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TESI DI LAUREA

Effects of a simulated heat wave on spatial cognitive abilities and associative learning in the guppy, *Poecilia reticulata*

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Riassunto

Il riscaldamento globale sta portando ad un aumento della frequenza di eventi meteorologici estremi come le heat waves, le ondate di calore. Queste possono essere generalmente definite come un incremento della temperatura massima di almeno 5°C (rispetto alla temperatura massima prevista per quel periodo), per almeno 5 giorni consecutivi. Le ondate di calore non solo hanno un impatto diretto sulla mortalità negli animali, ma possono anche avere conseguenze su tratti comportamentali, compresi quelli cognitivi, che a loro volta possono andare a influenzare mortalità e successo riproduttivo. Le conseguenze dello stress termico sui tratti comportamentali nei teleostei d'acqua dolce sono ben documentate e, come la maggior parte degli organismi, le comunità di acqua dolce dovranno affrontare un numero sempre crescente di eventi estremi come le ondate di calore nei prossimi decenni. Di conseguenza, è importante studiare l'effetto dello stress termico sulle loro abilità cognitive, per poter meglio comprendere l'effetto che questo può avere sulle popolazioni animali. In questo studio sono stati testati gli effetti di un ondata di calore simulata sulle capacità cognitive spaziali e sull'apprendimento associativo nell'organismo modello *Poecilia reticulata*. In studi precedenti è stata dimostrata la capacità dei guppy di apprendere e navigare un labirinto. Pertanto in questo studio è stato deciso di utilizzare un labirinto per testare le abilità cognitive spaziali dei guppy e verificare se esse potessero essere influenzate da un ondata di calore. Le abilità cognitive di maschi recentemente esposti ad un ondata di calore simulata sono state confrontate con quelle dei maschi tenuti ad una temperatura di controllo. Mentre nei maschi tenuti ad una temperatura di controllo il tempo impiegato per raggiungere la fine del labirinto e il numero di errori commessi sono mediamente diminuiti con l'aumentare del numero di prove, nei maschi esposti ad un ondata di calore questo miglioramento non è stato osservato. Questo risultato, in linea con i risultati ottenuti in altri organismi, suggerisce che anche in *Poecilia reticulata* lo stess termico, in particolare quello causato da un ondata di calore, può influire negativamente sulle abilità cognitive.

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1. ABSTRACT

Global warming is leading to a greater frequency of heat waves, generally defined as an increase in temperature of at least 5°C compared to the average maximum temperature for at least 5 days. Heat waves do not only increase mortality in animals, but can also have important consequences on behavioral traits, including cognitively demanding ones, which in turn can influence mortality and reproductive success. Thermal conditions are well documented to affect behavioral traits in freshwater teleosts, and like most organisms, freshwater communities will have to deal with an increasing number of extreme heat events in the coming decades. Thus, it is important to study the effect of heat stress on cognitive behaviors to better understand the consequences on animal populations. Here, we tested the effects of a simulated heat wave on spatial cognitive abilities and associative learning in the model organism *Poecilia reticulata*. Previous studies have demonstrated the ability of guppies to learn to navigate a simple maze. Therefore, we used maze learning as an indicator of spatial cognitive abilities in order to test whether heat waves affect this aspect of cognition. Using this test, we compared the cognitive abilities of males recently exposed to a simulated heat wave to males maintained at a control temperature. The results will provide experimental evidence for the effects of heat waves on cognition and, specifically, may help us better understand the consequences of events of extreme heat on freshwater species.

2. INTRODUCTION

2.1 Heat waves

Climate change is increasing the frequency and magnitude of extreme weather events such as droughts, heavy precipitations, and cold and heat waves (Robinson et al., 2021), that are only projected to increase in the coming years (Coffel et al., 2018). Despite evidence that climate change can have adverse effects on animal populations (Laloë et al., 2021; Stillman, 2019) the focus when studying the impact of climate change is largely on the increasing mean temperature, while the influence of extreme events is often neglected. One challenge in those studies is the lack of a standardized definition for a heat wave. The World Meteorological Organization (WMO) defines a heat wave as "five or more consecutive days during which the daily maximum temperature surpasses the average maximum temperature by 5°C or more" (www.britannica.com), but some countries have adopted their own definitions. One problem with local standards is that they have been created considering the climate of the region, and are therefore deficient when comparing studies from different areas of the world. Definitions vary not only in the threshold temperature, either as a specific temperature, as a set number of degrees above the average/maximum temperature or as a temperature above a specific percentile (usually the 90th or the 95th) of the maximum temperature, but also in the minimum number of consecutive days for it to be considered an extreme weather event, usually between 2 and 7 (Xu et al., 2016). For example, in Greece a heat wave is defined as a period of at least 3 consecutive days when temperatures above 39°C are recorded in widespread areas (www.emy.gr), while in Denmark as a period of 3 consecutive days at or above 28°C (www.dmi.dk). In other countries, the standard varies even between regions: in Italy, the definition varies between cities (although temperatures above the 90th percentile are usually considered) (www.salute.gov.it). In Germany, the definition varies among states, and considers a temperature above the 98th percentile for more than 3 days, or a temperature above 28°C (www.dwd.de). In the UK, a heat wave is defined as a period of 3 or more days in which the daily maximum temperature meets or exceeds the heat wave temperature threshold, which varies among UK counties (www.metoffice.gov.uk).

2.2 Effect of Heat Stress on Animals

Both the steady increase in global air and water temperature and frequency (and intensity) of extreme weather events are having an effect on animal populations, modifying their demography and phenology: populations migrate to a different latitude or altitude and anticipate or delay their migratory or hibernation periods (Ozgul et al., 2010; Pecl et al., 2017). Those changes do not only modify the interspecific interaction between species, but also their ability to forage and in general to obtain resources, which impacts reproductive success and survival rates and, in turn, the population demography (Soravia et al., 2021). In the case of migration, for many migratory birds of Nord America the mean arrival date of migration is changing due to the increase in spring temperatures, changing more drastically in species with longer migratory routes (Hurlbert & Liang, 2012). On the other hand, some organisms may be limited in their ability to range shift if their physiology requires environmental parameters that are unlikely to change due to climate change, as is the case for reef-building corals. These corals often inhabit locations where the increase in temperature during a heat wave is beyond their termal tolerance, resulting in coral bleaching. While they could slowly range shift to higher latitudes, their physiology requires the tropical photoperiod all year round to support their photosymbiosis. Therefore, their ability to range shift towards cooler areas is limited, and in the meantime their habitat shrinks as corals retract from hotter areas, resulting in a lower range size and a decline in population (Palumbi eta al., 2014; Stillman, 2019). The effect that this steady increase in air and water temperatures and heat waves have on animal populations is intertwined: as the global temperature increase, so does the frequency and intensity of heat waves, affecting similar aspect of animal life in different time frames. In the American black bear (*Ursus americanus*) the duration of hibernation is shortening due to the higher temperature during winter compared to the past; one study (Johnson et al., 2018) shows that for each 1°C increase in the winter minimum temperature, the hibernation was reduced by an average of 6 days, which means that the animal may emerge from hibernation before spring food resources are available in sufficient quantity. In the Richardson’s ground squirrel (*Urocitellus richardsonii*), an heat wave registered in march 2012 in Winnipeg, Manitoba (Canada) led to an earlier emergence from hibernation of females, who commenced breeding earlier than usual, while not all males were physiologically prepared to breed, as 58.6% of them had non-motile sperm (Kucheravy et al., 2021). Heat stress also has an effect on the life cycle of many animals. In the sea urchin (*Heliocidaris erythrogramma)* the effect of an heat wave across the metamorphic transition from larvae to juvenile was shown to be detrimental after 10 days of constant heat stress. Sea urchins showed high tolerance if the duration was reduced to 5 to 7 days (Gall et al., 2006).

