Scuola Superiore Sant'Anna

the BioRobotics Institute

PhD Program in

Biorobotics

Mixed societies and biohybrid systems as tools for biological investigation and bioinspired design

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To the love of my life.

Contents

Abstract

Animal-robot biohybrid systems represent an emergent discipline proposing unconventional scientific challenges (e.g. collective cognition, non-symbolic communication channels, interspecific interaction), requesting non-traditional approaches that will contribute to the development of research and technology areas in both engineering and biology.

These animal-robot mixed societies are dynamic biohybrid systems where a biomimetic animal replica is located in a definite place and time simultaneously with authentic animals, establishing a biohybrid ecological interaction (e.g. sociality, gregariousness, competition, predator-prey interaction, parasitic interaction).

In this PhD thesis I focused on innovative approaches to establish animal-robot biohybrid interactions to successfully investigate and manipulate unexplored complex behaviours in animals.

The robotic platforms developed here can effectively modulate different behaviours of a species after an in-depth analysis of the animal model behaviour, and the subsequent designing of the artifact presenting relevant bioinspired features.

Herein, I modulated successfully several behaviours that play a key role in the energetics and the physiology of a species (e.g. the escalation of aggressive behaviours, the intensity of courtship displays; the coalescence of animal aggregations and their location in the space), thus potentially affecting the fitness of a species. Furthermore, many of these behavioural displays have been controlled by using cues that are inedited to animal communication

systems (e.g. light stimuli). These results can greatly contribute to the management of natural systems and to control animals used as biosensors in the environment, pushing beyond the current state of the art in animal-robot mixed societies, as well as in multi-agent systems.

This thesis also provides a new paradigm of neuro-robotics by introducing biorobotic artifacts in neuroethological studies, and in particular in investigation focusing on laterality of several arthropod species. New scientific discoveries have been carried out by using the biorobotic approach, such as fascinating relationships between the evolution of the brain lateralization in vertebrates and invertebrates. In addition, the new scientific knowledge provided here can be exploited to design optimized control strategies in artificial systems endowed with a synthetic lateralized neural system.

A further contribution of this thesis is represented by the first biohybrid interaction involving a parasite and a robotic agent delivering host-borne cues. Biorobotics can produce new extraordinary opportunities to parasitology-oriented investigation, and in particular to the development of advanced and sustainable bioinspired devices for the control of vectors, parasites and pathogens of relevant medical and veterinary interest.

Therefore, one of the aims of this thesis is to pave the way to animal-robot biohybrid systems for real-world applications. In addition to new knowledge, this scientific field has a remarkable socio-economic impact on human being daily lives. Finally, I would like to highlight the importance to train novel scientists with a multidisciplinary background as a strategy to significantly advance this research field, and in general biorobotics, with advantages to both engineering and biology contexts.

Chapter 1: Introduction

Biorobotics (bioinspired and biomimetic robotics), is a relatively novel scientific field that merges disciplines such as robotics, biology, medicine, neuroscience and micro-nanotechnology. The ubiquity and the high impact on the society that biorobotics has shown in the last decades, is reflected in the countless and extremely creative emerging research areas in which bioinspired and biomimetic agents are applied.

Among the contexts of biorobotics and bionics, animal–robot interactive systems represent a fascinating and unique multidisciplinary research field (Romano et al. 2019a). This innovative field is opening up to new opportunities for multiple scientific and technological purposes, including biological investigations, as well as bioinspired engineering design (Krause et al. 2011; Garnier 2011; Halloy et al. 2013).

By following the principles of social behaviour and ecological interactions, living animals and robotic agents create a biohybrid intelligent system representing a new paradigm for bionics.

In animal-robot biohybrid systems a biomimetic animal replica is located in a definite place and time simultaneously with authentic animals, establishing a biohybrid ecological interaction (e.g. sociality, gregariousness, competition, predator-prey interaction, parasitic interaction).

These animal-robot mixed societies are dynamic systems where artificial agents are no longer simple dummies triggering specific reactions in animals, but they can evoke behavioural responses by adjusting the behaviour according with the animal's one (Halloy et al. 2013). Cognitive traits, including perception, learning, memory and decision making, play an important role in biological adaptations and conservation of an animal species.

Robots are resulting advanced allies in the study of these behavioural adaptations, since they are fully controllable if compared to real animals, and it is possible to adjust their position in the environment, allowing a highly standardized and reproducible experimental design.

To respect conventional ethology, robots allow to obtain a desired interaction at a desired time by producing and control life-like stimuli (Krause et al., 2011; Miklósi and Gerencsér 2012). In addition, they provide three-dimensional playbacks, producing stronger and realistic responses to respect more traditional synthetic methods (e.g. dummies and video-playbacks).

This novel approach for behavioural ecology investigation, merging robotics with ethology, is also known as ethorobotics (Partan et al. 2011; Kopman et al. 2013). Experiments in behavioural ecology have been revolutionized by the introduction of robotic models providing important methodological advantages.

Recent technology has made it possible by developing interactive robots, which can perform complex behavioural tasks, adapting their behaviour to signals from living animals and the environment (Garnier 2011).

This research field represents a sophisticated approach contributing to the study of behaviour in animals, with potential applications in the control of animal populations in agriculture or in the improvement of animal farming conditions, as well as in preserving wildlife (Garnier 2011; Halloy et al. 2013).

In addition, these biohybrid systems can act as distributed networks of sensors and actuators in which animals and robots take the best from each other and thus producing advanced bio-artificial multiagent systems (Mondada et al. 2013). In this case, animal-robot biohybrid systems can push beyond the potentiality of swarms composed by just robots, for instance during the exploration of hostile/dangerous environments.

How animals take and process information about the environment and decide to act on it during these biohybrid interactions, also represents a remarkable source of inspiration offering novel paradigms to bioinspired engineers, aimed to produce advanced robots performing the same effective strategies evolved by animals (Webb 2000; Ijspeert et al., 2005; Stefanini et al., 2012; Bonsignori et al., 2013).

1.1 AN OVERVIEW OF THE CURRENT STATE OF THE ART IN ANIMAL-ROBOT MIXED SOCIETIES

In recent years, robots have been increasingly proposed to shed light on hot topics in animal behavioural ecology, with special reference to the study of social interactions between animals and robots perceived as conspecifics. These robots allow to deliver selected cues triggering animal responses or evoking the animal collective behaviour.

Robotics devices in mixed society can be exploited for several reasons, i.e., to validate *in silico* systems, creating a closed-loop society with real animals, or to observe and modify the collective behaviour.

In the following paragraphs, I examined several attempts to design and establish controllable animal-robot biohybrid systems, according to different interactions that were stagedn (Romano et al. 2019a).

1.1.1 Mixed societies: animal perceiving biomimetic robots as conspecifics

Robot features are extremely important for their acceptance by single individual and animal groups as well. In order to elicit interactions with single individuals or group, the robot has to be perceived as a conspecific, and this is particularly important especially in social and gregarious animals. In this context, the use of robots may help to determine which features and signals are critical to trigger conspecific attraction.

Motor patterns seem to play an important role in conspecific attraction. For example, Marras and Porfiri (2012) determined the effect of tail-beat frequency of a robotic replica on individual golden shiners, *Notemigonus crysoleucas* Mitchill (Cypriniformes: Cyprinidae). They found that locomotion is a determinant cue to evoke fish preference, since it can provided considerable hydrodynamic advantages.

Another study showed that the water flow is determinant for schooling, because the coordinated swimming reduced energy expenditure (Polverino et al. 2013; Kopman et al. 2013; Bonnet et al. 2016a, b). Bonnet et al. (2016b, 2017a, b), showed that a robotic zebrafish replica is able to attract a shoal of zebrafish inside of a circular corridor according with the speed of the device, even if this effect was not enough to guarantee a full integration.

Another important issue in conspecific attraction is the visual information, which seems to be crucial in many species, especially colour and shape features. Interestingly, to understand how the body size affects the social behaviour in zebrafish, behavioural responses of zebrafish to shoals of 3D printed conspecifics of different size were investigated (Bartolini et al. 2016). In addition, it was found that the variations of morphophysiological and locomotory features were determinant of attraction towards the robotic fish (Abaid et al. 2012; Polverino et al. 2012). The behavioural response of zebrafish individuals to small shoals changed according to the variation of the aspect-ratio, colour pattern, tail-beat frequency and speed of the robotic fish. The attraction is maximized when the robotic fish replicated the animal counterpart in aspect ratio and colour (Bonnet et al. 2014). In contrast with the aforementioned observations on zebrafish, results on mosquitofish, *Gambusia affinis* Baird &Girard (Cyprinodontiformes: Poeciliidae), showed that they were repelled by mosquitofish-inspired robotic replica, independently of its aspect ratio or swimming depth (Polverino and Porfiri 2013). A recent study on the acceptance of biomimetic replica of the Trinidadian guppy, *Poecilia* *reticulata* Peters (Cyprinodontiformes: Poeciliidae), provides a description of the effect triggered by different appearance, motion patterns and interaction modes on the acceptance of the artificial fish replica. Integration of realistic eye dummies along with natural motion patterns led to a significant improvement of the robotic replica acceptance level (Landgraf et al. 2016). Bierbach et al. (2018a), investigated which cues produced by a robotic fish evoke acceptance in fish species. Authors tested the effect of the biomimetic artefact on two populations of *Poecilia mexicana* Steindachner (Cyprinodontiformes: Poeciliidae): the first population was adapted in dark habitats (e.g., caves), while the second population was adapted to natural light conditions. In light conditions, the acceptance of the robotic fish occurred in both populations, while in dark conditions the robotic fish did not have effects, since probably other cues are needed in the absence of light (e.g., chemical cues, auditory stimuli). In addition, Bierbach et al. (2018b), studied individual responsiveness to social stimuli in *P. reticulata* individuals, by controlling a biomimetic robot, to avoid influences rising from mutual interactions among fish (Fig 1A, B). Authors observed that responses to social stimuli are independent traits, not correlated with other individual behavioural displays.

Since zebrafish behaviours depend on social interactions as well as on their position in the environment, a multilevel model describing the zebrafish collective behaviours was developed to control a robot that was integrated socially in zebrafish group (Cazenille et al. 2017). In this case, optimization methods to calibrate automatically the controllers of a robotic agent according to the animal behaviour were developed (Cazenille et al. 2017).

However, the reconstruction of 3D trajectories should be considered (Macrì et al. 2017), since traditional behavioural displays observed in 2D can undermine data integrity. Ruberto et al. (2016-2017) studied the zebrafish response to a 3D-printed conspecific replica moving along realistic trajectories (Ruberto et al. 2017).

As observed, the design of biomimetic robots able to interact with fish is complex since it should ensure a luring capability, as well as the acceptatance of the robots by the animals as a conspecific. Stochastic model based behaviours of the robot were proposed by Cazenille et al. (2018a), to integrate it in a group of *Danio rerio* Hamilton (Cypriniformes: Cyprinidae).

Animal-robot interactions are benefitting from progress in technology that provides more complex systems to be exploited in animal behavioural studies. Worth to be mentioned is the recent use of virtual reality to control the animal behaviour (Stowers et al. 2017). This strategy boosts detailed surveys into neural and behavioural functions through the accurate control of sensorimotor feedbacks in animals moving in 3D scenarios. Besides the study of morphological and mobility features, Worm et al. (2014), investigated the acceptance of a robotic counterpart of the weak electric fish *Mormyrus rume* Valenciennes (Osteoglossiformes: Mormiridae). This fish is able to communicate by using electric signals, and for this reason, the dummy fish was equipped with electrodes for the generation and reception of signals. Results showed that the animals preferred to interact with the dummy fish in presence of electric signals (Donati et al. 2016). Worm et al. (2018), observed an enhanced interaction between *M. rume* individuals and a robotic fish when the artificial agent generated dynamic echo playback of biomimetic electric organ discharges compared to inter-discharge intervals displays statically and randomly produced.

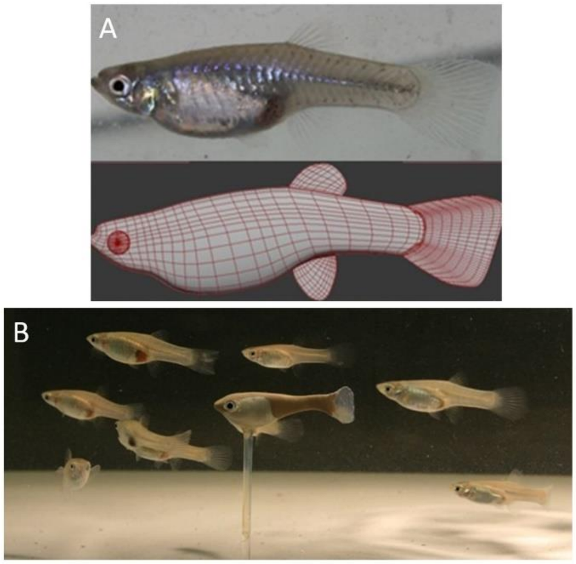


Figure 1. (A) An authentic guppy female and the virtual 3D model.(B) Guppy-replica with a group of female guppies. (From Bierbach et al. 2018b).

1.1.2 Robots evoking selected behaviours through different biomimetic stimuli

Animals are endowed with different senses to perceive environmental stimuli. A complex sensory system allows to perceive the surrounding scenario and its dynamic changes. Therefore, understanding how it works is a fundamental question to which the animal cognition must answer. Robots can represent an effective solution to this question, creating different combinations of perceptual cues, by modifying their features, which can elicit different responses in animal behaviour. Michelsen et al. (1992) used a mechanical honeybee model to understand the role of dance in the transfer of foraging information to follower bees. The robot was able to separate waggle dance and sound-producing wing-vibration of the dance, allowing to demonstrate that the waggle dance is important to transmit information about distance and direction of a given food source. Close-related studies have been conducted subsequently to investigate the acceptance of the robot in the hive (Landgraf et al. 2010, 2012).

The possibility to deliver different kind of cues separately through robots interacting with animals allows us to dissect the relative importance of the different stimuli triggering a response in living organisms. This is hard in classical ethological observations because all the cues are presented together and the response to a single feature in a complex (multi-stimuli) behaviour is not measurable. Robots make possible the deconstruction of different behavioural components to measure the individual responses. For example, a study focused on display modification to visual signals in the lizard *Sceloporus graciosus* Baird & Girard (Squamata: Phrynosomatidae), used a robotic counterpart to investigate two types of headbob displays (special-typical and unusual) both in short- and long-term assays (Martins et al. 2005). No evidence for immediate changes in signal structure or long-term changes were detected. However, the lizard was more agitated and produced highly aggressive displays when exposed to unusual headbob displays. Another study focused on the impact of different displays in *Anolis sagrei* Duméril & Bibron (Squamata: Dactyloidae) (Partan et al. 2011), showing that the social response to the movement of the robot was higher in signature patter than alternate pattern, despite they had a high degree of variability in signature bobbing display. In another study, the frog *Epipedobates femoralis* (Anura: Dendrobatidae), defended their territory and emit visual cues (i.e. vocal-sac pulsations) and auditory cues (i.e. advertisement cues). In this study, the two stimuli were presented with spatial disparity or/and time delay and the result showed that bistimuli with temporal overlap evoked aggressive behaviour while with lacking overlap they were ineffective. Similar studies have been conducted on ground squirrel, *Spermophilus beecheyi* Richardson (Rodentia: Sciuridae) (Rundus et al. 2007), where the importance of infrared signal to deter rattlesnake predator was investigated. Natural observation showed that squirrels, when confronting infrared sensitive rattlesnakes, add an infrared component to their snake-directed tail-flapping signals whereas, when confronting infrared-insensitive snakes, the tail-flagging is without augmenting infrared emission. The robot squirrel simulated the same behaviour and the results showed a greater shift from predatory to defensive behaviour of the rattlesnake when the infrared was present. In a similar study on multi-stimuli in *Sciurus carolinensis* Gmelin (Rodentia: Sciuridae) (Partan et al. 2009-2010), a biomimetic robot displayed different combination of alarm cues (i.e. visual and acoustic ones), investigating the response of wild squirrels. Multimodal signals showed higher response to cues' combinations over separate signals.

Interestingly, a biomimetic soft robot, able to 3D move in underwater environments was recently developed by Katzschmann et al. (2018), to be perceived as a fish and thus approaching and studying the aquatic life by avoiding aversion in animals (Fig. 2).

Figure 2. A biomimetic robot performing a closeup exploration of underwater life (from Katzschmann et al. 2018).

1.1.3 Social interacting robots

Animals live in a social environment that is highly dynamic. Therfore, they continuously update the information they hold from conspecifics (e.g. foraging for food and mates). The transfer of information between conspecifics is a complex task that remain poorly understood and difficult to manage. In this case, biomimetic robots can play an important role, allowing independent control of specific aspects of this behavior .

The relevance of social interactions was investigated in a study on dogs interacting with commercially available quadrupedal robots (Kubinyi et al. 2004), where the latter elicited either aggressive or playful responses from live dogs. It was shown that age and context influence the social behaviour of dogs. A laboratory study on learning showed that rats followed a remote-controlled electromechanical rat (WM-2) to sources of food (Takanishi et al. 1998). Live rats recognized the movement of robot and the latest helped them to learn response to stimulations. Later, the authors increased the complexity of the interaction by using a legged rat robot, which successfully taught the rat a lever pushing task to get food (Laschi et al. 2006; Patanè et al. 2007). Recently, Shi et al. (2010, 2013, 2015) investigated the interaction between a robotic rat and living rats, observing that rats with more active behavioural parameters are more susceptible to being adjusted by the robot (Fig. 3).

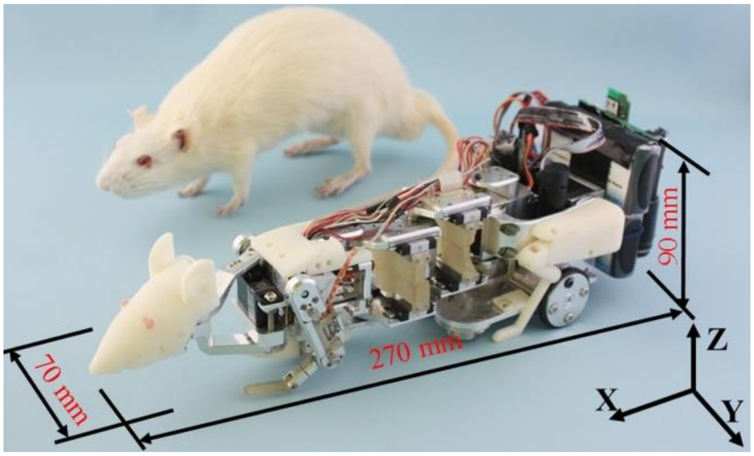


Figure 3. Rat-robot WR-4 used to modulate the behaviour in rats (from Shi et al. 2013).

In animal interactions, it is possible to manipulate the behaviour of individuals by using biomimetic robots (Fernández-Juricic et al. 2006). In this context, the foraging and scanning behaviour of the house finch *Carpodacus mexicanus* Muller (Passeriformes: Fringillidae), in response to different types of behaviours from artificial flocks was studied. Finches spent more time foraging when the robots simulated body movement that could be associated with successful foraging behaviour. A comparison on social information transfer in three different bird species (i.e. European starling, house finch,brown-heaaded cowbird) (Fernández-Juricic and Kowalski 2011) showed a nonlinear decrease in social information flow with increasing distance between the robots and live birds. This was more pronounced in species with lower visual acuity, because animals with higher visual acuity can detect changes in the behaviour of conspecifics from farther distances, which may have consequence in spatial distance between individuals within a flocks. High visual acuity is due to regions of the retina with a localized high density. These regions project into a visual space, which may require, in order to detect changes, to move the body and modify the position within the group (Butler and Fernández-Juricic 2014). Robots can be used to study the early learning occurring in several species, known as "imprinting". There is a brief receptive period, typically soon after birth or hatching, in which animal are capable of rapid learning and establish a long-lasting response to a specific stimuli (Lorenz 1935). The input can be a visual, auditory, or tactile experience that create the attachment with a given object. Usually, in nature, this object is a parent but in experimental conditions other animals and inanimate objects (e.g. a robot), can be used. It was shown that young individuals of *Gallus gallus domesticus* Linnaeus (Galliformes: Phasianidae), can be imprinted on robot introduced as a surrogate hen which can spatially interact (Gribovskiy et al. 2010, 2015, 2018). In a social experiment with the Japanese quail chick *Coturnix coturnix japonica* Temminck & Schlegel (Galliformes: Phasianidae), a mobile robot carrying a heat source was used to control the motion of quail chicks. Chicks showed better spatial abilities when raised with heated mobile robot than when exposed to an immobile heater. These experiments demonstrated that there was a measurable attachment to the robot (favored when the chick encounters the robot early after the birth), and this attachment was also combined with a synchronization of chick and robot activity (Jolly et al. 2016). Finally, in a study assessing social cues in the Australian brush-turkey *Alectura lathami* Gray (Galliformes: Megapodiidae), a serie of robot chicks was built with the same characteristics of the real one, except for one made with pecking movements. Chicks preferred a pecking model over static or scanning models (Göth and Evans 2004), suggesting that social responses of chicks depend upon conspecific motion patterns.

Animal behaviour may vary post-exposure to different traits. This is particularly true for courtship behaviour, where males often differ in their courtship sequence and these differences modify the rate of success at convincing female to mate. Interactive robots can provide different combinations of mate features to investigate their effect during courthsip and mating interactions. A study on courtship in fish (*Lucania goodei* Jordan; Cyprinodontiformes: Fundulidae) (Phamduy et al. 2014) measured the preference of a fertile female for male robot movements by varying aspect parameters (e.g. colour: red, yellow or blue skin) during motion of classical courtship behaviour. In satin bowerbirds *Ptilonorhynchus violaceus* Vieillot (Passeriformes: Ptilonorhynchidae) (Patricelli et al. 2002-2006), a female-mimicking robot was used to study the male response and to test how and when the male reduce the intensity of his courtship displays after females starling. Another example is the study of head-bobbing movements in the lizard *S. graciosus* that can elicit both aggressive and mating display (Martins et al. 2005). Results showed that two aspects of head-bobbing displays are independently-meaningful components interpreted different by different receivers. Males are attended to posture and females to number of head-bobbing, using it to distinguish male courtship. Multimodal signals during courtship was also investigated in Tùngara frogs (*Physalaemus pustulosus* Cope; Anura: Leptodactylidae) (Taylor et al. 2008). Females preferentially respond to multimodal stimuli (i.e. auditory plus visual cues) over a unimodal stimulus (i.e. auditory cues only). In this study, the females interacted with a robotic calling male frog, validating the theory that females positively select multimodal cue combinations over the same stimuli tested singly. Another recent example is a study on fiddler crabs (*Uca mjoebergi* Rathbun; Decapoda: Ocypodidae), and how they move their arms during mating. Four male-mimicking robots were used to produce asynchronous and synchronous movements of the arms during the courtship dance (Reaney et al. 2008). In a study on cricket courtship behaviour, (Kawabata et al. 2013), the real cricket interacted with a mobile robot, and the behaviour was evaluated on the basis of the animal pose. In a subsequent study (Kawabata et al. 2014) the robot robes the cricket's pheromone and its position was controlled by designing motion patterns based on visual motion tracking of both agents.

1.1.4 Biohybrid collective behaviours

A further application of artificial agents in animal-robot mixed societies is to investigate and influence the collective behaviour in social and gregarious species (Mondada et al. 2013). The collective behaviour is a complex system that presents several levels of organization (i.e. hierarchical organization). One of the major challenge in mixed society is to design robots that can modulate the natural society towards a desired behaviour influencing the decision-making process. The result is a closed-loop feedback system between artificial and authentic animals in which robots can react to sensory input triggered by the animals. To investigate the mechanism of shelter-seeking in the cockroache *Periplaneta americana* Linnaeus (Blattodea: Blattidae) (Halloy et al. 2007), a robot with the same behaviour of a real counterpart was fabricated. The robot was autonomous, and although it had not a biomimetic shape, it was perceived as a conspecific due to its pheromone-scented body. It was able to recognize the shelters and to interact with real cockroaches, and to elicit novel collective decisions (Fig. 4). They were programmed to lead cockroaches from the favorite shelter into an unsafe one in open area.

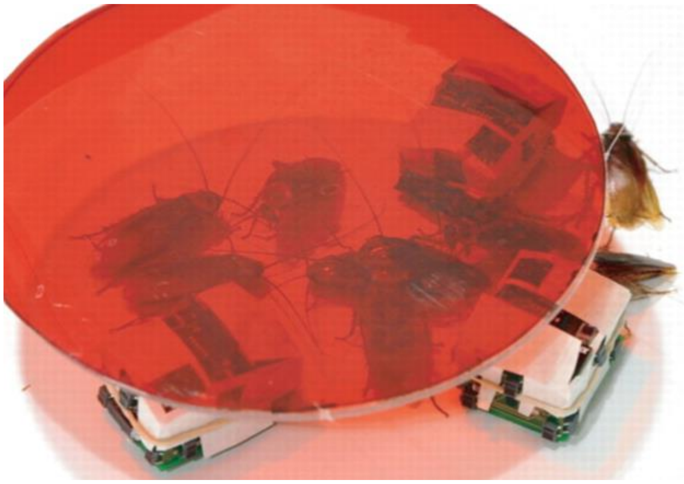


Figure 4. Robots used to investigate the decision-making behaviour in cockroaches (from Halloy et al. 2007).

