UNIVERSITÀ
DEGLI STUDI
DI PADOVA

Head office: Università degli Studi di Padova

Department of General Psychology

Ph.D. Course in: Psychological Sciences

Cycle: XXXVI

Visual illusions as potential enrichment tool for captive fish.

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Preface

This thesis is the result of three years during which my goal has been to expand our knowledge on some of the several unknown aspects of animals' cognition, focusing in particular on the behavior of aquarium fish and, more precisely, on a renowned animal model, the teleost fish *Danio rerio*, commonly called zebrafish.

The topic under consideration is remarkably delicate: it presents all the complexities of the study of the behavior of a living organism, the ultimate outcome of an incredibly complex process (even in the simplest creatures) that leads from the onset of sensorial perception to multiple parallel circles of cognitive elaborations that temporally intersect the uninterrupted stream of action necessary for each organism to conduct its life in its specific environment.

The actions are performed by the living creatures in response to the ecological challenges posed by the inescapable relation between them and the physical world in the form of spaces (habitats), qualities of these spaces (extent, temperature, etc.) and elements present in these spaces, either inanimate (water, rocks, air, light) and animate (the other creatures). The animate elements introduce a further element of complexity that each creature must deal with in order to better adjust to the ecological demands and to unknowingly try to influence the outcome of the evolutionary process. Each other animate element could represent a resource, an obstacle, or a threat, depending on its identity (prey, predator, relative, partner) in relation to other contextual variables (mating season, pre-post copulatory period, availability of food). Finally, the main cause of the combinatory explosion of possibilities is the equally active role of the "other" in the interaction, which generates a never-ending reciprocal influence.

All these features of the external world must, in the first place, be perceived through fine-tuned sensorial receptors that provide a first form of organization, and thus categorization, of the inputs. The categorization process is divided into multiple steps connected with memory processing and with the monitoring of the organism's internal state. The combination of information from inside and outside the system converges to form what we call the "motivational state", the precursor of the actions that will be performed in the absence of possible impediments. The behavior is the synthesis of all these variables in action and thus hides enormous complexities even behind the simplest action.

Differently from humans, considered until very recently the only complex living organisms worthy of attention for their ability to process information, animals don’t talk. The aforementioned complexities in the study of behavior are further complicated by the lack of reciprocal comprehension in communication.

Despite the humane limits in knowing and correctly expressing most of the time incognizable internal states, a direct form of communication through human language can be very useful for the scientific exploration of cognitive phenomena. Personal reports of feelings, memories, motivations, and plans, although sometimes unprecise or even misleading, are used as behavioral indexes of internal functions that are missing in animal studies. Moreover, reciprocal communication serves the purpose of precisely assigning tasks, checking the comprehension of these tasks, and establishing the promised consequences of behaviors during the tasks. All these necessary requirements, so naturally obtained in studies on humans, are one of the biggest challenges in the study of animal behavior because they constitute the base for the further construction of knowledge, but can be easily neglected by preconcepts or habits.

This difficulty must be overcome through careful design, multiple repetitions of experiments, and constant confrontations with those whose opinions disagree with ours. The willingness to reach solid, definitive, and fully explanatory results should not rush our interpretations and should push us to a state of mental openness that will allow us to integrate sometimes-contradictory information or information coming from different fields.

In the introduction of this thesis, I will present the results that biologists, psychologists, and neuroscientists working in the field of animal cognition have found in the last decades to provide a picture of the mental abilities of fish. These cognitive skills are necessary for these animals to cope with the challenges posed by their complex life conditions, as described above.

A particular focus will be on the perception of illusions. These phenomena are accidental products of the particular configuration of the perceptive systems that are triggered by special patterns present in nature or created by humans. Illusions are instances in which the goal of perception, that is to give the percipient the most accurate representation of the perceived, is markedly failed.

This failure produces a sense of "distortion of reality" that most of the time has detrimental effects on the fitness of the organisms because it is exploited by other organisms to take advantage of them. But the distortion of reality could also be of great use to entertain the organisms, if used appropriately: it can provide, to a certain limit, what it is for any reason impossible to supply otherwise; or it can manipulate the perception without resorting to invasive techniques.

In the two sets of experiments, I employed some visual illusion to try to provide what the species I took in exam usually actively look for: particular configurations of the space of the tank in which they live and the presence of alive conspecifics. Both features of the environment, the physical and social domains, have potentially a great impact on the quality of life of the fish but are inevitably compromised by captive conditions of maintenance.

Hopefully, these studies will help to better understand the mechanics of these needs in the species observed and to formulate new, adaptable, and cheap solutions to improve their well-being conditions.

In the first part of the thesis, before illustrating the experiments, I presented a frame of reference that will help understand the contour of the work. Firstly, I drew up a panoramic view of the complex cognitive life that these animals experience, listing their cognitive abilities. Secondly, an introduction on the theme of fish welfare exposes some of the problematics that led me to a reflection that inspired the work on this thesis: the need to develop new enrichment tools derived from visual illusions that would improve the often-insufficient quality of life of a vast number of living creatures.

PART I

THEORETICAL FRAMEWORK

Chapter 1

Fish cognition

1.1 Reasons to study fish cognition

Animal cognition is the process by which animals acquire, process, store, and act on information gathered from the environment (Shettleworth, 2009). Since the beginning of comparative cognition research, approximately a century ago (Churchill, 1916), fish cognitive abilities have been studied alongside the most well-known laboratory species, such as rats and pigeons. Despite this longstanding tradition and our constant interactions with the fish group in farming, research, and recreational activities, the general public has not gained much awareness of the great amount of information we possess on the fish's intelligence and peculiar characteristics, and this has had a deep impact on the generation of welfare protection policies (Brown, 2015).

The lack of knowledge of the abilities of these animals, so distant both in terms of living habitat, habits, and phylogeny, could cause the misperception of these creatures as mindless robots that act mechanically (Kenny, 1970), or better, react to stimuli without any further elaboration or consciousness of their actions. The presumed lack of consciousness automatically disqualifies any entity, preventing them from entering the "moral circle" (Lund et al., 2007) and from deserving empathy and attention to their living conditions just for their sake (Singer, 1981). Pain perception is usually the balancer swing in the diatribe of animal consciousness (Garcia-Larrea & Bastuji, 2018; Bradford, 2022), but it is not the only one. Consciousness is a far broader concept and includes sentience, intelligence, and self-awareness (Brown, 2015). In fact, consciousness is thought to originate as an emergent property of advanced cognitive capacities and associated complex neural circuitry (Macphail, 1998). Thus, to evaluate the potential for and growth of an animal group's consciousness, the entirety of its cognitive capacities should be examined (Kirkwood & Hubrecht, 2001; Huntingford, 2006).

The amount of research on fish cognitive abilities has grown considerably in the last decade (Pouca & Brown, 2017). The studies conducted from 2010 to 2019 account for around 70% of the total studies on fish cognition (Salena et al., 2021).

The studies on this newly established standard research animal model taxa focus especially on spatial cognition (Figure 1.1, Salena et al., 2021). Other areas deeply explored are social cognition and simple learning, and in the last position, we find studies on numerical abilities.

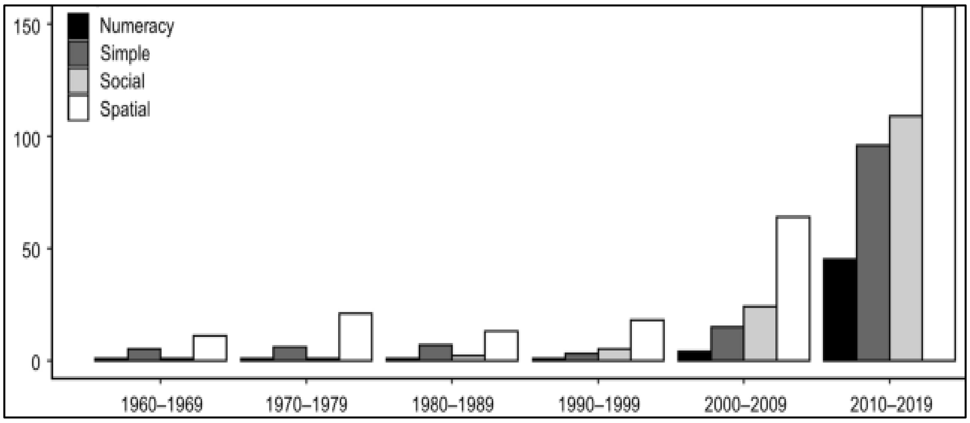

Figure 1.1. Number of studies aggregated per decade and subject (from Salena et al., 2021).

The knowledge obtained by this mass of work indicated that fish are just as good perceivers and elaborators of information as other taxa, including mammals, once overcome the difficulties inherent in comparing such different organisms. According to Brown et al., they demonstrate the full spectrum of cognitive capacities possessed by "superior" animals, including simple learning, plasticity, memory, spatial and numerical skills, and complex social cognition (Brow et al., 2011; Bshary & Brown, 2014; Sneddon & Brown, 2020).

A truly comparative approach to cognition would help to gather information from different taxa and use it to infer the ecological factors that stimulated the emergence of certain cognitive abilities, the underlying brain areas and processes, and new possibilities of solving cognitive challenges without recurring to complex mammalian-exclusive cognitive architectures, a sort of “null-hypothesis” for the primate brain (Bshary et al., 2002). We could compare several phenomena present in fish and primates, but the usual intuitive rules stated in the arguments of “Occam’s razor” (Thorburn, 1918) and Morgan’s

Canon (Karin-D'Arcy, 2005) are applied with a double standard towards taxa (Brown, 2015).

In the next paragraphs, the sum of the current knowledge on the various areas of fish cognition will be presented to depict a more precise image of the skills they possess and to catch a glimpse of their complex internal world that will help us in advancing the research hypotheses and developing fairer system to keep them in captivity.

It should be kept in mind that fish species exhibit the greatest species diversity of any other group of vertebrates, counting around 34 thousand species, due to their diffusion in world's water (FishBase, 2023). The majority of living fish is composed of teleost fish (96%) that account for around 50% of all vertebrate species (Nelson et al., 2016). This diversity makes any generalization about the group impossible, but it will serve as a starting point for further insights. Anyway, most of the research available on fish cognition has been conducted on a restricted number of species (Salema et al., 2021).

1.2 Current knowledge on fish cognition

1.2.1 Sensory perception

The first step in outlining the internal world and cognitive abilities of fish is to describe the acquisition process of information that the animal will further elaborate in its cognitive processes. Animals living in such a different environment as water could have vastly different needs and possibilities that shape their sensory perceptions. To better understand their internal world and design appropriate experimental stimuli, we should have an overview of the commonalities and differences between our and their perception.

Fish possess tetrachromatic vertebrate eyes that make them able to distinguish color nuances better than humans (Losey et al., 1999; Guthrie, 1986). Moreover, some species are capable of perceiving UV light to better detect plankton and sexual partners (Novales Flamarique, 2013; Garcia & de Perera, 2002) and polarized light to communicate, hunt, and orient themselves (Kamermans & Hawryshyn, 2011).

Visual stimuli such as video playbacks, immersive virtual reality, and visual illusions are often used in cognitive experiments (Chouinard-Thuly et al., 2017; Agrillo et al., 2020).

The latter, visual illusions, will be examined in further detail because it is more relevant to the thesis' focus.

Chemical sensing through olfaction and taste is of major ecological importance for fish. It plays a crucial role in reproductive, parental, feeding, defensive, territorial, schooling, and migration behaviour (Kasumyan, 2004). Four major classes of chemicals have been identified as specific olfactory stimuli and classified by their stimulatory effectiveness: amino acids, sex steroids, bile acids and salts, and prostaglandins (Hara, 1994). It is worth noticing that fish possess external taste buds spread all over the body (Gomahr et al., 1992), suggesting that the olfactory experience could be quite different from that of other animals and that extra caution should be adopted when designing experiments on this sense.

Another sensory experience in common with terrestrial animals is hearing. Many of the structures involved in fish hearing are identical to the ones of other vertebrates, but some of them are slightly different (Popper & Lu, 2000). Even if the hearing abilities of fish are less powerful than those of most mammals, they are greatly used in many aspects of their lives, including localization and communication (Brown, 2015). Different species develop hearing specializations tuned to the relevant sounds of their environment (Amoser & Ladich, 2005). The addition of vibration perception through the swim bladder and the lateral line greatly increases the sensitivity and allows them to swim in coordinated groups very efficiently (Wiernicki et al., 2020; Jiang et al., 2019).

The discovery that some species of fish possess mechanisms to perceive electromagnetic fields and use them to orient in space and locate objects (Graff et al., 2004; von der Emde & Fetz, 2007; Jung & Engelmann, 2019) acts as a reminder of the possibility that animal species so diverse from each other and from us could use radically different sensory channels and thus live in a completely different phenomenological world.

1.2.2 Perception of visual illusions

When the perceptual abilities of fish were examined in the previous paragraph, no mention was made of the actual correspondence between sensory responses and the "reality" of the underlying physical world. That's because sensory systems are tuned to the specific environment in which they evolved.

The sensory organs are useful tools to extract information from the physical world, but they offer only an economic and inaccurate version of it, suited to serve the purposes of the perceivers. Sometimes, some peculiar inaccuracies of the perceptual systems become evident. A discrepancy between the perceived world and the "real" world leading to noticeable errors in the acquisition of sensory information is called "illusion". The reliance on the concept of "noticeable error" is necessary, considering that there is no such thing as a "direct perception of the world". It should be noted that the interpretative nature of every brain elaboration implies that every subjective experience is, to a certain degree, an illusion (Eagleman, 2001). However, for the purpose of this thesis, we will consider only the acknowledged misperceptions that stem from assumptions or active recalibrations made by our sensory systems (Eagleman, 2011). The inaccuracy reflects the characteristics of our visual system, and visual illusions are useful tools for examining these characteristics (Watanabe, 2021).

It is also a non-invasive way to study the perceptual systems of animals and the quality of their perceptual experiences, bypassing the impossibility of communication. From the early days of comparative psychology (Révész, 1924; Warden & Baar, 1929) to today, a great variety of illusions (Figure 2.1), mostly visual, have been presented to many taxa across the animal kingdom (Kelley & Kelley, 2014; Parrish, 2021). The results of these investigations showed that in some cases, the experimental species in exam perceived the visual illusions similarly to humans, while in other cases, the illusions triggered an opposite perception or no effects at all (Feng et al., 2017). The existence of illusory perception in animal species has also been attested by the fact that some species naturally use illusory phenomena to deceive partners, prey, and predators (Kelley & Kelley, 2014).

Fish have now become the third animal group in terms of studies on visual illusions (Agrillo et al., 2020) after primates and birds. The most studied visual illusions in fish have been geometric illusions, in which some features of the stimuli distort the perception of their geometrical properties, such as length or size. A famous example is the Delboeuf illusion (Figure 2.1a), in which the size of two identical objects encircled by a smaller and a larger ring is misperceived. The Ebbinghaus illusion (Figure 2.1b) instead consists of encircling two identical objects with several big or small circles. The effect of the two is similar: in Delboeuf illusion, humans perceive a bigger object when encircled by a tight circle than by a wide one; in Ebbinghaus illusion, the same happens for wider outside circles.

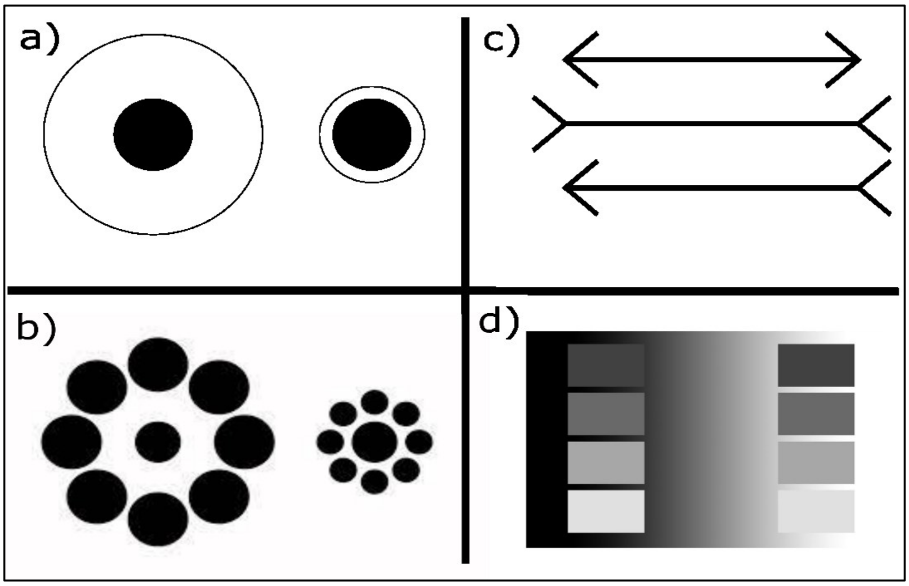

Figure 2.1. Representation of some of the illusions tested on fish: a) Delboeuf illusion, b) Ebbinghaus illusion, c) Muller-Lyer illusion, d) Simultaneous contrast illusion.

Damselfish perceive both illusions in the same way as humans (Fuss & Schluessel, 2017). Redtail splitfin fish perceive a human-like Ebbinghaus illusion too (Sovrano et al., 2015). On the contrary, bamboo sharks perceive the two illusions in the opposite way, as is the case for guppies Delboeuf illusion perception (Fuss & Schluessel, 2017; Lucon-Xiccato et al., 2019), along with zebrafish, angelfish, three-spot gourami, and Siamese fighting fish (Santacà et al., 2020b).

The Muller-Lyer illusion (Figure 2.1c) instead, consisting of two equal parallel segments that appear different in length because of the inward or outward pointing arrows at their extremities, is not perceived at all by bamboo sharks (Fuss et al., 2014), but it is perceived in a human-like fashion by redtail splitfin fish and guppies (Sovrano et al., 2016; Santaca & Agrillo, 2020).

Other illusions investigated in fish are the Rotating snakes illusion, the Kanisza triangle, and the Simultaneous contrast, respectively belonging to motion, illusory contours, and brightness illusions. The Rotating snakes illusion is a static, bidimensional pattern that triggers the perception of a circling motion in humans. In the same way, guppies and zebrafish are fooled by the peculiar pattern and will consider it as if it was rotating (Gori et al., 2014). The illusory contours of the Kanisza triangle pop out from three Pacman-like circular sectors oriented in a coherent way. Like humans, goldfish, redtail splitfin fish, and bamboo shark have been shown to perceive the non-existent triangle (Wyzisk & Neumeyer, 2007; Sovrano & Bisazza, 2009; Fuss et al., 2014).

The Simultaneous contrast illusion (Figure 2.1d) instead resembles Delboeuf and Ebbinghaus in the way that two identical stimuli are perceived differently relative to the surrounding context. Guppies and coral reef fish perceive differently the color of two identically grey rectangles depending on the background being of a darker or lighter grey (Agrillo et al., 2016; Simpson et al., 2016).

Inter- and intraspecific differences in the perception of the illusions come from different perceptual mechanisms, or styles. One of the principal candidates to explain the variations is the focus of the perceptual processes. It has been shown that in humans, the global features of a stimulus are more relevant than the local details (Navon, 1977). In different animals, the focus of attention could be directed toward other, more local features (Santacà et al., 2020c). It is worth suggesting that methodological issues could affect these perceptual processes (Watanabe, 2021). A trivial example are the dimensions of the stimuli presented. Would humans perceive visual illusions of the dimensions of a wall?

1.2.3 Spatial cognition

As we just saw, fish possess acute senses to perceive the world and have adapted to control the environment they live in to obtain better evolutionary fitness.

One of the most urgent needs that fish must satisfy is the necessity of orientation. For many organisms, finding new or tracing back old places with higher levels of resources and protection is vital. Sometimes the task could be challenging because the habitat could be missing any landmarks, or could be subject to variation. So, it is advantageous to possess mechanisms that allow orientation in different circumstances.

There are basically two non-mutually exclusive strategies to orient in space: egocentric (self-centered) and allocentric (world-centered) coordinate systems, which define relative directions, distances, and bearings (Meilinger & Vosgerau, 2010).

Egocentric strategies of orientation are based on idiothetic, self-centered cues coming from proprioceptive senses. The direction of turns and distance traveled are extracted from the reference coordinates encoded in several brain structures. In fish, the optic tectum networks are in charge of integrating many body-centered sensory modalities and translating them into muscular output, while the vestibular system offers a sense of position rooted in the invariant gravity field (Rodriguez et al., 2021). The appropriate use of these coordinates could by itself make an organism able to efficiently trace back its steps and constitute the basis for the mechanisms involved in path integration (Etienne & Jeffery, 2004; Collett & Graham, 2004; Heinze et al., 2018).

Despite the importance of self-center cues in orientation, no organism could efficiently perform any spatial task without recurring to external (allocentric) cues to some degree: the noisiness of environments and the possibility of an unintended displacement would make the task fail with no possibility of correction. Allocentric strategies instead involve the self-localization of the organism inside a wider system of coordinates independent from the individual features, based on local cues such as beacons and landmarks, or based on the spatial representation of environmental information about terrains placed beyond the current range of perception, forming cognitive maps (Tolman, 1948; Eden, 1988). The use of cognitive maps allows the use of "wayfinding" strategies (i.e., topological navigation, surveying, or metric navigation) (Wiltschko & Wiltschko, 2023), but requires extended learning and memory skills (Odling-Smee & Braithwaite, 2003). Fish hippocampal pallium is essential for processing and encoding complex spatial information to form map-like representations of the environment (Broglio et al., 2003).

Besides the distinction between the two strategies and the preference of each particular species, it is reasonable to think that complex and crucial behaviours like navigation and orientation are sustained by multiple redundant mechanisms to ensure the best results. In the study by Rodriguez et al. (1994), it was shown that goldfish could use either of the two strategies or both, depending on their previous training. The role of previous experience emerges in Brown (2007) too, even without any specific training. The rearing environment is in fact sufficient to affect the dominance of the use of featural (color) and geometric (shape) cues in a cichlid species. Another factor influencing the use of different strategies is the actual availability of one or another orientational cue.

Fish have a good sense of vision, and they use it to extract information from the environment to orient themselves (Warburton, 1990; Braithwaite & de Perera, 2006). But as it is described above, fish possess other refined senses that can be useful in the process. Salmons heavily rely on olfaction to recognize the odors associated with their natal site that they will use for homing successfully later in adulthood (Dittman & Quinn, 1996).

Surprisingly, the sound background of the natal reef is also used as a distinctive mark for orientation by young coral fish that have been dispersed over many kilometers in their larval state (Simpson et al., 2005). On a larger scale, astronomic or geologic positioning systems can be the best solutions to rely on. The use of the position of the sun or the perception of the polarization of light guides movements in some species of fish (Goodyear & Bennett, 1979; Hawryshyn et al., 1990), while others seem to be able to use the geomagnetic field to swim in open waters (Kalmijn, 1978).

In order to perform the complex navigation tasks requested by their ecological habits, fish must be able to use all the information available and integrate it in a coherent way (Huijbers et al., 2012). The role of the hippocampal pallium is crucial not only for the integration of different sensory modalities but also for the integration of the spatial with the temporal dimension of relational memories (Rodriguez et al., 2021).

Thanks to the several systems utilized in navigation, fish are able to accurately estimate distances, sizes, and volumes (Santacà et al., 2020a; Holbrook & de Perera, 2009), even in the absence of vision (Jung & Engelmann, 2019). With adequate training, they can solve complex mazes consisting of six consecutive T-junctions (Lucon-Xiccato & Bisazza, 2017) and efficiently orient themselves using geometric and non-geometric cues (Baratti et al., 2022).

1.2.4 Social cognition

Social cognition refers to the mechanisms by which animals acquire, process, store, and act on information from other individuals (Shettleworth, 2009). Social behaviour, in some of its manifestations, is considered to be a special kind of activity. The value that sociality has played in human development is well known and considered the spark that started the great cultural revolution that led us to diverge so deeply from our closest primate relatives (Dunbar, 1998; Oesch, 2018). The so-called "Social brain hypothesis" argues that the great evolutionary pressure put on creatures by living in complex social groups led to an increase in brain volume. This hypothesis holds only for primates. It seems to be a generalization of the relationship existing between large brain sizes and pair-bonded mating systems (Dunbar, 2009). Regardless of the causal role played by extended sociality in the development of skills and specializations, it seems undeniable that it is at least an indicator of complex cognitive abilities.

Like in many other areas of cognition, a wide range of possibilities of instantiation of a social group exist, differing by the strength of the group bond and by the awareness of belonging to a group that manifests itself in terms of the quality of the relationships with other members or non-members.

Some authors have found that certain highly coordinated group behaviours that at first glance seem to be the expression of high-order cognition could be emergent properties of multiple individual local rules of thumb acting together (Couzin et al., 2002; Buhl et al., 2006; Couzin, 2007). These behaviours do not belong exclusively to sociality but rather merge into it after originating from other domains.

Some other complex social interactions may require specialized cognitive processes such as joint attention (Siposova & Carpenter, 2019), shared intentions (Tomasello & Carpenter, 2007), the ability to imitate (Bates & Byrne, 2010), and emotional processes like empathy and the ability to maintain close, stable bonds (Bshary et al., 2014). Empathy, in particular, is regarded as the highest form of social cognition because it seems to imply some sort of identification with another subject in most of the various definitions given (Cuff et al., 2016). A partially overlapping construct is “theory of mind”. The ability to make inferences about the status of a particular informant's knowledge could be thought of as the cognitive counterpart of empathy because it involves the interiorization of other entities’ perspectives (Carlson et al., 2013), but it does not imply nor require the emotional component, although the two can act in synergy to maximize the level of social competence reached (Orpinas, 2010; Seyfarth & Cheney, 2013).

With the possible exception that there are no known eusocial fish, the range of social patterns in fish spans essentially the whole variety of sociality observed in animals. Fish have a variety of social structures, from loose groups with constantly changing membership that typically serve a single purpose to complex, long-lasting groups made up of members with a range of ages, sexes, reproductive, and social roles (Taborsky & Wong, 2017). Among the peculiarities of fish that shuffle the traditional mechanisms of sociality, like indeterminate growth and flexibility of sex determination (Bruintjes & Taborsky, 2011; Kobayashi et al., 2012), a crucial difference is the low levels of relatedness within groups, which reduce the importance of rigid kin selection mechanisms (Hamilton, 1964) and increase the importance of reciprocity.

One element of social competence is social recognition: fish are able to distinguish between conspecifics and non-conspecifics and between familiar or non-familiar individuals based on chemical cues or even solely vision (Griffiths & Ward, 2011; Sovrano, 2004). Fish usually prefer to swim with conspecifics, or in their absence, with familiar heterospecifics (Al-Imari & Gerlai, 2008; Ward et al., 2003). Familiarity with unrelated individuals is obviously based on experience, but visual recognition of conspecific features seems to be learned too, indicating an active role of memory in the process (Griffiths, 2003; Engeszer et al., 2004; Gerlach & Lysiak, 2006). Another ecologically important social information to obtain is the social rank in a dominance hierarchy (Bessa et al., 2021). Living in a social hierarchy presents several cognitive challenges associated with the maintenance of order through social signals (Chen & Fernald, 2011; Fernald, 2014). Furthermore, it has been shown that fish can infer the implied hierarchy vicariously by simply observing fights between rivals arranged around them in separate tank units (Grosenik et al., 2007; Hotta, 2020). The practice of eavesdropping has been studied in several works on zebrafish and Siamese fighting fish (Abril-de-Abreu et al., 2015; Doutrelant & McGregor, 2000).

The presence of others does not represent only a source of information but has an important effect on the behaviours of the observer fish too, called the audience effect (Plath et al., 2008a; Doutrelant et al., 2001), of particular relevance to intrasexual communication and mate choice. The “public” signaling behaviour emitted when the subject is aware of being observed could lead to honest, exaggerated behaviours (Desjardins et al., 2012; Pinto et al., 2011) or dishonest, deceiving behaviours (Bshary, 2002; Plath et al., 2008b). Some authors claimed that a coral reef fish is able to produce referential gestures to the multi-specific partners of a collective hunt (Vail et al., 2013). Referential gestures are considered a key element in language development and have been observed only in apes and ravens so far.

The observational skills of fish could help the process of information transmission and lead to socially enhanced learning in antipredatory (Suboski et al., 1990), orientation (Lindeyer & Reader, 2010), foraging (Laland & Williams, 1997; Thonhauser et al., 2013), and mate choice behaviours (Witte & Ryan, 2002). These results contribute to correcting the idea of social learning from a prerogative of "higher" mammals into a regular feature of vertebrate life (Brown & Laland, 2003).

Realizing that even simpler cognitive systems could sustain complex social systems through a variety of strategies more or less similar to mammals' is a productive approach to comparative social cognition that could produce a rationale applicable across species with varying degrees of complexity in their social structures. (Oliveira, 2013).

1.2.5 Numerical cognition

Numerical abilities are another example of skills that have for a long time been considered exclusive to humans as base for a greatly refined cultural product as mathematics, but that are instead widespread in virtually all the animal kingdom, from other mammals to insects (Bortot et al., 2021; Agrillo & Bisazza, 2018; Nieder, 2021).

The diffusion of these competences and the precocity with which they develop in many animal species led to the belief that they belong to some inborn core knowledge mechanism, along with physical and geometric competence, that is fundamental to facing life challenges and from which to build more complex and specialized abilities (Vallortigara, 2016).

Numerical competence is indeed a fundamental skill in many ecological occurrences like foraging, defensive responses, reproductive and safety strategies, and parental care (Nieder, 2020; Messina et al., 2022).

A well-known example of this idea is the spontaneous preference, observed in many fish species, to swim together with other conspecifics in shoals of variable sizes, to reduce the possibility of being predated and enhance foraging efficiency (Pitcher, 1993). The safety obtained is directly proportional to the size of the group joined, and in fact, when presented with two options, fish will swim with the largest group (Ogi et al., 2021; Agrillo & Bisazza, 2018). This spontaneous tendency has been utilized to assess the boundaries of competence in several species of fish.

The few species examined showed the ability to discriminate the numerosity of two shoals of conspecifics, choosing the largest, differing by just one element. While the guppies managed to distinguish between four and five fish (Lucon-Xiccato et al., 2017), the mosquitofish could not and only reached the easier discrimination of three against four (Agrillo et al., 2008). Lastly, the angelfish and the redtail splitfin demonstrated the ability to tell apart only until the limit of two versus three conspecifics (Gómez-Laplaza & Gerlai, 2011a; Stancher et al., 2013). But the redtail splitfin succeeded in discriminating quantities that spanned the boundary of the supposed discrimination limit, that is, 1 versus 4 and 2 versus 4, showing that the cognitive system is probably influenced by the ratio between the two numerosities in addition to an actual count based on object identification, which was thought to be the only system operating for small numerosities (Feigenson et al., 2004; Agrillo et al., 2012). The difficulty in number discrimination seems to increase as the numerical ratio between the two quantities approaches one. For larger quantities, ratio-dependent accuracy becomes the only alternative available, allowing guppies, angelfish, zebrafish, and mosquitofish to discriminate up to a ratio of 0.5 when one group is twice as large as the other (Agrillo et al., 2008, 2011; Gómez-Laplaza & Gerlai, 2011b; Seguin & Gerlai, 2017). The only recorded case of fish exceeding this ratio is the three-spined stickleback, which managed to reach an exceptional 0.87 (Mehlis et al., 2015).

The other ecological drives, like foraging and parental care, have been used to test numerical competence in fish too. Guppies spontaneously select the larger number of food items with contrasts of 1 vs. 4 and 2 vs. 4 (ratio 0.5, Lucon-Xiccato et al., 2015), while angelfish discriminate between identically sized food quantities with a numerical ratio of up to 0.67 (Gómez-Laplaza et al., 2018). 0.67 ratio discrimination was reached by females of convict cichlids trying to recover fry from the larger of two groups displaced from the nest too (6 vs. 9, Forsatkar et al., 2016).

If using spontaneous choices for natural preferences confers a great advantage in terms of ecological validity and time saved, this approach also has critical limitations that can be overcome with the recurrence of training procedures. Even if these procedures are time-consuming and hard to implement both in their manual and automatic form (Gatto et al., 2021), they offer the possibility to easily control for non-numerical quantity factors, like the size of the stimuli to count, the overall surface they occupy (convex hull), or their quantity of movement. In nature, most of the time these variables covary, making the disentanglement between the concrete and abstract properties of numbers impossible. The design of controlled and standardized stimuli could be easily achieved with some computer programs (Zanon et al., 2022). Accounting for these confounding continuous variables leads to a drop in the performances of fish (Messina et al., 2022), but the same is true when the discrete variable is not available, meaning that the conjunction of the two redundant sources of information is required for better performance (Agrillo et al., 2011). In addition to the opportunity to control the features of the stimuli, training procedures on numerical preference raise the number of correct choices up to 90%, a performance comparable to that of primates (Bisazza et al., 2014; De Long et al., 2017; Hanus & Call, 2007). Moreover, training procedures allow for precise determination of the focal stimulus. In this way, it is possible to control the criteria of choice used. For example, Miletto Petrazzini and colleagues (2015) found that the guppies will not choose the absolute numerosity of the stimulus targeted for reinforcement in a double choice paradigm, but its relative numerosity compared to the other option. The same generalization of relative numerosity was found in archerfish (Potrich et al., 2022).

1.3 Problematics and open questions in fish cognition

As we saw, the rise in interest in fish cognitive abilities led to an accumulation of information about various domains of intelligence that will be relevant in several comparative fields.

Nonetheless, the precision of this knowledge should not be taken for granted. Besides the usual problematics of many scientific fields (restricted sample size, low power, lack of preregistration, and replication), comparative cognition must face its own peculiar problematics.

In particular, there is an open debate on the ecological relevance of the information that has been collected on fish cognition. Salena and colleagues (2021) found that over 90% of the studies conducted on fish cognition took place in a laboratory (Figure 3.1). The laboratory setting is a fundamental help to control the variables involved in the experimentation but represents a radical change from the natural world in which the fish live, where multiple stimuli and motivational drives are present simultaneously and produce unique complex effects that can be isolated only on condition of losing the overall picture (Patton & Braithwaite, 2015; Leavell & Bernal, 2019).

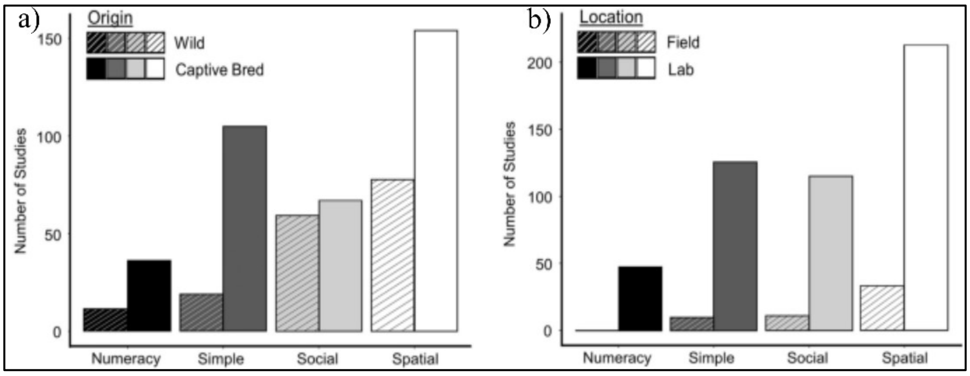

Figure 3.1. Number of studies on fish cognition divided by subject indicating a) the origin of the experimental subjects and b) the location where the studies have been conducted (from Salena et al. 2021).

