). Each CT value used for the calculations is the mean of three replicates of the same reaction.

Table 1. List of forward and reverse primers used for the qPCR analysis. *c-fos* and *egr-1* were the genes of interest for the brain activity analysis; *ef1a* and *18S* were used as housekeeping genes.

Genes	Forward	Reverse
c-fos	GTATTACCCGCTCAACCAGAC	TCCAGTAACCCTCATTTTGGG
egr-1	AGTTTGATCACCTTGCTGGAG	AACGGCCTGTGTAAGATATGG
ef1a	GACAAGAGAACCATCGAG	CCTCAAACTCACCGACAC
18S	ACCACCCACAGAATCGAGAAA	GCCTGCGGCTTAATTTGACT

Learning test

We assayed 47 zebrafish (control treatment: N = 15; PE: N = 17; PBAT = 15) in the learning test. An additional subject begun the experiment but did not show interest in the food and was discarded before collecting the data. During the test, the subjects had to discriminate between two colour stimuli to receive a food reward (Lucon-Xiccato *et al.*, 2022a; Montalbano *et al.*, 2022). The stimuli to be discriminated consisted of a card with a red circle and a card with a yellow circle (size of the card: 4 × 4 cm; size of the circle: ø 1.8 cm). We kept the subjects individually in aquaria (25 cm × 40 cm, h 25 cm; ure 1) provided with gravel on the bottom and an air stone (removed during the tests to avoid disturbance). The water in the aquaria was kept at 28 ± 1 °C and each tank was illuminated by a led strip set with a photoperiod of 12 hours of light and 12 hours of dark. Along each larger wall of the aquarium, we built a trapezoidal sector with transparent plastic, containing a small aquatic plant as enrichment (Figure 1). Because of these two sectors, the apparatus had the shape of an hourglass, with a central corridor and two larger sectors in correspondence of the short walls of the aquarium.

Before commencing the experiment, we assigned a rewarded colour to each fish, counterbalancing between the subjects of the experimental groups. The colour assigned had no significant effect on fish performance (two-samples t test on the number of days to reach the learning criterion: $t = 1.226$, $P = 0.229$). We then started the first phase of the experiment, the pre-test phase, which lasted two days. On day 1 of the pre-test phase, the experimenter presented the rewarded colour to the subject by attaching the card to a transparent stick (length 20 cm) that could be blocked onto the short wall of the apparatus. When the subject was in one of the two main sectors of the apparatus, the experimenter inserted the stimulus in the water in front of the opposed short walls of the apparatus. Then, the experimenter waited motionless until the subject approached the card; when this occurred, the experimenter delivered a drop of water containing live *A. salina* in front of the card using a Pasteur pipette. The experimenter administered eight pre-test trials with this procedure to each subject, half in the morning and half in the afternoon, alternating the presentation of the stimulus between the two sides of the apparatus.

On the second day of the pre-test phase, the experimenter presented the subject with both the rewarded and the non-rewarded colour stimulus. Each of the two cards was placed in correspondence of the corners of the short wall of the apparatus. The subject was therefore equally distant from the two stimuli when approaching them via the central corridor. The experimenter delivered the food if the fish approached the rewarded colour within 10 minutes (i.e., even if the initial choice was for the incorrect stimulus). In case of no approach, the trial was repeated. The zebrafish underwent 12 trials with this procedure, six trials in the morning and six in the afternoon, with an inter-trial interval of 10 minutes. The left-right position of the rewarded stimulus was randomly alternated between trials.

The third day of the experiment, the fish that consumed the food in all the training trials entered the test phase. The trials of the test phase followed the procedure described above. However, in case the subject did not choose the rewarded card at first, the experimenter removed both stimuli without delivering the food reward. The subjects underwent 12 trials per day. The test phase continued until the subject reached a learning criterion set at 17 correct choices out of 24 trials in 2 consecutive days (70 % correct choices). For subjects that did not pass the learning criterion within 30 days of experimenter, we interrupted the test. Therefore, the length of the test phase varied between subjects according to their learning speed.