Also, the Robot Sheep dog project developed a mobile robot to control a flock of ducks to go towards a specific place (Vaughan et al. 2000). The robot moved round behind ducks with respect to the goal and the flock moved away from the robot to the goal. When the flock reached the goal started the repulsive phase and the robot was less attracted.

Studies on collective behaviour in fish shoals are rapidly increasing. One of the reasons is the possibility to have easy access to fish and the limited space they needed. Zebrafish is one of the main species currently studied, due to their high reproduction rate, short intergeneration time and evident shoaling tendency. In Butail et al. (2013) a robot able to move at different speed by varying tail-beating was used to investigate the fish responses. It has been shown that for group cohesion, speed is a determinant feature. Indeed, the relative distance increases with speed of the robot. Further experiments investigated the influence in shoal size and configuration of fish increasing the number of robots (Butail et al. 2014a). Stress measurements showed increased value in presence of more robots, or with fast robot swimming alone instead of slow swimming of two robots. Other experiments focused on the collective behaviour of different fish species, including the analysis of self-organization and aggregation in Giant danios, *Devario aequipinnatus* McClelland (Cypriniformes: Cyprinidae), (Aureli et al. 2012). A similar study with robotic replica but different specie of fish (i.e. sticklebacks and guppies) involved one robot that moved according to the position of fish detected through an external camera (Landgraf et al. 2013). Risk-taking behaviour of individual golden shiners (*N. crysoleucas*) was investigated in the presence of a self-propelled robotic fish to test the hypothesis whether the behaviour of fish can be modulated by varying the behaviour of a robotic fish and to investigate whether such response depended on fish individual boldness (Abaid et al. 2013).

Robots can be used to survey how animals select the leader, and in which contests they follow it. Leadership occours when one or more subjects initiate a new directions of locomotion, followed by other group members. For example, in Faria et al. (2010) a fish-like replica was used to visually attract and drive single fish out of a refuge and to initiate new swimming directions in both individuals and groups. Ward et al. (2008) investigated the decision-making process in a Y-maze by using a robotic replica moved on a guide line, to demonstrate the role of "quorum responses" in the movement of the fish *Gasterosteus aculeatus* Linnaeus (Gasterosteiformes: Gasterosteidae). An information-theoretic approach to infer leadership starting from positional data of fish was also proposed (Butail et al. 2016, 2017). Rashid et al. (2012) presented another work about leadership, which involved the use of mobile light sources for guiding swarms of brine shrimp larvae (*Artemia salina* Linnaeus; Anostraca: Artemiidae).

Recently, computer vision and real-time control have enabled the development of closed-loop control systems that boost the degree of biomimicry of the artifacts, by improving the attraction and the interaction of *D. rerio* with the robotic stimuli (kim et al 2018). These artifacts that are perceived as conspecifics can be used to study social processes and to affect collective decision in the fish (Bonnet et al. 2018). Interestingly, Cazenille et al. (2018a, b), presented a strategy for real-time calibration of behavioural models based on an evolutionary algorithm, to improve the integration of the robot into the shoal.

1.1.5 Robots as tools for scientific validation

Robotic agents can be exploited for testing and validating behavioural models in embodied simulators. This possibility is defined *"in artefacto"*, compared to *in vivo*, *in vitro* and *in silico* well established approaches. Interactive robots can be used to assess mechanistic models and the assumption they are based on (Krause et al. 2011; Manfredi et al. 2013), allowing scientists to test hypotheses about mechanical behaviours and interactions with external cues (Fig. 5). They can be effective tools for validating theories and biological models, thanks to the achievement of high accessibility to the environment. It is possible to embed the biological knowledge and models directly on the robots, thus the observations can be made on such a complete physical model behaving in the real environment.

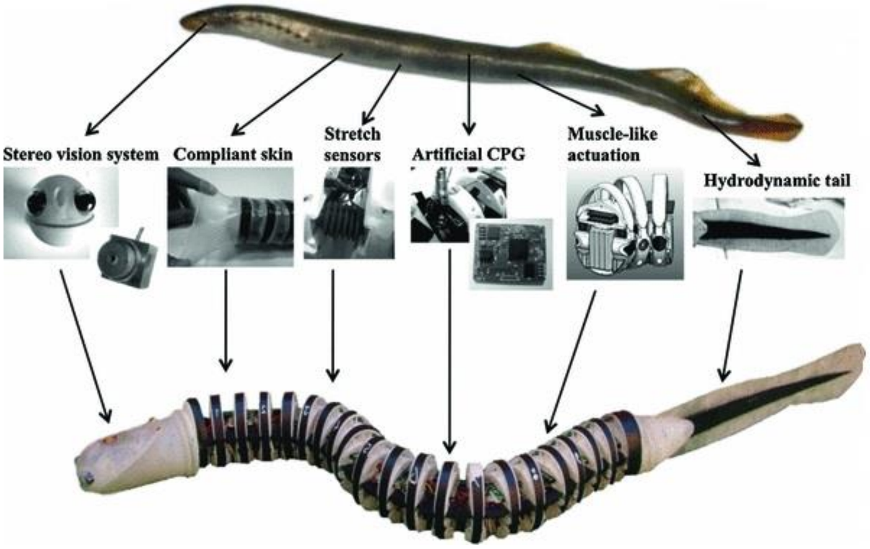


Figure 5. Scheme describing the similarities between the animal model and the artefact developed by Manfredi et al. 2013.

1.2 “ME-FIRST” INVESTIGATIONS ON ANIMAL-ROBOT INTERACTION: A RESEARCH AREA “FULL OF SECRETS”

Animal-robot biohybrid system is an emergent discipline proposing unconventional scientific challenges (e.g. collective cognition, non-symbolic communication channels, interspecific interaction), requesting non-traditional approaches that importantly will contribute to the development of research and technology areas in both engineering and biology.

The methodology design of these biohybrid systems often is focused on a number of rules in order to develop simple and formal models for closed-loop interactions. The result is that we neglect that animals, although simpler that human beings, are not machines and that complex processes are involved in an interaction.

In addition, although the amount of studies on animal-robot interaction is increasing, attracting a growing number of scientists from different scientific fields, most of the studies currently available investigate just group and collective behaviours. Nevertheless, biomimetic robots can play a pivotal role for further progress in investigating highly flexible and complex behaviours, both in intraspecific and interspecific interactions, that still remain unexplored.

A thorough knowledge of the cognitive dynamic, at individual level, of a species represent the main challenge to face in order to establish a successful biohybrid interaction.

The analysis of the animal model behaviour, by identifying cues and communication channels (e.g. based on chemical, tactile, auditory or visual stimuli), that are relevant for the interaction, is a key step for the design of an effective biomimetic artifact.

These biomimetic robotic platforms are advanced tools to study and modulate the behaviour of a species by displaying selected biomimetic cues reproduced accurately.

In addition, animals are forced to live in an increasingly anthropized world. Therefore, stimuli that are inedited to many animal species' communication channels (e.g. artificial light, electric fields, magnetic fields), are more and more present in the environment. How animals perceive, adapt, and exploit this stimuli is a crucial and overlooked phenomenon that could greatly benefit from the use of robotic agents.

Furthermore, biomimetic robots can be introduced in neuroethology for investigating selected neural circuits and basic principles of the brain organization (e.g. lateralization), during interactions between animal and and artificial agents.

The use of biorobotic artifacts in neuroscience could provide new perspectives and research contexts in neurorobotics, as well as could exploit biological findings to design optimized control strategies in artificial systems.

1.2.1 Modulating different behavioural traits through multimodal biorobotic cues

Ethorobotics has only scratched its potential, although this discipline can have a fundamental role in unravelling mechanisms involved in many flexible and highly evolved behavioural traits possessed by animals. Of interest is the possibility to control the behavioural dynamics of a species during interaction contexts in order to trigger a desired display in a precise time. This would have significant repercussions in both technological and scientific areas.

An interesting ecological interaction in which robots can provide a great contribution is represented by agonistic interactions occuring among individuals of the same species.

Aggressive behaviour is widespread across the animal kingdom since it has a key role in acquiring and defending limited resources. Game theory predicts that evolutionarily stable strategies for conflicts occurring between conspecifics, may involve stereotyped contests featured by the ritualized exchange of agonistic cues.

The Siamese fighting fish, *Betta splendens* (Regan) (Perciformes: Osphronemidae), is a perfect biological model to stage a biohybrid agonistic interaction, and to explore the interactive effects of a robot inducing aggression in the aquatic environment. *B. splendens* have territorial males performing highly stereotyped and vigorous aggressive displays towards conspecific males.

The combinations of dissected cues commonly perceived by these fish during fighting, (reproduced accurately in a robotic fish), were used to elicit aggressive responses, as well as to modulate the escalation of aggressive displays in real Siamese fighting fish.

In addition, no efforts focused on the effect of a light source mimicking a specific behavioural or a colour pattern of the body in animals. I endowed the robotic fish with LEDs producing light stimuli. This species performs gill flaring display (the erection of gill covers, often accompanied by the dropping of the brightly red branchiostegal membranes). The robotic fish produced a red bright signal (inedit to the real fish), that was perceived successfully by real fish.

Furthermore, in territorial species, to achieve the maximum mating success and to minimize injuries, both males and females have evolved specialized morphological features and behaviours that are important for sex recognition, and that robotics can help to identify.

In *B. splendens*, during courtship, males display several behaviours that are identical to male-male not-physical agonistic displays, as well as courtship-specific displays. Too aggressive males would chase the female away or would increase the risk of injuries. Conversely, too passive males would cause female loss of interest. In this scenario, I focused on the colour pattern exhibited by reproductive *B. splendens* females consisting in horizontal darker stripes along their bodies with lighter stripes in between. The effect of a light source mimicking the specific colour pattern of a reproductive female was evaluated. A robotic apparatus moving biomimetic fish replicas inspired by female *B. splendens* was developed to investigate biomimetic visual stimuli that are important to switch aggressive Siamese fighting fish males in courting subjects, thus deeply controlling their behavioural status. In particular, Siamese fighting fish males were exposed to a reproductive female-mimicking fish replica, exhibiting a luminescent colour pattern as well as to a reproductive female-mimicking fish replica with a painted colour pattern.

Another interesting trait arising from aggressive interaction is represented by animal aggregate formations as a collective behaviour emerging to exhibit cooperative antipredator strategies that allow animals to be safer in groups rather than isolated individuals. It is interesting the schooling behaviour emerging earlier in young fish in species not providing parental care but rather cannibalizing their offspring. Studying the capability of newborn schooling fish species to aggregate along with identifying which predator cues activate this collective behaviour in naïve individuals represent a context of investigation which remains unexplored. Biomimetic mechatronic agents offer the possibility of having a harmless tool with fully settable controls, exhibiting more realistic visual and physical predator-borne cues in a given ecological niche. Their multimodal influence is an advanced tool to investigate and modulate the coalescence of the fish group, with advantages in understanding basic mechanisms of self-organization and collective intelligence.

1.2.2 Using biomimetic robots in neuroethology

Biorobotic artifacts offer new paradigms of experimental manipulations of intraspecific and interspecific interactions that can be exploited in neuroethology investigations. Specialized neural circuits, allowing the accurate conversion of sensory signals into optimized motor outputs, have been implemented in a number of animal species. These neural networks contribute significantly to biological fitness in many animals, conferring important functional advantages.

Lateralization is the different specialization of the right and left sides of the nervous system reflected in left–right behavioural asymmetries. It can increase neural capacity in cognitive tasks, enabling the brain to perform simultaneous processing by avoiding duplications of functions in the two hemispheres. Lateralization represents a fundamental principle of the brain organization widespread among vertebrates.

Recent findings have demonstrated that also invertebrates (e.g. arthropods), endowed with simpler nervous systems, shows lateralized traits.

This is revolutionizing some theories related to vertebrate and invertebrate evolution.

In addition, the neural architecture of insects, compared to that of more complex organisms (e.g. vertebrates), is easier to study. Lateralization in these simpler organisms can provide innovative insights to implement into bioinspired artificial systems.

Indeed, it would add significant information to formulate advanced mathematical models on visuo-motor neuro-machinery involving sensory processing, brain specialization, cognition, as well as learning.

However, the study of asymmetrical interactions is complex and limited by many experimental limitations (e.g. directional cues, difficult management of multimple animals involved in the experiment, impact of the experimenter on the animal behaviour).

Biorobotic artifacts enable the achievement of highly standardized and controllable testing in these kind of researches by providing perfectly symmetric stimuli, in appearance and movement, as well as by avoiding mutual influences and/or feedbacks between multiple animals.

Although there is a range of evidences from vertebrates demonstrating lateralized responses to predators, in insects asymmetrical responses to predators have not been investigated. Predator-prey interactions are key selective mechanisms affecting both morphological and behavioural features in the animal kingdom.

Locusts use cryptic colourations and/or behavioural traits to go unnoticed during predator scanning. They avoid predatory attacks by using their powerful hind legs, well adapted for jumping. Thus, they are valuable models to test lateralized responses to a predator-mimicking artifact. A robot predator inspired to an avian natural enemy of locusts, allowed to engineer a biohybrid predator-prey interaction producing symmetric and readily controllable life-like stimuli with a degree of repeatability and standardization impossible to get with a real predator or with a dummy moved by an operator.

Besides, escape and surveillance responses to predators represent profitable models to increase basic knowledge on how these sensory-motor sequences are affected by visual experience and how this influences lateralization. Understanding how prior context experience affects the processing of sensory stimuli by an asymmetrical nervous system (e.g. to generate complex actions such as anti-predator behaviours), can represent an important goal in computational neuroscience. Furthermore, it can provide important insights on the predator-prey interaction dynamics. The hypothesis to "program" the locust jumping escape direction as well as surveillance orientation, as an adaptive consequence of prior exposure to the robotic predator in lateralized training sessions, would push beyond the current use of robots in behavioural ecology studies. Indeed, this would represent a new way to engineer a natural nervous system, based on behavioural interactions, by avoiding any invasive approaches. Also in this case, the development of a robotic apparatus actuating an artificial agent mimicking a predator of locusts (e.g. a gecko), allowed to manipulate a predator-prey interaction via a biohybrid approach.

Furthermore, a biomimetic mechatronic apparatus allowed to display a realistic combination of host-borne cues (i.e., both visual and olfactory stimuli) to the *Ixodes ricinus* (L.) (Ixodiidae) tick, to evaluate the lateralization of legs during questing (e.g. a particular host seeking behaviour showed by ticks waiting for hosts with their forelegs outstretched), as a response to a biomimetic stimulus. This study represent the first attempt in using a biomimetic agent in the scientific field of parasitology and medical entomology.

Chapter 2: Evoking and modulating complex behaviours via biorobotic artifacts delivering multimodal cues

2.1 CASE STUDY 1: A ROBOTIC FISH PRODUCING MULTIMODAL SIGNALING MODULATES ESCALATING LEVELS OF AGGRESSION IN SIAMESE FIGHTING FISH

In this study, I investigated biomimetic aggressive interactions involving the Siamese fighting fish, *Betta splendens* (Regan) (Perciformes: Osphronemidae), and a conspecific-mimicking robotic fish in an agonistic interaction (Romano et al. 2017a). I selected this species as an animal model to investigate which biomimetic cues produced by a robot induce aggression in the aquatic environment. Indeed, Siamese fighting fish males are territorial and perform highly stereotyped and vigorous aggressive displays towards conspecific males (Simpson 1968; Dzieweczynski et al. 2006). I developed a robotic platform including a fish replica that was inspired to the male of this species during fin spreading behaviour (an aggressive visual cue displayed by *B. splendens* males), to trigger aggressive responses in *B. splendens* males.

Furthermore, I proposed a novel animal-robot interaction paradigm, by incorporating two red light-emitting diodes (LEDs) in the fish replica, located close to gill regions. LEDs provided luminescence as artificial surrogate of the opercular gill flaring behaviour (a common aggressive display in Siamese fighting fish, consisting in the erection of gill covers and the dropping of the brightly red branchiostegal membranes, correlated with behavioural dominance).

The aim of this research was to use a robot to elicit aggressive responses in real Siamese fighting fish, post-exposure to combinations of dissected cues commonly perceived by these fish during fighting. Furthermore, I attempted to create a biomimetic fighting interaction enabling me to modulate the escalation of aggressive displays in *B. splendens* males. To achieve these purposes, male Siamese fighting fish were exposed to our robotic fish replica displaying a series of stimuli singularly and combined. As a general trend in the animal kingdom, aggressive physical interaction would result in a high loss of time and energy as well as risk of injury. Starting from these assumptions, I predicted a variation in the escalating level of aggression that characterizes the Siamese fighting fish when different animal-robot contests were presented.

2.1.1 Methods

2.1.1.1 Animal rearing and general observations

Male Siamese fighting fish (veil tail strain), were purchased from a local aquarium store in Pontedera, Pisa, Italy. All fish matched in size and had a blue livery, although the shade of blue was not perfectly homogeneous among subjects. Siamese fighting fish were reared individually in tanks (28 × 14 × 15 cm), filled with dechlorinated tap water, which was completely replaced every third day. Opaque partitions were placed between tanks to avoid fish seeing each other before the testing phase. Fish were maintained under controlled conditions [25 ± 1 °C, 16:8 (L:D) photoperiod] at the Institute of BioRobotics (Scuola Superiore Sant'Anna, Pisa) and fed twice daily using a diet of Tetramine flake food.

Experiments were carried out from March to June 2016 in laboratory conditions (25 ± 1 °C) in a room illuminated with overhead fluorescent daylight tubes (Philips 30 W/33) [16:8 (L:D) photoperiod, lights on at 06:00]. The light intensity in close proximity of the testing arena was approximately 1000 lux, estimated over the 300–1100 nm waveband using a LI-1800 spectroradiometer (LI-COR Inc., Lincoln, NE, USA), equipped with a remote cosine receptor. Directional light cues were avoided by using diffused laboratory lighting to reduce possible reflection and phototaxis.

All fish were tested in tanks (40 × 30 × 20 cm) with their sidewalls shielded with screens of white filter paper (42 ashless, Whatman Limited, Maidstone, Kent, United Kingdom) to prevent environmental cues. In each experiment, the behaviour of *B. splendens* was directly recorded by an observer dressed with a white coat, in order to minimize his impact. For each replicate, the test tank was carefully washed for about 30 s with warm water at 35–40 °C, then cleaned using water plus mild soap for about 5 min, rinsed with hot water for about 60 s, then rinsed with tap water at room temperature, and finally refilled with dechlorinated tap water at 25 ± 1 °C. Both the fish replica and the cylindrical dummy were carefully washed for about 30 s with warm water at 35–40 °C, then cleaned using water plus mild soap for about 5 min, rinsed with hot water for about 30 s, then rinsed with distilled water at room temperature, before starting each replicate.

2.1.1.2 Animal replica and experimental apparatus

Fish-replica design is inspired to the shape and size of *B. splendens* males during the fin spreading behaviour and includes fish appendages such as a dorsal fin, an anal fin, a caudal fin, two ventral fins and two ocular protuberances. Total length, height and width were 80 mm, 35 mm and 13 mm respectively. I designed the fish replica mold in SolidWorks (Dassault

Systemes, Vélizy-Villacoublay, France) and printed it in a rigid acrylonitrile butadiene styrene (ABS) plastic, manufactured in rapid prototyping (Fig. 6A). Two red LEDs were positioned close to the gills region in the mold (Fig. 6B). Afterwards, I melded a transparent liquid silicone rubber with a nontoxic blue pigment, since all the fish I tested had blue pigmentation, and injected it in the mold until the silicon rubber dried. Fish that we tested had variable shade of blue and generally, body colouration varies considerably in this species (Blakeslee et al. 2009), so we did not colour the fish replica perfectly like real fish. Concerning eyes, our fish replica had two small ocular protuberances that had the same colour of the body, although realistic coloured eyes (missing in our model), were found to significantly improve the acceptance level of a robotic fish during social interactions in some fish species.

Colour measurements of the fish replica body and of incorporated LEDs (Table 1) were recorded using standard CIELab colour space coordinates determined using a spectrometer Ocean Optic HR2000-UV–VIS-NIR (Ocean Optics, USA). I believed that the body in silicone rubber of the fish replica, to respect a dummy in ABS plastic, would improve the biomimicry of the aggressive interaction as it is soft and compliant as relatively similar is the body of the fish.

Tested cue	L*	a*	b*
Fish replica body	41.0 ± 2.31	-7.77 ± 0.81	-22.9 ± 2.11
Red LED	71.1 ±3.52	77.2 ± 3.85	39.5 ± 7.89

Table 1. Colour measurements of the fish body and incorporated LEDs ± standard error. L* represent the lightness component, a* (from red to green) and b* (from blue to yellow) are the two chromatic components.

The fish replica is anchored to the external apparatus by a plastic tube (length 225 mm; diameter 3,15 mm) light grey coloured, vertically inserted in the mold immediately forward the dorsal fin (Fig. 6A, B, C). The external apparatus is composed by a Robbe FS 100 Servo that was mounted on a plexiglass base plate (100 × 420 × 4,3 mm), by two threaded rods (length 180 mm; diameter 4,9 mm). This servomotor actuated the twist of the fish replica, by means of the plastic tube of the dummy.

I positioned the fish replica 30 mm below the water surface (Earley et al. 2000), approximately at the center of the robot zone of the test tank, since *B. splendens* guard their floating nest close to the water surface, and often, during fights, they exhibit surface breathing (Simpson 1968; Doutrelant et al. 2001; Dzieweczynski et al. 2006; Arnott et al. 2016). Both the servo and the LEDs were controlled by an external microcontroller (Arduino Mega 2560).

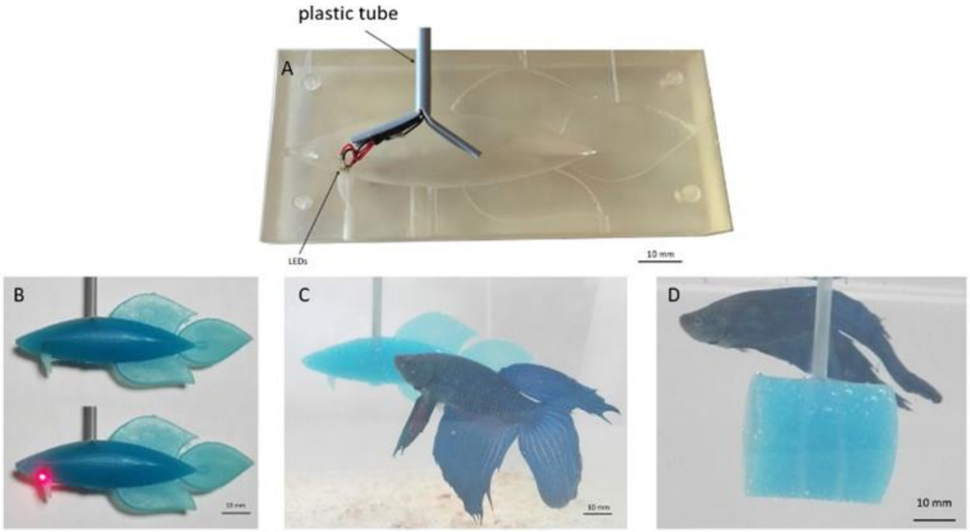


Figure 6. Snapshots from different *Betta splendens* fish replica development phases and the cylindrical dummy. (A) Right side of the fish replica's mold in ABS during the positioning of the plastic tube and LEDs. LEDs are located close to the gill region of the fish replica shape and their wires are housed in the plastic tube. (B) Fish replica with LEDs off (upper) and LEDs on (lower). (C) Fish replica static, in the test tank, evoking the fin spreading behaviour in a *B. splendens* male. (D) Cylindrical dummy static, in the test tank with a *B. splendens* male.

2.1.1.3 Interactive biohybrid experiments

B. *splendens* males were gently placed in the test tank individually at least 24 h prior performing experiments or until they build a bubble nest, since the nest presence has been found to be crucial to produce territorial males (Dzieweczynski et al. 2006). The test tank was virtually divided in three zones: nest, middle and robot zone. Test tanks were provided with a 7 × 7 cm square of bubble wrap (replaced after each replicate) on the surface of the water in one corner of the nest zone of the tank to facilitate Siamese fighting fish in building the nest and to control the nest location. The fish replica was placed in the center of the robot zone of the tank (Fig. 7).