Moreover, almost 70% of the studies use captive-bred rather than wild fish (Salena et al., 2021). This fact is of great relevance because it exposes the subjects to environments that are most of the time not properly enriched and to selective processes that are different from the natural ones. The effects of captivity are measurable in a few months, within a single generation of a fast reproduction cycle (Christie et al., 2012). Using captive fish would avoid these effects, along with the ones caused by the plasticity of fish that readily adapts to the new conditions (Ebbeson & Braithwaite, 2012).

The employment of enrichment techniques and ecologically relevant stimuli could help improve the performance on cognitive tests in fish (Strand et al., 2010; Bshary & Triki, 2022).

This ecological approach to ontogenetic variation will help to better understand the cognitive abilities of organisms that evolved and adapted to a specific wild environment (Salena et al., 2021).

1.3.1 Sources of variation: sexual differences, personality and lateralization

One problematic feature that could alter our perception of fish cognition is the relative ignorance of many aspects that affect the behaviours we are measuring. In most of the studies on cognition and behaviour is assumed that the experimental subjects come from a homogeneous group and that each one is an adequate representative of the species. But, as all biological phenomena, cognition is a complicated study topic because of its great variability among different species and within each species' population. Every trait, in fact, manifests itself in a continuous manner in each individual, depending on several factors. Some of the sources of variability are strictly biological; others are more exposed to experience. Fish are no exception.

One of the main sources of individual variation is sex. Sex deeply influences hormonal production and morphological characteristics. Moreover, in an evolutionary context, the different sexual roles impose differential selective pressures favoring certain cognitive traits in one sex depending on their function (Wallace et al., 2020). Sex influences several domains of cognition in fish. Like in many other species (Jones et al., 2003), the spatial abilities measured differed between the two sexes in geometric orientation tasks (Sovrano et al., 2003) or in solving complex mazes (Lucon-Xiccato & Bisazza, 2017). In the same study, females and males expressed similar performance in a detouring task. Detour is not merely a spatial problem because the transparency of the obstacle makes the final goal visible to the experimental subject. The task is then transformed, involving the inhibitory control abilities of the prepotent response to a highly desired outcome. Sex affects performance in detour tasks (Wallace et al., 2020; Triki & Bshary, 2021), object recognition (Wallace & Hoffman, 2021), problem solving (Lucon-Xiccato et al., 2020), and more generally affects the whole set of executive functions (Lucon-Xiccato, 2022). Nevertheless, in most studies on mature individuals, the sex of the specimens is not reported (Salena et al., 2021).

Sex seems also to be the greatest factor influencing the relationship between cognition and personality traits across different species (Dougherty & Guillette, 2018). The term personality trait, sometimes called temperament, includes a repeatable behavioural difference over time and across situations. There are still some methodological and terminological problematics in this relatively new field of inquiry. The many different labels could indicate the same core trait, or one label could refer to multiple constructs that do not consistently fit together, and the tests designed to measure these traits (open field, novel object, scototaxis, emergence test) substantially overlap in the intended measurement but without strongly correlating (Carter et al., 2013). That said, a generally accepted framework in current personality research considers five broad axes of animal personality: shyness-boldness, exploration-avoidance, activity, aggressiveness, and sociability (Réale et al., 2007).

Despite the pervasiveness of personality traits in affecting behaviours, it is still unclear which consequences these traits have on cognition and fitness in general (Dougherty & Guillette, 2018) and how deeply the different traits are influenced by genetic predispositions and by experience (Balestri et al., 2014).

Another factor influencing behaviours in otherwise similar members of the same group is cerebral lateralization. The phenomenon has been found across all taxa of vertebrates and has been studied extensively on fish through the use of a visual task in a detour test (Bisazza & Brown, 2011). The cerebral lateralization manifests itself in an asymmetrical processing of cognitive functions that often becomes noticeable in observable behaviour. The classic methodology to assess laterality in fish is to determine the preferred eye through which it observes a stimulus of different nature, like conspecifics, predators, or novel objects. The preferred eye projects to the contralateral hemisphere, which is thought to be dominant for the specific elaboration triggered by the stimulus.

The basic behavioural asymmetries are simple turning biases that can transform themselves into choice biases when a stimulus is presented or cumulatively become rotational biases. Due to the lateralization of many personality traits, emotions, and cognitive functions, the unbalance in hemispherical symmetry could cause important effects in several kinds of social behaviours (Berlinghieri et al., 2021) and enhance cognitive performance thanks to a parsimonious avoidance of duplication of functions and allowing parallel processing and divided attention (Vallortigara & Rogers, 2005). The causes of lateralization can be of various natures: genetic mechanisms, hormones, light stimulations, and rearing conditions (Miletto Petrazzini et al., 2020). So, it would be important to assess the magnitude of the laterality of each experimental subject before drawing conclusions on its performance on each cognitive test.

The more information is collected on the experimental subjects, the more complete picture of their real cognitive abilities we will have.

Chapter 2

The wellbeing of captive fish

Very little of the great cruelty shown by humans can really be attributed to cruel instinct. Most of it comes from thoughtlessness or inherited habit. The roots of cruelty, therefore, are not so much strong as widespread. But the time must come when inhumanity protected by custom and thoughtlessness will succumb before humanity championed by thought. Let us work that this time may come.

Albert Schweitzer, Reverence for Life

I see shining fish struggling within tight nets, while I hear orioles singing carefree tunes. Even trivial creatures know the difference between freedom and bondage. Sympathy and compassion should be but natural to the human heart.

Du Fu (712–770 AD)

The experimental subjects involved in this thesis are a few representatives of an enormous category, captive fish, with which they share many differences in terms of biology, ecology, scope and characteristics of their captivity state, and the problematics involved in it. But the core of their condition, besides each peculiarity, is the same: the insufficient attention and improving measures towards their well-being.

This chapter will provide an introduction to the size of the phenomenon of aquaculture, some operative definitions of wellbeing, and some possible solutions experimented to improve it.

2.1 Diffusion of fish and raise of awareness.

Despite the lack of precise measurements on the actual number, various indicators suggest that the number of fish being raised and kept in captivity is constantly growing. The 2022 report from the Food and Agriculture Organisation of the United Nations (FAO, 2022) describes a steady 4-5% yearly increase in the number of aquatic animals produced by aquaculture in the last decade. The exponential growth of this sector is even clearer when comparing the 0.3 tons of global fish production in 1950 to the 54 million tons in 2016. The number of fish produced by aquaculture is now equal to the fish captured, thanks to new technical and industrial developments that helped us humans cope with the difficulties of offering animals such different from us (land living) the appropriate conditions to thrive and reproduce (Lucas & Southgate, 2012).

More than half of this production is carried out on inland farms using purpose-built facilities, whose features are necessarily a compromise between economic efficiency and the respect of the pleasant living conditions of the animals. These numbers only consider the production of species for alimentary purposes, missing the count of those who are kept as pets and the species that are employed in scientific research.

When compared to the ones aimed at consumption, these small and colorful fish are surely insignificant in size but not in number. A conservative estimate of around two million amatorial fish keepers were spread around the world at the beginning of the century (Wabnitz, 2003), a figure deduced from the economic revenue of the industry (Andrews, 1990; Tlusty, 2002). A decade later, the Ornamental Fish International (OFI, 2023) reported that more than 120 countries are involved in the collection, breeding, import, and export of ornamental fish and the 2010 FAO report (Monticini, 2010) estimates that 1.5 billion fish are traded annually. Around 90% of the fish traded are captive-bred freshwater fish.

One last growing sector involved in the increase in the number of captive fish is research. Once again, there are no precise reports available, only estimates. Many countries do not report their use of research animals, such as the USA, which lists fish under the label "other animals", and the ones that do report it are quite approximate. The European Union used around 1.3 million fishes for research purposes in 2016 alone (Communication and Information Resource Center for Administrations, Businesses, and Citizens (CIRCABC), 2020). Norway alone, possessing a large aquaculture industry, used roughly the same number of fish for research, mostly aimed at developing fish vaccines to further improve the sanitary aspects of large-scale aquaculture for alimentary purposes (Mattilsynet, 2019). The same is true for Canada (Speaking of Research, 2019) and Australia (Humane Research Australia, 2016).

Fish are used for research into fundamental biology, genetics, cancer research, vaccine production, physiopathology, and diagnostic work. Additionally, fish are employed for telemetric monitoring in the wild and for biocide testing, but these applications make up a relatively minor portion of all fish utilized in experimental research. (Kristiansen et al., 2020).

Together with carp, salmon and trout are the largest single fish commodity by value in world trade (FAO, 2022). The huge economic importance of salmonids led them to be the most studied group and to find new ways to increase production and improve the efficiency of the farming industry.

Zebrafish (*Danio rerio*), on the other hand, is the most studied fish species, counting tens of thousands of publications in the last eighty years (Kinth et al., 2013), followed by medaka, guppy, stickleback, and goldfish (Oltova, 2022). These model species are typically robust, small, easily bred fish with a short generation interval. They do not require much space, as they are small and prefer to be in large groups or shoals. Among these species, zebrafish is particularly suited for biomedical observations because their eggs and larvae are transparent and relatively large, which allows microscopic observations and manipulations of embryos as they grow into fully formed larvae. The larvae grow rapidly, with all major organs developing within 36 hours (Kimmel et al., 1995).

Its genome was sequenced in 2013, allowing a crescent precision in genetic studies that capitalize on the extended homologies between it and the humane one (Howe et al., 2013).

This trend contributed to the increase in the number of specimens in circulation to the order of tens of millions (Kinth et al., 2013). The prolific biomedical research field is constantly increasing the number of fish used as animal models in place of mammals, as suggested by the 3R Principle (Maestri, 2021). In particular, the zebrafish (*Danio rerio*) is nowadays adopted as one of the best animal models for pharmacological, genetic, and neuroscientific research (Kinth et al., 2013; Harper & Lawrence, 2016).

Similarly to what happened for the intensive farm production of mammals and birds after the Second World War, the abrupt increase in the number of farmed fish in the last decades of the past century triggered the concern of animal protection groups in the early 1990s that started filing reports on the numbers and on the conditions of farmed fish (Lymbery, 2002). This awareness campaign affected the public, causing a transformation in the moral, political, and scientific landscape, especially in Europe. Nowadays, fish welfare is a critical aspect of sustainable aquaculture for nearly 30% of European consumers (Zander & Feucht, 2018). The shift in public opinion towards increased attention to the thematic influenced European and national authorities to create control organisms and funding institutions devoted to the care of fish conditions. New welfare standards for Atlantic salmon and rainbow trout were created in the United Kingdom (Royal Society for the Prevention of Cruelty to Animals (RSPCA) welfare standards, 2018). Other widely adopted standards, such as the Aquaculture Stewardship Council (ASC, 2023) Farm Standards for Sustainable Aquaculture and the Global Good Agricultural Practice Aquaculture Standard (GlobalGAP Aquaculture, 2023), do not specifically address animal welfare but rather place a greater emphasis on sustainability and minimizing the environmental impact of fish farming. However, their fish welfare checklists include crucial criteria for guaranteeing fish wellbeing and are a useful resource for farmers.

The international organization in charge of the control of the ornamental fish trade field is the World Organisation for Animal Health (OIE), established in 1924 in Paris and ratified by 167 countries. Its principal objectives are to protect global trade, guarantee the health and well-being of products of animal origin, publish health standards for live animals, and provide practical experience in epizootic controls. The foundational regulations of this organism derive from the Aquatic Animal Health Code (Speare, 2006) and from the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2009). The accent in this case is almost entirely on the avoidance of disease transmission across countries and on transportation measures that resemble more advice directed towards goods that must not be damaged. There is no mention of the interior state of the animal.

The legislation and guidelines relative to animal research, at least in the EU (Council of Europe, 1986, 2010), seem instead to be more comprehensive in terms of the different dimensions of the animal's well-being. In the first place, providers and users must have sufficient people on site and ensure that they are properly qualified and educated before performing every possible step of the research, such as designing and carrying out procedures on animals, taking care of them, and eventually killing them. Fish are offered the same protection as other vertebrates, but the indications relative to care and accommodation and basic and superior needs (such as "proper environmental enrichment"), even if present for the first time in all the documents we examined up to now, are quite brief compared to other vertebrates and undifferentiated in terms of species-specificity and situation-specificity. In fact, no species except zebrafish (but only in Annex I, which contains the list of species that must be purpose-bred) are individually mentioned. The 2014 revision council of Europe's Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Anderson, 2014) instead provides indications relative to the ten most widely used fish species, but the background documents produced by the dedicated working groups and the relative references to the scientific literature are not publicly available.

Nevertheless, this document expresses a much more inclusive concept of animal well-being that emphasizes the role of appropriate living conditions in terms of housing and enrichment to reduce stress-induced behaviors and extend the range of occupations *"...available to the animals and increase their coping activities including physical exercise, foraging, manipulative and cognitive activities, as appropriate to the species…calibrated on the species and individual needs of the animal concerned."*.

There is still a lot of work to do to improve the current state of protection for fish in Europe, where the legislation is already quite advanced, and in the rest of the world. China in fact accounts for almost two-thirds of global aquaculture production by weight, compared to a tiny 1% for Europe (Lucas et al., 2012). One important step to creating international guidelines or making the existing international legislation effective is to reach a consensus on the definitions of welfare as widely as possible around the world.

2.2 *Wellbeing definitions*

As previously illustrated, not only different countries can have different sensitivities regarding what is considered an appropriate caring attitude towards captive fish, but often different sectors of society, or even the same institution, depending on different tradeoffs, could vary its approach and regulations on the same topic. The enormous economic interests behind the fish farming industry, for example, demand the maximization of profits, derived in turn by the reduction of costs. All the measures to improve welfare imply in one way or another an increase in costs, so they are limited to the bare minimum.

The sector that is less tied to economic motives is research. Its productivity is less improved than in other sectors by the simple reduction of expenses. Moreover, the foundation of the scientific collective enterprise is staying true to what has been proven. The continuous attention of several branches of science towards other living beings made it hard to not realize the continuity that binds us humans to other species and thus probably shifted the moral compass closer to what are the conclusions to reasonable assumptions and testable/tested hypothesis.

The first systematic works on the topic in the sixties (Brambell, 1965; Thorpe, 1965) directly inspired the newly created Farm Animal Welfare Advisory Council (FAWAC) to establish five conditions that all the animals should obtain when kept under the responsibility of humans, the “Five freedoms”, that are still in use today (FAWAC, 2009), because of their wide consensus among professionals, animal protection NGO’s and governmental groups such as RSPCA in Australia and the UK; the Animal Welfare Institute in the USA (but not the Humane Society); the World Organisation for Animal Health; as well as the World Society for the Protection of Animals (WSPA), who make reference to them in the draft text for a UN Declaration on the Welfare of Animals (McCausland, 2014).

The “Five Freedoms” are (FAWAC, 2009):

1) Freedom from hunger and thirst—by ready access to water and a diet to maintain health and vigor.

2) Freedom from discomfort—by providing an appropriate environment including shelter and a comfortable resting area.

3) Freedom from pain, injury, and disease—by prevention or rapid diagnosis and treatment.

4) Freedom to express normal behavior—by providing sufficient space, proper facilities, and appropriate company of the animal's own kind.

5) Freedom from fear and distress—by ensuring conditions and treatment, which avoid mental suffering.

Along with the great success of these statements, there has also been some criticism. Some authors (Webster, 2005; McCulloch, 2013) evidenced that the definitions are based on negative aspects of well-being ("freedom from"), while they should include positive constructs to aim for, that some freedoms refer to multiple or complex conditions, and that the provisions that accompany the freedoms are mostly constitute by general words, susceptible to interpretations.

The two authors agree on these characteristics of the guidelines, but they differ in their opinions towards them. In Webster (2005), the "Five Freedoms" are not sufficient for a comprehensive framework because the list is judged incomplete. In McCulloch (2013), instead, the freedoms are judged to be individually necessary, because a lack of each one of them would cause inevitably suffering, and jointly sufficient, even though they must remain revisable in response to scientific advancements, as a framework for the analysis of animal welfare. The overlapping of the definitions of some freedoms, like discomfort and hunger/thirst, is an indispensable redundancy to ensure the complete satisfaction of interconnected needs. For this author, the negative form of the affirmations of the freedoms could be correctly reversed in most cases, such as freedom to eat and drink versus freedom from hunger and thirst, but not in other cases in which there is no positive counterpart to the prohibition. For example, freedom from pain cannot be expressed by the right to experience pleasure because they are not complementary sensations (McCulloch, 2012).

The lack of focus on positive experiences has also been criticized by Mellor (Mellor, 2016; Mellor et al., 2020). The authors suggest that negative sensations can never be eliminated because they are genetically embedded to be the fundamental drive of motivation for any living being. So, the effort should be towards the constant temporary neutralization of these negative experiences and the provision of positive experiences, such as comfort, pleasure, interest, confidence, and a sense of control. On the same line, Korte et al. (2007) proposed a different conceptualization of welfare that considers the adaptation of an organism in response to a just amount of environmental challenges as positive for the well-being of the animal, in contrast with both hyper and hypo stimulation (Figure 1.2).

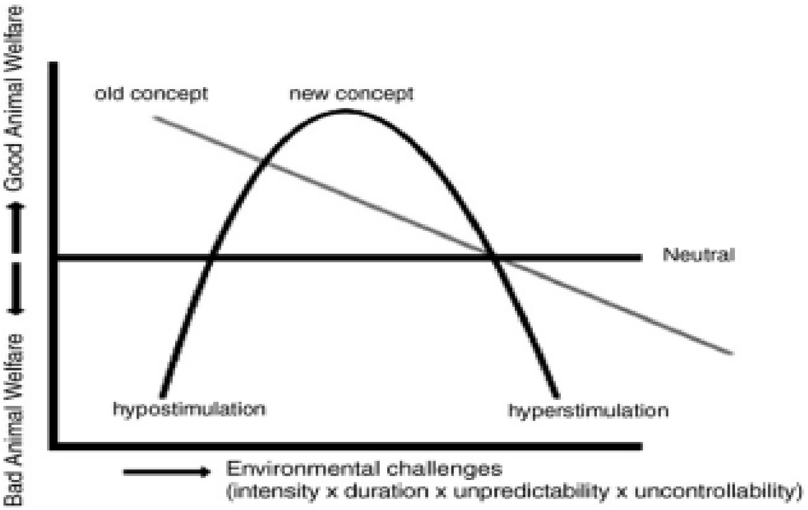

Figure 1.2. Illustration of the new conception of welfare (inverted U-curve) compared to the old conception (straight line) in relation to environmental challenges (from Korte et al., 2007).

Mellor et al. (2020) proposed an alternative "five domains" model, with four physical and functional domains (nutrition, environment, physical health, and behavior) that jointly influence the fifth domain, the animals' mental state, in response to the criticisms of the focus on negative characteristics. Both the "five freedoms" and the "five domains" models have been criticized for placing inadequate emphasis on prosperous welfare states (Mellor, 2016). As a result, the "five domains" concept has recently been expanded to take into account experiences that animals might have that could produce good effects (Mellor & Beausoleil, 2015).

However, the generality of the definitions is mandatory: the conditions formulated must refer to every possible species and cover virtually every possible occurrence, so their definition must be broader and more comprehensive than possible. Tailor-made tautological definitions are of no help in designing a comprehensive framework that is plastically adaptable to new advancements. The downside of this idealistic formulation is the structural contrast with the precise verification of the single adoptable measures that these recommendations can inspire.

Clearly, every definition of animal welfare is necessarily a product of human-made reflections based on the presupposition that it is possible for us to understand the relevant domains of animal well-being. An unconscious anthropomorphism usually makes humans focus on what is relevant to their own senses and motivations instead of the animals'.

The definitions of the animals' needs could be based on one or more elements of the following:

Physical/function-based definitions: the physiological and behavioral systems should function appropriately to guarantee the animal's complete overall health.

Feelings-based definitions: the internal emotive states of the animal should be generally positive, free from prolonged negative states such as fear and pain.

Nature-based definitions: the lives of the captive animal should resemble to the best the natural conditions, being able to use and develop their natural adaptations and skills.

2.2.1 Nature-based definitions

These definitions are partially overlapping and, in the modern view, should be integrated into a broad and plastic definition of animal welfare (Fraser et al., 2008; Hewson, 2003). A naturalistic environment should, in fact, lead the animal to express its natural set of behaviors, regulated by adequate levels of physiologic activations. A successful response to the environment should give the animal a sense of satisfaction and general positive feelings. But the nature-based concept of well-being, although linear in line of principle, hides problematics that can make its application difficult or even undesirable.

In the first place, most of the time we do not know extensively the natural conditions in which captive animals live, and even if we possessed that information, we are far from understanding the complex relations inside an ecosystem, such as the role of unexplored habitats (Pitcher et al., 2007; Hunter, 1991) or unexplored elements of the biology of the animal (Reese & Dunn, 2018). For example, olfactory enrichments are widely used as a form of enrichment in zoos, but it has been shown that the misuse of these enrichments can lead to counterproductive effects, such as behavioral stereotypies (Clark & King, 2008). These effects are caused by a lack of understanding of the meaning of naturalistic scents and their consequent bad implementation.

Besides that, the concepts of nature and naturality are not univocal. They depend on different ecological paradigms that could prefer to focus on some aspects more than others and result in different approaches to the conservation of naturality (Swart et al., 2001), balancing between a functional approach that wants to capitalize on the positive effects of naturality only, eliminating the other useless or negative aspects, and an approach focused on wilderness that instead values the pure self-regulating ecological mechanisms on the ecosystem and avoids any human intervention to soften some aspects. Some authors (Tomasik, 2015; Musschenga, 2002) focus on the importance of animal suffering in nature and consider natural life as an intrinsic value, despite the possibility that its effects could be enriching or harmful at the same time. Even though recently some authors tried to challenge the view of a cruel nature that provokes mainly enormous suffering to animals (Palmer, 2022; Browning & Veit, 2023), it is undeniable that exposure to uncontrolled environments can and will eventually cause suffering to the animals (Johannsen, 2020; Horta, 2010).

It is thus evident that there is a discrepancy between the consequences of natural life and the requirements of wellbeing definitions in captivity. Most of the definitions do not include the possibility for the animal to suffer, which is an ineliminable regularity in nature. So, the two disciplines, animal welfare and ecology, should be tackled separately because they differ in their objectives and scopes for the lives of animals (Soryl et al., 2021). An intermediate point between the two is what is called an “arcadian approach”: the maintenance of a balanced semi-natural condition in which human influence is considered, under some circumstances, to be a positive element because it may enhance wellbeing and may lead to a harmonious life (Swart et al., 2001).

2.2.2. Feelings-based definitions

The feeling-based definitions of animal wellbeing are, in principle, the most correct ones. The focus is, in fact, on the internal world of the animal, which is, ultimately, the best metric of well-being, at least in theory. The feelings-based approaches admit the possibility of a discrepancy between pain and suffering and between pleasure and enjoyment (Rowan, 1988), that is between known, observable inputs and their hidden and still not fully understood elaborations. The subjective content of experience should be the metric to adopt to judge and intervene on animal welfare (Duncan, 1993), because it is the most proximate construct to what we call “wellbeing”. Unfortunately, a subjective state is, by definition, refractory to objective exploration, which is needed in order to evaluate it with confidence. The inner mental life of each organism is in fact hard to access in its core (or unknowable; see Nagel, 1980; Bonjour, 2013). Nevertheless, the root of this perspective is to at least try to approximate it better than previously, when attention was focused almost exclusively on physical health rather than psychological health (Veasey, 2017). The challenge of accurately measuring internal states through the use of behavioral and physiological methods is hard (Desire et al., 2002), but its advancements would represent the best possible approximation to the reality of the internal experience of the animals that we keep in captivity. New frameworks of interpretation will help to have a better and more holistic comprehension of animal emotions (Mendl et al., 2010; Boissy et al., 2007) from a singular and comparative perspective (Anderson & Adolphs, 2014), through the discovery of new behavioral and physiological indicators (Boissy et al., 2007; Briefer, 2012; Descovich et al., 2017; Dolensek et al., 2020; Waller & Micheletta, 2013) and the implementation of neuroscientific knowledge on emotions (Alexander et al., 2021; Adolphs & Anderson, 2018).

2.2.3. Physical/function-based definitions

As mentioned before, the most widespread approach to animal welfare is based on observable and quantifiable features, such as biological and behavioral parameters. Pragmatically, it has been assumed that these measures correlate adequately with welfare conditions experienced by the animals (Broom, 2011). Moreover, observability and quantifiability make these definitions more tied to the scientific domain than the moral one.

One possible way of objectively judging animals’ captive life conditions is through life history traits. These factors, such as longevity, growth pattern, and features of progeny, are heritable but at the same time greatly influenced by life experience (Oli & Coulson, 2016; Nylin & Gotthard, 1998). The measurement of these parameters is the main reference point for production settings that mostly care about performance (Curtis, 2007), but it is also a useful tool to compare captive and wild conditions in the absence of other indicators (Wich et al., 2009; Wiese & Willis, 2004; Robeck et al., 2015).

Another quantitative assessment of animals' wellbeing, widespread in both research and industry, is neurochemical and endocrine measurement. Many neurochemical and endocrine changes occur in response to stressful events, but unfortunately, the precise role of these neurotransmitters and hormones in determining the internal wellbeing state of the organisms is unknown or poorly understood (Clark et al., 1997a). Some reactions can be categorized as adaptive coping mechanisms that successfully manage the dangers and thus contribute to wellbeing. Different reactions might be categorized as aberrant, maladaptive, or affective disorders. An ongoing danger may cause the neuroendocrine system to become chronically hyperactive, which harms rather than helps with wellbeing (Clark et al., 1997b).

Nevertheless, some of the most popular methods to assess stress levels in animals are the measurements of stress hormone metabolites, such as glucocorticoids (Palme, 2012), or neuroendocrine hormones, such as ACTH, noradrenaline, and adrenaline, which are more sensitive to acute stress (Hickman, 2018). One advantage of these measures is that they can also assess long-lasting effects of poor welfare, such as chronic stress (Trevisi & Bertoni, 2009).

The last quantitative measure of well-being in this section is behavioral observation. Behaviors are the final product of the means by which most functions of the organism are carried out. Differently from hormonal activation, which reflects a complex miscellaneous state, behaviors are usually quite directly associated with the functions and needs they are related to. Behaviors are good indicators of basic functions (eat, drink, sleep) and more complex ones (social, recreational). They are good tools for assessing bot animal health and motivational state at the clinical and pre-clinical level (Dawkins, 2003) and show great sensitivity in response to external conditions (Veasey et al., 1996). A careful observation, when a sufficient amount of species-specific information is collected, can become a useful tool to assess psychological states (Mitchell & Hamm, 1997), especially in context when a relationship with a human caregiver is in action and can become the basis for establishing some form of communication through behaviors (Wemelsfelder et al., 2000; Meagher, 2009; Fleming et al., 2016; Waiblinger et al., 2006).

Recently, attention has grown towards classic communicative behaviors such as vocalizations (Briefer, 2012) and facial expressions (Dolensek et al., 2020; Waller &

Micheletta, 2013) in animals. Other behaviors that are thought to be indicative of the general affective state of the animal are anticipatory behavior towards the possibility of reinforcement (Watters et al., 2014) and positive “judgment bias”, the tendency to interpret ambiguous stimuli as positive and react accordingly (Bethell, 2015; Seligman, 2006). Negative states such as pain (Landa, 2012), anxiety (Steimer, 2022), and boredom (Meagher, 2019) are expressed through behaviors too, and we can refine our attention and ability to interpret them.

In fact, the immediacy and transparency of behaviors can become double-edged qualities. The relative ease of acquiring information could lead to superficial or rushed interpretations (Watters et al., 2021) or anthropomorphizations (Urquiza-Haas & Kotrschal, 2015). In conclusion, the indirectness of the measures of wellbeing, even the quantifiable ones, is hard to interpret.

How each variable responds to a stressor can be affected by several factors of the stimuli, and the responses differ between species, between individuals, and within the individual (Mason & Mandy, 1993). It is thus a good practice to combine several different kinds of measurements to obtain a precise picture of the animal’s welfare state (Broom, 1988).

2.3 Fish problems and enrichment strategies

From our mammalian perspective, fish are relatively unfamiliar creatures. Their living conditions and environment are difficult to imagine and relate to. A lack of identification does not help the development of empathy, causing a delay in public attention to their welfare conditions compared to more familiar mammals and birds (Driessen, 2013). Sometimes the identification effort could come from unexpected subjects, like anglers (Bear & Eden, 2011), but it does not always translate into a change of consciousness (BBC News, 2009). One of the first codes of conduct for hobby and trade was promulgated by the trade organization Ornamental Aquatic Trade Organization (OATA, 2015).

Due to their exoticness, fish are often excluded from the moral circle that would oblige human moral agents to actively care for them (Bovenkerk & Meijboom, 2012), or they are considered less significant than humans for their inferior cognitive abilities

(DeGrazia, 1999) and for their classic place in the nowadays outdated but still relevant "Scala Naturae" (Hodos & Campbell, 1969; Diogo et al., 2015).

One particularly relevant topic in the discussion is whether fish could experience pain. Nociceptors are present in fish nervous systems, granting an unconscious reflex response to aversive stimuli (Braithwaite, 2010), but in order to truly experience pain, the pain signal should be sent and elaborated in the brain (Bovenkerk & Meijboom, 2012). The principal objection to this possibility is that fish lack neocortical areas, so the responses are "just" automatic behavioral programs controlled by lower levels of the central nervous system (Rose et al., 2014). But the coherence with which simple (hormonal) and complex (behavioral) activation reacts to aversive stimuli seems to be reasonably good reasons to infer that fish experience suffering (Huntingford, 2006; Lund et al., 2007; Whittaker & Brown, 2023; Sneddon & Roques, 2023) and probably have systemic circuitry elaborating pain stimuli different from neocortex (Chandroo et al., 2004). Admitting a "complex" experience of pain would mean acknowledging some degree of conscious state (Garcia-Larrea & Bastuji, 2018), one of the most agreed-upon requirements to be a welfare subject (Bradford, 2022; Dawkins, 2017).

Let's now try to consider all the possible sources of distress that captive fish could experience. All the stages of the life of a captive fish, from capture to the trading process to the eventual final stable condition, are constellated with possible different welfare problems, originating from the quality of water, from the physical and functional properties of the aquaria, from the concentration and compatibility of the other occupants of the tank, and from the quality of food they are offered (Kristiansen et al., 2020). Each threatening situation leads to a psychophysiological response, the stress response, which activates two hormonal systems, leading to the production of corticosteroids and catecholamines. The cascade effect alters the allocation of resources to vital areas of the organism causing a general imbalance that negatively affects other necessary life functions, especially if the exposure to stressors is prolonged and/or repeated (Schreck & Tort, 2016).

The aversive effects of stress can be mitigated, or even better, prevented, by the implementation of environmental enrichment. Environmental enrichment can be described as an improvement in the biological functioning of captive animals resulting from modifications to their environment (Newberry, 1995). Enrichment is now considered a fundamental part of zoo animal husbandry, becoming more complex in its form and mode of presentation (Young, 2013). Research on environmental enrichment has grown consistently in the last decades, targeting for the most part research animals (70%), followed by farm animals (15%) and zoo animals (10%). Ninety percent of the studies targeted mammals (de Azevedo et al., 2007). Behavior is generally preferred to hormonal and neurological assessments as indicators of improved well-being (Binding et al., 2020).

For the purpose of this thesis, we will focus on the implementation of environmental enrichment techniques on captive fish, which represent only a small percentage of the animals subject to enrichment studies. Being the most widespread research animal category for this kind of study, most of the results come from the most widespread research animal fish, the zebrafish (Kinth et al., 2013).

Following Bloomsmith and colleagues (1991), we can divide environmental enrichment into five domains: dietary, physical, sensory, occupational, and social.

2.3.1 Dietary enrichment

Dietary enrichment does not refer to the composition of the diet (nutritional enrichment). It refers to the food type and to the features of feeding distribution, like quantity and periodicity. Every species has distinct species-specific feeding strategies that make them prefer a certain type of food (floating flocks, sinking pellets) depending on their feeding habit. The species could be surface or bottom feeders and prefer to feed at certain hours of the day (López-Olmeda et al., 2012).

It has been proposed that living prey could constitute an additional form of enrichment, but while the effects on growth and survival have been assessed (Monteiro et al., 2018; Fowler et al., 2019), only verbal reports of improved general welfare exist (Lidster et al., 2017).

2.3.2 Physical enrichment

Physical enrichment is by far the most studied form of enrichment (Naslund & Johnsson, 2016). The term physical enrichment includes every possible increase in environmental complexity through the insertion of structures and substrates. These enrichments could serve as shelter for a number of widespread farmed fish species (Coulibaly et al., 2007; Pounder et al., 2016; Cogliati et al.l 2019; Kistler et al., 2011), as repair to reduce aggression rate and damages (Mork et al., 1999; Basquill & Grant, 1998; Kadry & Barreto, 2010), or just to increase the complexity of the environment, making it more similar to the naturalistic one and stimulating cognitive functions (Salvanes et al., 2013; Spence et al., 2011; Strand et al. 2010). Substrates, in particular, are of great importance, especially for fish that interact extensively with the bottom surface (Hardy & Hale, 2020). They can ease the camouflage process, thus improving the sense of protection (Howell & Canario, 1987; Marcon et al., 2018) the prophylactic process (Ottesen et al., 2007; Arndt et al., 2001; Kang & Kim, 2013). and the reproductive process, serving as incubators (Benhaïm et al., 2009; Spence et al., 2007a).

2.3.3 Sensory enrichment

Although many of the aforementioned physical enrichments could be considered appropriately as sensory enrichment on a visual and tactile level, their main function is not sensory stimulation per se. Sensory stimulation has been extensively employed to enrich the conditions of many animal species, but often fish are excluded from the list of possible targets of these treatments (Wells, 2009). This may be due to inattention, because fish show great sensitivity to basic sensory conditions such as lighting, noise, and temperature. (Arechavala-Lopez et al., 2022).

Studies on farm and laboratory fish showed that system-lighting conditions (intensity, spectrum, and periodicity) could greatly affect all aspects of fish's lives (Sánchez-Vázquez et al., 2019; Güller et al., 2020; Williams et al., 2009).

The sound environment should also be controlled to avoid the detrimental effects of noise. Some species are more affected than others (Popper & Fay, 1993), but it is important to reduce the amount of environmental noise that fish are exposed to, to allow stress-free performance of biological functions (Popper & Hastings, 2009; Ladich, 2004) in both captive (Davidson et al., 2009) and wild conditions (Kusku, 2020).

Chemical senses are probably the most relevant among the others for their ecological impact (Valentinčič, 2004). They are extremely important for intra-specific communication in reproductive (Sorensen & Wisenden, 2015) and dominance (Keller-

Costa et al., 2016) contexts. They are the most relevant signals during foraging (Hansen & Reutter, 2004) and predation (Arvigo et al., 2019). Thus, great attention to the regulation of the accumulation of these olfactory cues is crucial for maintaining adequate living conditions for captive fish (Mota et al., 2014) and avoiding negative chain effects (Toa et al., 2004).

Tactile senses are almost as relevant as chemical ones (Kasumyan, 2011), as they are deputed to the same ecological functions, and they may be easier to understand because of the visibility of the tactile triggers.