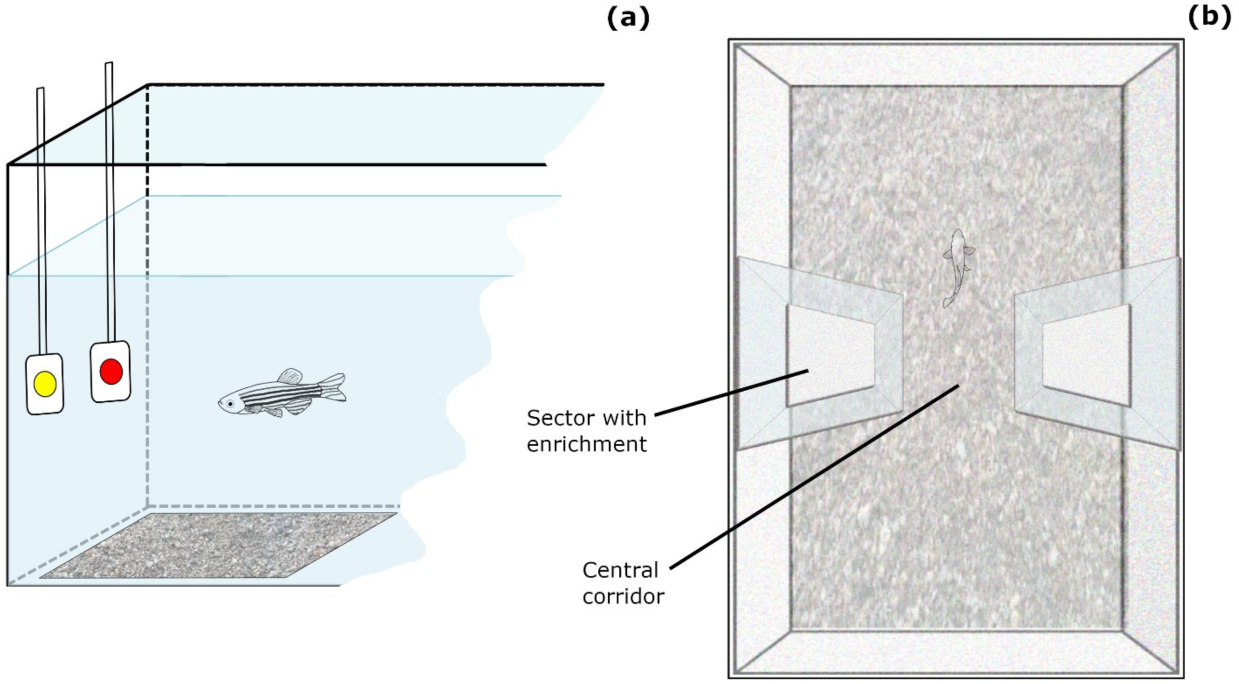

Figure 1. Diagrams of the experimental apparatus used in the cognitive phenotyping. (**a**) Lateral view of the sector with the stimuli and (**b**) top view of the entire apparatus.

Cognitive flexibility test

After the learning task, the 38 subjects that reached the learning criterion (control treatment: N = 11; PE: N = 13; PBAT = 14) performed the reversal learning task to measure cognitive flexibility (Montalbano *et al.*, 2022). The reversal learning task began the day after a subject reached the learning criterion of the learning task. The subjects remained in the same tank in which they performed the learning test. The general procedure of the reversal learning resembled the one described in the test phase of the learning test. Yet, the experimenter reversed the reward contingent. Therefore, each subject had to choose the colour that was previously unrewarded. The criterion to complete the reversal learning task was set as in the previous test (70% correct choices).

Statistical analysis

We performed the statistical analysis in R version 4.0.1 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). Significance level was set at P = 0.05 and statistical tests were two-tailed if not stated otherwise. To examine changes in brain activity among the three experimental groups, we analysed the relative gene expression (log transformed due to right-skewed distribution) of *c-fos* and *egr-1* using one-way ANOVAs and Tukey post-hoc test.

For the learning and reversal learning tasks, we analysed three dependent variables with three different models fitted with treatment as fixed effect. First, we checked for differences between treatments in the number of subjects that reached the criterion. We assigned the value "1" to subjects that reached the criterion and the value "0" to subjects that did not reach the criterion. Given the binomial distribution of this dependent variable, we analysed it with a generalised linear model with binomial error distribution. Second, we compared the number of days necessary to reach the criterion with ANOVAs. To include the subjects that did not reach the learning criterion, we assigned them the maximum possible value (i.e., 30 days) and we rank transformed the data before running the model. Last, we analysed the number of errors made by each subject that solved the task in each day of the test phase. This dependent variable had a repeated measures structure and different number of observations per each subject (due to the different number of days taken to reach the criterion). We therefore used linear mixed-effects models that can handle this type of data. In this last model, we additionally fitted individual ID as random effect to account for the repeated measurements and day as fixed effect. We kept the treatment by day interaction in the model because, if significant, would indicate different learning rates between treatments.

Because we did not detect significant effects of the treatment in the learning and reversal learning task, we used a Bayesian approach to provide a more robust evidence of performance similarity between fish from the different treatments. From the models described above, we obtained an approximate Bayes factor (BF) following Wagenmakers (2007). The BF provided an estimation of the relative strength of evidence in favour of the absence of a treatment effect in our data (Dienes, 2014).

To compare cognitive variance between the different experimental groups, we followed a two-step approach. First, we used Bartlett tests to compare variance in the main performance scores of our experiment (number of days to criterion and number of errors in the learning and the reversal learning task). Then, we tested for covariation within individuals in the learning and the cognitive flexibility performance using Spearman rank correlation tests separated per each treatment.

Results

Brain activity

The expression of *c-fos* was significantly affected by the treatment (ANOVA: $F_{2,9} = 4.560$, $P = 0.043$; Figure 2a). The Tukey post-hoc test revealed that the fish exposed to PE displayed downregulation of *c-fos* compared to control subjects ($t = -2.985$, $P = 0.037$), while the remaining contrasts were not significant (PBAT treatment versus control: $t = -1.891$, $P = 0.197$; PE treatment versus PBAT treatment: $t = -1.094$, P = 0.541). The expression of *egr-1* was not significantly affected by the treatment ($F_{2,9}$ = 0.367, P = 0.703; Figure 2b).