When experiencing heat stress during a heat wave, animals can modify their behavior to seek shelter and move to cooler areas, but this may bring to increased predation risk, lowering the rates of reproduction and the opportunities to forage resources (Bartholomew 1964). It is to note that the effect of heat stress on organisms may vary widely not only because of different environmental conditions, but also because of the different thermal tolerance of the organisms, as this is affected by factors such as body mass, life stage and sex even within the same species. In the *Littorina obtusata,* an intertidal gastropod, thermal tolerance varies during life stages: early life stages are more sensitive to high temperature compared to adults, but only if the increase in temperature is short, while if that occurs over a longer period of time it is the opposite, with the adult stage being more sensitive to heat stress of longer duration (Truebano et al., 2018). One important thing to note is that during development, and to a lesser extent adulthood, individuals exposed to an increase in the environmental temperature may increase their thermal tolerance, but this is achieved only in the case of slow and gradual temperature increase such as seasonal changes, and not in the case of harsh and unpredictable events such as heat waves (Cooper et al., 2019).

All this means that, when assessing the effects of heat stress on behavior on an organism, it is not only necessary to measure environmental temperature and exposure duration in a relevant scale, but also to consider all individual traits that could influence the organism's performance, such as sex, size and life stage. If possible, it may also be good to consider the thermal conditions experienced during development by the individuals, as they can influence the thermal tolerance of the adult individual.

2.3 Cognition

Cognition may be defined as the neuronal processes involved with attainment, retention and use of information (Dukas, 2004). Information acquired by an organism about its environment through its sensory organs must be refined before it can be used to make behavioral decisions, which necessitate multiple cognitive mechanisms: attentional mechanisms and inhibitory control to focus only on relevant stimuli; learning mechanisms to associate stimuli to form predictions; memory to retain information; and decision making mechanisms to evaluate the appropriate behavioral response to the stimuli (Dukas, 2004). Cognition processes are not directly observable, thus it is necessary to infer them through experimentation. By measuring the responses of the organism in a particular task, we can obtain a measure of its cognitive performance (Thornton et al., 2014). Cognitive performance is also often associated with fitness, in particular when considering spatial abilities and memories. These give an individual an advantage in finding and remembering shelter and food location, as is the case in *Poecile gambeli,* the mountain chickadee. It has been found that individuals who performed better in a spatial memory test had better chances of surviving the winter. This is explained by their habit of hiding huge quantities of food before winter to retrieve it during the colder months, a task requiring spatial memory abilities (Sonnenberg et al., 2019). Reproductive success may also be impacted by cognitive performance, as in the case of the New Zealand robin (*Petroica longipes*); individuals who showed higher cognitive performance (i.e., performed better in the tested cognitive task) raised on average more offspring per brood (Shaw et al., 2019). On the other hand, this is not always the case, as there may be a tradeoff between fitness-related traits and the higher investment in more energetically-costly brain tissue associated with higher cognitive performance (Burger et al., 2008). Nonetheless, even when no correlation between higher cognitive performance and fitness is found, below average performance could still negatively impact fitness.

The main cognitive traits include perception, attention, learning, memory and decision making. Perception is the translation of signals from the environment, gathered through sensory organs, into neuronal representation. Attention refers to the neuronal activation at any given time. Memory is the passive representation of already acquired information. Decision making is the ability to measure the already acquired information and the environmental variables to take an action (Dukas, 2004). Finally, learning is an animal's ability to employ information acquired in past experiences and use it to inform future behaviors (Cauchoix & Chaine, 2016). This includes both non-associative forms of learning, such as habituation and sensitization, and associative learning. Associative learning is a form of learning in which connections are made either between unconditioned and conditioned stimuli, as is the case for classical conditioning, or between a specific behavior and some stimuli, as is the case for operant conditioning (Salena et al., 2021). In classical conditioning an association is made between a neutral stimuli, such as a specific color, with a biologically potent one, such as food, while in operant conditioning the strength of a behavior is altered through punishment or reinforcement.

One specific aspect of cognition that is being extensively investigated is the field of spatial cognition. Spatial cognition is defined as the ability to acquire and reorganize spatial information to make sense of an environment, and is involved in many behaviors such as mating, foraging, migrating and predatory avoidance, and can be inferred through tests such as a maze learning task (Salena et al., 2021). Maze learning is defined as "a learning process which involves circumnavigating a subject being presented with a variety of false routes into the core subject, however over time the learner, if successful, can reach the end goal or aim in the least amount of time by applying their previously held knowledge, and it is considered a good indicator of spatial cognitive abilities and associative learning abilities" (www.PsychologyDictionary.org). The test consists of a maze, a system of paths and dead ends that can be simple or more complex depending on its structure and number of dead ends, and a reward at the end. Usually, the test starts by releasing the studied individual in a defined "starting point" and letting it navigate the maze until it reaches the end where a reward, usually food, shelter, a social reward and/or a partner is given to it. The process is repeated multiple times in a given amount of time, usually days, recording the search time and/or the number of errors in each attempt.

2.4 Effect of Heat Stress on Cognition

Heat stress has been documented to negatively affect cognitive abilities in both human and non-human animals (Mazloumi et al., 2014, Soravia et al., 2021). Being a stressor, heat can lower attention and impair decision making by diverting resources from cognitive processes. These resources are instead employed to monitor the threat presented by heat. The attentional shift can lower survival rates, as in the wild even a small decrease in attention and reaction speed may prove fatal when reacting to predators (Maille & Schradin, 2016; Maille & Schradin 2017). As previously noted, a gradual temperature increase can be mitigated by organisms thanks to adaptive responses to thermal stress, but sudden and intense heat stress has been found to have dramatic effects such as necrosis in goldfish and convulsions in mice (Cerf & Otis, 1957; Kane & Jarvik, 1970). Nonetheless, the effects on cognition are manifested even before the appearance of severe symptoms: in studies spanning multiple taxa, it has been found that intense heat stress may negatively impact memory and spatial learning. Western Australian Magpies (*Cracticus tibicen dorsalis*) during heat stress conditions display markers of heat dissipation, such as wings splaying and panting. Magpies displaying markers of heat dissipation during a heat wave showed lower performance in an associative learning task. Interestingly, it was found that there was a threshold temperature (31,83°C) after which the performance dropped abruptly, with only 14% of individuals passing the test above this temperature compared to the 81% able to pass it when the temperature was lower (Blackburn et al., 2022). This shows how heat stress may not have a linear effect on cognitive performance in some organisms. In mice (*Mus musculus*), individuals exposed to high temperatures (43°C) for 7, 14 or 42 days showed impaired long term memory and learning abilities, tested with an Y-maze, a passive avoidance test and a novel object recognition task (Lee et al., 2015). This is also true for invertebrates: in fruit flies (*Drosophila melanogaster*) even a short exposure (19 minutes) to a temperature 9°C above the control impairs aversive olfactory memory retention (Zhang et al., 2008). Bumblebees (*Bombus terrestris*), a species requiring a wide array of cognitive abilities to forage effectively, showed lower performance in an associative learning and memory test under the effect of heat stress. Similarly, both their foraging performance and flying ability diminished at higher temperature (Gérard et al., 2022). The effects may be temporary, since the decrease in cognitive performance may last only as long as the temperature is increased and shortly after, or be sustained for a longer period of time, but no conclusive studies tackled this subject. Lastly, heat stress may have long-lasting effects on organisms by altering brain development during early life stages. Memory, spatial learning and motor skills in later life stages may be impacted. In reptiles, incubation temperature experienced by embryos may influence their learning ability, and due to global warming many reptile species eggs are subjected to higher-than-average temperatures. For example, in the Velvet Gecko (*Amalosia lesueurii*), hatchlings born from eggs incubated at higher temperature showed decreased spatial learning abilities compared to hatchlings born from eggs incubated at lower temperature. Furthermore, the same hatchlings born from eggs incubated at higher temperature had less probability of surviving in the wild, as spatial learning was directly correlated with survival in the wild (Dayananda et al., 2017). Similar results have been found in some invertebrates: in *Drosophila,* exposure to a daily episode of heat stress during the larval stage correlates with associative odor learning impairment (Wang et al., 2007).