Besides these basic maintenance conditions that try to avoid fish exposition to noxious or unpleasant stimuli, other studies focused on positive forms of sensory enrichment, trying to provide positive, pleasant stimuli instead of simply avoiding and removing the bad ones (Lawrence et al., 2019).

For example, many studies tried to discover which color preference fish may have (Siregar et al., 2020) and color the tank wall in the most pleasant way for the fish (McLean, 2021; Okada et al., 2015). Another way to provide positive visual stimulation is through the use of mirrors (Desjardins & Fernald, 2010), although their effect could often be opposite than expected (Balzarini et al., 2014), and monitors that project interesting images to the fish, such as moving conspecifics (Qin et al., 2014).

A positive auditory stimulus that has been proposed for several species is music (Alworth & Buerkle, 2013), including fish. The few works that exist on the topic point towards the positive effects of music for growth (Vasantha et al., 2003) and for the reduction of anxiety indicators (Barcellos et al., 2018). A special type of artificial sound presented to captive fish could be their habitat soundscape (Filiciotto et al., 2013). The techniques employed in positive tactile stimulation fall into the physical enrichment category, as mentioned above. The roughness of substrate and structure is, in fact, supposed to be appreciated for its function of helping reproductive or prophylactic purposes, although it could also be perceived as a general positive sensation (Gauy et al., 2021). Another popular technique is the use of waterflows (Arechavala-Lopez, 2022), but it will be examined under the category of "Occupational enrichment".

2.3.4 Occupational enrichment

As just mentioned, providing tanks with a regular stream of water could be beneficial for fish well-being. A water flow would stimulate the motor function of the fish that could be inhibited in a restricted tank with still or turbulent water (Liao & Cotel, 2012). The swimming activity would represent a form of physical exercise (Palstra & Planas, 2011; Huntingford & Kadri, 2012) and a possible way to cope with stress (Schirmer et al., 2013).

Some authors (Burghardt, 2015; Oliveira et al., 2010) have suggested that fish have the ability to play, defined as the voluntary and repetitive manipulation of a non-food object, excluding instances of intention or purpose, for the only sake of a pleasurable experience by itself, and some include human interactions as an instance of it (Fife-Cook & Franks, 2021).

Lastly, training is being explored as a form of occupational enrichment able to give the animal more control over its environment, add behavioral choices, promote species-appropriate repertoires, and empower the animal to deal adequately with challenges (Westlund, 2014; Fernandez, 2022). To date, unfortunately, it has never been tested on fish.

2.3.5 Social enrichment

Social enrichment is probably the most complicated form of enrichment examined. It is strongly ambivalent in its effects and yet to be fully understood. Social life in fact represents a great source of advantages in nature, but at the same time, it presents several costs (Ward & Webster, 2016). It is very hard to draw the exact lines to avoid isolation on one side and overcrowding on the other (Adams et al., 2007; Baldwin, 2011). Due to economic reasons, excessive stocking density is one of the major problems of commercial aquaculture and a certain source of stress for captive fish (Ramsay et al., 2006; Arends et al., 1999), but undercrowding could lead to severe negative effects as well (Gilmour et al., 2005), like the rise of the level of aggression to establish dominance hierarchies (Harper & Lawrence, 2016; Galhardo et al., 2009). Besides varying the stocking density to the appropriate concentration, the use of virtual reality has been tested as a possible alternative to provide the needed sociality while avoiding its drawbacks (Stowers et al., 2017; Soyka et al., 2016).

2.4 Generalizations and specifications

Fish is the largest existent group of vertebrates (Facey et al., 2022) and shows remarkable diversity in the different evolutionary adaptations produced by the species included in it. Each one of these species could have more or less slight differences in terms of their basic and advanced needs, which must be assessed with precision. It is thus important to consider the specificity of each finding and the adequacy of its implementation in each species.

Moreover, when studying and applying enrichment techniques, particular attention must be drawn to the so-called **DETAILS** (**D**imensions, **E**cological rationale, **T**iming of enrichment, **A**mount, **I**nputs, **L**ighting and **S**ocial environment) (Jones et al., 2021), a set of specifications on the precise modalities of presentation of enrichment that will greatly help in the standardization of the results obtained by the research in the field.

The number of animal species used in aquaculture systems is greater than in terrestrial systems. Although each species possesses unique behavioral and physiological traits and environmental requirements, the overall results suggest that the trend of the effects of environmental enrichment on growth, activity, aggression, stress, and disease resistance within the captive welfare trait, growth, activity, sheltering behavior, competitive ability, foraging behavior, and cognitive abilities, is positive (Zhang et al., 2022).

Fish that receive enrichment have been shown to develop their brains more than fish kept in sterile environments (Marchetti and Nevitt, 2003; Kihslinger and Nevitt, 2006; Salvanes et al., 2013); experience less stress, have better foraging ability, and have improved post-recovery behavior (Brown et al., 2003; Strand et al., 2010; Rodewald et al., 2011).

Despite the overall consensus on the importance of providing fish with a complex, custom-made environment, it must be mentioned that several studies have reported conflicting results regarding the effects of environmental enrichment. For example, Brydges & Braithwaite (2019) found no significant differences between the behavior of three-spined sticklebacks exposed to ten months of enrichment compared to conspecifics kept in a state of unenriched captivity. Moreover, it is possible that enrichment could affect differently physiological aspects of fish, like growth rate, stress-related hormonal expression, and behavioral response to stressors, as reported by Zhang and colleagues (2019).

2.4.1 Conclusive remarks on zebrafish

All the experiments in this thesis include zebrafish as experimental subjects. Each new finding on this species could have a cascade effect on all the fields that use it as a model. Despite the diffusion of these creatures and the increasing amount of knowledge on their ideal lifestyle and sensitivity to external conditions, many relevant details regarding their captive habitat preferences are missing, and even when they are present (Stevens et al., 2021), they are often ignored, even by experts (Message & Greenhough, 2019).

Figure 2.2. Picture of the standardized tanks in which the great majority of zebrafish raised for research purposes live.

For example, while it has been shown in this chapter that zebrafish like large, enriched tanks with structures and substrates necessary to carry out their natural behavioral repertoire and regulate their stress levels, the standard maintenance conditions for zebrafish ignore this body of research. The zebrafish are kept in completely transparent tanks, whose dimensions are just a few body lengths with no structure or substrate (Figure 2.2).

It is important to keep these valuable animals in the best conditions possible to ensure their wellbeing and obtain clean results, unaltered by the limiting effects that captive life could cause.

The use of zebrafish in the neuroscientific and biomedical fields is of capital importance for the advancements of medicine and our general understanding of living systems, but it comes at a high price for the species we exploit. A compassionate perspective that tries to overcome the suffering and distress of the animals should be a primary focus.

PART II

EXPERIMENTAL SECTION

Chapter 3

Depth preference for real and illusory surfaces

As mentioned in the previous chapters, it is clear that the physical space in which fish live affects their lifestyle extensively. They are able to perceive small differences in the geometry and size of aquariums (Braithwarte & de Perera, 2006; Baratti et al., 2022) and prefer the most suitable environments for their needs (Huijbers et al., 2012; Näslund & Johnsson, 2016).

The size of the tanks in which fish live is greatly relevant in relation to the number of fish they are living with. Overpopulation is one of the biggest problems in aquaculture because it relates directly to the efficiency of farming. The less space and resources pro capita allocated, the lower the expenses. But living at too high densities implies a dramatic increase in aggressiveness and fights among fish, which in turn produces different other negative effects (Ramsay et al., 2006; Arends et al., 1999; Galhardo et al., 2009). Low densities could be detrimental for the wellbeing of captive fish too (Gilmour et al., 2005). It is thus important to consider different possibilities when it comes to the design of the physical environment.

Several other features of the physical space are important for the quality of life of captive fish in terms of functional aspects of behavior, such as the opportunity to seek shelter using structures or camouflaging with the bottom of the aquarium (Pounder et al., 2016; Cogliati et al., 2019; Hardy & Hale, 2020). For example, it is known that different species vary in time spent in proximity to the water surface or to the bottom of the tank, especially when they are frightened (Cachat et al., 2010) or when specific tasks of their lives, like foraging or mating, must be performed (Sessa et al., 2008; Sims et al., 2005). The preferences for certain types of environments are in fact influenced by the ecological conditions in which the different species live. Evolutionary pressures shaped the morphological, cognitive, and behavioral habits of the animals, making them adapt to a particular niche (Bergmüller & Taborsky, 2010; Chase & Leibold, 2009). In the first part of the study (Experiment A), we wanted to quantitatively measure novel and known but anecdotal information regarding the depth preferences of four globally widespread species for research and recreational purposes.

Unfortunately, quite often the information available on some species' preferences is ignored (Message & Greenhough, 2019), citing economical or practical reasons like the money, time, and personal resources needed to implement those measures.

In the second part of the study (Experiment B), we wanted to use the data collected to test the possibility of using a visual illusion to enrich the physical environment of these species. Freshwater fish in general have a good sense of vision (Caves et al., 2017) that is used to collect information from the surrounding environment. As resumed before, they are also sensitive to some visual illusions to which they have been exposed to (Agrillo et al., 2020). With these experiments, we could assess the efficacy of a visual illusion based on pictorial cues on the visual system of these species. The illusion should trigger a false sense of difference in the altitudes of two sectors of the tank, which should orient the fish's decision towards one area. In the case of positive results, the stimulus could be used to create fake levels that nonetheless exert a positive effect on the fish without resorting to resource-consuming techniques.

3.1 Experiment A: Assessing inter-specific differences in depth-preference during free swimming

3.1.1 Introduction

In the present work, we wanted to study a specific domain of habitat preference, the preference for water depth, in four different species widely spread for ornamental and research use: Siamese fighting fish (*Betta splendens*), zebrafish (*Danio rerio*), guppies (*Poecilia reticulata*), and honey gouramis (*Trichogaster chuna*). We tried to select species separated by a similar phylogenetic distance (Betancur-R et al., 2017) that shared features implicated in practical details of the experiment (body size, preferred water temperature, and salinity) and are among the most common aquarium fish.

Each one of these species has its own peculiarities regarding important aspects of their ecology, such as foraging, reproduction, and predation avoidance. These differences could have a deep influence on the preference for positioning in the water column. I will now describe them to have an interpretation key for the data we collected in the experiment.

All the species employed in the study are commonly found in rivers and ponds in warm tropical areas, not particularly deep waters rich in vegetation. The zebrafish, the honey gourami, and the Siamese fighting fish are native to the southern Asian continent; the first two are from India and Bangladesh, and the last one is from the territory of the drainage basin of the Mekong River. The guppy, instead, comes from the South American continent.

The intensive practice of aquaculture led to the modification of many traits of these species, especially the accentuation of the sexual dimorphism of flashy male secondary sexual traits such as colors and tail length or the improvement in genetic homogeneity, through dozens of generations of domestication (Milla et al., 2021). This process could lead to uncontrolled genetic and subsequent behavioral variation between the domesticated and wild varieties.

Besides the genetic artificial selection, the lifestyle adopted by these species in captivity is probably mitigated by the lack of ecological inputs that cannot be provided in aquariums. Although it is necessary to clarify it, this fact will not have repercussions on our work because our focus is on the living conditions of captive fish. Nevertheless, as mentioned before, the ecological information that we possess on these species could address our hypothesis on their behavior in captivity, especially for the features that do not change between the natural and laboratory environments.

One invariable characteristic in and out of captivity is the ability for Siamese fighting fish and honey gourami to directly breathe atmospheric air through an organ called the labyrinth (Rüber et al., 2006). This allows anabantoid fish to live in waters with low levels of dissolved oxygen, which naturally drives them to spend time close to the surface of the water.

Another stable reason for Siamese fighting fish and honey gourami to spend time close to the surface is their reproductive technique (Jaroensutasinee & Jaroensutansinee, 2001; Swain et al., 2021). The males of both species create a bubble nest at the water surface, in which they will position the fertilized eggs in need of aeration received from the females after the mating process. Zebrafish and guppies, on the contrary, are not able to breathe air from the atmosphere and do not form a floating bubble nest. Guppies are, in fact, ovoviviparous and can spawn differently in any place (Shahjahan et al., 2014). Zebrafish are ovuliparous (i.e., the fertilization of the eggs is external), like the Siamese fighting fish and Honey Gourami, but after the courtship, the females lay the eggs on the substrate of the river or ponds in which they live. As observed in laboratories, the preferred spawning site is a shallow area, probably to make it easier for the males to fertilize the eggs (Sessa et al., 2008). Therefore, they are attracted to shallow areas where the bottoms are close to the surface.

A third dimension to consider is feeding behavior. Although in the lab the feeding (or hunting) strategies could be altered by the specific administration of food by the animal keepers (usually dry food from the bottom and *Artemia salina* in the water column), it is reasonable to think that the abundance of food provided in captive conditions compared to its availability in the wild could lead to the maintenance of the preferred feeding style. For example, the Siamese fighting fish is a carnivorous species that feeds on bloodworms and mosquito larvae that come from above the water surface, thanks to the morphology of its mouth end and to the food-processing behavior exhibited during feeding (Konow et al., 2013; da Silva Souza et al., 2020). The honey gourami is preferentially a surface feeder too, but often feeds in the water column (Jearld, 1975). The zebrafish seems to have wider feeding possibilities: they feed on planktonic items in the water column, but they also practice surface feeding towards small insects and occasionally bottom feeding, as suggested by the presence of debris in their guts (McClure et al., 2006; Spence et al., 2007b). Lastly, guppies are opportunistic benthopelagic omnivorous feeders, preferring to feed from the substrate, varying their food preference with season (Lawall et al., 2012; Magurran, 2005).

One last element to take into consideration is predator avoidance. The prevalence of danger coming from outside of water should push the fish to swim in deeper regions, far from the surface. Conversely, a bottom-feeding predator would encourage them to swim closer to the surface. All of the species examined live in shallow ponds where it is unlikely to meet a bigger predator fish, except the guppy, which lives in slightly larger water basins or rivers (Reznik & Travis, 2019). In honey gourami, an initial general arousal to a chemical stimulus of predation is replaced by extensive freezing (Seigel et al., 2022). The zebrafish is known to engage in escape behaviors when presented with its natural predators or even robotic versions of them (Bass & Gerlai, 2008; Ladu et al., 2015). Due to the material presentation of danger, we only know that the zebrafish escaped at a certain distance from the threatening stimulus, wherever it was necessary. But other studies on alarm cues and anxiety behavior showed that the zebrafish tend to swim close to the bottom when they are in stressful situations (Levin et al., 2007).

The role of predation in affecting guppies' behavior has been studied by many authors, but the results seem to show that the avoidance behavior is quite plastic, depending on the environmental conditions (Reznik & Travis, 2019). For these reasons, we think that in the lab, the effect of phylogenetic traits of predator avoidance could disappear after the initial habituation to the new tanks.

Lastly, one of the main sources of individual variation is sex. Sex deeply influences hormonal production and morphological characteristics. Moreover, in an evolutionary context, the different sexual roles impose differential selective pressures favoring certain cognitive traits in one sex depending on their function (Wallace et al., 2020). Sex influences several domains of cognition in fish. Like in many other species (Jones et al., 2003), the spatial abilities measured differed between the two sexes in geometric orientation tasks (Sovrano et al., 2003) or in solving complex mazes (Lucon-Xiccato & Bisazza, 2017). Sex affects performance in detour tasks (Wallace et al., 2020; Triki & Bshary, 2021), object recognition (Wallace & Hoffman, 2021), problem solving (Lucon-Xiccato et al., 2020a), and more generally affects the whole set of executive functions (Lucon-Xiccato, 2022).

The aim of the study was to quantitatively assess the preferences for these species in a controlled laboratory environment, similar to possible tanks used in research facilities or in domestic aquariums.

3.1.2 Methods

Subjects

We tested 32 mixed-sex adult fish for each species, for a total of 128. Fish were maintained at the comparative psychology laboratory (Animal Behavior and Cognition

Lab) of the Department of General Psychology (University of Padova) in mixed-sex groups of 20–30 individuals. The aquariums were grey plastic tanks (70x45x55 cm) provided with air filters, natural gravel, and live plants at a temperature of 25 ± 1 °C). The only exception to this accommodation was made for the Siamese fighting fish. The high aggressiveness of the males towards other males forced us to house each male in a separate, smaller tank with two females. Fish were fed daily twice: once with commercial food flakes and once with live brine shrimps (*Artemia salina*), on a 12:12 h light-to-dark (L:D) photoperiod and an 18 W fluorescent light.

Apparatus

The experimental tank was a 40x60x30 cm glass tank (Figure 1.3.a). A green plastic stairway was placed into a 40x60 cm aquarium. Each step was 16 cm wide and 8 cm high. No other elements were present in the tank to avoid possible interactions between features. The short side opposite to the stairway was covered with green, opaque plastic to prevent from seeing outside. The two long sides were instead covered with a grey fish net to alleviate the reflection on the glass and prevent the fish from seeing outside while at the same time allowing us to record from the side.

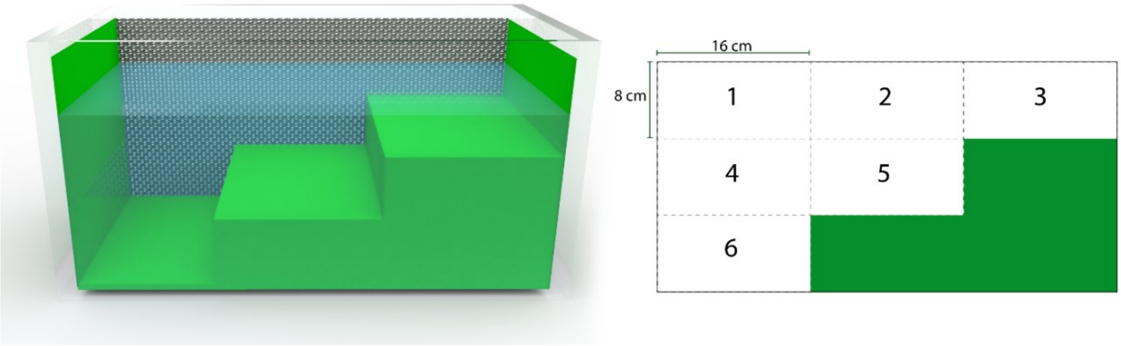

Figure 1.3. a) lateral view of the apparatus, b) schematic of the division in sectors

The camera was placed at a one-meter distance, pointing at the center of one of the long sides. One 16-watt LED light (746 lm) was positioned one meter above the water surface at the center of the tank.

Procedure

The experiment was recorded through a camera placed at a one-meter distance from the experimental tank. Experiments were performed during the day, ranging from 10:00 (2 hours after morning feeding) to 15:00 (2 hours before evening feeding). Each subject was selected from the stock tanks and put into a small (1L) white plastic recipient for transportation in the experimental room. After approximately one minute, the subject was gently inserted into the experimental tank. When the experimental time elapsed (30 minutes), the subject was removed from the experimental tank by a fish net, transported through the plastic recipient back into the maintenance room, and put into a new, post-experimental tank. After the removal of the subject, 70 percent of the water in the experimental tank was replaced with water coming from three 150-liter tanks enriched with gravel, plants, lamps, and a filter system to ensure the same quality and chemical composition of the water and minimize possible interference of the previous subjects' scent (e.g., hormones). Lastly, the positions of the experimental and control stimuli were inverted.

Video analysis

The size of the steps was used to virtually subdivide the arena into six sectors (8 cm x 16 cm each, Figure 1.3.b). The time in each sector of the tank was taken as a dependent variable. The videos were analyzed offline by three different trained observers. Each video consisted of preparatory routines (showing the ID of the subject and the position of stimuli and controls in the tank), one minute of habituation, 30 minutes of experimental recording, and the final removal of the fish. The time spent in each sector was scored using the program Boris (Friard & Gamba, 2016). The change of sector was determined by the passage of half of the body of the subject.

Data analysis

Analyses were performed in R version 3.5.3 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project. org). For each subject, we calculated the percentage of time spent in each area. The percentage of time spent in each area was then used as the dependent variable in a linear mixed model with Area (6 levels), Sex, and Species as predictors, and Subject as random effect. We subsequently aggregated different areas in sectors on the basis of three factors: proximity to the surface or bottom (SURFACE= Area 1+2+3, BOTTOM= Area 3+5+6), extension of the water column (LONG= Area 1+4+6, INTERMEDIATE= Area 2+5, SHORT= Area 3) and absolute depth (SHALLOW= Area 1+2+3, MID= Area 4+5, DEEP= Area 6) and ran the same model. To assess the magnitude of interspecific differences, we ran contrast analysis between pairs of species, adjusted with Tukey's honest significance test. At the intraspecific level, Student's t-tests were performed to assess if the eventual preference for an area was statistically significant when compared to chance levels and if sexual differences played a role in it.

3.1.3 Results

The results obtained from the video scoring, reported in Table 1.3, evidence substantial differences in the preference for positioning in the water column among the four different species examined and between sexes.

Area	Betta splendens		Danio rerio		Poecilia reticulata		Trichogaster chuna	
	Females	Males	Females	Males	Females	Males	Females	Males
Area 1	14.13% (3.28)	17.59% (4.49)	6.80% (3.91)	5.92% (3.51)	22.35% (8.06)	19.08% (6.95)	12.66% (5.48)	10.65% (5.34)
Area 2	13.88% (3.14)	19.22% (3.58)	7.78% (3.55)	7.88% (4.36)	13.73% (4.63)	11.10% (5.10)	10.54% (3.70)	9.93% (4.67)
Area 3	32.69% (4.82)	35.56% (5.32)	23.11% (11.61)	19.63% (8.05)	20.55% (7.17)	16.65% (7.80)	14.15% (4.43)	13.46% (8.23)
Area 4	6.90% (1.59)	7.79% (3.45)	11.76% (4.14)	12.88% (4.41)	14.58% (5.01)	15.48% (7.04)	16.68% (4.01)	16.64% (5.14)
Area 5	10.24% (3.12)	7.32% (2.26)	16.67% (5.31)	19.36% (6.93)	9.53% (5.26)	12.55% (6.19)	16.31% (3.63)	19.19% (5.49)
Area 6	22.01% (5.59)	12.61% (5.33)	33.82% (12.15)	34.25% (11.36)	19.26% (9.03)	25.17% (7.54)	28.95% (9.82)	30.10% (16.38)

Table 1.3. Percentage of time spent in each sector divided by species and sex. Inside the brackets are reported the standard deviations.

A more intuitive graphical representation of the results of our observations is provided in Figure 2.3.

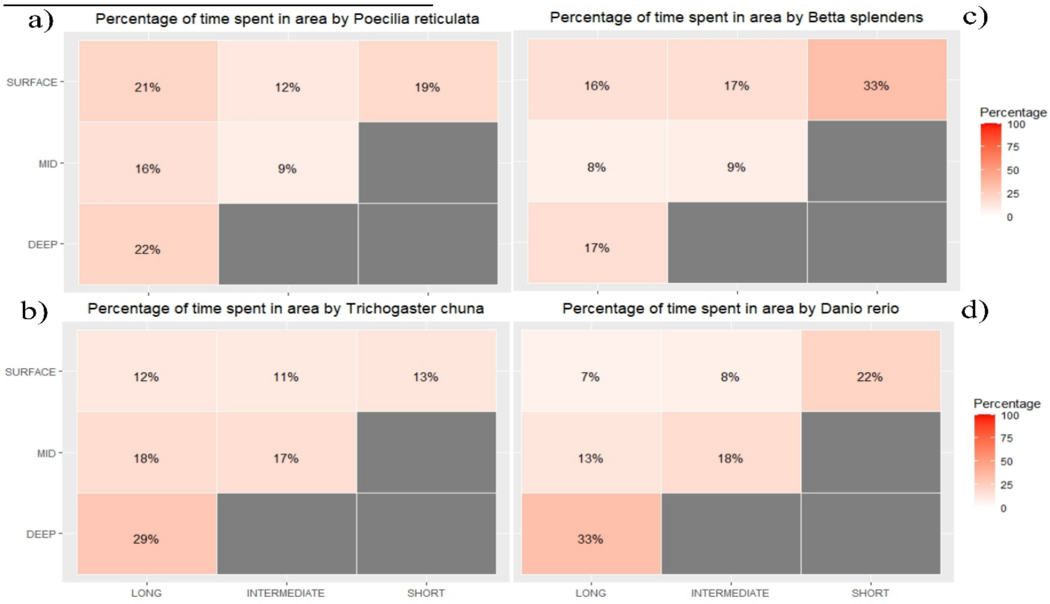

Figure 2.3. Spatial representation of the percentage of time spent in each sector by a) guppies, b) honey gourami, c) Siamese fighting fish, and d) zebrafish.

The results of the linear model evidence a strong effect of the Area (F = 89.32, p < 0.0001), meaning that, as expected, the subjects preferred to swim in some areas rather than others. The significant interaction between Area and Species (F = 29.49, p< 0.0001) indicates that different species preferred to swim in different areas, and the further interaction of these two with Sex (F= 2.65, p<0.001), together with the lack of simple interaction Sex*Area, suggests that a sexual difference in preference influenced the outcomes, but only in some species.

Activity levels

Besides all the differences examined with regard to depth preferences, we derived further information from the count of switches between areas made by our subjects as a good proxy for determining activity levels.

We found huge interspecific differences in the activity levels (Table 3.3, Figure 3.3). Zebrafish was by far the most active species, followed by honey gourami, guppy, and Siamese fighting fish, each significantly less active than the previous. On the other hand, there were unexpectedly small intraspecific sexual differences: the only significant one was found in guppies, where the males were more active (F = 6.77, p = 0.014) than the females, at a level comparable to honey gourami's activity.

Betta splendens		**Danio rerio**		**Poecilia reticulata**		**Trichogaster chuna**	
Females	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**
233.28 (90.74)	281.81 (119.57)	686.81 (171.23)	627.68 (130.87)	290.28 (102.29)	432.97 (194.05)	509.12 (172.05)	436.31 (170.22)
7.77 per min.	9.39 per min.	22.89 per min.	20.92 per min.	9.68 per min.	14.43 per min.	16.97 per min.	14.54 per min.

Table 2.3. Overall number of switches between sectors divided by species and sex in the 30-minutes sessions and mean number of switches per minute. Inside the brackets are reported the standard deviations.

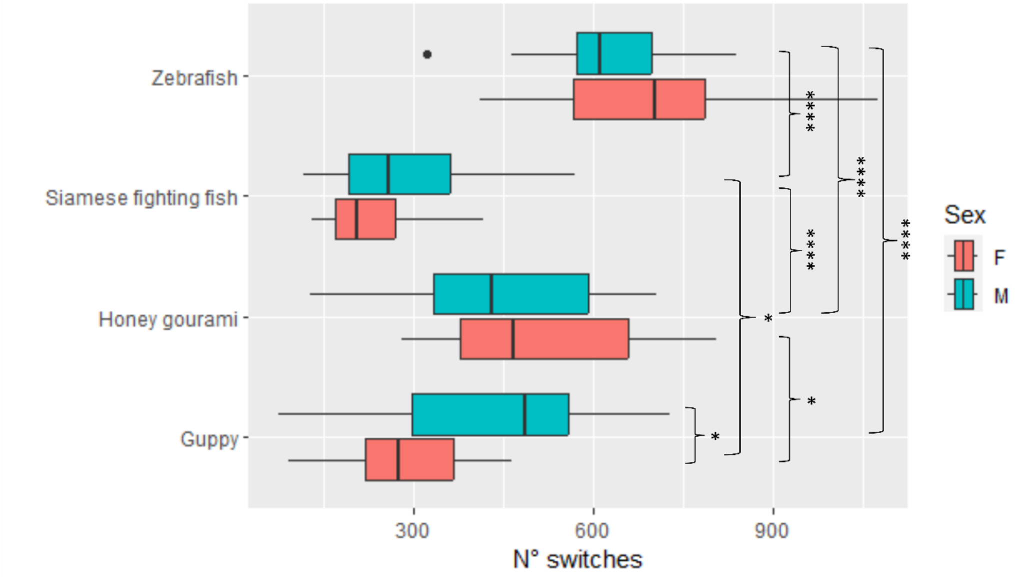

Figure 3.3. Activity levels measured by number of switches between sectors, divided by species and sex. The asterisks indicate significance levels (* = p< 0.5, ** = p< 0.01, *** = p< 0.001, **** = p< 0.0001).

Proximity to surface or bottom

Figure 4.3 shows the percentage of time spent by the subjects of the four species in the SURFACE and BOTTOM sectors (see Figure 1.3: SURFACE = Area 1+2+3, BOTTOM = Area 3+5+6).

Regarding the SURFACE sector (Figure 4.3a), a strong effect of the factor Species (F = 50.59, p< 0.0001) and an interaction between Sex and Species (F = 3.49, p = 0.018) were found. The analysis of the contrasts between pairs of species revealed that both Siamese fighting fish and guppies spent significantly more time than the other two species in the SURFACE sector, but Siamese fighting fish spent more time than guppies.

Siamese fighting swam in the SURFACE sector for more than 50% of the time (t (31) = 10.40, p = 1.229e-11); on the contrary, both zebrafish and honey gourami spent significantly less time than chance in it (zebrafish: t(31) = -6.39, p-value = 4.046e-07; honey gourami: t(31) = -6.18, p-value = 7.308e-07).

The analysis from the BOTTOM sector (Figure 4.3b) instead showed opposite trends. While the factor Species (F = 34.58, p = 3.524e-16) and its interaction with Sex (F = 5.39, p = 0.0016) were still significant, the pairwise comparisons showed a predominance in the BOTTOM sector of zebrafish, whose percentages are greater than both Siamese fighting fish and honey gourami's ones. The two are, in turn, more prone to the BOTTOM sector than guppies.

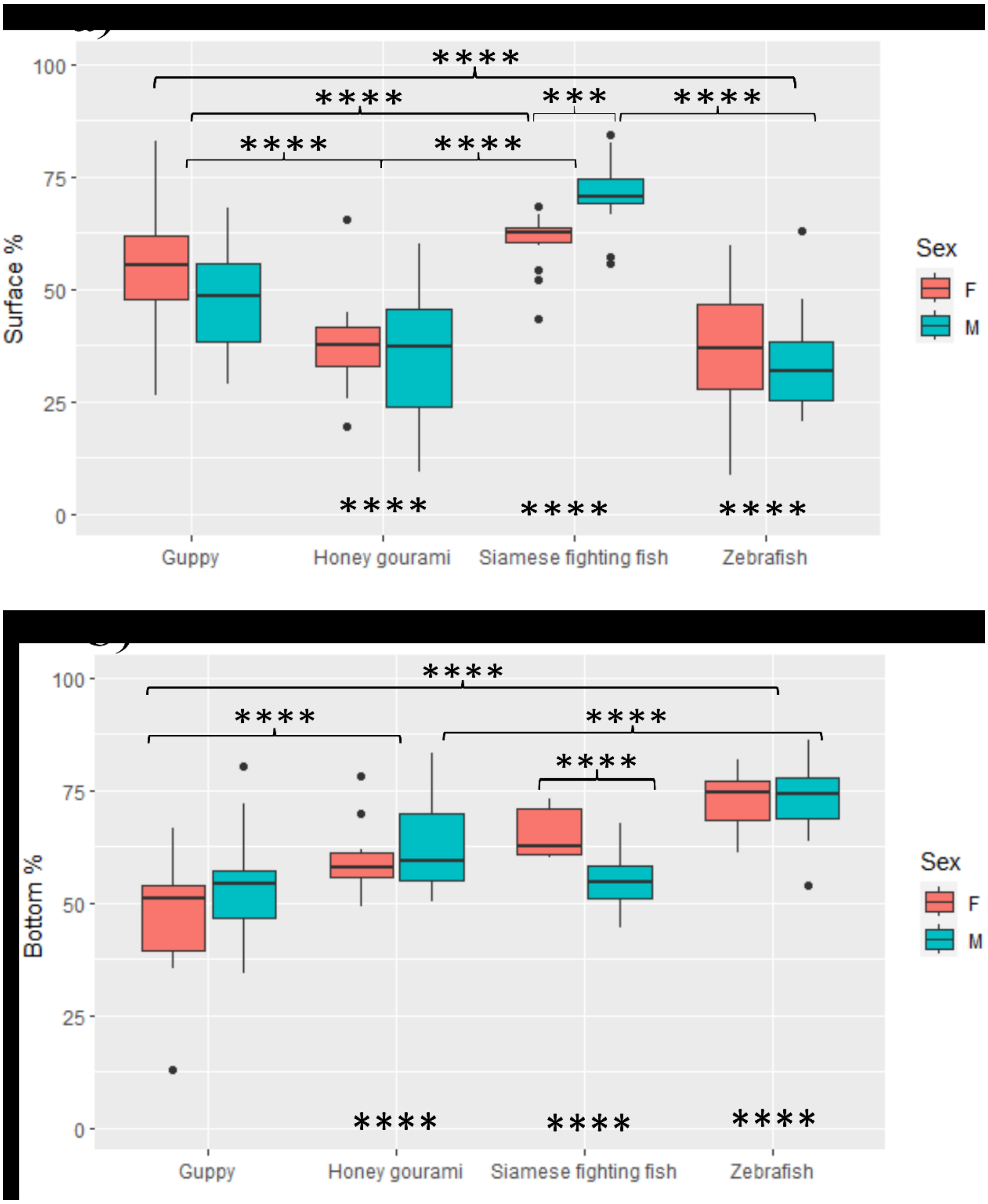

Figure 4.3. Percentage of time spent in SURFACE sector and b) in the BOTTOM sector. The asterisks indicate significance levels (* = p< 0.5, ** = p< 0.01, *** = p< 0.001, **** = p< 0.0001). The brackets indicate differences between groups, free asterisks indicate difference from chance level.

All the species swam in the BOTTOM sector for more than 50% of the time (zebrafish: t(31) = 18.14, p < 2.2e-16; honey gourami: t(31) = 7.01, p = 7.146e-08; and Siamese fighting fish: t(31) = 7.26, p = 3.665e-08), with the exception of guppies.

As it is clear from the graph, the only sexual difference present has been found in Siamese fighting fish. Females spent less time close to the SURFACE sector (F = 18.27, p< 0.001), but more time in the BOTTOM sector (F = 26.13, p< 0.0001) than male Siamese fighting fish.

Extension of the water column

Figure 5.3 shows the percentage of time spent by the subjects of the four species in the different columns of water (see Figure 1.3 LONG = Area 1+4+6, INTERMEDIATE = Area 2+5, and SHORT = Area 3).

No interspecific differences have been found for the presence in the INTERMEDIATE sector (Figure 5.3b): every species generally avoided this central sector, spending significantly less time than chance in it. On the contrary, “Species” exerted influence on the presence in the LONG (F = 12.25, p = 4.758e-07) and SHORT sector (F = 41.34, p <2e-16). In the LONG sector (Figure 5.3a), Siamese fighting fish were less present than every other species, swimming significantly below chance levels, while zebrafish and guppies both swam on it at chance levels. Honey gourami was the only species swimming in the LONG sector above chance levels, but not significantly more than zebrafish and guppies.