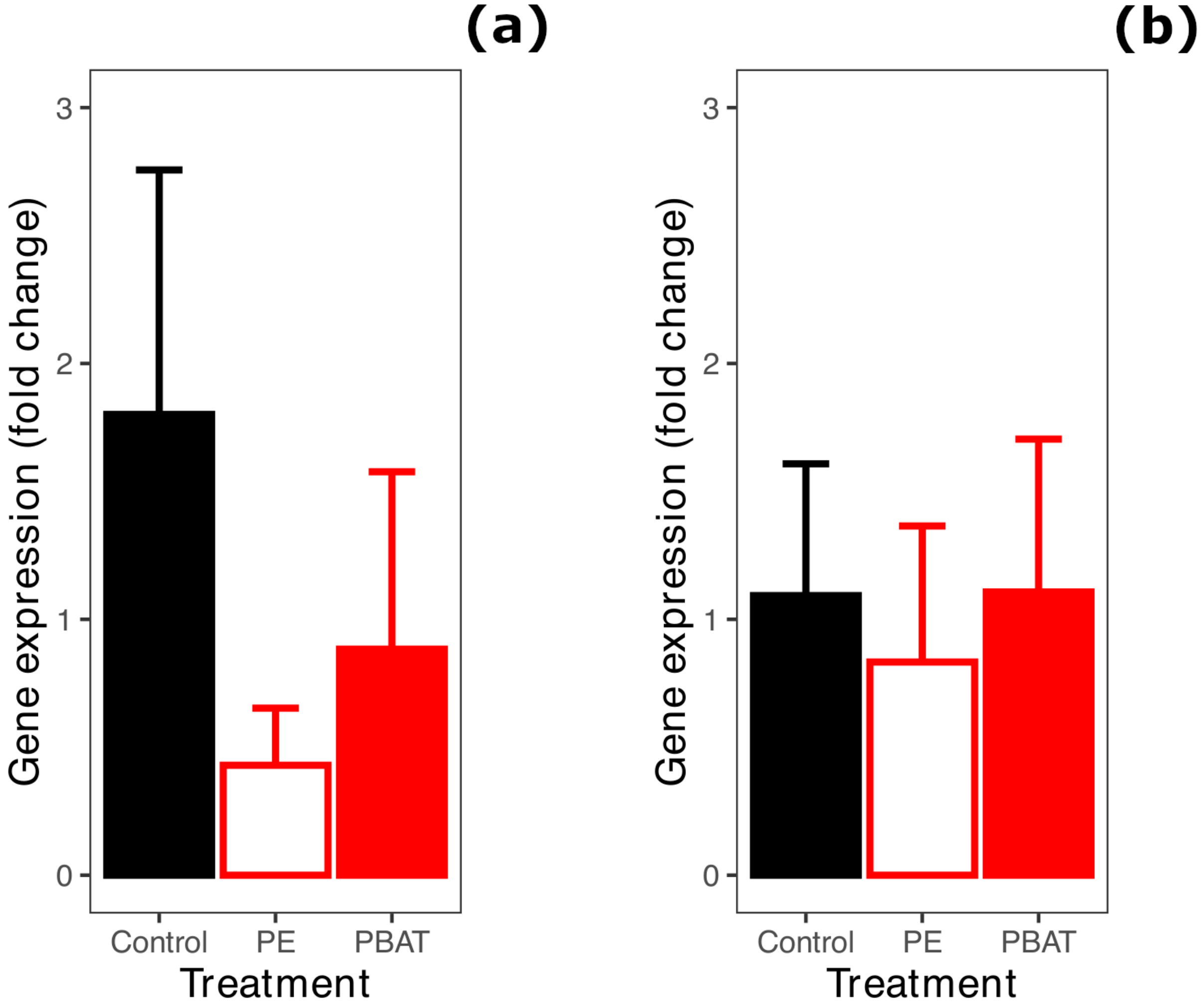

Figure 2. Results of brain gene expression. (**a**) *c-fos* and (**b**) *egr-1* expression in the brain of zebrafish from the three treatments; bars represent means and error bars represent standard errors. Control = zebrafish fed with normal food; PE = zebrafish fed with food supplemented with microplastics of a synthetic polymer; PBAT = zebrafish fed with food supplemented with microplastics of a biodegradable polymer.

Learning

Most of the subjects (38 out of 47; 80.85 %) learned the colour discrimination task according to established criterion. The number of learner subjects varied between treatments as follows: control = 11 out of 15 (73.33 %); PE treatment = 13 out of 17 (76.47 %); PBAT treatment = 14 out of 15 (93.33 %). There was no significant difference in the likelihood to solve the colour discrimination task due to the treatment (Table 2; BF = 12.73). The ANOVA on the number of days necessary to reach the learning criterion indicated no difference due to the treatment (Table 2; BF = 7.00; Figure 3a). The analysis on the daily number of errors of the learner subjects indicated a decrease across days of training (Table 2), as expected due to learning. However, the analysis found no significant effect of the treatment (Table 2; BF = 383.61; Figure 3b) and no significant treatment by day interaction (Table 2).