While there is a sizable library of studies investigating the effects of heat stress on cognition, most of them only focused on the effects of very short-duration (usually spanning only a few minutes) and high-intensity heat stress (Soravia et al., 2021). In the context of climate change, a perhaps more relevant ecological significance would be to test the effects of heat stress of longer duration, as in a heat wave. On the other hand, when possible, the best option is studying the effect of an actual heat wave in the field, as was the case for magpies (Blackburn et al., 2022), but this is not always achievable.

2.5 Aims of the Study

As illustrated above, the effects of climate change are well documented as having an impact on animal communities on our planet, and there is an important focus by the scientific community in understanding this phenomenon and its effects. But while a vast number of studies have been published in this regard, only a fraction of those investigates the effect on cognitive abilities. In this context, the aim of the present study was to gauge the effect that heat waves may have on cognitive abilities using an ectotherm, *Poecilia reticulata, as a model organism.* This could help better understand the effects of extreme weather events on animal populations and how to mitigate them. This study focused on studying the effects of heat waves on some cognitive abilities, i.e., spatial and associative learning. To do so, the fish were subjected to a 5-day-long heat wave and then tested for a week. The test was a maze learning task, carried out in a simple maze with a reward at the end, in the form of a female. This species was chosen for multiple reasons; being a model species, there is a vast literature on its behavior and, more crucial for this study, cognitive abilities. In particular, several past studies documented this species abilities in spatial learning. Beyond this, guppies are small, easy to handle and care for, allowing for a high number of tests in a short period. Due to previous studies showing the ability of guppies to learn both simple and complex mazes (Kellogg & Gavin, 1960; Kotrshal et al., 2015; Lucon-Xiccato & Bisazza, 2017), we expected the control fish, not subjected to the heat wave, to gradually reduce the time required to exit (successfully complete) the maze and reach the reward after each trial, while also simultaneously decreasing the number of errors. On the other hand, we expected the fish from the group subjected to the heat wave to either: 1) not be able to complete the maze; 2) be able to complete the maze but without improving the time after each trial; or 3) to improve the time with each trial but to be a slower learner than the control group.

There is a lack of studies on the effects of heat waves on guppies, with the exception of a study (Breedveld et al., unpublished data) that found sex-specific effect of heat wave exposure on survival, behavior and fertility. Due to the lack of knowledge, this study will be crucial as a first step towards the development of a better understanding on the consequences of heat waves on guppies' cognitive abilities. And as seen above, cognitive performance may be associated with higher fitness, although no study confirming this hypothesis has been performed on guppies.

2.6 *Poecilia reticulata* and Maze Learning

The guppy, *P. reticulata,* is a freshwater tropical fish of the family Poecilidae, originating from northern South America but nowadays found in many environments all over the world. They are used as model organisms in the fields of evolutionary biology, ecology and behavioural ecology. Wild guppies live in a wide range of temperatures, from 23°C to 32°C, and have been reported to experience fluctuation up to 7°C daily (Reeve et al., 2014). Guppies from different populations may have adapted to withstand different temperature ranges and not all of them may be able to endure the same amount of heat stress. Environmental perturbations such as pollutant and heat stress have been shown to influence behaviour in guppies, in particular reproductive and shoaling behaviour (Breedveld et al., unpublished data; Mason et al., 2021), but less attention has been placed on the effects on cognition. Despite the lack of studies about the effect of heat stress on guppy's cognition, the literature on this organism's cognitive abilities is ample, with a wide range of studies investigating learning, memory, inhibitory control, numerical abilities (Burns et al., 2008; Dugatkin et al., 2003; Kniel et al., 2020; Agrillo et al., 2012). The focus of this study is guppies' spatial learning ability. Guppies have shown the ability to navigate both simple and complex mazes (Kellogg & Gavin, 1960; Kotrshal et al., 2015; Lucon-Xiccato & Bisazza, 2017), an ability associable to their natural habitat, characterized by rivers fragmenting into riffles and pools and an abundant presence of stones, plants and roots, an environment that requires high spatial cognitive abilities to navigate. Maze learning has already been used in guppy to study the cognitive abilities. For example, maze learning has been used to investigate the effect of brain size in males, with the conclusion that bigger brains are associated with better performance in the maze, as the search time decreased faster after each trial in big-brain males compared to small-brain males (Kotrshal et al, 2015).

3. MATERIALS AND METHODS

3.1 Overview of the experimental design

In this experiment a simple maze was used to evaluate the spatial abilities of guppies, comparing the performance in a maze learning task of the control group with the performance of the group that experienced a simulated heat wave. The heat wave group was composed of male adult guppies that were exposed to a 5° temperature increase for 5 days. Fish from both groups were tested three times per day for 4 consecutive days, albeit the first day was only used to acclimate the fish and those tests were not considered in the final analysis. The testing consisted of a maze learning task where the males had to reach the end of a simple maze with a social reward at the end, i.e., a virgin adult female (as in Kotrshal et al, 2015). The performance of each fish was measured and recorded; specifically, the measures considered where the ability to complete a trial, the search time, the number of errors, and the latency time.

3.2 Experimental subjects

For this experiment, 82 male guppies were used. The guppies used are descendant of a wild-caught population in Trinidad (original habitat of the species) and maintained in semi-natural conditions in a large outdoor pond in Padua's Giardino Botanico, where they have been introduced in 2012. The collected guppies, used in this experiment as adults, were then kept indoor in 120 litre-tanks with a gravel bottom, plants, aerator and water filter, and raised till adulthood. In the lab, fish are maintained at a light-dark cycle of 12:12 h, and fed twice per day commercial food flakes or live *Artemia salina* nauplii. The guppies used were born in two different months, in October 2021 and in January 2022. Guppies of wild descent were used instead of commercial ones, despite previous study demonstrating better performance in commercial guppies in this kind of tests and a lower propensity to freezing behaviour (i.e., to lay still at the bottom of the tank for more than 2s, immobile except for eyes and gills) (Lucon-Xiccato & Bisazza, 2017), because commercial guppies may have been selected, intentionally or not, for traits that could impact their performance in this test (Salena et al., 2021).

