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Caught by a heatwave: effects on behavior, mate choice and life history

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RIASSUNTO

Negli ultimi decenni le ondate di calore marine (MHW), effetto del cambiamento climatico, sono aumentate notevolmente. Inoltre, come riportato da numerosi studi, si prevede che questa tendenza continuerà in futuro nella quasi totalità dei bacini idrici.

Numerosi lavori hanno evidenziato gli impatti che le MHW hanno sugli organismi marini, tuttavia la letteratura presenta ancora carenze per quanto riguarda gli effetti che questi eventi hanno sulla fauna ittica. Lo scopo di questo studio è quindi quello di valutare gli effetti che le MHW hanno su pesci teleostei per avere una migliore comprensione di come questi eventi possono influenzare la biodiversità marina. Per questo scopo ho utilizzato un pesce di acqua dolce, *Poecilia reticulata*. Specie d'acqua dolce/salmastra possono infatti essere modelli preziosi per studiare le MHW per la loro semplicità di stabulazione.

Femmine gravide e giovanili sono stati sottoposti a un’ondata di calore artificiale a seguito della quale alcuni tratti di life history e comportamentali sono stati confrontati con quelli degli individui appartenenti al gruppo di controllo. Le ondate di calore hanno provocato una significativa diminuzione della fecondità delle femmine. Le femmine sottoposte all’ondata di calore hanno prodotto meno nidiate rispetto alle femmine di controllo, covate di dimensioni inferiori con periodi di gestazione più lunghi. Inoltre, le femmine sottoposte all’ondata di calore hanno presentato una mortalità significativamente maggiore rispetto al controllo. In seguito al primo parto, le femmine, nuovamente recettive, sono state sottoposte ad un test di motivazione sociale/sessuale in cui potevano scegliere se stare con femmine o con maschi. In queste condizioni le femmine sottoposte a ondata di calore hanno speso significativamente più tempo assieme ad altre femmine rispetto al gruppo di controllo. Per quanto riguarda l’esperimento con i giovanili questi non hanno presentato differenze significative tra individui trattati e individui di controllo per quanto riguarda dimensioni corporee, peso, indice di condizione corporea e mortalità. Il giorno successivo al trattamento termico i giovanili sono stati sottoposti a tre diversi test. Nel primo test è stato misurato il comportamento anti-predatorio in risposta a due stimoli differenti. In risposta ad uno stimolo visivo gli individui sottoposti all’ondata di calore si sono immobilizzati più spesso e più a lungo rispetto ai controlli. Quando sottoposti a stimolo chimico gli individui sottoposti all’ondata di calore hanno risposto in maniera analoga agli individui di controllo. Entrambi i gruppi hanno aumentato la tigmotassi, tuttavia, l’aumento è stato meno marcato nel gruppo sottoposto ad ondata di calore rispetto a quello di controllo. Il secondo test a cui sono stati sottoposti i giovanili era un test di cattura. In questo test non sono state riscontrate differenze tra il gruppo trattato con ondata di calore e il gruppo di controllo. Infine, i giovanili sono stati sottoposti ad un test che ne misurava la velocità critica di nuoto e la resistenza sfruttando la loro innata propensione a nuotare controcorrente. In questo caso non sono risultate differenze significative nella velocità critica di nuoto tra il gruppo sottoposto ad ondata di calore e il

gruppo di controllo; tuttavia i due gruppi hanno differito nel tempo medio di resistenza avendo il gruppo di controllo tempi di resistenza maggiori.

Da questo studio si evince che le ondate di calore hanno un forte impatto su molteplici caratteristiche, anche in una specie eutermica quale *P. reticulata.* Questo studio dimostra inoltre che le MHW possono avere effetti diversi su individui che si trovano in fasi di vita differenti. Le MHW si presentano quindi come una forte minaccia per la resilienza delle popolazioni e più in generale per la biodiversità ittica nel prossimo futuro.

1. INTRODUCTION

2. MATERIALS AND METHODS

3. RESULTS

4. DISCUSSION

1. INTRODUCTION

1.1 Heatwaves

Climate change is a well documented phenomenon nowadays and global warming is one of many effects that climate change has on the human society and the environment. Since 1900 the global average temperature has increased by 0.89°C with the major identified cause being human activities (Hartmann et al. 2013). With the increase in the average temperature comes a disproportional increase in extreme events such as heatwaves. In the past years, different definitions of heatwaves have been used by different authors and studies. Yet commonly heatwaves are referred to as prolonged, discrete periods with anomalously high temperatures (e.g. Oliver et al. 2021). Perkins et al. in 2012 have shown that since 1950, regardless of the definition used to identify heatwaves, these events have increased in intensity, duration and frequency. Moreover, many other studies have consistently reported increases in heatwaves in the last decades, although with differences between regions (Perkins 2015). Models for the future have also shown an increasing trend in heatwave occurrence, especially maintaining a high or in this current CO_2 emission scenario, with a positive feedback loop mechanism between atmospheric heating and further drying of the soil (Perkins 2015).

As the frequency and intensity of heatwaves increase, also the marine environment is affected. Marine heatwaves (MHWs) have indeed also increased and the trend is expected to continue in the future for almost all water basins (e.g. Frölicher et al. 2018; Darmaraki et al. 2019; Smale et al. 2019; Oliver et al. 2019). Oliver et al. in 2019 have also found that, if the emissions will continue under a “business as usual scenario”, by 2100 about 50% of the oceans will be in a permanent MHW state; if the emissions will instead continue to rise, by the end of the century the entire globe is predicted to experience a permanent MHW state.

MHWs are usually described in the same manner as heatwaves, that is prolonged, discrete periods with anomalously high surface sea temperatures, but as for their terrestrial counterparts, a common definition has still to be acknowledged (Oliver et al. 2021). In 2016, Hobday et al. proposed to define MHW as a period during which seawater temperatures exceed a seasonally varying threshold (usually the 90th percentile) for at least 5 consecutive days. Following this definition successive heatwaves with gaps of 2 days or less are considered part of the same event.

MHWs are caused by a combination of high-pressure systems with air-sea heat fluxes, low wind speeds, low cloud coverage, horizontal and vertical mixing often connected with upwelling and downwelling processes (see Oliver et al. 2021). Moreover, MHWs are also connected to large-scale climate drivers, such as the El Niño Southern Oscillation.

MHWs are impacting many ecosystem services (benefits that humans obtain from the natural environment) and they represent a major treat to global biodiversity (Smale et al. 2019). For example, a MHW was responsible for the first ever recorded coral bleaching event off the coast of Western Australia in 2010-2011 (Smale and Wernberg 2013; Wernberg et al. 2013). Other recorded impacts on ecosystems include, but are not limited to, widespread mortality, reduced growth, local extinctions, range shifts, changes in abundances, reduced carbon sequestration, decreased phytoplankton production, and disruption of carbon and nitrogen cycling (Smale et al. 2019). MHWs have also impacted human activities, with socio-economic repercussions such as fishery closures or quota changes and even tensions between nations (Oliver et al. 2018).

Heatwaves are also influencing freshwater ecosystems. In particular Woolway et al. in 2021 found out that the past and future trends of lake heatwaves look very similar to the ones that Oliver et al. in 2019 found for MHWs with some lakes reaching a permanent lake heatwave state by the end of the century.

Despite the growing number on studies on the effects heatwaves on natural environments, not much is known about their effects in aquatic ecosystems and, specifically, in marine ecosystems. To better understand how heatwaves will impact marine species in the future and consequently human activities it is therefore very important to increase the number of studies regarding MHWs. Because of the similarities between freshwater ecosystems and marine ecosystems, the first could serve as models to better understand the effects of heatwaves in all aquatic ecosystems. Moreover, the former has practical advantages for research purposes such as smaller scale, higher availability, and ease of rearing of the organisms in laboratory facilities.