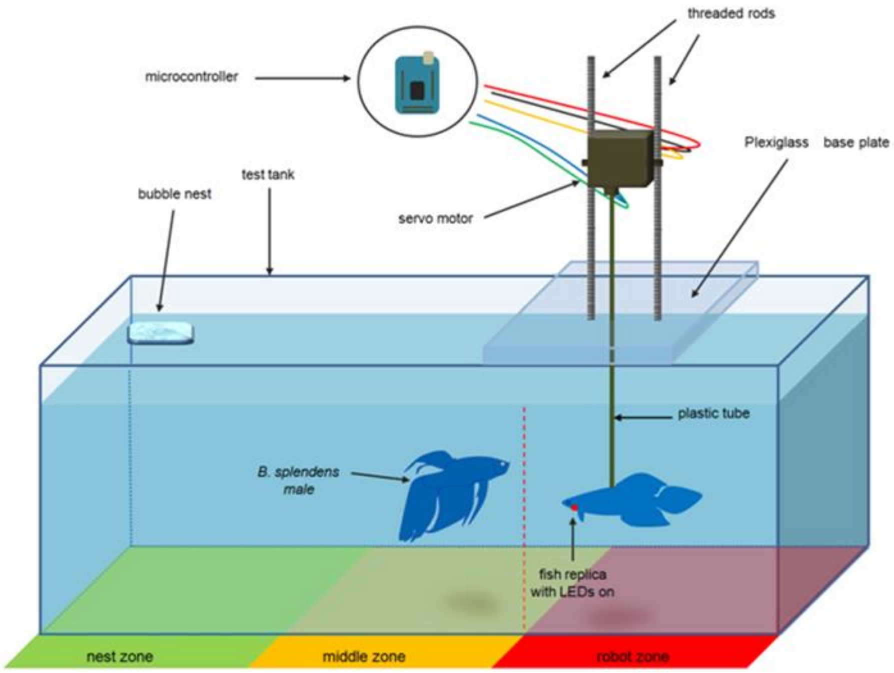


Figure 7. Experimental setup. The virtual division of the test tank in nest (green), middle (yellow), and robot (red) zone, is depicted below. The bubble nest is located in the nest zone. The fish replica is located in the robot zone. Tests start once an opaque partition (red dashed line), between the middle and the robot zone, is removed and the Siamese fighting fish, *Betta splendens* male can see and approach the fish replica. The fish replica is coupled to the servomotor by the plastic tube. The servomotor can be adjusted along the threaded rods to change the fish replica depth. A microcontroller was used to control both the servomotor and LEDs. The green line and the blue one, indicate wires connecting LEDs to the microcontroller. Orange, black and red line, indicate wires connecting the servomotor to the microcontroller.

An opaque partition (30 × 20 cm) prevented fish to view the stimulus until the test began and was removed after 10 min from the fish replica insertion allowing visual and physical contact.

15 subjects were tested and each of which interacted with the fish replica in the following contexts: (i) fish replica static; (ii) fish replica static with LEDs on; (iii) twisting fish replica; (iv) twisting fish replica with LEDs on. In the third and fourth context, the fish replica twisted of an angle of 25° and with a

frequency of 0,2 Hz. As indicated by preliminary observations, these are the most suitable values to avoid suspicion and aversion in this fish as well as they are pretty similar to the body movements that we observed during not physical aggressive displays (Simpson 1968), when a mutual assessment process occurs between two opponents in *B. splendens*. Recent studies provided evidence of the role of motor patterns on fish-robot interactions, based on that observed in live fish (Ruberto et al. 2016, 2017; Butail et al. 2014b; Ladu et al. 2015).

Nevertheless, agonistic interactions are flexible and unpredictable (Anderson et al. 2013; Benelli et al. 2015a; Brown et al. 2007; Vallortigara & Rogers 2005), thus it is difficult to evaluate or reproduce the trajectory of a fish during fights. In addition, since this was the first attempt in investigating biomimetic agonistic interactions in *B. splendens*, I preferred to move the fish replica in a 2-dimensional regular way to have a standardized moving stimulus that does not compromise the elicitation of aggressive interactions. However, the exact role of different motor patterns needs to be further studied in *B. splendens* agonistic interactions.

Each context was recorded when fish started to explore the fish replica and lasted 25 min. The sequence of contexts was randomized over the experiments. Each fish was involved in subsequent experiments at least after 7 days, in order to reduce any effect due to prior contexts experiences, indeed the effects of context outcome appear to disappear between 24 and 48 h in Siamese fighting fish (Dzieweczynski & Forrette 2013).

For each context I noted: 1. duration of initial exploration (when fish noticed the fish replica and started to swim toward it and around it without performing aggressive displays); 2. duration of fin spreading display towards the fish replica, defined as the full erection of all fins; 3. number of fin spreading events; 4. duration of gill flaring display towards the fish replica (the erection of gill covers, often accompanied by the dropping of the branchiostegal membranes), 5. number of gill flaring displays; 6. number of tail beats directed to the fish replica; 7. number of bites to the fish replica; 8. whole duration of the interaction, i.e. from the exploration until the end of the aggressive interaction. The behaviour of *B. splendens* was focally recorded.

As control, I performed two experiments: (a) fish interaction with a non-biomimetic object, and (b) fish interactions with other fish. In order to observe if the shape of the fish replica had a relevant role to evoke aggression, the same experimental procedure was adopted to test interactions between the fish and a cylindrical dummy (length 30 mm; radius 20 mm). I positioned two red LEDs in the centre of a cylindrical mold in ABS plastic and subsequently a transparent liquid silicone rubber melded with the same nontoxic blue pigment used for the fish replica, was injected in the mold, to obtain a cylindrical dummy with the same colour and material of the fish replica (Fig. 6D).

The cylindrical dummy was mounted on the robotic platform instead of the fish replica. All the fish interacted with the cylindrical dummy in the same four contexts described above.

Concerning fish vs. fish interaction (used as control treatment), a male was individually placed in the testing tank until he built a bubble nest. Afterwards an intruder male was inserted into the same tank, in the robot zone that was initially separated by two partitions (e.g. an opaque partition and a transparent one). After 10 min from the intruder insertion, only the opaque partition was removed, to avoid fish injuries, and the male–male interaction was observed for a period of 25 min. Overall, 9 treatments were performed: four contexts involving fish-fish replica interaction, four contexts involving fish-cylindrical dummy interaction, one context involving fish-fish interaction.

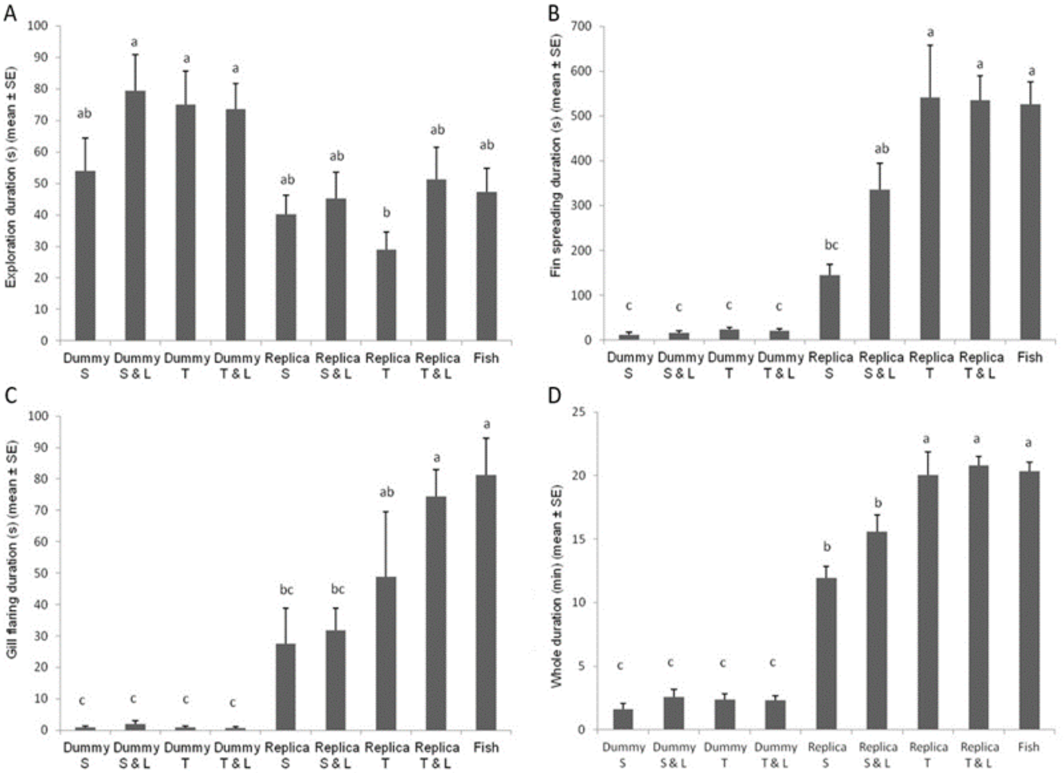
2.1.1.4 Statistical analysis

B. splendens fighting data (i.e. exploration duration, fin spreading duration, number of fin spreading acts, gill flaring duration, number of gill flaring displays, number of tail beats, number of bites, or fighting whole duration) were analyzed by JMP 10 (SAS) using a general linear mixed model (GLMM): I used a GLMM with a fixed factor (i.e. the tested cue/combination of cues), which also considered ID_w as the w-th random effect of the individual over repeated testing phases. Averages were separated by the Tukey's HSD test. A probability level of $P < 0.05$ was used to test significance of differences between means.

2.1.2 Results

Results showed that *B. splendens* males responded differently to various combinations of cues. Duration of exploration was significantly different in the twisting fish replica context ($F_{8,112} = 3.8501$; $P = 0.0005$). Siamese fighting fish explored marginally longer the cylindrical dummy for contexts in which it was static with LEDs on, twisting and twisting with LEDs on (Fig. 8A). Exploration lasted slightly shorter in other treatments except for the twisting fish replica context that was explored significantly shorter. Fin spreading duration was significantly affected by tested combination of cues ($F_{8,112} = 26.2142$; $P < 0.0001$). Duration of fin spreading display occurring in fish replica twisting and twisting with LEDs on contexts was comparable with the duration of fin spreading occurring during fish vs. fish context and it was longer to respect contexts in which the fish replica was static and static with LEDs on (Fig. 8B). Spreading fin duration was significantly shorter when fish were exposed to any contexts involving the cylindrical dummy.

Gill flaring duration was significantly affected by tested combination of cues ($F_{8,112} = 11.4135$; $P < 0.0001$). Gill flaring behaviour lasted significantly longer when a fish interacted with another fish and with the fish replica which exhibited the overlap of twisting and LEDs on stimuli to respect contexts in which the fish replica just was static, static with LEDs on or twisting (Fig. 8C). The duration of gill flaring display was almost nothing when we presented the four contexts replacing the fish replica with the cylindrical dummy.

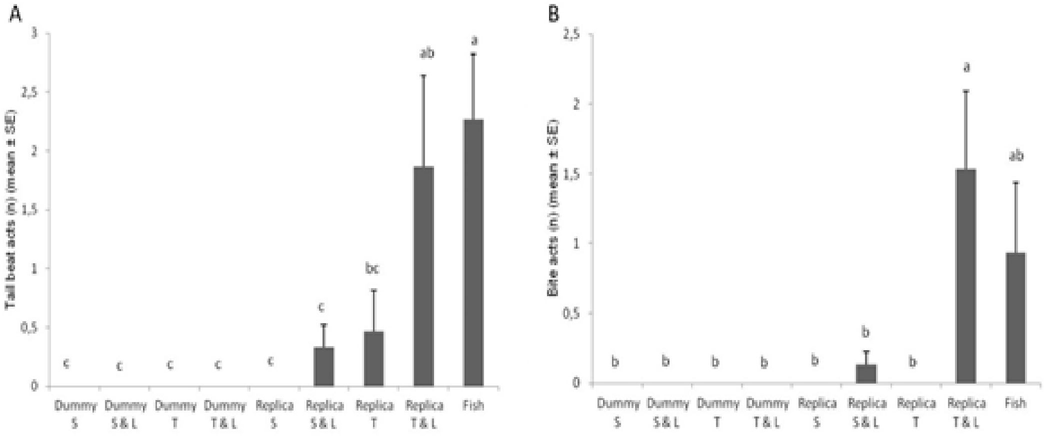


Dummy S = cylindrical dummy static. Dummy S & L = cylindrical dummy static with LEDs on. Dummy T = dummy twisting. Dummy T & L = cylindrical dummy twisting with LEDs on. Replica S = fish replica static. Replica S & L = fish replica static with LEDs on. Replica T = fish replica twisting. Replica T & L = fish replica twisting with LEDs on. Fish = fish vs. fish.

Figure 8. Duration of *Betta splendens* (A) exploration, (B) fin spreading, (C) gill flaring, as well as (D) the whole aggressive interactions, post-exposure to different robot-borne combinations of fighting cues. Different letters above each bar indicated significant differences. T-bars are standard errors.

The number of tail beat displays was significantly affected by tested combination of cues ($F_{8,112} = 7.1096$; $P < 0.0001$). Tail beat number was significantly higher when testing the fish replica twisting with LEDs on, while no significant differences were found in fish vs. fish context (Fig. 9A). When fish interacted with the twisting fish replica, they exhibited a lower number of tail beats than when fish replica presented together the twisting movement with

LEDs on. The number of tail beats displayed by fish towards the fish replica was lower when the replica was static or static with LEDs on, as well as towards the cylindrical dummy when it was static, static with LEDs on, twisting and twisting with LEDs on. The number of biting acts was significantly affected by the tested combination of cues ($F_{8,112} = 4.7611$; $P < 0.0001$). The number of bites towards the fish replica twisting with LEDs on was significantly higher and slightly exceed the number of bites towards real fish during fish vs. fish context (Fig. 9B). Among the rest of treatments, no significant differences, regarding the number of beats, were found.



Dummy S = cylindrical dummy static. Dummy S & L = cylindrical dummy static with LEDs on. Dummy T = dummy twisting. Dummy T & L = cylindrical dummy twisting with LEDs on. Replica S = fish replica static. Replica S & L = fish replica static with LEDs on. Replica T = fish replica twisting. Replica T & L = fish replica twisting with LEDs on. Fish = fish vs. fish.

Figure 9. Number of *Betta splendens* (A) tail beat acts, and (B) bites, post-exposure to different robot-borne combinations of fighting cues. Different letters above each bar indicated significant differences. T-bars are standard errors.

The whole aggression duration was significantly affected by the tested combination of cues ($F_{8,112} = 89.3610$; $P < 0.0001$). Duration was significantly longer when testing fish replica twisting, fish replica twisting with LEDs on and fish vs. fish. In contexts where the fish replica was static and static with LEDs on, the whole duration of the aggressive interaction was significantly lower. The whole duration of aggressive interactions was significantly lower in all the contexts where the cylindrical dummy was involved (Fig. 8D).

2.1.3 Discussion

I teased Siamese fighting fish males, triggering and modifying their aggressive behaviour, through the robotic approach, that is an elegant and innovative method to investigate animal behaviour. As predicted, significant differences were obtained testing different combination of cues, highlighting the communication functions that visual cues, sent by the fish replica, and how much their changes/overlapping affect specific action patterns.

My observations revealed that the *B. splendens* male, before starting an agonistic encounter, explored the intruder agent (e.g., fish replica, cylindrical dummy, fish) for a variable period without displaying aggressive behaviours. The duration of this period depended marginally to the tested cues. Particularly, the cylindrical dummy was inspected slightly longer over other treatments, but basically no main differences were found, except for the twisting fish replica. It may suggest that any foreign object in the fish territory is worth to be overseen and in the case of the twisting fish replica context, fish felt more threatened thus they preferred to exhibit aggressive displays in advance. This is also supported by the whole duration of the aggressive behaviour sequence, where Siamese fighting fish males performed agonistic interactions, significantly longer towards the fish replica.

Fin spreading and gill flaring duration was longer in contexts in which the fish replica was used to respect the cylindrical dummy. Interestingly, a *crescendo* in duration of both displays was observed as the number of signals simultaneously emitted by the fish replica increased in the different contexts, achieving the same duration observed in the real fish context. The number of fin spreading events observed in each fish replica context was comparable with those in fish-fish encounters showing as the spread of fins was easily evoked by the biomimetic shape of the fish replica. Conversely, the number of gill flaring displays was affected by the fish replica different signalling. In addition, the effect of LEDs as surrogate of the gill flaring display was more appreciable in conjunction with the twist of the fish replica. My data revealed that probably, the gill flaring display represents a more selective behavioural reaction over the fin spreading behaviour, probably because the ability of fish to extract oxygen from water, by ventilating their gills, is drastically limited during gill flaring (Abrahams et al. 2005).

Therefore, I hypothesize that gill flaring display needs a higher level of sensory information for being evoked and for increasing its performance. This showed that multiple signal systems represent an important evolved communication strategy to unlock energy costly intraspecific behavioural patterns.

However, success in evoking and modulating both these displays did not guarantee the achievement of my aim in triggering aggressive behaviour. Indeed, *B. splendens* males perform these behaviours both in agonistic encounters, to threaten other opponent males, and in courtship interactions, to persuade females (Simpson 1968; Robertson & Sale 1975).

Evidences of my aim achievement can be confirmed relying on triggering aggressive physical acts that *B. splendens* displayed towards the fish replica. In fact, aggressive behaviours are supported only if benefits of territoriality exceed costs, therefore, physical combats are generally reserved to rare events in nature, in order to reduce the loss of energy, time and to avoid the risk of injuries (Hsu et al. 2006; Benelli et al. 2015a).

Physical acts occurred when the fish replica delivered at least two stimuli simultaneously (e.g., biomimetic shape in conjunction with LEDs on) and not when the fish replica was static like a standard dummy. Interestingly, although the cylindrical dummy was able to turn on LEDs, twist and twist in conjunction with LEDs on, as the fish replica did, no physical aggressive displays were directed to it. My results showed a significant lower response regarding both not physical and physical aggressive displays towards any context in which the cylindrical dummy was used. This confirms the key role played by the biomimetic shape in the acceptance of the fish replica as an opponent. Nevertheless, also the effect of realistic eyes, missing in my fish replica, is worth studying in further investigations concerning agonistic interaction, since realistic eyes led to a significant improvement of the acceptance level in a fish replica during social interactions with *P. reticulata* (Landgraf et al. 2016). In addition, the luminescence of LEDs as surrogate of the gill flaring behaviour and the movement of the fish replica also had a significant role, in conjunction with the shape, to modulate and increase the level of aggression in Siamese fighting fish. As further confirmation, the number of tail beats grew as the fish replica displayed more stimuli concurrently, approximating the number of acts of this behaviour displayed towards a real opponent.

Remarkable were responses in terms of number of bites. Biting acts represent the culmination of the aggressive display in *B. splendens*males (Simpson 1968). Accordingly, it would be triggered by a cogent and complex communicative system. In my case, a significant number of bites was obtained when the fish replica fully displayed its stimuli concurrently (e.g., shape, LEDs on, twisting) over other fish replica and cylindrical dummy contexts, and were comparable to the number of bites displayed towards real fish. Although experimental conditions were slightly different in fish-fish context (e.g., a transparent partition was placed between real fish opponents to avoid injuries due to physical contacts), it can be assumed that these results are close to my predictions.

In recent years, several studies were performed in order to investigate animal social interactions trough the use of robotic replicas and many of these studies involved fish. Surprisingly, few researches using robots to investigate aggressive interactions were carried out, although aggressive behaviour represent a crucial factor in optimizing the fitness of a species.

To the best of my knowledge, the present study represents the first attempt to use a robotic replica to investigate intraspecific aggressive behaviour in fish and provides basic knowledge for the use of light signals mimicking specific behavioural displays during interaction with animals.

Overall, I showed that my fish replica elicited aggressive behaviour in *B. splendens*, that escalated as the cues overlapping (e.g., shape, light, twisting), increased. The combination of stimuli emitted by the fish replica allowed me to confirm my second hypothesis, where I predicted the possibility to modulate the escalating level of aggression in *B. splendens* males. From an ecological point of view, my results add basic knowledge to understand key aspects of territorial aggression in Siamese fighting fish, and may also help to develop novel reliable methods, based on a biomimetic approach, to investigate aggressive displays in aquatic animals.

2.2 CASE STUDY 2: MULTIPLE FEMALE-MIMICKING ROBOTIC STIMULI EVOKE COURTHSIP BEHAVIOUR IN MALES OF A HIGHLY TERRITORIAL FISH

In this study, I developed a robotic apparatus moving biomimetic fish replicas inspired by female *B. splendens* to investigate visual stimuli that are important for Siamese fighting fish males to discriminate females from other males (Romano et al. 2019b). In this species the risk of injuries to females as well as to males is significant (Clotfelter et al., 2006). I focused on the colour pattern exhibited by reproductive *B. splendens* females consisting in horizontal darker stripes along their bodies with lighter stripes in between (Rainwater, 1967). Our fish replica was endowed with 3 bright stripes per side of its body (e.g. 2 dorsal, 2 median, and 2 ventral), each of them obtained by using light emitting diodes (LEDs), in order to have 2 darker longitudinal areas along the body (i.e. between the dorsal and the median bright stripes and between the median and the ventral bright stripes), and mimicking the colour pattern of a receptive female.

In this scenario, I tested two hypotheses: (*a*) The cues delivered by fish replicas were of different attractiveness to males, e.g. male and female conspecifics, fish replica with activated LEDs, painted and neutral fish replica ; and (*b*) Light stimuli reproducing the colour pattern of reproductive females increase the consistence of courtship displays in *B. splendens* males.

To address these issues, Siamese fighting fish males were exposed to a reproductive female-mimicking fish replica, exhibiting a luminescent colour pattern as well as to a reproductive female-mimicking fish replica with a painted colour pattern. In addition, I evaluated the degree of biomimicry of our artefacts by comparing B. splendens male responses to the fish replicas with those obtained in the interactions of authentic male-male and male-female interactions.

2.2.1 Methods

2.2.1.1 Animal rearing and general observations

Siamese fighting fish were maintained as described earlier in the "case study 1", also available in Romano et al. (2017a). Observations were carried out from January to June 2017 in laboratory conditions (25±1 °C), with a 16:8 (L:D) photoperiod. The test tanks sidewalls were screened by using white filter paper (42 ashless, Whatman Limited, United Kingdom) to avoid external cues. Before starting an experimental replication, the test tank was carefully washed as described earlier in the "case study 1".

2.2.1.2 Animal replica and experimental apparatus

The process used to fabricate the fish replicas as well as the external apparatus actuating them is similar to that used in the "case study 1", with some modifications concerning the fish replicas. Fish replicas reproduce a Siamese fighting fish females, having less gaudy colours and shorter fins than males (Rainwater, 1967; Clotfelter et al., 2006), (Fig. 10A, B).

A liquid silicone rubber (Dragon Skin F/X PRO), mixed with a non-toxic pigment similar to the colour of a *B. splendens* female, more faintly coloured than in males (Rainwater, 1967), was injected in the mould, in order to cast the fish replica. In this species, the colouration of the body varies considerably among individuals (Romano et al. 2017a), so the colour of the fish replicas did not reproduce accurately the colour of real fish. Fish replicas were 70 mm long, with a height of 35 mm, and 13 mm wide. According to previous findings demonstrating an increased level of acceptance by real fish of robotic fish that included realistically coloured eyes (Ruberto et al., 2017), I endowed our fish replicas with two nickel-plated birdshots (diameter 2.5 mm), since they are visually similar to *B. splendens* eyes.

The colour pattern exhibited by reproductive *B. splendens* females (2 horizontal darker stripes along their bodies with lighter stripes in between), was reproduced in the fish replica with a luminescent colour pattern by locating 6 bright stripes (e.g., 2 dorsal, 2 median, 2 ventral ones), in the mold, prior to inject the silicone rubber, in order to have 3 bright stripes along each side of the fish replica body (Fig. 10C). The fish replica with LEDs off (neutral fish replica) and with activated LEDs is shown in Fig. 10D, E respectively. This, as mentioned earlier, allows me to have a darker longitudinal area between the dorsal and the median bright stripes and another darker longitudinal area between the median and the ventral bright stripes on the body of the fish replica. Each dorsal and ventral bright strip consisted of 6 LEDs connected in series. Each median bright strip consisted of 9 LEDs connected in series. The 6 LEDs stripes were connected in parallel.

In the fish replica with a painted colour pattern, two horizontal darker stripes were painted with a non-toxic pigment (Fig. 10F).