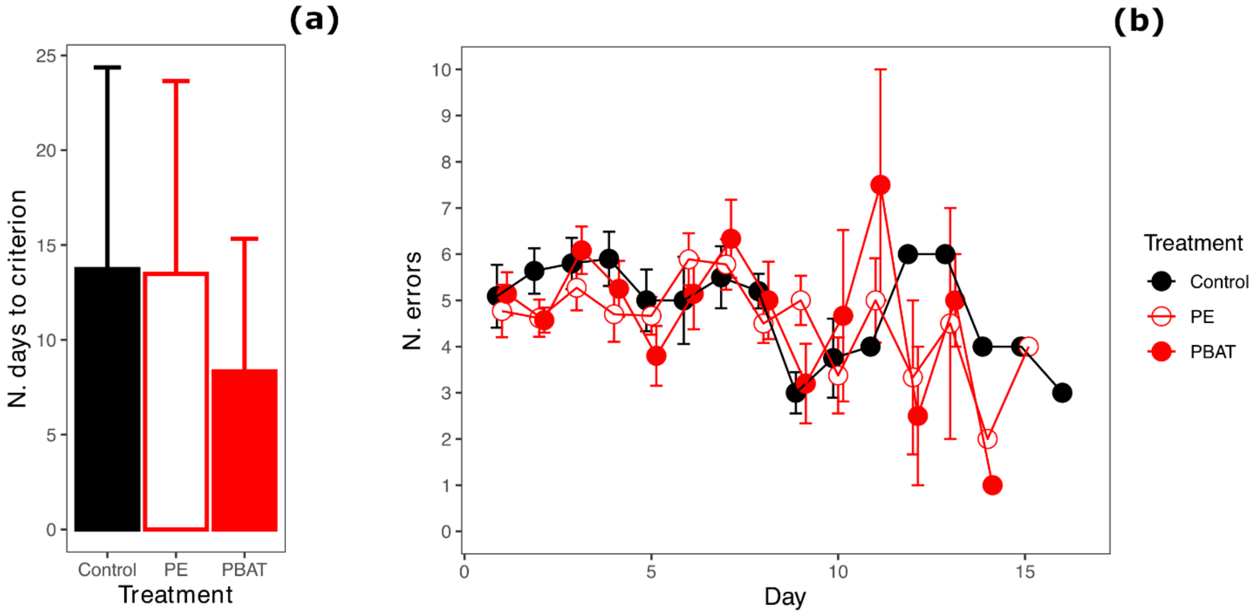

Figure 3. Results of the colour discrimination task used to assess learning. (**a**) Number of days to reach the criterion of all the subjects (N = 47) including those that did not reach the criterion (value assigned: 30 days); bars represent means and error bars represent standard errors. (**b**) Number of errors per each day of training of the subjects that reached the criterion (N = 38); points represent means and error bars represent standard errors; points without error bars indicate that only one subject had to pass the criterion. In both panels, Control = zebrafish fed with normal food; PE = zebrafish fed with food supplemented with microplastics of a synthetic polymer; PBAT = zebrafish fed with food supplemented with microplastics of a biodegradable polymer.

Table 2. Outputs of the three models used to analyse fish learning performance in the colour discrimination task. DV = dependent variable.

Term	DV 1: likelihood to learn	DV 2: days to criterion	DV 3: errors per day
Treatment	$\chi^2_2 = 2.612$, $P = 0.271$	$F_{2,44} = 1.857$, $P = 0.168$	$F_{2,35} = 0.774$, $P = 0.469$
Day of training	-	-	$F_{1,249} = 8.370$, $P = 0.004$
Treatment × day	-	-	$F_{2,249} = 0.397$, $P = 0.673$

Cognitive flexibility

Twenty-six out of 38 subjects (68.42 %) that underwent the reversal learning task reached the criterion. The number of subjects from the different treatments that solved the reversal learning task was as follows: control = 8 out of 11 (72.72 %); PE treatment = 9 out of 13 (69.23 %); PBAT treatment = 9 out of 14 (64.29 %). There was no significant effect of the treatment on the likelihood to solve the reversal learning task (Table 3; BF = 34.22). Similarly, the ANOVA on the number of days to the criterion indicated no difference due to the treatment (Table 3; BF = 36.91; Figure 4a). The analysis on the daily number of errors of the learner subjects indicated no significant decrease across days of training (Table 3), no significant effect of the treatment (Table 3; BF = 58.54; Figure 4b) and no significant treatment by day interaction (Table 3).

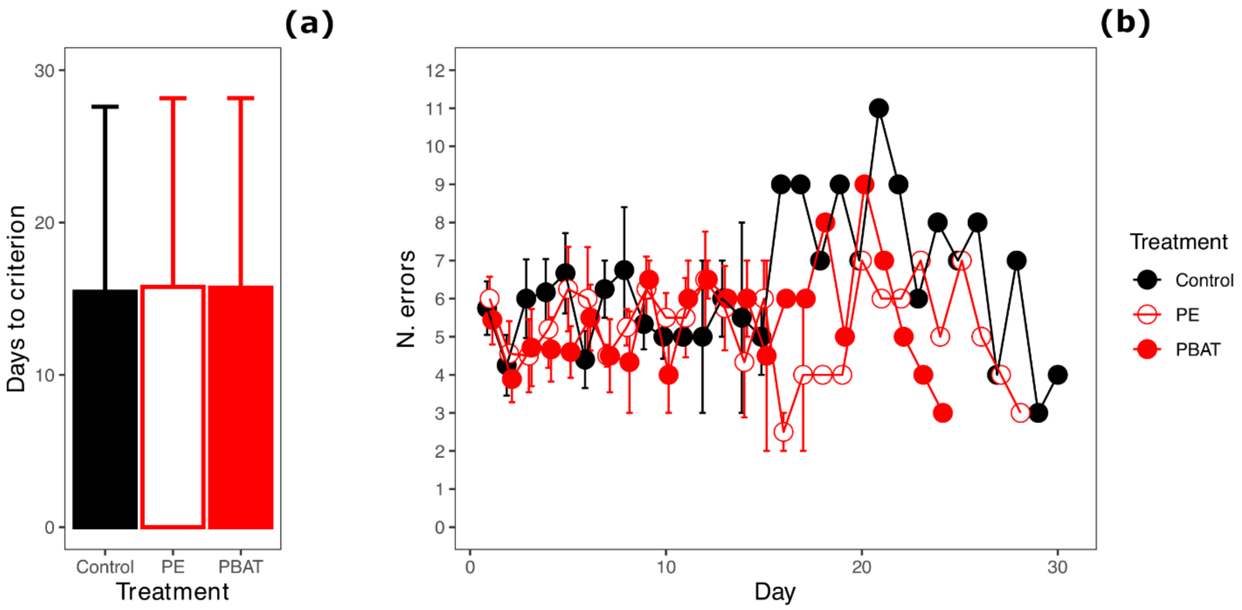

Figure 4. Results of the reversal learning task used to assess cognitive flexibility. (**a**) Number of days to reach the criterion of all the subjects (N = 38) including those that did not reach the criterion (value assigned: 30 days); bars represent means and error bars represent standard errors. (**b**) Number of errors per each day of training of the subjects that reached the criterion (N = 26); points represent means and error bars represent standard errors; points without error bars indicate that only one subject had to pass the criterion. In both panels, Control = zebrafish fed with normal food; PE = zebrafish fed with food supplemented with microplastics of a synthetic polymer; PBAT = zebrafish fed with food supplemented with microplastics of a biodegradable polymer.