1.2 Effects of heatwaves on fish

Many studies have focused on the various effects that temperature has on organisms. However, most of them focus either on the effects of the increasing average temperatures or on the critical thermal limit, the latter of which is usually well above the temperatures that organisms experience in the wild.

Mortality is probably the most reported effect that extreme temperatures have on fish species, but an increasing number of studies have shown that, within species, the thermal tolerance changes with the ontogenesis of the individuals. In particular, Brewer (1976) for *Engraulis mordax* and Rijnsdorp et al. (2009) for *Solea solea* have shown that bottlenecks in the thermal tolerance are present during the early life stages (especially larval stages) and the spawning stages. Moreover, both studies reported that juveniles are the stages that are more temperature tolerant (Pörtner and Peck 2010). Thermal tolerance is low in early life stages due to the insufficient capacity of the undeveloped organs to make up for the increased needs of the organism caused by the thermal stress (Pörtner et al. 2006). Thermal tolerance then improves in juveniles where the organs capacity is high, but the body size is still small. Finally, in the spawning stages the tolerance declines again because of the increased oxygen demand caused by the gametes or the embryos (e.g. Pörtner and Peck 2010; Pörtner et al. 2017).

Within and across species thermal tolerance usually changes with the latitude that a particular population inhabits. Generally speaking, species inhabiting mid-latitudes, where seasonal differences are high, usually show a high thermal tolerance. In contrast, for species that inhabit low or high latitudes the thermal range is usually narrow (Pörtner and Peck 2010). It is also important to note that marine species are usually less susceptible to high temperatures because they can more easily change their habitat, both horizontally (across latitudes) and vertically (in the water column) compared to freshwater species.

Indeed, freshwater species are more susceptible to high temperatures, as in *Poecilia reticulata* where Dzikowski et al. (2001) reported that persisting high temperatures (32°C, 70 days) increased mortality, shortened the gestation length and reduced the brood size (with ovaries degeneration). In the same species, Karayücel and co-authors (2006) found similar results with high temperatures, over a 2-week period, impacting the fecundity of the females and the survival of the fry. As late as 2021 Auer and co-authors investigated the upper thermal tolerance of *P. reticulata* at different reproductive stages concluding that females in late pregnancy stages have a significantly lower critical thermal maxima compared to other females.

However, studies that are using critical thermal limits need to be interpreted with caution since experimental protocols usually do not account for the time dependence of the lethal threshold (Pörtner and Peck 2010). That means that high temperatures, but still lower than the critical thermal limit, can also be lethal if the exposure is long enough. It is therefore important to test the effect of extreme events, such as heatwaves, closely mimicking natural events, considering both the intensity of the phenomenon and the duration of it.

Over the past few years several studies have been conducted on the effect of heatwaves on fish species (e.g. Kikuchi et al. 2019; Smith et al. 2019; Spinks et al. 2019; Arimitsu et al 2020; Madeira et al. 2020; Mameri et al. 2020; van der Walt et al. 2021). Spinks et al. (2019) tested the effects of MHWs, during the early life stages, on the damselfish *Acanthochromis polyacanthus.* Newly hatched fish were exposed to a MHW for 3, 7, 30 and 108 days. Fish exposed to high temperatures for 30 and 108 days showed a higher escape mean speed and distance covered during the escape compared to the controls. Moreover, in the group exposed to the longer treatment MHWs also negatively affected the length and the weight of the individuals, with fish both shorter and lighter than the controls. Mameri et al (2020) also found that heatwaves, over a period of six days, reduced activity level and boldness of the Iberian barbel, *Luciobarbus bocagei*. Madeira et al. (2020) showed how heatwaves effects the levels of numerous cellular stress response biomarkers (namely glutathione-S-transferase, superoxide dismutase, lipid peroxidation, heat shock protein 70 kDa, ubiquitin) in *Sparus aurata*. They also reported increased mortality in both larvae and adults and tissue damage in all life stages (larvae, juveniles and adults). Finally, van der Walt and co-authors (2021) found that the water temperatures reached during the 2015-2016 MHW off the coast of South Africa were high enough to cause cardiac arrhythmia in adults of *Diplodus capensis.*

1.3 Biology of the model species used

Poecilia reticulata is one of the world’s most widely distributed freshwater tropical fish and one of the most popular among aquarium hobbyists*. P. reticulata* is therefore commonly known with a variety of different names such as guppies or millions fish.

Guppies are members of the family Poeciliidae, a group of fish including 190 species characterized by internal fertilization and ovoviviparity (the only exception being *Tomeurus gracilis,* Keith et al. 2000) and the presence in males of a copulatory organ named ‘gonopodium’ (Magurran 2005). *P. reticulata* was first described from Venezuela by Wilhelm Peters in 1859 but changed name several times during the following years until 1963 when the original name was restored. Its natural habitats are streams and pools of Venezuela, Trinidad and Tobago, Guyana, Surinam and possibly Barbados, Cuba, Grenada (its uncertain if the species was introduced there by humans). Today guppies are found in every continent, excluding Antarctica, and its range is continuously expanding through fish trade, as a mean of controlling malaria mosquito populations and thanks to their ability to survive in polluted and brackish waters (Magurran 2005). Reeve et al. (2014) have reported that wild guppies can live in a wide range of temperatures and can experience fluctuations in temperature up to 7°C during the same day. Some populations of guppies have been reported living at temperatures as low as 23°C meanwhile others can experience peaks up to 32°C (Reeve et al. 2014). Furthermore, in captivity, critical thermal maxima up to 40.6°C have been reported with death points being between 41.2 and 42.9°C (Chung 2001).

Guppies are opportunistic, omnivorous feeders. Their diet consists of insect larvae and other small invertebrates, algae, their own young, eggs and young of other species of fish and benthic detritus (Dussault and Kramer 1981).

Guppies exhibit phenotypical differences between populations due to different predatory pressure (Strauss 1990; Endler 1995), genetic drift and sexual selection (Houde 1997; Breden and Lindholm in Evans, Pilastro and Schlupp 2011). Body size varies between populations, but generally males are smaller than females, with the latter sex reaching a few centimeters in body length (Magurran 2005; Fishbase). Male coloration also varies between populations, with populations under higher predation risk having less colored males (Endler 1995). Overall, wild-type females are grey/yellowish in body color for a better camouflage with the background. Males, on the other hand, have spots and stripes, typically black, orange and iridescent. Many studies have confirmed that the color pattern of the males is the target of female choice during courtship behaviour. Carotenoid colors (red, orange and yellow) are especially important in mate choice because they are a cue of the nutritional and overall health status of the individual (Houde 1997). Brooks and Endler (2001) also found that females prefer a large fin area and larger iridescent color spots. In contrast, black spots are not used during female choice (Magurran 2005).

Guppies, as all poecilids, are internal fertilizers (Wourms 1981). Spermatozoa are packed into discrete bundles called spermatozeugmata and transferred to the female through the gonopodium. The gonopodium is the male’s copulatory organ and originates from the thickening of the rays 3, 4 and 5 of the anal fin (Hopper 1949). The gonopodium presents spines both ventrally and dorsally, a hook located in the apical position of ray 5 dorsally and a hood that originates ventrally from ray 3. During copulation the gonopodium enters the gonopore of the female (Weishaupt 1925) and the spermatozeugmata are transferred trough a groove formed by the rays of the anal fin (Philippi 1908).