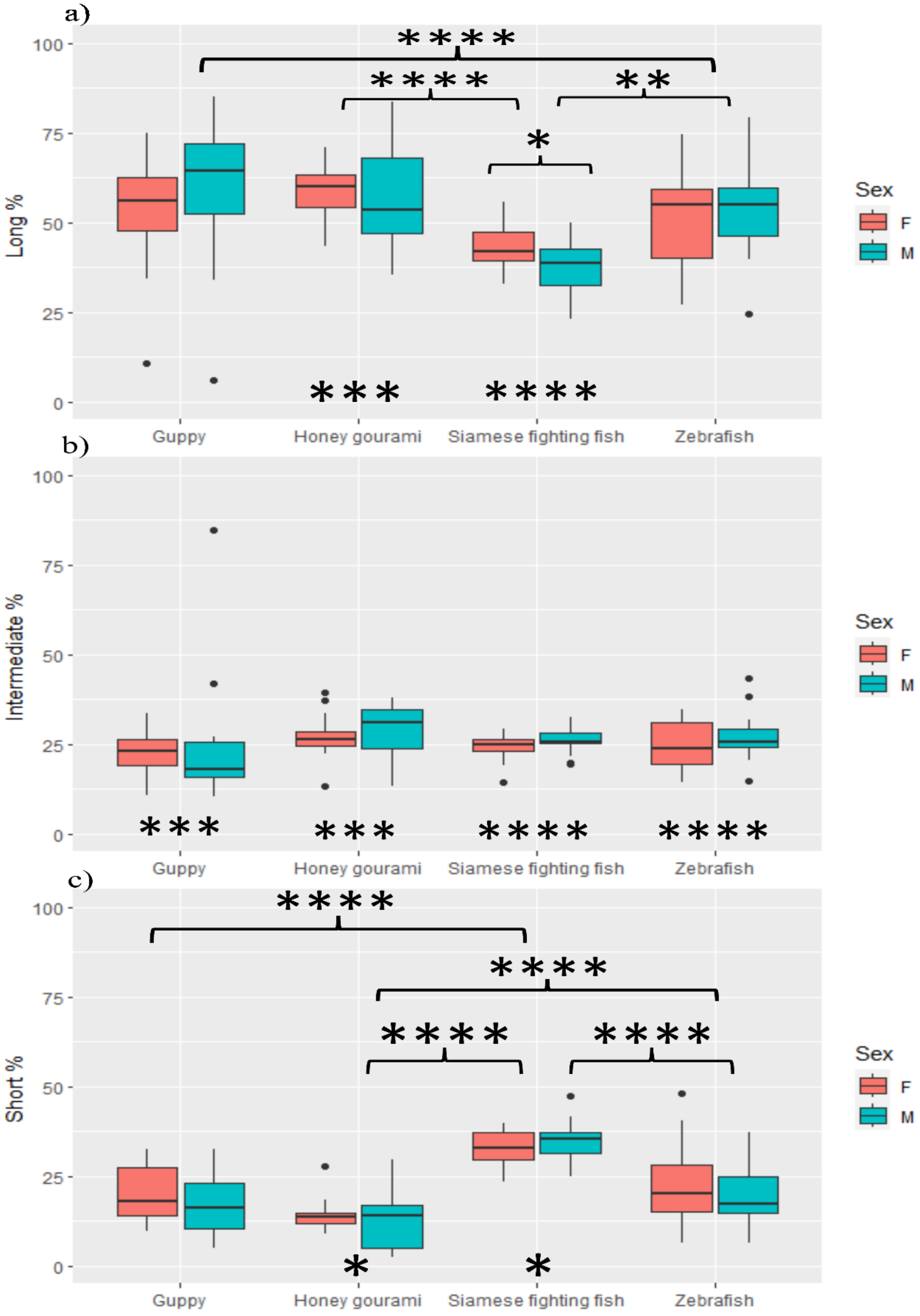

Figure 5.3. Percentage of time spent in the a) Long, b) Intermediate, and c) Short sector. The asterisks indicate significance levels (* = p< 0.5, ** = p< 0.01, *** = p< 0.001, **** = p< 0.0001).

The brackets indicate differences between groups, free asterisks indicate difference from chance level.

In the SHORT sector (Figure 5.3c), Siamese fighting fish significantly spent more time than any other species, and zebrafish spent more time than honey gourami, being the first significantly above chance levels and the second one below. Chance levels were set to 50% for the LONG sector (3/6 areas), 33.3% periodic for the INTERMEDIATE (2/6 areas) and 16.6% periodic for the SHORT sector (1/6 area). Once again, Siamese fighting fish presented sex differences, as females spent more time in the LONG sector than males (F = 5.84, p = 0.02). No other sex differences emerged from this partitioning.

Absolute depth

Figure 6.3 shows the percentage of time spent by the subjects of the four species in the different depth sectors (see Figure 1.3: SHALLOW = Area 1+2+3, MID = Area 4+5, and DEEP= Area 6).

The SHALLOW sector (Figure 6.3a) corresponds identically to the SURFACE sector of the second paragraph of the results section, so everything that has been told before will not be repeated.

Siamese fighting fish were the species that spent less time in the MID sector (Figure 6.3b) compared to the others. Furthermore, honey gourami, the only species that did not swim in the MID sector below chance levels, spent more time in it than guppies.

With regard to the DEEP sector (Figure 6.3c), zebrafish and honey gourami's percentages were significantly higher than Siamese fighting fish and guppies', although every species, except Siamese fighting fish, spent significantly more time than chance in this sector.

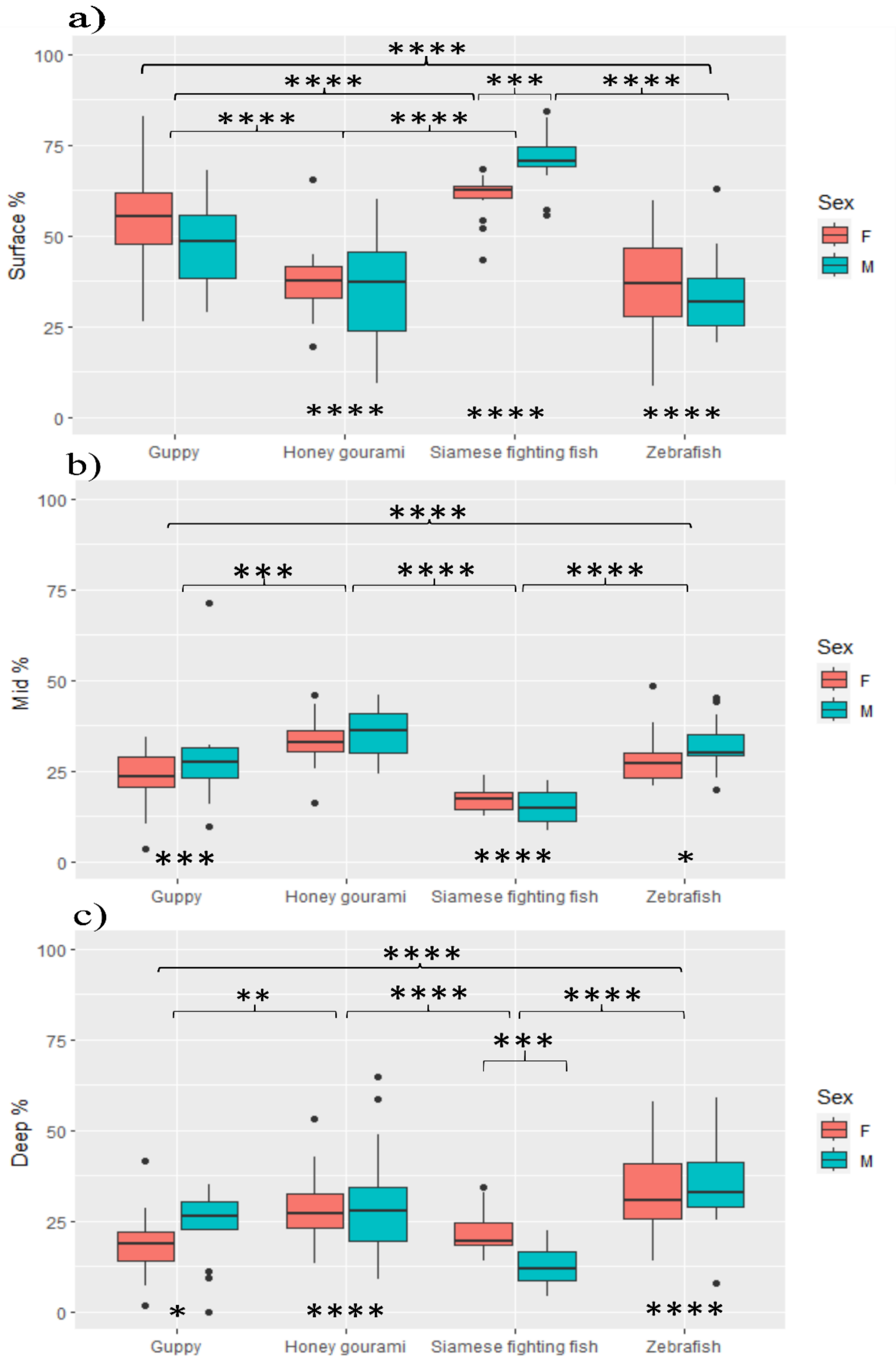

Figure 6.3. Percentage of time spent in the a) Surface/Shallow, b) Mid, and c) Deep sector. The asterisks indicate significance levels (* = $p < 0.5$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$). The brackets indicate differences between groups, free asterisks indicate difference from chance level.

No sexual differences were found in the intraspecific comparisons on the MID and DEEP sectors, except for a higher propensity of female Siamese fighting fish over the males for the DEEP sector (F = 24.62, p = 2.594e-05). Chance levels were set to 50% for the SURFACE sector (3/6 areas), 33.3% periodic for the MID sector (2/6 areas), and 16.6% periodic for the DEEP sector (1/6 areas).

3.1.4 Discussion

The aim of his study was to assess inter- and intra-specific differences in the positioning in the water column of a three-stepped aquarium. The new information regarding the preferences provided by our results will help improve the maintenance conditions of these four widespread species.

Each one of these species had a precise, distinct pattern of movement compared to the others, which reflects their peculiar motivations. Siamese fighting fish (*Betta splendens*) was the most attracted by the water surface, especially in an area where it could access a close bottom as well. The Siamese fighting fish showed the lowest level of activity and a tendency to rest on solid surfaces without swimming. This movement style is coherent with the time spent in Area 3 and Area 6, but contrasts with the avoidance of Area 5 and the presence in Area 1 and Area 2, denoting a strong motivation to stay close to the surface. Despite the general trend of this species, the degree of willingness to stay at the surface level is variable between sexes. The strong sexual differences observed showed us that males prefer to stay at surface level even more than females, who spent considerable time in the deepest area, Area 6. This reflects the natural behavior of Siamese fighting fish in aquariums. Both sexes feed and breathe from the surface, but males are the nest builders and keepers. Their constant patrolling of the nesting area and their aggressivity towards anything that moves nearby probably explain this intersexual difference.

The second species, the zebrafish *Danio rerio*, showed a complete preference for the BOTTOM sector. The favorite among the three areas that form it is Area 6, the deepest and safest one. The diving response, that is the tendency of zebrafish to swim in the deepest area of the tanks when in distress, is a well-known phenomenon used as a proxy for anxiety in pharmacological studies (Egan et al., 2009; Fontana et al., 2022). However, as observed by Blaser and Goldsteinholm (2012), bottom areas are preferred by zebrafish not only for their absolute depth but also for their proximity to the bottom itself, even when close to the surface level. We know that zebrafish is the only species among the four examined here to lay eggs on a substrate to make the male fertilize them, but in this case the fish were isolated, so there was no need to proceed with mating behavior. An additional explanation could be an extreme lower-body thigmotaxis. Staying close to a substrate could increase the possibilities for camouflage (even if in our study the steps were green), avoiding potential dangers coming from inside or outside the water.

The guppy, *Poecilia reticulate,* was the least "choosy" species. All the areas were almost equally explored, except the MID sector (Area 2 and Area 5). These areas were significantly less explored, indicating a possible attraction toward the vertical walls of the aquarium. Despite the well-known morphological and cognitive sexual differences in guppies (Piyapong et al., 2010; Lucon-Xiccato et al., 2016), there were no significant differences between the two. Males showed a greater tendency to swim deeper, but not at a significant level. Like the Siamese fighting fish, this species was domesticated a long time ago (Fernando & Phang, 1985; Kwon et al., 2022). The domestication process could have erased the ecological pressures involved in the development of habitat preferences (Teletchea, 2016). However, contrary to the Siamese fighting fish, this species has no reproductive or feeding/breathing reason to prefer either the top or bottom sectors.

Lastly, *Trichogaster Chuna*, the honey gourami. We do not possess much information from the scientific literature on this species. It is more widespread as a pet than it is as an animal research model, and the information we based our predictions on seems to be in contrast with what we observed. In fact, its reproductive, feeding, and breathing techniques were quite similar to the ones of Siamese fighting, but the behavioral outcome was the opposite. The honey gourami in fact showed a stark preference for the three deepest areas and a clear avoidance of the surface ones, including Area 3, the one with the closest bottom too. This could be explained in terms of anxiety for the test. The activity level was comparable to that of the zebrafish, but if in the maintenance tanks the zebrafish is quite motile, honey gourami is instead most of the time stuck at mid-water, moving in short bouts every now and then. This different behavior could reveal an increased arousal by this species during the test, which led to the need to seek repair in the deepest, darkest areas of the apparatus. Differently from what we hypothesized for the zebrafish (the defense technique of staying close to the bottom wall, whichever absolute depth), the less camouflageable honey gourami could adopt a slightly different strategy.

In the end, a general, consistent result is the confirmation of the thigmotactic behavior (Lucon-Xiccato et al., 2020b; Correia et al., 2022) in these species too. The less explored areas were Area 2, Area 4, and Area 5. The INTERMEDIATE and MID sectors were, in fact, the less explored by all the species that preferred to avoid swimming in them. The MID sector is the result of the combination of Area 2 and Area 5, while the INTERMEDIATE the combination of Area 4 and Area 5. By themselves, these areas did not possess any special features, except for the fact that they were the furthest from the lateral and inferior walls of the aquarium, or from the surface "wall". The proximity to the surface and the absolute depth of Area 2 were the same as those of Area 1 and Area 3, which were explored extensively. Likewise, the proximity to the bottom of Area 5 was the same as the two most explored areas, area 3 and Area 6.

All the species, regardless of their preference for shallow or deep water, for areas close to the surface or to the bottom, and for LONG or SHORT water sectors, generally avoided these middle areas, which probably did not satisfy any of the motivational drives of behavior. These areas, together with Area 1, were the most exposed, and in an arena with no vegetation or other objects nor central walls, they are generally avoided.

Area 3 and Area 6 were instead the preferred ones by far. Area 6 offers the best repair in terms of wall enclosure, distance from the source of light, and from possible predators, so it is quite obvious. Area 3, on the other hand, was clearly appreciated by all the species, except for honey gourami, which avoided any contact with the shallow areas. Anyway, it was the preferred zone of the three surface areas. This could be a precious hint to develop new aquarium structures or enrichments that provide raised platforms and barriers that break the continuity of water, which are considered threatening for most of the species examined.

It is necessary to say that behavioral studies of this kind, while providing important information on the behavior of the animals, do not provide a complete framework for the animal’s overall state. Analysis of the physiological markers for stress (Pottinger, 2008) would tell us more about the arousal state of the experimental subjects and the potential benefit of the adaptation of the tanks proposed for the well-being of the animals.

Moreover, in order to better depict the effects of the proposed enrichment, it should be considered the role of prolonged exposure and group life in affecting the preferences for the stimuli and the effects on the well-being state of the fish. The presence of conspecifics could trigger the formation of a social hierarchy in which the more dominant individuals monopolize the valuable resource of the enriched environment (Grant & Kramer, 1992; Iwata et al., 2008; Paull et al., 2010), causing suffering to the less dominant ones.

Besides this, another area for future studies would be the examination of the underlying mechanisms of depth perception in these species. We still do not know if the cognitive mechanisms involved in the repositioning of the animals at the desired depth are exclusively dependent on proprioceptive cues, like hydrostatic pressure (Davis et al., 2021), or if they rely on visual cues too (Jeffery et al., 2013). The spontaneous preference expressed by the species could serve as a baseline for studies on visual illusions as tools for investigating visual processing and enrichment.

3.2 Experiment B: testing the effects of a depth illusion on swimming preferences

3.2.1 Introduction

As emerges from Experiment A, the four species expressed different swimming behaviors in terms of general activity and positioning in the volume of the tank. The two species that exhibited the most pronounced preferences were the Siamese fighting fish *Betta splendens* and the zebrafish *Danio rerio*. The Siamese fighting fish spent 66% of the time in the “Surface” area, which was the closest to the water surface. It most predominantly swam in the surface area that had a closer bottom, indicating a possible need for repair from below or the opportunity to rest, laying its body on the floor while staying close to the source of food and fresh air. The zebrafish showed a different pattern. The most explored areas were the ones closer to the bottom surface, which cumulatively accounted for 73% of the time. The preferred zone was also the deepest, but in general, shallower areas were preferred to some deeper or equally deep ones because of their greater proximity to a bottom surface.

The pronounced values of preference for real depth levels of these two species were taken as baseline for the subsequent step of the inquiry, which was to examine the possibility of misleading the visual system, assuming that the visual component in the estimation of distances played an important role in the positioning behavior through the use of optical illusions that fake depth levels.

The visual illusion tested was based on the principle of size constancy. Size constancy is a type of perceptual constancy that makes an object perceived to be the same size despite the fact that the size of its retinal image changes depending on its distance from the observer (VandenBos & Association, 2015). This perceptual ability to adjust for distances comes from the preexisting knowledge of equality between objects or from a correct distance estimation dependent on cues external to the object (Sperandio & Chouinard, 2015).

When the two objects are of unknown dimensions and/or the distances are incorrectly estimated (like in the sun-moon coincidental illusion or the man-made “Ames room” illusion , see Figure 7.3), then the perceptual mechanism can be easily and powerfully fooled. The stimuli employed in this experiment were checks and stones of unknown dimensions, because our subjects had never experienced them before the tests. Moreover, there were no other cues to evaluate the distance at which they were placed. In absence of other information, the increasing size of seemingly identical stimuli should lead to estimate an increasing distance of the stimulus. This illusion could be generally called “Size inconstancy”. It is a derivation of the more general Emmert’s Law (Boring, 1940).

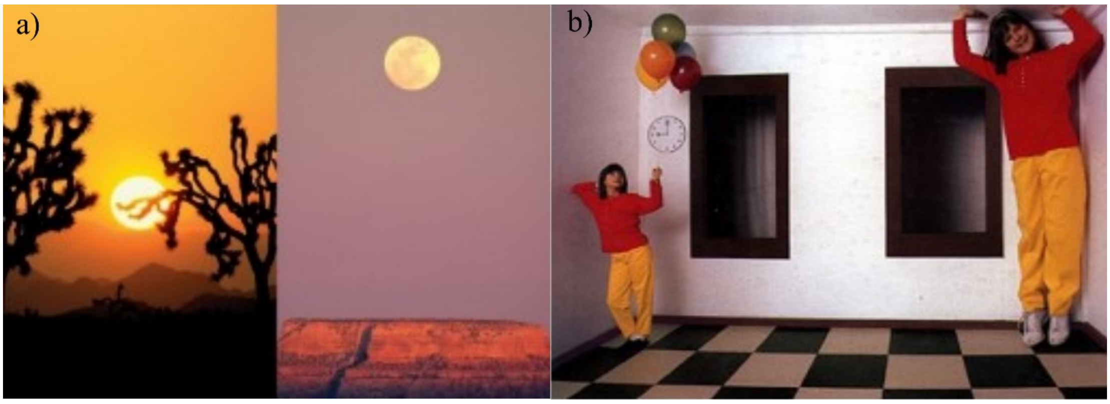

Figure 6.3. Example of Emmert's Law in which a) the different sizes of two objects seem equal when they are not, and b) the equal sizes of two objects seem different when they are equal (from Sweet & Kaiser, 2011).

The black-and-white checkerboard pattern can be used to give a sense of both the absolute scale of the environment and the relative scale of nearby objects in augmented reality settings (Wither & Hollerer, 2005). The ability to link dimensions and distances from pictorial cues is present in human infants before the age of six months (Kavšek et al., 2012) and has been demonstrated in primates and monkeys (Imura et al., 2008; Ungerleider et al., 1977), other mammals like horses (Timney & Keil, 1996), dogs (Müller et al., 2011), and possibly even in bats' echo-acoustic perception (Heinrich & Wiegrebe, 2013). Size constancy has also been attested in birds (Pastore, 1958), in bees (Horridge et al., 1992), and lastly, in fish (Douglas et al., 1988; Schuster, 2004).

All the mentioned studies have been conducted in laboratories, but this phenomenon has been observed in nature and exploited by some animals as an adaptive advantage. The most appalling example is the Great Bowerbird, which arranges the features on a positive of the mating site in the "Ames Room" style, following a distance-size gradient to appear bigger when observed by the female (Kelley & Endler, 2012; 2017).

Our hypothesis is that these two species will prefer to spend time in the area above the big checks or stones, because both of them prefer to swim close to a bottom surface, even if the Siamese fighting fish prefers to do so when close to the surface while the zebrafish tends to swim at greater depths.

3.2.2 Methods

The same methods as in Experiment A were used in these tests, with an exception made for the number of subjects, a component of the apparatus, and the analyzed areas.

Subjects

We tested 16 mixed-sex adult fish for each species (2) and each condition (2), for a total of 64. We only employed the two species that showed the most marked preference for the top sector (Siamese fighting fish – *Betta splendens*) and for the bottom sector (zebrafish – *Danio rerio*).

Fish were maintained at the comparative psychology laboratory (Animal Behavior and Cognition Lab) of the Department of General Psychology (University of Padova) in mixed-sex groups of 20–30 individuals. The aquariums were grey plastic tanks (70x45x55 cm) provided with air filters, natural gravel, and live plants at a temperature of 25 ± 1 °C. The Siamese fighting fish were accommodated differently because of the high aggressiveness of the males towards other males. So, we housed each male in a separate, smaller tank, together with two females. Fish were fed daily twice: once with commercial food flakes and once with live brine shrimps (*Artemia salina*), on a 12:12 h light-to-dark (L:D) photoperiod and an 18-watt fluorescent light.

Apparatus

All the features of the tank were identical to the previously reported ones. The experimental tank was a 40x60x30 cm glass tank (Figure 8.3c). The two long sides were instead covered with a grey fish net to alleviate the reflection on the glass and prevent the fish from seeing outside while at the same time allowing us to record from the side. The camera was placed at a one-meter distance, pointing at the center of one of the long sides.

One 16-watt LED light (746 lm) was positioned one meter above the water surface at the center of the tank. The only exception was that the staircase was completely removed, so the tank was left empty. The tank was lifted 5 cm above the table by wooden supports covered in black plastic. The visual illusion was placed below the transparent floor of the tank.

In one case, the illusion consisted of a picture of a checkerboard (Figure 8.3a) divided into three parts: big checkers (4x4 cm), small checkers (1x1 cm), and an intermediate zone that connects the borders with continuity, enhancing the illusory cues of depth.

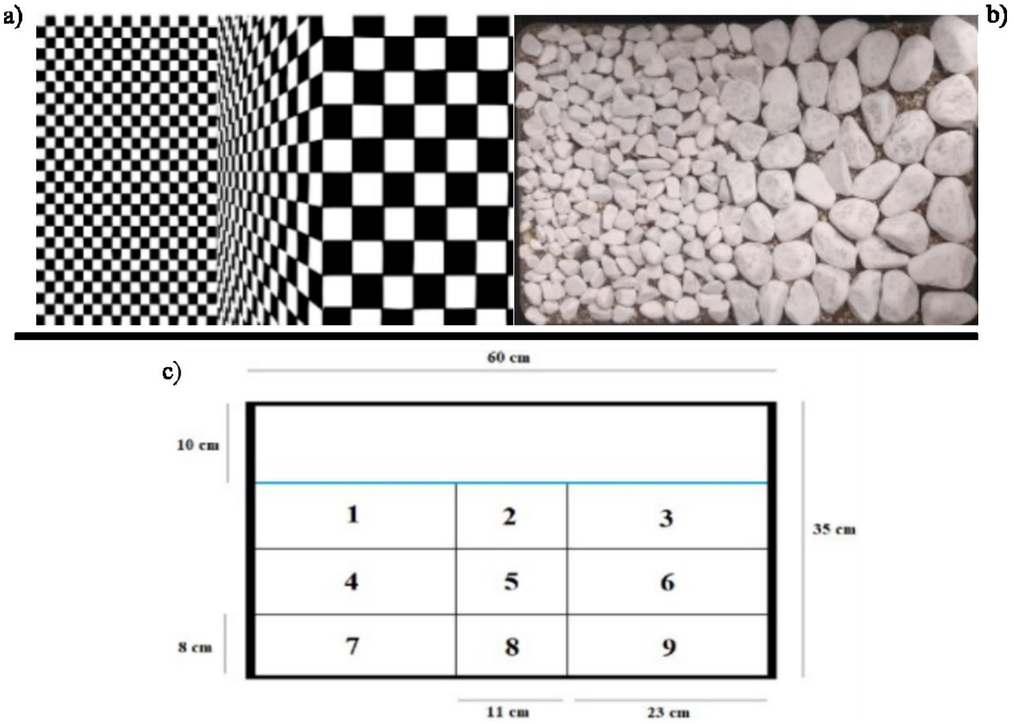

Figure 8.3. Experimental stimulus placed below the bottom of the tank in condition a) “Checks”, and b) “Stones”. c) Virtual subdivision of the sectors of the tank

The second stimulus was a tray filled with white stones (Figure 8.3b) of increasing size. The biggest and smallest stones on both sides were nearly the same size as big and small checkers. There was no intermediate sector because of the continuity of the stones dimensions. Nonetheless, the same regions of interest were used in the analysis to improve comparability.

Procedure

The experiment was recorded through a camera placed at a one-meter distance from the experimental tank. Experiments were performed during the day, ranging from 10:00 (2 hours after morning feeding) to 15:00 (2 hours before evening feeding). Each subject was selected from the stock tanks and put into a small, 1-liter, white plastic recipient for transportation in the experimental room. After approximately one minute, the subject was gently inserted into the experimental tank. When the experimental time elapsed (30 minutes), the subject was removed from the experimental tank by a fish net, transported through the plastic recipient back in the maintenance room, and put into a new, post-experimental tank. After the removal of the subject, 70 percent of the water in the experimental tank was replaced with water coming from three 150-liter tanks enriched with gravel, plants, lamps and a filter system to ensure the same quality and chemical composition of the water and minimize possible interference of the previous subjects' scent (e.g., hormones). Lastly, the positions of experimental and control stimuli were inverted when half of the subjects had completed the test.

Video analysis

We used the size of the sectors with different patterns on the ground (Figure 8.3a) to virtually subdivide the arena into nine areas (Figure 8.3c), six lateral ones (23 cm x 8 cm), and three central ones (11 cm x 8 cm). The time in each area of the tank was taken as the dependent variable. The videos were analyzed offline by three different trained observers. Each video consisted of preparatory routines (showing the ID of the subject and the position of stimuli and controls in the tank), one minute of habituation, 30 minutes of experimental recording, and the final removal of the fish. The time spent in each area was scored using the program Boris (Friard & Gamba, 2016). The change in area was determined by the passage of half of the body of the subject.

Data analysis

Analyses were performed in RStudio version 2023.06.2 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project. org). For each subject, we calculated the percentage of time spent in each area. The lateral sectors of the subjects that underwent the experiments with the illusory pattern on the opposite side to prevent any effect of side bias were switched in order to make them represent the same underlying stimulus. In this way, the three right areas indicate that the fish was swimming above big stimuli, either checks or stones, while the three left areas indicate the areas above the small stimuli. The virtual configuration is the same as depicted in Figure 8.3 a/b)

Each species has been examined separately because of the known great differences between them that would have a confounding effect on the analysis. The percentage of time spent in each area was then used as the dependent variable in a linear mixed effects model with Sex, Side, and Height as fixed effects predictors, and Subject as random effect variable.

A preference index was obtained by calculating the proportion of time spent in the area that should be perceived as closer over the time spent in both lateral sectors with the following formula:

Preference for the big elements = time above the big elements / (time above the big elements + time above the small elements).

A linear model was performed to evaluate the preference trend over the thirty minutes time.

3.2.3 Results

The results obtained from the video scoring, reported in Table 3.3, confirm the difference in positioning in the water column between the two species and the two sexes.

Area	***Betta splendens***				***Danio rerio***			
	Checks		**Stones**		**Checks**		**Stones**	
	Females	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**
Area1	31.46% (8.32)	31.33% (14.60)	30.05% (11.84)	33.57% (20.91)	10.70% (5.11)	12.78% (6.50)	9.48% (3.60)	10.69% (5.98)
Area2	6,25% (3.17)	7.27% (3.51)	11.13% (1.74)	5.42% (2.16)	5.14% (2.54)	5.59% (4.14)	5.24% (2.73)	2.74% (1.68)
Area3	29.61% (11.99)	30.11% (14.69)	38.60% (10.73)	39.74% (24.92)	11.25% (3.07)	16.69% (6.85)	11.67% (8.50)	7.83% (4.35)
Area4	5.10% (2.55)	4.24% (2.92)	6.33% (2.96)	4.62% (4.52)	9.64% (3.48)	11.15% (4.83)	7.25% (1.68)	11.96% (5.89)
Area5	2.01% (1.53)	2.00% (1.01)	2.01% (1.34)	1.23% (1.18)	5.69% (2.98)	6.39% (1.85)	4.35% (2.11)	4.87% (2.39)
Area6	5.55% (3.45)	6.06% (2.71)	5.19% (3.04)	4.53% (3.26)	11.87% (3.69)	10.17% (2.19)	11.68% (6.23)	9.26% (2.35)
Area7	10.06% (6.22)	5.07% (4.98)	6.86% (3.13)	8.12% (6.26)	19.14% (7.26)	17.03% (6.86)	18.18% (6.51)	24.83% (10.93)
Area8	2.67% (1.80)	1.76% (1.54)	2.28% (1.75)	0.79% (0.49)	9.67% (3.38)	8.31% (3.46)	8.45% (2.54)	9.65% (2.07)
Area9	9.49% (4.41)	6.10% (4.20)	6.31% (4.42)	4.90% (4.72)	16.87% (7.96)	16.88% (10.53)	18.65% (5.10)	18.11% (6.10)

Table 3.3. Percentage of time spent in each sector divided by species, condition, and sex. Inside the brackets are reported the standard deviations.

A more intuitive graphical representation of the results of our observations is provided in Figure 9.3.

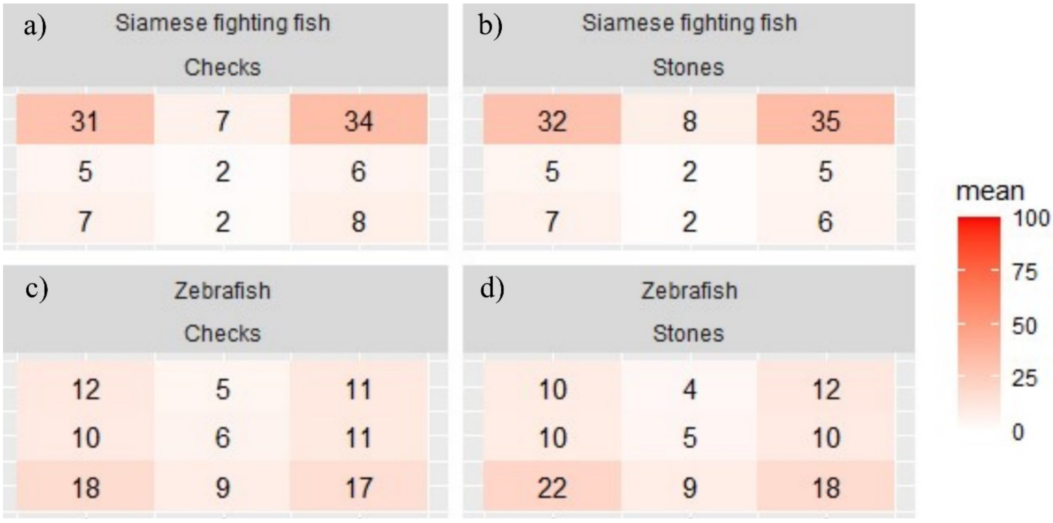

Figure 9.3. Spatial representation of the percentage of time spent in each sector divided per species and condition a) Siamese fighting fish "Checks", b) Siamese fighting fish "Stones", guppies, c) zebrafish "Checks", and d) zebrafish "Stones".

Activity levels

Besides all the differences examined with regard to depth preferences, we derived further information from the count of switches between areas made by our subjects as a good proxy for determining activity levels.

In Table 4.3, the activity levels are reported counting the number of switches between sectors made by the experimental subjects during the 30 minutes.

Betta splendens				***Danio rerio***			
Checks		**Stones**		**Checks**		**Stones**	
Females	**Males**	**Females**	**Males**	**Females**	**Males**	**Females**	**Males**
151.375 (97.81)	93.62 (42.46)	212.31 (110.56)	67.25 (27.31)	860.43 (154.26)	820.37 (186.14)	757.75 (201.06)	750.19 (207.20)
5.04 per min.	3.12 per min.	7.07 per min.	2.24 per min.	28.68 x minute	25.25 x minute	27.34 per min.	25.01 per min.

Table 4.3. Overall number of switches between sectors divided by species, condition, and sex in the 30 minutes sessions and mean number of switches per minute. Inside the brackets are reported the standard deviations.

As in the previous experiment, zebrafish were definitely more active than the Siamese fighting fish ($F = 341.78$, $p < 2*10^{-16}$). While there was no overall effect of sex and type of illusion (i.e. checks or stones), specific analysis revealed the marked difference in activity between males and females Siamese fighting fish, but no difference related to the stimulus. Females are in fact significantly more active than males ($F = 12.98$, $p = 0.003$).

We also compared the levels of activity between this experiment and the previous with a real stairway through t-tests and surprisingly, we found two different effects on the two species: *Siamese fighting fish significantly* decreased its activity levels (t(60) = 5.02, p = 4.673 $*10^{-6}$), while zebrafish markedly increased them (t(60) = -3.30, p = 0.002).