Table 3. Outputs of the three models used to analyse fish cognitive flexibility in the reversal learning task. DV = dependent variable.

Term	DV 1: likelihood to learn	DV 2: days to criterion	DV 3: errors per day
Treatment	$\chi^2_2 = 0.209$, $P = 0.901$	$F_{2,35} = 3.328$, $P = 0.974$	$F_{2,23} = 0.696$, $P = 0.509$
Day of training	-	-	$F_{1,206} = 2.025$, $P = 0.156$
Treatment × day	-	-	$F_{2,206} = 0.468$, $P = 0.627$

Cognitive variability

The interindividual variability in cognitive performance did not differ between the three experimental groups both considering the learning (days to criterion: Bartlett $K^2_2 = 0.355$, $P = 0.838$; number of errors: $K^2_2 = 0.103$, $P = 0.950$) and the reversal learning task (days to criterion: $K^2_2 = 0.007$, $P = 0.996$; number of errors: $K^2_2 = 0.020$, $P = 0.990$). Moreover, there was no evidence of covariation between learning and reversal learning performance in any of the experimental groups both considering the number of days to the criterion (control: Spearman rank correlation $\rho = -0.066$, $P = 0.847$; PE: $\rho = -0.279$, $P = 0.356$; PBAT: $\rho = -0.266$, $P = 0.377$) and the number of errors (control: $\rho = -0.241$, $P = 0.474$; PE: $\rho = -0.213$, $P = 0.484$; PBAT: $\rho = -0.291$, $P = 0.313$).

Discussion

We assessed whether ingestion of microplastics derived from synthetic or biodegradable polymers affects cognition in a teleost fish, the zebrafish. Results indicated that synthetic microplastics reduced the expression of *c-fos*, a proxy of neural activation, in the brain. However, we found no evidence of phenotypic changes in learning performance measured with a visual discrimination task and no changes in cognitive flexibility measured with a reversal learning task.

c-fos expression in the brain of control zebrafish was more than twice that in the group of subjects exposed to PE microplastic. In line with what observed in our study, alterations in basal *c-fos* expression have been previously reported for fish (Baraban *et al.*, 2005; Salierno *et al.*, 2006; Salierno *et al.*, 2007; Topal *et al.*, 2015) and mammals (Hidaka *et al.*, 2011; Ryabinin *et al.*, 1997; Zhang *et al.*, 2002) exposed to stressors. Most neurons do not express *c-fos* under baseline condition (Morgan & Curran, 1989), they do so after a stimulation (Bosh *et al.*, 1995) and in the early phases of neuronal plasticity (Kaczmarek, 1993; Minatohara *et al.*, 2016). Considering that we measured *c-fos* RNA in the whole brain and in absence of specific stimulations, our data likely reflects general brain activation and rate of neuronal plasticity. We conclude that these two aspects were reduced in zebrafish exposed to the synthetic microplastics. Additionally, as *c-fos* has been linked to neuronal survival (Zhang *et al.*, 2002), our data suggests that synthetic microplastics might determine a whole-brain decrease in neuronal survival. Interestingly, the second immediate early gene considered, *egr-1*, did not show downregulation due to microplastic exposure. The two results likely reflect the different roles of these two genes (Calvo & Schluessel, 2021). For instance, *egr-1* expression is also constitutively expressed (Worley *et al.*, 1991).

None of our analyses on the cognitive phenotype provided evidence of an effect due to microplastic ingestion. The absence of significant differences between the experimental groups appears robust to the well-known problems of the null-hypothesis testing approach. Indeed, the approximate Bayes factors indicated that the models without the effect of the treatment were from 7 to 380 times more likely to explain our data, thereby providing positive to very strong evidence in favour of similarity between the experimental groups. It has been found that ingested PE microplastics determined no learning and memory impairments in the honeybees (Balzani *et al.*, 2022), which seems to align with our results. Additionally, one study reported that microplastic did not affect intraspecific behavioural variability in a teleost fish (Tosetto *et al.*, 2017), as in our study it did not affect cognitive phenotypic variability. It is worth noting that the effects of microplastics on cognition were expected to be indirect. The average size of microplastics used in our study might prevent crossing of the blood-brain barrier (Campanale *et al.*, 2020). Therefore, an eventual cognitive plasticity should be the by-product of other responses. For example, productions of hormones of the stress axis, which might be triggered by microplastic exposures, have large effects on cognition (Lindau *et al.*, 2016; Lupien *et al.*, 2007), and so have extended inflammatory responses (Sun *et al.*, 2017) and alterations in intestinal homoeostasis (Alemohammad *et al.*, 2022; Davidson *et al.*, 2018). The indirect nature of the effect might be responsible of small effect sizes, which might be difficult to detect with cognitive assays in animals. This hypothesis should be evaluated before completely rejecting the idea that learning and cognitive flexibility are affected by microplastic ingestion.