Male guppies have two different tactics to obtain a copulation: performing a courting behavior, called sigmoid display, or mating forcingly with a sneaking behavior called gonopodial thrust. All males perform both tactics but there is variation in the relative frequencies between individuals (Magurran and Seghers 1990) and populations (Luyten and Liley 1985). Before performing a display, the male usually follows the female. During the display the male's body assumes an S shape (from which the name "sigmoid display"), the body quivers and the black spots became more vivid. Sometimes the fins are extended and, if the display protracts enough, the male moves a few millimeters up and down (Houde 1997; Magurran 2005). The whole display can last for several seconds (Magurran 2005). A positive female's response to a mating attempt is indicated by the female performing a gliding motion towards the male that ends into copulation. In situations where displays are expected to not be effective (e.g. when females are not sexually receptive or in the presence of other males or better males) or too costly (e.g. because of the presence of predators or due to poor visibility) the sneaking tactic becomes the most frequent tactic (Houde 1997). After any successful copulation the male jerks up and forward several times (Houde 1997). Sperm can be stored for several months by females in ampulla-like extensions of the ovarian cavity (Jalabert and Billard 1969). Therefore, with a single successful mating a female can produce up to 8 successive broods (Winge 1937) for which brood size declines over time (Magurran 2005; Greven in Evans, Pilastro and Schlupp 2011; Gasparini and Evans 2018). Because guppies are promiscuous, sperm from different males can be stored by the females and multiple paternity is very common (Becher and Magurran 2004; Hain and Neff 2007; Neff et al. 2008). Neff et al. in 2008 reported that across 10 populations 95% of the broods had multiple sires with on average 3.5 sires per brood (range 1-9) but this value depends on the population examined. Gestation usually lasts 25-30 days (Haskins et al. 1961; Houde 1997), even though there can be significant variation (Evans and Magurran 2000). Reproduction continues all year round (Alkins-Koo 2000) with some seasonal variation (Reznick 1989). Females are receptive for two or three days after giving birth or when virgin (Houde 1997).

In poecilids parturition coincides with ovulation, young from the same brood are born within a few hours (Greven in Evans, Pilastro and Schlupp 2011) with brood sizes that can vary from 1 to over 50 fry (mean 12 from Neff et al. 2008). The fry are born without the yolk sac (which has already been reabsorbed) and they are completely self-sufficient.

1.4 Aim of the study

Climate change is nowadays well documented and each year numerous studies from all fields of science try to better understand the impact that this phenomenon has on the planet, society and biodiversity. Within this framework, the aim of this study was to assess the impacts that MHWs have on fishes and ultimately to better understand how these events can affect marine ecosystems.

As previously mentioned, freshwater species can be valuable models to study MHWs because of the similarities between ecosystems and organisms. *P. reticulata* was used because it well suited the needs of these experiments. First, the extensive literature present for this species allows having detailed knowledge of its life history and behavior, which is fundamental to understand the effects of a temperature increase. Secondly, the short life cycle and high fecundity is critical to be able to conduct experiments on successive generations in a relatively short period of time. Moreover, the water parameters tolerance and freshwater nature of *P. reticulata* allow minimizing the effect of other stressors (pH, water hardness) and simplifying the rearing, eliminating the need of using saltwater. Lastly, because guppies are internal fertilizers and livebearers, the brood survival is usually very high. Therefore, the effects of the treatment could be discussed on a conservative model (the effects would be stronger on less resistant species) and any mortality could be easily linked to the treatment effect.

To meet this aim, we focused on the effects of heatwaves on survival, fecundity and behavior of adult females. Because of anisogamy (difference in gametes sizes between males and females), females are commonly the sex that invests more energy in reproduction and thus are more susceptible in this particular life stage. The effects of heatwaves were also investigated on survivability, growth rate, condition and anti-predatory behavior of their offspring, to understand the effects of heatwaves exposure during early life stages. For this experiment guppies were exposed to a heatwave of 32°C for 5 days as suggested by Hobday and co-authors (2016).

We predict that individuals that face a heatwave event will show reduced fecundity, higher mortality, and an overall worse condition compared to the fish in the control group.

2. MATERIALS AND METHODS

2.1 Females' experimental treatment

The experiment consisted of exposing fish to a simulated heatwave (5 days) or no heatwave (control group). At the start of the experimental period females and males were housed together to allow free matings. After 10 days, females were isolated physically from the males (but in visual and olfactory contact) and the treatment (heatwave or no heatwave) started for 5 days. After experiencing the heatwave, female where isolated till parturition when some of their life-history traits were measured and their behavior tested.

The experiment was conducted using 60 six-month-old females of *P. reticulata*. Individuals were descendants of wild caught fish from Tacarigua River, Trinidad and Tobago (2002). Since 2013, the laboratory population has been living in semi-natural conditions at the Botanical Garden of the University of Padova. The experimental females were captured as newborns and reared in aquariums in which no males were present to ensure their virginity (and thus avoiding stored sperm from previous reproductive events). All tanks in the facility were filled with 48h rested tap water; lights were set to a 12h day/night cycle. All individuals were fed *ad libitum* 6 days a week, with a mixed diet of dry food (Duplarin) and freshly hatched *Artemia salina* nauplii.

The experiment was divided into 6 blocks to spread the workload. Each block consisted of 2 tanks with the capacity of 25l (one tank for the control and one for the treatment) for a total of 12 tanks. On day 1 in each tank 5 virgin females were released together with 5 males for 10 days at a temperature of 26±1°C, to allow mating between individuals. The females were randomly assigned to either one of the two treatments to minimize differences between the two groups.

In the morning of day 11, the females were moved inside two perforated containers which were immersed in the experimental tank, allowing visual and chemical contact between females and males but preventing copulations during the treatment week. The temperature of the tanks that simulated the heatwave was then gradually raised to 32°C: at 9:00 am an aquarium heater (NEWA Therm VTP 100W) was added to the tank and set to 28°C and at 12:00 the heater was set to 32°C. The control tanks were kept at room temperature (26±1°C).

The heater was removed around 9 am on the 17th day, and the tank was left to cool down gradually to room temperature. In the morning of day 18 the females were moved to the Tecniplast system (a rack system in which all tanks all connected to a single filtration system) waiting for the first broods. The females were housed in a tank individually to prevent family mismatching. When females were within one week from giving birth (average pregnancy duration 25 days) a birthing cage was added to the Tecniplast tank in order to isolates the female from the fry as soon as these are born to prevent cannibalism; females were checked twice daily to spot any newborn for a minimum of 70 days.

The morning after the first newborn was spotted (day 1 since birth), the brood was separated from the female and housed in a separate tank. From this moment onward the female and the brood were handled differently (see sections 2.1.1 and 2.1.2).

2.1.1 Females

In the morning of day 1 or 2 after giving birth (i.e. when females are receptive, Houde 1997) females underwent a social/sexual motivation test. The test was performed before 11:00 am, in order to ensure that the test was performed during the period of maximum activity of the fish. The female was fed and left to acclimatize in the observation room (ambient temperature 26±1°C) for one hour before testing started. The female was then transferred into the experimental tank (water temperature 26±1°C) and her behavior was recorded for 30 minutes with a camera (Panasonic HC-V180) mounted above the tank. The experimental tank consisted of a 29x39x32cm aquarium divided in 3 watertight compartments (water level 15cm): half of the tank was reserved to the focal female, 1/4 contained 3 stimulus males and the last 1/4 contained 3 stimulus females. The portion of the tank with no stimuli fish was divided into 3 choice areas: no-choice, male-choice and female-choice (*Figure 1*). The side walls of the tank were covered with opaque film to remove any external visual stimuli and the bottom was layered with white gravel. Guppies can be easily scared by large fish silhouettes because these can be perceived as predators so in order to exclude a possible bias, females of the same size of males were used. The side in which stimulus males and females were located was randomly chosen each test.

The experimental tank allowed visual contact between the focal female and the stimulus individuals. Stimulus fish compartments were divided from each other with a black panel in order to prevent visual contact between stimulus males and stimulus females. The black panel extended partially into the focal female’s compartment so that the tested female was not able to see the fish in both compartments at the same time when she was close to the glass, thus forcing her to make a clear choice between the two compartments.