3.3 Treatment

The experiment was structured in 9 blocks. Each block lasted for 2 weeks, and consisted in a treatment phase and a testing phase, during the first and second week, respectively. The testing phase of each block overlapped with the treatment phase of the successive block. During the treatment phase the fish were either exposed to a simulated heat wave or, in the case of the control group, kept at a constant temperature. This phase lasted from Day 1 to Day 7. The day after, on Day 8, the testing phase began, lasting 4 days until Day 11. In each block, nine to ten fish were tested. One week before the testing the males were moved to smaller tanks where the temperature could be easily controlled. Five males, together with five females for social enrichment, were moved to the "heat wave" tank on Day 1. Here, the temperature was maintained at 26± 1°C. On Day 2, the temperature was gradually increased up to 31± 1°C, and kept for 5 more days until Day 7, when the temperature was gradually lowered to 26± 1°C again. Five other males, together with five other females, were instead assigned to the "control" tank where the temperature was kept at 26± 1°C. The temperatures were recorded using data loggers (Figure 1). During treatment and testing all fish were fed ad libitum twice per day with commercial food flakes. After the treatment, each male fish was moved to a separate tank where it would stay, alone for individual identification, for the next week when the testing phase would take place. The water temperature of these tanks was likewise kept at 26± 1°C.

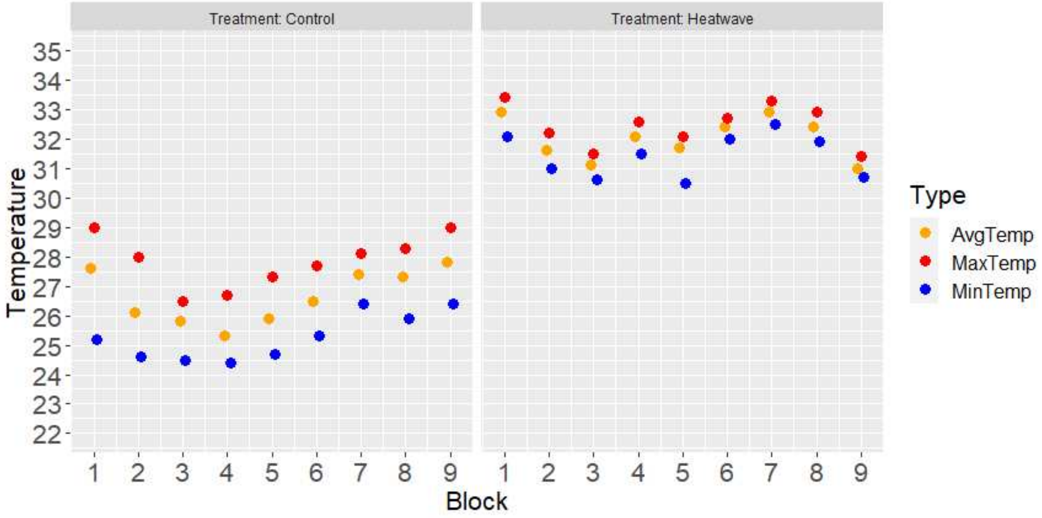

Figure 1: Average (yellow), Maximum (Red) and Minimum (Blue) temperatures recorded by data loggers across the 9 blocks.

3.4 Apparatus

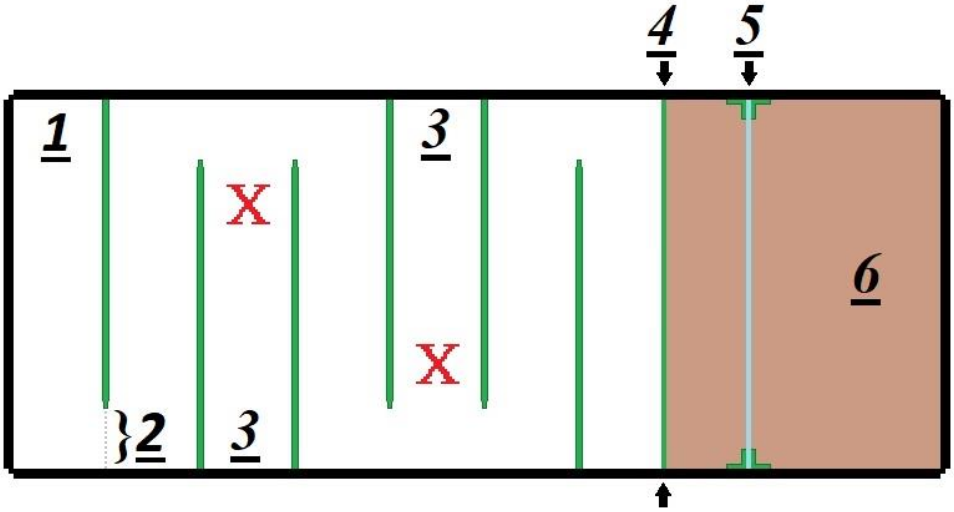

Figure 2: Maze diagram.

1)Test male starting position *2) First section threshold* *3) Dead ends*
4) Final threshold *5) Liftable clear plastic door* *6) Reward(virgin female)*

The maze was built out of a 19 cm x 47 cm glass aquarium, with walls covered by green plastic to hide the surrounding from the inside, and the bottom covered by white sand for ¾ of the length (see Figure 2). This was done to provide a better contrast between the fish and the background for the recording device. The final part of the maze floor was instead covered with gray gravel instead and some vegetation was present to provide a more appealing environment, similar to that of the housing tanks. This is the part of the maze were the female would later be situated. A low green plastic barrier was placed between the first and final part of the maze (Figure 2, indicated by the arrows) to divide the white sand from the gravel and, more importantly, was used as the finish line for the test, and will be called thereafter the Final Threshold. To form the corridors the fish had to navigate, 6 green plastic barriers were made with corrugated plastic placed at regular intervals (5 cm), glued on one side, leaving an opening on the other side where the fish could swim through. This formed a corridor the fish had to swim through to reach the end, and divided the maze in 7 sections. Each section was delimited by two plastic barriers except for the first one, delimited by a barrier and the tank glass wall, and the last one, delimited by a barrier and the final threshold. To increase the difficulty, the maze also contained 2 dead ends. The width of the corridor and the dead ends was 5 cm. This width was chosen as previous studies demonstrated an increased willingness of guppies to explore a maze and reach the end if the corridors were narrow (Kellogg & Gavin, 1960). The structure of the apparatus was constructed to mimic the shallow waters the males have to navigate to reach the females in their natural environment. At the end of the maze, a receptive virgin female was placed behind a clear plastic wall that was lifted once the male passed the final threshold. At the starting point a plastic container was placed, made of clear plastic, where the fish was placed right before the start of the test. The container was liftable with the help of a thread and open on the top and the bottom. While the container was lowered in the maze, a fish placed inside (thanks to the opening on the top) could not escape as the bottom opening was closed by the sand at the bottom of the aquarium and the top was a couple of centimetres above the surface of the water. A webcam (Panasonic HC-V180) was mounted 1 meter above the apparatus to record all the fish movements and to watch in real time the movements without having to look directly at the maze, to avoid scaring the fish during testing. The maze was illuminated by 2 led light placed on the sides of the maze, but oriented in such a way for the light to not be reflected by the water surface, to not distract the fish during testing. When no tests were in progress, an heater was placed in the maze to keep the temperature of the maze the same as the temperature of the tanks (26± 1°C).

During a preliminary testing, a different maze design was used. This prototype was more complex, with a higher number of dead ends and possible routes, and also 3 clear plastic windows in the middle part. The plastic windows allowed the tested subject to see the reward from its starting position and while exploring the maze. Ultimately, the design was discarded as it was too complex, since fish not subjected to the heat wave were not able to complete it.