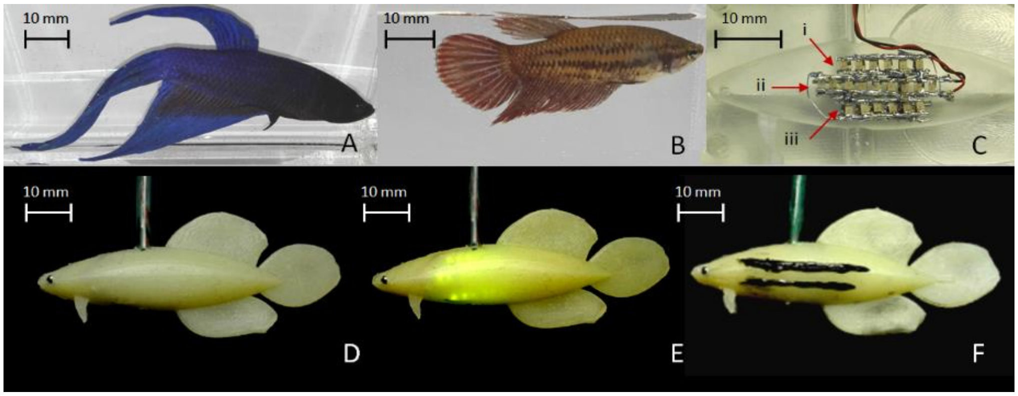


Figure 10. Different stimuli presented to males of Siamese fighting fishes during the experiments. (A) *Betta splendens* male; (B) reproductive *B. splendens* female exhibiting horizontal darker stripes along her body with lighter stripes between; (C) dorsal (*i*), median (*ii*) and ventral (*iii*) bright stripes located in the mould, before casting the fish replica; (D) neutral fish replica; (E) fish replica with activated LEDs; (F) painted fish replica.

Colour measurements of the fish replicas are shown in Table 2 , and were obtained by using a spectrometer Ocean Optic HR2000-UV–VIS-NIR (Ocean Optics, USA). During tests, fish replicas were individually positioned at a depth of 30 mm, in the middle of the robot zone (b) (see next section) of the test tank (Romano et al., 2017). An external microcontroller (Arduino Mega 2560) was used to activate both the servo and the LEDs.

Tested cue	L*	a*	b*
Fish replica body	47.2 ±1.52	-15.0 ± 2.25	0.6 ± 1.51
Painted stripe	2.11±0.09	-0.68±0.32	-0.71±0.1
LEDs on	48.6 ± 3.08	-37.1 ± 1.99	19.1 ± 3.69

Table 2. Colour measurements of the fish replica body, painted stripes and incorporated LEDs ± standard error. L* represent the lightness component, a* (from red to green) and b* (from blue to yellow) are the two chromatic components.

2.2.1.3 Interactive biohybrid experiments

The tank used as experimental arena (500 × 300 × 200 mm), consisted of three virtual zones: nest, middle and robot (a), (b) zone (Fig. 11).

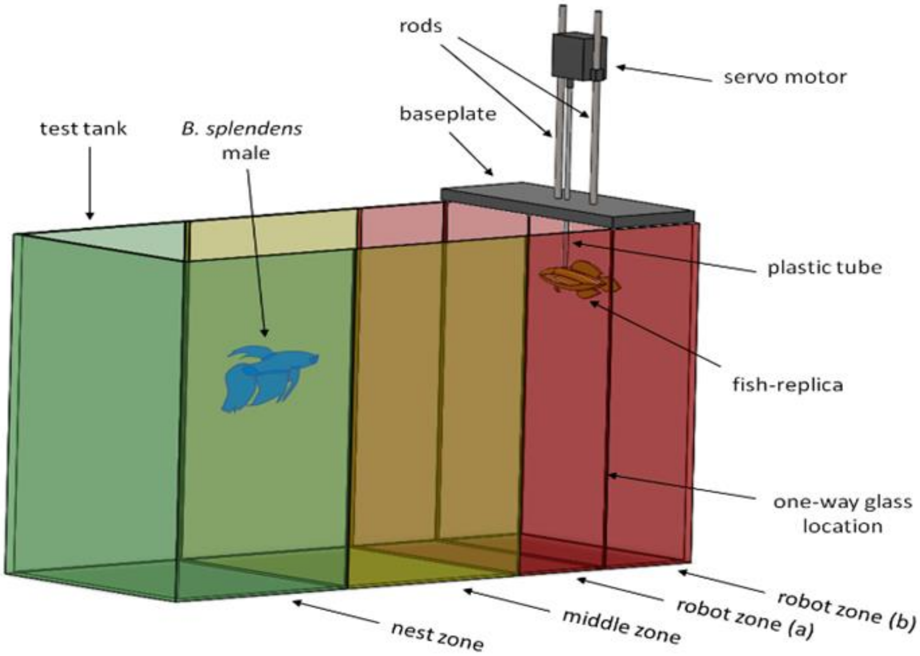


Figure 11. Experimental setup. Different colours of the test tank indicate its virtual division in nest zone (green), middle zone (yellow), and robot zone (red).

A square of bubble wrap was located in the nest zone of the tank, since it speeds up the bubble nest building in *B. splendens* and allows me to control the nest position (Dzieweczynski et al., 2006). Prior to perform a test, Siamese fighting fish males were individually placed in the test tank until they build a bubble nest (i.e., usually within 24-48 h), (Dzieweczynski et al., 2006).

The stimulus (e.g., fish replicas or real fish), was placed in the centre of the robot zone (b) of the tank, that was isolated from the other zones by a one-way glass. The one-way glass isolates the stimulus fish so that cannot see the focal fish, to prevent visual feedback between the conspecifics (Ruberto et al., 2017) and thus ensuring standard conditions during experiments with living or artificial stimuli.

An opaque partition (30 × 20 cm) prevented the fish from viewing the stimulus until the test began and was removed to allow visual contact with the stimulus after 10 min from its insertion.

Stimuli presented to the tested subjects included: (*i*) neutral fish replica (e.g. without stripes), see Fig. 10D; (*ii*) fish replica with activated LEDs (e.g. luminescent stripes), see Fig. 10E; (*iii*) painted fish replica (e.g. painted stripes), see Fig. 10F; (*iv*) female (as control), see Fig. 10B; (v) male (as control), see Fig. 10A.

In each fish replica context, the dummy displayed its body axis orthogonal to the central longitudinal line of the tank, to exhibit the lateral colour pattern of its body. In addition, the fish replica was twisted in an angle of 30° and with a frequency of 0.5 Hz to emulate the decreased locomotor activity of real *B. splendens* behaving individually in a tank (e.g. a fish stimulus used as control, having no visual contact with the conspecific), as well as a female starting mate evaluation or eavesdropping (Doutrelant et al., 2001; Clotfelter et al., 2006).

The fish replica autonomously yawed 180° every 5 min to invert the head-tail orientation, to avoid positional bias. 15 sexually mature *B. splendens* males were analysed and each of them was exposed to the stimuli listed above.

Observations lasted 30 minutes and started when fish noted the proposed stimulus. The sequence of stimuli was randomized over the experiments. To limit prior context experience effects (Hsu et al., 2006), each fish was involved in subsequent experiments that were separate for at least 7 days (Arnott et al., 2016), since the effects of context outcomes are drastically reduced between 24 and 48 h in Siamese fighting fish (Dzieweczynski & Forrette, 2013).

For each stimulus, I noted behaviours identically displayed in both agonistic and courtship interactions, including: (*i*) the fin spreading behaviour duration towards the stimulus, corresponding to all fins outspreaded (Simpson, 1968); (*ii*) the gill flaring duration towards the stimulus, consisting in the erection of gill covers (Simpson, 1968); (*iii*) time-to-the-stimulus defined as the duration of *B. splendens* males swimming inside the robot zone (a).

Behaviours used only in male-female interactions, such as: (*iv*) number of zigzag displays (the male move away from the female in a zigzag way to magnify its colouration and body size, as described by Simpson, 1968); (*v*) time spent by males stopping upwards the nest (e.g. still males with head-caudal orientation towards the nest), and undulating their bodies in order to show the nest to females, as described by Simpson (1968); (vi) bubbling acts (intermittently work on the nest by adding bubbles to encourage the female to come closer), (Rainwater, 1967), were recorded as well.

The behavioural parameters characterizing *B. splendens* responses were focally recorded.

2.2.1.4 Statistical analysis

Courtship data concerning identical displays performed in both agonistic and courtship interactions (i.e., fin spreading duration, gill flaring duration, time to the stimulus) as well as courtship data related to specific male-female interactions (i.e., number of zigzag displays, upwards the nest duration, bubbling acts) were analysed by JMP 9 (SAS). I used a general linear mixed model (GLMM) with a fixed factor (i.e. the tested cue/combination of cues), which also considered ID_w as the w-th random effect of individual over repeated testing phases. Averages were separated by Tukey's HSD test. A probability level of $P < 0.05$ was used to test significance of differences between means.

2.2.2 Results

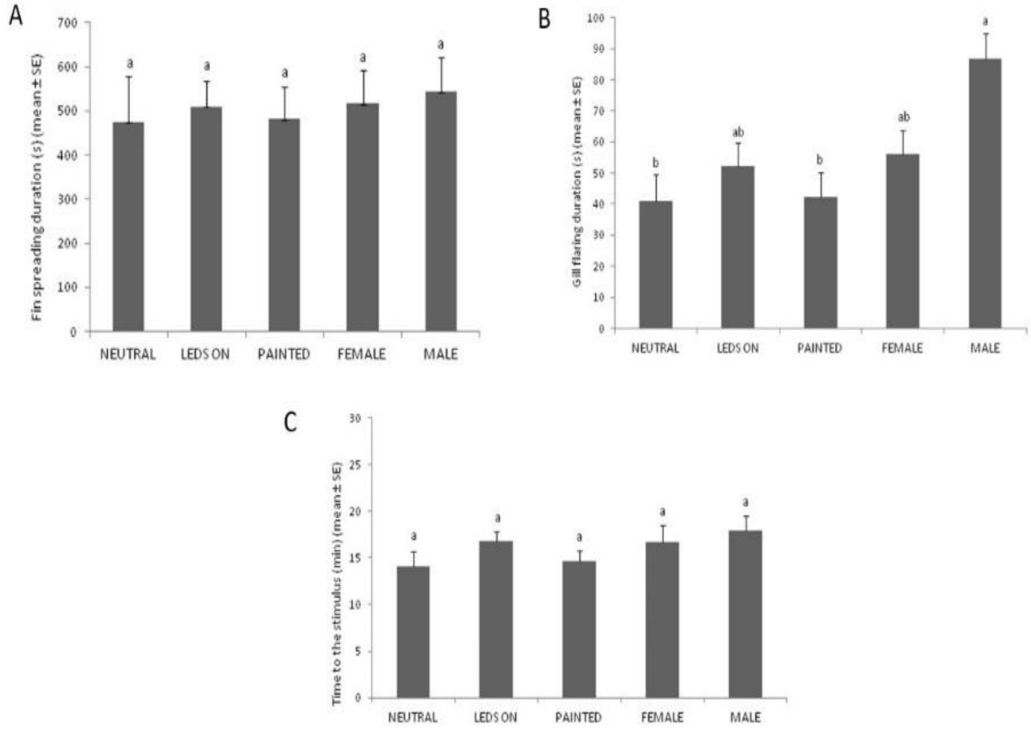
Visual cues produced by different agents (e.g. living agents and artificial agents), marginally modulated male displays used both in agonistic and courtship interactions.

Fin spreading duration was not affected by different stimuli ($F_{4,56} = 0.1309$; $P = 0.9705$). The duration of the fin spreading behaviour was not significantly different in male-male and male-female contexts as well as in contexts involving the neutral fish replica, the fish replica with activated LEDs and the painted fish replica (Fig. 12A).

Duration of gill flaring display was marginally influenced by different stimuli ($F_{4,56} = 4.5939$; $P = 0.0028$). Gill flaring was performed slightly longer towards conspecific males, compared to conspecific females and to the fish replica with activated LEDs. Gill flaring duration was shorter in contexts involving the neutral fish replica and the painted fish replica (Fig. 12B).

Time spent by males in the robot zone (a) was not significantly affected by the different stimuli that were proposed ($F_{4,56} = 1.4324$; $P = 0.2353$), as in Fig. 12C.

Visual cues produced by different agents (e.g. living agents and artificial agents), significantly affected specific courtship displays performed by *B. splendens* males.



NEUTRAL = neutral fish replica (without stripes). LEDs ON = fish replica with activated LEDs (luminescent stripes). PAINTED = painted fish replica (painted stripes). FEMALE = female conspecific. MALE = male conspecific.

Figure 12. Duration of *Betta splendens* (A) fin spreading, (B) gill flaring, and (C) swimming in the robot zone (a), evoked by different agents. Same letters above each column indicated not significant differences (P>0.05). T-bars are standard errors.

The number of zigzag displays was significantly affected by different agents ($F_{4,56}$ = 46.9644; P = 0.0001). The number of zigzag displays performed by males during male-female interactions were comparable with those performed in contexts involving the fish replica with activated LEDs (Fig. 13A). A significantly lower number of zigzag displays were performed in contexts involving the painted fish replica. The number of zigzag displays was significantly different in context involving the neutral fish replica and in male-male contexts.

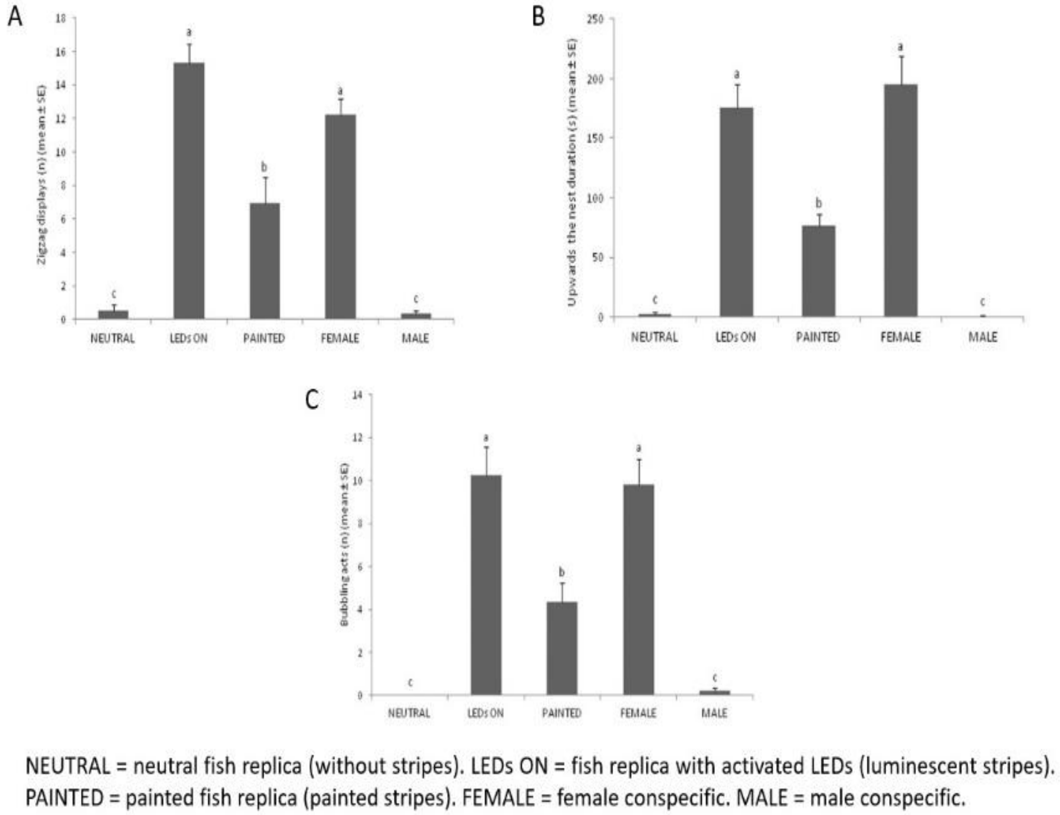


Figure 13. Number of *Betta splendens* (A) zigzag displays, (B) duration upwards the nest, and (C) number of bubbling acts evoked by different agents. Different letters above each column indicated significant differences (P<0.05). T-bars are standard errors.

Time spent by males upwards the nest was significantly influenced by the different stimuli proposed ($F_{4,56}$ = 39.4586; P = 0.0001). Males spent a longer period upwards the nest in contexts including a female conspecific as well as the fish replica with activated LEDs compared to a context involving the painted fish replica. In addition, the neutral fish replica and male conspecifics produced shorter periods spent upwards the nest (Fig. 13B).

The number of bubbling acts was significantly affected by different agents ($F_{4,56} = 27.2202$; $P = 0.0001$). *B. splendens* males displayed a comparable number of bubbling acts during interactions with a female conspecific and with the fish replica with activated LEDs (Fig. 13C). The painted fish replica evoked a significantly lower number of bubbling acts in Siamese fighting fish males, compared to other agents. A non-significant response, concerning the number of bubbling acts, has been recorded in male-male interactions as well as in interactions involving the neutral fish replica.

2.2.3 Discussion

The robotic system presented in this study provided an important contribution in unveiling the decision-making process of *B. splendens* males during sexual recognition and courtship behaviour.

An early study by Phamduy et al. (2014) investigated female mating preferences of bluefin killifish for differently coloured male-mimicking robotic fish.

However, in highly aggressive species, how male courtship displays are elicited by receptive female signals, is a key aspect poorly explored that can greatly profit from using robots, performing highly reliable and standardized behaviours.

In Siamese fighting fish, mating success is ensured by the rapid recognition of the mate, that is mainly affected by specific visual cues delivered by the two mating fish, and this avoids the risk of injuries as well (Simpson, 1968; Rainwater, 1967; Clotfelter et al., 2006).

The results presented here showed no significant differences in the fin spreading duration towards males, females and female-mimicking agents. Generally, larger males are socially dominant and they build larger nests (Jaroensutasinee & Jaroensutasinee, 2001). Thus, fin spreading could be a strategy used by males to appear larger to threaten conspecific males, and in the same time to attract conspecific females during courtship (Simpson, 1968; Robertson & Sale, 1975).

The gill flaring display was marginally longer in male-male compared to the case of male-female and male-fish replicas with activated LED interactions, and significantly longer compared to the interactions involving the painted fish replica and the neutral fish replica (Fig. 12B). During this display, oxygen extraction from water, is drastically limited in fish.

However, Siamese fighting fish have evolved a particular organ (e.g. the labyrinth organ), which acts functionally like a lung (Tate et al., 2017). This enables *B. splendens* and the other anabantoids (a group of air-breathing fishes living in Africa and south Asia), to persist in extremely hypoxic situations where gill breathing would be ineffective anyways. The gill flaring longer persistence in male-male interactions suggest that gill flaring behaviour is cost efficient as not-physical aggressive display in defending the nest from other intruders. Indeed, the gill flaring behaviour as a courtship display seems to be not correlated with male parental quality but with tolerance to hypoxia, and the relevance of this to female reproductive success is unknown (Abrahams et al., 2005; Clotfelter et al., 2006). However, the similarity of the intensity of male behaviours used both in courtship and agonistic interactions can be explained by *B. splendens* female selection of males that are aggressive and large enough to protect the offspring.

The intensity of courtship-specific behaviours (e.g., zigzag displays, time spent by males upwards the nest, bubbling acts), significantly increased in the following stimuli sequence: conspecific males, neutral fish replica, painted fish replica, fish replica with activated LEDs and conspecific females. The extreme difference of male courtship-specific responses displayed to female conspecifics compared to other male conspecifics allows us to use robotic stimuli to map which cues are crucially displayed by females to be considered potential by sexual mates.

The painted fish replica always evoked courtship-specific behaviours, indicating the pivotal role of the longitudinal stripes in triggering these highly selective responses.

Noteworthy, the novel animal-robot interaction paradigm I introduced, based on a light emitting communication strategy, (e.g. the fish replica with activated LEDs), enabled me to evoke a significantly higher intensity of these responses compared to the painted fish replica, and triggered similar reactions in males to those evoked by conspecific females, revealing its closer biomimicry with a *B. splendens* female.

Concerning the role of light stimuli in the courtship responses produced in *B. splendens* males, although the fish replica with activated LEDs has less marked dark stripes compared to the painted fish replica, the former exhibits a brighter appearance that can be perceived by males as an indication of a healthy female (Vershinin, 1999). For instance, carotenoid pigments, procured by fish through foraging, accomplish several physiological roles (Vershinin, 1999; Clotfelter et al., 2007). In addition, carotenoids have an important role in animal communication, in the context of carotenoid-based signals (Svensson & Wong, 2011). Thus, fish that have high levels of carotenoids in their diet display a brighter colouration, and are perceived as high quality subjects (Clotfelter et al., 2007; Svensson & Wong, 2011).

Overall, my study reports for the first time which cues, produced by an artificial agent, are important in eliciting courtship behaviours in a high territorial species such as *B. splendens* during sexual recognition. In addition, I demonstrated that light stimuli, mimicking the colour pattern of reproductive females, boost the consistence of courtship displays in *B. splendens* males, probably because it indicates a female that is a good forager, parasite free, and producing a high number of quality-eggs.

Our robotic approach to establish bio-hybrid individual interaction can represent an advanced tool for trait-based ecology, that is a rapidly developing context of ecology merging evolutionary with traditional population and community ecology (Kiørboe et al., 2018).

2.3 CASE STUDY 3: ANALYSING AND CONTROLLING SCHOOLING BEHAVIOUR IN NAÏVE NEWBORN GUPPIES THROUGH BIOROBOTIC PREDATORS

A major advantage of aggregations in animals concerns cooperative antipredator strategies allowing animals to be safer in groups rather than isolate individuals (Parrish et al. 1989). Interestingly, schooling behaviour emerges earlier in many species, especially in fish not providing parental care but rather cannibalizing their offspring (Brown 1984). In several fish species, it has been reported that schooling behaviour gradually increases during postembryonic development (Shaw 1961). Therefore, learning mechanisms relying on previous experience have been proved to play a fundamental role in the development of antipredator strategies, including shoals. Furthermore, there are evidences describing fish aggregation as a flexible self-organized behaviour, where the coalescence of the group is increased by the presence of a predator, and decreased when limited resources trigger competition (Hoare et al. 2004). Environmental architecture (e.g., natural shelters, vegetation, background colour), also influences the coalescence of the aggregation (Sinopoli et al. 2015).

However, studying the capability of newborn schooling fish species to aggregate along with identifying which predator cues activate this collective behaviour in naive individuals represent a context of investigation which remains unexplored.

In this framework, the role of filial-preying adults as well as the colour background of the environment on the innate schooling formation and collective spatial distribution in newborn fish was explored. Herein, the Trinidadian guppy, *Poecilia reticulata* Peters (Cyprinodontiformes:

Poeciliidae), was selected as biological model, since this schooling species does not provide parental cares and its predatory action on conspecific juveniles has been documented in detail (Magurran & Seghers 1990).

I developed a robotized system moving different biomimetic agents (e.g., female and male adult guppies and a *Lithobates catesbeianus* Shaw-mimicking tadpole with developed legs) in an arena presenting a chessboard drawing pattern, with white and brown areas. The effect of these robotic stimuli and the impact of the colour background triggering group behaviours in newborn *P. reticulata* was investigated. Furthermore, olfactory stimuli in guppy adults (Chapman et al. 2007), and in other species (Tollrian et al. 1999), have been reported as a sufficient condition to produce morphological and behavioural antipredator responses in juvenile fish. On this basis, experiments involving robotic cues in combination with adult guppy-borne olfactory cues were carried out to study their multimodal influence on the coalescence of the fish group.

2.3.1 Methods

2.3.1.1 Animal rearing and general observations

Trinidadian guppy adults were provided by an aquarium centre in Abu Dhabi, UAE, and housed in laboratory conditions (24 ± 1° C, 12:12 (L:D) photoperiod by using overhead fluorescent tubes, Philips 30 W/33), in tanks ensuring about 1.2 L/1 fish, and with a 1:1 sex ratio. Flake food (TetraMin) was employed for *ad libitum* daily feeding. Gravid females were isolated in nursery tanks (200 x 100 x 150 mm), with the same environmental and feeding conditions described earlier. *P. reticulata* is a livebearer species, so once *P. reticulata* were born, they were immediately transferred in another tank to limit early experience with adult females, and they were fed by using micro flakes (TetraMin baby). Newborn fish were tested within 12 h from birth, in groups of 7 individuals, in dedicated tanks (described in the next section), surrounded by a white filter paper to reduce cues perturbing fish behaviour.

2.3.1.2 Animal replica and experimental apparatus

A female-mimicking robotic guppy, a male-mimicking robotic guppy, and an heterospecific-mimicking robotic replica (e.g., a tadpole mimicking young instars of the species *L. catesbeianus*), were casted in a liquid silicone rubber by using 3D printed moulds (Fig. 14). Each robotic agent reproduced morphological and size features similar to its mimicked animal model and was endowed with two metallic spheres (diameter 2.5 mm), as realistic eyes.

