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Does thermal adaptation enhances mosquito male tolerance to heatwaves?

L'adattamento termico aumenta la tolleranza dei maschi di zanzara alle
ondate di calore?

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ABSTRACT

Aedes albopictus, the Asian tiger mosquito, is a highly invasive species and a major arboviral vector. Because mosquitoes are ectotherm organisms, climate change, including global warming and the increase in extreme weather events are expected to affect *Ae. albopictus*. Its epidemiological relevance by conditioning its distribution and abundance. Current control strategies targeting adult mosquitoes rely on the release of appropriately-modified males, which should successfully locate a female and transfer enough viable sperm to fertilize eggs throughout the female lifespan, because *Aedes* spp. mosquitoes tend to be monandrous, but can re-mate if the first mating is not successful. While aspects of sperm maintenance in female spermathecae and fecundity have been studied, aspects influencing male reproductive capacity are less understood.

This thesis investigated whether prior exposure to heat improves male tolerance to an heatwaves (HW), and whether repeated HW exposures produces additive detrimental effects on male fitness.

I found that prior exposure to heat enhance negative impacts of exposure to HWs both at the pupal and adult stages. Exposure to a second HW had an additive impact on male longevity. On the contrary I did not observe significant changes in female fecundity and fertility when females had mated with HW-exposed males. These findings indicate that thermal history does not straightforwardly improve male heat tolerance and underscore a stage-specific vulnerability to HWs.

ABSTRACT

Aedes albopictus, la zanzara tigre asiatica, è una specie altamente invasiva e un importante vettore di arbovirus. Poiché le zanzare sono organismi ectotermici, si prevede che i cambiamenti climatici, tra cui il riscaldamento globale e l'aumento degli eventi meteorologici estremi, influenzino *l'Ae. albopictus*, incidendo sulla sua rilevanza epidemiologica attraverso la modifica della sua distribuzione e abbondanza. Le attuali strategie di controllo rivolte alle zanzare adulte si basano sul rilascio di maschi opportunamente modificati, che dovrebbero riuscire a individuare una femmina e a trasferire una quantità sufficiente di spermatozoi vitali per fecondare le uova per tutta la durata della vita della femmina, poiché le zanzare del genere *Aedes* tendono ad essere monandriche, ma possono accoppiarsi nuovamente se il primo accoppiamento non va a buon fine. Mentre sono stati studiati alcuni aspetti relativi alla conservazione dello sperma nella spermateca femminile e alla fecondità, quelli che influenzano la capacità riproduttiva dei maschi sono meno conosciuti.

La presente tesi ha indagato se una precedente esposizione al calore migliori la tolleranza dei maschi alle ondate di calore (HW) e se esposizioni ripetute alle HW producano effetti negativi additivi sull'idoneità dei maschi.

Ho riscontrato che l'esposizione preventiva al calore accentua gli effetti negativi dell'esposizione alle ondate di calore sia allo stadio pupale che a quello adulto. L'esposizione a una seconda ondata di calore ha avuto un impatto additivo sulla longevità dei maschi. Al contrario, non ho osservato cambiamenti significativi nella fecondità e nella fertilità delle femmine quando queste si erano accoppiate con maschi esposti alle ondate di calore.

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1. INTRODUCTION

1.1 *Aedes albopictus*

Aedes albopictus, also known as the Asian tiger mosquito, is a species capable of transmitting more than 20 different viruses, overall called arboviruses, threatening many people worldwide, partly due to its rapid global spread (1,2). *Aedes albopictus* is the main vector of arboviruses in Europe, where it became established during the 1990s and has since caused recurrent local outbreaks of dengue and chikungunya (2-4).

Aedes albopictus is a holometabolous insect. Growth begins as eggs, which hatch into larvae. Larvae move through four different developmental stages before pupation. Pupae undergo metamorphosis leading to adult emergence. Eggs are oval-shaped, and females lay them at the edge of bodies of water. Eggs hatch approximately 48 hours after deposition, giving rise to first-instar larvae. Throughout their four stages, larvae feed actively. After each molt, they increase in size until they pupate. Pupae, do not feed. Adults emerge two to three days after the pupal stage. The size of an adult mosquito ranges from two to ten millimeters in relation to crowding conditions, feeding opportunities and rearing temperature, males are always approximately 20% smaller than females. Adults are characterized by silvery-white scales on the palp, tarsi, and tarsal segment of the legs (Figure 1). An important feature is a central white line running from the dorsal surface of the head across the thorax (5,6). In addition to their smaller size, males are clearly distinguished by their feathery antennae. Additionally, while males feed exclusively on nectar, females require a blood meal from a vertebrate host to complete eggs developmental. Female *Aedes spp.* mosquitoes are aggressive diurnal biters with a preference for outdoor environments.

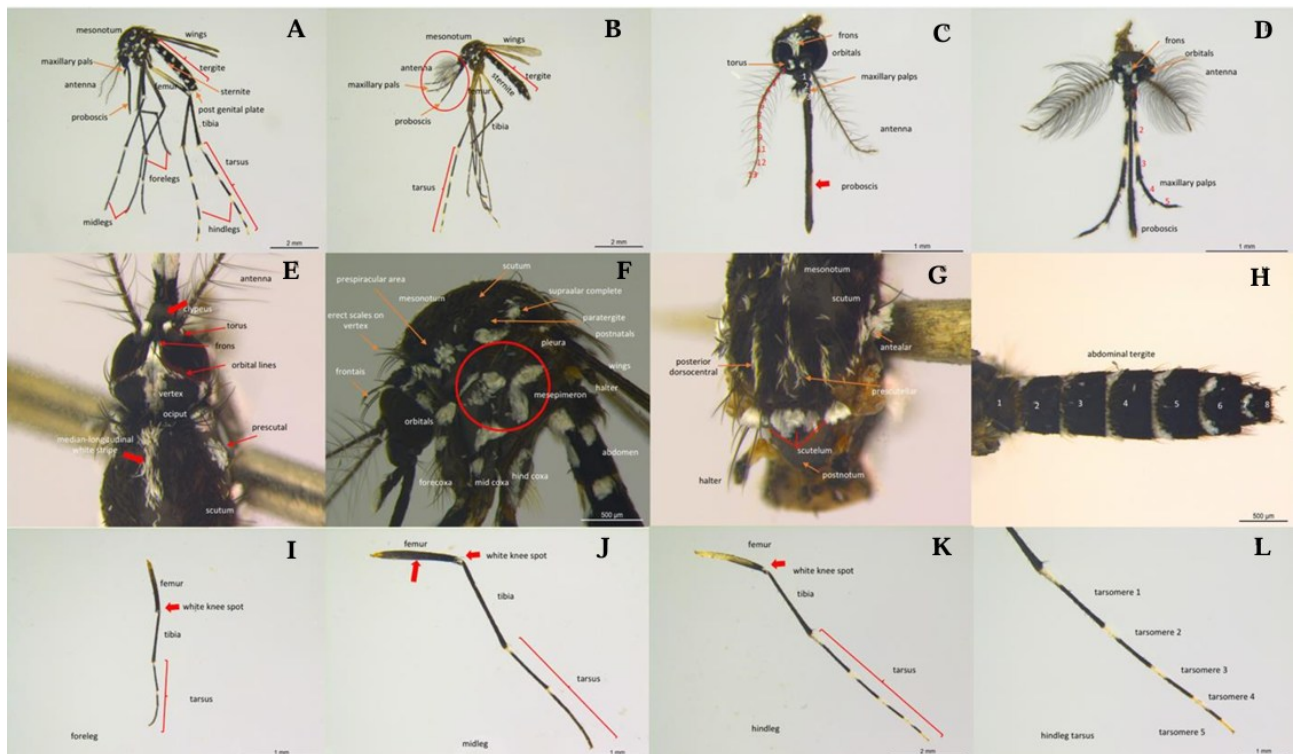


Figure 1. *Aedes albopictus* adults. (A-B) female and male, (C-D) head of a female, head of a male, (E-G) different view of thorax, (H) dorsal abdomen, (I-K) fore, mid and hind legs, (L) tarsus of hind leg. (85)

1.2 Invasion of *Aedes albopictus*

Aedes albopictus is a highly invasive species that has spread worldwide over the past 60 years (Figure 2). This species is native to Southeast Asia, where it was among the zoophilic species found in tropical forests. From there, *Ae. albopictus* migrated toward suburban and rural areas, where most vegetation is in close contact with humans. These environments offered greater opportunities for blood feeding, while also providing ideal ecological conditions for increasing developmental speed and survival rates (10-12).

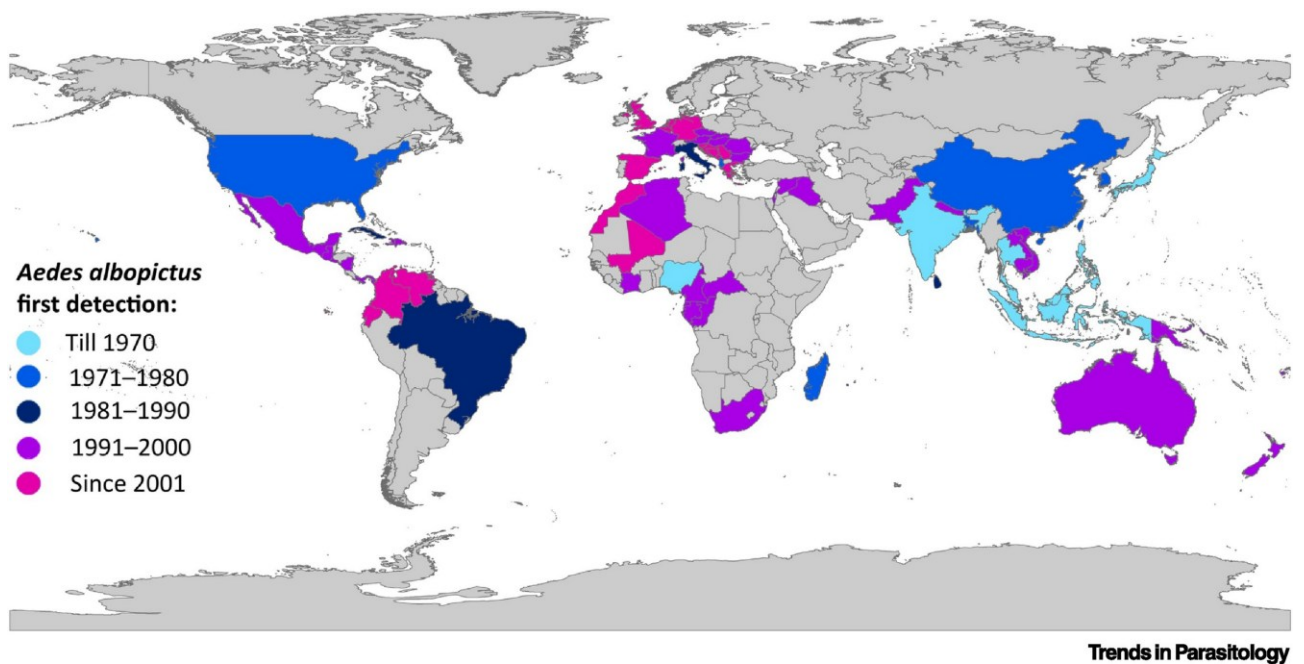


Figure 2. Global distribution of *Ae. albopictus* colored according to the first detection year in the area. Modified from (12).

Global expansion occurred in two phases. The first wave took place in the 1800s, when human migration between the Indo-Malay peninsula and the Indian Ocean facilitated the spread of *Ae. albopictus* to island of the Indian Ocean such as La Reunion (13). The second wave began in the latter half of the last century, when *Ae. albopictus* spread across the globe, primarily through passive movement associated with human mobility and intercontinental trade (14). Based on these two invasion waves, *Ae. albopictus* populations are classified as native, old, or invasive, depending on their origin within the native range or in regions colonized during the first or second expansion wave.

In Europe, *Ae. albopictus* was first reported in 1979 in Albania. In Italy, it was first detected in Genoa in 1990 (15). Shortly thereafter, it was documented in 22 other Italian provinces in northern Italy, including regions of Friuli-Venezia Giulia, Veneto, Lombardy, Emilia-Romagna, and the coastal areas of Lazio (16). Today, *Ae. albopictus* is ubiquitously present across all Mediterranean countries, also in northern European countries such as Switzerland, Belgium, the Netherlands, Bulgaria, and Russia (17,18). In the Americas, the species was first reported in Texas in 1985 and is now established in more than 32 states (16). It spread to Brazil and Mexico within three years. Its presence was also documented, during the same period, in Bolivia, Venezuela, Uruguay, Argentina, Colombia, Paraguay, and various Caribbean islands (1). In Africa, the first sighting of

Ae. albopictus occurred in Cape Town in 1989, linked to the trade of tires imported from Japan. Shortly afterward, it was also documented in Cameroon, Gabon, Nigeria, and Equatorial Guinea (3,19). Clear genetic differences exist among *Ae. albopictus* populations originating from different regions. Invasive population tend to be genetically admixed because invasion generally occurs through numerous introductions from different regions (20).