3.5 Females (social reward)

A virgin female guppy was used as a social reward and placed at the end of the maze. Virgin female guppies are more likely to accept male mating attempts and male guppies are more likely to copulate with a virgin than with a mated female (Guevara-Fiore et al., 2009), therefore only virgin females of reproductive age were used. 15 virgin females were kept in a tank with gravel bottom, plants, aerator and water filter. 10 minutes before testing, a female from the tank was transferred to the end of the maze (with the plastic door closed) and let to acclimate. When the male reached the end of the maze, females where allowed to interact with it for 120s by lifting the plastic door. During this time they were observed to make sure no mating occurred, to ensure that the female where still virgin and fit for the following trials. After all fish were tested once, the female was removed and placed in a secondary tank until the end of the week. Then, another female from the main tank was taken and placed at the end of the maze following the same procedure as before. This was repeated 3 times in total each day, so that each male would see each female only once per day, and maximum once per week. At the end of the week, all females were moved back to the main tank. During preliminary testing we experimented with a slightly different social reward: beyond the final plastic door were placed three males and three females, and the door remained closed and the fish could not directly interact with them. Despite a previous study with a similar setup suggested that this reward could work (Prentice et al., 2021), during testing the control fish did not improve their time and showed low interest in the reward.

3.6 Testing procedures

The fish were split into individual tanks on Day 7, when the treatment (heat wave or control) ended, and the testing started the day after. Each fish was fed right before the first test and after the last test of the day. The testing took place between 10:00 AM and 02:00 PM each day, from Day 8 to Day 11. Each fish was tested 3 times each day with one hour of gap between the trials. This was done to avoid stress on the fish , as in preliminary testing we found that the performance drastically worsened if the trials were too close in time. The order in which the fish were tested was kept constant during the 12 trials. During the first 3 trials, on Day 8, the times were recorded but not included in the final calculations. This was done to leave the first day for the fish to acclimate and explore the maze. In all calculations and plots, the first trial of the second day of testing, Day 9, have been then considered as the first trial, so that by the end of the week the dataset is composed of 9 trials. Before the trial, the fish was captured from its tank and placed in a beaker with water from the maze and from the tank, for 60s, to acclimate and for easier placement in the maze. Then, at the beginning of each trial the fish was placed in the plastic container at the beginning of the maze. After 60s of acclimation, the container was lifted and the fish was free to explore the maze. During the exploration the number of error made by the fish was noted and scored. Two types of error where considered: wrong turn and backtracking. Each time the fish entered a dead end was considered a wrong turn. Each time the fish swam back for at least a section of the maze was considered a backtracking error (Figure 3). When the fish crossed the threshold line at the end of the maze (Figure 2, indicated by the arrows), the time was stopped and the plastic door lifted, releasing the female. The two fish were then allowed to interact for 120 seconds. After 120s passed, the male was removed with a net and placed back in his tank. The female was instead chased back into the final section and the plastic door was closed. Two times were recorded during the trials: latency time and total time. Total time was the time from the lifting of the plastic container to the crossing of the final threshold, while latency time was the time spent by the fish in the first section before starting to explore the maze right after the lifting of the plastic container and before crossing the first threshold (Figure 2). If the fish could not complete the trial in 10 minutes total (after the plastic container was lifted), it was removed and the trial considered a failure. The same was done if the fish did not exit the first section of the maze after 7 minutes after the lifting of the plastic container. In both cases, failures were considered as the maximum possible time of 10 minutes during data analysis. After the time was up, the fish was guided towards the end of the maze and rewarded with access to the female anyway. Fish that died throughout the week or failed to complete the maze in half or more of the trials were removed from the data pool. During data analysis the latency time was subtracted from the total time to obtain the partial time. This is a more representative measurement of search time, also used in similar maze studies (Kotrshal et al, 2015), and will be referred as such form this point on. This partial time is a better representation of search time as it represents the totality of the time the fish spent in the maze exploring it without the latency, time spent in the starting area that is more correlated with personality and boldness (Salena et al., 2021).

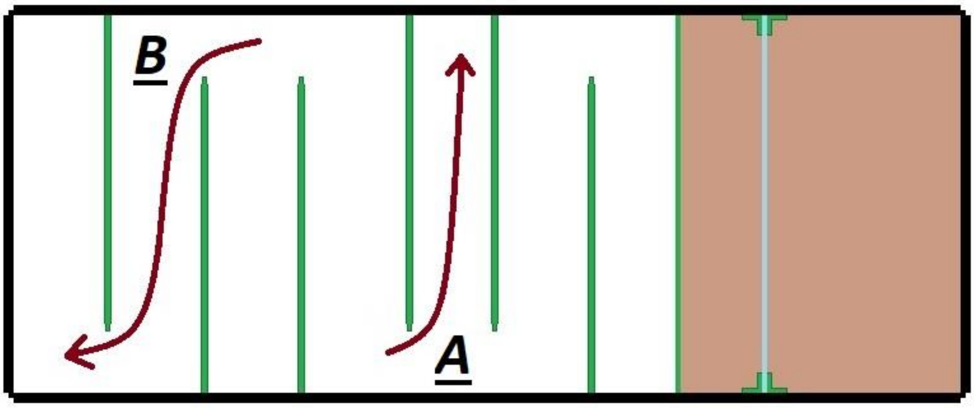

Figure 3: Examples of errors:

A) Wrong Turn: the test male enters a dead end

B) Backtracking: the test male swims back for at least a whole section of the maze

Four variables were considered and analyzed: the ability of a fish to complete the trial, the search time, the total number of errors and the latency time. The ability to complete a trial refers to a binary value that identify if the fish was or was not able to finish the trial in 10 minutes. The search time refers to the time spent by the male in the maze after its exit from the starting section and before reaching the end. This measure, together with the number of errors, was not particularly useful alone, as those measure are strongly individual dependant and cannot be compared with one another. Instead, they can be plotted against the trial number to show if the fish is improving or not, and this plot can be considered a measure of the spatial maze learning performance of the tested individual, i.e., how much the male is exhibiting the ability to learn how to reach the end of the maze faster (or more accurately in the case of total errors). The last measure taken was the latency time, that is the time spent in the first section of the maze after its release, before it starts exploring the maze. This measure is not correlated with maze learning abilities but with neophobia, the aversion to a novel stimuli, in this case the novel environment presented by the maze.

3.7 Statistical Analysis

Data analysis was conducted using RStudio 2022.07.2 build 576

All data were analysed using either linear mixed models (LMMs) using "lmer" function or generalized linear mixed models (GLMMs) via "glmer" function from the "stats" and "lme4" packages (Bates et al., 2014). In all models trial number, treatment and the interaction trial number*treatment were included as fixed factors. The fish ID was instead included as a random factor. The effects of the fixed variables were tested using the "Anova" function from "car" package.

4. RESULTS

Out of the 82 fish used, 74 survived and completed the testing phase (The number of fish that died was the same in the heat wave and in the control group).