P. reticulata is a sexual dimorphic species, in addition, this fish present a striking intrasexual polymorphism in colour patterns, which make it difficult to identify a model livery. However, I coloured the female-mimicking robotic guppy with a yellowish-brown pigment (colour code: PANTONE PQ-7753C), to visually resemble the observed colour in many of them. The male-mimicking robotic guppy was coloured similar to the female, yet the only difference lies in terms of its tail which was larger in size and of an orange colour (code: PANTONE PQ-3556C). Indeed, the orange pigment in male *P. reticulata* plays an important role in social interactions in this species, particularly in the female choice mechanisms (Houde & Endler 1990). The robotic tadpole had a dark green colour (code: PANTONE PQ-3537C).

All robotic agents were positioned 30 mm below the water surface. They were hinged to a plastic cylinder (acrylonitrile butadiene styrene, ABS), by a transparent rod. This cylinder held a magnet placed on the floor of the arena. Below the arena, a servomotor (Robbe FS 100 Servo) was connected with a circular plate holding another magnet. The servomotor, activated by a microcontroller, rotated the plate allowing the magnet coupling with the robotic agent in the arena.

The experimental arena (300 x 200 x 150 mm), presented 3 virtual compartments: (*i*) the agent compartment, where the robotic agents were positioned; (*ii*) the exploration compartment, in the middle of the arena; (*iii*) the escaping compartment, that was the furthest area from the agent compartment. Each of these compartments had one half of the floor white coloured (code: PQ-11-0601TCX) and one half brown coloured (code: PQ-8580C). This was achieved by placing white paper and brown cardboard squares (100 x 100 mm), below the arena floor. The experimental setup is depicted in Fig. 14. These white and brown squares had a chessboard pattern along the different compartments. After each replicate, the experimental arena was rotated clockwise 90°, as well as the position of the white and the brown squares was alternated to avoid directional bias.

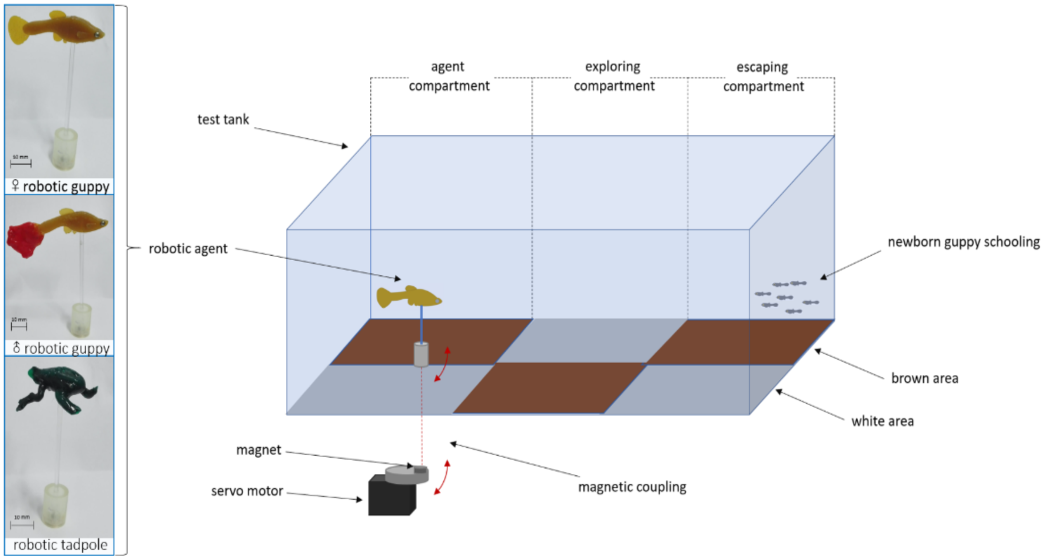


Figure 14. Schematic representation of the experimental setup and the robotic agents used to study schooling in newborn guppies, *Poecilia reticulata*.

2.3.1.3 Interactive biohybrid experiments

Newborn guppies were gently introduced in groups of 7 individuals in the exploration compartment of the arena. After 10 min, a robotic agent, connected with the cylinder holding the magnet, was dropped in the middle of the agent compartment that was immediately caught and moved by magnetic coupling with the servomotor below the arena. The robotic agents moved on a semi-circumference (diameter 50 mm), with a frequency of 0.166 Hz.

Fish were exposed to different treatments including: (*i*) no agent, (*ii*) no agent plus adult guppy olfactory cues, (*iii*) female-mimicking robotic fish, (*iv*) female-mimicking robotic fish plus adult guppy olfactory cues, (*v*) male-mimicking robotic fish, (vi) male-mimicking robotic fish plus adult guppy olfactory cues, (*vii*) robotic tadpole, and (*viii*) robotic tadpole plus adult guppy olfactory cues.

During the treatments which involve olfactory stimuli, the water obtained from an aquarium containing an equal number of adult females and males *P. reticulata* was used. Each observation lasted 15 min.

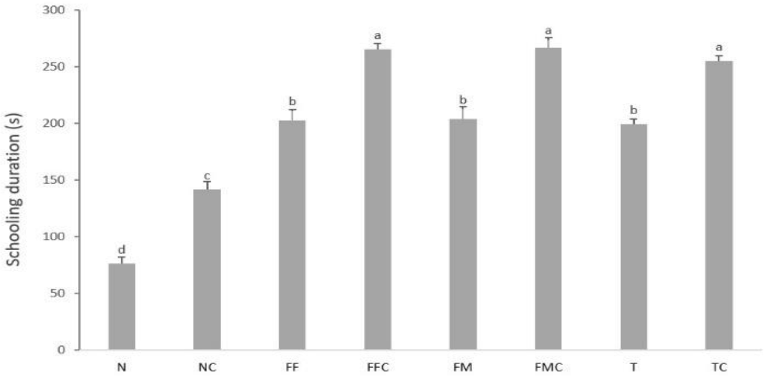
For each treatment, I measured the schooling behaviour duration exhibited by newborn fish. Schooling behaviour is defined as the tendency of fish to aggregate at a distance of at least 5 body lengths from each other and the ability to move collectively (Magurran & Seghers 1994). However, in my experiments, and according also to the observations led by Magurran & Seghers (1994), fish schooled generally closer in the afore-mentioned distance. In addition, the time spent by newborn fish schooling in each compartment as well as in different coloured backgrounds was recorded.

2.3.1.4 Statistical analysis

Differences in the mean duration of schooling behaviour in response to different proposed stimuli (robotic and chemical cues), as well as the mean duration of schooling behaviour in each arena compartment post-exposure to the selected cues, were analyzed by JMP 9 (SAS) using one-way ANOVA where the factor was the treatment (from *i* to *viii*). Means were separated by Tukey's HSD test. A probability level of $P < 0.05$ was used to assess the significance of differences among means.

2.3.2 Results

The schooling time spent by newborn guppies was significantly affected by the tested combination of cues ($F_{7,79} = 53.912$; $P < 0.0001$). Newborn fish spent more time schooling when robotic stimuli were combined with adult guppy olfactory cues. When no robotic agents were tested, schooling duration was significantly longer if adult guppy olfactory cues were presented (Fig. 15).

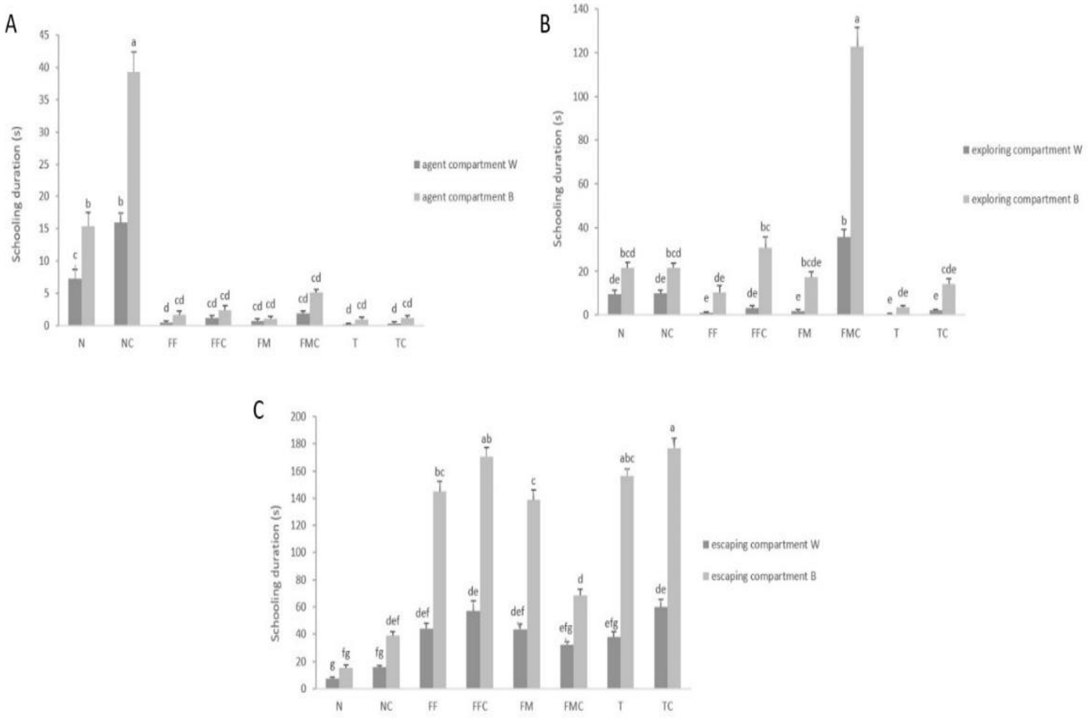


N = no agent; NC = no agent plus adult guppy olfactory cues; FF = female-mimicking robotic fish; FFC = female-mimicking robotic fish plus adult guppy olfactory cues; FM = male-mimicking robotic fish; FMC = male-mimicking robotic fish plus adult guppy olfactory cues; T = robotic tadpole; TC = robotic tadpole plus adult guppy olfactory cues.

Figure 15. Duration of schooling behaviour of *Poecilia reticulata* newborns during interactions with different combination of cues. T-bars are standard errors. Above each column, different letters indicated significant differences (P<0.05).

Fish schooling duration in the agent compartment was significantly affected by the tested combination of cues ($F_{15,159} = 55.150$; $P < 0.0001$). Newborn fish swarm in schooling for a longer time in the robot compartment on the brown area, and only when olfactory cues typical of adult guppies were provided. Schooling duration in this compartment into the white area plus adult guppy olfactory cues as well as on the brown area without adult guppy olfactory cues was significantly shorter. Differences in schooling duration within the robot compartment were marginal among context including different robotic stimuli and their combination with adult guppy olfactory cues. Exceptions were the female-mimicking robotic fish, the robotic tadpole and the robotic tadpole in combination with adult guppy olfactory cues, which led to a shorter time of schooling newborn fish swimming in the white area of the robot compartment (Fig. 16A).

Schooling duration in the exploration compartment was significantly influenced by the tested combination of cues ($F_{15,159} = 62.535$; $P < 0.0001$). The male-mimicking robotic fish in combination with adult guppy olfactory cues evoked the longest schooling duration in the exploring compartment, and it was longer in its brown area over the white one. Schooling in the brown area of the exploring compartment with no agent, as well as in presence of adult guppy olfactory cues, female-mimicking robotic fish, female-mimicking robotic fish plus adult guppy olfactory cues, male-mimicking robotic fish, robotic tadpole, as well as robotic tadpole plus adult guppy olfactory cues, was marginally shorter. Further, the permanence in the white area of the exploring compartment was marginally shorter in presence of no agents, no agent plus adult guppy olfactory cues, and female-mimicking robotic fish plus guppy olfactory cues. Schooling duration in the exploring compartment was significantly shorter within the white area during interactions with the female, male and tadpole robotic replicas as well as robotic tadpole plus guppy olfactory cues (Fig. 16B).



N = no agent; NC = no agent plus adult guppy olfactory cues; FF = female-mimicking robotic fish; FFC = female-mimicking robotic fish plus adult guppy olfactory cues; FM = male-mimicking robotic fish; FMC = male-mimicking robotic fish plus adult guppy olfactory cues; T = robotic tadpole; TC = robotic tadpole plus adult guppy olfactory cues.

Figure 16. Duration of schooling behaviour of *Poecilia reticulata* newborns in white [W] and brown [B] zones of: (A) the agent compartment, (B) the exploration compartment and, (C) the escaping compartment, during interactions with different combination of cues. T-bars are standard errors. Above each column, different letters indicated significant differences (*P*<0.05).

Schooling duration in the escaping compartment was significantly affected by the tested combination of cues ($F_{15,159} = 92.644$; $P < 0.0001$). Schooling behaviour lasted more in the brown area post-exposure to the robotic tadpole plus adult guppy olfactory cues. Schooling behaviour was slightly shorter in the brown area of the escaping compartment during interactions with the female and male-mimicking robotic fish. Little differences were observed concerning the schooling duration in the brown area of the escaping compartment post-exposure to the male-mimicking robotic fish plus adult guppy olfactory cues, no agent plus adult guppy olfactory cues, as well as in the white area of the escaping compartment post-exposure to the female-mimicking robotic fish, the female or male-mimicking robotic fish plus olfactory cues, as well as the robotic tadpole plus adult guppy olfactory cues. The shortest schooling behaviour was observed post-exposure to no agent plus adult guppy olfactory cues (in the white area) and no agents (both in the white and the brown area) (Fig. 16C). The proportion of time spent by *P. reticulata* newborns performing schooling behaviour in both white and brown areas of the arena compartments during interactions with different combination of predator-borne cues, is detailed in Fig. 17.

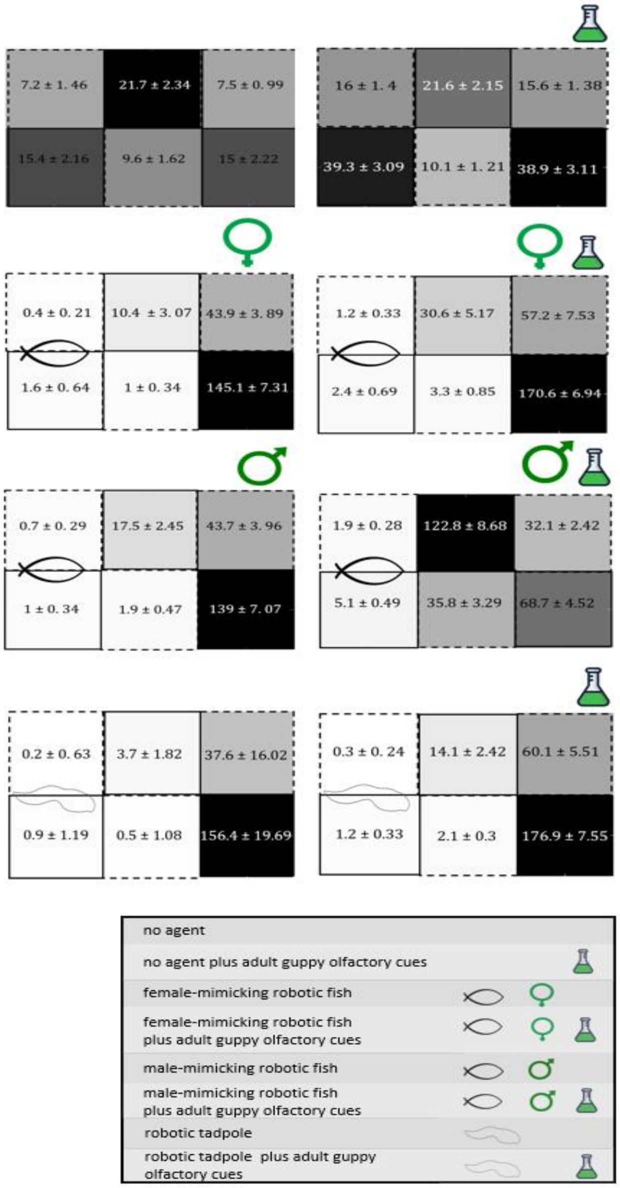


Figure 17. Schooling behaviour duration of *Poecilia reticulata* newborns in white (dashed line squares) and brown (continuous line squares) areas of each compartment of the testing arena during interactions with different combination of cues. The intensity of colour of each square indicates the proportion of time (s ± SE) spent by newborns performing schooling behaviour.

2.3.3 Discussion

Although fish aggregations as antipredator strategies are importantly affected by experience during development, the present investigation demonstrates that *P. reticulata* newborns are innately able to aggregate with other newborn conspecifics immediately after birth. This is in contrast with a relatively recent study on *D. rerio* (Buske & Gerlai 2011), which showed a low tendency to shoal during the first week of zebrafish life.

Physical cues, such as artificial agents moving in the arena, as well as chemical stimuli, including adult guppy olfactory cues, triggered aggregations in *P. reticulata* newborns (Fig. 15). Generally, predation is the major factor of mortality of young individuals in several fish species. In filial-preying species such as *P. reticulata*, the additional selective pressure exerted by cannibalism can bring out the early and innate tendency to aggregate in newborns.

Schooling behaviour, in the absence of artificial agents, significantly increased with the presence of adult guppy olfactory cues, confirming earlier research on guppies (Chapman et al. 2007), and other cannibalistic species (Tollrian et al. 1999), reporting olfactory stimuli from adults as sufficient cues to trigger antipredator behaviour in young fish. In addition, the combination of physical cues with chemical cues increased the duration of schooling behaviour displayed by newborns.

In this research, I also investigated the spatial distribution of the schooling fish into the arena, according to the predator-mimicking stimuli presented, as well as, to the colour background of the arena (Fig. 17). When newborn fish were not exposed to robotic agents, they showed schooling behaviour preferentially in the middle of the arena (e.g., exploring compartment), while in the case of no biorobotic agent tested but in presence of adult guppy olfactory cues, fish aggregation swam longer at the ends of the arena (e.g., agent compartment and escaping compartment). The presence of predator-borne olfactory cues without visual cues may induce newborn guppies to station close to the ends of the arena, to have just one side exposed to possible predators, thus improving surveillance.

When a predator-mimicking robotic device was placed in the agent compartment, schooling behaviour was observed in the escaping compartment. This represents a consistent response, being the escaping compartment the furthest zone from the predator-mimicking agent. However, the combination of the male-mimicking robotic fish and adult guppy olfactory cues, led to an unexpected response, where guppy newborn aggregation stationed more in the exploring compartment. The orange-coloured tail of the male-mimicking robotic fish in combination with adult guppies olfactory cues induced newborn fish to swim closer to the agent. The orange pigment in male *P. reticulata* is recognized as one of the most important indicators of male quality and it has an important role during courtship (Houde & Endler 1990).

However, no explanations exist in the literature clarifying the signaling role of this pigment between *P. reticulata* adults and newborns. One hypothesis is that the orange pigment of adult *P. reticulata* males, in combination with adult olfactory cues, are used by newborn conspecifics to recognize adults of the same species to swim in their vicinity (i.e., to find common resources), but at safe distance to avoid cannibalism.

Beside cues directly triggering schooling behaviour, (e.g., robotic agents, adult guppy olfactory cues, and their binary combinations), I also reported the interesting effect produced by the colour of the background in the arena. Schooling behaviour has been observed to occur mostly where the background was brown. The shared need to be less visible to possible predators may drive *P. reticulata* newborns towards darker backgrounds, raising the chance of their encounters and indirectly leading to the emergence of the self-organized schooling behaviour in naïve newborns.

Overall, this research provides novel insights on the early cognitive ability of newborn guppy fish, as well as on the cues innately triggering schooling behaviour in newborns. Furthermore, basic knowledge presented here can be helpful to boost the use of biomimetic robots in predator-prey interaction researches as substitutes for real predators in experiments that are compliant with ethical issues (Huntingfor 1984; ASAB/ABS 2004).

Chapter 3: Observation and encoding of behavioural asymmetries in invertebrates via biorobotic artifacts

3.1 CASE STUDY 4: LATERALIZATION OF ESCAPE AND SURVEILLANCE BEHAVIOURS IN LOCUSTS EXPOSED TO A PREDATOR-MIMICKING ROBOT

Recently, I reported, in pioneer researches with my research group, several findings on lateralized traits in different insect orders (e.g. Romano et al. 2015, 2016a, b, 2018a; Benelli et al 2015b).

A range of evidences from vertebrates demonstrate lateralized traits (i.e., left-right asymmetries in the brain and behaviour), during interactions with predators (Vallortigara et al. 1999; Rogers 2002). This is probably due to the specialization of one hemisphere of the brain for a particular function allowing the other hemisphere to perform different functions in parallel (Vallortigara & Rogers 2005).

However, in insects, asymmetrical responses to predators have not been investigated.

In this case study, I developed a robot predator inspired to the helmeted Guinea fowl, *Numida meleagris*(Linnaeus) (Galliformes: Numididae), a natural enemy of locusts, to simulate a predation event evoking escape and surveillance behaviours in locusts (Romano et al. 2017b). In particular, lateralized escape and predator surveillance behaviours in neanids, nymphs and adults of *Locusta migratoria* Linnaeus (Orthoptera: Acrididae) at gregarious phase, were investigated at individual and at population-level during interactions with the predator-mimicking robot.

It has been proposed that lateralization at population-level is more likely to evolve in social/gregarious species (Vallortigara & Rogers 2005), although lateralization at population-level has been reported in several solitary species (Benelli et al 2015b; Romano et al. 2015, 2018a).

I evaluated: (*i*) if *L. migratoria* shows any lateral bias when – startled by an approaching predator – it jumps off, and (*ii*) if there is an eye preference used for overseeing a potentially threatening animal. In addition, in insects post-embryonic development affects numerous morphological, physiological and behavioural features (Edwards 1970). So, I also investigated if these lateralized responses to a predator varied among the different developmental stages.

3.1.1 *Methods*

3.1.1.1 Animal rearing and general observations

Experiments were conducted on second-instar, fourth-instar, and adult *L. migratoria*. Locusts were maintained under controlled conditions (25 ± 1 °C, 55 ± 5% R.H., 16:8 h L:D) at the BioRobotics Institute laboratories (Scuola Superiore Sant'Anna, Pontedera). They were fed by wheat, vegetables and water. Only animals with intact eyes, legs, wings and antennae were used for experiments. Experiments were conducted in the laboratory (25 ± 1 °C, 55 ± 5% R.H.) during December 2016-March 2017 in a room illuminated with fluorescent daylight tubes (16:8 h L:D, lights on at 6:00). Neon tubes (Philips 30 W/33) were used; light intensity around the test arena was ca. 1000 lx, estimated over the 300–1100 nm waveband with an LI-1800 spectroradiometer (LICOR Inc., Lincoln, NE, U.S.A.), equipped with a remote cosine receptor. Directional light cues were avoided by using diffuse laboratory lighting to reduce reflection and phototaxis. For each experiment, the behaviour of locusts was directly recorded by an observer.

A white wall of filter paper (Whatman) surrounded both the arena and the robot, the observer was dressed in a white coat, to minimize his impact on *L. migratoria* behaviour and was placed symmetrically behind the robot at a reasonably distance from the arena.

3.1.1.2 Animal replica and experimental apparatus

To simulate a predator of locusts, a *N. meleagris* head in acrylonitrile butadiene styrene (ABS) (Fig. 18A) was designed in SolidWorks (Dassault Systemes, Vélizy-Villacoublay, France) and fabricated by rapid prototyping.

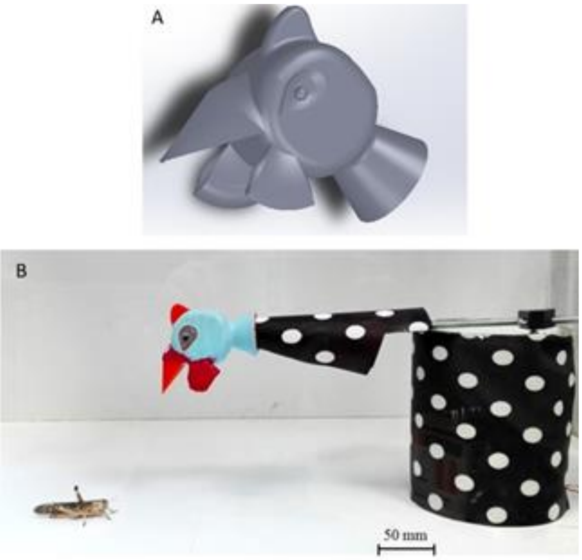


Figure 18. (A) Helmeted Guinea fowl head-replica design, and (B) the Guinea fowl-mimicking robotic predator with an adult locust.

The *N. meleagris* head-replica had a diameter of 40 mm a thickness of 30 mm and a total length, including the beak, of 75 mm. The bird head (except the beak and helmet), was covered by a thin layer of silicone rubber (Dragon Skin), by turning molding and then coloured, reproducing the colour pattern of real *N. meleagris* birds. A 300-mm steel rod, connected the bird head to a DC motor (Precision Microdrives: 225–202), producing a simple robotic arm. The DC motor was placed in a Plexiglas pipe section (height 150 mm, diameter 120 mm), partially filled with iron weights, as a support body. A grey-black sheet with white polka dots covered both the support body and partially the rod, to improve the resemblance with the *N. meleagris* plumage (Fig. 18B). A microcontroller (Arduino, Mega 2560) was used to control the movement of the robotic arm, capped with the plastic bird head. Depending on the experiment, the robotic stimulus can be moved in an upright way or horizontally by changing the support base of the pipe.