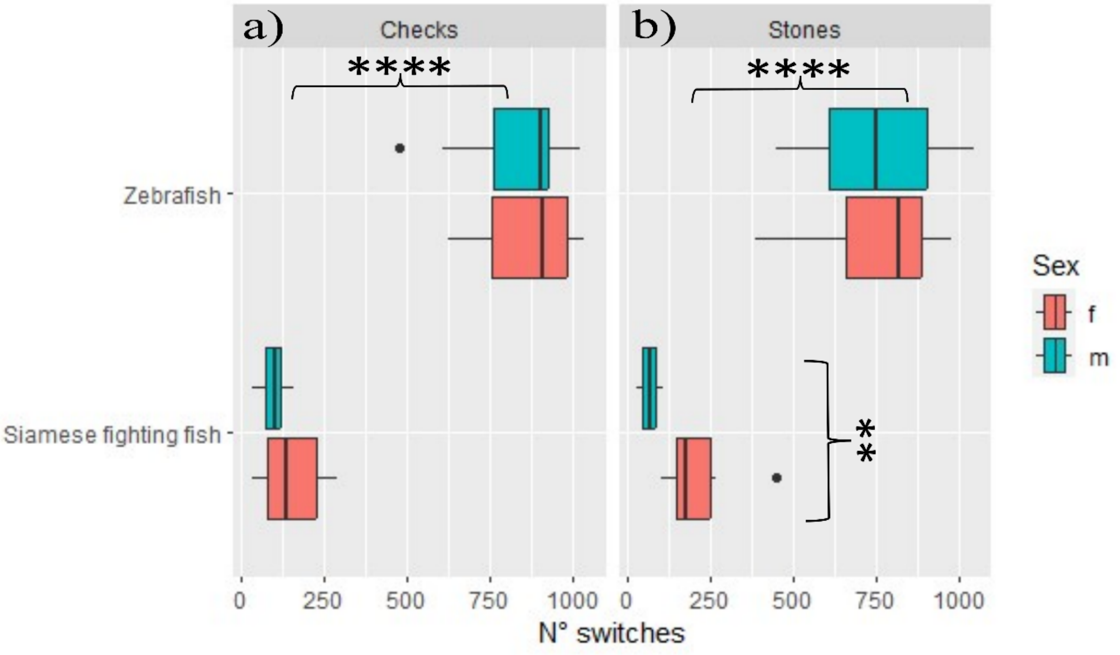

Figure 10.3. Activity levels measured by number of switches between sectors for a) Siamese fighting fish, and b) zebrafish, divided by sex and condition. The asterisks indicate significance levels (* = p< 0.5, ** = p< 0.01, *** = p< 0.001, **** = p< 0.0001).

Overall Side preference

While the central sector was clearly avoided by both species (t(63) = -6.02, p< 0.0001) when compared to chance levels (20%, that corresponds to the volume of the central sectors over the total), the results evidence no preference for the “Big” stimuli sector compared to the small one in both Siamese fighting fish and zebrafish, under either condition. This is clear from watching the Figure 11.3, which shows slight differences that are widely compensated by the variability of the behaviors. The only significant difference is between female and male zebrafish (F = 7.66, p = 0.015). Females spent significantly more time swimming over the big stones (t(14) = 2.70, p = 0.017), while males did the opposite, swimming more on the small stones t(14) = -2.72, p = 0.017)

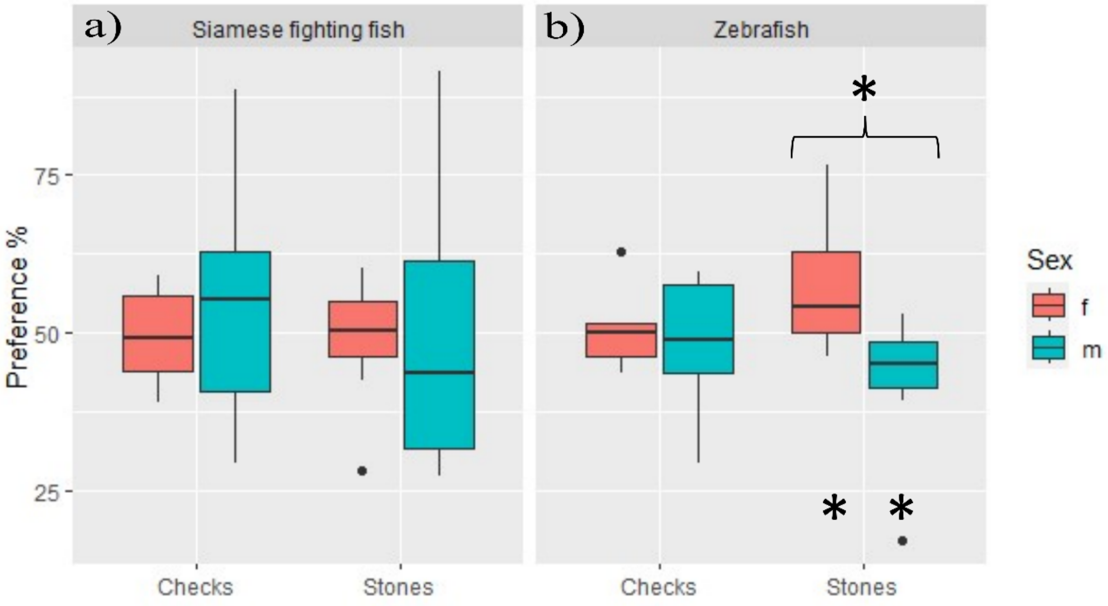

Figure 11.3. Percentage of preference for the "Big elements" sector for a) Siamese fighting fish, and b) zebrafish, divided by sex and condition. The asterisks indicate significance levels (* = p< 0.5, ** = p< 0.01, *** = p< 0.001, **** = p< 0.0001). the brackets indicate differences between groups, free asterisks indicate difference from chance level.

Siamese fighting fish: checks

The sixteen Siamese fighting fish swam in their usual manner for the thirty minutes of the test when exposed to the visual illusion produced by the checks. As it is shown in Figure 12.3a, both sexes avoided the central sector and spent more time in the highest parts of the lateral sectors. The model revealed, in fact, a significant effect of Height (F = 104.77, p< 0.0001), and the contrast realized with Tukey corrections evidenced a significant preference for the top sector (p< 0.0001). No significant sexual or side differences were found. Despite the time trend in Figure 12.3b, which shows wide fluctuations in the preference, no effect of time is present in both sexes.

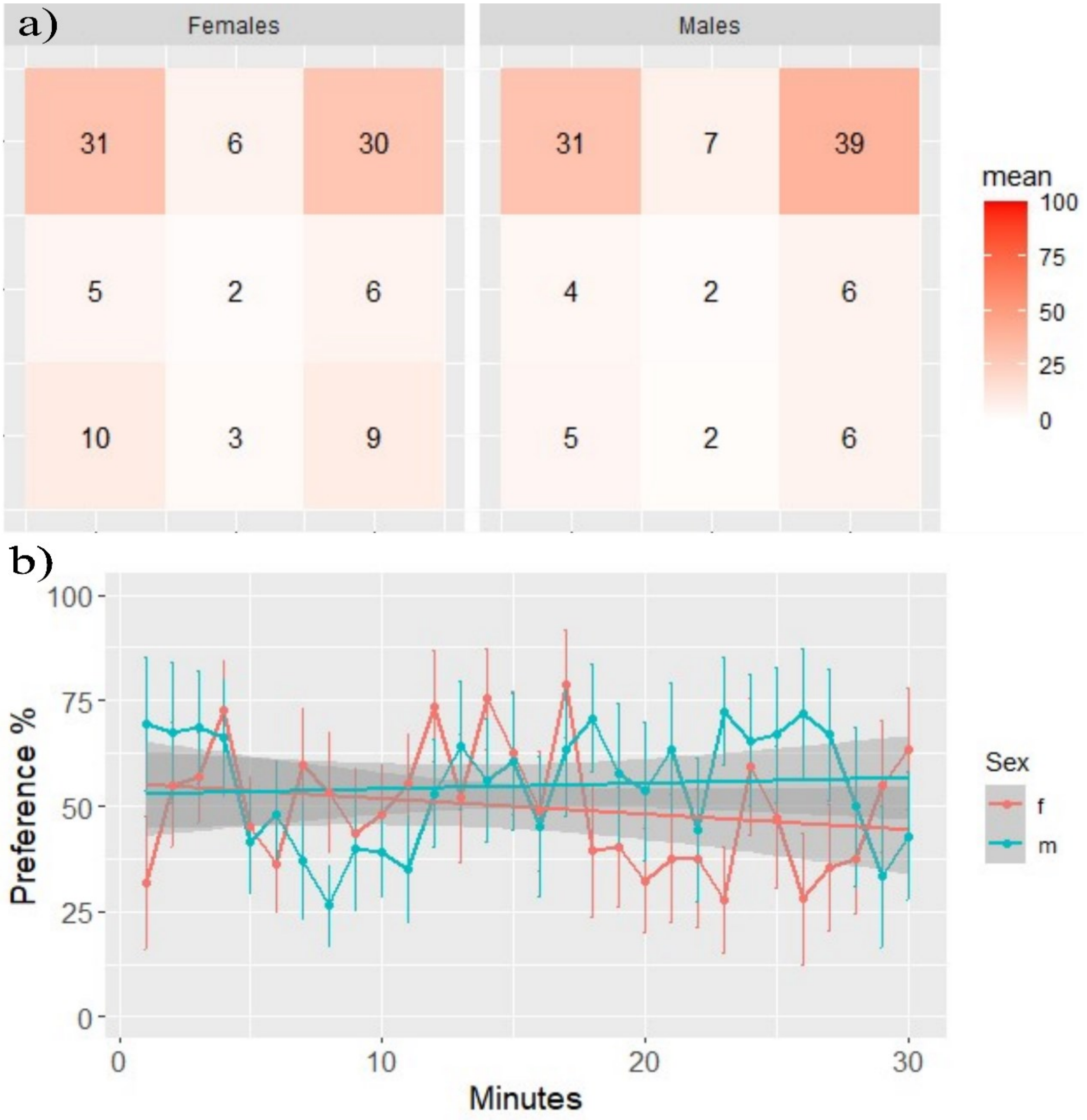

Figure 12.3. a) Percentage of time spent in the different sectors, divided by sex, and b) Percentage of preference for the “Big elements” sector, divided by sex. In b), bars indicate S.D. The red and blue lines and the dark grey shades indicate a smoothing function operated on the data obtained with the package ggplot2 (method = ”lm”) and their confidence intervals.

Siamese fighting fish: stones

Similar results were observed in Siamese fighting fish swimming patterns when exposed to the visual illusion produced by stones. As it is shown in Figure 13.3a, both sexes avoided the central sector and spent more time in the highest parts of the lateral sectors. The model revealed, in fact, a significant effect of Height (F = 62.68, p< 0.0001), and the contrast realized with Tukey corrections evidenced a significant preference for the top sector (p< 0.0001). No significant sexual or side differences were found. The linear model revealed an interaction between Time and Sex (F = 4.30, p = 0.043). In fact, as is shown in Figure 13.3b, with the passage of time, male *Siamese fighting fish* tend to swim more on the side with bigger stones, differently from females that remain stable.

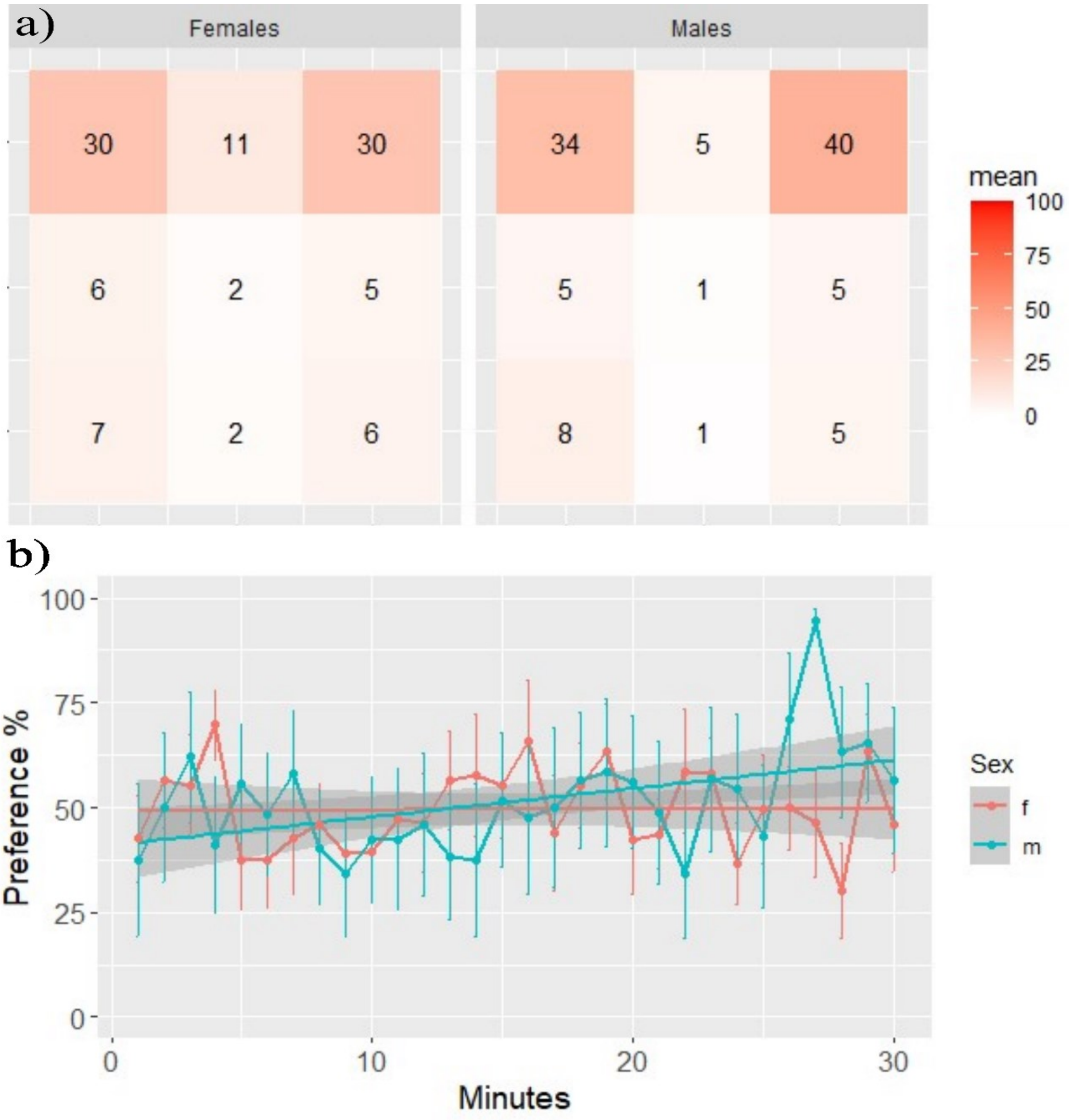

Figure 13.3. a) Percentage of time spent in the different sectors, divided by sex, and b) Percentage of preference for the "Big elements" sector, divided by sex. In b), bars indicate S.D. The red and blue lines and the dark grey shades indicate a smoothing function operated on the data obtained with the package ggplot2 (method ="lm") and their confidence intervals.

Zebrafish: checks

The zebrafish swimming patterns when exposed to the visual illusion produced by checks were similar to those produced by the zebrafish freely swimming, therefore opposite to the Siamese fighting fish's pattern. In fact, as shown in Figure 14.3a, both sexes preferred the lowest parts of the lateral sectors. The model revealed, in fact, a significant effect of Height (F = 11.48, p< 0.0001), and the contrast realized with Tukey corrections evidenced a significant preference for the lower sector (p< 0.001). No significant sexual or side differences were found. The linear model revealed an interaction between Time and Sex (F = 10.42, p = 0.002). Females gradually increased their preference for the side with big checks, while males gradually got worse, as shown in Figure 14.3b.

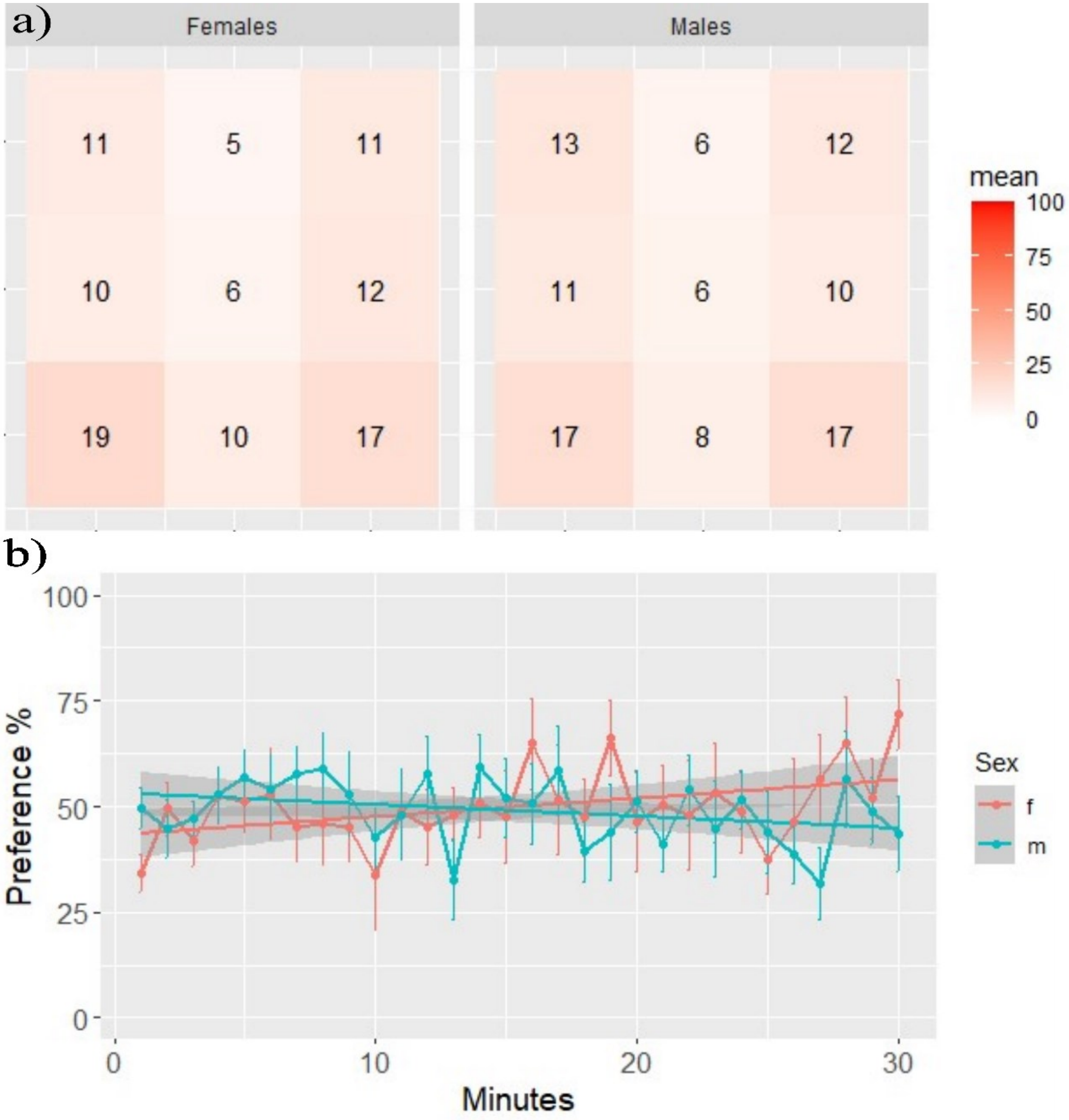

Figure 14.3. a) Percentage of time spent in the different sectors, divided by sex, and b) Percentage of preference for the "Big elements" sector, divided by sex. In b), bars indicate S.D. The red and

blue lines and the dark grey shades indicate a smoothing function operated on the data obtained with the package ggplot2 (method =”lm”) and their confidence intervals.

Zebrafish: stones

The zebrafish swimming patterns, when exposed to the visual illusion produced by checks, were similar to those produced by the zebrafish freely swimming. As shown in Figure 15.3a, both sexes preferred the lowest parts of the lateral sectors. Besides the usual effect of Height (F = 25.23, p< 0.0001) with a significant preference for the lower sector (p< 0.0001), there was also found an interaction effect between Sex and Side (F= 10.64, p = 0.0016). As mentioned before, the females spent significantly more time swimming over the big stones (t(14) = 2.70, p = 0.017), while the males did the opposite, swimming more on the small stones (t(14) = -2.72, p = 0.017). This difference grew with time (Figure 15.3b), as attested by the significant interaction between Sex and Time (F = 9.06, p = 0.004) in the linear model.

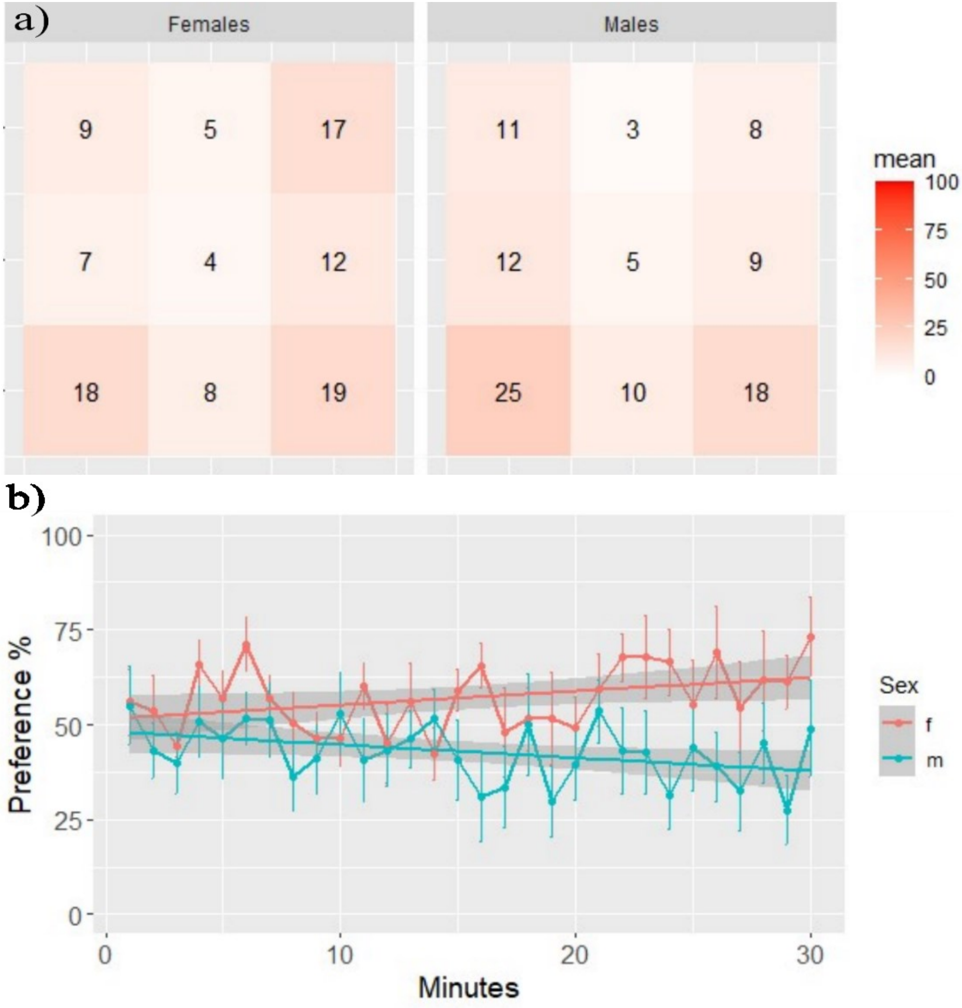

Figure 15.3. a) Percentage of time spent in the different sectors, divided by sex, and b) Percentage of preference for the "Big elements" sector, divided by sex. In b), bars indicate S.D. The red and blue lines and the dark grey shades indicate a smoothing function operated on the data obtained with the package ggplot2 (method ="lm") and their confidence intervals.

3.2.4 Discussion

The aim of this study was to test the possibility of enriching the physical environment of two widespread aquarium species through the use of special patterns able to fool the perceptive system of the fish, producing a visual illusion. Each stimulus was made by two different sides. The contrast between the two sides of the pattern positioned below the bottom floor should have misdirected the size constancy principle, used by perceptual systems of animals across several taxa (Imura et al., 2008; Timney & Keil, 1996; Pastore, 1958; Horridge et al., 1992; Schuster et al., 2004), to perceive a discrepancy in the depth levels of the two sides, perceiving one (the side that presented smaller components) as further away from the perceiver and the other (the side that presented bigger components) as closer, thus shallower. Two different components were arranged to produce the illusion. The first one was a checkerboard made of black and white squares. The high contrast enhances the performance of humans in many visual tasks (Westheimer et al., 1999) and usually helps in eliciting perceptual phenomena such as illusions (Pina & Spillmann, 2005), even if not in all illusions (Westheimer et al., 1999). On the other hand, the pattern was poorly ecological. The second element was instead generic white stones of the same dimensions as the squares. While the visual contrast between two adjacent elements was certainly lower than the one produced by the checkerboard, the ecology of the flooring was greatly improved. Based on the results of the previous experiment, we expected that both species would have expressed a preference for swimming on the seemingly closer side paved with greater elements: the Siamese fighting fish in the highest parts of the aquarium and the zebrafish in the lowest parts, closer to the bottom. The tendencies for positioning in the water columns expressed in the presence of a real stairway were replicated here. Siamese fighting fish expressed a clear preference for the higher sectors of the aquarium, and the zebrafish preferred the lower ones.

The Siamese fighting fish did not express any preference for the different sides of both illusory stimuli. Both the high-contrast and the high-ecology nature of the two patterns failed to elicit the expected preference for the bigger components (i.e., the "closest"). The lack of any effect could be caused by the failed perception of the illusion. Although it has been proven that goldfish and archerfish can use the size constancy principle to determine the size and distance of objects (Douglas & Guttridge, 1988; Schuster et al., 2004), we cannot safely generalize those results to each of the many existing species of fish. Moreover, the two experiments mentioned were based on reinforcement procedures that ensure a high level of motivation and focused attention towards the proposed stimuli. While the consistency of the results in Experiment A suggests that the subjects were motivated to follow their natural swimming behavior, it cannot be excluded that their attention was not directed towards the bottom of the tank. It is possible that the water surface is used as the most prominent cue for positioning in the water column, both for its stability and for the other vital purposes it serves in the life of the Siamese fighting fish. The highly domesticated strains of this ornamental fish rarely display fear of predation or human contact and spend most of their time waiting for food, caring for their bubble nest, or breathing from the air. In all these activities, their attention and gaze are directed towards the water surface.

Their behavior does not vary during the thirty minutes, signaling no effect of reaction and subsequent habituation to a novel environment. It is possible then that the activity level, already extremely low in Experiment A, further decreased in both conditions, down to an average of 2.5 sector changes per minute in males and around 6 sector changes per minute, signaling an apparent calm. Conversely, the already frenetic zebrafish increased their activity to more than 25 sector switches per minute in both males and females.

The state of continuous movement made the zebrafish in our experiment explore more thoroughly all the sectors of the tank, even if they preferred the lower ones. In both conditions, the zebrafish of each sex strengthened their preference for one side with the passage of the thirty minutes. This effect of time could suggest that after an initial habituation, the zebrafish started to process the visual stimuli and to react accordingly to their preferences. In the "Checks" condition, no overall preference for any side emerged in both sexes. On the other hand, in the "Stones" condition, it has been observed that there is a significant preference for the big components side, at least limited to females. Males, on the contrary, showed an unexpectedly significant preference for the opposite side, the furthest one, following the principle of the illusion. This result could indicate a greater effectiveness of the realistic stimulus over the abstract one, even if it is more defined in its properties. The naturalness of the gravel substrate made of stones of variable size could have triggered different processes in the perceptual and exploratory mechanisms. While there is no reason in the literature to hypothesize differences in the visual systems of the two sexes, it is known that male and female zebrafish differ in some of their behaviors because of their sexual roles. These differences could influence the exploration style of the subjects, making each sex focus on particular relevant cues.

We know that females are attracted to shallow areas to be able to lay eggs. The fast ovarian cycle lasts precisely five days at 26 °C (Hisaoka & Firlit, 1962), so we can assume that many of our female experimental subjects could have been inside or proximate to their breeding period. Female zebrafish are responsible for the choice and defense of the spawning site, while males' occupation is to chase the females, and eventually engage in mate-guarding behaviors (Hutter et al., 2010). Female zebrafish prefer a thick-grained gravel substrate to silt as a depositing site because it better protects eggs from lack of oxygenation, parasitism, and cannibalism, substantially raising their survival rate (Spence et al., 2007a). Thus, the suitable nature of the stimuli proposed could have triggered the ecological behavior of site-seeking in females. In the absence of females, the tendency for males to set in shallower areas could have been reduced, making them focus on other priorities, such as seeking repair in the deepest areas of the tank. This different pattern of behavior has not been observed in Experiment A, probably because of the sub-optimal quality of the bottom surface texture.

To resume, the tests of Experiment B showed no particular influence of the visual illusion that we call "Virtual Cliff", except for zebrafish in the "Stones" condition.

It is not clear if Siamese fighting fish can or cannot perceive the illusion. It is possible that the focus of visual attention in this species is on the water surface, making the bottom texture irrelevant. To prove this, it would be necessary to expose *Siamese fighting fish* to the illusion in their visual range, above the water level. However, it seems that this method would be of no use in environmental enrichment practices. Conversely, zebrafish are known to pay great attention to the substrate features of the tanks they live in (Marcon et al., 2018; DePasquale et al., 2019). In fact, they showed a marked side preference, at least in the condition where the illusion placed under the tank was built with realistic features. While it is not completely clear how to interpret the sexual differences found, it is a first proof of the perception of this illusion and thus of the use of the size constancy principle in zebrafish. Further studies will study the efficacy of this illusory pattern compared to a homogeneous one and its impact on other physiological indicators of well-being like hormone levels and egg production and deposition.

As mentioned in the discussion of Experiment A, the interpretation of the results obtained from this experiment would have greatly benefited from the analysis of the physiological markers for stress (Pottinger, 2008), which would have allowed a comparison between the attraction toward a stimulus (or the lack of it) and the actual stress-related activation. Once again, prolonged exposure and group life could lead to unexpected interacting effects that are not predictable from our data and need ad hoc testing to be assessed.

Chapter 4

Shoal illusions

*parts of this chapter were adapted from the publication “Mair, A., Dadda, M., Kitaoka, A., & Agrillo, C. (2023). Illu-Shoal Choice: An Exploration of Different Means for Enrichment of Captive Zebrafish. Animals, 13(16), 2640.”

In this chapter, all the experiments will focus entirely on the zebrafish’s sociality.

The zebrafish is a well-renowned model, and, over time, the number of zebrafish held captive in laboratories across the world has risen to several millions in over 100 countries (Kinth et al., 2013) due to the peculiar biological and morphological features that this animal model possesses. As mentioned before, the robustness and small dimensions of this animal, matched with the great observability of their developmental stages, made it one of the most appreciated animal models in biomedical and neuroscientific research. They do not require much space, as they are small and prefer to be in large groups or shoals. The relative ease of maintaining zebrafish populations and the apparent normal behavioral patterns displayed by the animals during their lives can sometimes lead to neglect toward important aspects of their lifestyle (Message & Greenhough, 2019).

Furthermore, the results from different studies collected in a recent review (Stevens et al., 2021) sometimes show discordant conclusions, indicating a possible lack of a comprehensive approach orparadigm to understand zebrafish well-being. Sociality is no exception.

For example, zebrafish is widely considered a gregarious species because it naturally lives in groups ranging from tens to hundreds of individuals (Suriyampola et al., 2016). The onset of social contact is very precocious (Dreosti et al., 2015; Stedniz & Washbourne, 2020), around 10 days post-fertilization (dpf), shortly after being able to move and when the larvae’s perceptual system is still immature. During development, the shoaling behavior of zebrafish grows gradually (Buske & Gerlai, 2011) thanks to the development of recognition mechanisms based on multiple sensory modalities like sight and smell (Saverino & Gerlai, 2008; Gerlach et al., 2008) that allow to discriminate conspecifics from non-conspecifics, kins (Hinz et al., 2013), and even precise individuals over time (Madeira & Oliveira, 2017). The morphological features of the other members are learned with experience and used as a basis for future recognition (Engeszer et al., 2004). Other important contextual information is extracted vicariously from other conspecifics living in the same group through eavesdropping (Abril-de-Abreu et al., 2015) and used as a cue to modulate the behavior, decreasing (social buffering) or increasing (social contagion) the fear response (Akinrinade et al., 2023).

The innateness and efficiency of social skills suggest that group life is a key element for this species' survival. Group living in fact grants a number of advantages, like protection and access to resources (Ward & Webster, 2016).

At the same time, however, living close to conspecifics implies several costs to bear, connected to overcrowding, opposite-sex conflicts for reproduction, and, more generally, dominance conflicts for resources such as sexual partners, food, and sheltering areas in the tank (Grant & Kramer, 1992; Spence & Smith, 2006; Paull et al., 2010). In zebrafish, dominance hierarchies cause many conflicts that result in aggressions towards the weaker members (Oliveira et al., 2011; Zabegalov et al., 2019). Aggression rates increase when the zebrafish are housed in dyads (Mukherjee & Bath, 2021), and in extreme cases, when other aggression-modulating features like habitat complexity are lacking, these aggressions could even lead to death (Keck et al., 2015). The stressful components of social life emerge in the paradoxically lower level of stress hormones observed in isolated fish (Giacomini et al., 2015; Shams et al., 2017; Forsaktar et al., 2017).

The effects of sociality could be modulated by the personality tendencies of certain individuals' (Ariyomo et al., 2013; Nunes et al., 2017) that could prefer less contact with conspecifics. Prolonged forced isolation of otherwise social fish alters their behavioral and brain activity patterns, making them resemble asocial fish, but the effect is temporary and reversible (Tunbak et al., 2020). After the initial increased anxiety expression when presented with conspecifics, the behavioral and hormonal activation levels return to normal.

It is important to improve the current state of knowledge on sociality in zebrafish for several reasons. In the first place, a deeper understanding of this crucial dimension in this species' life will improve the maintenance conditions of the captive individuals, reducing the negative effects of other stressors and preventing the insurgence of aggressive behaviors (White et al., 2017). A positive social environment will reduce stress levels and keep the animals healthy and reliable as experimental subjects. Stress can in fact be an important confounding factor in the performance of several tasks (Strand et al., 2010; Fontana et al., 2021; Buenhombre et al., 2023).

Secondly, increased competence in the social world of the zebrafish could help in improving their ability to produce highly effective artificial dummies and act as social reinforcers. While it is already used as a common methodology to raise the motivational levels of the experimental subjects in performing some tasks (Agrillo & Bisazza, 2014; Baratti et al., 2020; Mair et al., 2021), it could be perfected by creating a super-normal stimulus (Staddon, 1975; Barett, 2010), more effective than real conspecifics and food (Daggett et al., 2019).

Lastly, sociality is a complex, multifaceted ability that is grounded in many different parts of the brain (Stednitz et al., 2018; Chou et al., 2016; Teles et al., 2016). The dissection of different components of sociality could help us shed light on the specific circuitry involved and possibly generalize it to different animal models, including humans (Oliveira, 2013; Geng & Peterson, 2019).

In both experiments, we used a spontaneous choice paradigm: the experimental subjects were let free to swim without any previous training towards the preferred stimulus. Two different stimuli are presented on opposite sides of the experimental tank, and the fish should swim in proximity to the preferred stimulus, which should be socially more attractive in this case (Ogi et al., 2021). Several factors could influence the preference for one group of conspecifics over the other: number (Agrillo et al., 2008), size (Lucon-Xiccato et al., 2017), activity (Pritchard et al., 2001,) and even water temperature (Angiulli et al., 2020). We would like to add some useful pieces of information to this growing literature.

4.1 Experiment A: bidimensional static illusions of movement

4.1.1 Introduction

The company of conspecifics is certainly a natural and necessary feature for a healthy environment, but it presents a variability that could alter the desired enriching effects. Besides the potential undesired effects of group living, some experimental procedures, such as training, require extensive periods of isolation.