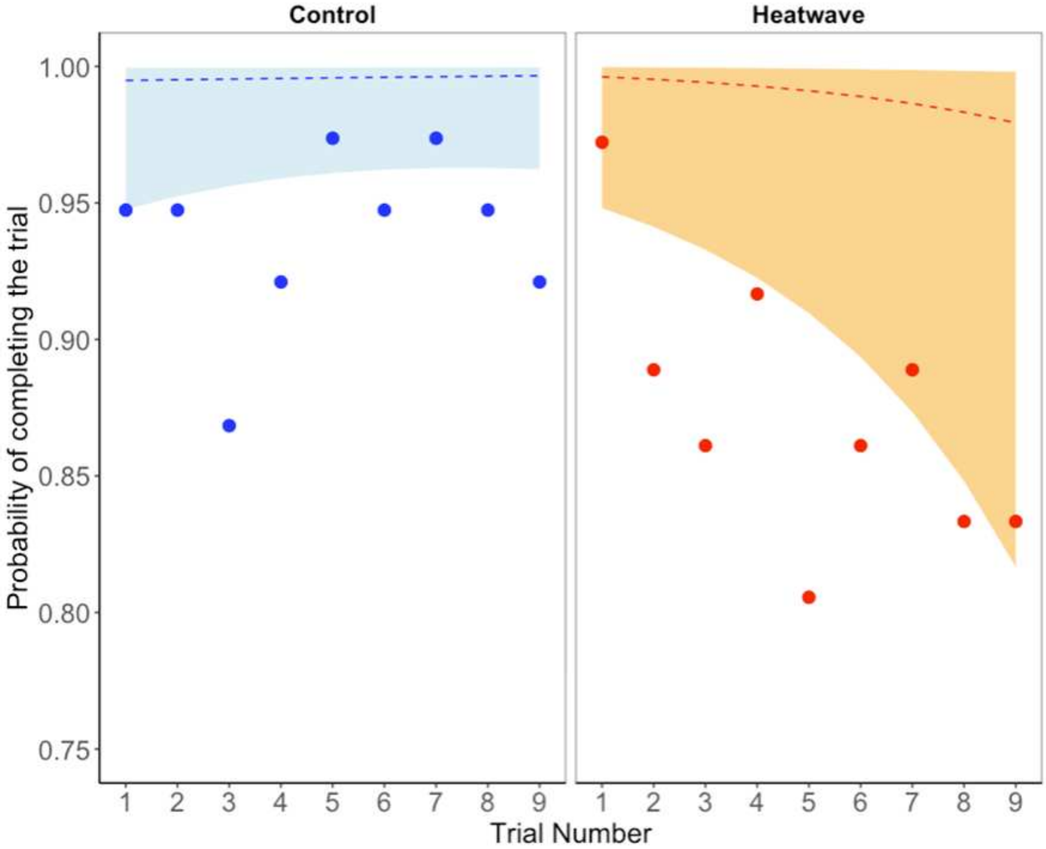

Figure 4: Plot of the probability of completing the trial in the control group (left) and the heat wave group (right) during the experiment.

The probability that a male completed a trial, i.e., reached the goal zone within 10 minutes, was not significantly affected by treatment ($\chi^2_{(d.f.)}$ = 0.81$_{(1)}$, p = 0.368) or trial number (χ^2 = 1.63$_{(1)}$, p = 0.202). However, there was a non significant trend for the interaction (χ^2 = 3.583$_{(1)}$, p = 0.058), suggesting that, with time or experience, heat wave males were less likely to complete a trial, while control males showed no change (Figure 4).

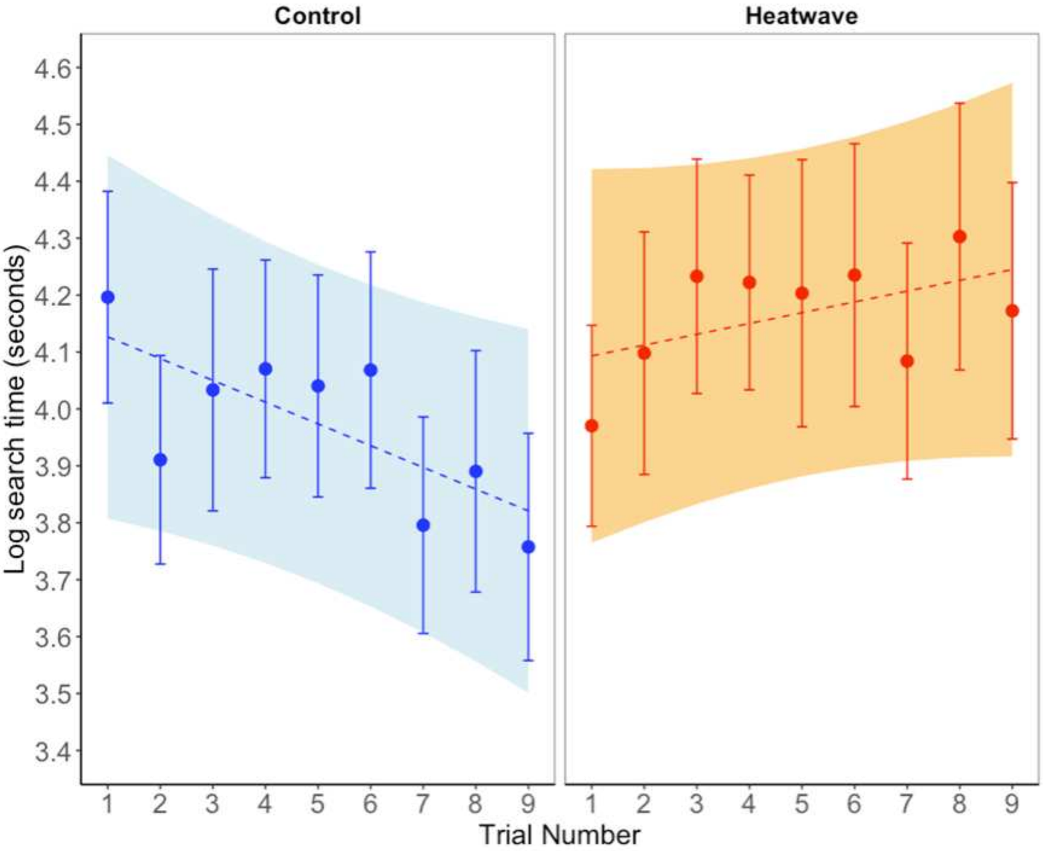

Figure 5: Plot of the search time, the time spent by the fish in the maze after crossing the first threshold and before the last one. On the Y axis: Log of the search time (seconds); on the X axis: trial number.

Search time, i.e. the time from leaving the start area till reaching the goal zone, was significantly affected by the interaction between treatment and trial number ($F_{(1,590)}$ = 4.126, p = 0.043), indicating that males from the control group reduced their search time with experience in the maze, while heat wave males did not (Figure 5). Treatment ($F_{(1,152)}$ = 0.133, p = 0.716) and trial number ($F_{(1,590)}$ = 0.546, p = 0.460) were not significant.