1.3 The relevance of reproduction for a species' invasion success

Following its introduction, any species must establish to be considered an invader. This means that invading populations must adapt to the new environment through interactions with native predators and similar resident species. Regardless of taxon, invasive species, tend to show high reproductive capacity (a greater number of offspring) and very short generation times (24-27). Reproduction is a complex trait governed by both genetic and physiological factors. Additionally, both reproductive development and reproductive physiology tend to be non-static traits (28). Based on the genetic diversity and physiological plasticity of *Ae. albopictus* populations, it is expected that their reproductive capacity may differ as well. Understanding these dynamics is important to highlight the invasion capacity of the species and possibly identify its most invasive populations.

Differences in timing and resource allocation to reproduction were identified across *Ae. albopictus* populations (90).

Furthermore, crosses between different populations have shown that offspring from these matings display superior reproductive performance compared to the parental populations, providing evidence for heterosis. Thus, genetic admixture between populations originating from independent invasion events can increase overall progeny fitness, improving reproductive efficiency and, consequently, enhancing the species' expansion potential (90). Reproductive traits

thus emerge as a determining factor in the invasion process. In the context of invasive success of the species the male contribution to reproduction, has not been extensively studied apart from the understanding of the anatomy of the reproductive apparatus, and in relation to vector control applications such as SIT and Wolbachia-based approaches.

1.4 Male reproductive apparatus and physiology

Most of current knowledge of the *Aedes spp.* mosquito reproductive system is based on *Ae. aegypti*, where the male reproductive system (MRS) consists of a pair of testes, one vas deferens per side, a pair of seminal vesicles, a pair of accessory glands, and a common ejaculatory duct — an anatomical arrangement consistent with the general organization of the MRS described in insects (91). Full development of the apparatus is not synchronized across all components but follows a well-defined post-embryonic sequence. Already at the last larval stage (L4), the testis is present and shows an initial, slight degree of spiralization of the testicular follicle, associated with a thin vas deferens; at this stage, neither the accessory glands nor a functionally mature seminal vesicle are yet distinguishable, although the structure remains histologically recognizable, characterized by a thin epithelium and the absence of spermatozoa in the lumen (91). At the transition to the pupal stage, the MRS reaches its full structural configuration, with the appearance of the accessory glands and the formation, in the proximal portion of the vas deferens, of a funnel-shaped dilation (termed the "vas deferens goblet") that converges, together with the seminal vesicles and accessory glands, into the common ejaculatory duct (Figure 3) (91).

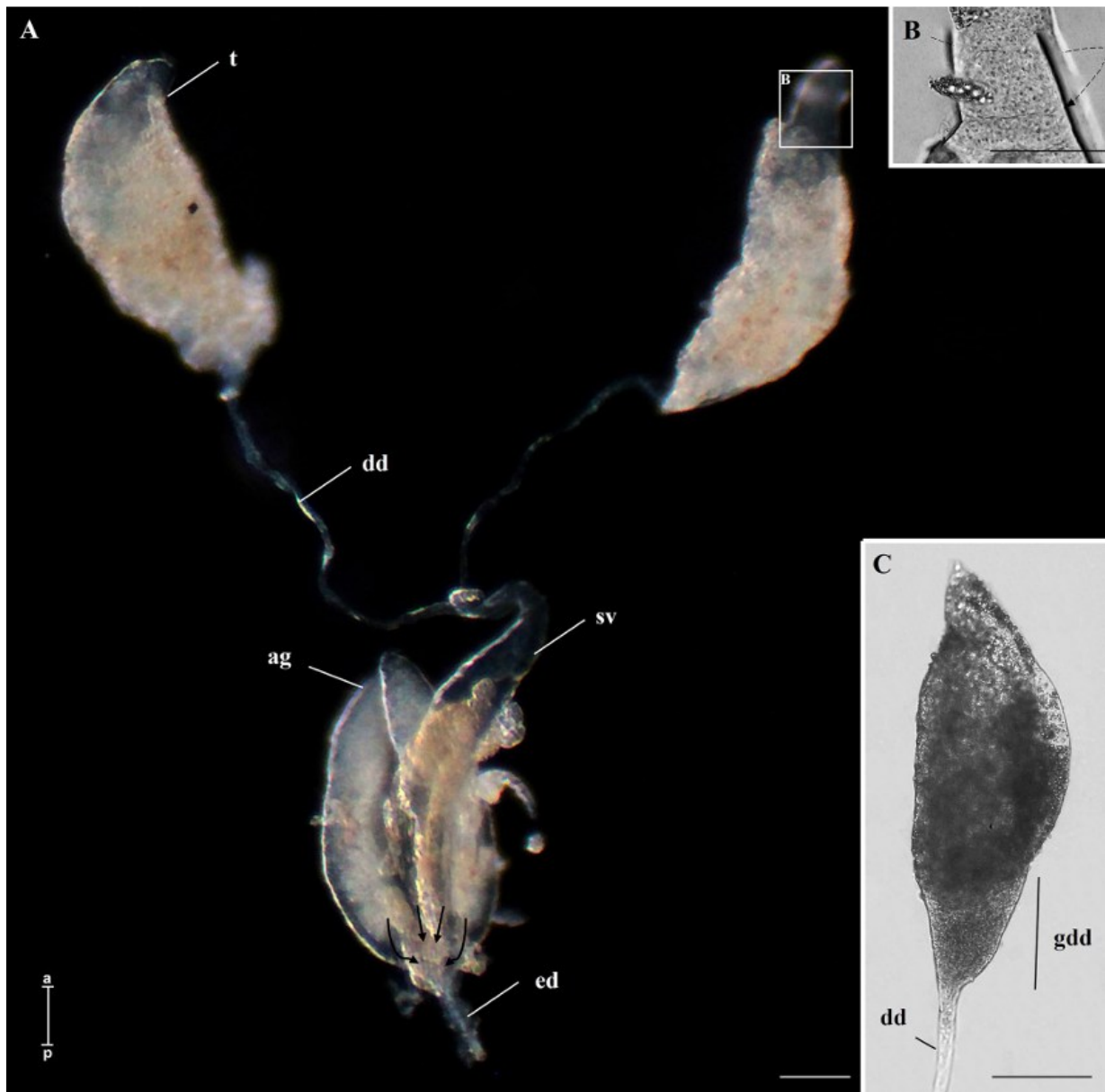


Figure 3. Whole-mount of MRS of *A. aegypti* adults. **A** Testis (t), deferent duct (dd), seminal vesicles (sv), accessory glands (ag), and ejaculatory duct (ed). The arrows indicate that the accessory glands and the seminal vesicles empty into the ejaculatory duct. **B** The spiral shape of the testis (dotted arrow). **C** Detail of the testis showing the goblet of the deferent duct (gdd). Scale bars: 100 μ m. a, anterior region; p, posterior region. Adapted from (92)

The seminal vesicles, which serve as the site of sperm accumulation and storage, are histologically observable as early as the L4 stage; in adults, they are already full of spermatozoa immediately after emergence (91). The accessory glands, of the apocrine secretory type, become morphologically distinguishable starting from the pupal stage and increase in volume in the adult,

likely due to progressive accumulation of secretory material; together with the seminal vesicles, they converge into the ejaculatory duct, which has an internal cuticular lining (91).

Overall, the available data on *Ae. aegypti* indicate that structural development of the male reproductive tract is closely linked to the onset and maintenance of spermatogenesis, following a sequential maturation pattern (larva → pupa → adult) with continuous sperm production in the adult. These findings provide an essential biological reference for understanding the target mechanisms of vector control strategies based on altering male fertility or reproductive competitiveness, which will be discussed in the following section.

1.5 Control strategies against *Ae. albopictus* adults focus

All innovative strategies to control adult mosquitoes, including the Sterile Insect Technique (SIT) and strategies based on the endosymbiotic bacterium *Wolbachia pipientis*, are based on the release of appropriately modified males.

SIT is based on releasing large numbers of males rendered sterile, traditionally through irradiation. Once released sterile males should compete with wild males for mating with females, producing non-viable eggs and thereby reducing the density of the target population (83). The effectiveness of this approach depends critically on the ability of sterilized males to transfer enough sterile sperm to females to inhibit re-mating and be as competitive as wild males in mating with females. In this regard, an experimental study conducted on an *Ae. albopictus* population originating from Rimini examined in detail the reproductive strategy and sperm management in males of the species, comparing untreated individuals with individuals sterilized by X-ray irradiation (83). The results indicate that, at the level of a single mating, sterilized males did not differ significantly from untreated ones in copula duration, insemination success (approximately 80% in both groups), or the approximate amount of sperm transferred, estimated by measuring the surface area of the female *bursa inseminalis* (83). This results is particularly relevant for SIT, as it suggests that the sterilization treatment does not compromise, at least in the short term, the reproductive competitiveness of released males relative to wild males.

The study also showed that the contents of the *bursa in seminalis* — a mixture of spermatozoa and male accessory gland secretions — progressively solidify in the minutes following copulation, acting as a temporary physical barrier that limits the possibility of a second effective insemination: sperm competition between two males is limited to closely timed copulations within an interval of about 40 minutes, beyond which the progeny produced in subsequent gonotrophic cycles is generated exclusively by the first mating male (83). A particularly relevant aspect for planning SIT programs concerns the ability of males to replenish their sperm reserves after depletion induced by repeated matings: while untreated males show a distinct recovery pattern after a rest period of a few days, sterilized males do not, resulting in a lower maximum number of females they can fully inseminate over their lifetime compared with untreated males (83). This has real implications for the operational design of release programs, since the reduced replicative capacity of the sperm stock in sterilized males requires carefully calibrating the frequency and scale of releases to maintain sufficient competitiveness over time.

Wolbachia pipientis is an obligate intracellular endosymbiotic bacterium naturally present in numerous arthropod species, including *Ae. albopictus* (91). The biological mechanism underlying the use of *Wolbachia pipientis* is cytoplasmic incompatibility (CI), a phenomenon where mating between a *Wolbachia*-infected male and a female, that is not infected or is infected with a different strain than males, results in non-viable embryos, females carrying the same strain as the male produce viable, *Wolbachia* infected offspring (91). This mechanism gives infected females a selective reproductive advantage that, once a critical frequency threshold is reached in the population, makes the spread of the infection self-sustaining.

Based on this mechanism, two distinct operational strategies have been developed. The first, known as population replacement, involves releasing individuals of both sexes carrying *Wolbachia*, with the aim of permanently replacing the uninfected wild population. This strategy works with insect species, which do not carry *Wolbachia* naturally.

The second strategy, known as population suppression or the Incompatible Insect Technique (IIT), instead involves releasing only infected males in order to induce sterility in uninfected wild females, thereby reducing the overall density of the vector population; unlike the first strategy, this one depends on continuous releases over time and does not lead to permanent population replacement (91).

From a comparative standpoint, the two *Wolbachia*-based strategies offer complementary advantages and limitations relative to classical SIT. Population replacement is generally more

economically sustainable over the medium-to-long term, as infection stability in the target population has been documented for periods of up to 7–10 years following a single intervention, whereas both population suppression and traditional SIT require ongoing releases to maintain the control effect (91).

Overall, SIT and Wolbachia-based strategies share the central approach of releasing males to interfere with the reproduction of the target population, but they differ in the biological mechanism exploited, artificially induced sterility in the former, naturally occurring cytoplasmic incompatibility in the latter, and in their respective logistical implications, long-term sustainability, and reversibility of intervention. Both approaches involve high implementation costs, estimated at approximately seven times higher than those of traditional control methods, and require dedicated infrastructure, specialized personnel, and a high degree of coordination among the agencies involved (91). Additionally, the efficacy observed in one geographic context is not directly transferable to others, since the prevalence of the transmitted disease is strongly influenced by the local conditions under which releases are carried out (91).

1.6 Impact of climate change on *Ae. albopictus*

Being *Ae. albopictus* ectotherm, environmental temperature (T_a) controls its phenology and population dynamics. As such T_a is the main abiotic factor affecting the geographic range of *Ae. albopictus*. The thermal breath of a species is defined by its critical thermal minimum and maximum, C_{tmin} and C_{tmax} , respectively, which are the temperature below or above which the species cannot complete its biological cycle (29,30).

In response to global warming, a redistribution of the species' range is expected, with populations expanding or contracting depending on local conditions relative to the species C_{tmin} and C_{tmax} . This redistribution will not depend exclusively on changes in mean T_a but will also be significantly influenced by occurrence of extreme thermal events, whose effects on populations can diverge substantially from those of global warming (31,32).

As a direct consequence of the changes in the distribution of *Ae. albopictus*, a change in the geographical extent of risk of arboviral transmission is expected. Therefore, the effects of global warming and extreme weather events on the distribution of *Ae. albopictus* should be explicitly incorporated into models predicting risk of arboviral disease.

1.7 Climate change and heatwaves (HW)

As the planet becomes warmer, the frequency and intensity of extreme weather events are expected to increase proportionally (33). Under global warming, heat waves (HWs) are more prevalent than cold waves, and this trend is expected to persist over time (34).