Once the test ended, females were weighted and photographed following a light sedation using the anesthetic MS222 (tricaine methanesulfonate). After they recovered from anesthesia they were returned to their Tecniplast tank to be monitored again for the next broods. If the female did not give birth within 70 days from the last brood (more than two average gestations), she was supposed to not have any other brood to deliver.

The video recordings of the test were used to realize an ethogram through BORIS software v. 10.7.5 (Friard and Gamba 2016). In the video, the experimental tank was divided into three areas of choice as shown in *Figure 1* via EpicPen software version 3.9.116 using two refences in the tank. Dimensions of the male-choice and female-choice areas were 6x14.5cm, and the width of the area was approximately twice the body length of *P. reticulata* (Cattelan et al. 2017). In the ethogram the focal female was considered to be choosing when she was actively swimming against the glass, trying to reach either the stimuli females or males. The position of the head of the fish was used to assign the precise position to the fish when she was moving across different areas. The time the focal female spent in the female-choice and male-choice area was later analyzed as a proxy of her social and sexual motivation respectively.

Figure 1. Experimental tank for the female's social/sexual motivation test. The two shoals of stimulus fish are separated between them by the opaque divider (in black) and from the focal female by a see-through divider (in grey). Choice area outlines (in blue) were added in postproduction via

software. In the example the female (red silhouette) is located in the female-choice area.

2.1.2 Broods

One day old fry belonging to the first brood were photographed using a stereo microscope (10x magnification) in a Petri dish with water; the individuals were then classified in one of the following categories: normal, deformed, stillborn or dead. Once photographed, the broods were then moved into 1 l tanks for the first 10 days. Each brood was checked daily to assess mortality.

On day 10 (±2 days) four random fry were selected from each brood (excluding deformed individuals) to perform a schooling test following a previously established protocol (Cattelan et al. 2020). The test was preceded by 5 minutes of acclimatization in the arena (a round dish: diameter 17.5cm, volume 300ml). The fry were recorded via a high definition camera (Panasonic HC-V180) mounted directly above the arena (to prevent perspective distortion) for 20 minutes at 25 frames per second. The test consisted of 10 minutes of free swimming followed by a stimulus to invoke an anti-predatory response further followed by another 10 minutes of free swimming. The stimulus consisted of a chemical stimulus obtained by adding 15 µl of a liquid *predator cue* to the arena, coupled with a visual stimulus created by the shadow of the hand of the operator. The stimulus was supposed to simulate a successful predator attack with the rupture of the prey's epidermis. The chemical predator cue was prepared and dosed following previously established protocols (final concentration 1:200000, Evans et al. 2007; Heathcote et al. 2017; Cattelan et al. 2020). Briefly, female guppies were euthanized and part of the body homogenized with distilled water. The liquid was then filtered to remove any particles, centrifuged and the supernatant collected. The liquid was prepared in a large batch before the experiment started and stored in the freezer at -20°C. On the day of a schooling test, 15 minutes before the test started, a small aliquot of the liquid was defrosted at room temperature and then used as the predator cue in the testing arena. After the test, the four selected fry were photographed and housed together in a Tecniplast tank.

The video recordings of the schooling test were cut, cropped and converted in grayscale using Handbrake Version 1.3.3. and used to track the fry movement in the arena through idTracker (Version 2.1, Pérez-Escudero et al. 2014).

On days 20 and 30 (±2 days) the four fry were photographed again in order to evaluate their growth rate from four different time points (birth, 10 days, 20 days, 30 days). From the photos, using ImageJ (v. 1.53) software, the standard length of the fish, brain area and brain width (following Näslund 2014 protocol) were measured.

Unfortunately, due to the fact that females from the heatwave treatment produced hardly any broods the comparison of the schooling behavior between offspring from the heatwave and control females could not be performed. Specifically, only 4 first broods from the females that underwent the heatwave were collected and tested (N = 24 for the control group). Due to the poor numerosity of the offspring heatwaves group the analysis on the growth (i.e. standard length and brain size) and the mortality was also not performed.

2.2 Juveniles' experimental treatment

During the second phase of the experiment, I tested the effects of a heatwave experienced during development on the behavior and condition of juveniles. The individuals used for this phase of the experiment were juveniles belonging to the first brood of the control females. By doing so, the rearing conditions of each individual was already standardized and the morphological data previously collected could be used.

Similarly to the female treatment, the juveniles were exposed, at 35 days of age, to 5 days simulated heatwave (heatwave group) or standard temperature (control group). Then their behavior and morphology measured. The fish were maintained in the Tecniplast recirculating system (see above) until the treatment phase that took place into experimental tank. Two experimental tanks (15 l) were set up as follows:

- a control tank in which the water was kept at a constant temperature of 26±1°C;
- a heatwave tank with water temperature maintained at 32±1°C using a 300W (NEWA Therm VTX) aquarium heater.

At the age of 35 days (±3 days) the individuals of each brood (from the same mother) were split into an even number of pairs (2 or 4 pairs). Each pair was then transferred into a dedicated container (transparent bottles) and randomly assigned to one of the two treatments (day 1). At 10:00 in the morning the containers were submerged into the experimental tanks, maintaining the same number of bottles in the two treatments (i.e. heatwave and control). The containers were partially perforated to allow water exchange between the container and the water in the experimental tank. At 16:00 pm of day 5, the bottles were taken out of the experimental tanks and left to cool down to room temperature (i.e. 26°C) until the following morning.

On the morning of day 6, each individual was tested using the following three successive behavioral assays.

2.2.1 Open-field test

In the first test, each individual was released in a round arena with a water volume of 300ml (17.5cm in diameter) to measure swimming behavior and anti-predator behavior. In order to trigger the response two different stimuli were used:

- By quickly switching on and off all the lights of the room a dark stimulus was given (~2 sec). This stimulus was used to collect data on short term response to an unknown and unpredictable event. The dark stimulus is meant to resemble the passing shadow casted by an avian predator.
- To test long term response and olfactory perception a chemical stimulus (which has already been described in paragraph 2.1.2) was used.

The test was performed at a temperature of 26±1°C. Individuals were recorded using a high-definition camera set at 25 frames per second. The camera was mounted perpendicularly above the arena in order to minimize the perspective distortion. The test was divided in successive periods as follows: 5 minutes of acclimatization; 10 minutes of free swimming during a control period with no external stimuli; dark stimulus; 1 minute of swimming after the dark stimulus; chemical stimulus; 10 minutes of swimming following the chemical predator stimulus. At the end of the experiment the videos were cut, cropped and converted in grayscale using Handbrake Version 1.3.3. The prepared videos where then used to track the individuals using idTracker Version 2.1 (Pérez-Escudero et al. 2014). The output of the tracking software consisted of a table with cartesian coordinates corresponding to the position of the fish in the arena. Using RStudio the outputs of the tracking software were refined (e.g. the frames where the software lost the tracked individual were removed), realigned and rescaled so that they could be finally analyzed (*Figure 2*).

The effect of the dark stimulus was analyzed considering the first 10 seconds after the stimulus. The effects of the chemical stimulus where instead analyzed by comparing the 10 minutes of free swimming during the control period and the 10 minutes of swimming following the chemical predator stimulus, which from now on will be called “pre chemical” and “post chemical” respectively. Using the tracking output, knowing the position of the fish at each time point, five variables were calculated to evaluate the effect of both stimuli:

- *Average velocity:* the swimming velocity, calculated for each individual while actively swimming. The swimming velocity was used as a proxy of the activity level.
- *Total distance travelled:* the distance travelled during the entire analyzed period was also used as a proxy of the activity level.
- *Number of squares explored*: the area of the arena was divided in 1cm^2 and the number of unique squares explored by each individual was counted as a proxy of its exploratory behavior.
- *Time on the edge / time in the center:* the proportion of time the fish spent near the edge of the arena (distance from the center > 7.2 cm) over the time the fish spent close to the center of the arena (distance from the center < 7.2 cm) was used as a proxy of anxiety level (Sharma et al. 2008).
- *Time freezing / time moving:* the proportion of time that the individual spent frozen (i.e. the fish stay still, with a velocity of less than 1cm/s) over the time that the individual spent moving was calculated. This variable was then used as a proxy of anti-predatory behavior.