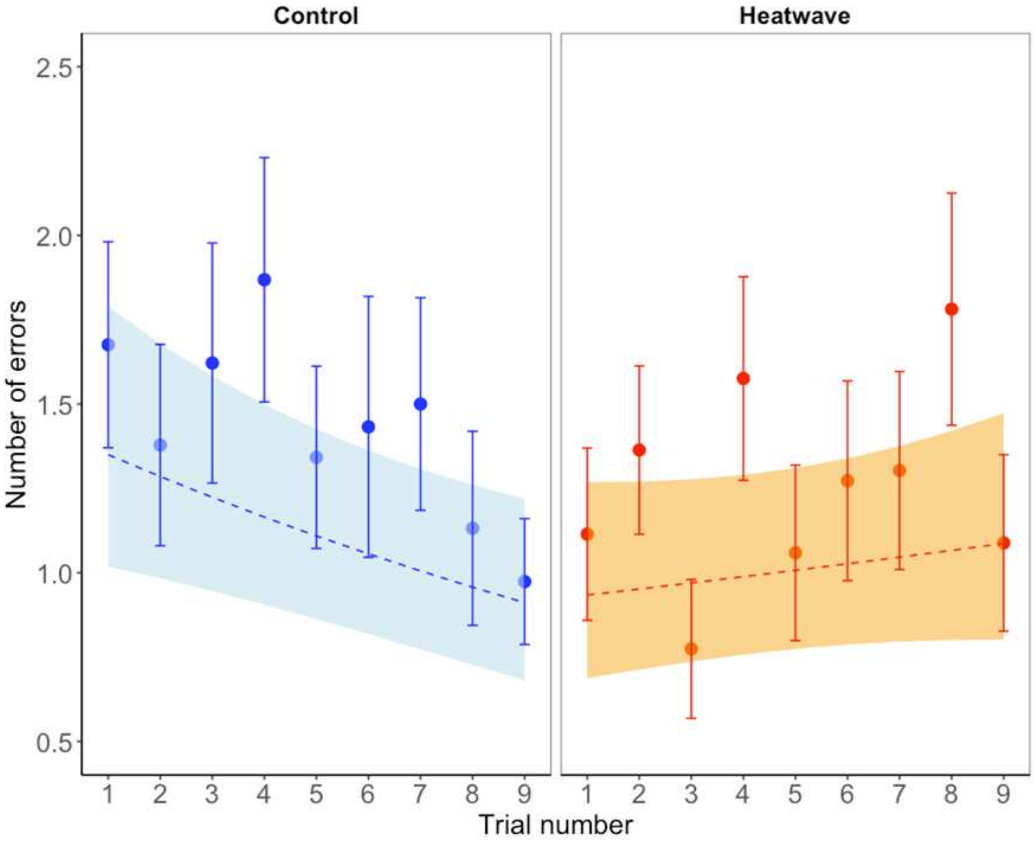

Figure 6: Plot of the number of errors, sum of both wrong turns and backtracking errors committed by the fish while in the maze. On the Y axis: average number of errors; on the X axis: trial number.

The total number of errors, i.e., the number of times a fish took a wrong turn or swam backwards, was also significantly affected by the interaction between treatment and trial number (χ^2 = 6.65 $_{(1)}$, *p* = 0.010), indicating that males from the control group also reduced the number of errors committed with experience in the maze, while heatwave males did not (Figure 6). Treatment (χ^2 = 0.31 $_{(1)}$, *p* = 0.577) and trial number (χ^2 = 2.16 $_{(1)}$, *p* = 0.141) were not significant.

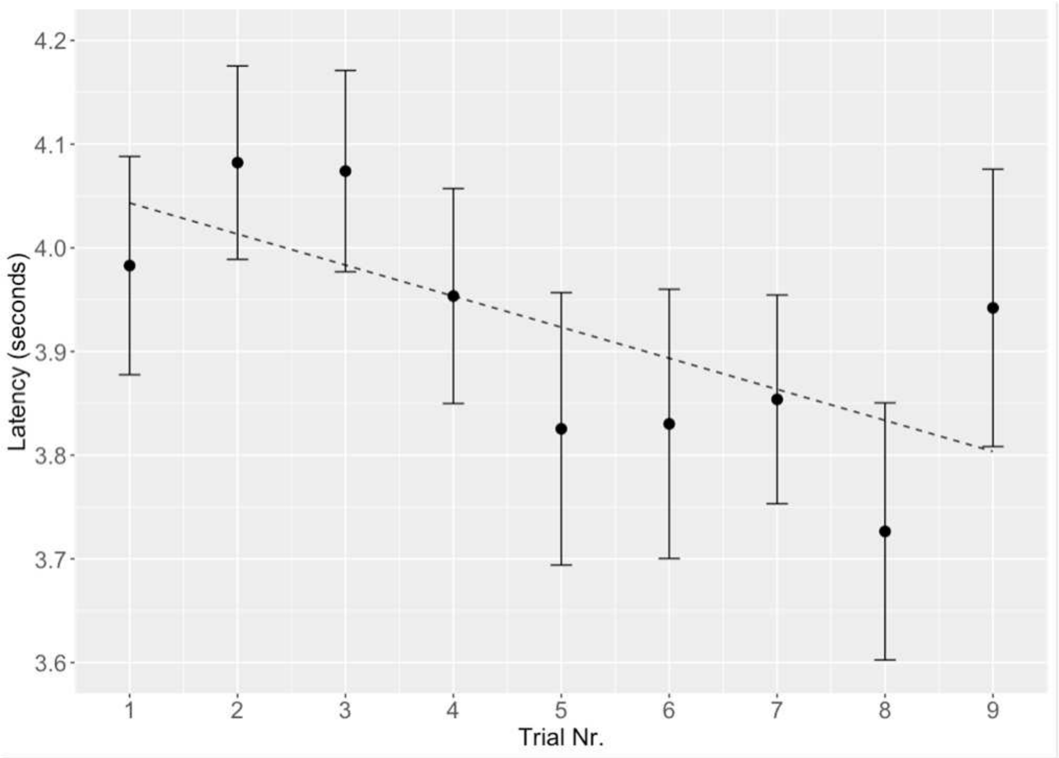

*Figure 7: Plot of the latency time. On the Y axis, the latency in seconds (the time spent in the first section of the maze before crossing the first threshold), on the X axis the trial number. As no effect was found between latency and treatment or treatment*trial number, the control and heatwave datasets were combined to better show the influence of trial number.*

Lastly, the latency to leave the starting area, i.e., the time spent by the fish in the first section of the maze at the beginning of the trial, was not significantly affected by treatment ($F_{(1,72)}$ = 0.276, p = 0.601) or by the interaction between treatment and trial number ($F_{(1,564)}$ = 0.003, p = 0.959), but was significantly affected by trial number ($F_{(1,565)}$ = 5.631, p = 0.018), indicating that both males from the control group and from the heatwave group decreased their latency time with time (Figure 7).

5. DISCUSSION

This study demonstrates that when subjected to a heat wave male guppies suffer a decrease in their maze learning ability compared to males from a control group. The number of fish that managed to reach the end of the maze remained constant for the control group, but decreased with trial number in the heatwave group . Fish that failed the trials, i.e., spent more than 7 minutes in the starting section or more than 10 total minutes in the maze, where substantially more represented in the heat wave group (47 total trials were not completed in heat wave males) than in the control group (28 total trials were not completed in the control group). This result may have two possible, non mutually exclusive explanations. First, heatwave males may have had less interesting the reward (the female) and so did not have enough motivation to complete the maze. Second, heat wave males' spatial memory may have been affected in such a way that they were not able to navigate the maze properly. Whatever the underlying reason, this result show that the heat wave group is, either, not motivated enough to reach the end of the maze or not able to do so, on average when compared to the control group.