In general terms, a HW occurs when temperatures exceed by 5 degrees the average T_a for a given area/season over several consecutive days. However, this broad definition overlooks important parameters characterizing HWs and influencing HW biological impacts on organisms (35). First, not all HWs have the same intensity, in terms of both deviation from the mean T_a and, especially, duration. Second, daily thermal fluctuations are also expected during a HW, with thermal deviations above the mean T_a being usually followed by periods of lower temperature. These

moments of lower temperature are known as recovery periods, which depending also on the frequency between thermal shifts, can mitigate the effects of a HW, by providing organism a buffer to the stress of exposure to extreme temperatures (36-38). Finally, the geographic region is essential for determining the average thermal distribution and for establishing whether an event qualifies as a HW. The absence of a truly standardized definition for a HW has resulted in a wide variety of variables used across studies, which complicates meaningful comparison of results (Table 1). The most common approach to define a HW is established temperature thresholds based on specific percentiles of the thermal distribution for a given area/season, which must persist for three or more days (39-42). Some studies simply define a HW as a T_a exceeding the upper physiological thresholds of a given organism (34). Because formulating a fixed set of parameters is inherently complicated, focusing on events that have been meteorologically recorded and fall within the thermal limits of the species under study may provide the most realistic approach which also carries ecological relevance.

HWs are expected to cause deleterious effects on holometabolous insects, including behavioral alterations, severe mortality and disruption of physiological functions, to the point of failure to emerge from the pupal stage, if organisms are pushed towards their C_{tmax} during extreme thermal events (33). Despite the general assumption that HWs are detrimental, quantifying their biological effects is genuinely difficult due to HW heterogeneous nature of a HW remains, as well as their unpredictability.

Table 1. Examples of HW definitions

	HW definition	References
1	Environmental temperatures exceeding the upper physiological limits of an organism.	34, 43, 44
2	Three or more days where the combination of excess heat and heat stress are atypical with local climate.	45
3	Five or more days in which the thermal maximum exceeds the average local maximum by 5°C.	39–42
4	Two or more days when the daily maximum exceeds the 90-99th percentile of its distribution.	46–50
5	Simulated high temperatures meteorologically recorded in the species' area.	37, 51–56

1.8 Insect responses to thermal changes

Insects have developed various strategies to cope with rapid and drastic thermal changes (57). They can simply move to an area with favorable conditions, a strategy known as range shift. Other behavioral changes include altering posture or using the tracheal system to favor ventilation (58). For instance, *Colias* butterflies, can adjust their posture relative to the sun to modulate T_a exposure (57; Figure 4). Evaporative thermoregulation has been reported in the orders Diptera, Hymenoptera, Orthoptera, and Hemiptera (59). However, ventilation is a costly mechanism because it can increase dehydration (57,59). Some insect species beat their limbs or wings to promote airflow. Insects can also activate metabolic and physiological responses in response to thermal stress to avoid associated cellular damages.

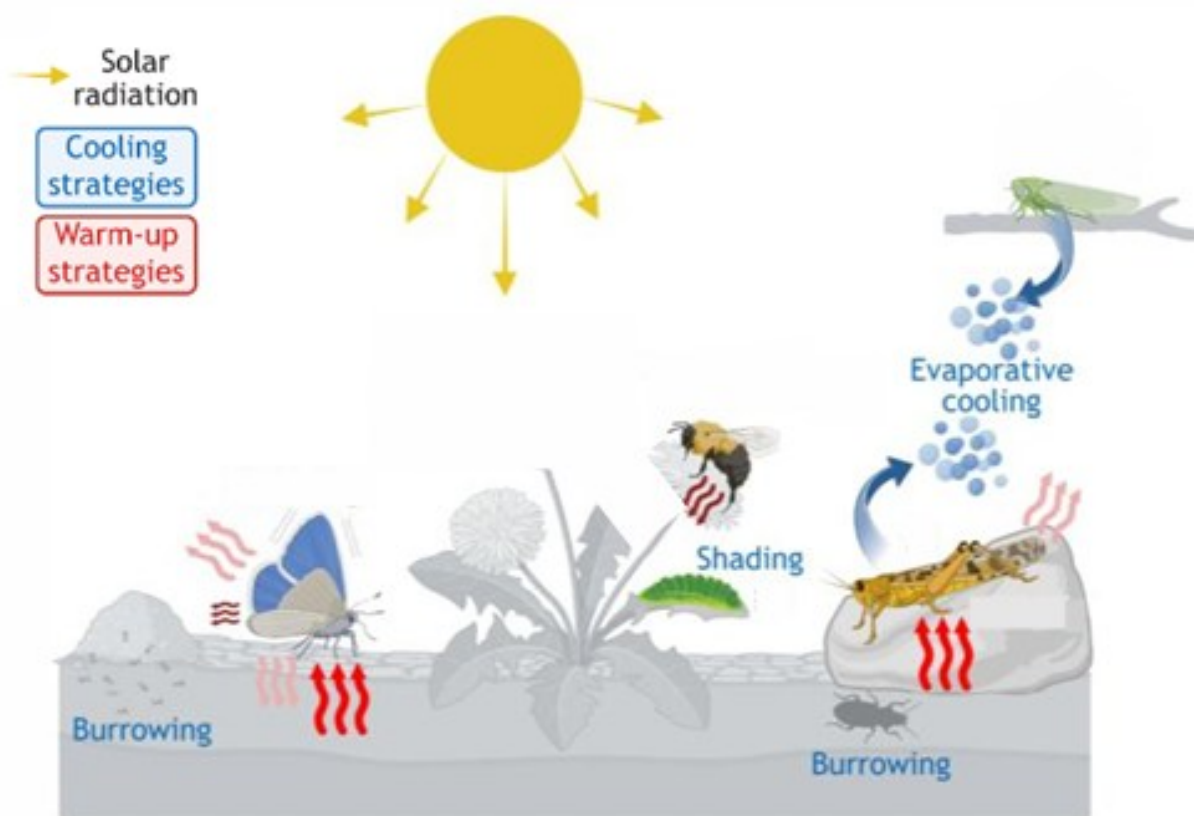


Figure 4. Mechanisms for thermoregulation in insects. Cooling strategies are shown in blue, while warm up strategies are shown in red. Modified from (59).

These responses involve the synthesis of heat shock proteins (HSPs), the regulation of energy reserves, and the production of detoxifying enzymes to counteract the oxidative stress associated with free radical production resulting from metabolic alterations (57,60-62). HSPs are a group of molecular chaperones activated under stress conditions. Their role is to prevent the denaturation of non-functional proteins at elevated temperatures (63).

Insect stress responses differ not only between species but also across developmental stages (33,64). Different life stages have distinct thermal sensitivities, also related to body size, with smaller insects being more susceptible to dehydration or overheating than larger ones (62,65,66). Multiple factors influence the specific nature of these responses, including changes in the environment and microclimate to which the different stages are exposed. This variability becomes particularly important in insects with complex life cycles, such as mosquitoes, in which larvae live in aquatic environments while adults are terrestrial (57).

1.9 HW effect on insect populations

The frequency of HWs is expected to increase in spring, autumn, and winter, intensifying the effects of global warming, which in turn favors earlier spring onset and warmer autumn and winter seasons (33,67). In diapausing insects, where the main cues for diapause are T_a and day length, autumn HWs can disrupt diapause induction. As a result, overwintering survival could be compromised, ultimately affecting spring population growth (68). Similarly, a winter HW could affect both diapause itself and post-diapause development (69).

HW activated responses are often energetically costly, resulting in fitness costs (57,70). In general, the more intense or prolonged the HW, the greater the costs (33,41,64). Fitness costs vary between direct and indirect costs depending on the life stage of the insect exposed to a HW. Result in reduced survival and body size, as well as alterations in the insect reproductive capacity (57,64).

Following a HW, reduction or loss of fertility has been recorded in several *Drosophila* species, in beetles and aphids (37,39,54,71,72). The most drastic reductions in fertility typically occur when HW exposure coincides with gonadal development, likely due to incomplete organ growth or impaired offspring production (37,39,43,73-76). Reduced fertility is further compounded by

behavioral changes. For example, pheromone-based communication is disrupted (77), abnormal courtship behaviors occur (42,78), mating frequency may be reduced (39), and typical oviposition patterns are altered (46,79). Changes in fertility are particularly important for population dynamics.

Whether parental exposure to a HW affects offspring reproductive performance and fitness remains unclear. In *Drosophila suzukii*, parental HW exposure improves offspring thermal tolerance (44). In contrast, in *Tribolium castaneum*, offspring of HW-exposed individuals subsequently showed reduced fertility (41). The mechanisms underlying transgenerational effects of HWs exposure, and whether these effects are beneficial or harmful, remain largely unexplored (57).

While global warming can lead to profound and lasting physiological, behavioral, and in some cases genetic changes, enabling a species to adapt, develop, survive, and reproduce under unfavorable conditions (40,80-82), exposure to extreme events entails immediate responses. Only a few studies have begun to link global warming with exposure to extreme weather events in scenarios of ecological relevance. For instance, in the burying beetle *Nicrophorus vespilloides* (53). Individuals were reared at a standard temperature of 20°C or at an elevated temperature of 23°C, and then exposed to a HW of 37°C. Fecundity was significantly reduced in adults reared under the stressful 23°C condition who were subsequently also exposed to a HW. These results highlight that studying effects of global warming and HWs in isolation could underestimate climate change impacts. Another study in *T. castaneum* used a similar approach, exposing lines, which had been experimentally evolved at either 30°C or 38°C for 45 generations to a HW of 42°C (40). Beetles that had evolved at 38°C were significantly more resilient to the HW, in terms of both survival and fertility than those that had been kept at 30°C (40). This result indicates that in beetle warm-evolution improved HW resilience. Both studies highlight the close relationship between rising mean Ta and HWs. Future studies should place greater emphasis on integrating these factors to more fully address insect survival and population dynamics under climate change.

2. AIMS OF THE WORK

Given the epidemiological relevance of *Ae. albopictus*, the absence of therapeutic treatment for arboviruses and limited vaccines, control interventions to limit vector abundance are essential to reduce the risk of arboviral transmission. All current innovative strategies to control adult mosquitoes are based on the release of appropriately modified males. Males that have been manipulated to be sterile or carry endosymbionts such as Wolbachia are expected to mate with wild females either decreasing the size of the target population or modifying female vector competence, thereby preventing further pathogen transmission (87). These strategies rely on the ability of released males to efficiently locate a partner and transfer sufficient viable sperm to fertilize eggs throughout the female lifespan. This because *Aedes spp.* females tend to be monoandrous (87), but they can mate multiple times if the first mating results in incomplete insemination (87). While aspects of maintenance of sperm viability in female spermathecae and egg fertilization have been studied (87), little is known about the male contributions to fertility and consequently to mosquito population dynamics. This is particularly important considering global climate change as previous work in my laboratory has shown that males are less thermal resilient than females when both exposed to a HW and a thermal challenge perpetuated across generations mimicking global warming (108). On this basis, for my thesis I focused on males and had two main objectives:

1. Test if previous adaptation to heat positively condition male responses to a HW
2. Test for additive effect of HWs by exposing males to two consecutive thermal stresses
3. Develop a protocol to extract and visualize sperm from *Ae. albopictus*

3. MATERIALS AND METHOD

3.1 Mosquito strains and rearing conditions

I used four different *Aedes albopictus* lines, both originating from the Foshan genome-reference strain. The Foshan strain was established in 1981 in Guangzhou, at the Guangdong Provincial Center for Disease Control and Prevention, China. It has been reared in the insectary of the University of Pavia since 2015, when a batch of eggs was received from Southern Medical University in Guangzhou.

The lines I used are called Line 1, Line 2, Line 4 and Line 5. Lines 1 and Line 2 have been maintained in the insectary of the University of Pavia under standard conditions, which include 28°C for 14h followed by 26°C for 10h. Lines 5 and 4 have been reared under tropical conditions (32°C 14h followed by 26°C for 10h) for more than 20 generations, hence, they are called warm-evolved lines. All lines are maintained at 70% relative humidity, thus the only difference across the four lines is the thermal regime under which they are reared. Mosquitoes of all four lines are maintained in parallel as follows.

Larvae are reared in plastic containers at a controlled density to avoid competition. Food consisting of fish food (Tetra Goldfish Gold Colour) is provided daily. Upon emergence, mosquitoes are fed using cotton soaked in 0.1 g/mL sucrose solution. For the blood meal, adult females were fed mutton blood (Biolife Italiana) using a Hemotek blood membrane feeding apparatus.

3.2 Thermal stress in the form of a HW

Male and female pupae can be distinguished by size, so at the pupal stage, I separated males from females. Each pupa was transferred individually into sterile 50mL polypropylene conical tubes (Falcon®) before HW exposure.

Using a Binder KBWF, I mimicked a HW that lasted two days and consisted of a cycle of 12h at 37°C, followed by 12h at 26°C (light:dark, 70% relative humidity). After HW exposure, mosquitoes were moved back to the rearing conditions of the respective lines. I performed single and double HW exposure. In the latter, I exposed males to a HW as before, 24h after the first exposure.

3.3 Fitness trait assessment

For each line, I was recorded: immediate mortality after HW exposure, longevity of males surviving HW exposure and their reproductive capacity. All experiments were performed for each of the four previously cited lines.

3.3.1 Quantification of HW immediate mortality in pupae

Three groups of 30–35 individually separated male pupae were subjected to HW. After the HW, the percentage of pupal mortality, referred to as HW-related mortality, was recorded, after which pupae were returned to standard rearing conditions. In parallel, three additional groups of 30–35 individually separated male pupae were maintained under standard rearing conditions.

Comparison of mosquito immediate mortality/lines was assessed with two-way ANOVA followed by Tukey's multiple comparison test. All statistical analyses were conducted using Prism 10 (GraphPad Software, San Diego, CA, USA).