Overall, the cognitive plasticity detected by our study was limited to the molecular analysis of *c-fos* expression, which suggested potential alterations in general brain activation and/or in neuronal plasticity and survival. The question is now whether this will translate into fitness impairments in nature, when animals are exposed to microplastic pollution. One way for the brain activity alterations to impact fitness could be by affecting the cognitive phenotype, which is a relevant determinant of individuals' fitness in many vertebrate species (e.g., Cauchard *et al.*, 2013; Smith *et al.*, 2015). However, our study detected no phenotypic alterations in two cognitive assays. In rodent models, experimental reduction of the *c-fos* pathway with mutational and pharmacological procedures decreases cognitive performance in several tasks (Clements *et al.*, 2005; Fleischmann *et al.*, 2003; Grimm *et al.*, 1997). Based on the evidence of similar functions of *c-fos* between fish and mammals (Calvo & Schluessel, 2021; Matsuoka *et al.*, 1998), we speculate that fish exposed to microplastics might show impairments in cognitive functions different from those measured in our tasks. Alternatively, it is worth noting that alterations in neural plasticity and survival might determine changes in the brain circuitry that do not affect the cognitive phenotype in the short term (e.g., Zhang *et al.*, 2002) but can be detrimental during development or in case of prolonged exposure, calling for studies of these situations. Moreover, it should be considered that our experiments likely underestimated the cognitive impact of microplastics in nature. Microplastic indeed absorb chemical pollutants dissolved in the aquatic environments, and some of these chemicals are known to impact cognitive processes (e.g., heavy metals: Brennecke *et al.*, 2016; Hildebrandt *et al.*, 2021; Strungaru *et al.*, 2018). Therefore, in nature, microplastic ingestion also exposes the animals to other substances that might affect cognition. In conclusion, in spite of the limited evidence of effects directly associable to fitness in our study, a fitness impairment due to microplastic impact on cognition remains rather plausible and deserves attention in future studies.

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7. General discussion

In the studies of this thesis, my aim was to identify potential sources of cognitive plasticity in teleost fish and consequently various environmental factors that can influence cognition. The factors investigated were: general habitat enrichment, resource predictability, social environment, seasonality, and a form of pollution. Results of the studies overall indicate high plasticity of the fish cognitive system. Indeed, I found evidence of cognitive plasticity for all the aforementioned factors but not for the pollutant.

In the first study, guppies exposed to a treatment that increased the complexity of the habitat (enrichment treatment) showed greater learning in a colour discrimination task compared to guppies exposed to a barren habitat. As mentioned before in the introduction, environmental enrichment may influence the capacity of learning in several species of fish (Arechavala-Lopez *et al.*, 2020; Kotrschal & Taborsky, 2010; Salvanes *et al.*, 2013), then our results are consistent with the literature. I did not find differences between treatments in executive functions (inhibitory control and cognitive flexibility). This was at least in part unexpected because in mice researchers found effects in executive function, such as cognitive flexibility, linked to enriched condition (Zeleznikow-Johnston *et al.*, 2017). Moreover, the findings in learning is aligned with literature because Chapman and colleagues found that in guppy the social learning ability is affected by social environment (Chapman *et al.*, 2008) and the phenotypic plasticity in guppies is also affected by environment as suggested by Reddon and colleagues, in particular by level of risk of predation. Indeed, they found a strong correlation between brain mass size of male guppy in a high predation population than the male living in habitat with lower risk of predation (Reddon *et al.*, 2018), confirming that also factor as predaction risk can affect the phenotypic plasticity on morphology in this species.

In the second study, I investigated the role of living in a habitat with predictable and unpredictable food resources. In a colour discrimination learning task, guppies reared in a condition in which food was disposed in the same foraging area in a specific predetermined time passed the learning criterion faster than the fish in an unpredictable foraging situation. We can attribute this finding to saying that living in a predictable environment enhances the learning capability to cope with the necessity to learn the precise spatial and temporal values to decrease the cost of foraging in terms of metabolic effort and time spent to find the food resources (Riveros & Gronenberg, 2012). On the other hand, I found in guppies with the unpredictable situation, an increased ability to inhibit foraging and to reverse the just learn association between colour and food reward in the learning task. This evidence is in line with our previous hypothesis because in an environment in which the food occurs in different locations and time during the day, fish has to inhibit the behaviour to search for food in the same place to research it and explore the territory and also reverse the previous response given due to changing environment to search and obtain food. Moreover, this unpredictable situation can be seen as a environmental enrichment that can shape the cognitive plasticity of animals, for example in birds (van Horik *et al.*, 2019). The treatments also affected fish behaviour, but our analysis revealed that this was not the mechanism that determined the cognitive change. In the social preference task, we used like stimuli five conspecific and a barren compartment, and we did not find any difference between treatment, maybe because the stimulus of conspecifics has a stroger effect respect the other one due to the intrinsic characteristic of guppy to be a social species. On the other hand, changing stimuli with a smaller shoal instead of the barren compartment versus a larger group of conspecifics (e.g. 2 versus 8) do will not change the result of the investigation because Lucon-Xiccato and colleagues in their work on guppy, delineated a clear capacity of the fish to discriminate a shoal of four conspecifics from a shoal of six ones and also a shoal of five from a shoal of six fish (Lucon-Xiccato, Dadda, *et al.*, 2017).

In the third paper, I focused on the social environment, and I investigated how various manipulations of this factor influence the inhibitory control in guppies. Our results suggest that fish raised in the absence of a social group and in a stable social group had a higher capacity to inhibit foraging behaviour. In other words, being in a smaller and stable group may improve the capacity of inhibitory control. The result of this study did not align with the "social brain hypothesis", in which is describe a correlation between a complex habitat and a major capacity in cognitive abilities during evolution (Dunbar, 1998). A possible explanation would be that this hypothesis is valid only in specific taxa. Considering that guppies in nature are highly competitive, I also suggest that having a higher capacity to inhibit a behaviour can lead the individual to reach a food resource even if there are competitors, and this can help explain the findings of my study. Moreover, there is the evidence that support our finding in guppy described by Day and co-workers in which they involved guppy in a task to quantify the foraging efficienty. They found that in larger shoal fish located the food faster than the smaller one without any obstacle in experimental tank between fish and food. On the other hand, when they subjected fish in a tank in which the fish to reach the food had to pass throught a hole set in an opaque wall, they found the opposite pattern and smaller shoal reached the foraging areas faster than the larger groups. Lastly, when they exposed the individuals to the same experimental tank but with a transparent wall instead of an opaque one, they highlighted the greater capacity of larger shoals to solve the foraging task (Day *et al.*, 2001). They concluded saying that there was a positive interaction between shoal size and conformity.