These problems lead to the necessity of developing a neutral social enrichment free of the potential negative effects of real physical interaction. Artificial stimuli are another valid enrichment tool. An experiment by Saverino and Gerlai (2008) showed that video playback of moving conspecifics attracts spontaneously zebrafish that prefer them to static images and to moving images of abstract figures. The relevance of these video dummies can be altered by manipulating the aesthetic features of the stimulus. For instance, a strong elongation of the fish silhouette stimulates avoidance behaviors due to the increasing similarity to a possible predator shape. On the contrary, a yellow coloration, possibly associated with good health, increases the attraction to the conspecifics' dummy (Saverino & Gerlai, 2008).

We saw that the video presentation of artificial stimuli is a valid method to elicit social responses in zebrafish. But the main problem with video presentations is their practical implementation as an everyday tool to improve the well-being of captive zebrafish. Using a monitor for every tank that necessitates the treatment could be costly for the laboratory's facilities.

Here we aimed to create a non-expensive 2D printable image of a zebrafish capable of triggering a positive social response that can be used in laboratories, regardless of their economic possibilities. In our preliminary investigation, simple, realistic images of zebrafish printed on paper failed to trigger any form of attraction; therefore, we decided to enhance the realism of the stimulus by modifying the internal livery. The visual system of zebrafish is in many ways similar to humans', and they demonstrated on several occasions susceptibility to visual illusions and other properties of the visual system comparable to mammals' (Gori et al., 2014; Najafian et al., 2014; Sovrano et al., 2022).

We chose different patterns and tried to mesh them into the livery in order to recreate the artificial features, such as motion and pictorial cues of depth that we thought could have been relevant for zebrafish. We selected black and white striped patterns, similar to zebrafish skin, to create a stimulus that was as realistic as possible. We then confronted them with non-realistic control stimuli and grey ellipses with the same dimension and overall shape.

Our hypothesis was that dummies boosted-up with illusory features of movement and tridimensionality would have been considered more attractive than the controls, resulting in more time spent in their proximity.

4.1.2 Methods

Subjects

We tested 56 mixed-sex adult zebrafish: 16 zebrafish (approximately 12–16 months old) were tested in the first experiment (Ouchi-Spillmann illusion) and 20 in each of the other two experiments.

Fish were born and maintained at the comparative psychology laboratory (Animal Behavior and Cognition Lab) of the Department of General Psychology (University of Padova) in mixed-sex groups of 20–30 individuals (150 liters in each tank). The aquariums were grey plastic tanks (70 cm x 45 cm x 55 cm) provided with air filters, natural gravel, and live plants at a temperature of 25 ± 1 °C. The test tanks had no heating mechanism, as did all the tanks in our lab. The water temperature was maintained by ensuring a constant room temperature roughly 2 degrees above the desired water temperature. Fish were fed twice daily: once with commercial food flakes and once with live brine shrimps (*Artemia salina*), on a 12:12 h light-to-dark photoperiod and an 18-watt fluorescent light.

Apparatus

The experimental tank was a 40 cm x 60 cm x 30 cm glass tank. The whole tank, except the two short sides, was covered with green opaque plastic to prevent the fish from seeing the outside (Figure 1.4a). Moreover, two walls of green plastic were inserted into the tank. The narrow space between two sectors was 10 cm wide. The reason for this, following previous shoal choice methods (Saverino & Gerlai, 2008), was to partially prevent the simultaneous sight of the two stimuli. The fish's choice of compartment had to imply a strong effect of the stimuli's visual availability. The corners of the tank were rounded using the same green plastic to reduce the fish's tendency to seek shelter when placed in a novel environment. All of these components together concurred to form an "hourglass" shape (Figure 1.4b).

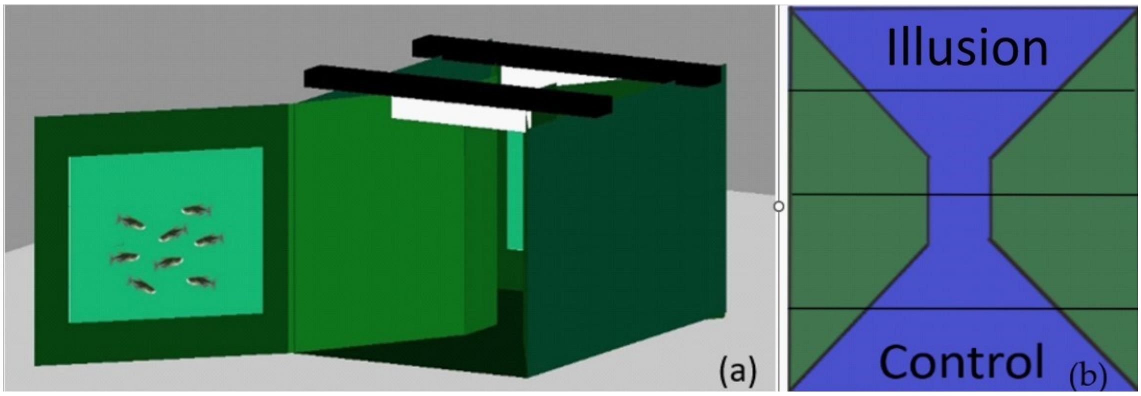

Figure 1.4. Visualisation of the apparatus **a)** from the side (one of the two short sides is open for illustrative purposes) and **b)** from above, evidencing the hourglass shape.

The experimental stimulus and the control stimulus were glued to two panels of the same green plastic and were attached to the two short sides of the tank during the experiment, alternating the presentation between the two positions.

Two 16-watt LED lights (746 lm) were positioned on the top of the long sides of the tank to homogeneously illuminate the arena. The recording camera was placed 50 cm above the water surface at the center of the tank.

Stimuli

The aim of the experiments was to create a dummy conspecific that was able to elicit a spontaneous positive social reaction in the experimental subject. To obtain this result, we tried to mesh the artificial features that could be relevant for zebrafish into an overall semi-realistic dummy. All of the artificial stimuli selected were black and white striped patterns, similar to zebrafish skin. The three stimuli were produced using the vector graphic software Inkscape version 0.92.3 (https://www.inkscape.org, accessed on 15/06/2023). All three stimuli were created by inserting the artificial pattern into the body of the fish figure. The other features (head, fins, and size) were maintained.

Eight artificial fish of a particular illusory pattern (see Figure 2.4) were arranged with different orientations and inclinations on a rectangular sheet (30x21 cm) and subsequently glued to the panels.

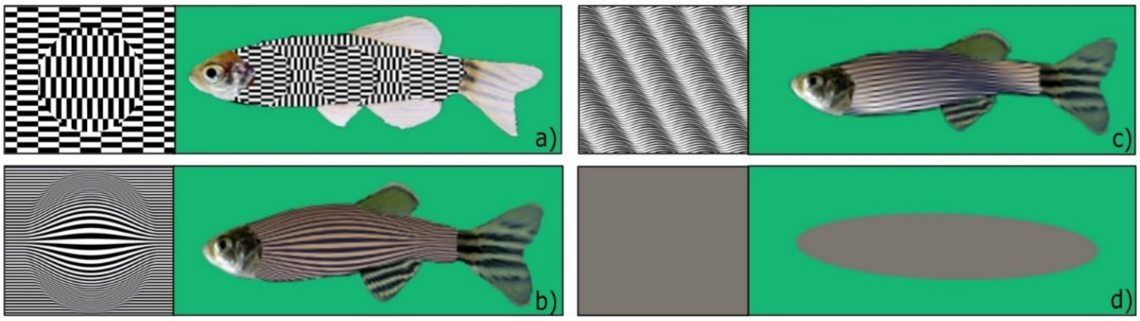

Figure 2.4. The four types of dummies used in our experiment: **a**) Ouchi–Spillmann illusion, **b**) depth illusion #1, **c**) depth illusion #2, **d**) grey ellipse as control.

- Experiment 1: Ouchi-Spillmann illusion (Spillmann, 2013) (Figure 2.4a)

This visual illusion consists of a disk made of horizontally oriented checks centered on a vertically oriented checked surround. The center appears to slide relative to its surroundings according to subtle eye and/or image movement. This motion illusion is quite robust in humans: it is enhanced by movement, it is also perceived monocularly, and, when blurred, it persists when presented near the periphery of the visual field and when the surroundings correspond to the length of a check only. We thought that these features could be promising for effectiveness in fish.

- Experiment 2: Depth illusion #1 (Figure 2.4b)

We chose this black-and-white pattern to simulate the 3D effect of depth. The pictorial cue consists of the enlargement of the zebrafish body's central stripes to render the effect of greater proximity. The movement of the stimuli produces a flickering that could contribute to drawing the subjects' attention.

- Experiment 3: Depth illusion #2 (Figure 2.4c)

We chose this black-and-white pattern to simulate the 3D effect of depth. The pictorial cue consists of the non-linearity of zebrafish stripes. The different luminances could reflect the various light effects produced by a source of light impacting the body of a swimming zebrafish. The shades on the body produce an effect of greater proximity, of tridimensional movement, and of flickering that could contribute to drawing the subjects' attention.

- Control stimulus for Exp. 1-3 (Figure 2.4d)

The control was a grey ellipse (RGB: 123 119 120) of the same dimensions as a 4 cm-long zebrafish. The ellipse had no illusory movement, no body patterns to resemble a conspecific, and no distinctive fish features such as eyes, tail, and fins, considered of high importance in shape discriminations. Also, the control stimulus provided no depth cues, nor was it able to elicit any illusory motion in human observers.

Procedure

Experiments were performed during the day, ranging from 10:00 (2 hours after morning feeding) to 15:00 (2 hours before evening feeding). Each subject was selected from the stock tanks and put into a small (1-liter) plastic bucket for transportation to the experimental room.

After the time strictly necessary to move the fish from the maintenance to the experimental room (approximately 1 minute), the subject was gently inserted into the experimental tank. After 60 seconds, the experiment started, and the time spent near the stimuli at the two short sides of the tank was taken as the dependent variable. We decided for a short habituation period to balance the subjects' need for alleviation to netting and air exposition stress on one side and the necessity to avoid excessive habituation that would have mitigated the motivation of this test, namely the need to shoal with conspecifics when placed in a new, empty, and thus slightly stressful environment. No subject showed signs of extreme stress in their behavior, such as freezing.

When the experimental time elapsed (20 minutes), the subject was removed from the experimental tank using a fish net, transported through the plastic bucket back to the maintenance room, and placed into a new, post-experimental tank.

After removing the subject, 70% of the water in the experimental tank was replaced with water coming from three 150-L tanks enriched with gravel, plants, lamps, and a filter system to ensure the same quality and chemical composition of the water and minimize possible interference of the previous subjects' scent (e.g., hormones). Lastly, the positions of experimental and control stimuli were switched to prevent any possible bias originated by side preferences.

Video analysis

The experiment was recorded through a camera placed above the experimental tank. Three trained observers analyzed the videos offline. Each video consisted of preparatory routines (showing the ID of the subject and the position of stimuli and controls in the tank), 1 minute of habituation, 20 minutes of experimental recording, and the final removal of the fish.

The region of interest were the two rectangles (10 cm x 40 cm each, following previous works (Gatto et al., 2019; Lucon-Xiccato et al., 2016) adjacent to the walls where the stimuli were hung, and the time spent in each sector was scored using the program Ciclic Timer. The change of sector was determined by the passage of half of the subject's body.

Data analysis

Analyses were performed in R version 3.5.3 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org). Preference for the target stimulus was evaluated by calculating the proportion of time spent by the subject in the choice area associated with the target stimulus, in detail:

Preference for the target stimulus = time near the target stimulus / (time near the target stimulus + time near the control stimulus)

In the same way, the preference for the lateral sectors was calculated and confronted with the central sector. Student's t-tests were performed to assess if the eventual preference for a sector was statistically significant against the null hypothesis of $\mu = 0.5$.

Additionally, we performed the same t-tests using a Bayesian approach using the R package BEST to obtain a less dichotomous evaluation of the results.

The fluctuation of the preference across the 20 minutes of experiments was tested through a linear model with ID as the random factor and minutes as the predictor.

4.1.3 Results

In Table 1.4 are listed the percentage of time spent in the three sectors by our experimental subjects.

	Ouchi-Spillmann illusion	Depth illusion #1	Depth illusion #2
Illusion	34.36 % ± 30.36	37.58 % ± 31.44	37.87 % ± 32.63
Center	30.82 % ± 21.72	22.76 % ± 16.26	21.06 % ± 17.86
Control	34.53 % ± 30.82	39.67 % ± 31.76	41.85 % ± 34.17

Table 1.4. Mean percentages of time the spent in the different zones by the subjects.

Do fish find the stimuli attractive? Lateral sectors vs. central sector.

In each experiment, the lateral sectors were preferred to the central one, especially considering that the area of the lateral sectors was only 40% of the total, the other 60% being the central sector.

The average percentage of time spent in the two lateral sectors compared to the central one was 69.18 ± 14.72 for the traditional version of the Ouchi-Spillmann illusion (t = 7.928, df = 15, p-value = 9.617e-07, Cohen's d = 1.98), 77.24 ± 7.88 for the Depth illusion #1 (t = 21.134, df = 19, p-value = 1.166e-14, Cohen's d = 4.73), and 78.78 ± 10.86 for the Depth illusion #2 (t = 16.024, df = 19, p-value = 1.713e-12, Cohen's d = 3.58). This ensured us that the stimuli were explored by the fish for an extended period of time (Figure 3.4); thus, we had the opportunity to adequately compare them.

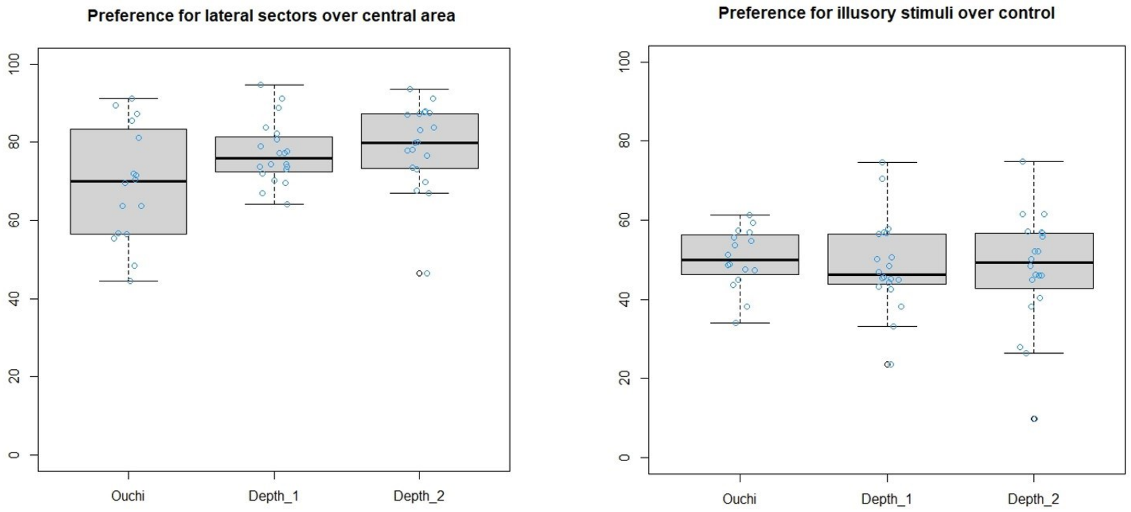

Figure 3.4. Boxplots representing the preference for lateral sectors over central area (left) and illusory stimuli over control (right).

Do fish find the illusory pattern attractive? Illusory stimulus vs. control stimulus

On the other hand, no preference for any stimulus was found. The average percentage of time spent in the sector close to the target stimulus was 50.24 ± 7.58 for the Ouchi-Spillmann illusion (t = 0.1283, df = 15, p-value = 0.8996, Cohen's d = 0.03), 48.78 ± 11.55 for the Depth illusion #1 (t = -0.4705, df = 19, p-value = 0.6433, Cohen's d = -0.105), and 47.72 ± 14.38 for the Depth illusion #2 (t = -0.7085, df = 19, p-value = 0.4872, Cohen's d = -0.158).

As mentioned in the method section, we tried to evaluate the weight of the slight differences in preference for the stimuli using Bayesian t-tests. Figure 4.4 depicts the distributions of the differences between our results and the null hypothesis after applying the Markov Chain Monte Carlo (MCMC) method with 100,000 simulations.

The tests revealed no preference for the illusory stimulus in the Ouchi-Spillmann illusion experiment.

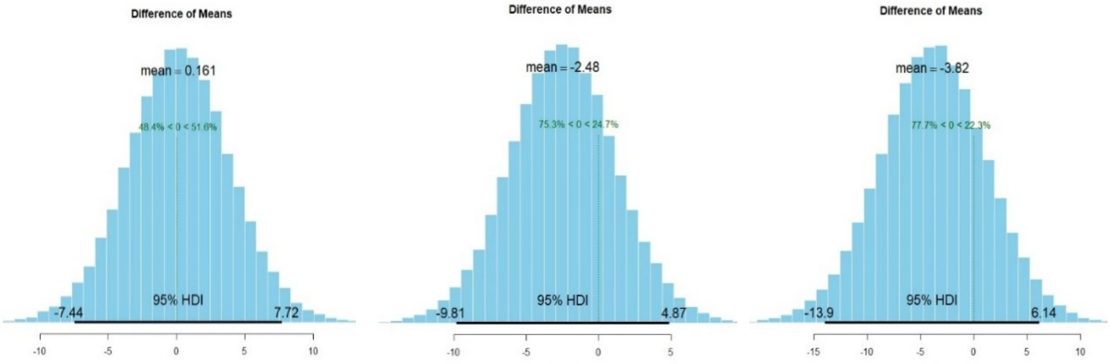

Figure 4.4. Posterior distributions of the MCMC simulations for Ouchi-Spillmann illusion (left), Depth illusion #1 (center), Depth illusion #2 (right) preferences over the null hypothesis.

The mean difference between the percentage of time spent in the region adjacent to the stimuli and the control is $\mu = 0.161$. The probability that the illusory stimuli are preferred to the control is 51.6%, very close to chance. In fact, the Bayes factor (BF) calculated to compare the likelihood of the presence of an effect against the null hypothesis is extremely low (BF = 0.25). Of comparably small size is the BF of the depth illusion #1 (BF = 0.25) and depth illusion #2 (BF = 0.3), indicating evidence for the absence of the stimuli's effect. Nevertheless, the distributions of MCMC simulations of the two depth illusions result in negative differences of means between stimuli and control preference, indicating a tendency towards an effect that is opposite to the expected one. The difference in means for depth illusion #1 is μ = −2.28, resulting in a probability of preference for the control stimuli of 75.3%. The difference of means for depth illusion #2 is μ = −3.82, resulting in a probability of preference for the control stimuli of 77.7%.

Finally, we assessed if fish preferences changed as a function of time. The linear models showed that fish preferences did not change across time (Ouchi-Spillmann: F = 0.023 Pr(>F) = 0.879, Depth illusion #1: F = 0.842 Pr(>F) = 0.359, Depth illusion #2: F = 0.314 Pr(>F) = 0.575). As shown in Figure 5.4, the fluctuations of preference above and below the mean seem to be quite randomly distributed both within and between the experiments.

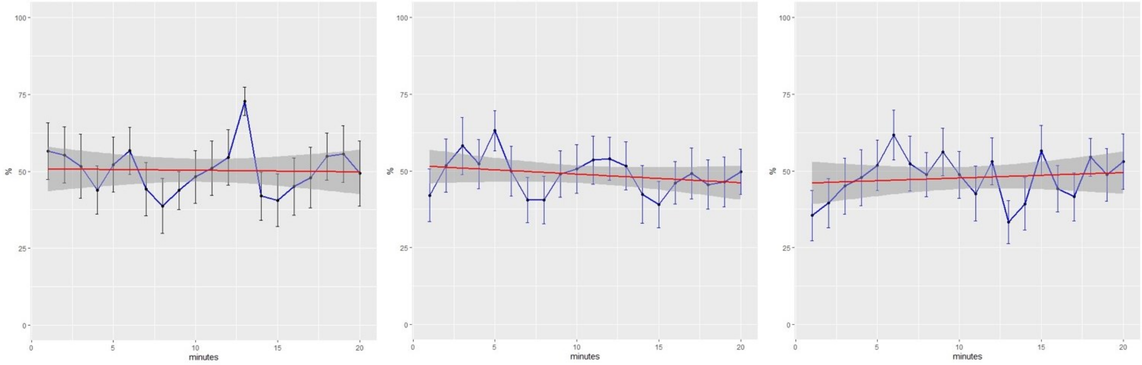

Figure 5.4. Percentage of time spent in the illusory sector close to Ouchi–Spillmann illusion (**left**), depth illusion #1 (**centre**), depth illusion #2 (**right**), over the 20 min of observation. Bars indicate S.D. The red lines and the dark grey shades indicate a smoothing function operated on the data obtained with the package ggplot2 (method =”lm”) and their confidence intervals

4.1.4 Discussion

This study aimed to create 2D dummies of conspecifics that could be attractive to zebrafish. This would represent the first step to assess the validity of an efficient, social, visual enrichment. In particular, we focused on two of the most important visual features of conspecifics: the presence of (illusory) movement and depth cues. To obtain these features on a 2D image, we deployed black and white patterns to create a potentially realistic image of a conspecific that could attract the experimental subjects left in isolation. Our data analyses combined both p-value and Bayesian approaches and showed that our target stimuli were not more attractive than a control stimulus that does not include any feature of conspecifics. In the presence of null results, we can only speculate on the lack of difference in the proportion of time spent near target and control stimuli. First, fish could be poorly attracted by the target stimuli, as these do not accurately represent real conspecifics. Previous studies have shown that visual stimuli with no odor or acoustic cues are effective as a proxy for conspecifics (Saverino & Gerlai, 2008; Ruhl & McRobert, 2005; Nunes et al., 2020; Spence & Smith, 2007). However, in these studies, body features (e.g., color, texture, etc.) were similar to those of conspecifics. Here, in the attempt to add depth cues and illusory motion, we largely modified the inner features of the dummy social companions, probably disrupting the perception of real, healthy conspecifics.

Other methodological issues may have played a role. The scoring technique only allowed us to count the time spent in each sector and calculate the preferences, possibly missing other relevant kinematic features such as a frame-by-frame position and a speed that could be captured by implementing efficient tracking technology. Depth and speed both have biological meaning (Blaser & Rosemberg, 2012) in zebrafish and could reflect dispositions that might be missed by analyzing only the x and y positions of the subjects. Distance from the target stimuli is the most common dependent variable adopted in this field, but focusing only on this variable might have caused us to neglect other aspects of fish behavior that could have indirectly revealed their preference for either stimulus. Another index that may reveal underlying processes is stress levels. In the mildly stressful situation of being alone in an empty tank with no enrichment, the (illusory) presence of conspecifics would mediate the production of stress-related hormones such as cortisol (Forsatkar et al., 2017). Measuring the quantity of cortisol in the water removed after each trial and matching it with the precise kinematic profile would give us a wider picture to interpret the results.

Alternatively, the illusions could fail to reproduce the desired effect of realism for several reasons. First, zebrafish perceive UV light and are naturally attracted to it (Nava & Hamil, 2011). A real conspecific could have a distinctive UV reflectance pattern when staying still and when swimming, which may help in the recognition of biological entities in an otherwise possibly turbid environment full of nonbiological distractors.

Secondly, the illusion presented could be based on some perceptual mechanism that zebrafish do not possess. In humans, the illusion requires checks of low spatial frequency; it persists with low luminance contrast and blurring of the inducing pattern but is absent at equiluminance (Spillmann, 2013). The perception of the Ouchi-Spillmann illusion is thought to be independent from some magnocellular neuronal mechanisms yet to be identified that could be absent or reduced in the visual pathways of zebrafish.

Moreover, an important parameter for the strength of this illusion is the dimension of the circle compared to the dimension of the checks (Ashida, 2002). To obtain a visible central circle and inner sectors that could match the dimensions of zebrafish's stripes, we had to expand it, limiting the space.

Another possible hypothesis for our experimental subjects' lack of reaction to these stimuli is that these patterns produce effects localized inside the body of the conspecific. No effects are producing a shift in the fish's body shape relative to the background. Zebrafish and many other animals use immobility (e.g., freezing) to hide from predators. During freezing behavior, a zebrafish continues to move its pectoral fins to maintain its position in the water. Thus, the global movement of the fish in the environment seems to be more relevant from a biological evolutionary perspective than small local movements, even if they are constant and rapid.

Lastly, we did not assess the influences of personality trait scores with tests measuring relevant sources of individual variation (Sih & Del Giudice, 2012). Thus, we cannot rule out the possibility that different predispositions to sociality could have existed in our sample and affected the behavioral response and effectiveness of our enrichment stimuli.

The route is long, and further investigation is required in this field. In the absence of any preference by fish for our target stimuli, we must be cautious in assuming that stimuli with an overall shape of conspecifics and depth cues and illusory motion in the inner part could be the powerful social enrichment we were hoping for.

The quest for refining animal welfare conditions is never-ending and brings with it possible advancements in the comprehension of the perceptual mechanisms of the study's animal subjects. Some natural preferences could be hard-wired in the brain and senses of the animal and trigger a series of behaviors and conditions that enhance or diminish the animal's well-being.

In this study, we tried to capitalize on the natural predisposition of zebrafish for shoaling, or at least the preference for group living. This is part of a set of studies aiming to trace the fundamental features of visual social recognition in zebrafish. We tried to simulate some realistic features, such as movement and depth, using some visual illusions, but the behavioral results we collected are pretty inconclusive. We cannot firmly rule out the occurrence of methodological issues in stimulus presentation, but the most likely explanation is that the illusions are not perceived by the subjects, or if they are perceived, they fail to produce the effect of a realistic conspecific.

Future studies should disentangle this issue and continue to advance the availability of information regarding zebrafish social and perceptual systems.

4.2 Experiment B: Kinegrams based on Moiré patterns

4.2.1 Introduction

The stimuli tested in Experiment A were ineffective, indicating that shape is not a sufficient element to trigger social attraction in the zebrafish. The Ouchi-Spillmann illusion, capable of triggering the perception of apparent motion in human observers, was used as a semi-realistic illusory pattern but failed to obtain the desired effect. Besides the possibility of a failed perception of the illusory effect, it is also possible that the apparent motion is instead perceived, but not considered attractive. Extreme anxiety in an aversive situation is in fact expressed by the zebrafish by freezing behaviors (Speedie & Gerlai, 2008; Maximino et al., 2010b), that is, the absence of any movement beside respiration, signaled by the movement of the gills. The view of a zebrafish that moves some part of its body but does not move in the whole visual field could signal a sick or scared fish, and as we saw in the general introduction of this chapter, the zebrafish is able to infer contextual information by observing other conspecifics (Abril-de-Abreu et al., 2015; Akinrinade et al., 2023).

Movement is known to be a crucial feature of the stimulus that triggers gregarious behaviors in the zebrafish, but it must possess some features that make it realistic, a "biological motion" (Larsch & Baier, 2018; Nunes et al., 2020; Kappel et al., 2022; Kuroda et al., 2023). To obtain this effect, we used a different illusion that, at least for the first steps of its investigation, will be based on a video presentation. The use of the computer will reduce the cost of using it purely as an enrichment tool, but it will also allow us to study perceptual phenomena in a model species whose nervous system has been extensively studied.

To create these stimuli, we tried to adapt an illusory visual phenomenon based on geometric features called "Moiré Patterns". The Moiré patterns are patterns of interference produced by the superimposition of at least one periodic visual stimulus that presents transparent intervals, e.g., a grating, and shifting it over another pattern. The basic case is when two identical simple patterns are displaced or rotated on top of each other. In the case depicted in Figure 6.4, the patterns emerge as two vertical lines. If the superimposed pattern continues to move, the interference changes in terms of direction, frequency, and shape.

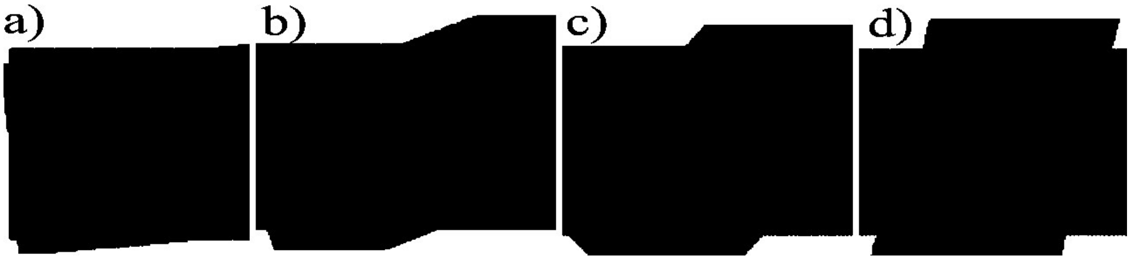

Figure 6.4. Moiré pattern, formed by two sets of parallel lines, one set inclined at an angle of a) 5°, b) 20° c) 45°, d) 75° to the other.

Even the smallest differences in the two images are magnified, presenting emergent patterns that can lead to the perception of stereoscopic effects (Spillmann, 1993). This principle can be exploited by carefully designing the static "background" image. Segmenting it accordingly to the pattern of the superimposed "grid" allows it to produce stop-motion-like effects when the grid is moved, sometimes called "Kinegrams" (Kinegram ("Scanimation"), 2014). While siding the grating on top of the background image, the cyclical animation repeats itself for each period of the grid. Each step of each period reveals a different background image, giving the impression of movement of these fast transitive shapes.

The properties of the animation are dependent on several parameters of the grating (Balas, 2021): the width of the bars and intervals, the frequency of intervals over subsequent bars, and the direction and speed of movement of the grating. The distance between the grating and background image could be varied too, obtaining interesting effects like the perception of the illusion from the observer' point of view, but it is of no interest for the purpose of these experiments.

The aim of these tests was to verify the perception of the visual illusion by the zebrafish, to assess the ability of the zebrafish to integrate different information from the stimulus, and to check the influence of some general properties of the stimulus not directly related to the illusion, such as brightness and contrast.

4.2.2 Methods

Subjects

We tested 92 mixed-sex adult zebrafish: 32 zebrafish (approximately 12–16 months old) were tested for the first two conditions, to assess the illusion validity (16 each), 24 in the two conditions studying the relevance of higher-level properties of the illusory image (12 each), and 36 in the three conditions aimed at study the effect of light properties of luminance and contrast on the illusion.

Fish were born and maintained at the comparative psychology laboratory (Animal Behavior and Cognition Lab) of the Department of General Psychology (University of Padova) in mixed-sex groups of 20–30 individuals (150 liters in each tank). The aquariums were grey plastic tanks (70 cm x 45 cm x 55 cm) provided with air filters, natural gravel, and live plants at a temperature of 25 ± 1 °C. The test tanks had no heating mechanism, as did all the tanks in our lab. The water temperature was maintained by ensuring a constant room temperature, roughly 2 degrees above the desired water temperature. Fish were fed twice daily: once with commercial food flakes and once with live brine shrimps (*Artemia salina*), on a 12:12 h light-to-dark photoperiod and an 18-watt fluorescent light.

Apparatus

The experimental tank depicted in Figure 7.4 was a 40 cm x 60 cm x 30 cm glass tank. The whole tank, except the two short sides, was covered with green, opaque plastic to prevent the fish from seeing the outside. Moreover, two walls of green plastic were inserted into the tank. The narrow space between two sectors was 10 cm wide. The reason for this, following previous shoal choice methods (Saverino & Gerlai, 2008), was to partially prevent the simultaneous sight of the two stimuli. The fish's choice of compartment had to imply a strong effect of the stimuli's visual availability. The corners of the tank were rounded using the same green plastic to reduce the fish's tendency to seek shelter when placed in a novel environment. All of these components together converged to form an "hourglass" shape.

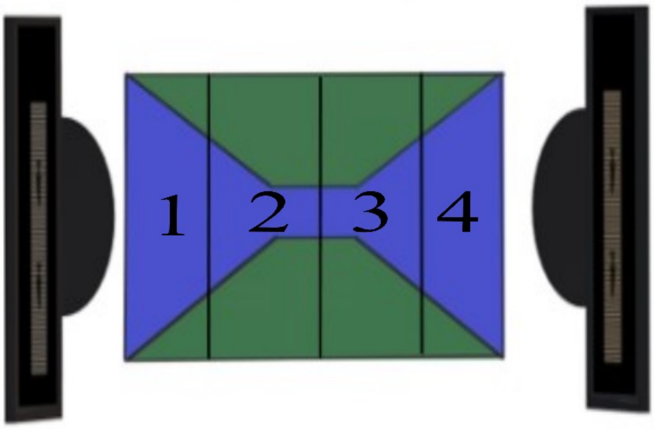

Figure 7.4. Vision of the apparatus from above. The areas used for the video analysis are numbered from left to right

The experimental stimulus and the control stimulus were shown on the monitors placed at a distance of 5 cm from each of the two short sides of the tank during the experiment, alternating the presentation between the two positions. The camera was placed at a-one meter distance, pointing at the center of one of the long sides. One 16-watt LED light (746 lm) was positioned one meter above the water surface at the center of the tank.

Stimuli

All the stimuli have been produced using the vector graphic software Inkscape (Version 0.92.3), following the principles of Moiré patterns. This procedure was carried out to create the standard version of the stimulus, while the variations will be illustrated when the other conditions are explained.

The grid (Figure 8.4) was created by attaching elementary components several times. Each elementary component can be divided into three parts: two black bars and one transparent bar. Each bar was 1mm wide, so each period of 3 bars measured 3mm.

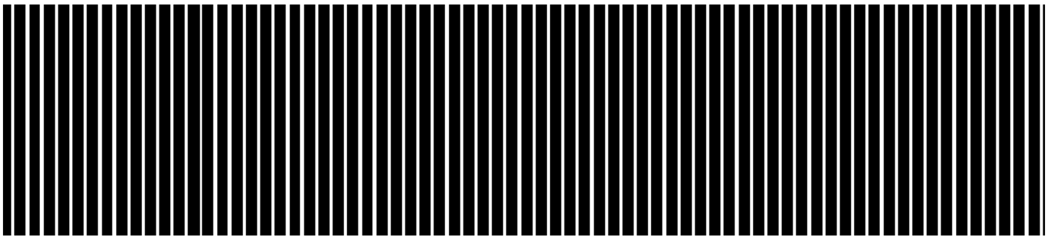

Figure 8.4. Representation of the sliding grid used in all the experiments.

The background image was made of a series of real-size images of zebrafish (3.5 cm long). Three images of zebrafish are needed to obtain the movement of one zebrafish image in a 3-step period grating. Each image was placed at a horizontal distance of 2.5 cm, thus partially overlapping (Figure 9.4). This was made to obtain greater continuity in the illusory movement.

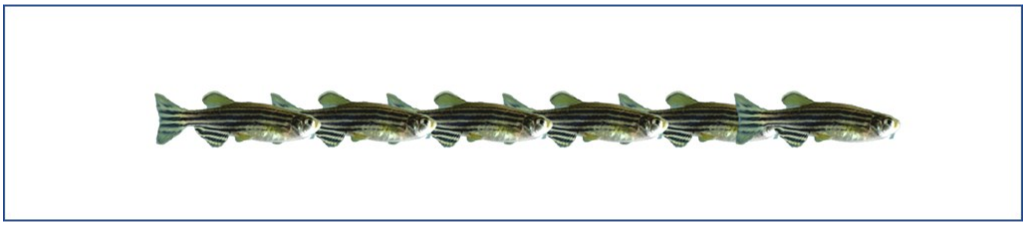

Figure 9.4. Sequence of six real images of 3.5 cm long zebrafish at a distance of 2.5 cm. Intermediate step for the creation of the illusory stimulus.