3.3.2 HW effects on adults

For each strain, three groups of 30–35 individually separated male pupae were subjected to HW. After the first HW, pupae were kept under standard rearing conditions for 24h. In this timeframe surviving pupae emerged into adults, which were exposed to a second HW at second day after emergence. After the second HW, the percentage of individual mortality, HW-related mortality, was recorded, and individuals were returned to standard rearing conditions.

3.3.3 Quantification of male longevity

After HW exposure, Falcon containing males were moved back to the rearing condition of the respective lines. A sugar-soaked cotton ball was placed on top of the mesh covering each Falcon, with 10% sucrose solution refreshed daily and the cotton replaced weekly. To assess adult longevity, adult cups were checked daily, and the number of dead individuals was recorded until all mosquitoes had died. Longevity was assessed for 20-23 males/line. Comparisons of mosquito longevity/lines were performed using Kaplan–Meier survival analysis with the log-rank (Mantel–Cox) test. Differences in median lifespan across lines and HW-exposure were assessed with one-way ANOVA followed by Tukey’s multiple comparisons test. All statistical analyses were conducted using Prism 10 (GraphPad Software, San Diego, CA, USA).

Longevity was assessed both in individuals emerged from pupae that survived the first HW, and in individuals that survived the second HW, by counting the total number of days until the death of all individuals in each group. In parallel, longevity was also assessed in groups maintained under standard rearing conditions from the outset, without any exposure to HW.

3.3.4 Quantification of male reproductive capacity

A total of 60-65 males/line surviving either one or double HW exposure(s) were allowed to mate with unexposed virgin females. I set up a total of 60-65 single pair crosses. After three days, I offered a blood meal using a Hemotek membrane feeding apparatus, and sampled a total of 60 engorge females, which were gently transferred two days later to mini-cups containing a piece of moist absorbent paper to serve as an oviposition substrate. On the fourth day post blood meal, females were removed, and the number of eggs laid was recorded to measure fecundity and assess sterility, that is the percentage of females that laid no eggs. After two further days, the eggs laid by each female were hatched for four days. The total number of larvae that emerged for all eggs deposited by a female was counted as a measure of fertility.

Comparisons of mosquito fecundity/lines were assessed first with Shapiro-Wilk test, to control normality of distribution, and with Kruskal Wallis test, followed by Dunn's multiple comparisons test.

Comparisons of mosquito fertility/lines were assessed first with Shapiro-Wilk test, to control normality of distribution, and with Mann-Whitney test.

All statistical analyses were conducted using Prism 10 (GraphPad Software, San Diego, CA, USA).

3.4 Visualization of sperm

Males were collected from a rearing cage and placed on ice to prevent flight. Males were individually transferred to a petri dish and placed in a drop of 1x PBS under a Leica EZ4 stereomicroscope for dissection. I removed the last body segment to extract the entire male reproductive tract, including testes, vas deferens and seminal vesicles, using surgical forceps. The accessory glands do not contain spermatozoa, so their presence in the dissection is irrelevant. The need to remove the entire reproductive tract stems from the pathway followed by spermatozoa prior to ejaculation; from the sites of production (testes), to transport (vas deferens), and temporary pre-copulatory storage (seminal vesicles). Once dissected, the reproductive tract was transferred into 0.5 mL microcentrifuge tubes (Eppendorf®) containing 8 μ L of 1x PBS. Next, I tried either to pipet up and down eight to ten times or use a sterile needle to disrupt the various components of the reproductive tract; both methods were effective in allowing spermatozoa to exit the testis, seminal vesicles and deferent duct. Finally, I added 2 μ L of pure H₂O to the tube. This last step serves to slow, rather than stop, sperm motility, making the spermatozoa easier to observe. Lastly, I placed 2 μ L of the final volume/solution on a microscopy slide, and covered it with a coverslip before, visualization using an Olympus CKX53 microscope. The microscope was fitted with an Olympus SC50 camera, connected to a dedicated computer software for live visualization and image acquisition called “cellSens”.

4. RESULTS

I studied four different *Ae. albopictus* populations line, all originating from the genome reference Foshan strain. Two lines, namely Line 4 and 5 were obtained through an experimental evolution approach by rearing them under “tropical” conditions consisting of $32^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 14 hours followed by 26°C , 70% relative humidity, and a 12h:12h light:dark photoperiod. Two lines, namely Line 1 and 2 are the line 4 and 5 corresponding lines maintained under standard rearing conditions, that is $28^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 14 hours followed by 10 hours at 26°C and 70% relative humidity.

For each line, I separated male pupae and performed two types of experiments (Figure 5). In Exp. 1, I exposed 90-102 male pupae/line to one HW and assessed pupal viability after 48 hours, along with the longevity and the reproductive capacity of males surviving HW exposure. In Exp 2, I exposed 90-102 male pupae/line to a HW, scored immediate mortality, and then exposed surviving males to a second HW. Males were 2 days old when exposed to the second HW and I scored their immediate mortality after HW exposure, along with the longevity and the reproductive capacity of surviving males. For Exp 1, I used pupae from lines 4 and 5 that had 27 generation of experimental evolution; whereas pupae from for lines 1 and 2 were collected at the 15 generation. For Exp 2, I used pupae at the 28 generation of experimental evolution for lines 4 and 5 and at the 16 generation for lines 1 and 2.

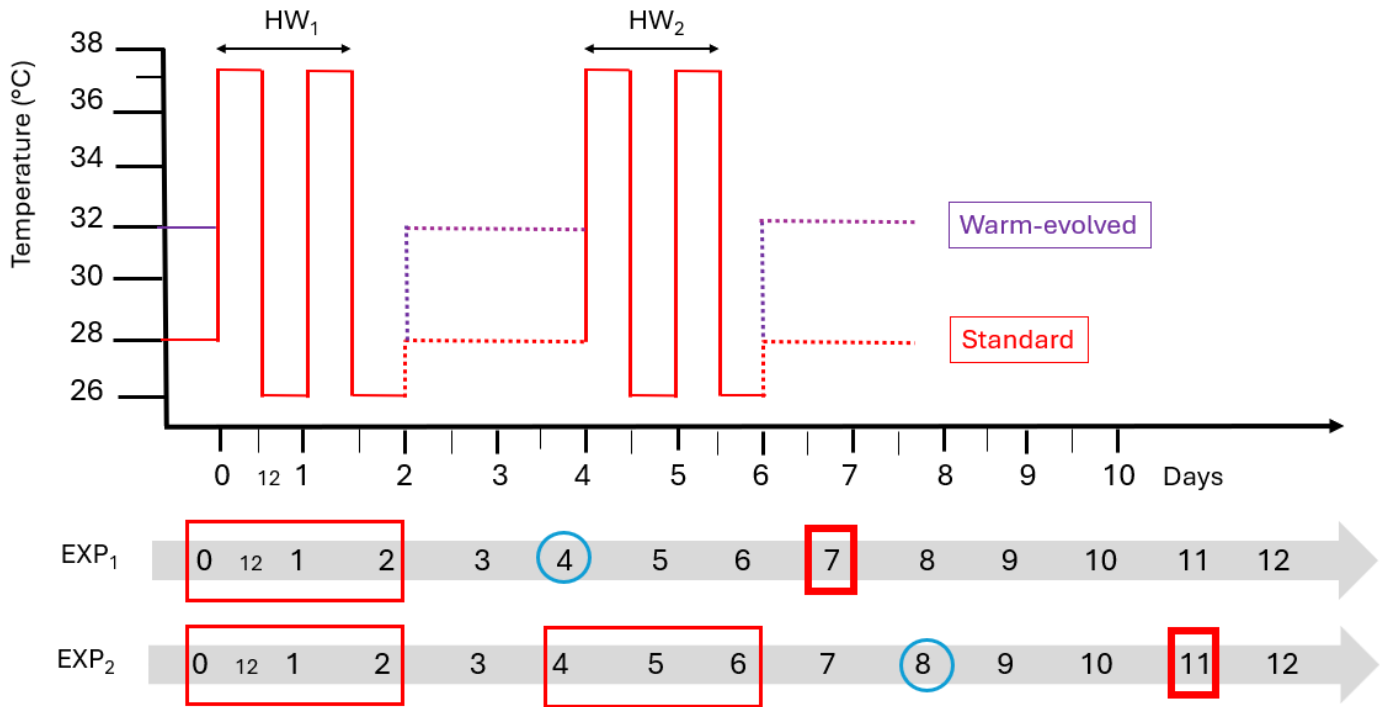


Figure 5. Study design. The top panel shows timeline of HWs and the two different thermal regimes (i.e. tropical in purple and standard in red) in which mosquitoes were kept. Exp1 and Exp2 refers to the two types of experiments that were conducted. In each “Exp” line, numbers refer to days from pupae separation. The red box indicates the days in which mosquitoes were exposed to the HW. The blue circle refers to the day in which males were exposed to virgin females and the thicker red box refers to the day in which females were offered a blood meal.

4.1 Experiment 1

4.1.1 Male fitness after one HW exposure

Mortality after one HW exposure at the pupal stage (Exp 1) was $2.00\% \pm 0.20\%$ in males from control lines (lines 1 and 2), and $26.50\% \pm 0.06\%$ in males from warm-evolved lines (lines 4 and 5). I observed a significant difference in the pupal mortality after HW exposure between control and stress-condition in the warm-evolved lines were Line 5 (p-value: 0.01) and Line 4 (p-value: 0.006). I observed also a significant difference between both stress-condition for control lines and warm-evolved lines, were Line 1 and Line 5 (p-value: 0.01), Line 1 and Line 4 (p-value: 0.013), Line 2 and Line 5 (p-value: 0.009), and Line 2 and 4 (p-value: 0.011).

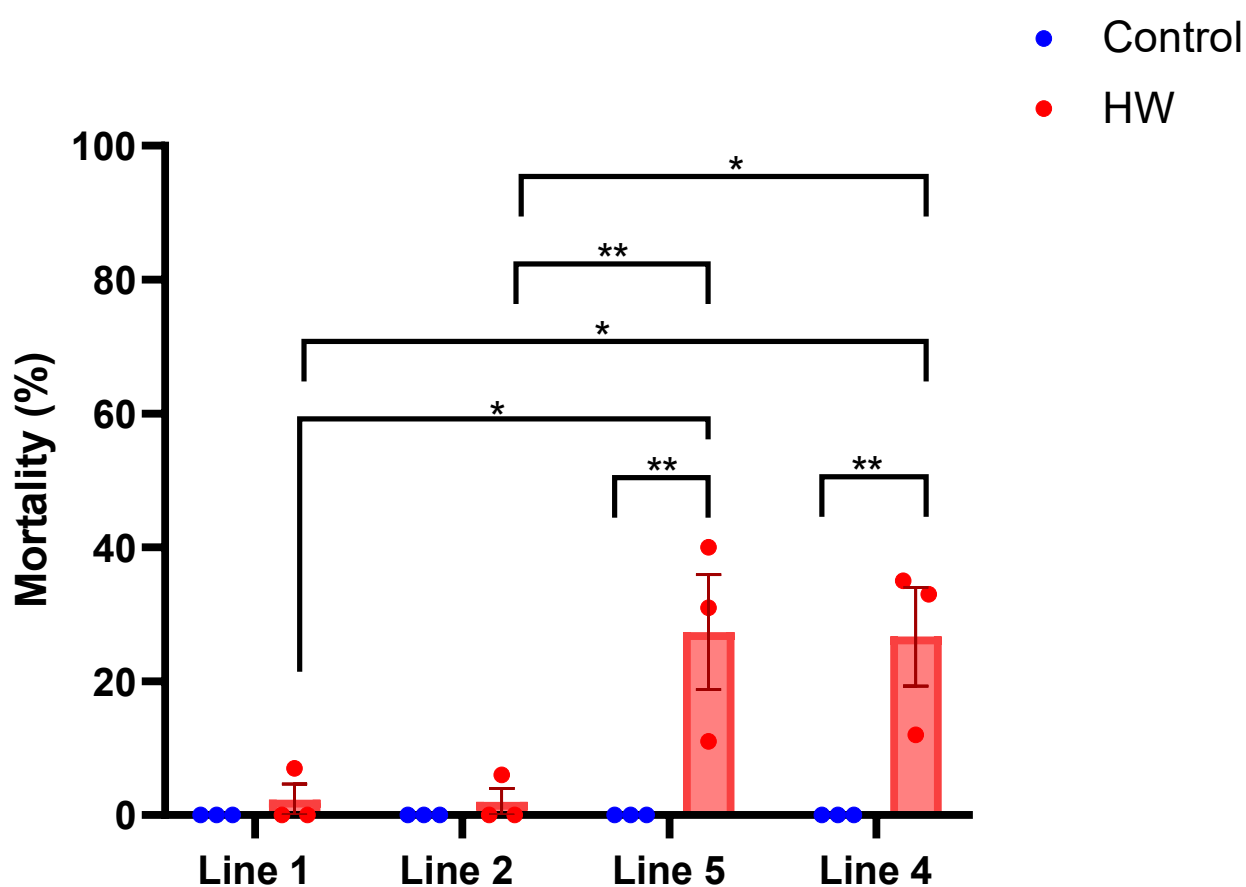


Figura 6. Pupal mortality after HW exposure. Viability of pupae was scored 48 hours after HW exposure in each line. Between 90-102 pupae were tested for each line in 3 independent experiments. Error bars show the standard error of the mean (SEM). An * refers to a p-value <0.05 and ** to a p-value <0.01.