In the fourth paper, I considered another species, *O. latipes*, commonly called medaka. I decided to study this species for its characteristic of being a seasonal fish that can respond to alteration of photoperiod. I subjected medaka into two different treatments, with a summer photoperiod and a winter light photoperiod, finding in inhibitory and learning tasks there were differences between the two treatments. As we supposed from the literature, the capacity of inhibitory control decreased in the summer photoperiod because to cope with mating and foraging and suppressing this executive function can be advantageous and can increase the fitness of individuals. Indeed, I found that medaka of the summer photoperiod attacked more the unreachable prey than the other fish in the winter photoperiod, suggesting a low capacity of inhibition in foraging. In discrimination of colour and shape, I found a lower ability to learn the task in summer medaka than the fish in winter treatment. I can address the same explanations given in the inhibition task, saying that the ecological needs of the season do not require spending energy in learning something new but well in mating and foraging. This situation is surely linked with a change of hormonal factors implied in priory in mating. Indeed, also in meadow voles (*Microtus pennsylvanicus*), a rodent, it was found that the photoperiod affected the social behaviour and in winter the meadow voles tend to form group when are in the wild. Under lab conditions, researchers found that during shorter days the individual were more affiliative with foreigns, and consequently, less aggressive (Lee *et al.*, 2019). On the other hand, during the summer, the mating period, characterized by loger days, the rodents became more territorial. Indeed, I also found an upregulation of *tshβ* and *gh* and the transcription factor *tef* in medaka maintained in the summer photoperiod.

The results of these four studies highlight a large potential of cognitive plasticity in teleost fish. First, it is worth noting that the data involved two species, which both responded with plasticity to the treatments administered. This suggests that potentially several other species of teleosts may display cognitive plasticity. Previous data in the literature support this hypothesis. Plasticity in cognition has been reported in salmon for environmental enrichment conditions (Salvanes *et al.*, 2013) and in cichlid (Kotrschal & Taborsky, 2010b). Future studies should increase the number of species investigated to address how common are the veracious forms of plasticity across the fish phylogeny. Notably, we may expect large interspecific differences. For instance, some species might have evolved in more variable environments and therefore be hardwired with greater plasticity. Fish are a great model to answer this question given their ecological diversification and the possibility to conduct large screenings.

A second notable conclusion from the first four studies reported is related to the functions targeted by plasticity. For both the enrichment and predictability treatments, I found that the effect of plasticity was not generalised. In other words, I did not observe a general increase or decrease in cognition but rather function specific effects. This conclusion tells us that plasticity is potentially very specific in its effect. Mechanisms that permit to a specific function to be altered should have evolved. A direct consequence of this finding has an impact on comparative research. Very often scholars have used gross morphological measures as indexes of cognition, such as the size of the whole brain. My study indicates that cognition is more likely composed of many, partially independent modules. Only by adding more complexity to the comparative investigations (e.g., including data on multiple functions or detailed brain areas), it is possible to correctly investigate cognitive evolution.

The finding of function specificity also aligns with the idea of the adaptive value of the observed plasticity. If a form of plasticity were not due to adaptive selection, it is difficult to imagine a function-specific effect. I can therefore conclude that cognitive plasticity is likely an important ‘tool’ that permits fish to match the requirements of their habitats. This conclusion bears further questions. For instance, what are the costs of cognitive plasticity? Is it possible that plasticity determined by a certain factor has deleterious effects due to interactions with other environmental factors? Future research should attempt to study these potential consequences of cognitive plasticity.

A question that arises from my studies is what mechanism controls the observed plasticity, especially in light of its complex function-specific action. Several hypotheses have been proposed in each specific study, and in the case of seasonal variability a potential hormonal mechanism has been also indirectly investigated. However, a goal of close future research should be to try to add analysis of the brain at the molecular level. Additionally, genetics should be added to the work. For instance, we currently do not know whether all individuals display the same form of plasticity or whether some genotypes are more prone to cognitive change.

The last paper presented in this thesis deserves a discussion on its own. I focused on the effects of microplastics on the cognition of zebrafish. In both cognitive tasks, I did not find any difference between the three treatments, revealing that microplastics used during the treatment are not involved in a direct impairment of learning and cognitive flexibility. This result is unique in my thesis, first because it involves non-adaptive plasticity and second because I did not find the expected plasticity. A first interpretation could be that the zebrafish is less plastic compared to the species used in the previous studies. A second interpretation could be that non-adaptive plasticity is less common than adaptive plasticity in the cognitive system. However, accepting these explanations is currently impossible because other low-level explanations cannot be ruled out. For instance, the size of the microplastics used may prevent them to pass the blood-brain barrier (Campanale *et al.*, 2020). Consequently, the microplastic used may not affect directly cognition or the microplastic effects could be mediated through mechanism that does not rely on blood-brain-barrier passage, for example endocrine disruption. From the molecular analysis, I observed a reduction of expression of *c-fos* in the brain due to microplastic exposure, which may indicate some alteration at the molecular level. An interesting future investigation can include the quantification of the immediate early genes (IEG) in response to other specific tasks different from learning, such as an inhibitory control task to observe how the expression of these IEG change linked with inhibitory performance. Yet, this alteration might have been too small to be detected with the cognitive tasks. Therefore, it is difficult to draw general conclusions on non-adaptive plasticity in fish cognition based on my thesis work, and more studies are required.