Last, the most common responses triggered by the dark stimulus were either a “jump” (i.e. the individual steeply increased its swimming speed for a fraction of time) or a freeze. For the dark stimulus the *number of freezing responses* obtained in the two groups was also taken into consideration as an anxiety proxy.

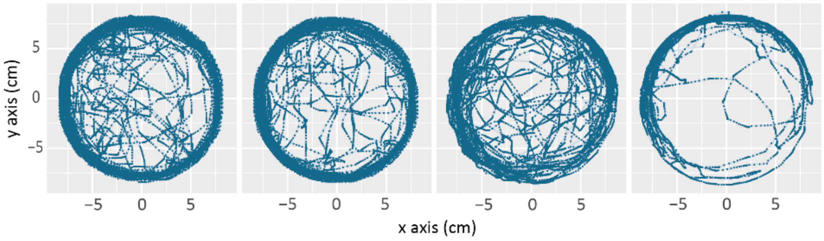

Figure 2. Examples of the tracking trajectories obtained after realignment and rescaling.

2.2.2 <u>*Capture test*</u>

To measure the ability of the fish to escape from a predator, a capture test was performed following Devigili et al. 2015. Before the test started, the specimen was introduced into a circular arena (49cm in diameter, 11cm of water) and left to acclimatize for 2 minutes. Subsequently, the operator began the test inserting an aquarium net (7.5x10cm) on the opposite side to where the individual was. Keeping the speed of the net constant, the operator approached the fish from behind trying to capture it. Time (in seconds) was measured from when the net was introduced in the arena to when the juvenile was in the net.

2.2.3 <u>*Flow chamber test*</u>

To estimate the juveniles' overall condition, a flow chamber was used. The test exploits the propension of fish to swim against the water flow and was used to measure the critical swimming speed (i.e. max speed flow endured by the individual) and stamina (i.e. test duration) using a previously established protocol (Nicoletto 1991). To achieve a steady laminar flow, water was pumped via an aquarium pump into a clear PVC pipe (1.5cm inner diameter, 50cm long) in which the tested individual could swim against the current (*Figure 3*). Time started when the fish was introduced in the pipe and ended when it fell from the pipe into the tank below. The flow was increased by 3 cm/s increments every minute (starting flow speed 7cm/s) through a valve that was connected to the system; the total resistance time and the critical speed were recorded. To account for the different swimming ability due to fish size, the critical speed was corrected dividing it by the standard length of the fish in centimeters (Stahlberg and Peckmann 1987, Nicoletto 1991) for the statistical analysis.

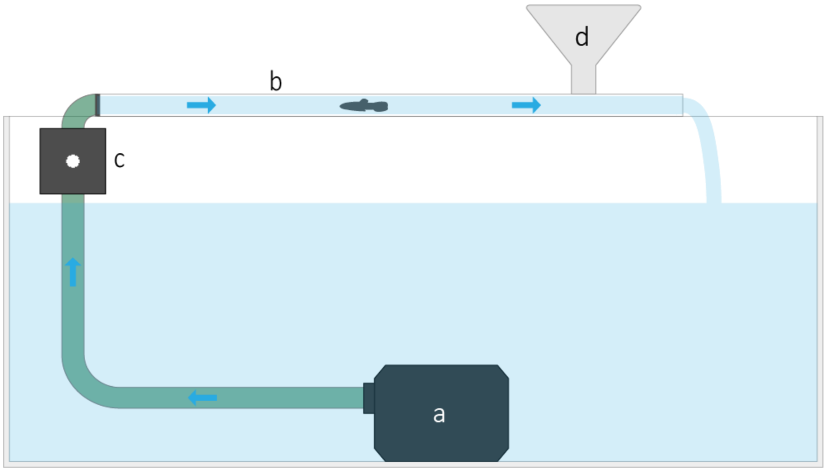

Figure 3. Experimental setup of the flow chamber test. A pump (a) was used to create a constant flow of water into a clear plexiglass pipe (b). The flow speed was controlled through a valve (c) located between the pump and the pipe. The tested individual was introduced into the setup through a funnel (d) connected to the pipe.

At the end of the experiment, each individual was photographed and weighted.

2.3 Statistical analysis

Data analysis was conducted using RStudio 2021.09.0 build 351. All data were analyzed using either linear models (LMs) with “lm” function, 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 the models, the treatment and period (i.e. pre or post stimulus) taken into consideration (where needed) were included as fixed factors. The fish ID, the tank where the fish was housed, the bottle where the individual was kept during the treatment and the mother’s ID were included as random factors when needed. The effects of the fixed variables were tested using the “Anova” function from “car” package. Capture test data were log transformed to achieve the normality of residuals distribution. The survival analysis was performed using “survival” and “survminer” packages using Kaplan-Meier method to fit data and performing a log-rank test (In and Lee 2018; In and Lee 2019).

Data means, in the results section, are reported with their standard error.

3. RESULTS

3.1 Heat waves effects on females' life-history and behavior

3.1.1 Fecundity and Survivability

The heatwave group produced significantly fewer broods compared to the control group ($X^2_{1,60} = 24.174$, $p < 0.001$). Only 4 out of 30 heatwave females produced broods, compared to 24 out of 30 control females. Moreover, from the heatwave group, 3 females produced only 1 brood and 1 female produced 2 broods, while many of the females from the control group produced up to 4 broods in the experimental time frame. Broods' size varied between a minimum of 2 fry and a maximum of 7 (mean 4.8 ± 1.0) for the heatwave group and between 2 and 14 (mean 6.4 ± 0.4) for the control (*Figure 4)*. As previously mentioned, data analysis for the fecundity was not performed due to poor numerosity. In total, 333 fry from the control group and 24 from the heatwave group were obtained. It is also worth mentioning that the gestation length of the females that underwent the heatwave (36.8 ± 9.8 days) appears to be longer compared to the control females (29.5 ± 1.6 days).

Survivability in the two groups significantly differed, with control females surviving longer after the treatment compared to the heatwave ones ($X^2_{1,60} = 5.200$, $p = 0.022$). There was a significant difference in survival at 7 and 14 days after the treatment started; the difference was no longer significant after 90 days when females where approximately 9 months old and natural mortality occurs (*Table 1, Figure 5*).

Table 1. Results of the survival test of the females at 3 different time points.

Time point	Survival control ± SE	Survival heatwave ± SE	Z value	p-value
7	1.000±0.000	0.733±0.081	3.303	**< 0.001**
14	0.967±0.033	0.567±0.091	4.157	**< 0.001**
90	0.400±0.089	0.2233±0.077	1.41	0.15

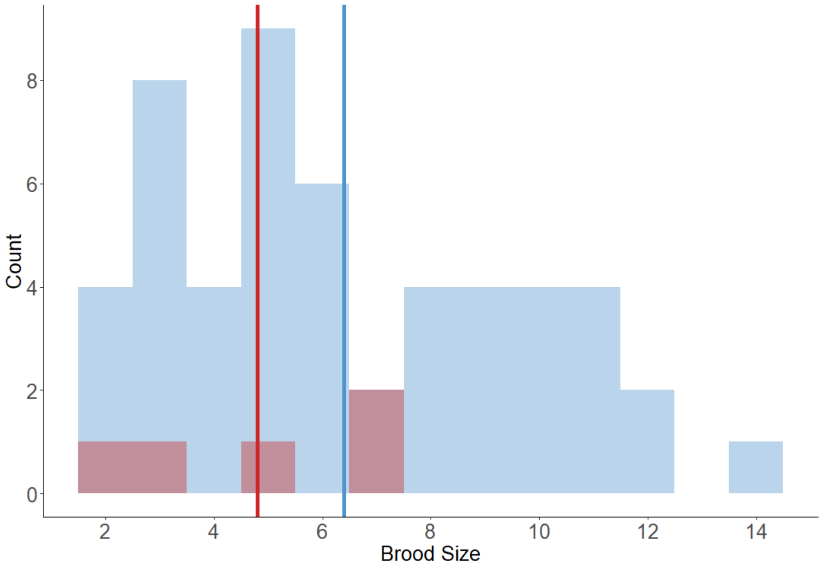

Figure 4. Broods' sizes distribution (shaded areas) and means (vertical lines). Control group (blue) produced on average bigger broods compared to the heatwaves group (red).