4.1.2 Quantification of male longevity after one HW exposure

Male longevity was measured following HW exposure. Adult males were transferred into cups and monitored daily to record the number of individuals that died each day, until the last day before all mosquitoes had died. In Exp1, I observed that the mean longevity was 18 in both lines 1 and 2 and 14.5 ± 2.5 in lines 4 and 5, respectively. Longevity was compared to test the effects of HW-exposure in each lines (Figure 7). I observed a significant reduction in longevity of HW-exposed mosquitoes in both control (Figure 7, A and C) (p-value: <0.001 in both comparison) and warm-evolved lines (Figure 7, B and D) (p-value: <0.0001 in both comparison).

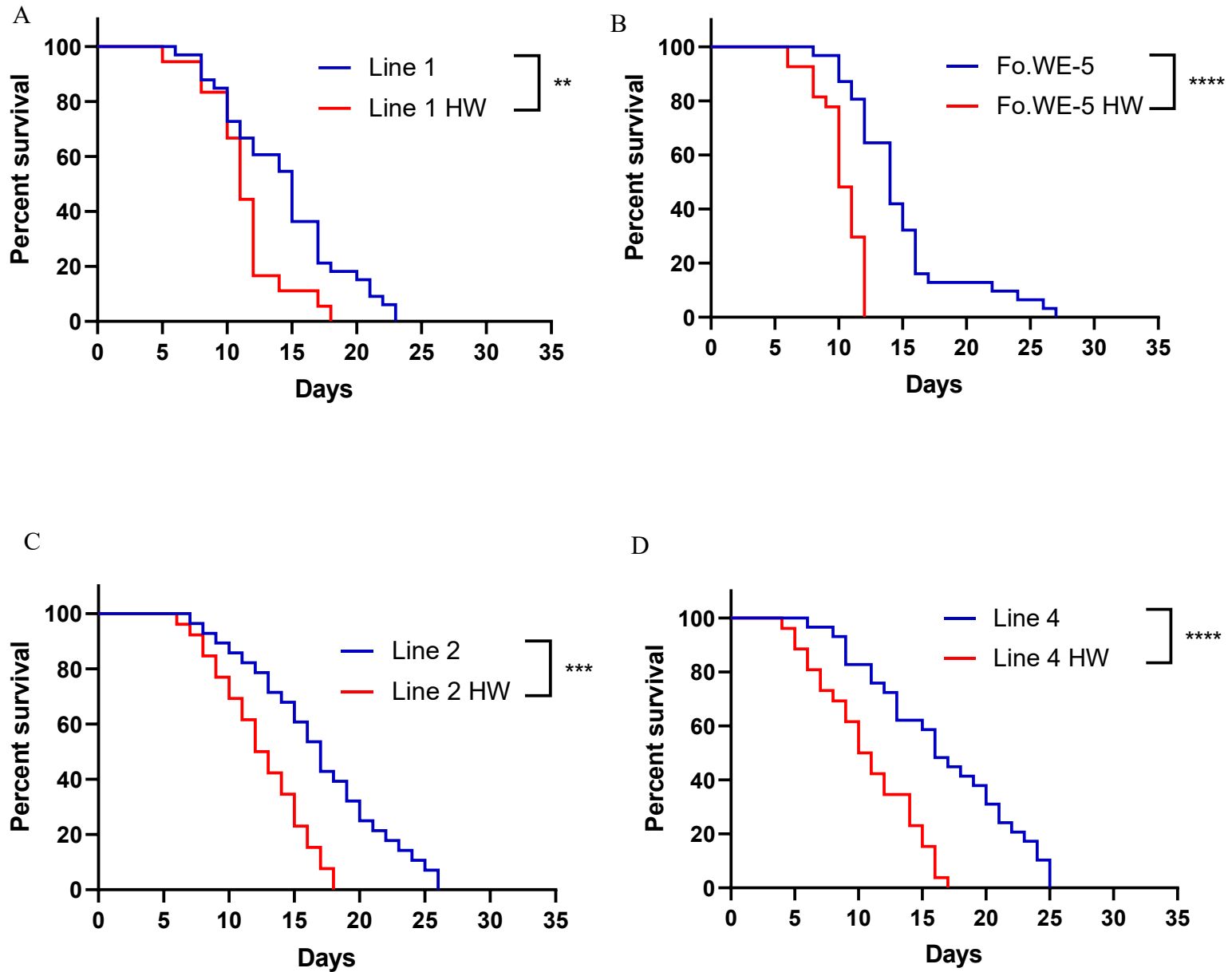


Figure 7. Longevity after an HW exposure. Longevity was scored day by day after HW exposure in each line. Between 20-24 adults were tested for each line in 3 independent experiments. An * refers to a p-value < 0.05 , ** to a p-value < 0.01 , *** to a p-value < 0.001 , **** to a p-value < 0.0001 .

4.1.3 Quantification of male reproductive capacity after one HW exposure

To quantify male reproductive capacity, fecundity and fertility were measured for each female paired individually with one male. Fecundity was assessed by counting the eggs laid by each female, while fertility was calculated as the percentage of eggs laid by each female that successfully hatched into larvae. In addition, I measured female sterility, as the percentage of females that did not lay eggs after having mated with males and acquired a blood-meal.

Fecundity was 62.75 ± 2.15 in control lines and 67.05 ± 0.45 in warm-evolved lines, when males were not exposed to HW. In contrast, fecundity was 47.2 ± 0.3 in control lines and 53.85 ± 1.15 in warm-evolved lines, when males had been exposed to HW. I observed no statistical difference (by Kruskal-Wallis test, p-value: non significant/ns) in the fecundity of control line (Line 1), but I observed significant difference (p-value: 0.0008) in the fecundity of control Line 2. And no statistical difference in the fecundity of warm-evolved lines comparing data from HW-exposed and not exposed males.

Sterility was 2.60 ± 0.10 in control lines and 10.60 ± 2.00 in warm-evolved lines, when males were not exposed to HW. In contrast, sterility was 9.50 ± 3.50 in control lines and 16.60 ± 0.10 in warm-evolved lines, when males had been exposed to HW. I observed no statistical difference (by 2way-ANOVA test, p-value: ns) in the sterility of control lines, and no statistical difference in the sterility of warm-evolved lines.

Fertility was 74.35 ± 1.45 in control lines and 83.20 ± 0.10 in warm-evolved lines, when males were not exposed to HW. In contrast, fertility was 75.00 ± 0.50 in control lines and 63.63 ± 0.13 in warm-evolved lines, when males had been exposed to HW. I observed no statistical difference (by Mann-Whitney test, p-value: ns) in the fertility of control lines, and no statistical difference in the fertility of warm-evolved lines.

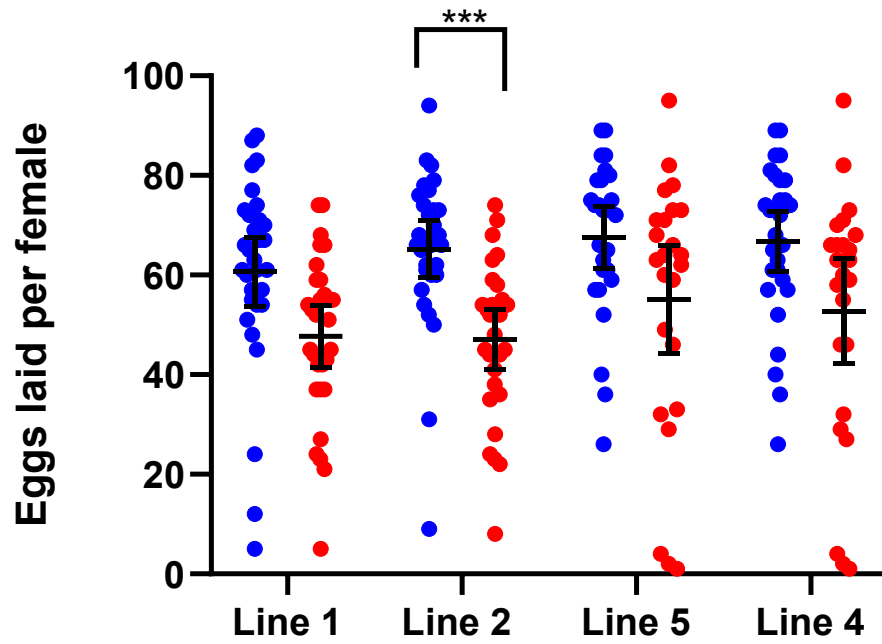


Figure 8. Fecundity. Fecundity was scored after oviposition in each line. Between 60-65 females were tested for each line in 3 independent experiments. Error bars show the standard error of the mean (SEM).

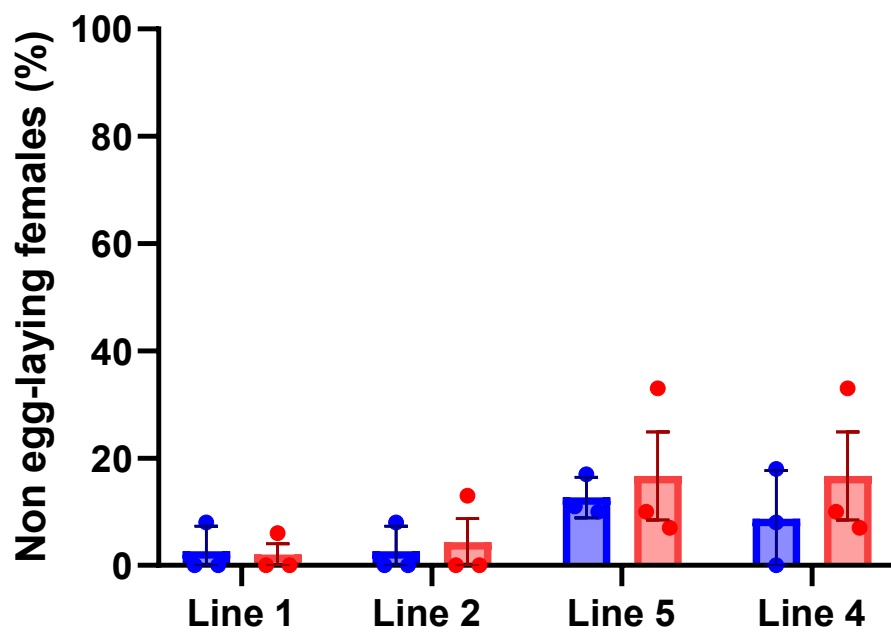


Figure 9. Sterility. Sterility was scored after oviposition in each line. Between 60-65 females were tested for each line in 3 independent experiments. Error bars show the standard error of the mean (SEM).

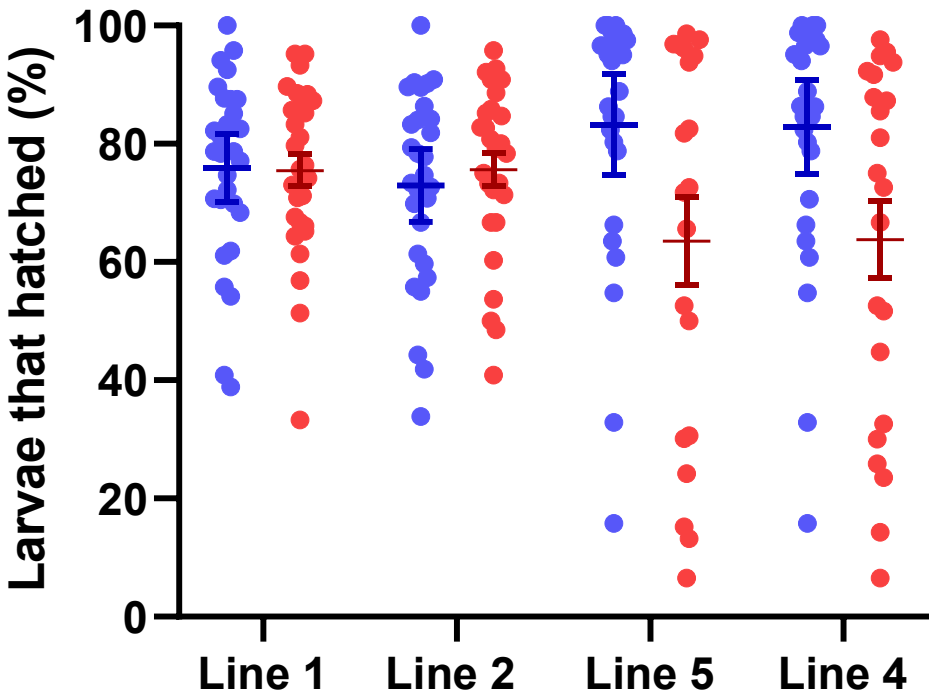


Figure 10. Fertility. Fertility was scored after all the eggs hatched. Between 60-65 females were tested for each line in 3 independent experiments. Error bars show the standard error of the mean (SEM).