3.1.1.3 Interactive biohybrid experiment 1: laterality of escape responses in locusts

I evaluated if locusts showed a bias in jumping to the left or to the right when the robot-predator approached them frontally. Individual *L. migratoria* were gently placed on a cubic platform (100 × 100 × 100 mm) of white cardboard exactly centered with respect to the robotic stimulus, in the centre of a rectangular white arena (800 × 600 × 600 mm). Insects were placed to an identical distance from the right and left side of the arena. The robotic stimulus was visually isolated from the tested subjects by a wall of the arena (600 × 600 mm) fitted with a white curtain with a vertical slot in the centre, which allowed the robotic arm, capped with the bird head, to enter in the arena when simulating predation (Fig. 19A).

A jumping escape was evoked when the robot predator simulated an attack, striking moving from top to bottom, 100 mm from the locust head, and then returning. The robot was perfectly symmetric in appearance and movement to avoid any lateral bias. The top of the arena was covered with a transparent partition, to allow focal observations of the locust behaviour. For laterality observations, I considered only locusts which were approached by the robot predator when they were perfectly centered with respect to the robot predator. For each insect, the laterality of 30 jumps was recorded. Each jump was evoked by the Guinea fowl-mimicking robot predator after 10 min from the previous one. 30 II instar, 30 IV instar and 30 adult locusts that jumped after a perfectly symmetric robotic stimulus were analyzed in this experiment.

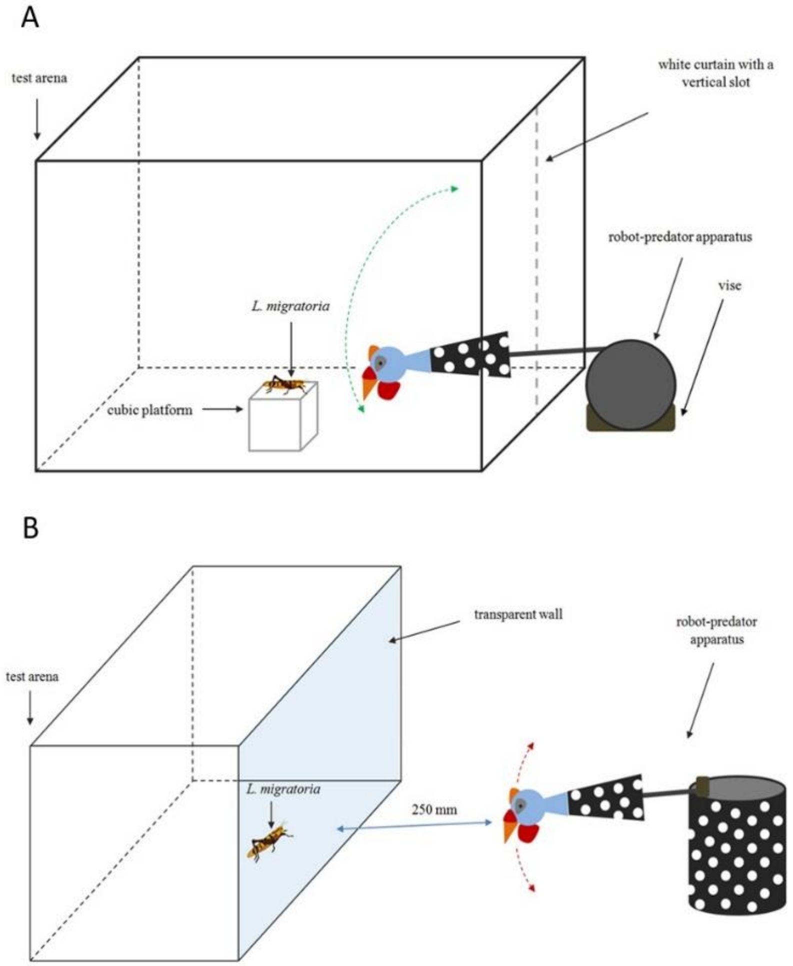


Figure 19. (A) Experiment 1 setup. The Guinea fowl-mimicking robot-predator moves vertically from top to bottom and then backing on, evoking a jumping escape of the locust. **(B) Experiment 2 setup.** The Guinea fowl-mimicking robot is yawed in front of the transparent wall of the test arena to be overseen by the locust.

3.1.1.4 Interactive biohybrid experiment 2: laterality of predator surveillance in locusts

Here, I investigated the eye preference used by locusts to scan the Guinea fowl-mimicking robotic predator. Locusts were placed individually in the centre of a rectangular white arena (800 × 600 × 600 mm), with their body axis orthogonal to a transparent wall (800 × 600 mm, Plexiglas). Insects were placed to an identical distance from the right and left side of the arena. The robot predator was placed outside the arena in correspondence of its middle, with the bird head 250 mm from the transparent wall. The rod capped with the bird head was yawed by the DC motor, 45° to the left and 45° to the right with a frequency of 0.5 Hz, to be perfectly symmetric in appearance and movement. Tests (lasting 30 minutes) started 5 minutes after the locust was introduced in the arena, removing an opaque partition from the transparent wall, that allowed visual contact with the robotic stimulus (Fig. 19B). At the beginning of the experiment, the locust was placed head on to the robotic stimulus and was able to orientate the body, according to its overseeing of the robot predator.

For each locusts, I recorded how long a given side of the insect's body (e.g., steered body axis forming an angle >45°, with the initial orientation of the body axis perfectly centred with the stimulus, to have just one eye able to see the robot (Kral & Poteser 1997; Horridge 1977), was exposed to the Guinea fowl-mimicking robot predator. Furthermore, since locusts assume a static pose to go unnoticed when a predator is nearby (Ruxton et al. 2004), I recorded the number of jumps and the duration of the walking behaviour, to evaluate if the cryptic behaviour of subjects overseeing the predator was affected by lateral bias. The further distance of the robot predator and its slow movement, if compared to the previous experiment (e.g. "laterality of escape responses in locusts"), was predicted to evoke the cryptic behaviour in locusts over the jumping escape. 30 II instars, 30 IV instars and 30 adults of *L. migratoria* were tested.

3.1.1.4 Statistical analysis

A laterality index (LI) was calculated for each insect, to analyse the differences in the direction of jumping escape responses (Frasnelli et al. 2012):

LI = [(number of jumps to the right − number of jumps to the left)/(number of jumps to the right + number of jumps to the left)].

We calculated a LI for each insect evaluating bias in the use of the right and left eye during predator surveillance:

LI = [(duration of surveillance with the right eye − duration of surveillance with the left eye)/(duration of surveillance with the right eye + duration of surveillance with the left eye)].

Individual asymmetrical dominance was determined by comparing the size of the LI value (ranging from −1 to + 1), with a threshold (LI_{TH}) set to 0.3 (e.g. LI >0.3 right-biased; LI <−0.3 left-biased). Furthermore, the absolute value of the laterality index (ABLI) was considered, to discriminate individuals with a bilateral dominance from individuals with a lateral dominance, regardless the left or the right direction of the bias.

Laterality differences among the numbers of locusts (II young instars, IV young instars and adults) displaying right- or left-biased jumping escapes, as well as right- or left-biased eye use during surveillance were analysed by JMP 9 (SAS) using a weighted generalized linear model (glm): $y = X\beta + \varepsilon$ where y is the vector of the observations (i.e., escape response or surveillance), X is the incidence matrix linking observations to fixed effects, β is the vector of fixed effects (i.e., the tested instar and laterality) and ε is the vector of the random residual effects. A probability level of $P < 0.05$ was used for the significance of differences between means. Furthermore, differences in the (*i*) mean duration of walking response as well as (*ii*) the number of jumps during the surveillance of a robotic predator were analysed using the glm described above with normal distribution, fixed effects were the tested instar and laterality of the behavioural response. Averages were separated by Tukey's HSD test. A probability level of $P < 0.05$ was used for the significance of differences between means.

Within each locust instar, the difference in the number of locusts using left or right eyes during the exposure to the predator was analyzed using a χ^2 test with Yates correction ($P < 0.05$).

3.1.2 Results

3.1.2.1 Interactive biohybrid experiment 1: laterality of escape responses in locusts

The jumping escape response to a Guinea fowl-mimicking robotic predator was lateralized at the individual level, while the same was not true at the population level ($F_{2,89} = 0.312$; $P < 0.7330$). Right-biased adult locusts were not significantly more abundant than left-biased adult locusts, the same was true for IV and II instars.

The presence of a jumping escape response at the individual level (regardless the left or the right direction), was significantly affected by the insect instar ($F_{2,89} = 95.151$; $P < 0.0001$). It was higher in adults and IV instar locusts over II instar ones (Fig. 20A).

The absolute values of the laterality index (ABLI) were significantly influenced by the locust instar ($F_{2,89} = 207.404$; $P < 0.0001$). ABLI in adults was significantly stronger over ABLI in IV instar locusts; ABLI in IV instars was stronger than the one calculated for II instars (Fig. 20B).

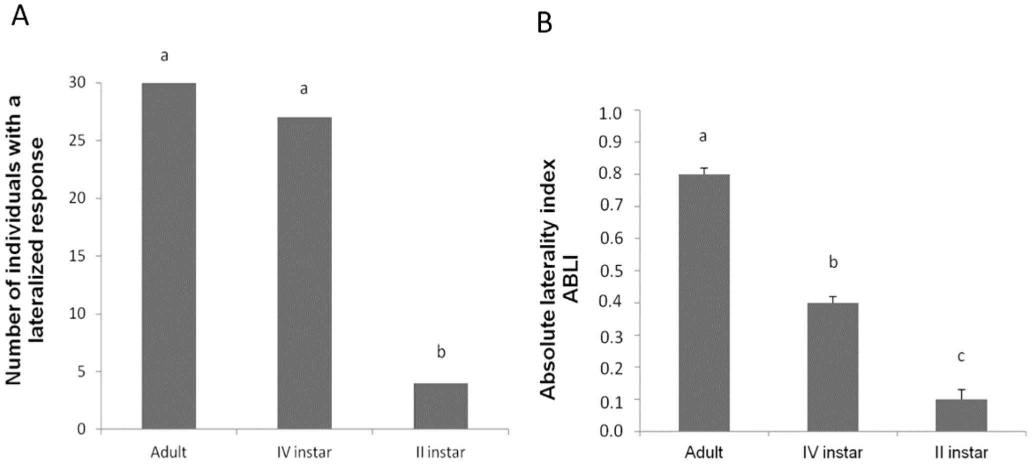


Figure 20. (A) *Locusta migratoria* individuals that exhibit a lateralized jumping escape response at individual level during the exposure to a Guinea fowl-mimicking robotic predator. Different letters above each column indicate significant differences. **(B) Absolute value of ABLI for the jumping escape response of *L. migratoria* during the exposure to a Guinea fowl-mimicking robotic predator.** Different letters above each column indicate significant differences. T-bars represent standard errors.

3.1.2.2 Interactive biohybrid experiment 2: laterality of predator surveillance in locusts

Locusta migratoria adults and young instars showed lateralized eye use during the surveillance of the Guinea fowl-mimicking robotic predator. The laterality index (LI) value significantly differed between left- and right-biased individuals ($F_{1,84} = 1319.947$; $P < 0.0001$). No significant differences were detected among adult, IV instar and II instar locusts showing the same lateral bias (Fig. 21A). The absolute value of ABLI concerning the use of a compound eye for predator surveillance did not differ among tested instars

($F_{2,87} = 1.561$; $P = 0.216$). A right-biased lateral dominance was observed in adult, IV instar and II instar locusts (Fig. 21B).

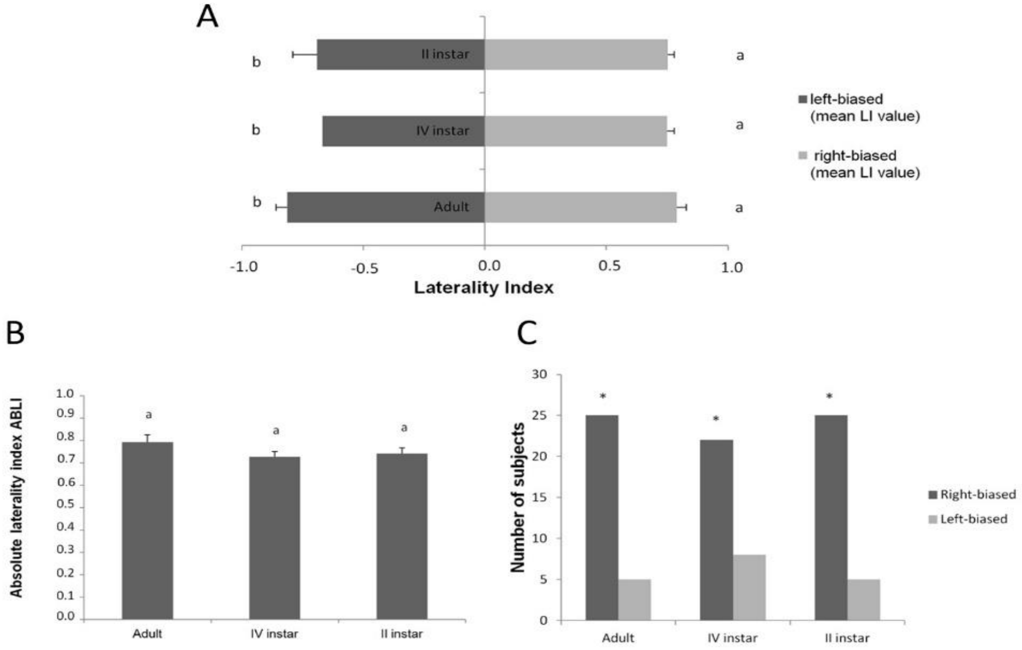


Figure 21. (A) LI values for *Locusta migratoria* surveillance during the exposure to a Guinea fowl-mimicking robotic predator. Different letters near of each bar indicate significant differences. T-bars represent standard errors. **(B) Absolute value of ABLI for the eye use during *L. migratoria* surveillance during the exposure to a Guinea fowl-mimicking robot.** Different letters above each column indicate significant differences. T-bars represent standard errors. **(C) Left- and right-biased use of eyes in *L. migratoria* surveillance during the exposure to a Guinea fowl-mimicking robotic predator.** Asterisks indicate significant differences between left- and right-biased subjects.

Lateralized eye use for predator surveillance was noted at population-level by adult, IV instar and II instar locusts. Adult locusts preferentially use the right eye over the left one for predator surveillance (right vs. left: 25 vs. 5; $\chi^2_1 = 52.61$; $P < 0.0001$). The same was true for IV instars (right vs. left: 22 vs. 8; $\chi^2_1 = 50.69$; $P < 0.0001$) and II instars (right vs. left: 25 vs. 5; $\chi^2_1 = 52.61$; $P < 0.0001$), (Fig. 21C).

The number of locust jumps ($F_{2,87} = 1.0136$; $P < 0.3672$) (Fig. 22A) and the walking time spent during predator surveillance ($F_{2,87} = 0.1987$; $P < 0.8253$) (Fig. 22B) were not significantly affected by the tested instar. The number of jumps ($F_{1,84} = 60.161$; $P < 0.0001$) (Fig. 22A) and the walking time spent during predator surveillance ($F_{1,86} = 30.410$; $P < 0.0001$) (Fig. 22B) showed significant differences between right- and left-biased lateralized responses. Locusts with preferential use of the left eye for predator surveillance showed higher jumping and walking activity over right-biased individuals, without significant differences among developmental stages (Fig. 22A, B).

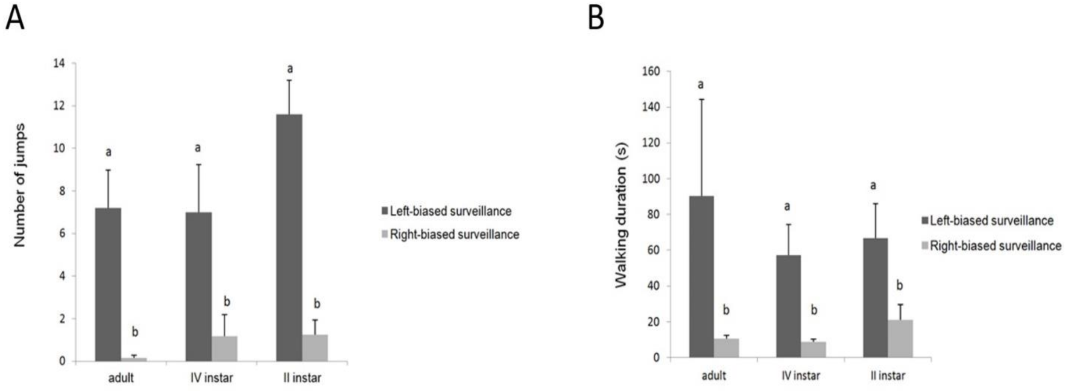


Figure 22. (A) Number of *Locusta migratoria* lateralized jumps during surveillance of a Guinea fowl-mimicking robotic predator. Different letters above each column indicate significant differences. T-bars represent standard errors. **(B) Duration of *L. migratoria* walking during surveillance of a Guinea fowl-mimicking robotic predator.** Different letters above each bar indicate significant differences. T-bars represent standard errors.

3.1.3 Discussion

Here, I investigated the lateralization of escape and surveillance responses in young instars and adults of *L. migratoria* during interactions with a biomimetic robot-predator inspired by the helmeted Guinea fowl. Results showed an individual-level lateralization in the jumping escape of *L. migratoria* exposed to a simulated predator attack. The laterality of this response increased in *L. migratoria* adults if compared to young instars.

We noted that the *L. migratoria* escape response was lateralized at individual-level in adults and IV instar individuals, although lateralization at population level was not detected. Previous studies also reported lateralization at individual level for forelimb use of *S. gregaria* (Bell & Niven 2016), showing that stronger lateralization provides an advantage in terms of boosted motor control. Furthermore, II instar locusts did not show lateralized jumping escape responses. One of the main disadvantage of lateralization is predictability, which may be used for predation by other species or cannibalization by individuals of the same species (Vallortigara & Rogers 2005; Bazazi et al. 2008). The presence of individual-level lateralization in locusts could be a strategy to avoid the cost of predictability, everyone has a different lateral bias when approached by the predator. Moreover, individual-level lateralization in the escape behaviour can contribute to the jumping performance thanks to doubled reinforcement by experience (e.g., improvement of the jumping escape performance over time centred on one of the two forelegs), as reported for other insect species (Romano et al. 2016a, 2018a; Benelli et al 2015b; Bell & Niven 2016).

The role of experience in producing stronger lateralized locusts can contribute to explain our data reporting a significant increase of lateralization strength in jumping escape from II instar to adult locusts. Indeed, locusts can exhibit motor learning at single ganglia-level (Bell & Niven 2016).

In addition, it has been proposed that the specialization of forelimb movements control can be related to motor circuits and mechano-sensory reflexes within the prothoracic ganglion of locusts (Rowell 1961). Lastly, I cannot exclude the effect that the post-embryonic development of the neural system can play, leading to insects with a stronger lateralization as they grow.

Furthermore, population-level lateralization of predator surveillance was found testing both adults and young instars, showing that *L. migratoria* used the right compound eye to oversee the Guinea fowl-mimicking predator.

Static cryptic behaviour of locusts overseeing the predator was efficiently performed by right-biased insects over left-biased ones. The latter was “more visible” to a potential predator by exhibiting a higher number of jumps and a longer walking activity.

Locusts show several physiological and morphological changes during the gregarious phase, reflecting a modulation of the individual’s metabolism to favour greater mobility (Dempster 1963). It should be noted that, in terms of jumping, population level laterality would not evolve in this gregarious species, since this would make the group movements more predictable than if only the single individuals had lateralized jumping performances. The population-level lateralization in the eye use in *L. migratoria* could be partially linked to the need to perform specific group tasks, such as swarm coordination, while predictability can be avoided by individual-level lateralization of escape responses. In addition, while the surveillance is mainly accomplished by visual sense, more stimuli are involved in the escape behaviour, such as mechano-receptive hairs that sense air displacement around insects and alert them when a predator is attacking (Li et al. 2010).

This can be related to different reactions of locusts to predators, concerning lateralized responses during surveillance and escape. Further research is needed to investigate how these lateralized traits can be connected each other in *L. migratoria*, and how they change during different social phases.

This study represents the first report of lateralized predator-prey interactions in insects. Findings reported here outline the possibility of relying on biomimetic robotic predators to study predator-prey interactions in arthropods, avoiding the use of real predators, thus achieving highly standardized experimental conditions to investigate complex and flexible behaviours.

3.2 CASE STUDY 5: ENCODING LATERALIZATION OF JUMP KINEMATICS AND EYE USE IN A LOCUST EXPOSED TO A BIOROBOTIC ARTIFACT

The sub-order Caelifera (Orthoptera) includes remarkable biological models to assess how sensory-motor sequences are affected by visual experience and how this influences lateralization (Romano et al. 2019c). The brain neuro-architecture is well described in these insects (Santer et al. 2006; Kurylas et al. 2008; Fotowat et al. 2011). Particularly, in Caelifera - such as locusts - each optic lobe (included in the protocerebrum), contains a neuron (the lobula giant movement detector, LGMD), at level of the third neuropil, that responds specifically to looming stimuli (e.g., an approaching predator), (Santer et al. 2006; Fotowat et al. 2011), by producing trains of spikes transmitted to the descending contralateral movement detector (DCMD) that conveys visual information to motor centres (O'shea et al. 1974; Simmons 1980; Fotowat et al. 2011).

An important issue concerns the basic knowledge on the network connecting sensory signals with asymmetrical motor outputs/orientations, and how they are modulated by experience.

To manipulate a predator-prey interaction, I developed a robotic apparatus that actuates an artificial agent mimicking a predator of locusts, the leopard gecko, *Eublepharis macularius* Blyth (Squamata: Eublepharidae) (Thorogood and Whimsterf 1979; Cooper and Williams 2014).

Herein, gregarious individuals of *L. migratoria* were trained by using the robotic leopard gecko, to observe directional biased predator approaches (Romano et al. 2019c). Since antipredator behaviour importantly contributes to survival and fitness boosting in animals, I investigated the hypothesis to "program" the locust jumping escape direction as well as surveillance orientation, as an adaptive consequence of prior exposure to the robotic predator in lateralized training sessions (Romano et al. 2019c). Furthermore, in locusts the jumping escape lateralization is age-related, while surveillance asymmetry is equally exhibited among different instars (Romano et al. 2017b). Based on this, herein I evaluated if the developmental stage has a sensible role in the modulation of antipredator responses in this species.

3.2.1 Methods

3.2.1.1 Animal rearing and general observations

Locusts were fed *ad libitum* with wheat, vegetables and water (Bell and Niven 2016), and maintained at 25±1°C, 55±5% R.H., 16:8h(L:D). Second-instar, fourth-instar, and adult *L. migratoria* individuals of both sexes were tested. Experiments were conducted in laboratory under the same experimental conditions described above, light intensity around the test arena was about 1000lux. The behaviour of locusts was directly recorded by an observer during the experiments (Romano et al. 2017b). A white wall of filter paper (Whatman) surrounded the arena, the observer was dressed in a white coat, to minimize his impact on *L. migratoria* behaviour.

3.2.1.2 Animal replica and experimental apparatus

A gecko replica inspired to *E. macularius* was designed in SolidWorks (Dassault Systemes, Vélizy-Villacoublay, France) and fabricated by rapid prototyping in acrylonitrile butadiene styrene (ABS). The biomimetic morphology of the *E. macularius* replica includes the head with the mouth, two eyes and nostrils, main body and the two forelegs with feet. The leopard gecko replica has a total length of 107 mm and a total width of 44 mm, reasonably corresponding to the size of *E. macularius* (Kratovíl and Frynta 2002), as it reproduces only a portion of the body of the authentic lizard.

To produce a similar colour pattern and the skin rough texture of a real *E. macularius*, the artifact was endowed with a skin-like coating, obtained by mixing a transparent liquid silicone rubber (Dragon Skin by Smooth-On, Pennsylvania, USA), with nontoxic pigments and used to cover the leopard gecko replica by turning molding (Romano et al. 2017b). The leopard gecko replica was connected with a DC gearmotor (model: 212-103), forming a robotic arm.

In order to integrate the robotic platform, the leopard gecko replica, was inserted in a dedicated slot in middle of one of the shorter sides of a support. The support includes a flat top surface (150x220 mm), and four cylindrical legs (120 mm, diameter 20 mm), in polyoxymethylene (POM), fabricated by using a CNC machine.