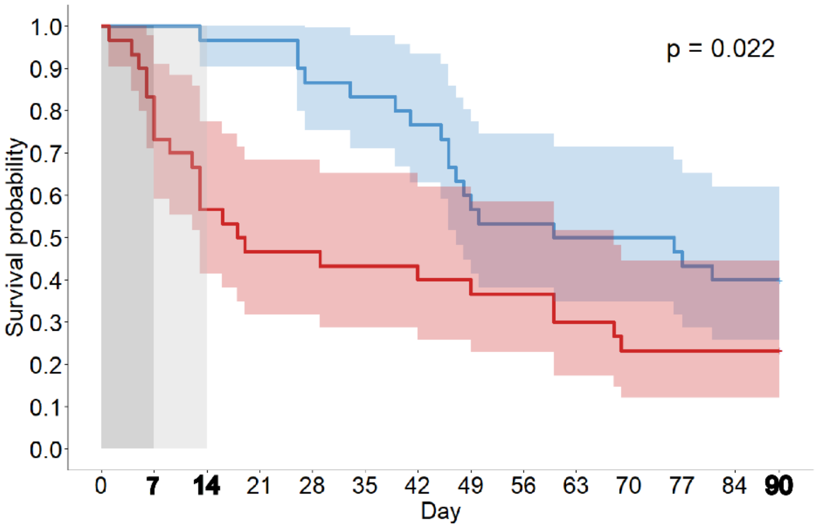

Figure 5. Female's survivability plot. On the x axis the number of days since the treatment started. Red line heatwave group; blue line control group; dark grey rectangle heatwave week; light grey rectangle 1st week after heatwave; shaded areas confidence intervals.

3.1.2 *Social/sexual motivation*

In order to perform the data analysis, the total duration of the test was divided into three different periods, each lasting 5 minutes (i.e. 1st period from 0 to 5th minute; 2nd period 5th-10th minute; 3rd period 10th-15th minute). The period of the trial taken into consideration and the interaction between the period and the treatment had a significant effect on the ratio of time the female spent in the male-choice area over the time spent in the female-choice area (*Table 2*). Generally, the control females spent less time in the female-choice area as the test progressed, while the heatwave group was more variable. Specifically, in the 2nd period heatwave females spent more time in the female-choice area compared to the control females (X^2_{28} = 9.060, p = 0.003); the same trend was present in the 3rd period (X^2_{28} = 2.985, p = 0.084) (*Figure 6*). The differences were statistically significant even between the 1st - 2nd period (X^2_{28} = 47.127, $p <$ 0.001) and the 2nd - 3rd period (X^2_{28} = 78.431, $p <$ 0.001) within the heatwave treatment. Lastly within the control treatment differences were statistically significant between the 1st - 3rd period (X^2_{28} = 54.799, $p <$ 0.001) and 2nd - 3rd period (X^2_{28} = 33.164, $p <$ 0.001).

Table 2. Results of the GLMM for the social/sexual motivation test. The period and the interaction between the treatment and the period had a significant effect on where the females spent their time.

Predictors	**DF**	X^2	**p-value**
Treatment	1	1.497	0.221
Period	2	73.124	**< 0.001**
Treatment:Period	2	54.076	**< 0.001**

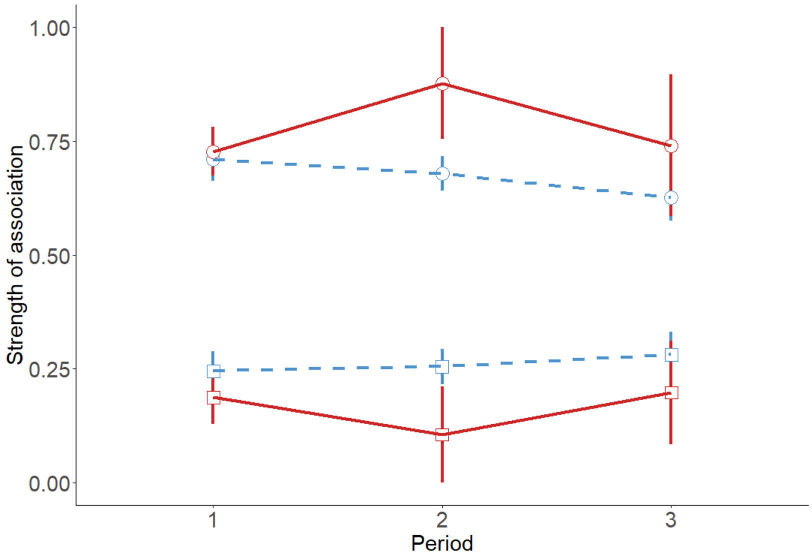

Figure 6. The figure represents the proportion of time that the females spent in the female-choice area (circles) and male-choice area (squares) over the total experiment time in each period. Red solid line: heatwave group; dashed blue line: control group. Vertical lines show the standard error at each point. The sum of the values in each period within treatment differs from 1 because the fraction of time spent in the no-choice area is not plotted.

3.2 Heat waves effects on juveniles' life-history and behavior

In total 102 juveniles, evenly split between heatwave and control groups, from 20 different mothers were tested. Post treatment comparison between the two groups shows no differences in standard length ($F_{1,102}$ = 0.011, p = 0.916), weight ($F_{1,102}$ = 0.011, p = 0.918) or body condition computed using Fulton's index (Kotrschal et al. 2015; $F_{1,102}$ = 0.972, p = 0.324). Mortality for both juvenile groups was equal to zero.

3.2.1 Open-field test results

Dark stimulus: The dark stimulus generally had no effect within 10 seconds on all the variables tested but two (even if not significatively so). The time the fish spent frozen after the dark stimulus tended to be higher in the heatwave group compared to the control group (X^2_{102} = 3.0726, p = 0.080). Furthermore, the heatwave group tended to freeze more often compared to the control group (X^2_{102} = 2.9266, p = 0.087).

Chemical stimulus: The time period (i.e. pre or post chemical stimulus) significantly affected all of the measured variables (*Table 4*), indicating that fish from both the control and the heatwave group reacted to the exposure to the chemical stimulus by changing their behavior. In general, there was a reduction in velocity and activity after the chemical stimulus *(Table 3)*. Treatment and the interaction between treatment and time period affected swimming velocity and the ratio of time that fish spent in the peripheral zone of the arena, respectively (*Table 4*). Specifically, the average swimming velocity of fish from the heatwave group tended to be higher than that of fish from the control group, independent of the time period *(Table 3 and 4, Figure 7).* Post hoc comparisons of the ratio of time fish spent in the periphery showed that while fish from both treatments increased time in the periphery after receiving the chemical stimulus, the increase was steeper in the control (X^2 = 210.39, $p <$ 0.001) compared to the heatwave group (X^2 = 9.813, p = 0.002, *Table 3, Figure 8*). Neither treatment nor its interaction with time period was significant in any of the other models (*Table 4*).

Table 3. Means and standard errors of the variables analyzed before (pre) and after (post) chemical stimulus.