4.2 Experiment 2

4.2.1 Male fitness after double HW exposure

Mortality after the second HW exposure (Exp 2) was $35.23\% \pm 1.14\%$ in control lines (lines 1 and 2), and $51.39\% \pm 1.39\%$ in males from warm-evolved lines (lines 4 and 5). The two populations of HW-exposed males were significantly different (assessed appropriately by ANOVA followed by Tukey's test). Two different populations were tested for each rearing condition.

Mortality was significantly different comparing results between HW-exposed and not exposed mosquitoes for both control (p-value: 0.001 in Line 1 and 0.003 in Line 2) and warm evolved lines (p-value: <0.0001 in Line 5 and <0.0001 in Line 4).

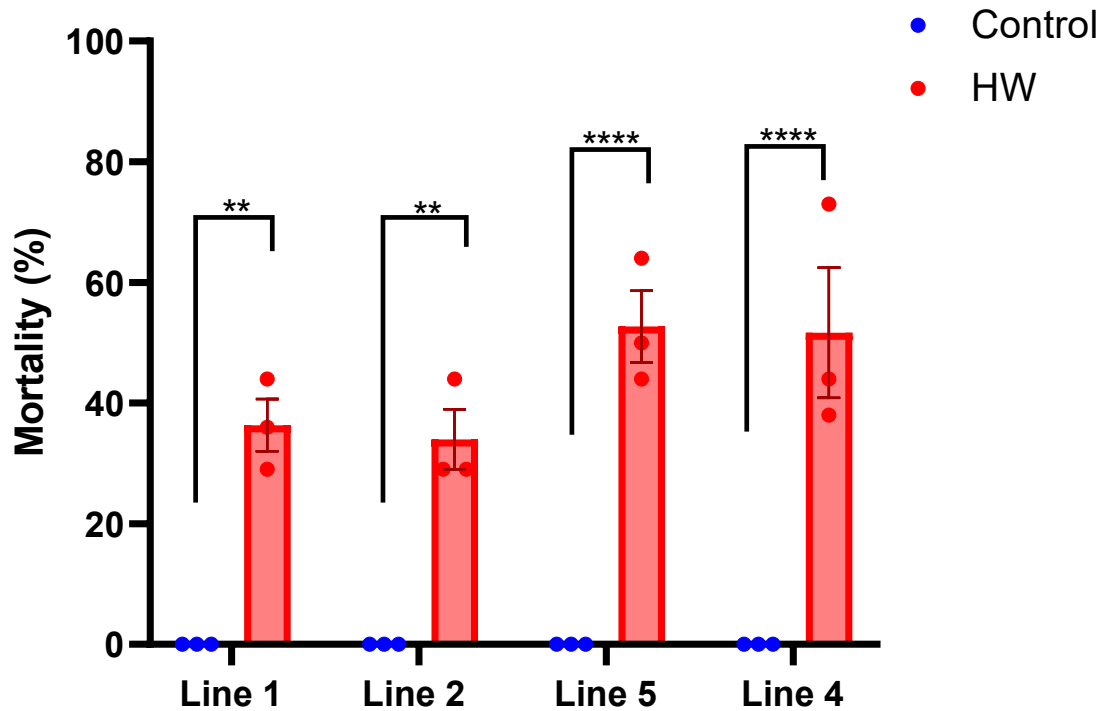


Figure 11. Pupal mortality after double HW exposure. Viability of pupae was scored 48 hours after the second HW exposure in each line. Between 90-102 mosquitoes pupae were tested for each line in 3 independent experiments. Error bars show the standard error of the mean (SEM). An * refers to a p-value <0.05 and ** to a p-value <0.01, *** to a p-value <0.001, **** to a p-value <0.0001.

4.2.2 Quantification of male longevity after double HW exposure

Male longevity was measured following the second HW exposure, with the same protocol used for Exp 1. Control lines lived 13 ± 1 days after the second HW exposure. Warm-evolved lines lived 10 ± 1 days after the second HW exposure. Comparison between HW-exposed and not exposed mosquitoes showed significant difference in each line (Line 1 p-value: 0.0004, Line 5 p-value: <0.0001, Line 2

p-value: <0.0001, Line 4 p-value: <0.0001. In contrast, I did not detect differences between the two tested warm-evolved lines, or between control lines and warm-evolved lines in longevity after double HW exposure.

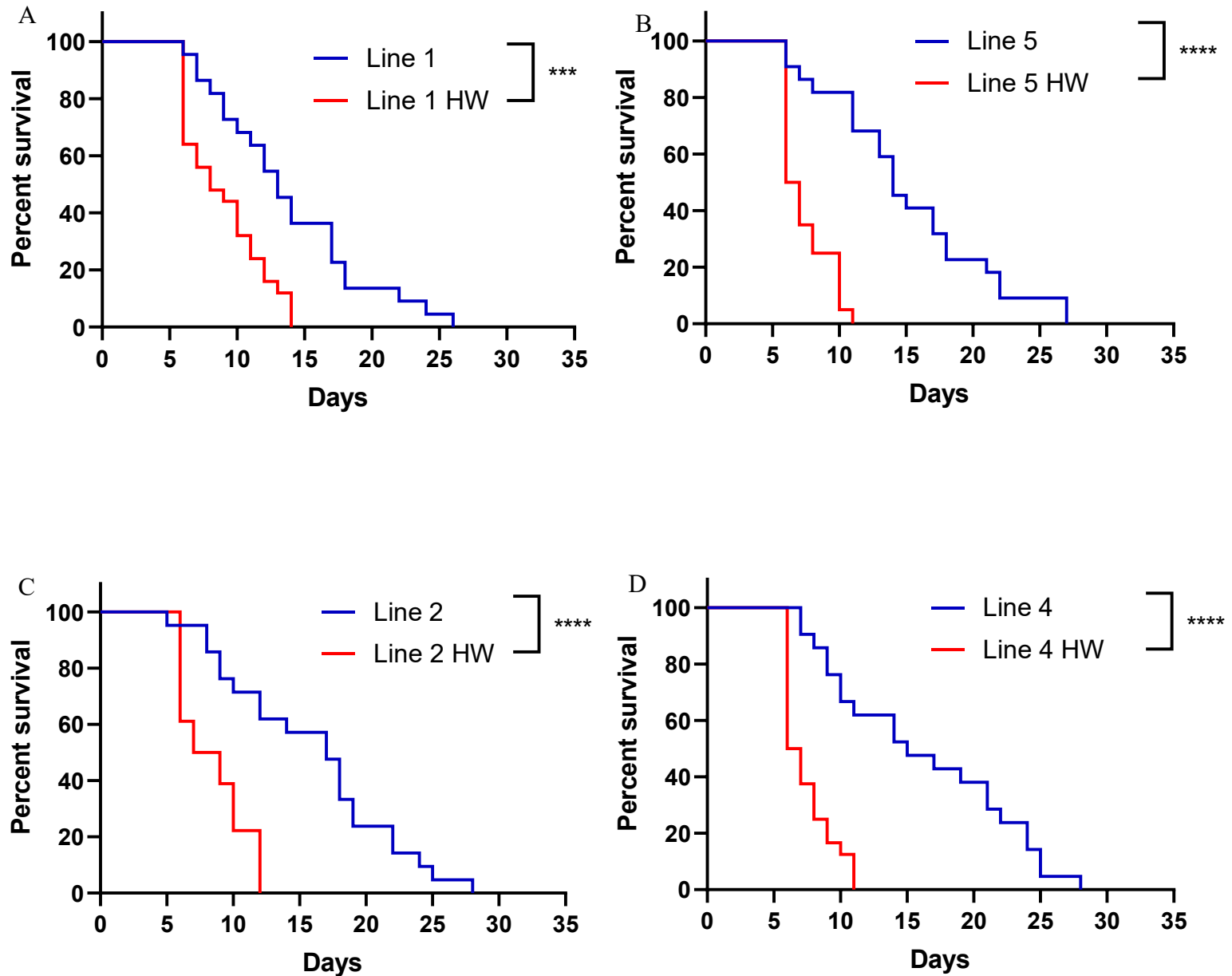


Figure 12. Longevity after double HW exposure. Longevity was scored day by day after HW exposure in each line. Between 20-24 adults were tested for each line in 3 independent

experiments. An * refers to a p-value <0.05, ** to a p-value <0.025, *** to a p-value <0.0002, **** to a p-value <0.0001.

4.2.3 Quantification of male reproductive capacity after double HW exposure

To quantify male reproductive capacity, fecundity and fertility were measured for each female paired individually with one male. Fecundity and fertility were assessed using the same protocol used for mosquitoes emerged from pupae exposed to one HW (Exp 1).

Fecundity was 65.95 ± 0.55 in control lines and 73.50 ± 0.80 in warm-evolved lines, when males were not exposed to HW. In contrast, fecundity was 59 ± 1 in control lines and 62.70 ± 0.04 in warm-evolved lines, when males had been exposed to double HW. I observed no statistical difference (by Kruskal-Wallis test, p-value: non significant/ns) in the fecundity of control lines, and no statistical difference in the fecundity of warm-evolved lines.

Sterility was 6.95 ± 1.35 in control lines and 9.60 ± 0.01 in warm-evolved lines, when males were not exposed to HW. In contrast, sterility was 5.49 ± 1.83 in control lines and 9.50 ± 1.50 in warm-evolved lines, when males had been exposed to double HW. I observed no statistical difference (by 2way-ANOVA test, p-value: ns) in the sterility of control lines, and no statistical difference in the sterility of warm-evolved lines.

Fertility was 84.75 ± 0.65 in control lines and 90.1 ± 1.40 in warm-evolved lines, when males were not exposed to HW. In contrast, fertility was 75.12 ± 4.87 in control lines and 57.45 ± 0.20 in warm-evolved lines, when males had been exposed to double HW. I observed no statistical difference (by Kruskal-Wallis test, p-value: ns) in the fertility of control lines, but statistical difference in the fertility of warm-evolved lines, Line 5 (p-value: <0.0001) and Line 4 (p-value: <0.0001).

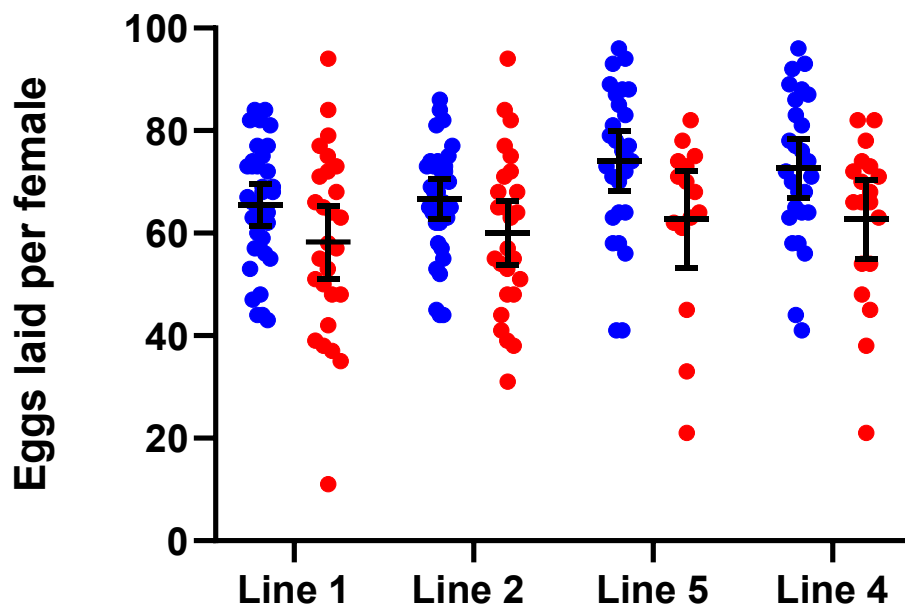


Figure 13. Fecundity after double HW exposure. Fecundity was scored after oviposition in each line. Between 60-65 females were tested for each line in 3 independent experiments. Error bars show the standard error of the mean (SEM).

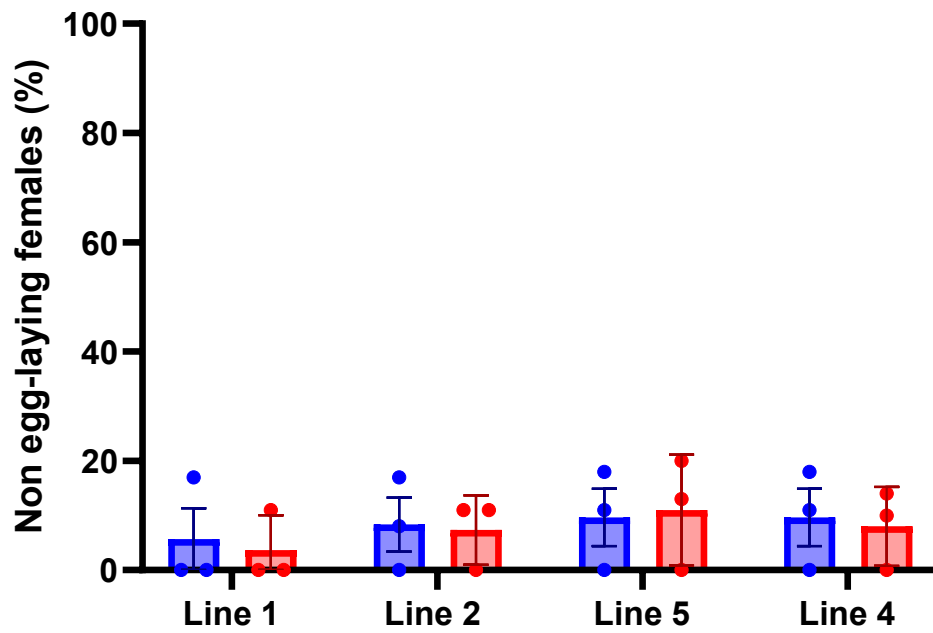


Figure 14. Sterility after double HW exposure. Sterility was scored after oviposition in each line. Between 60-65 females were tested for each line in 3 independent experiments. Error bars show the standard error of the mean (SEM).