Each image was segmented imposing the grid pattern on it and deleting all the parts of the image hidden behind the grating (Figure 10.4). The subsequent images were segmented in the same way, after translating the grid horizontally by 1 mm, exactly the same size as the transparent interval. In this way, with a 1mm movement of the grid, the grid would have revealed the segmented image of the background 2.5 cm to the right compared to the previous one. After a 3 mm movement, at the beginning of the new period, the background would have revealed the image again in the first position, 5 cm to the left.

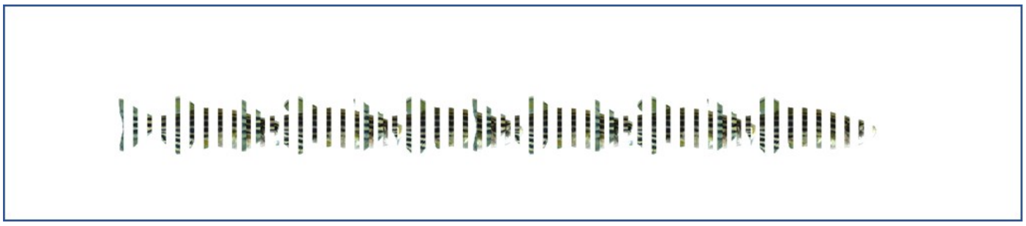

Figure 10.4. Sequence of six real images of 3.5 cm long zebrafish at a distance of 2.5 cm after the segmentation. Definitive background image that reveals the illusion when the grid of Figure 8.4 slides on it.

A total of six segmented zebrafish were placed on the same line, distanced by 2.5 cm intervals. In this way, at the onset of the new period, the image in position 3 would have appeared just after the one at position 2, giving a better sense of continuity (Figure 11.4). So, at any given time and position of the grid over the background, two zebrafish were observable behind it, making the stimulus more attractive and more spread into the space (from the beginning of the image in position 0 to the end of the image in position 5, the space is 13.5 cm).

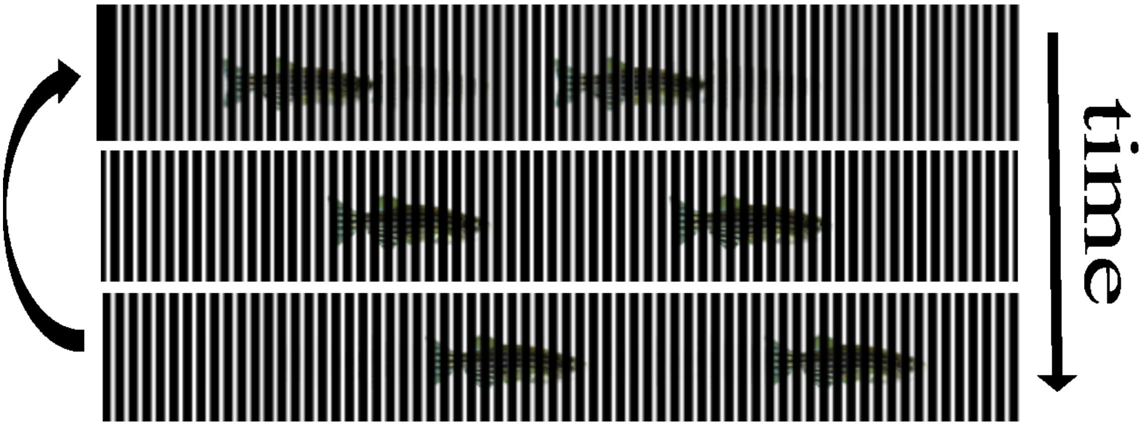

Figure 11.4. Serial representation of three periodic frames revealed by the constant sliding of the grid towards left. The arrows indicate the passage of time. The images of the zebrafish appear to move toward right two times before restarting from the first position.

The grid was moved at a speed of 4 mm/s. So, the image in the background would have moved in half a second from Time 0 to Time 2, at a distance of 5 cm. In this way, the speed of the illusory movement of the background image would have been 10 cm/s, considered slightly above the resting swimming velocity ($V_{rest} = 5$ cm/s) and greatly below the maximum velocity ($V_{max} = 40$ cm/s) in zebrafish (Wakamatsu et al., 2020).

- Experiment B.1: Effectiveness of the illusion

Is the illusory pattern attractive?

The first step after producing the visual illusion was to test its attractiveness. We compared the standard stimulus, which consisted of a black grid moving toward left at 4 mm/s and producing the illusory movement of two fish swimming towards right at 10 cm/s on a white background, with a static version of the stimulus where neither the grid nor the background image were moving. The static stimulus was just two fish images covered by the grid. Movement enhances the salience of the conspecific's image and triggers gregarious behavior (Nunes et al., 2020; Kuroda et al., 2023), so if the subjects prefer to swim close to the illusory stimulus, it can be assumed that the illusory motion is perceived.

Does fish preference depend on optomotor response?

The optomotor response (OMR) is a motor reflex executed to reorient the position of the animal, as described in different animal species. It is induced by a moving repetitive stimulus pattern, like a grid (Maaswinkel & Li, 2003), that alters the neuronal mechanism involved in the processing of the optic flow, which is necessary for visual guided self-motion (Kern et al., 2001). OMR has been extensively studied in zebrafish, down to the precise circuitry involved (Naumann et al., 2016,) and its effectiveness is even used to assess visual deficits in larvae and adults (LeFauve et al., 2021).

Since the illusory stimulus proposed to the subject involves a moving repetitive pattern, it is important to exclude that the movements that signal attraction to the stimulus are not induced by simple OMR.

Firstly, we compared the standard stimulus (grid moving towards left at 4 mm/s producing the illusory movement of two fish swimming towards right at 10 cm/s on a white background) with a stimulus in which the regular black grid slides at the usual speed (4 mm/s towards left) over the static full images of two fish. In this way, we canceled the illusion, leaving only the possible optomotor effects.

On the other hand, we want to test the efficacy of the stimulus, removing the possible effects of the optomotor response. To test that, we compared the illusory stimulus with a modified version of it, in which the background is painted in the same black as the bars of the grid (Figure 12.4). Canceling any contrast, the normally perceived black grid vanishes in the black background, leaving visible only the segmented image of the fish, appearing following the rules of the Kinegram. In this way, we aim to remove any effect of the OMR, leaving intact the perception of the illusion.

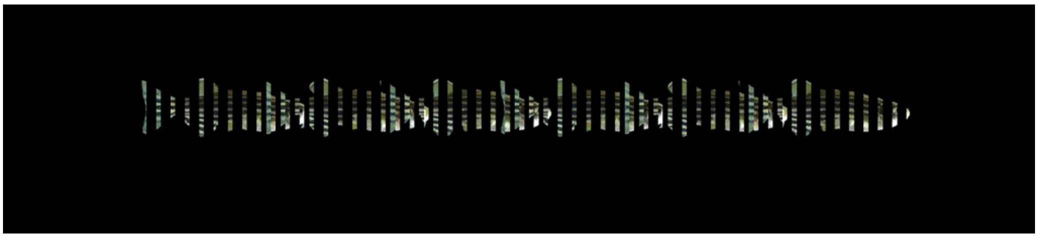

Figure 12.4. Background image used in the condition "Black on black". After the superimposition of the grid, only two zebrafish body are visible at any given time.

- Experiment B.2: Relevance of higher-level features of the illusion

Once it is verified that the illusion is perceived and attractive, the aim is to assess if some components of the illusion are more attractive in eliciting the gregarious responses of the subjects. How well the background image is perceived? Do the features of the image influence the attractiveness of the illusion?

To answer these questions, two different rounds of tests were performed: in the first one, the standard stimulus (a black grid moving towards left at 4 mm/s producing the illusory movement of two fish swimming towards right at 10 cm/s on a white background) was compared with a modified version of it, in which the segmented image consisted of the Moiré processing of a scrambled fish instead of an integer one. In this way, the same visual bits were present in the image but not arranged in a coherent way. The scrambled image of the fish was obtained by equally mixing vertical slices of a zebrafish looking toward right, one looking toward left, and a third one with the two halves of the image switched.

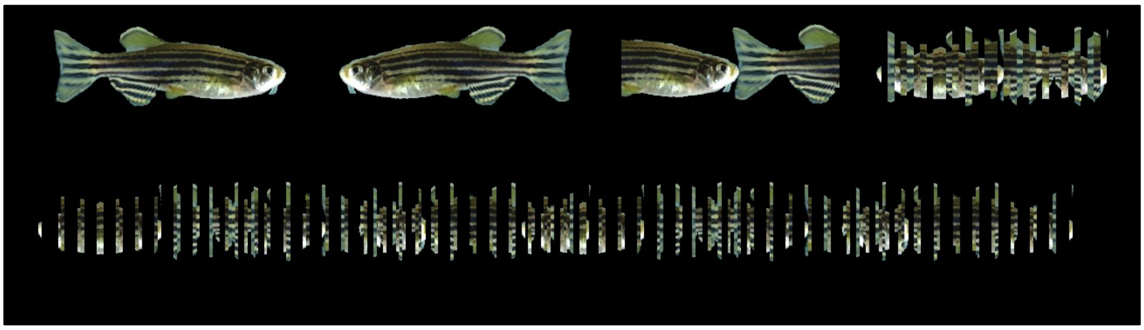

Figure 13.4. Images used to build the “Scrambled” condition. Segments from a a) right-oriented, b) left-oriented, c) switched-half body of zebrafish were mixed together to form d) the scrambled image. e) Background image used in the condition “Scrambled”. After the superimposition of the grid, only two zebrafish body are visible at any given time.

In the second test, the standard stimulus (black grid moving toward left at 4 mm/s producing the illusory movement of two fish swimming toward right at 10 cm/s on a white background) was compared with a modified version of it, in which the segmented image consisted of the Moiré processing of a grey ellipse of the same dimensions as the fish image. In this way, only the general shape of the segmented stimulus would be the same, without any species-specific trait (fins, head, livery patterns).

Figure 14.4. Background image used in the condition “Ellipse”. After the superimposition of the grid, only two ellipses are visible at any given time.

- <u>Experiment B.3: Effect of luminance and contrast in the perception of the illusion</u>

Other properties of the stimulus that could be relevant to enhancing the optimality of the perception of the illusion are brightness and contrast. It is known that zebrafish are generally scototaxic animals when in situations of mild distress (Maximino et al., 2010a; Maximino et al., 2011). Very bright stimuli could deter the exploratory behavior of the subjects.

At the same time, it is known that several illusions are influenced by the color contrast of the elements. An analysis of the similar “footsteps illusion”, in which two colored blocks “race” towards a side of the visual field when a grid is moving, reveals that properties of the illusion like speed, dimensions, and direction are strictly dependent on contrast features (Anstis, 2001; Kitaoka & Anstis, 2021). Furthermore, too much brightness and contrast in the stimuli could impair sight, damaging the retina (Glickman, 2002; Hunter et al., 2012). The interplay between color and contrasts is at the base of the principles of camouflaging, which many animals unconsciously take advantage of through their skin patterns (Stevens & Merilaita, 2009; Troscianko et al., 2009).

To study these effects, we compared four different versions of the standard stimulus, variating brightness and contrast. (Figure 15.4)

For the first comparison, we used the results of the test that compared the standard stimulus (black bars on white background, “Black on white”) with the “Black on black” version (black bars on black background) to exclude the role of optomotor response (Figure 15.4a) The “Black on black” stimulus had a null contrast and a lower brightness than the standard “Black on white” one.

Then, we compared the inversed versions of the two stimuli: a “White on black” (Figure 15.4d) versus a “White on white” (Figure 15.4b). The contrast of the “White on white” was null compared to the “white on black”, but its brightness was higher.

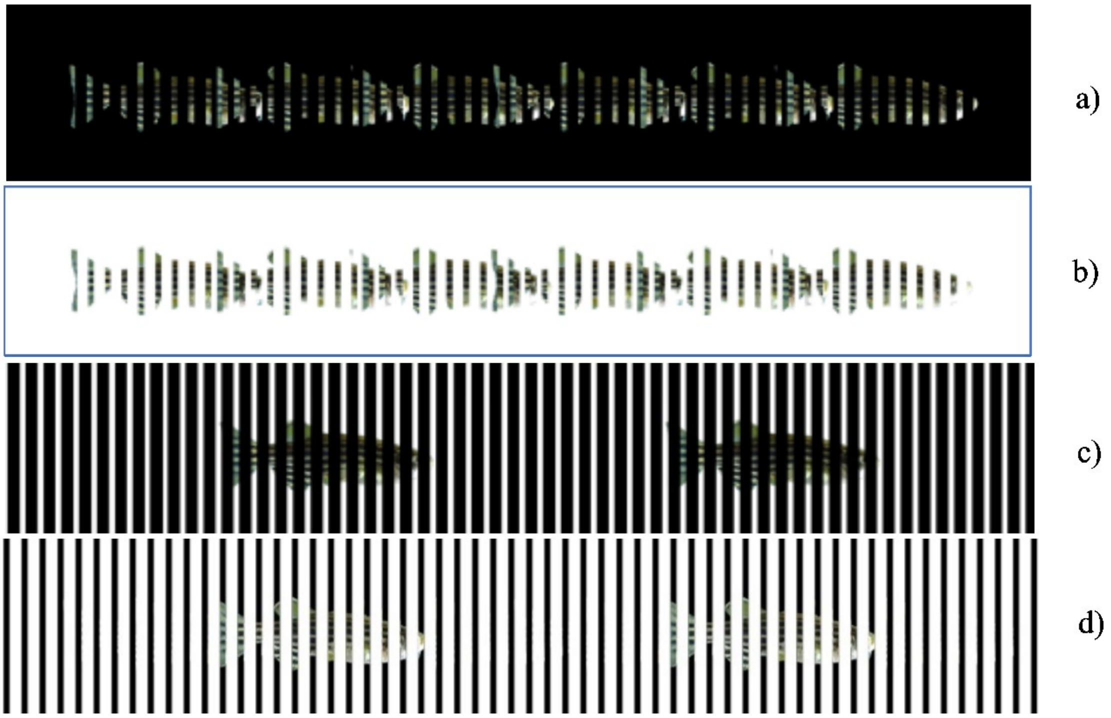

Figure 15.4. Background image used in the condition a) “Black on black”, b) “White on white”, c) “Black on white” (standard), d) “White on black”. In a) and b), no grid is superimposed, for illustrative purposes. After the superimposition of the grid, only two zebrafish body are visible at any given time, like in c) and d).

Lastly, we compared the “Black on black” to the “White on white”. The two different versions of stimuli had both null contrasts but very different brightness values.

Despite the effort, the best way to understand all the conditions is to watch them while they move (Figure 16.4).

Figure 16.4. QR code linking to a Drive folder containing 10 seconds snippets of the stimuli presented above, to have a better sense of the power of the illusion.

Procedure

Experiments were performed during the day, ranging from 10:00 (2 hours after morning feeding) to 15:00 (2 hours before evening feeding). Each subject was selected from the stock tanks and put into a small (1-liter) plastic bucket for transportation to the experimental room.

After the time strictly necessary to move the fish from the maintenance to the experimental room (approximately 1 minute), the subject was gently inserted into the experimental tank. The experiment started, and the time spent near the stimuli at the two short sides of the tank was taken as the dependent variable.

When the experimental time elapsed (20 minutes), the subject was removed from the experimental tank using a fish net, transported through the plastic bucket back to the maintenance room, and placed into a new, post-experimental tank.

After removing the subject, 70% of the water in the experimental tank was replaced with water coming from three 150-liter tanks enriched with gravel, plants, lamps, and a filter system to ensure the same quality and chemical composition of the water and minimize possible interference of the previous subjects' scent (e.g., hormones). Lastly, the positions of experimental and control stimuli were switched to prevent any possible bias originated by side preferences.

Video analysis

The experiment was recorded through a camera placed above the experimental tank. Three trained observers analyzed the videos offline. Each video consisted of preparatory routines (showing the ID of the subject and the position of stimuli and controls in the tank), 20 minutes of experimental recording, and the final removal of the fish.

The region of interest were the two rectangles (10 cm x 40 cm each, following previous works (Gatto et al., 2019; Lucon-Xiccato et al., 2016) adjacent to the walls where the stimuli were hung, and the time spent in each sector was scored using the program BORIS (Friard & Gamba, 2016). The change of sector was determined by the passage of half of the subject's body.

Data analysis

Analyses were performed in RStudio version 2023.06.2 (The R Foundation for Statistical Computing, Vienna, Austria, http://www.r-project.org).

The percentage of time spent in Area 1 (corrected for the position switching carried out during the procedure to avoid any position bias) was confronted with the percentage of time spent in Area 4 through a linear mixed model, without considering Area 2 and Area 3, because in all the tests the central sectors were avoided, accounting for around 10% of the total time, and Subject as a random effect.

Preference for the target stimulus was evaluated by calculating the proportion of time spent by the subject in the choice area associated with the target stimulus, in detail:

Preference for the target stimulus = time near the target stimulus / (time near the target stimulus + time near the control stimulus).

The fluctuation of the preference across the 20 minutes of experiments was tested through a linear model with minutes as predictor.

The activity levels were measured by counting the number of mean sector switches in the 20 minutes and analyzed through a linear model to assess differences in the tests.

4.2.3 Results

Standard (Area1) vs Still image (Area4)

The time spent in Area 1 was significantly greater than the time spent in Area 4 (F = 70.70, p = 2.199e-09). There was no influence of time on preference for Area 1 (F = 1.23, p = 0.27).

Area	Area1	Area2	Area3	Area4
Mean %	57.81	7.89	8.06	26.20
SD	11.61	3.23	3.56	9.56

Table 2.4. Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for “Standard” condition (“Black on white”) and Area4 for a static snapshot of the stimulus.

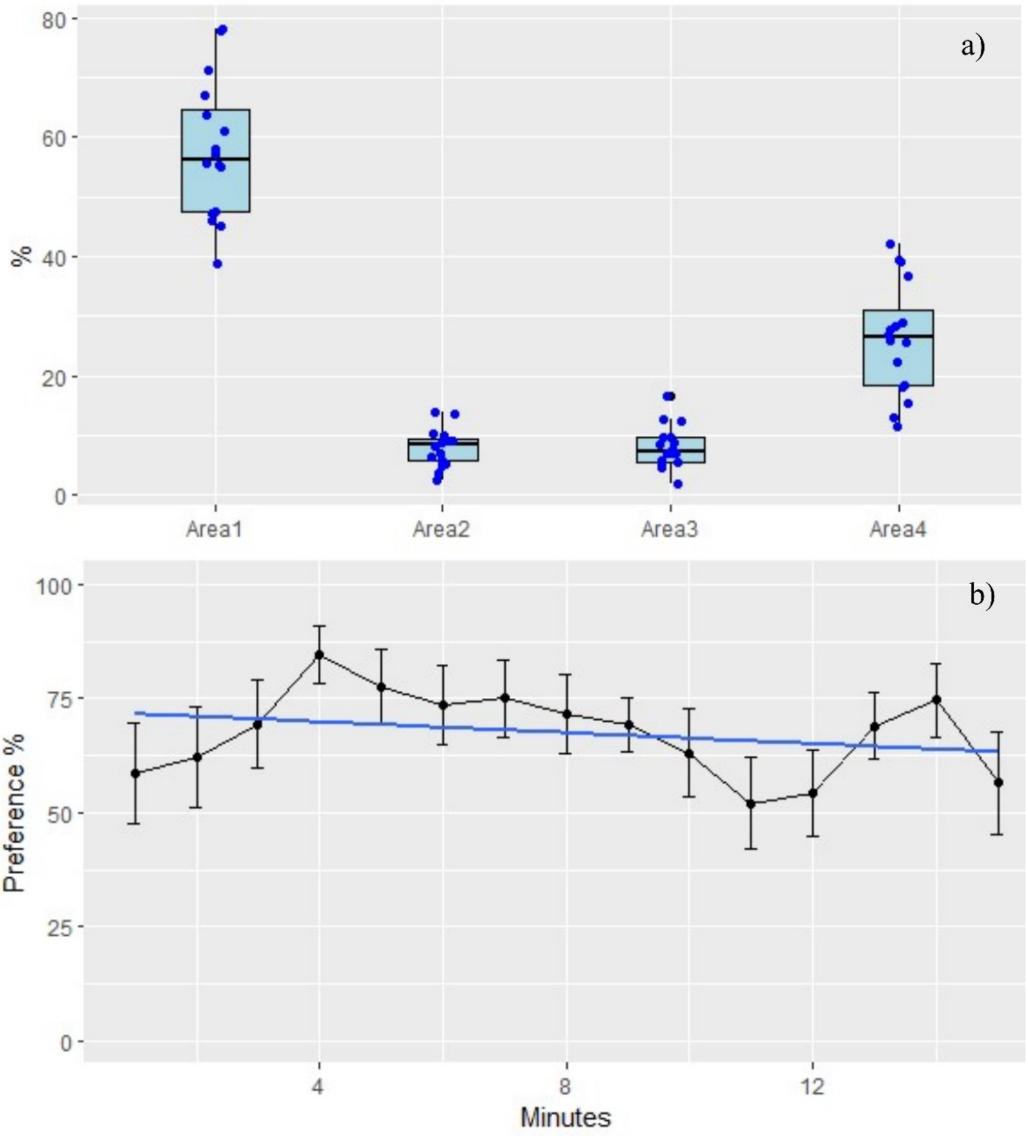

Figure 17.4. a) Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for "Standard" condition ("Black on white") and Area4 for a static snapshot of the stimulus. b) Percentage of time spent in the illusory sector close to the Area1 over the 12 minutes of observation. Bars indicate S.D. The blue line indicates a smoothing function operated on the data obtained with the package ggplot2 (method ="lm").

Standard (Area1) vs Only grid moving (Area4)

The time spent in Area 1 was significantly greater than the time spent in Area 4 (F = 38.12, p = 8.577e-07). There was a slight increase of preference for Area 1 during the 20 minutes, but it was not statistically significant (F = 2.98, p = 0.08).

Area	Area1	Area2	Area3	Area4
Mean %	65.79	6.50	5.43	22.24
SD	21.03	4.33	3.27	18.81

Table 3.4. Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for “Standard” condition (“Black on white”) and Area4 for the “Only grid” condition.

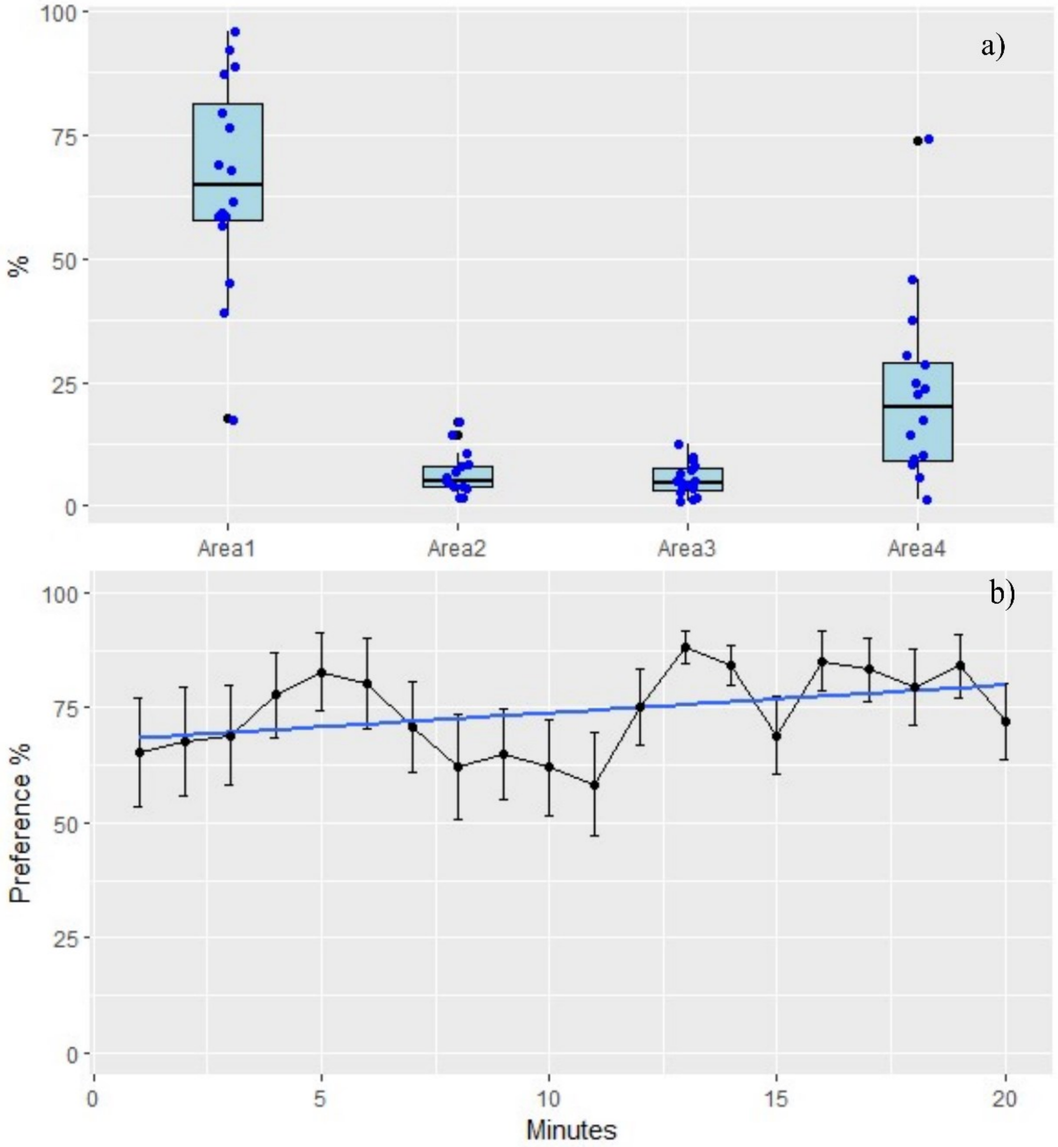

Figure 18.4. a) Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for “Standard” condition (“Black on white”) and Area4 for the “Only grid” condition. b) Percentage of time spent in the illusory sector close to the Area1 over the 20 minutes of observation. Bars indicate S.D. The blue line indicates a smoothing function operated on the data obtained with the package ggplot2 (method =”lm”).

Standard (Area1) vs "Black on black" (Area4)

The time spent in Area 1 was significantly smaller than the time spent in Area 4 (F = 10.87, p = 0.004). The preference for Area 4 greatly increased during the time of the test (F = 24.75, p = 1.323e-06).

Area %	**Area1**	**Area2**	**Area3**	**Area4**
Mean %	32.42	4.15	6.31	56.91
SD	16.99	3.78	3.97	17.85

Table 3.4 Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for "Standard" condition ("Black on white") and Area4 for the "Black on black" condition.

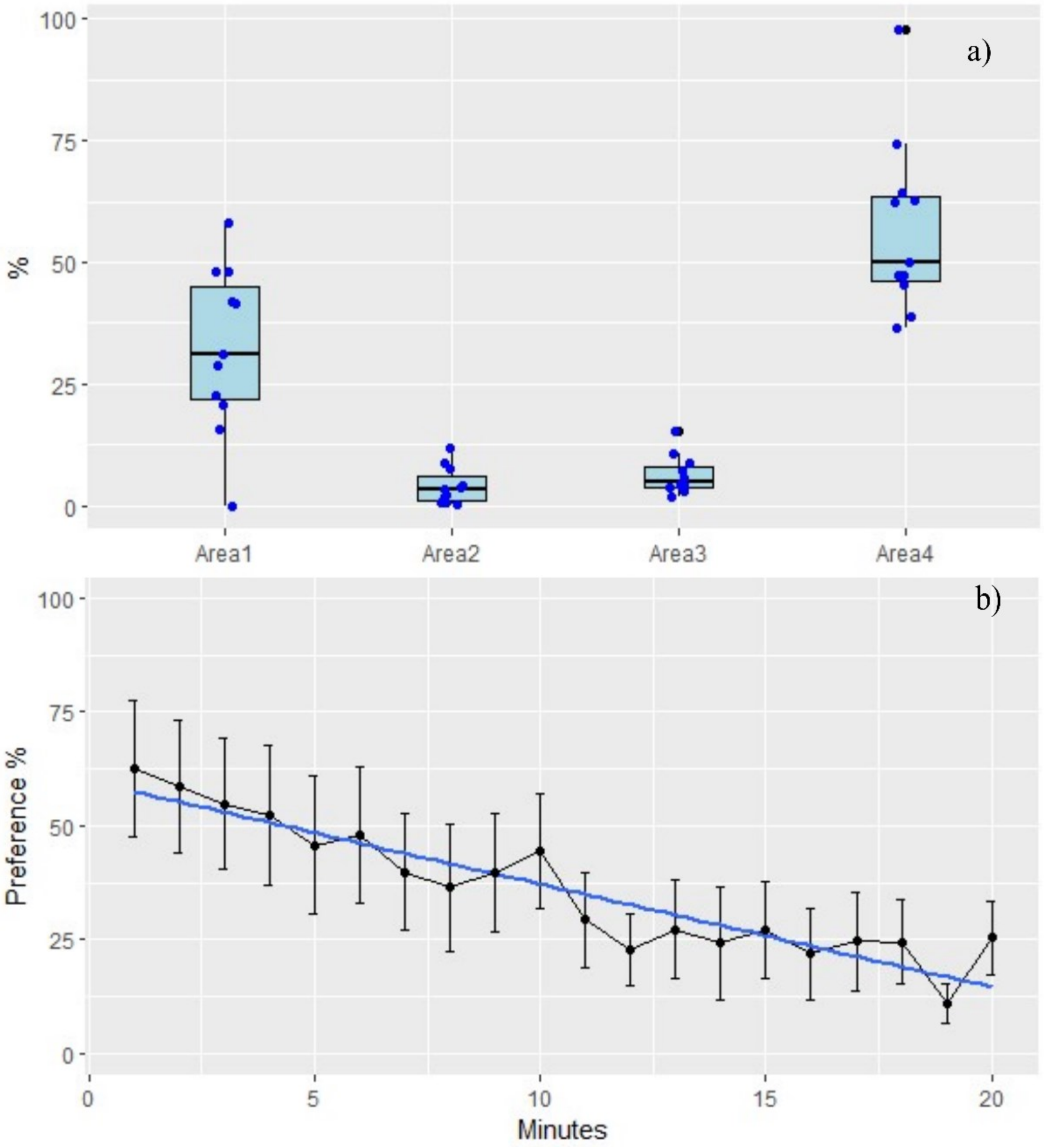

Figure 19.4. a) Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for "Standard" condition ("Black on white") and Area4 for the "Black on black" condition. b) Percentage of time spent in the illusory sector close to the Area1 over the 20 minutes of observation. Bars indicate S.D. The blue line indicates a smoothing function operated on the data obtained with the package ggplot2 (method ="lm").

Standard (Area1) vs Scrambled (Area4)

The time spent in Area 1 was not significantly different from the time spent in Area 4 (F = 0.81, p = 0.38). However, time had a significant effect on preference, indicating a substantial increase for choice of Area 1 during the test (F = 4.974, p = 0.03).

Area	**Area1**	**Area2**	**Area3**	**Area4**
Mean %	48.22	5.27	5.46	40.91
SD	19.23	3.16	3.27	20.53

Table 4.4. Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for "Standard" condition ("Black on white") and Area4 for the "Scrambled" condition.

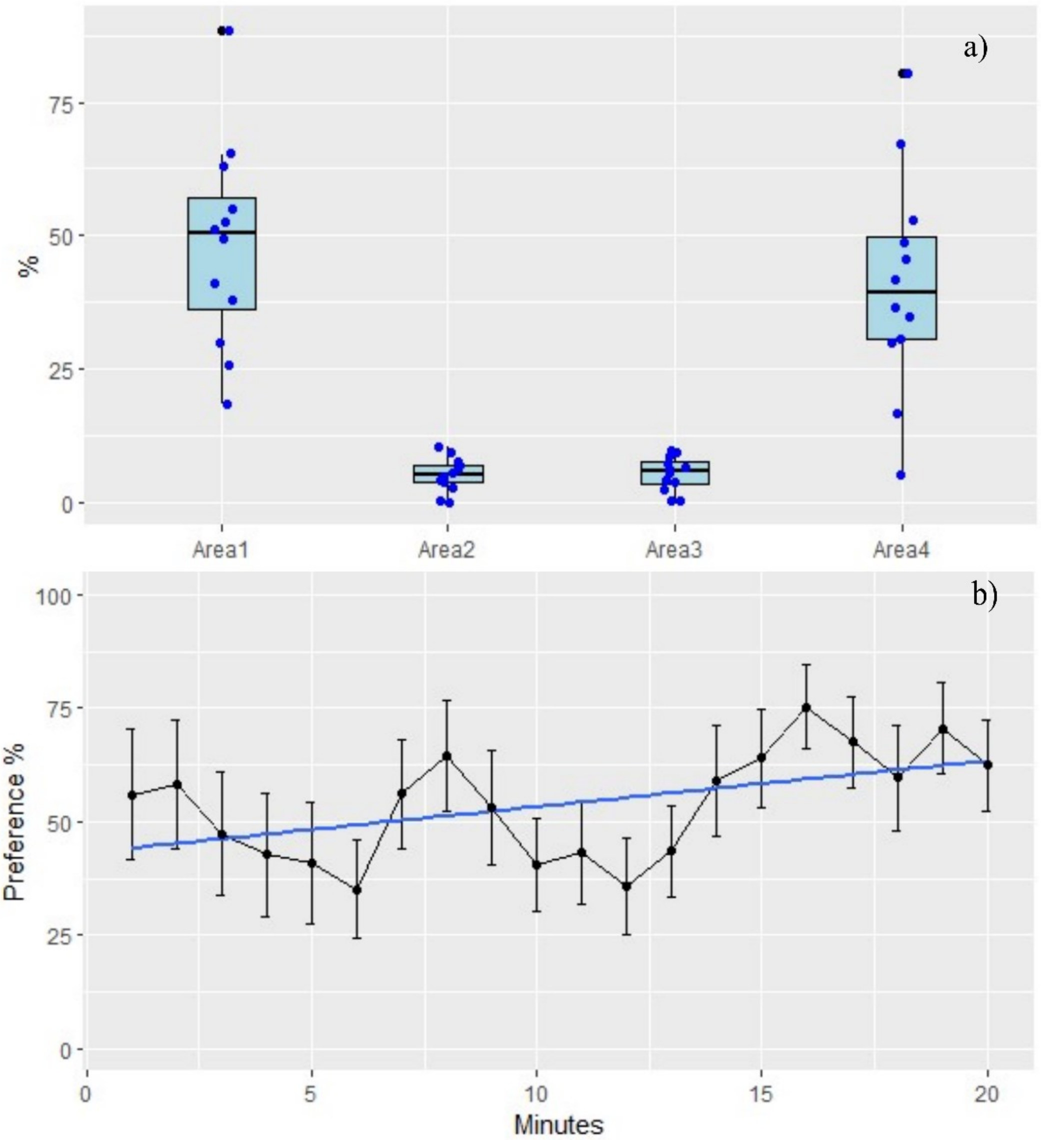

Figure 20.4. a) Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for “Standard” condition (“Black on white”) and Area4 for the “Scrambled” condition. b) Percentage of time spent in the illusory sector close to the Area1 over the 20 minutes of observation. Bars indicate S.D. The blue line indicates a smoothing function operated on the data obtained with the package ggplot2 (method =”lm”).