When the DC gearmotor was activated, the leopard gecko replica could be vertically rotated from the bottom of the test bench, to the horizontal plane on the top surface. Even if one can argue that this movement did not carbon-copy an attack by a living gecko, the predatory event displayed by the robotic leopard gecko was perfectly symmetric in appearance and movement to avoid any lateral bias in locusts during the experiments. Fig. 23A, B show the robotic leopard gecko lying on the flat top surface with a locust frontally placed, as well as the jumping escape of a locust during the robotic leopard gecko approach respectively. The activation of the leopard gecko replica's attack was performed via a microcontroller (Arduino, Mega 2560).

3.2.1.3 Training phase

Locusts were individually released in a transparent cage (80x15x80 mm for adults; 40x15x80 mm for neanids and nymphs), wide enough to prevent the constraining of their natural posture but significantly reducing insect locomotion. This enabled me to impose a given orientation during the simulated attacks by the robotic leopard gecko. The transparent cage containing the locust was placed on the top surface of the test bench, perpendicularly to the longitudinal body axis of the robotic leopard gecko at a distance > 70 mm from its mouth when it lays on the horizontal plane, see Fig. 23C. The posture of *L. migratoria* was constantly monitored during the training phase, to ensure correct orientation. The training phase lasted 60 min in which the robotic leopard gecko appeared from the bottom of the test bench (invisible to the locust), to the horizontal plane of the top surface, and then returning, at intervals of 30 s. According to the side of the locust that was exposed to the simulated predator, we obtained left/right trained locusts.

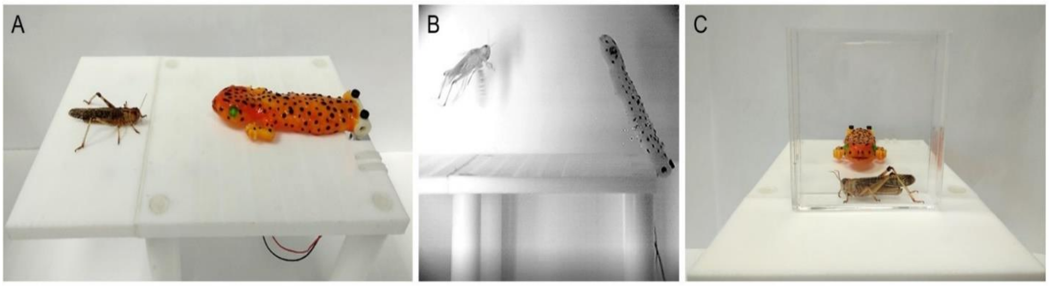


Figure 23. (A) The robotic leopard gecko and a locust (*Locusta migratoria*) on the top surface of the experimental apparatus. (B) Snapshot of a biohybrid predator-prey interaction that shows the approaching robotic leopard gecko and an escaping locust. (C) A locust in the transparent cage with the right side exposed to the predator-mimicking robot during the training phase.

It is well acknowledged that just the risk of predation can produce physiological stresses in a wide number of species. These stresses have been reported to have long-lasting influences on prey escape responses (Hawlena et al. 2011). Thus, although the robotic leopard gecko did not injure physically the locusts (this would not be acceptable from an ethical point of view), its presence as a potential predator and its simulated attack would affect the escape response of the locust. The control treatments were similarly conducted with the exceptions that the robotic leopard gecko was not visible to the locusts (i.e., hidden below the test bench), and no simulated attacks were displayed. The interval among the training phase and the experiments described below, was 120 min, where locusts were isolated from other subjects to avoid subsequent experiences affecting the effect of the training.

3.2.1.4 Interactive biohybrid experiment 1: modulating the jumping escape lateralization

Here, the possibility to condition the direction of the jumping escape response by providing a symmetric stimulus to previously trained locusts, was evaluated.

L. migratoria trained with presentation of the robotic stimulus to the left or right compound eye, as well as control subjects (naïve), were placed individually on the top surface of the test bench, with their longitudinal body axis accurately centred (± 0.5°), with the longitudinal body axis of the robotic leopard gecko, at a distance of ≃ 70 mm from its mouth when it lays on the horizontal plane. The robotic apparatus was placed in the centre of a rectangular white arena (800 x 600 x 600 mm), equidistant from the left and right side, to minimize external cues affecting the locust's behaviour. At the beginning of the test, the robotic leopard gecko was hidden below the test bench and thus not visible to the tested locust.

The jumping escape direction of the locust was recorded following the robotic leopard gecko rotation from the bottom of the test bench, to the horizontal plane on the top surface (ω= 4.97 rad/s), emulating a predator that comes out of a hiding place, see Fig. 24A. Locusts that were not accurately centred with the robotic leopard gecko when approached by it, were not considered for laterality observations.

For each replicate, the whole setup was rotated 90° horizontally, to randomize its orientation and to avoid positional effects.

For each developmental stage of *L. migratoria* considered in our study (e.g., II instar, IV instar and adult) 25 naïve subjects, 25 left trained subjects and 25 right trained subjects, escaping after a perfectly symmetric predator-prey interaction, were analysed. The direction of 30 jumps, delayed 10 minutes from each other, was recorded for each insect.

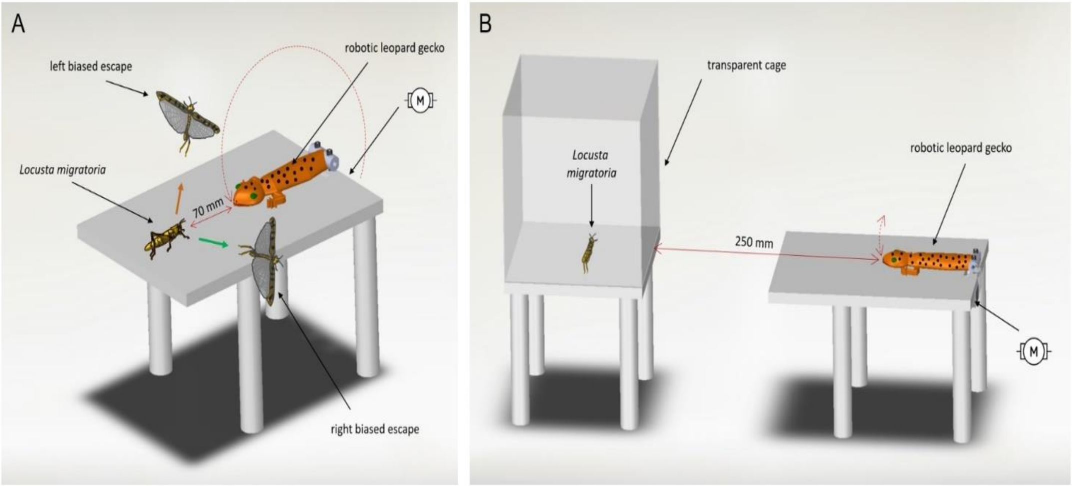


Figure 24. Schematic illustrations of (A) the biohybrid experiment 1, and (B) the biohybrid experiment 2.

3.2.1.5 Interactive biohybrid experiment 2: modulating the surveillance lateralization

Here, the assumption to manipulate the eye use preference during surveillance in locusts by a prior bio-robotic interaction was evaluated. A transparent cubic cage (150x150x150 mm) containing a locust, was placed in the middle of a rectangular white arena (1300x900x600 mm), at the same distance from the right and left side of the arena. The cubic cage was positioned at 250 mm from the robotic leopard gecko mouth when it lays on the horizontal plane. The floor of the cubic cage and the top surface of the test bench were positioned on the same horizontal plane.

Before starting the test, the robotic leopard gecko was not visible to the tested locust since it was hidden below the test bench. After 5 min from the introduction of the locust inside the transparent cage, the test started, and the robotic leopard gecko rotated from the bottom of the test bench, to the horizontal plane on the top surface. Then, for the whole duration of this experiment, a still phase lasting 15 seconds, in which the robotic leopard gecko was motionless on the top surface, was alternated with a pitch phase, lasting 5 seconds. During the pitch phase, the robotic leopard gecko rotated 30° from bottom to top and then returning (5 Hz).

The larger distance of the robotic agent from the locust, compared to the experiment 1, and the short angle and duration of the pitch phases, ensured to better select cryptic and surveillance behaviours (Ruxton et al. 2004) and to avoid an excessive threat triggering an escape response.

The test lasted 30 min, and the exposure duration of orientation of each side of the locust body to the robotic leopard gecko was recorded for each animal. In particular, to ensure the monocular vision of the robot by the locust, only locusts with their steered body axis forming an angle >45° with the initial orientation of their body axis aligned with the stimulus were considered for laterality observations (Horridge 1977; Kral and Poteser 1997). After each replicate, I rotated the whole setup 90° horizontally, to randomize its orientation and to avoid positional effects.

25 naïve subjects, 25 left trained subjects and 25 right trained subjects, were tested for each *L. migratoria* developmental stage observed in this research (e.g. II instar, IV instar and adult).

3.2.1.6 Statistical analysis

To analyse the differences in the direction of jumping escape responses, as well as in the use of the right and left eye during predator surveillance, a laterality index (LI) was calculated for each insect, following the method by Frasnelli et al., (2012):

LI=(R-L/R+L)

For the predator escape behaviour, R and L indicate, respectively, the normalized mean number of times in which each locust jumped to the right or to the left. A score of 1.0 indicated exclusive preference to jump on the right, while a score of −1.0 indicated exclusive preference to jump on the left. A score of 0 indicated equal numbers of right and left jumps during jumping escape acts.

For the predator surveillance behaviour, R and L indicate, respectively, the normalized mean value of the duration in which each locust used the right eye or the left eye to oversee the robotic predator. A score of 1.0 indicated exclusive use of the right eye, while a score of −1.0 indicated exclusive use of the left eye. A score of 0 indicated equal duration in using the right and left eye during surveillance.

Furthermore, the absolute value of the laterality index (ABLI) was considered, to discriminate individuals with a bilateral dominance from individuals with a lateral dominance, regardless the left or the right direction of the bias, and to evaluate the strength of lateralization.

Laterality differences among naïve, right trained and left trained locusts over different instars (II young instars, IV young instars and adults) displaying right- or left-biased jumping escapes, as well as right- or left-biased eye use during surveillance, were analysed by JMP 9 (SAS) using a general linear model with two factors, i.e., the tested naïve/trained instar and laterality. P<0.05 was used to assess the significance of differences between means. Moreover, for each treatment, the difference in the number of locusts jumping to the right or left as well as using left or right eyes during the interaction with the biomimetic gecko predator was analysed using a χ^2 test with Yates' correction (P<0.05).

3.2.2 Results

3.2.2.1 Interactive biohybrid experiment 1: modulating the jumping escape lateralization

The direction of motor outputs towards left or right, during the jumping escape from the robotic leopard gecko, was successfully manipulated in all locust instars following exposure in the training phase, and thus allowing to control the lateralization of the jumping escape at population level.

The population mean value of the jumping escape's laterality index (LI), was significantly modulated by different contexts of the training phase ($F_{8,224}$ = 41.077; P < 0.0001). The LI of each left trained locust instar shows the preference to jump to the right. The LI of each naïve instars shows an equal preference of these locusts to jump to the right and to the left. The LI of each right trained locust instars shows the preference to jump on the left (Fig. 25A).

The absolute value of the laterality index (ABLI) was significantly modulated by the training phase ($F_{8,224}$ = 31.684; P < 0.0001). In adults, the ABLI was marginally higher in naïve subjects compared to right trained locusts, and significantly higher compared to left trained locusts. In IV instar locusts, the ABLI of both right trained and left trained subjects was significantly higher compared to the ABLI of naïve locusts. In II instar locusts, the ABLI of right trained subjects as well as left trained insects was significantly higher compared to the ABLI of naïve locusts (Fig.25B).

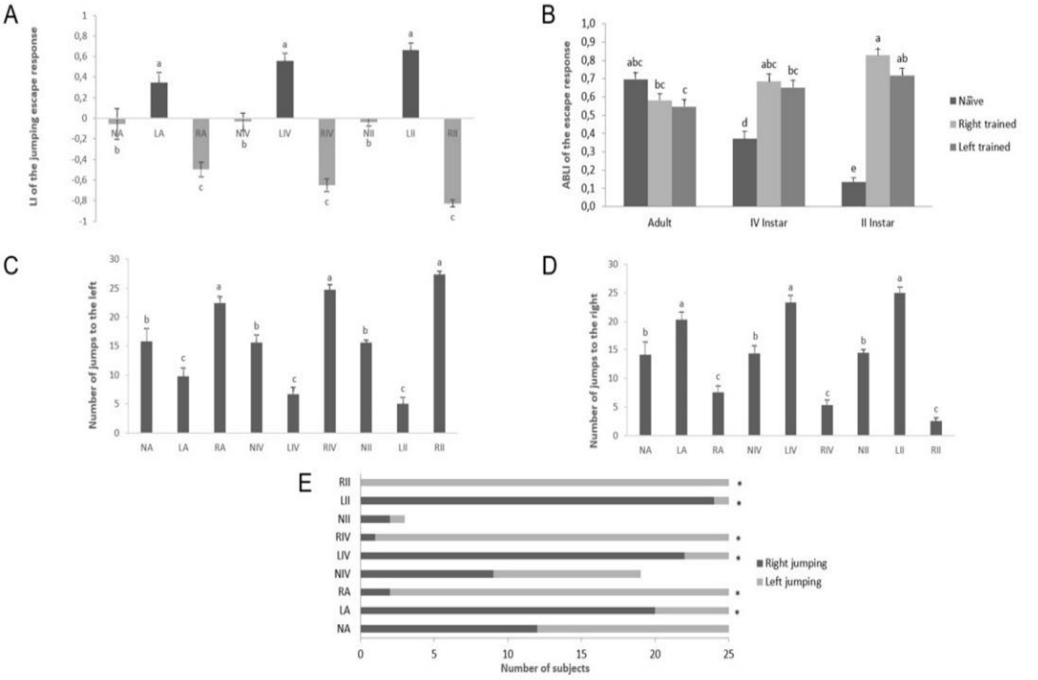
The number of jumps to the left was importantly affected by the training phase ($F_{8,224}$ = 41.080; $P < 0.0001$). For each locust instar considered, the number of left jumps was higher in right trained insects compared to naïve individuals. In addition, the number of left jumps was lower in left trained insects compared to naïve individuals (Fig. 25C).

The number of jumps to the right was significantly affected by the training phase ($F_{8,224}$ = 41.071; $P < 0.0001$). Regardless of the tested locust instars, the number of right jumps was higher in left trained individuals compared to naïve individuals, and the number of right jumps was lower in right trained locusts compared to naïve individuals (Fig. 25D).

The lateralization of the jumping escape at population level was successfully determined in each locust instar involved in a training context (Fig. 25E). The number of naïve adult locusts that preferentially jumped on the left did not significantly differ from the number of naïve adult locusts that preferentially jumped on the right (left vs. right: 13 vs. 12; χ^2_1 = 0.001; P = 0.99). The same results were observed testing naïve IV instar locusts (left vs. right: 10 vs. 9; χ^2_1 = 0.001; P = 0.99), as well as naïve II instar locusts (left vs. right: 2 vs. 1; χ^2_1 = 0.001; P = 0.99).

Right trained locusts preferentially jumped on the left, when approached by the robotic-borne combination of cues, in each instar considered, including adult locusts (left vs. right: 23 vs. 2; χ^2_1 = 16; $P < 0.0001$), IV instar locusts (left vs. right: 24 vs. 1; $\chi^2_1 = 19.36$; $P < 0.001$), and II instar locusts (left vs. right: 25 vs. 0; $\chi^2_1 = 23.04$; $P < 0.001$).

Left trained locusts preferentially jumped on the right when approached by the robotic leopard gecko. This was noted for adult locusts (left vs. right: 5 vs. 20; $\chi^2_1 = 7.84$; $P = 0.0051$), IV instar locusts (left vs. right: 3 vs. 22; $\chi^2_1 = 12.96$; $P = 0.0003$), and II instar locusts (left vs. right: 1 vs. 24; $\chi^2_1 = 19.36$; $P < 0.0001$).



LI = laterality index; ABLI = absolute value of the laterality index; NA = Naïve adult; LA = Left trained adult; RA = Right trained adult; NIV = Naïve IV instar; LIV = Left trained IV instar; RIV = Right trained IV instar; NII = Naïve II instar; LII = Left trained II instar; RII = Right trained II instar.

Figure 25. (A) LI, (B) ABLI, (C) left- (D) and right-biased jumps characterizing the jumping escape response of different trained *Locusta migratoria* during the symmetric exposure to the robotic leopard gecko. Different letters near each bar indicate significant differences (P<0.05). T-bars represent standard errors. **(E) Left- and right-biased *L. migratoria* showing a lateralized jumping escape during the symmetric exposure to a robotic leopard gecko.** Asterisks indicate significant differences between left- and right-biased locusts (P<0.05).

3.2.2.2 Interactive biohybrid experiment 2: modulating the surveillance lateralization

This experiment showed innate lateralization of *L. migratoria* using a preferential eye during surveillance. The poor plasticity of this feature to adapt to environmental perturbations (e.g., a predator-mimicking robotic stimulus), has been also reported.

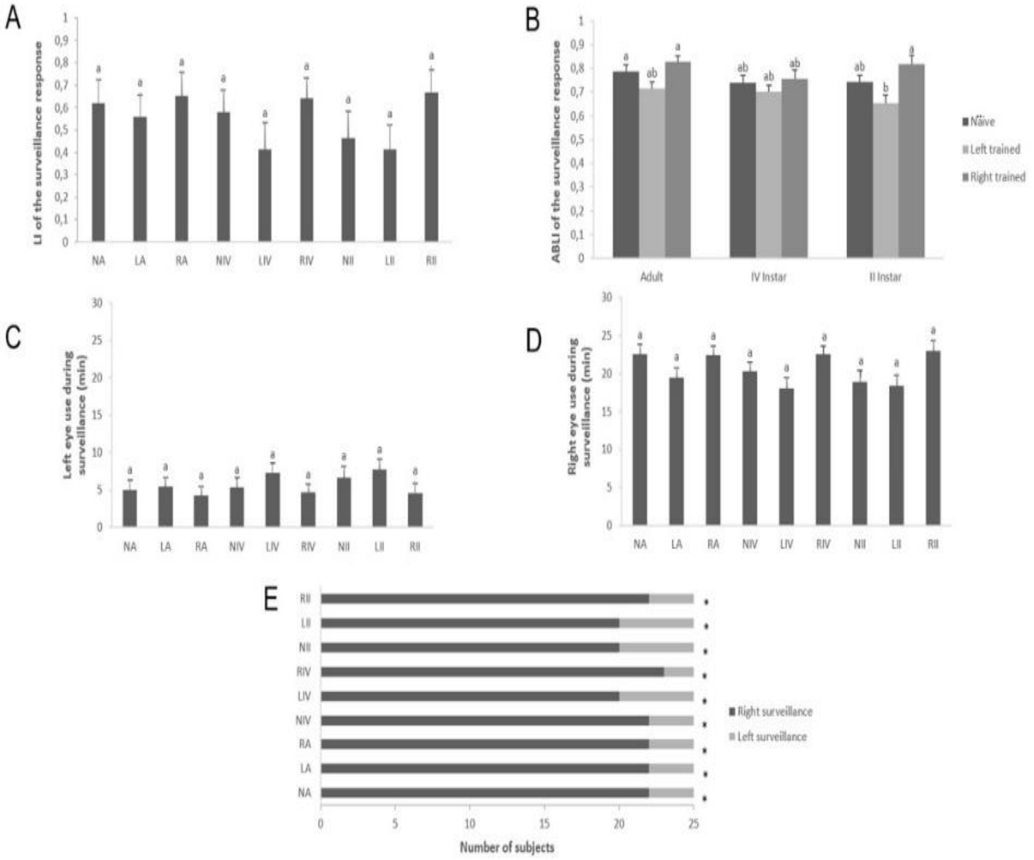
The population mean value of the laterality index (LI) of the eye use, was not significantly influenced by different contexts of the training phase ($F_{8,224}$ = 0.936; P = 0.486). Each training phase produced right-biased locusts, regardless of the instar of the insects (Fig.26A).

The absolute value of the laterality index (ABLI) was marginally modulated by the training phase ($F_{8,224}$ = 3.585; P = 0.0006). Compared to naïve subjects of each developmental stage, only left trained adults, as well as left trained and right trained II instar locusts had a marginally different ABLI (Fig. 26B).

The duration of time intervals in which each locust used the left eye to oversee the robotic predator was not importantly affected by the training phase ($F_{8,224}$ = 0.919; P = 0.501). For each locust instar, the duration of the left eye use to oversee the robotic leopard gecko was similar for naïve, left trained and right trained subjects (Fig. 26C).

The duration of time intervals in which each locust used the right eye to oversee the robotic predator was not significantly affected by the training phase ($F_{8,224}$ = 1.899; P = 0.061). Naïve, left trained and right trained locusts performed the surveillance with the right eye with a similar duration in each locust instar considered (Fig. 26D).

Surveillance lateralization at population level was not affected by training (Fig. 26E). The number of naïve adults (left vs. right: 3 vs. 22; $\chi^2_1 = 12.96$; $P = 0.00031$), left trained adults (left vs. right: 3 vs. 22; $\chi^2_1 = 12.96$; $P = 0.00031$), and right trained adults (left vs. right: 3 vs. 22; $\chi^2_1 = 12.96$; $P = 0.00031$), that preferentially used the right eye to oversee the robotic leopard gecko, was significantly higher compared to naïve, left trained and right trained adults that used preferentially the left eye during surveillance. Also naïve IV instar locusts (left vs. right: 3 vs. 22; $\chi^2_1 = 12.96$; $P = 0.0003$), left trained IV instar locusts (left vs. right: 5 vs. 20; $\chi^2_1 = 7.84$; $P = 0.0051$), and right trained IV instar locusts (left vs. right: 2 vs. 23; $\chi^2_1 = 16$; $P < 0.0001$), as well as naïve II instar locusts (left vs. right: 5 vs. 20; $\chi^2_1 = 7.84$; $P = 0.0051$), left trained II instar locusts (left vs. right: 5 vs. 20; $\chi^2_1 = 7.84$; $P = 0.0051$), and right trained II instar locusts (left vs. right: 3 vs. 22; $\chi^2_1 = 12.96$; $P = 0.00031$), used preferentially the right eye to oversee the robotic leopard gecko.



LI = laterality index; ABLI = absolute value of the laterality index; NA = Naïve adult; LA = Left trained adult; RA = Right trained adult; NIV = Naïve IV instar; LIV = Left trained IV instar; RIV = Right trained IV instar; NII = Naïve II instar; LII = Left trained II instar; RII = Right trained II instar.

Figure 26. (A) LI, (B) ABLI, (C) left- (D) and right-biased eye use characterizing surveillance of different trained *Locusta migratoria* during the symmetric exposure to the robotic leopard gecko. Different letters near each bar indicate significant differences ($P<0.05$). T-bars represent standard errors. **(E) Left- and right-biased *L. migratoria* during the surveillance of a robotic leopard gecko.** Asterisks indicate significant differences between left- and right-biased locusts ($P<0.05$).

3.2.3 Discussion

The results reported in this study offer new and fascinating insights on how a lateralized brain reacts and adapts to dynamic events that are crucial for survival, such as predator-prey interactions.

The most interesting fact is that the same visual cues (e.g., robotic-borne stimuli), laterally perceived during the training phase, were able to influence the jumping direction of subsequent escape responses to symmetric stimuli, but they did not affect the preferential eye use for surveillance. This indicates a high plasticity of those escape motor outputs, that are occurring almost in real time with the perceived stimuli, making them greatly adaptable and compliant to environmental changes, to be effective and reliable.

In particular, I observed that locusts can adapt the direction of the jumping escape to external asymmetric events repeatedly occurring, and to maintain this preference when no stimuli or symmetric stimuli are presented.

I found that the main factor conditioning training is vision, directing sensory information to motor centres and likely producing motor learning at the level of the prothoracic ganglion of locusts (Horridge 1962; Rowell 1961). In fact, motor action was not involved, being jumping escapes prevented by external constrains. Indeed, although insects could make limited movements inside the cage during the training phase, they could not display a real jumping escape.

Several vertebrate species exhibit a higher reactivity when they are approached by predators from their left visual field (controlled by their right hemisphere), (Lippolis et al. 2002; Austin and Rogers 2007), if compared to the right visual field. These studies confirm that in vertebrates the right hemisphere is specialised for the control of fear and escape responses, potentially representing a phylogenetic ancient trait.

Interestingly, my results are consistent with this right hemisphere specialization, although the biological model here was an invertebrate. Indeed, in insects each compound eye is connected with the ipsilateral optic lobe included in the protocerebrum (Strausfeld 2005). Therefore, since locusts preferentially used the right compound eye for surveillance, it can be assumed that also in insects the right hemisphere of the cerebrum controls fear and escape functions.

In addition, surveillance lateralization has been found to have a strict program to adhere to. This is probably part of higher level of brain organization that includes other functions to be carried out in parallel (Vallortigara and Rogers 2005; Romano et al. 2017b). Furthermore, surveillance lateralization is a population-level feature innately occurring in *L. migratoria* aggregations, (Romano et al. 2017b): its low forgeability by environmental factors would avoid disorganization at swarm level and would improve swarm coordination during group tasks.