Male guppies from the control group were able to improve their search time with each trial, as expected, and consistent with previous studies that tested the maze learning capabilities of guppies (Kellogg & Gavin, 1960; Kotrshal et al, 2015; Lucon-Xiccato & Bisazza, 2017). While not all control males improved their times, a significant decrease in the average time (indicating improvement) was found. The same pattern was observable in the total number of errors committed, which decreased with trial number in the control group, meaning that not only guppies were completing the maze faster but more accurately, too. On the contrary, male guppies subjected to the heat wave were not able to improve neither their time nor their number of errors. The heat stress experienced by the fish of the heat wave group had detrimental effects on maze learning abilities. While this may be correlated with a drop in cognitive abilities, further research is necessary to pinpoint the specific effect that this type of heat stress has on the fish. Stress may impact spatial learning, spatial memory, or there may be a decrease interest in the female. Thus, it is necessary to be careful when interpreting those results, as the actual effect of this type of heat stress on those animals may be complex and influencing multiple facets of their cognition. Having said that, heat stress was found to affect spatial abilities as learning and memory in multiple species (Lee et al., 2015; Dayananda et al., 2017; Soravia et al., 2021), and as such it is reasonable to associate the lower performance of the heat wave group in the maze learning task, at least partially, to a lowering of their spatial learning and/or spatial memory abilities. Likewise, guppies also showed lowered spatial learning abilities when under different form of stress, such as when raised in crowded conditions (Chapman et al. 2008), and so it may be possible that different forms of stress affect cognition similarly.

The fish were fed ad libitum during both the treatment phase and the testing phase, and in a previous study with the same conditions, male guppies were weighed before and after the heat wave treatment. No change in body weight was found, thus we can exclude the necessity of the fish to allocate resources away from cognition to more basal metabolic functions. The average latency time decreased in both groups across trials, which means that guppies tended to spend less time in the starting area as the trials progressed. This may correlate to a decrease in the neophobia experienced by the fish as it become more acquainted with the maze and become less fearful of going out of the initial section to seek his reward. Neophobia represent the fear and the consequential avoidance of a novel stimuli. Novel stimuli may represent a resource, but at the same time they may present the potential for danger, thus several species show avoidance for novel stimuli (Miller et al., 2022). This is also true for *P. reticulata*, (Feyten et al., 2022). In the case of the maze learning test, the neophobia is experienced in the context of a novel environment, i.e. the maze, and decrease the more the fish get acquainted to it. There was no effect of treatment, or its interaction with trial number, on latency time, suggesting that this variable was not influenced by heat stress. Similarity can be drawn between this study and the one investigating differences in maze learning performance due to brain size in guppies (Kotrshal et al, 2015). As in this study, the control group over performed compared to the heat wave group, in that study the big brained males outperformed the small brained ones. However, there are not enough clues to determine if this means that the differences in performance between those groups are due to those variables (heat stress experienced and brain size respectively) influencing cognition in a similar way. Nonetheless, in both studies the latency decreased with trial number in all groups, hinting that in both cases the variance in cognitive performance due to heat stress or brain size does may correlate with a different effect on neophobia, as all groups shortened their latency with time.

This study add to the already vast library of research about the effects that climate change is having on animals, and most importantly add to the scarcer collection of studies about the effects of heat waves on cognition. While some studies on this subject have been already made (Blackburn et al., 2022), most of them focus more generally on the effect on cognition of short (often only a few minutes) and intense heat stress (Soravia et al., 2021), which is less ecologically relevant. As the number of extreme weather events, such as heat waves, increases in both intensity and frequency, it is important to understand how animal populations will be affected. This is certainly interesting when assessed in the field, on the animal natural environment, but considering that this is not always possible, simulating those events in a laboratory setting has some important pros. For example, in a laboratory setting it is possible to avoid all confounding effects found when working on the field. For instance, in terrestrial animals, there is a correlation between temperature increase and dehydration (Jacobs et al., 2020), and so when studying the effects of heat stress on cognition it is necessary to account for the loss of water that the animal may experience due to the increase in temperature, in order to avoid misinterpreting the effect of dehydration as a consequence of the heat stress (Gérard et al., 2022). In laboratory studies it is possible to monitor the animal conditions and give them ad libitum water, something impracticable while working on the field.

While this study accomplished its objectives satisfyingly, in the future a more complete experimental protocol may be used to further increase the types of data obtained. The sample size was suitable for the type of study performed but increasing it may help understanding the results at the edge of the significant threshold, such as the probability of completing a trial in both groups. On the other hand, it would be interesting to add another treatment group, with fish experiencing the heat wave tested after a certain period of time from the heat wave, i.e., a month later, to see if there are any long-term effects beyond the short-term ones. This would be particularly interesting as most research on this subject is done by exposing the eggs, mothers or larvae to the heat stress and not the adults (Soravia et al., 2021). Similarly, it would be interesting to test the effect of heat wave of different intensity and duration, as while the simulated heat wave used here followed the definition of The World Meteorological Organization, in a natural setting the magnitude of this extreme weather may vary widely from event to event; therefore, it may be useful to simulate different intensity and duration to more precisely understand their consequences. Moreover, in future tests it may be useful to measure the fish tested to see if there is a correlation between the guppy’s size and how affected it is by the heat wave, as individuals with higher body mass could be more resistant that smaller ones. Lastly, this study focused only on adult male guppies of wild descent from a single population, but in the future more experiment could be performed to test the effect of heat waves at different life stages, in females and/or in different populations or in commercial guppies. Previous studies found differences between males and females cognitive performance in multiple tests (Lucon-Xiccato & Bisazza, 2014; Lucon-Xiccato & Bisazza, 2016), thus testing females could help in better understanding those differences. However, while for adult males a receptive female is a viable reward, it is unknown whether an adult male is a viable reward for a receptive female, as previous studies that tested females used different rewards that required more complex setups, such as placing the whole maze inside the living habitat of multiple guppies (recreated in a bigger tank), so that by reaching the end not only a social reward was present but also an environmental one, with the fish going back to its home tank (Lucon-Xiccato & Bisazza, 2017). Nonetheless, testing females could also help understand the ecological divergences underlying the differences in cognitive abilities between males and females. Testing guppies that experienced the heat wave during development could reveal, if any, long-term effects of heat stress, which in other species has been shown to be influential (Dayananda et al., 2017; Wang et al., 2007), and may be even more impactful on the ecology of the fish. Finally, testing different populations may help understand how much they have adapted to different environment and how differently they will be affected by a similar change in temperature. This study focused on testing important abilities for the guppies in a natural environment, in this case the necessity to navigate shallow waters to find a mate. Moreover, the laboratory environment does not reflect the complexity found in the natural environment, therefore the results, should be validated also in nature. An example of this is the fluctuation of temperature between daytime and nighttime (since temperatures decline during the night), that exists in the natural environment but we did not recreate in our set up. In the wild, guppies may also seek cooler waters during an heatwave, by moving in more shaded parts of the river. Thus, during a heat wave, wild guppies in a natural environment may be subjected to a less constant heat stress then the ones in the experiment, affecting their cognitive abilities differently. On the other hand, while guppies during the test were fed ad libitum, wild guppies have to forage and, as discussed previously, the change in behavior brought by the heat stress could affect fitness, too.

The effect of lowered cognitive performance on fitness must not be underestimated, as multiple studies investigating their relationship shown the correlation between them, and individuals that performed worse in tested cognitive task were associated with lower fitness, and of those spatial learning and memory were particularly influential (Dayananda et al., 2017; Sonnenberg et al., 2019; Shawl et al., 2019).

The increasing number of extreme weather events such as heat waves may have a negative impact on the fitness of animals, not only due to the physiological effect of heat stress but also due to the often underestimated effect on cognition and the consequential effects on behavior. As the temperature of our planet increases, so will the intensity and frequency of heat waves, affecting some animal population more drastically than others, which may ultimately impact the entire ecosystems. As such, it is vital to develop a deeper understanding of the effects that such events may have on all aspect of animal life, so that it will be possible to devise solutions to attenuate the damage that animal communities will inevitably suffer in the coming years due to climate change.

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