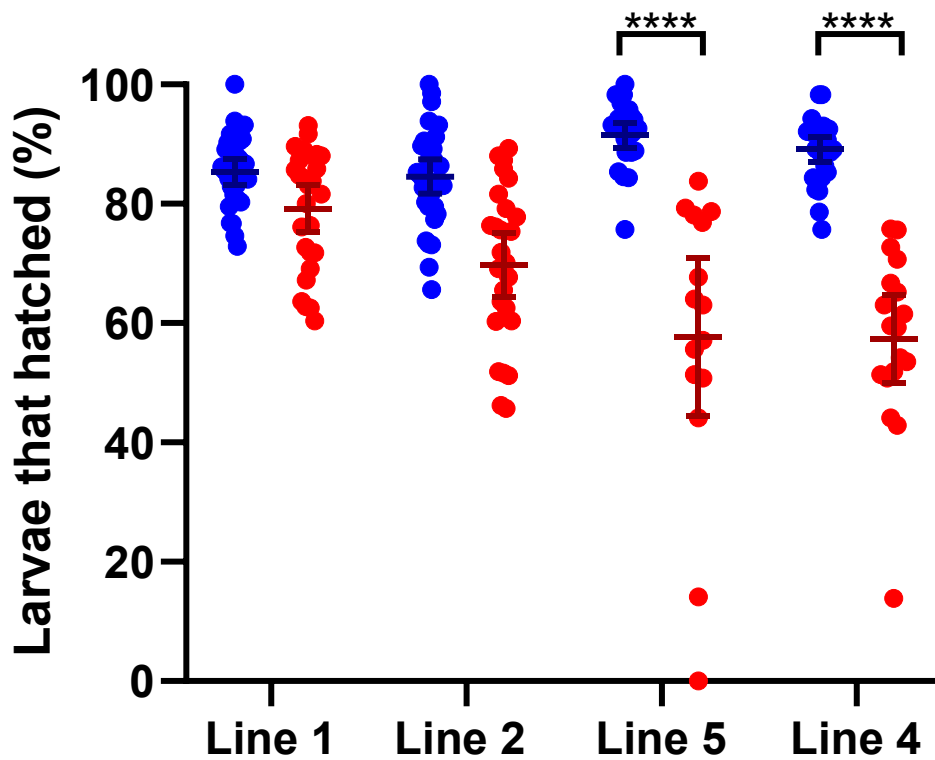


Figure 15. Fertility after double HW exposure. Fertility was scored after all the eggs hatched. Between 60-65 females were tested for each line in 3 independent experiments. Error bars show the standard error of the mean (SEM). **** refers to a p-value <0.0001.

4.3 Comparison between one-exposure and double-exposure to HW

Before proceeding with the direct comparison between the four lines subjected to an HW exposure and double HW exposure, the comparability of the respective controls without any exposure from the two experiments was assessed. This check is necessary because, although the controls originated from experiments conducted at different times, they should represent equivalent baseline conditions in the absence of HW exposure. The absence of significant difference between the controls is in fact a methodological prerequisite for attributing the differences observed between HW exposed males specifically to the effect of the different HW exposure applied, rather than to intrinsic variability between the two independent experiments.

Comparison of mortality between controls of one HW exposure and double HW exposure, assessed with 2way ANOVA and Sidak's multiple comparisons test, showed no significant difference in Line 1, and in Line 2, and I observed no statistical difference in Line 5, and also in Line 4 for warm-evolved lines.

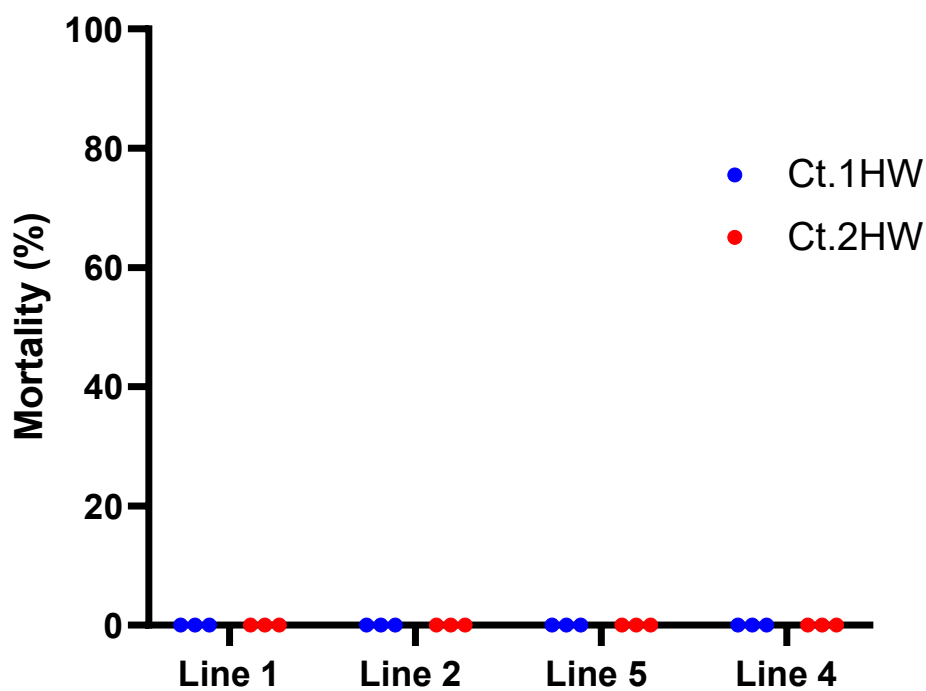


Figure 16. Comparison of mortality in controls between one HW exposure (blu) and two HW exposure (red).

Longevity was measured following emergence. I observed, with Mantel-Cox test, that the comparison of male longevity is not statistically different in Line 1 (p-value: 0.945) and in Line 2 (p-value: 0.905) for control lines, and in Line 5 (p-value: 0.524) and in Line 4 (p-value: 0.770) for warm-evolved lines.

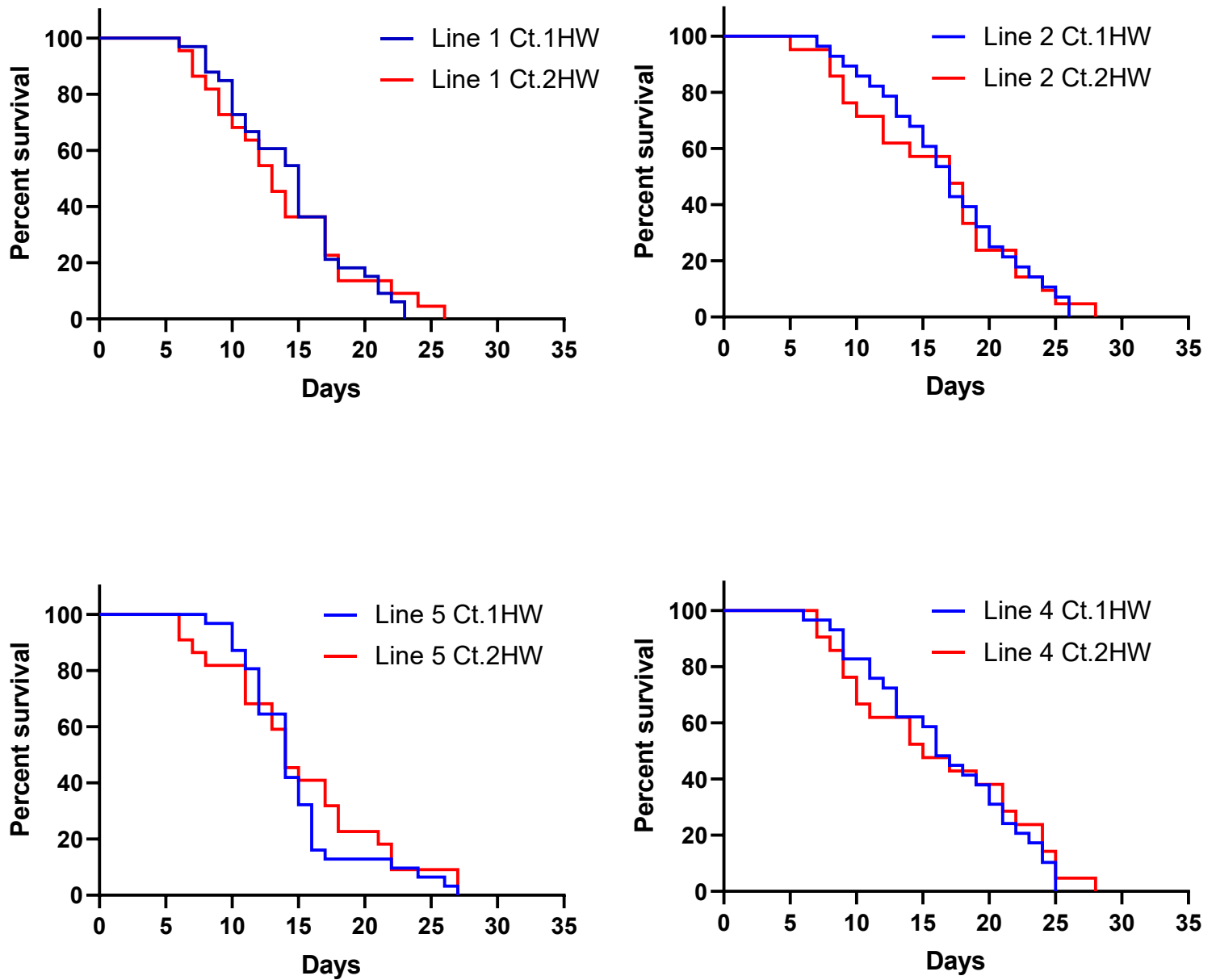


Figure 17. Comparison of longevity in controls between one HW exposure (blu) and two HW exposure (red). Longevity was scored day by day after emergence.

I also measured the comparison of traits to male reproductive capacity, fecundity, sterility and fertility, between controls of one HW exposure and two HW exposure.

I observed, with Kruskal-Wallis test and Dunn's multiple comparison test, that the fecundity has not statistical difference, in Line 1 (p-value: >0.999), in Line 2 (p-value: >0.999) for control lines, and also in Line 5 (p-value: >0.999) and in Line 4 (p-value: >0.999) for warm-evolved lines.

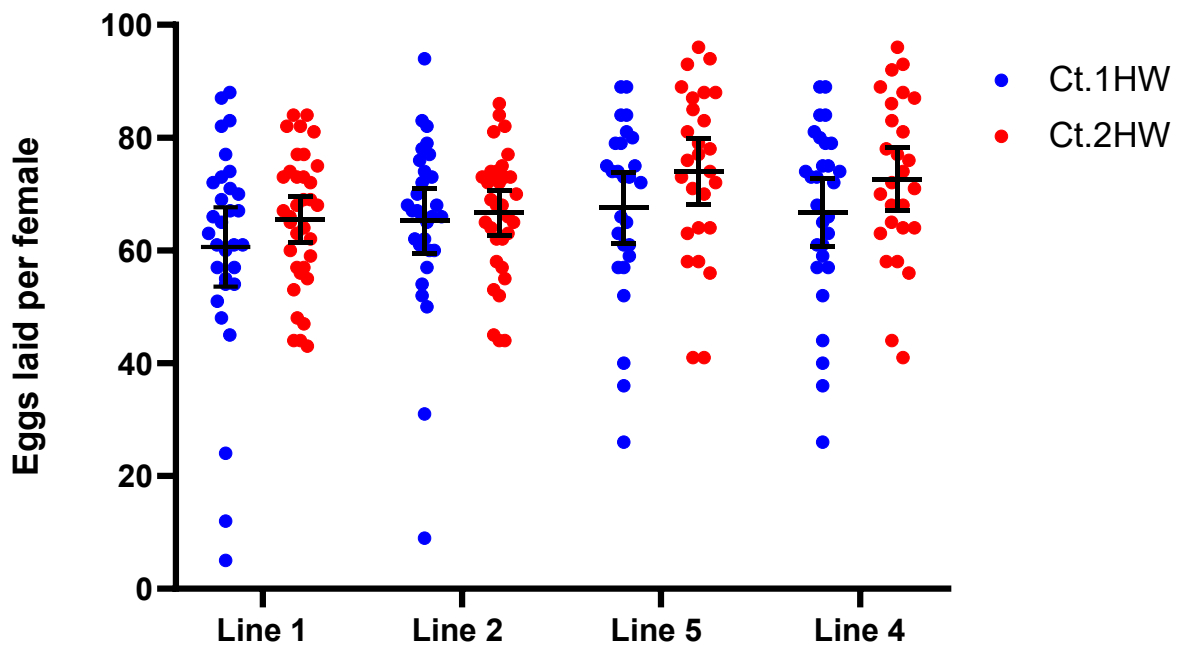


Figure 18. Comparison of fecundity in controls. Fecundity was scored after oviposition in each line. Error bars show the standard error of the mean (SEM).