The highly-lateralised response of young instars, after the training with a leopard gecko-mimicking robot, is particularly interesting, although naïve young individuals do not exhibit innately lateralized jumping escapes (Romano et al. 2017b). This is in contrast with recent evidence in rats (Kurzina et al. 2018), where lateralized motor behaviours in young subjects were little affected by previous learning compared to adults. A possible explanation is consistent with the hypothesis that young individuals belonging to several mammal species are largely depending on parental cares. These mammal species first develop cortical functions such as perception, language, and cognition (Merzenich 2001; Cornelissen et al. 2004; Shtyrov et al. 2010), consequently motor learning is developed slower. Although young locusts do not present innately lateralized traits in the jumping escape, they have shown an impressive reactive motor learning system producing lateralized adaptive behaviours. This is probably due to their greater vulnerability to predation compared to adults.

The very successful induction of lateralized jumping escape in young locusts, besides adults, can be related to an antipredator tactic based on early motor learning to environmental factors. Further efforts are needed to understand how motor learning is affected by lateralized stimuli in young insects belonging to species exhibiting parental care. My findings add fascinating insights to different hypothesis on lateralization in vertebrates and invertebrates that would be determined by a common ancestor or by convergent evolution (Ghirlanda and Vallortigara 2004; Frasnelli 2013).

3.3 CASE STUDY 6: LATERALIZED QUESTING OF A TICK TO A MECHATRONIC APPARATUS DELIVERING HOST-BORNE CUES

During my literature survey searching for behavioural asymmetries in ticks and other mites, I faced a severe lack of knowledge. No researches on the topics have been found for in whole subclass Acarina. More generally, only three studies are available for the Arachnida class (Heuts and Lambrechts 1999; Ades and Ramires 2002; Ruhland et al. 2017).

Ticks are considered among the most dangerous arthropod vectors of diseases agents to both humans and animals worldwide (Colwell et al. 2011; Jongejan and Uilenberg 2004), holding a vector competence surpassed only by mosquitoes (Bissinger and Roe 2010).

Both biotic (e.g., host density) and abiotic (e.g., weather) factors can play an important role in affecting the host-seeking activity in ticks, which is directly correlated with the host risk of contracting tick-borne diseases (Hubálek et al. 2003).

However, ticks cognitive endogenous factors are also worth to be investigated in the host seeking/contacting behaviour. During host seeking, hard ticks (Ixodidae) exhibit a display called questing (e.g. a tick with its forelegs outstretched) as a way of increasing the chances of coming in to contact with a suitable mammal host (Lees 1948).

To my mind, tick questing is an ideal display to investigate behavioural asymmetries, as well as to introduce biorobotics in parasitology investigations.

Here, I investigated if ticks have any lateral bias in the use of the forelegs when a robotic host was presented to a questing tick (Benelli et al. 2018a).

To evaluate if *Ixodes ricinus* (L.) (Ixodiidae) ticks have a preference in using the right or the left foreleg to climb on a host, I developed a mechatronic device that moved a tuft of fox skin with fur as host-mimicking combination of cues.

The mechatronic apparatus delivered host-borne cues frontally to the tick, to evaluate the leg preference during questing as response to a symmetrical stimulus.

Furthermore, I provided the host-borne cues laterally, in an equal proportion to the left and to the right of the tick, to investigate if the host direction affects the tick perception, thus questing behaviour.

3.3.1 Methods

3.3.1.1 Animal rearing and general observations

Ixodes ricinus adult females were obtained from fresh carcasses of *Vulpes Vulpes* L. of both genders shot during the regular hunting seasons in the Province of Pisa (43°N, 10–11° E). Ticks were identified relying to the systematic keys by Estrada-Peña et al., (2004), then stored in clean glass vials (diameter 10 mm, length 50 mm) for 6 hours, until the testing phase. Each tick was provided with a wet filter paper disc (diameter 10 mm) dipped in tap water. Damaged ticks were discarded and not used for behavioural experiments.

Engorged individuals were not selected for the experiments because of their reduced mobility and responsiveness.

Experiment were conducted in March 2017. All observations were carried out in a Petri dish arena (diameter 100 mm; height: 10 mm) from 11:00 to

19:00 hours, at 25 °C and 65% RH. The room was illuminated with fluorescent daylight tubes [16:8 (L:D) photoperiod, lights on at 6:00]. Neon tubes (Philips 30 W/33) were used, and the light intensity in close proximity of the testing arena was 1000 lux, estimated over the 300- to 1100-nm waveband using a LI-1800 spectroradiometer (LICOR, Lincoln, NE, USA), equipped with a remote cosine receptor.

3.3.1.2 Animal replica and experimental apparatus

The host-mimicking device delivering host-borne cues was composed by a servomotor (Hard HS 3004) connected to a rotor (diameter 50 mm) in acrylonitrile butadiene styrene (ABS), designed in SolidWorks and fabricated by rapid prototyping.

As a host-mimicking combination of visual and olfactory stimuli, a tuft of fox fur (length: 60 mm; width: 30 mm), tied to a hole close to the circumference of the rotor, was placed and moved by controlling the servomotor with a microcontroller (Arduino, Mega 2560).

This simple system was located on a suspended platform upon the test arena, to move the fox fur close to a tested tick.

3.3.1.3 Interactive biohybrid experiment

In the first experiment, I used the mechatronic apparatus described above to present the combination of host-mimicking cues to the tick frontally. Once the tick extended the forelegs exhibiting the questing posture, the robotic combination of cues was frontally brought about 5 mm from the tick palps and the leg used to transfer itself on the host-mimicking cues was noted.

Thirty replicates were performed for each tick. Ticks that did not display questing posture or were constrained to the side of the arena were not considered for laterality observations.

In the second experiment, a tick was placed in the testing arena and the combination of host-borne cues was swung perpendicularly to the longitudinal body axis of the tick, 5 mm from its palps, after the tick questing posture occurred.

In both experiments, for each tested tick, I recorded (*i*) the number of climbing attempts occurred when the cues were provided from the left and from the right of the tick, (*ii*) the first leg used to attempt a climbing, as well as (*iii*) the success in climbing (e.g., when a tick intercepted and fixed itself on delivered combination of cues).

Furthermore, the duration of the successful anchored ticks on the stimulus was recorded, evaluating the number of ticks fixed on the host-mimicking mechatronic device for more than 30 seconds. Ticks that were not involved in any seeking behaviour or that were constrained to the side of the arena were discarded. Each tick was tested thirty times. The experimental setup, including the mechatronic apparatus, is depicted in Fig. 27.

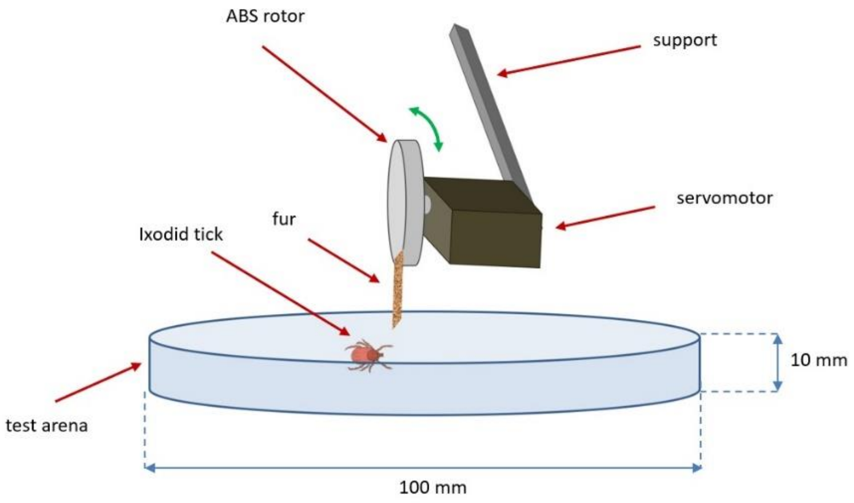


Figure 27. Schematic representation of the mechatronic apparatus used to deliver host-mimicking cues to *Ixodes ricinus* ticks, evoking questing behaviour.

3.3.1.4 Statistical analysis

Concerning *I. ricinus* lateralized questing data, population-level differences in the overall number of ticks displaying a lateralized response was analyzed using a likelihood ratio $\chi 2$ test with Yates' correction (Sokal and Rohlf 1981). Results were considered statistically significant using P=0.05 as threshold. Individual-level lateralization in the tested ticks was computed calculating the laterality index (LI) following the method by Frasnelli et al. (2012):

$LI = (R - L/R + L)$

Where R and L indicate, respectively, the normalized mean number of times in which each tick use the right or the left anterior leg during questing; a score of 1.0 indicated exclusive use of the right leg, while a score of −1.0

indicated exclusive use of the left leg. A score of 0 indicated equal numbers of right and left legs during questing acts.

3.3.2 Results

The lateralized questing behaviour in *I. ricinus* exposed frontally to a mechatronic apparatus delivering a combination of host-mimicking cues was described in Table 3, showing individual level lateralization, with left-biased questing displays (16 on a total of 25 ticks, each tested 30 times). Furthermore, left-biased LI (22 on a total of 25 ticks, each tested 30 times) were observed when the combination of host-mimicking stimuli was presented to the ticks from a lateral side (Table 4). In both experiments, it was observed that the large majority of the tested ticks exhibited left-biased questing acts, if compared to the ones showing right-biased LI values (Tables 3 and 4).

Population-level questing responses of *I. ricinus* were shown in Fig. 28. I noted significant left-biased questing in ticks post-exposure to a combination of host-mimicking cues provided frontally ($\chi 2$= 4.208; *d.f.*=1; $P<0.05$) as well as from the left or the right side ($\chi 2$= 15.423; *d.f.*=1; $P<0.05$) of the tick.

Table 3. Individual-level lateralized questing behaviour in *Ixodes ricinus* ticks exposed frontally to a mechatronic apparatus delivering host-mimicking cues. To calculate the laterality index (LI), each tick was repeatedly tested thirty times.

Tick ID	Right (n)	Left (n)	LI	Individual laterality
1	12	18	-0,2	L
2	3	27	-0,8	L
3	7	23	-0,5	L
4	19	11	0,3	R
5	20	10	0,3	R
6	12	18	-0,2	L
7	15	15	0	0
8	23	7	0,5	R
9	19	11	0,3	R
10	22	8	0,5	R
11	5	25	-0,7	L
12	7	23	-0,5	L
13	8	22	-0,5	L
14	6	24	-0,6	L
15	21	9	0,4	R
16	10	20	-0,3	L
17	10	20	-0,3	L
18	28	2	0,9	R
19	10	20	-0,3	L
20	9	21	-0,4	L
21	17	13	0,1	0
22	7	23	-0,5	L
23	11	19	-0,3	L
24	8	22	-0,5	L
25	3	27	-0,8	L

Table 4. Individual-level lateralized questing behaviour in *Ixodes ricinus* ticks exposed to left- or right-biased host-mimicking cues delivered by a mechatronic apparatus. To calculate the laterality index (LI), each tick was repeatedly tested thirty times.

Tick ID	R-biased cue (n)	L-biased cue (n)	LI cue	Lateralized cue	R leg (n)	L leg (n)	LI leg	Lateralized leg
1	13	17	-0,1	0	8	22	-0,5	L
2	16	14	0,1	0	3	27	-0,8	L
3	13	17	-0,1	0	8	22	-0,5	L
4	12	18	-0,2	L	8	22	-0,5	L
5	14	16	-0,1	0	9	21	-0,4	L
6	18	12	0,2	R	10	20	-0,3	L
7	14	16	-0,1	0	10	20	-0,3	L
8	8	22	-0,5	L	22	8	0,5	R
9	14	16	-0,1	0	2	28	-0,9	L
10	12	18	-0,2	L	11	19	-0,3	L
11	15	15	0	0	6	24	-0,6	L
12	15	15	0	0	7	23	-0,5	L
13	16	14	0,1	0	5	25	-0,7	L
14	15	15	0	0	7	23	-0,5	L
15	22	8	0,5	R	23	7	0,5	R
16	19	11	0,3	R	8	22	-0,5	L
17	15	15	0	0	9	21	-0,4	L
18	18	12	0,2	R	8	22	-0,5	L
19	19	11	0,3	R	22	8	0,5	R
20	16	14	0,1	0	8	22	-0,5	L
21	18	12	0,2	R	10	20	-0,3	L
22	14	16	-0,1	0	3	27	-0,8	L
23	16	14	0,1	0	8	22	-0,5	L
24	15	15	0	0	7	23	-0,5	L
25	18	12	0,2	R	8	22	-0,5	L

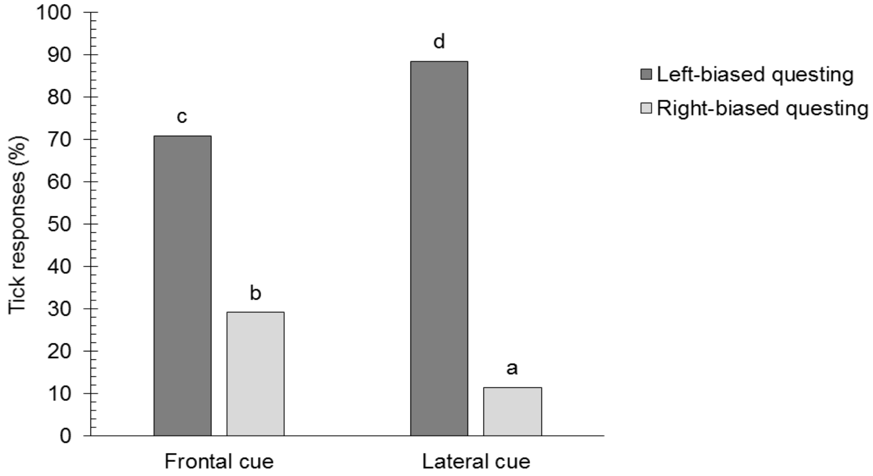


Figure 28. Population-level questing responses of *Ixodes ricinus* adult females postexposure to host-mimicking cues provided frontally or from the left or the right side of the tick, using a mechatronic apparatus. Above each column, different letters indicate significant differences (χ2 test with Yates' correction, P<0.05).

3.3.3 Discussion

A theoretical model on the evolution of asymmetries suggested that lateralization at the population-level is more likely to evolve in social species, while lateralization at an individual-level is more likely to evolve in solitary species (Ghirlanda and Vallortigara 2004; Ghirlanda et al. 2009). However, as outlined by recent evidences on the insects and spiders mentioned above, population-level asymmetric traits have been reported also in several gregarious and solitary species. It has been hypothesized that the population-level behavioural asymmetries found in these species relate to mating and other social interactions (e.g., fighting and escape responses), therefore can be explained by "pre-social" interactions occurring between members of these solitary species and their conspecifics or predators (Frasnelli et al. 2012; Benelli et al. 2015b).

In this scenario, the evidence of population-level lateralized questing in *I. ricinus* represent a peculiar finding. Indeed, the intraspecific interactions among these ticks are limited to strict time frames during their life (i.e., mating or sharing of highly infested hosts), therefore it looks conceivable to argue that the arising of population-level lateralized questing can be linked with the repeated interactions of ticks with their hosts, where lateralized questing allows ticks to enhance cognitive capacity and efficiency of the brain, thus counteracting the ecological disadvantages of lateral biases in behaviour (Vallortigara and Rogers 2005).

Overall, to the best of my knowledge, this is the first report showing evidence of behavioural asymmetries in ticks of medical and veterinary importance, with special reference to laterality in ixodiid questing. The biomimetic mechatronic apparatus presented here allows a repeated and standardized presentation of the cues to the animal, thus creating repeated testing sessions in sequence, a relatively rare result in laterality literature.

Moreover, the mechatronic apparatus developed in this research can be exploited to evaluate the impact of repellent products on tick questing in highly reproducible standardized conditions, which is currently a major challenge in tick management science (Tabari et al. 2017).

Further research is still needed on the proximate mechanisms leading to left-biased questing, shedding light on potential differences in muscular size, exoskeleton robustness and/or nervous innervations between left and right tick anterior legs.

Conclusions and future perspectives

Animal-robot biohybrid interactions can be used to bring new capabilities to current bioinspired systems by producing a thick network of natural and artificial agents that perceive and act in the environment by sharing their abilities collectively.

The scientific and technological potential that these biohybrid systems can have is huge, but still few pathways have been explored. The bulk of the research in this scientific field has been mainly devoted for investigating collective behaviour in social and gregarious species to establish the acceptance of the robotic agent in the animal group, and to study collective interactions from the inside.

This PhD thesis describes, in its different case studies, innovative approaches to establish animal-robot biohybrid interactions to successfully investigate and control unexplored complex and flexible behaviours, by exploiting the synergic contribution between engineering and biology.

The robotic platforms developed here, have relevant bioinspired design features enabling the effective modulation of different behaviours of a species after an in-depth analysis of the animal model behaviour.

Important results are represented by the possibility to modulate the escalation of aggressive behaviours, as well as the intensity of courtship displays, that are deeply involved in the energetics and the physiology of a species. Furthermore, these studies are also pioneers in the use of inedited stimuli to animals (e.g. light stimuli), to evoke and manipulate selected behaviours.

The control of the coalescence of animal aggregations and their location in the space is another big achievement of this thesis.

These findings can importantly be exploited to manage natural systems and to control animals used as biosensors in the environment, pushing beyond the current state of the art in animal-robot mixed societies.

In addition, I introduced biorobotics in neuroethological studies outlining what can be defined as a new paradigm of neuro-robotics. Through biohybrid behavioural interactions, involving animals and robots, it was possible to investigate lateralization in several arthropod species.

Here, biorobotic artifacts provided stimuli that are accurately controllable by humans and that overcome limits related to the direction/orientation of cues during laterality experiments.

This approach has successfully produced new science of considerable interest to the research community. In addition, the findings provided by these researches can be used to design optimized control strategies in artificial systems (Grillner et al. 2007), endowed with a synthetic lateralized neural system.

In addition, in this thesis the first parasitic biohybrid interaction, involving a parasitic species and a host-mimicking robotic apparatus, has been presented. Biorobotics can open new extraordinary opportunities to parasitology-oriented investigation. The employ of these agents as “artificial hosts” would be perfectly in line with the 3R principle “reduce, replace and refine” addressed to limit the use of experimental animals in research and thus avoiding the transmission of diseases, ethical problems regarding animal welfare, and the costs of animals rearing as well (Romano et al. 2018b).

Also interesting is the development of innovative and sustainable bioinspired devices for the control of vectors, parasites and pathogens representing a key threat for humans, and animals.

An important aim of my PhD activity was to understand the role of animal-robot biohybrid systems in the society and bring them out of the laboratory. I studied and designed solutions based on biohybrid systems providing new points of view and strategies to fields of application so far unexplored by biorobotics, such as parasitology. This is not just a good purpose for future perspectives. This is already reality.

The HUBILIFE® Company (Human centered and bio-inpsired ideas for daily life), has been recently founded by prof. Cesare Stefanini, me, and other 2 non-academic partners. HUBILIFE® aims to develop innovative strategies with reduced environmental impact for insect control through bioengineering solutions.

The first product that will be developed (patent application filed), concerns a relatively inexpensive biohybrid device, with electronic parts and "living" parts, able to attract and eliminate mosquitoes (important vectors of a wide range of infectious agents deleterious to humans and animals, such as malaria, dengue, Zika virus, chikungunya and yellow fever). The mechanism of action to eliminate mosquitoes will be based on a revolutionary biomimetic principle that will make the system extremely safe and usable in closed environments, even in the presence of children and/or pets, without releasing harmful substances into the home environment.

Indeed, animal-robot interactions, in addition to new knowledge, can hold to a remarkable socio-economic impact on our daily lives. Their potential application includes the control of animal populations in agriculture, the improvement of animal farming conditions, getting better breeding conditions, and the protection of endangered species.

However, although many robots have been developed for research purposes, robots available outside of the laboratories are very rare in these contexts. A good example of artificial agents interacting with animals in the "real world" can be represented by milking robots that are becoming more and more used in cattle husbandry and dairy production (Jacobs and Siegford 2012). In this case, although robots are not biomimetic, animals directly interact with the machine without involving humans (Pastell et al. 2006). Also smart collars, wearable devices mounted directly on animals, are very common in farms (King 2017). Furthermore, an impressive biomimetic robot (e.g. Robird), has been recently developed to be marketable as a sustainable product for bird control (e.g. to relocate birds around airports) (Folkertsma et al. 2017). This robot mimics in its appearance and flight a rapacious searching for a prey.

Service robots are having a great impact on the market in the domestic environment (Decker et al. 2011; Forlizzi and DiSalvo 2006), and innovative artificial agents used to interact/control animals would have a huge market potential as well.

One of the main challenges that could still limit the developing of these kinds of robots for real applications is represented by the design of biomimetic agents able to perform long-term interactions, and to behave in highly unstructured scenarios (Beer et al. 1997; Li et al. 2012; Stefanini et al. 2012). Further efforts on this are imperative, considering the potential that these devices would have.

Also, it is possible to identify some ethical limitations that can rise in this scientific context.

For instance, people might erroneously be concerned by the fact that scientists are building tools to potentially control all types of animals in the planet, as well as human being, using artificial devices. To provide a good answer to this issue, should be considered that traditional strategies to manage livestock, as well as to control pest and wildlife populations are often carried out by adopting unethical and non-eco-friendly methods (e.g. employ of pesticides and selection hunting). Thus, one of the aims of animal-robot interactions, concerning real-world applications, is to produce advancements in animal wellness and environmental sustainability. In addition, the approach of this scientific field is elegant and minimally stressful for animals as reported in the works presented in this thesis. An additional example is the use of predators in laboratory-staged predator-prey experiments, and real opponents in aggressive behavioural studies, that are no longer ethically acceptable (Huntingford 1984; ASAB/ABS 2004). The use of robotics in studies focused on predator-prey interactions and aggressive behaviour, can be an excellent solution to these issues (Ladu et al. 2015; Romano et al. 2017b; El Khoury et al. 2018).

Finally, my studies presented in this thesis, as well as numerous previous researches on this topic, highlighted the mutual benefit that animal-robot biohybrid systems ensure to both engineering and biology research communities. However, robotic devices are mainly used by few research groups that are highly multidisciplinary. We should not forget the importance that traditional dummies and mirrors had in the study of animal behaviour (Tinbergen 1948). Although these tools have many limitations compared to robots, they are still commonly used in laboratories, since they are easier to manufacture and to use, compared to mechatronic devices. Further efforts are needed in improving worldwide the number of collaborations involving researchers in engineering, and in biology. The increasing in the number of novel scientists with a multidisciplinary background would greatly advance this research field, and in general biorobotics, with advantages to both engineering and biology contexts.

OTHER CONTRIBUTIONS AND SCIENTIFIC DISCOVERIES BY THE AUTHOR DURING HIS PHD

During my PhD I was involved in many additional research activities that lead to important discoveries and to a copious scientific production.

Particularly, numerous studies focused on lateralization and courtship and mating behaviours in several insects species including *Leptomastidea abnormis* Girault (Romano et al. 2016a), *Anagyrus* sp. near *pseudococci* Girault (Romano et al. 2018a), *Lucilia sericata* Meigen (Benelli & Romano 2019), *Trogoderma granarium* Everts (Benelli et al. 2017a), *Tribolium confusum* Jacquelin and *Sitophilus oryzae* L. (Romano et al. 2016b; Benelli et al. 2017b), *Tribolium castaneum* Herbst (Boukouvala et al. 2019), *Ceratitis capitata* Wiedemann (Benelli & Romano 2018), as well as on important ecological interaction among organisms such as the mutual symbiosis between plants and pollinators (Benelli et al. 2017c; Martini et al. 2017).

Furthermore, the kinematics of jumping insects and the impact that different substrate roughness have on the jumping behaviour of *L. migratoria* have been investigated and described in a model (Mo et al. 2019). I contributed significantly to the study of parasitology and of arthropods of veterinary and medical importance (Benelli & Romano 2017; Benelli et al. 2018b), also by investigating innovative strategies and technologies for their management (Romano et al. 2018b), including nanotechnologies (Benelli et al. 2017d; Pavela et al. 2019).

Also other research activities were carried out during my PhD, including the development of a swarm of autonomous robots able to behave in challenging underwater habitats that are restricted from exploration and exploitation due to their vast size, physical (current, pressure, turbidity) and chemical (corrosion, incrustation, fragmentation) conditions (Thenius et al. 2016; Donati et al. 2017). These robots have been developed in the framework of the European Project subCULTron and they operate in out-of-the-lab, real world underwater habitats.

Furthermore, I conducted research activities carried out in the framework of recent advances in methods of using Terahertz (THz) frequency band to study the biohybrid interaction between radiation and biological molecules and tissues.

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