Variable	**PreChemical**		**PostChemical**	
	Control	**Heatwave**	**Control**	**Heatwave**
Average velocity	4.185±0.290	4.824±0.316	3.790±0.307	4.678±0.326
Total distance travelled	2349.820±159.344	2593.988±186.499	1807.351±147.171	2171.015±202.642
Nr of squares explored	220.098±4.184	214.961±6.004	196.157±5.329	196.529±6.467
Time edge / total time	0.754±0.022	0.744±0.021	0.800±0.020	0.754±0.023
Time freezing / total time	0.107±0.019	0.130±0.027	0.263±0.030	0.282±0.035

Table 4. Results from GLMM models testing various behaviors of juveniles in an open field test in response to the Treatment (control or heatwave), Period (pre or post receival of a chemical stimulus), and their interaction.

Variable	**Chemical stimulus**					
	Treatment		**Period**		**Interaction**	
	X^2	**p**	**X^2**	**p**	**X^2**	**p**
Average velocity (cm/s)	2.951	0.086	6.827	**< 0.001**	1.547	0.214
Total distance travelled (cm)	1.809	0.179	41.852	**< 0.001**	0.641	0.423
Nr of squares explored	0.112	0.738	34.823	**< 0.001**	0.589	0.443
Time edge / time center	1.122	0.29	150.582	**< 0.001**	69.623	**< 0.001**
Time freezing / time moving	0.451	0.502	5347.343	**< 0.001**	0.001	0.983

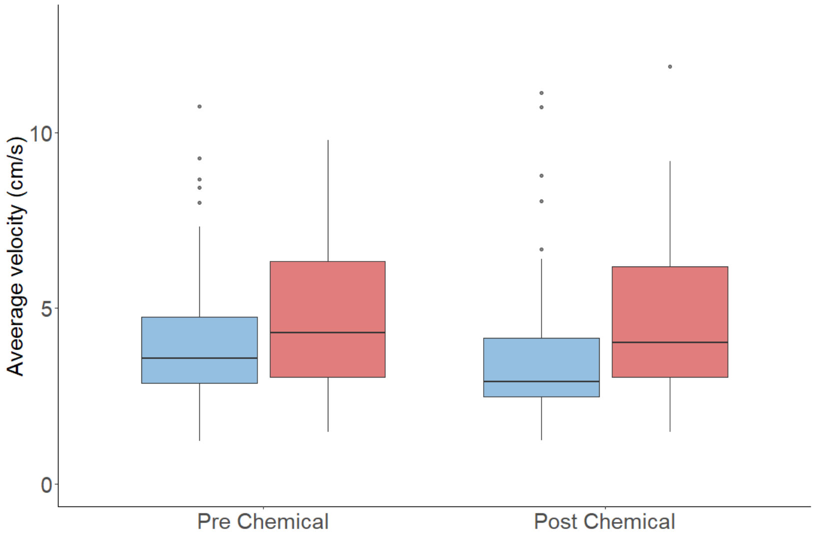

Figure 7. The boxplot represents the average velocities pre and post chemical stimulus.

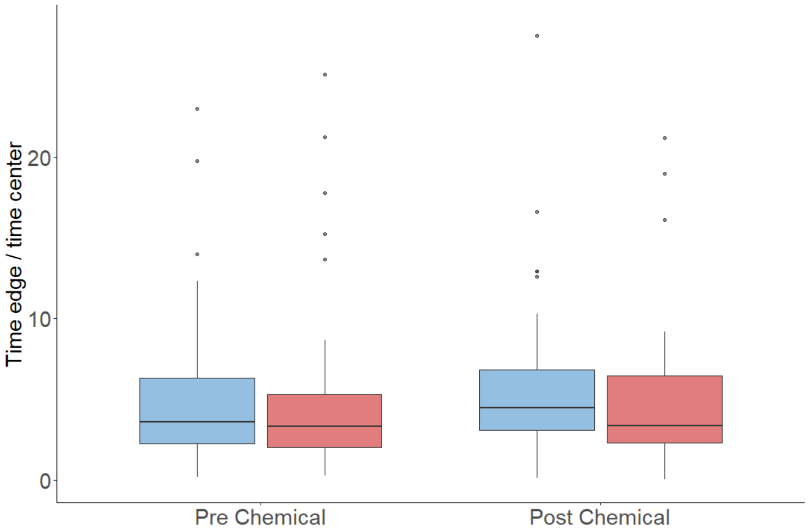

Figure 8. The boxplots represent the fraction of time the fish spent on the edge over the time spent in the center of the arena, pre and post chemical stimulus. Control group (blue) increases the time spent close to the edge in the post chemical relative to the pre chemical more than the heatwave group (red).

3.2.2 *Capture test results*

No significative differences were found between the average capture times of the control group and the treated group ($X^2_{102} = 0.997$, $p = 0.318$).

3.2.3 *Flow chamber test results*

No significant differences were found between the corrected critical speeds of the two groups ($X^2_{102} = 1.840$, $p = 0.175$). Same for the differences in resistance time between the control and the treatment individuals ($X^2_{102} = 1.330$, $p = 0.249$).

However, when removing from the dataset two outliers that scored very low resistance times, probably because these individuals were in poor health conditions, there was a trend for individuals in the heatwave group to have on average a lower resistance time compered to the control group ($X^2_{100} = 3.353$, $p = 0.067$, *Figure 9*).

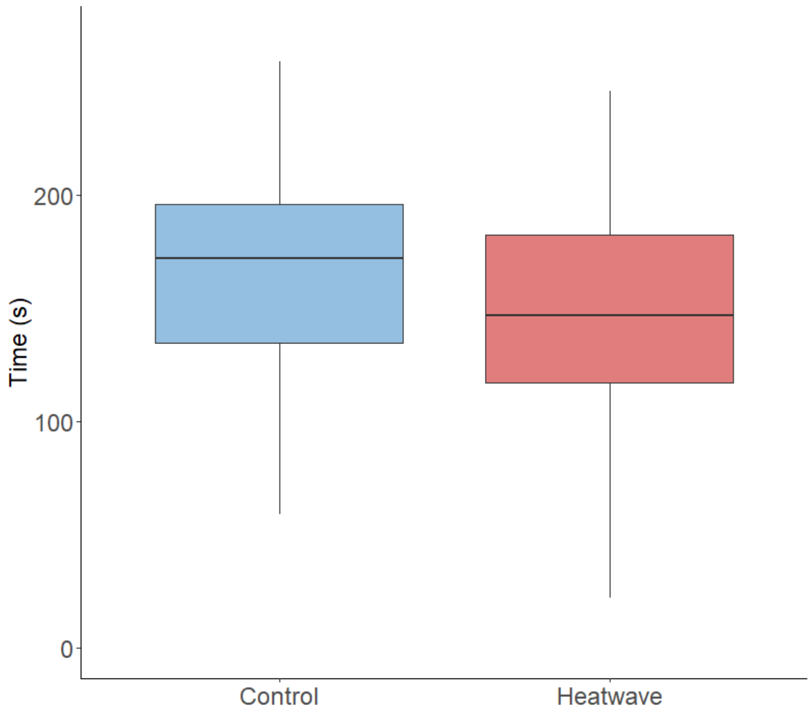

Figure 9. Boxplots show the resistance time without the outliers. Heatwave individuals tended to have lower resistance times compared to the control individuals.

4. DISCUSSION

This study demonstrates that MHW can have different effects on individuals that are in different life stages. The results obtained also show that heatwaves effect mortality, fertility and anti-predatory behavior.

4.1 The effects of heatwaves on females' fecundity, survivability and social/sexual motivation

Heatwaves negatively affected gravid females of *P. reticulata* decreasing their fecundity, increasing their mortality, and changing their social and sexual motivation.

In line with a previous study (Dzikowsky et al. 2001), despite some differences in the protocol used, this study shows that heatwaves do have an effect on the fecundity of the females. In particular, females that underwent the heatwave produced fewer and smaller broods compared to the control females. However, it is important to keep in mind that these results have to be interpreted with caution because the mortality rates differed in the two groups. In particular heatwave females had a higher mortality rate (see below) and therefore only four females produced any brood. Due to the lower numerosity of the females giving birth in the heatwave, analyses on the fecundity between the two groups will require further investigation in order to confirm the results.