Standard (Area1) vs Ellipses (Area4)

The time spent in Area 1 was not significantly different from the time spent in Area 4 (F = 0.02, p = 0.89). However, time had a significant effect on preference, indicating a substantial decrease for choice of Area 1 during the test (F = 8.49, p = 0.004).

Area	Area1	Area2	Area3	Area4
Mean %	50.23	4.48	6.26	48.94
SD	26.71	2.90	4.23	11.08

Table 5.4. Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for “Standard” condition (“Black on white”) and Area4 for the “Ellipses” condition.

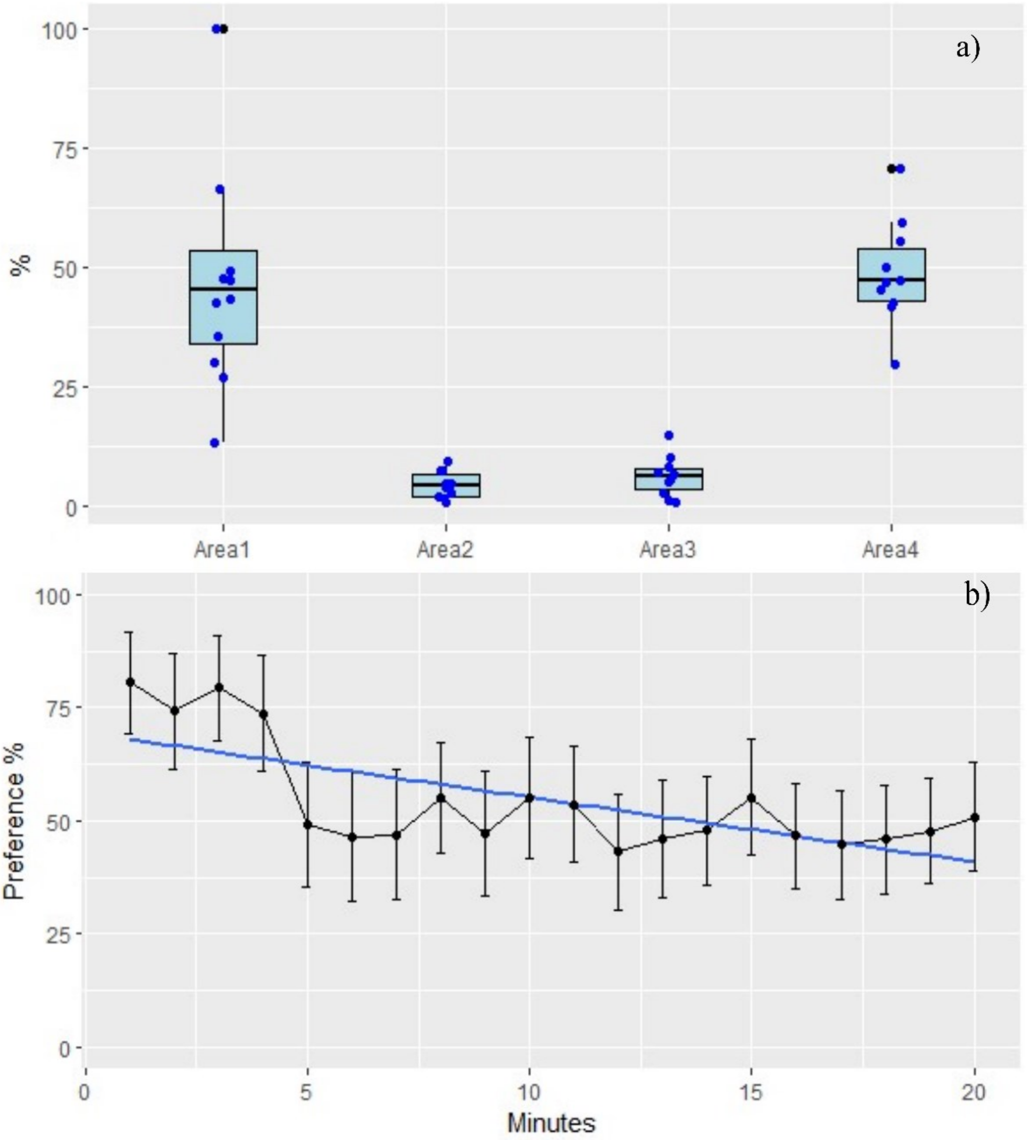

Figure 21.4. a) Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for “Standard” condition (“Black on white”) and Area4 for the “Ellipses” condition. b) Percentage of time spent in the illusory sector close to the Area1 over the 20 minutes of observation. Bars indicate S.D. The blue line indicates a smoothing function operated on the data obtained with the package ggplot2 (method = ”lm”).

"White on black" (Area1) vs "White on white" (Area4)

The time spent in Area 1 was significantly greater than the time spent in Area 4 (F = 5.69, p = 0.03), despite the significant decrease in preference during the time of the test (F = 7.33, p = 0.007).

Area	Area1	Area2	Area3	Area4
Mean %	59.30	6.18	5.38	34.49
SD	21.62	5.63	6.55	27.58

Table 6.4. Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for "White on black" condition and Area4 for the "White on white" condition.

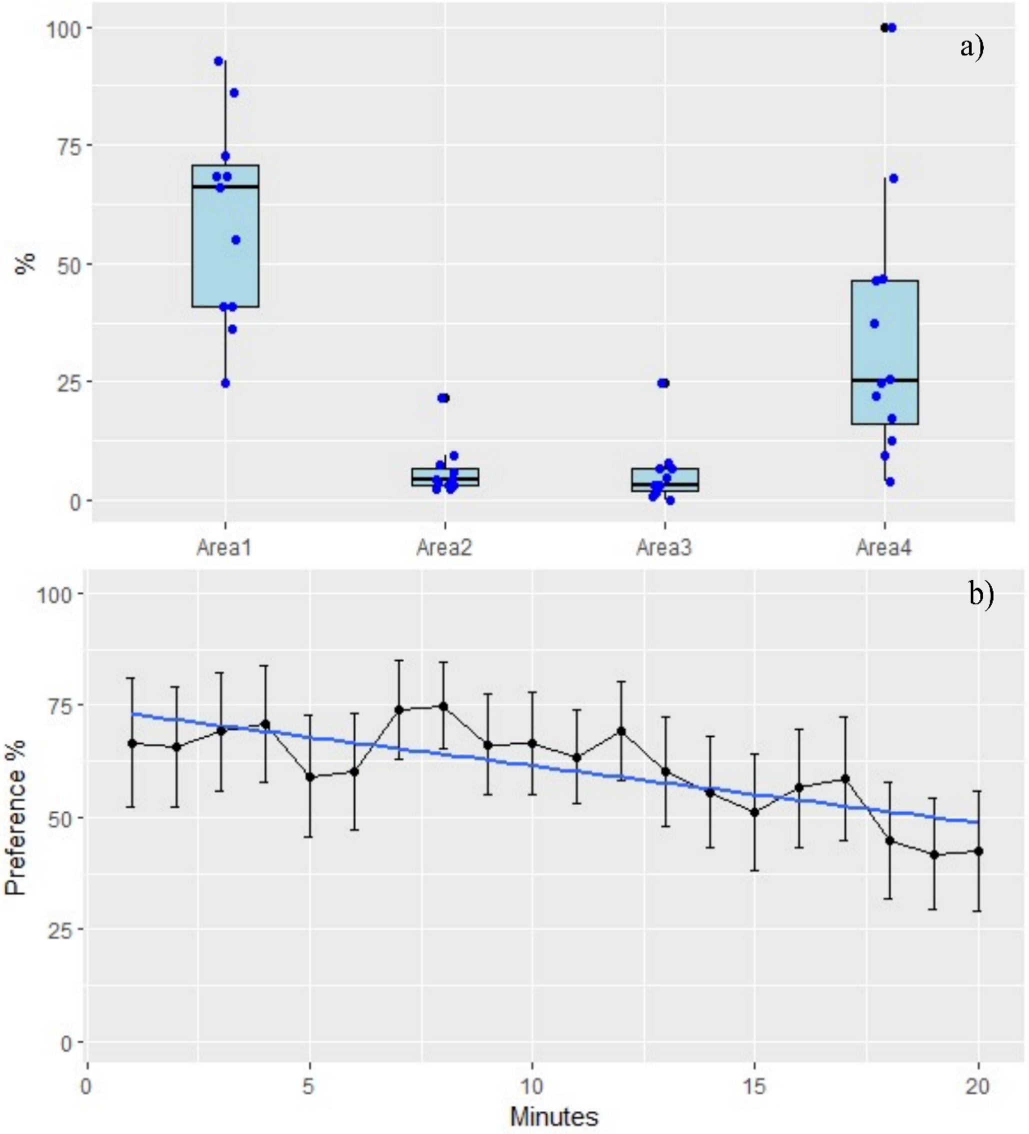

Figure 22.4. a) Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for “White on black” condition and Area4 for the “White on white” condition. b) Percentage of time spent in the illusory sector close to the Area1 over the 20 minutes of observation. Bars indicate S.D. The blue line indicates a smoothing function operated on the data obtained with the package ggplot2 (method = ”lm”).

“Black on black” (Area1) vs “White on white” (Area4)

The time spent in Area 1 was significantly greater than the time spent in Area 4 (F = 68.05, p = 3.534e-08). Despite the wide fluctuations, there was no effect of time on the preference (F = 0.22, p = 0.64).

Area	**Area1**	**Area2**	**Area3**	**Area4**
Mean %	61.77	7.28	6.55	24.37
SD	12.28	4.26	3.33	9.80

Table 6.4. Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for “Black on black” condition and Area4 for the “White on white” condition.

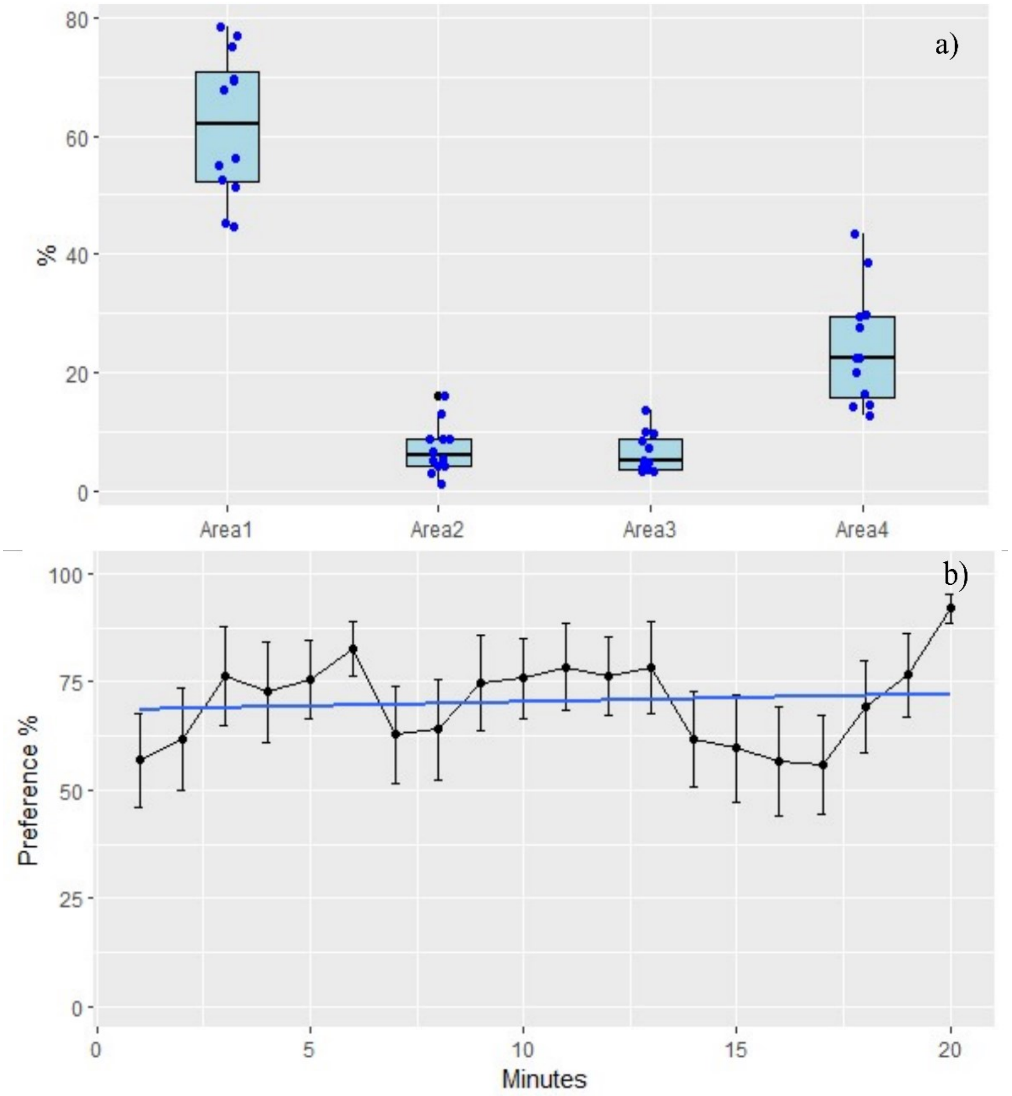

Figure 23.4. a) Percentage of time spent in the four areas of the tank, where Area1 is the region of interest for “Black on black” condition and Area4 for the “White on white” condition. b) Percentage of time spent in the illusory sector close to the Area1 over the 20 minutes of observation. Bars indicate S.D. The blue line indicates a smoothing function operated on the data obtained with the package ggplot2 (method = ”lm”).

4.2.4 Discussion

This study aimed to assess if a motion illusion could be a valid tool to create a stimulus that would be socially attractive to zebrafish. If perceived, the illusion adapted from the visual phenomenon called “Moiré pattern” should provide both the shape and the motor cues necessary for conspecific recognition and for triggering a gregarious behavior. The great preference expressed for the illusory stimulus when compared to a static version of it indicates a predilection for the moving stimuli. Further tests permitted us to understand which component (grid, motion, and brightness) is more relevant to attract fish toward the illusory pattern.

The likewise great preference for the illusion when compared to a grid sliding on a non-illusory image of two fish excludes the possibility that the movement of the grid and the connected optomotor response would be the only ones responsible for the attraction elicited in the experimental subjects. Moreover, when the standard version of the illusion, in which the sliding grid is clearly visible above the white background, is compared to a version with a black background that cancels the perception of the grid, the latter is substantially preferred, indicating a possible impairing effect of the high contrast between grid and background.

The effects of light properties on the perception of the illusory stimulus were assessed through three comparisons. The first one has just been mentioned. A low-contrast, low-brightness stimulus (“Black on black”) was preferred to the standard version of the stimulus. On the other hand, the stimulus with an equally low contrast (“White on white”) was avoided in favor of a higher-contrast, but lower-brightness stimulus (“White on black”). Excessive brightness seems to be a repellent factor for this species, known to often adopt scototaxic behaviors (Maximino et al., 2010a). Further confirmation of this comes from the strong preference for the “Black on black” stimuli over the “White on white”.

Lastly, the behavior could only be properly called “social” or “gregarious” if the subjects would have perceived the illusorily moving images as conspecifics. The results from the comparisons with the “Scrambled” and “Ellipses” stimuli are encouraging, although not conclusive. The standard version was not generally preferred to any of the two non-social stimuli, but with important fluctuations over time. The “Scrambled” version was gradually rejected by the subjects in favor of the standard version, suggesting a possible hard and time-consuming discrimination process. The standard version was instead greatly preferred to the “Ellipse” version to begin with, but the preference disappeared after the first few minutes.

Several studies have investigated the role of specific cues in triggering the social behavior of the zebrafish. The most important feature is considered to be movement. In particular, “self-like” biological motion is more attractive than simple linear movement (Nunes et al., 2020), even if performed by simple dots (Larsch & Baier, 2018), especially if it respects some natural features like the intermittency of movement (Larsch & Baier, 2018) and its compatibility with the force of gravity (Ma et al., 2022). Neither the shape of the moving object nor the reciprocity of its movement to the perceiver’s actions are necessary in triggering this innate behavioral pattern (Larsch & Baier, 2018), whose circuitry has been described in great detail and localized in projections from the optic tectum via the dorsal thalamus to other hypothalamic areas (Kappel et al., 2022). This tectothalamic pathway responsible for biological movement is responsible for the mechanism of social attraction to conspecifics (Kappel et al., 2022) and is affected by the disruption of oxytocin signaling, a neurotransmitter known to regulate social behaviors across species (Nunes et al., 2020).

On the other hand, vital complex functions such as social recognition and aggregation must rely on multiple redundant mechanisms that ensure greater efficiency. Another crucial visual cue for conspecific recognition and preference is conspecific form (Saverino & Gerlai, 2008; Nunes et al., 2020), while only one study has observed a relevant effect of the stripes of the livery (Rosenthal & Ryan, 2005). During the maturation of the visual system in the zebrafish from the larval to the juvenile stage, the core innate mechanisms are integrated with new ones (Harpaz et al., 2021), allowing more complex discriminations to occur. The concurrent availability of conspecific form and biological motion promotes the best engagement with the fish (Nunes et. al, 2020; Kuroda et al., 2023). For example, the movement of a conspecific image triggers the subject’s attraction when the direction matches the local orientation of the head (Neri, 2012), attesting to the occurrence of feature binding in the process of social recognition.

The perception of real or apparent movement, following modern interpretations (Nishida et al., 2018), grounds its basis in lower-level mechanisms that detect variations in the visual field, filtering the multiple spatial information bits through a time axis (Adelson & Bergen, 1985). These relatively simple filters could account for short-range motion detection but not for long-range motion (Braddik, 1974). Long-range motion detection constitutes the basis for elaborating on the modulations caused by higher-order motor features like contrast, flicker, orientation, direction, and depth (Nishida et al., 2018). The perception of movement in Kinegrams, the kind of illusion we proposed to the fish, is perceived by an artificial visual system relying on first-order motor perception mechanisms only (Battaje et al., 2023) based on the spatiotemporal energy model (Adelson & Bergen, 1985). However, it is likely that the two levels work in concert: the low level feeds the higher-level processing, which in turn constrains the low level (Nishida et al., 2018). Thus, the combination of the low- and high-level properties of the stimulus constitutes an element that should be considered jointly as a whole. This could explain the discrepancies between the findings that assess the relevance of different properties of the stimulus in eliciting a gregarious response.

Overall, the illusion proposed is perceived and appreciated by the zebrafish, even though it could be improved by varying the several parameters involved in the phenomenon, like the width of the bars and intervals, the frequency of intervals over subsequent bars, and the direction and speed of movement of the grating. All these parameters are interconnected and constitute the essence of this illusion. It is possible that the visual system of the zebrafish (and of other species) possesses slightly different properties, so it is important to fine-tune the stimuli proposed to them.

One parameter that would have a qualitatively different effect compared to the others would be the distance from the grid to the background, which, in the simplest cases, is proximate to 0. Detaching the two planes would cause a slight disruption of the illusion, caused by the interfering perception of multiple steps of the background image through the transparent intervals, previously impossible. On the other hand, this misalignment would allow the possibility of perceiving the illusory motion even when the normally sliding grid would instead be static. The movement of the observer's point of view would cause a minor apparent sliding of the grid in the background. This effect could be a turning point in the design of enriching stimuli because, after the precise determination of the optimal parameters, there would be no need for computer animation and monitors, which could represent an unbearable cost to sustain for many facilities. Moreover, the analysis of the swimming behavior of the subjects in this scenario would give us precious information to reverse engineer the optical properties of the visual system.

Physiological analyses of the levels of stress hormones and socially triggered neurotransmitters would corroborate the behavioral analysis of this study. In an already planned study, the cortisol levels of the experimental subjects exposed to isolation, dyadic relations, group life, and illusory enrichment will be compared to assess if the attractiveness of the illusion would represent a possible source of well-being as well.

Chapter 5

General discussion

The aim of this thesis was to research new and efficient ways to enrich the captive environment for fish kept in captivity for ornamental, farm, or research purposes, but the general principles could be applied to any other animal species.

The first step is to look at the peculiar natural life of the species, intended as the complex set of adaptations needed to cope with the environmental challenges in the wild habitat of origin. Each species is evolutionarily shaped by a particular environment and expresses this adaptation in different physical and behavioral manifestations. The starting point of the inquiry is a pure, naturalistic conception of well-being. The naturalistic point of view considers every component of the ecosystem necessary to define the life conditions of the organisms, being these components either positive or aversive for the ultimate survival outcome and for the experience of more or less transient states of comfort or discomfort for a particular individual. It is assumed that the totality of experiences shapes the overall complex of needs and adaptations that each species presents in different degrees, depending on individuals. Following this conception, the perfect way to counteract the effects of captivity should be to provide the animals with the exact same set of stimuli encountered in nature. Beyond the factual impossibility and senselessness of keeping captive animals in perfectly wild conditions, a more serious problem is posed: the ethical contradiction of inflicting aversive stimuli that induce negative physical or psychological experiences in the animal in order to keep it “healthy”.

On one hand it makes sense to consider difficulties stimulating, but it is inconceivable to voluntarily inflict suffering on any organism, even if we would have a complete comprehension of the whole system of checks and balances involved, which we do not have. We do not have a scientific description of whether and which aversive stimuli can result in a positive outcome, the ways and doses of administration of these stimuli, and the correct combination of them with other conditions that act as resilience factors.

On the contrary, most of the time, the study and implementation of well-being in animals is focused on the prevention of negative sensations like hunger, thirst, and pain in the first place, followed by other less physical states like fear and distress. These universally accepted basic conditions of captivity were first stated by the Farm Animal Welfare Advisory Council (FAWAC) in the famous “Five Freedoms” declaration. Nonetheless, the generality of this declaration allows the definitions to be opened to more refined interpretations of well-being, beyond the quite reductive “lack of sufferance”. The expression “freedom to express normal behavior” brings attention back to the naturalistic perspective of “normality”. Some species of animals, for example, do not just need to eat but also to hunt in order to express their normal behavior. Furthermore, the expression “freedom from distress” contributes to placing more emphasis on prosperous welfare states (Mellor, 2016), including the necessity of providing positive stimulations to the animals (Mellor & Beausoleil, 2015), once it is accepted that boredom could be a greatly aversive stimulus (Burn, 2017) and that the lack of environmental challenges can be just as bad as the hyperstimulation produced by an excess of these challenges (Korte et al., 2007).

After having scrutinized the positive and negative needs of the species we are focusing on, the next step would be to try providing them with powerful, appropriate stimulations in the domains of welfare that are ignored at the current time. To have a deeper practical impact, the enriching measures formulated must be effective in the first place but also cheap and easy to use for users of every expertise and disposable income.

Visual illusions could represent a precious tool to provide positive stimulation to the animals. The nature of illusions is to distort some quality of reality, clashing against some fundamental properties of the perceptual systems of the organisms that usually work appropriately in describing the external world. If used correctly, illusions could provide the needed stimulation without using expensive resources. Every additional adjustment to basic conditions (e.g., spaces, structures, positive social interactions, a dynamic environment) represents a cost and a possible source of variability, that, most of the time, needs to be avoided. The standardization of cheap enriching stimuli would represent a great advancement in fields that are based on animals’ exploitation.

This reflection has been applied to improve the environments in which humans live, which are often perceived as unpleasant for the limited space and the lack of design of their physical properties (Jaglarz, 2011, 2012). The attempt of using perceptual effects of particular geometries has been a constant in world architecture, from the entasis (slight swelling about a third of the way up to counteract a tendency of the eye to see them as curving inwards from either side of the columns) in Doric temples (Kosman, 2008), to the forced perspective of many buildings, like Palazzo Spada by the architect Borromini (Figure 5.1a), to the widespread use of *trompe-l'œil* techniques (Figure 5.1b) in contemporary art (Ebert-Schifferer, 2002). At the present time, the employment of these techniques could be used to improve the living conditions of many people that live in the small and constricted spaces of big metropolises (Alfirević, Đ., & Simonović Alfirević, 2019; Mahmood, 2021), or to improve the design of ubiquitous elements that could deeply affect people's life, like steps (Foster et al., 2016) or crosswalks (Figure 5.1c) (Wang, 2022).

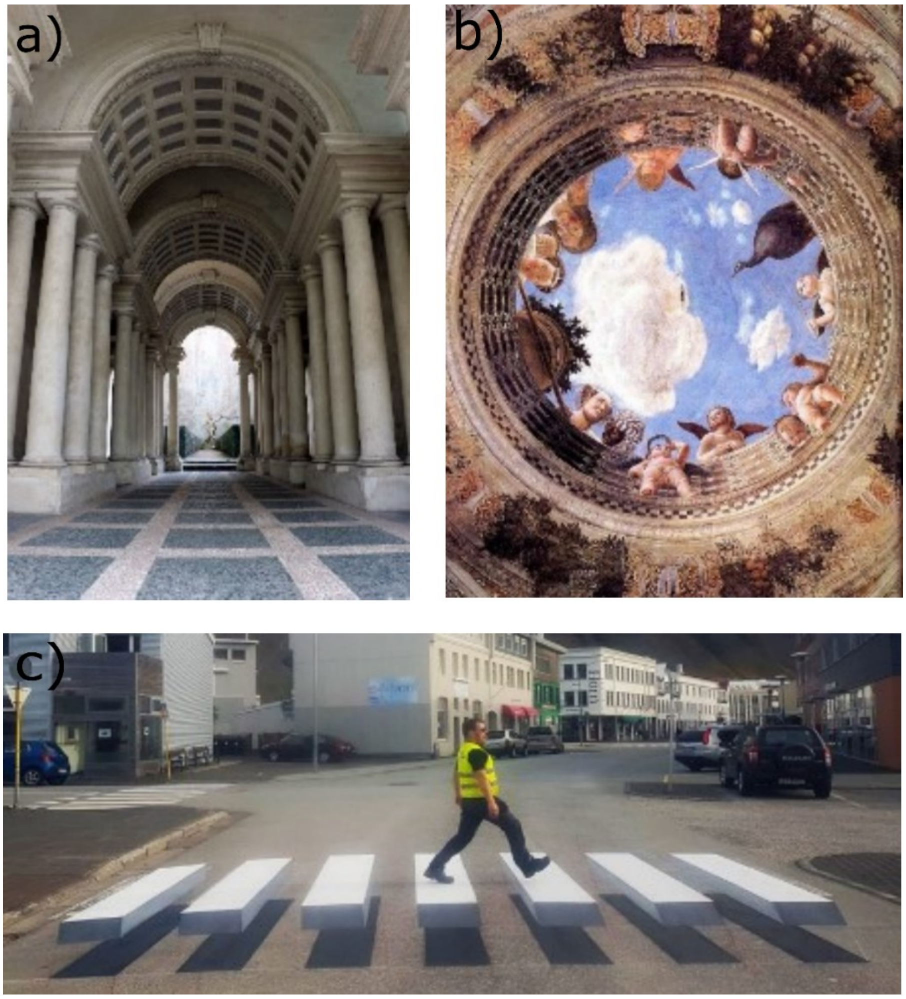

Figure 1.5. a) Palazzo Borromini, Rome: the corridor is only 8 meters long and the statue at the end is 60 cm tall. B) detail of "The Bridal Chamber" by Andrea Mantegna, c) a crosswalk in Iceland

A first attempt at implementing visual illusions to enrich the life conditions of animals forced into captivity had been made on tigers. The preliminary investigation concluded that the visual illusion of movement presented have an effect on the interactions with the stimuli and on the overall ethogram of the tigers (Regaioli et al., 2019).

In the experiments contained in the first part of the thesis, we focused on physical enrichment, in particular on the importance that different levels of water depth have on different species. To better understand the modalities of enrichment for these species, we tested if the predictions based on information obtained by the analysis of their adaptive ecological habits would have accurately described the behaviors of the experimental subjects in the captive setting. We used a total of 4 species of teleost fish: the Siamese fighting fish *(Betta splendens*), the honey gourami (*Trichogaster chuna*), the guppy (*Poecilia reticulata*), and the zebrafish (*Danio rerio*).

The Siamese fighting fish expressed a marked preference for the superficial areas of the tank, especially the shallow ones, according to their breathing, feeding, and reproductive behaviors. On the other hand, the honey gourami, which shares most of the aforementioned living features with the Siamese fighting fish, showed the complete opposite behavior, hiding in the deepest areas of the experimental tank. This discrepancy could be due to the different levels of domestication of the two species. The majestic morphology and coloration of the Siamese fighting fish are indicative of a heavy process of selection and domestication that ends up in selecting species almost totally irresponsive to external dangers. The exemplars of honey gourami tested in our experiment showed a high tendency to startle when approached by humans or objects, indicating a constantly alarmed state that could have influenced the preference for water depth. Guppies, which, like Siamese fighting fish, are highly domesticated species selected for their pleasant colors and attitude, did not show any particular preference, as expected by the apparent lack of any ecological advantage to swim close to the bottom or to the surface. Conversely, the ovuliparous (i.e., the fertilization of the eggs is external) zebrafish swam preferentially close to the bottom, even when the bottom is raised to be close to the surface, as already shown by Blaser and Goldsteinholm (2012).

In summary, while some ecological attitudes are preserved, other may change due to influencing factors that are not entirely known or understood. So, before implementing any measure derived from naturalistic presuppositions, it is necessary to assess the generalizability of these presuppositions to the captive environment.

Once the spontaneous tendencies towards the real features of the environment have been assessed, the subsequent step has been to try replacing these features with illusory ones. If effective, illusory physical enrichment could improve the maintenance conditions of captive animals. Providing different levels of water in each tank would mean building custom-made aquariums or inserting structures that could result in greater maintenance costs and possibly risks for the fish (e.g., remaining trapped in unintended spaces or being crashed by the structures). We then tested an illusion based on Emmert’s law, the principle of size constancy, to trick the experimental subjects into perceiving a cliff just by using a 2D stimulus placed underneath the tank. We chose the two species that expressed the most marked behavior: zebrafish and Siamese fighting fish. We hypothesized that both species would have chosen the side of the aquarium on which the illusion produces the effect of proximity, considering the fact that both species preferred shallow areas. The Siamese fighting fish did not express any preference for either of the sectors, seeming not interested in the floor of the aquarium, while the zebrafish showed to be affected by the perception of the illusion, but only in the naturalistic version of it (gravels rather than 2D checks). Males and females expressed the opposite behavior, being attracted, respectively, to the illusorily deeper and shallower sectors. The possible causes of the sexual differences in the reaction of zebrafish to the illusion could be determined by the different roles played in reproductive stages by the zebrafish. The different reaction registered between the abstract version and the naturalistic one could be attributed to the same reproductive mechanism searching for the most suitable areas for mating (Spence et al., 2007a), which is thick-grained gravel substrate.

These interpretations must be explored in greater detail in future investigations, but the present results indicate the possibility of profiting from this kind of illusion to enrich the constricted spaces in which zebrafish live. Probably, the illusory effect would even be greater if the illusion were applied to the lateral walls too, instead of just the bottom, to recreate a more immersive setting, similar to virtual reality simulations (Vidal et al., 2022). In fact, while the same illusion (checks) has not been found to be effective in mice when simply applied to the bottom, the combination of full tridimensional covering of each wall and the adjusting feedback based on the movement of the animal processed by the simulation fooled the animals (Stowers et al., 2017).

The mechanism of depth and distance estimation needs to be understood in further detail to create more effective environmental illusions that override the multiple other perceptual systems dedicated to the task, like the perception of hydrostatic pressure (Davis et al., 2021). In some species, like the Siamese fighting fish, the illusion could be perceived but still be ineffective because of the different modalities of environmental exploration, which are not strictly dependent on the visual system. Some fish species may rely more on lateral lines than visual mode. It is thus important to consider the different explorative styles in the interpretation of the behavioral reactions of the species.

The second part of the experimental section was instead focused on the social enrichment domain. The only species employed in this test was the zebrafish because of the extensive knowledge accumulated on its social attitude at the behavioral, genetic, and neurophysiological levels (Oliveira, 2013). The first set of stimuli were bidimensional static visual illusions of movement that could not trigger the behavioral response of shoaling. The tests could not reveal if the illusions were not perceived at all or if they created an illusory movement that does not resemble the swimming pattern of a real healthy fish.

The second set of illusions instead triggered a consistent and prolonged social response in our experimental subjects. The stimuli were several versions of kinegrams of zebrafish that varied in illusory features, background image features, and lighting properties. Although the illusion is generally perceived and found attractive, low-level properties of the stimulus like brightness and contrast could play an important role. Excessive brightness was avoided by the experimental subjects and probably caused a disruption of perception to some degree that could have hindered the perception of high-level features, such as the deformation of the background image. Once adjusted these parameters to the preferences of zebrafish, they could be profitably used as tools to enrich solitary animals, avoiding the possible downsides of forced cohabitation with conspecifics.

Currently, this illusion has been tested on a bidimensional screen to assess important issues necessary before further advancements. The next step is to build a material version of the illusion, separating the grid from the background image, in order to obtain the illusory motion effect without any additional movement to that of the fish. This version of the illusion would represent a true, affordable advancement in enriching techniques. In the meantime, the adaptable computer version of the illusion could be used as a tool for studying the visual mechanisms of the zebrafish. The mechanisms involved in visual processing in the zebrafish, known in great detail (Bollmann, 2019), could be further studied through the manipulation of the rate of the frames in terms of the frequencies and durations of the intervals and of the grid. The manipulation of these variables would allow the exploration of the different image reconstruction mechanisms that bind together shape and movement, which usually cannot be disjointed.

The value of the works presented in this thesis suffers from a lack of multidimensional evaluation of well-being. Although behavior is generally preferred to hormonal and neurological assessments as indicators of improved well-being (Binding et al., 2020), the measure of time spent in proximity to the presented stimulus is too reductive to infer its effectiveness as an enrichment condition. The results presented in the thesis indicate that the species perceive the illusions and prefer a certain condition. However, preference is only indirectly linked to improved well-being, and physiological evaluation of stress-related parameters such as cortisol levels in the blood and scales would improve the reliability of the findings. Another evaluation relevant to inferring the improvement of the animals' condition would be the measurement of growth rate, life span, and fertility, some of the most grounded indicators of success, relative to evolutionary theory. A whole-life longitudinal study would be needed to assess the relevance of the proposed enrichment techniques to these parameters. A long-lasting study of this kind would also reveal the durability of these enrichment techniques, as continuous, prolonged exposure could lead to frustration or indifference over time, something that needs to be excluded or at least controlled for.

In conclusion, there is evidence that visual illusions may represent a valuable tool to improve the wellbeing of captive animals like fish because they are often cheap substitutes for the real, costly, biologically-relevant stimuli. They also have the additional advantage of serving as preliminary investigations into the visual systems of the animals involved. The effectiveness of illusory stimulation based on ecological preferences demonstrates the perception of the illusion without recurring to intensive and complicated training procedures, usually adopted to assess if the illusion is perceived in the same way by the animal and thus if its visual perception or elaboration could be in some way comparable to humans' one.

It is difficult to imagine the possibility of completely avoiding the infliction of suffering on animals. While some fields could be revolutionized by the implementation of new techniques, others will remain anchored to the forced exploitation of animals for a long time. It is important to realize the deep commonalities that all animals share and their implications in the ethical sphere. A rational and ethical perspective imposes a continuous effort towards the research of ways to improve the living conditions of the organisms to which we owe so much.

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