In conclusion, in this thesis, I try to increase the knowledge on plasticity in teleost fish. As I aforementioned in the introduction, cognitive plasticity is commonplace in animals, and it is affected by external stimuli and can influence morphological, physiological and behavioural traits. From the literature, several studies prove the presence of plasticity in mammals, birds, reptiles, fish and in invertebrates (Réale *et al.*, 2003; Price *et al.*, 2003; Meuthen *et al.*, 2018; Middlemis Maher *et al.*, 2013; Das *et al.*, 2020; Androwski *et al.*, 2017). The capacity to be plastic is connected to the need to survive in changing conditions of the habitat at multiple levels, such as alteration of social structure in populations, variation of abiotic factors of the environment and availability of food resources (Pfennig, 2021). This mechanism is also crucial for the invasion process, in which the alien species in the first stage of the invasion have to cope with the new environment and being cognitively and behaviourally plastic can help the individuals to avoid the risk of extinction. As I aforementioned in the introduction, alien species tend to be more plastic in some traits (Hazlett *et al.*, 2002). The research about the link between invasions and cognitive plasticity is overlooked, especially in fish. This possibility to expand the consciousness about this field and fill this lack of information can be useful to understand better the processes during the invasion, the behaviour of the alien species and the effects on the native species and their action to coexist in the same habitat (Hoare *et al.*, 2007). Considering all variables of environment and the relation with the capacity of the individual to be plastic into cognitive traits, is common asking what the difference between species are and whether the same factor influences individuals from different species or taxa in the same way or if there are differences in cognitive response.

It is now well established that environmental enrichment, consisting of social composition, the complexity of the habitat, and food resources, can enhance cognitive performance in different executive functions and learning (Dunbar, 1998; Salvanes *et al.*, 2013; van Overveld *et al.*, 2018).

Phenotypic plasticity can be also related to the mechanism of epigenetic variation but in this thesis, this aspect was not investigated, even if is an important process to consider in this field. Epigenetics relates to modifications of the gene expression during mitosis and meiosis that can be inherited but do not involve changes in the genome (Delcuve *et al.*, 2009). These variations in the epigenetic mechanism are given mainly by methylations of the DNA and modifications of histones and can alter the structure of the chromatin in a specific gene and its modulation during the mechanism of transcription. Since epigenetics is commonplace, we can see various examples of it in animals, like in the threespined stickleback (*Gasterosteus aculeatus*). Researchers presented a group of gravid females to a predator, Northern pike (*Esox lucius*) and a control group without any risk of predation. Then, they collected data about the antipredator behaviour of the offspring and their survival when they were set alone in a condition of predation with the same predator. The offspring of the mothers not exposed to the Northern pike showed a capacity to orient avoiding contact with the predator more than the other group of newborn, suggesting a strong antipredator behaviour also because the “orientated” offspring had major possibility to survive. Moreover, the offspring of the mother conditioned with the presence of the predator were eaten faster by the predator. Finally, the predator influence experienced by the mother may affect the behaviour of the offspring and its survival due to the same predator. Then, the stressed condition can have a maternal effect with non-adaptive consequences in the offspring when they meet the predator (McGhee *et al.*, 2012).

In this thesis is suggested that an enriched habitat can enhance the learning ability in guppy, a result that is aligned with the literature. When I focused on social composition (value of environmental enrichment) and inhibitor control ability on the same species, the result that came out was not expected, suggesting that not all the components of the habitat led to the same response in teleost fish. Predictable food resource are also a value of enrichment and composition of a habitat and my initial hypothesis found confirm in my work, saying that executive functions are enhanced by the unpredictable characteristic of food resources in guppies.

When I focused on abiotic factors in the other two species investigated, medaka e zebrafish, we found a different pattern of response, suggesting that the environmental factor used, respectively different photoperiods and ingestion of two types of microplastics, impact in different ways cognition. This difference is given by different characteristics of the factors (natural and anthropic) and by different characteristics of these species. Indeed, medaka is a seasonal species (Koger *et al.*, 1999) and responds well to seasonal variation. On the other hand, zebrafish is a model organism for toxicology research and is perfect for finding impairment due to anthropic issues and pollutions, such as microplastics (Bhagat *et al.*, 2020).

To sum up, I found how species cognitively respond to environmental characteristics of the habitat and to an anthropic pollutant.

This result can help the next step of the cognitive research in teleost fish to understand better the risk for example of pollution in different forms due to global changing and anthropic factors and how can affect the life of animals. Moreover, we focused a lot on the environmental enrichment that can help to understand the mechanism behind phenotypic plasticity and welfare. As described in my works, I gave proof that phenotypic plasticity is in these teleost fish adaptive in and can affect cognition, as described in the work about guppy and medaka, and physiological elements in zebrafish.

Another interesting point to evaluate in future research is the studies about phenotypic plasticity in match-mismatch experiments linked to fitness, in which subjects are exposed to a condition, like the presence of predator, and another group in absence of this stimulus. In case of the presence of predator, the individual that belonged to the treatment with the predation risk should have a greater ability to escape from the predator and consequently live longer with higher fitness than the subject that did not have exposure to the predator as seen in a work in tadpoles (Albecker & Vance-Chalcraft, 2015). This kind of approach gives optimal results when is sought behavioural plasticity. This aspect is not as easy to prove and test when it comes to cognitive plasticity but in great tits, (*Parus major*) researchers found that birds of urban habitat had greater problem solving ability than birds in natural environment in a task in which the birds had to learn to remove obstacle to get food and in a foraging task (Preiszner *et al.*, 2017). Moreover, there was a positive correlation between the speed to solve the problem solving task and the fitness, seen as hatching and number of offsprings. These results suggest that being in an urban context can enhance the cognitive abilities and may lead to a higher fitness success.

I studied whether and how environmental factors, biotic and abiotic, can influence the behaviour and cognition of three species of teleost. My findings confirmed the presence of cognitive plasticity in most of the cases examined and provided interesting insights into how plasticity works. Cognitive plasticity might be common in teleost fish although poorly investigated in the past in this group. Other investigations are necessary to answer some questions deriving from my work.

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