Mortality caused by MHWs has been reported for numerous taxa and species over the past years (Darmaraki et al. 2019). Data collected in this study clearly shows that females that underwent the heatwave had a lower survival rate compared to control females. Mortality was particularly high during the heatwave and in the following week. Moreover, it is important to highlight that mortality due to the heatwave was almost absent in the males that were treated with the females (Breedveld et al, unpublished data). Therefore, the mortality is probably connected to the pregnancy, in line with pregnant females showing reduced upper thermal tolerance (Auer et al. 2021). Being fishes ectothermic organisms and being *P. reticulata* a small sized organism with high surface-volume ratio and therefore a low thermal inertia, the higher temperature of the water could rapidly increase the metabolic rate of the females and thus aging (Flouris and Piantoni 2015). This different response to the stressful environment could be the mechanistic cause of the observed difference between experimental groups. Moreover, the higher temperatures in the heatwave group may also have decreased the oxygen levels of the water adding another stressor to the already not optimal conditions.

In the social/sexual motivation test, control females increased the time they spent with males as the test progressed; contrarily heatwave females did not, spending more time close to other females. *P. reticulata* shows a strong social tendency when put into an unfamiliar tank (Dadda et al. 2015; Lucon-Xiccato et al. 2016). Control females over time get used to the tank and thus are more inclined to stay with the males. Heatwave females instead spent more time with other females compared to the control group probably because of a higher stress condition (caused by the heatwave). The potential stress and/or decreased condition of the heatwave females may therefore have led to a lack of sexual motivation. In this species males always attempt to mate (using the forced mating tactic, i.e. sneaking). Therefore, a receptive female that is not in optimal condition is expected to reduce the time with the males and increase the shoaling with other females in order to reduce the costs connected to reproduction and unwanted mating sexual attempts.

Alternative explanations can be given as to why heatwave females tend to avoid males especially in the 2nd period of the test. One of the explanations could be that the males show decreased interest in the heatwave females because they can perceive their overall poor condition leading to decreased mating/courtship attempts; the females will thus be less attracted to the males as a response to the male behaviour. Reversible plasticity (i.e. short-term environmental effects on a behavioral response) could be another explanation to the variability that the heatwave females of this study had in their social/sexual choices. Previous studies have reported that stressors can lead to incoherent or generally hard to explain behaviors in both invertebrates (as the stalk-eyed fly, *Cyrtodiopsis dalmanni,* Hingle et al. 2001) and vertebrates (in particular fishes as the green swordtail, *Xiphophorus helleri,* Pilakouta and Alonzo 2014). In these studies, the effect of food and predator stressors, respectively, heavily influenced the behavior of the individuals altering the strength of females' preferences. Other stressors, as the thermic stressor of the present study, could have a similar effect. Moreover, both studies have shown that the stressor did not cause any permanent change and that resulted in a high variability in behavior.

It is important, however, to keep in mind that the sample size obtained in the social/sexual motivation test was unbalanced. More studies are therefore needed to consolidate these results.

4.2 The effects of heatwaves on juveniles' condition and anti-predator behavior

Heatwaves also affected juveniles' behavior and overall condition without, however, effecting their growth rate or survival.

After the heatwave there were no differences in standard length, weight, body condition index and mortality between the heatwave group and the control group. As previously mentioned, higher temperatures correspond to a higher metabolic rate and thus energy consumption, hence a worse body condition index or slower growth rate could be expected in the heatwave group. However, the short duration of the treatment and the *ad libitum* feeding plan used in this study could have masked the effects of the heatwave on the juveniles' life-history traits examined. Future studies could test this hypothesis by testing heatwave effects on juveniles that are maintained in a more stressful environment, such as under a restricted diet regime. This is however good news as it shows that a single heatwave during development does not affect growth or that fish can efficiently catch up.

As Templeton and Shriner (2004) have shown, a common fish anti-predator strategy is freezing in order to avoid being detected. In the present study, the freezing time and number of freezing events were measured as proxies of the short-term response to the dark stimulus. The group that underwent the heatwave, in the ten seconds after the stimulus, froze more often and for longer compared to the control group. These results suggest that individuals that underwent the heatwave are more fearful, probably because of the stress recently suffered compared to the control individuals (Oswald et al. 2012).

All variables taken into consideration suggested that the chemical predator stimulus was highly effective in triggering a behavioral response. In general, the stimulus caused a reduction of velocity and activity levels in all tested individuals of both experimental groups leading to conclude that the heatwave did not interfere with the olfactory perception of the individuals. Nevertheless, the response that the two groups had to the stimulus differed. In particular, control individuals responded at the chemical predator stimulus spending more time close to the edge of the arena, a common behavior usually performed to avoid being detected by predators. Fish from the heatwave group on the other hand did not respond as strongly to the stimulus. They maintained a high average swimming velocity (indicating a high stress level) while at the same time not increasing thigmotaxis (“wall-hugging” behavior) as steeply as the control (often considered as index of anxiety in vertebrates e.g. Simons et al. 1994 and fishes e.g. Sharma et al. 2008). This mixed response could once more be consistent with poor choice consistency due to the high level of stress caused by the heatwave (Hingle et al. 2001; Pilakouta and Alonzo 2014). This sub-optimal response to a predation cue could expose individuals to a higher predation risk having obvious repercussions on fish survival, and hence communities (Bresson et al. 2020).

Both the capture test and the flow chamber test measure the condition of the individual although with different focuses. The capture test has a short duration and the endurance of the fish is therefore of secondary importance; moreover, the individual is tested in a “life or death” situation with the possibility to swim freely. In this test no differences were found between the control and the heatwave group suggesting that the ability to escape from a predator in the short term is not compromised by the heatwave. On the other hand, the flow chamber strictly measures the endurance of the individuals when swimming against a current for a much longer time. In this situation the fish that underwent the heatwave scored lower times. A possible explanation for this result is that growth was more demanding for fish that underwent the heatwave. If this is the case, we expect that even if they grew at the same rate as the control individuals they did not have a lot of energy available to perform well in the endurance test. Therefore, their “poor” anti-predator response may make heatwave fish more easily detectable by a predator.

4.3 Conclusions

This study clearly shows that heatwaves have a strong effect even on hardy species as *P. reticulata*, even when living in optimal conditions (ad libitum food, no predators, no parasites). This is not always the case in nature where many other factors come into play. Considering the controlled environment in which the individuals of this study were tested and the high thermal tolerance of guppies it is therefore probable that MHWs have an even stronger effects on other fish species.

It is also clear that MHWs do not impact all individuals of a population equally. As this study shows, individuals in a particular life stage or of a particular sex can be much more susceptible to MHWs compared to others; specifically, the mortality risk was significantly increased by the heatwave for gravid females but the same was not true for the juveniles or the males. MHWs could therefore differently impact species depending on when the MHW occurs in relation to the life stage of that particular species, and the sex ratio of the population. Juveniles that underwent the heatwave showed a normal growth rate. Nevertheless, they traded off condition against endurance, potentially exposing them to a higher predation risk.

Obviously very mobile species (as some fishes) can easily shift their habitats, but smaller species or species that require specific resources or symbiosis to survive, will probably be severely impacted by MHWs. Moreover, MHWs will have a major impact on populations that already inhabit areas close to their upper thermal limit (Smale et al. 2019).

The results obtained from the present work fit well in the increasing number of studies regarding MHWs, confirming and expanding knowledge on this particular topic. Extreme events such as heatwaves appear to be one of the major problems resulting from climate change and studies like the present one are extremely important to better understand how populations respond to this threat and how this might affect population resilience and, more generally, impact biodiversity.

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