I observed, with 2way ANOVA and Sidak's multiple comparisons test, no statistical difference in the comparison of sterility in controls between one HW exposure and double HW exposure, in Line 1 (p-value: 0.983) and in Line 2 (p-value: 0.851) for control lines, and in Line 5 (p-value: 0.983), Line 4 (p-value: 0.999) for warm-evolved lines.

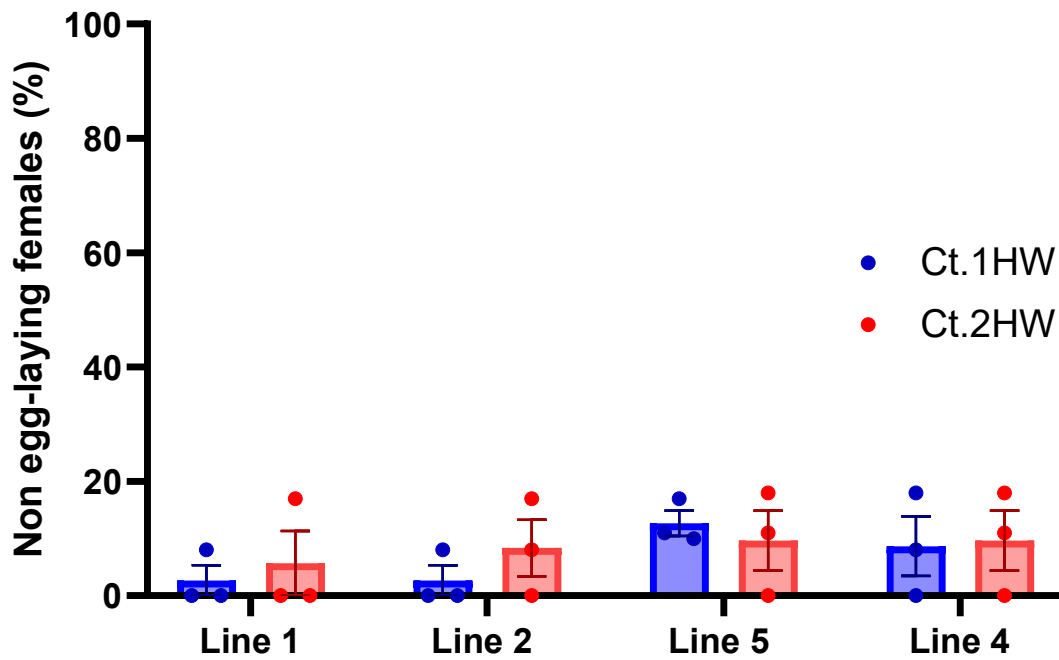


Figure 19. Comparison of sterility in controls. Sterility was scored after oviposition in each line. Error bars show the standard error of the mean (SEM).

I observed, with Kruskal-Wallis test, that is statistical difference in Line 1 (p-value: 0.006) and in Line 2 (p-value: 0.003) for fertility of control lines. But I observed no statistical difference in warm-evolved lines, in Line 5 (p-value: 0.969) and in Line 4 (p-value: 0.785).

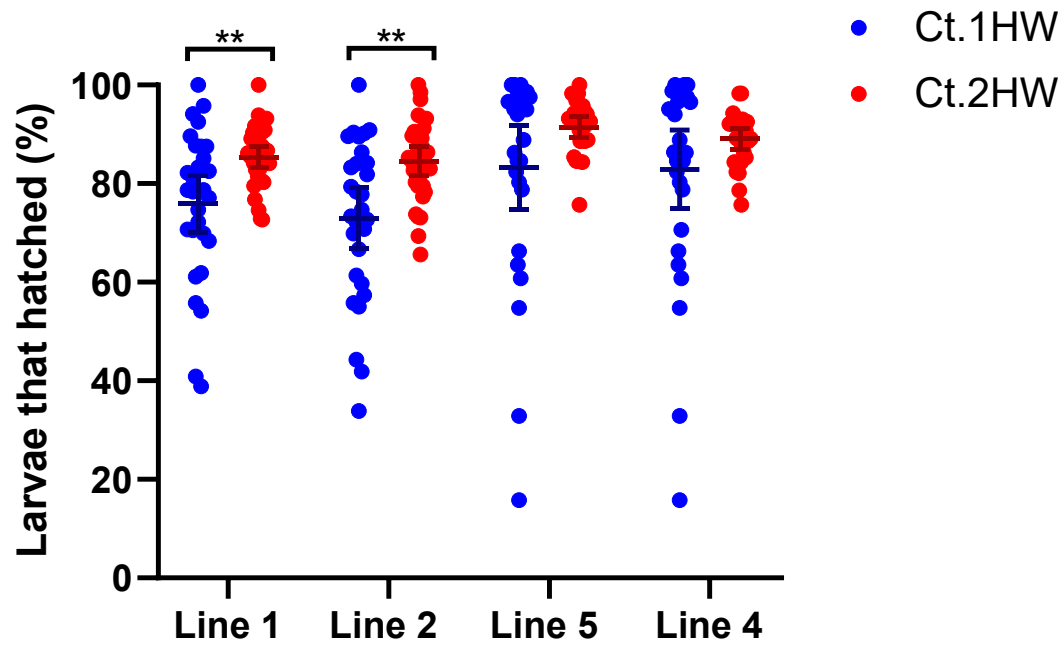


Figure 20. Comparison of fertility in controls. Fertility was scored after all the eggs hatched. Error bars show the standard error of the mean (SEM).

After all the comparison between controls for one HW exposure and two HW exposure, it was then possible to proceed with the direct comparison of the effects induced by the two different HW-exposure across the four lines.

Comparison of mortality between one HW exposure and double HW exposure, assessed with 2way ANOVA and Sidak's multiple comparisons test, showed significant difference in Line 1 (p-value: 0.007), and in Line 2 (p-value: 0.011) for control lines, but I observed no statistical difference in Line 5 (p-value: 0.052), and also in Line 4 (p-value: 0.056) for warm-evolved lines.

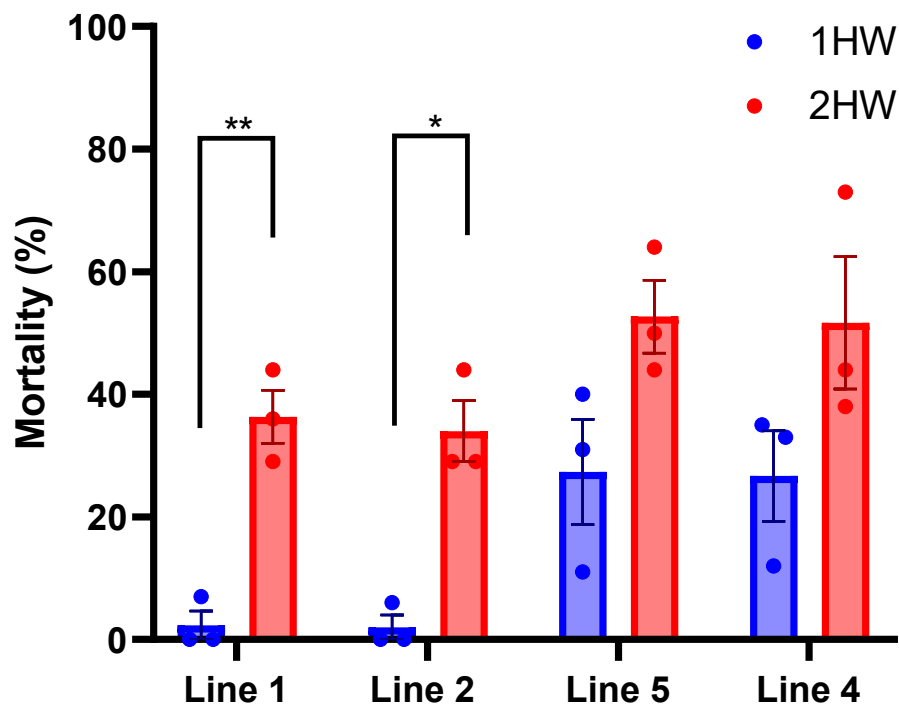


Figure 21. Comparison of mortality after one HW exposure (blue) and after two HW exposure (red). Error bars show the standard error of the mean (SEM). An * refers to a p-value < 0.05 and ** to a p-value < 0.01.

Longevity was measured following each HW exposure. I observed, with Mantel-Cox test, that the comparison of male longevity is not statistically different in Line 1 (p-value: 0.0502) but showed statistical difference in Line 2 (p-value: < 0.0001) for control lines, and in Line 5 (p-value: < 0.0001) and in Line 4 (p-value: 0.0002) for warm-evolved lines.

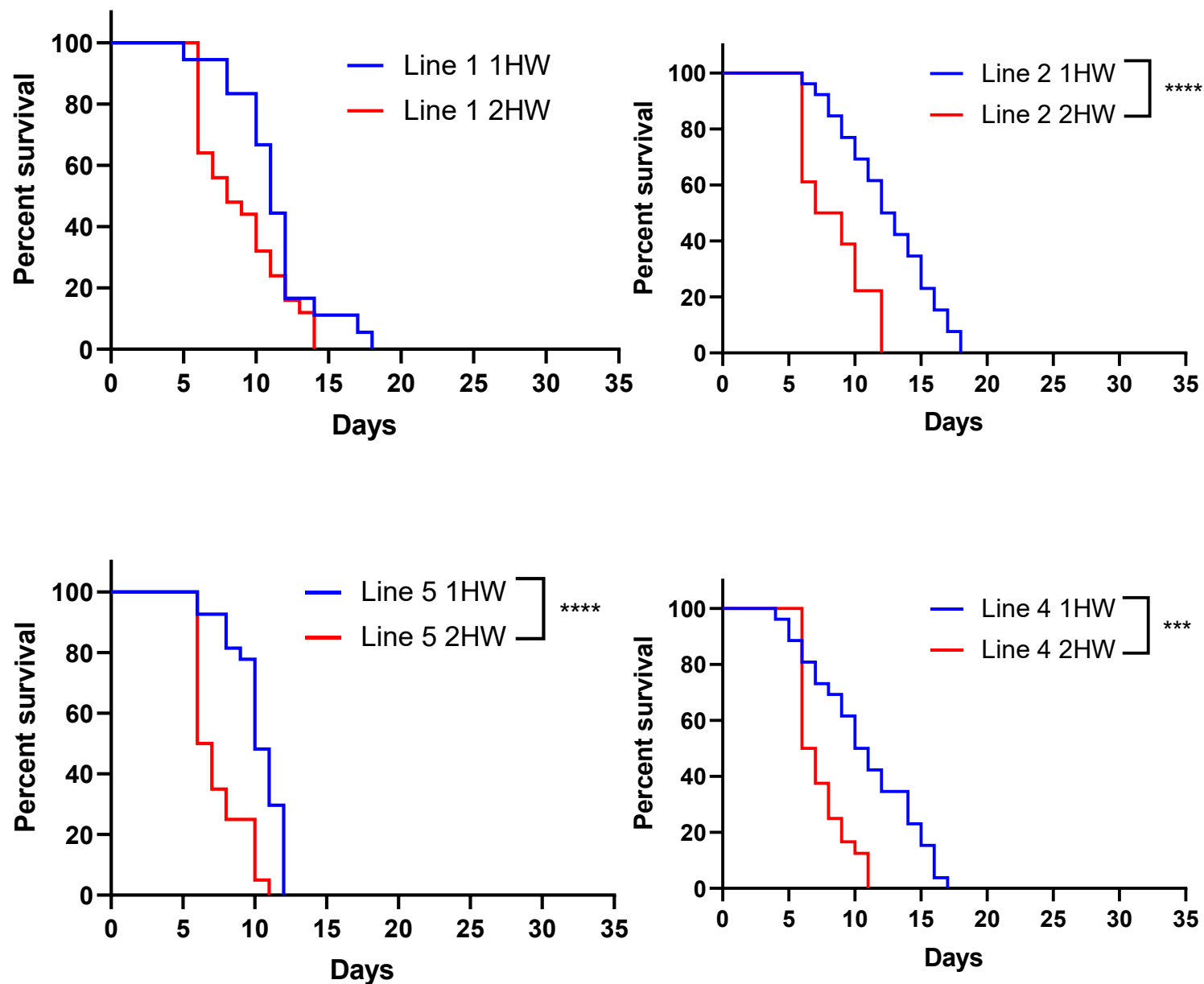


Figure 22. Comparison of longevity after one HW exposure (blue) and double HW exposure (red). Longevity was scored day by day after HW exposure in each line. An * refers to a p-value <0.05, ** to a p-value <0.01, *** to a p-value <0.001, **** to a p-value <0.0001.

I measured also the comparison of traits to male reproductive capacity, that means fecundity, sterility and fertility, between one HW exposure and double HW exposure.

I observed, properly with Kruskal-Wallis test and Dunn's multiple comparisons test, that the fecundity has not statistical difference, in Line 1 (p-value: 0.793), in Line 2 (p-value: 0.277) for control lines, and also in Line 5 (p-value: >0.999) and in Line 4 (p-value: >0.999) for warm-evolved lines.

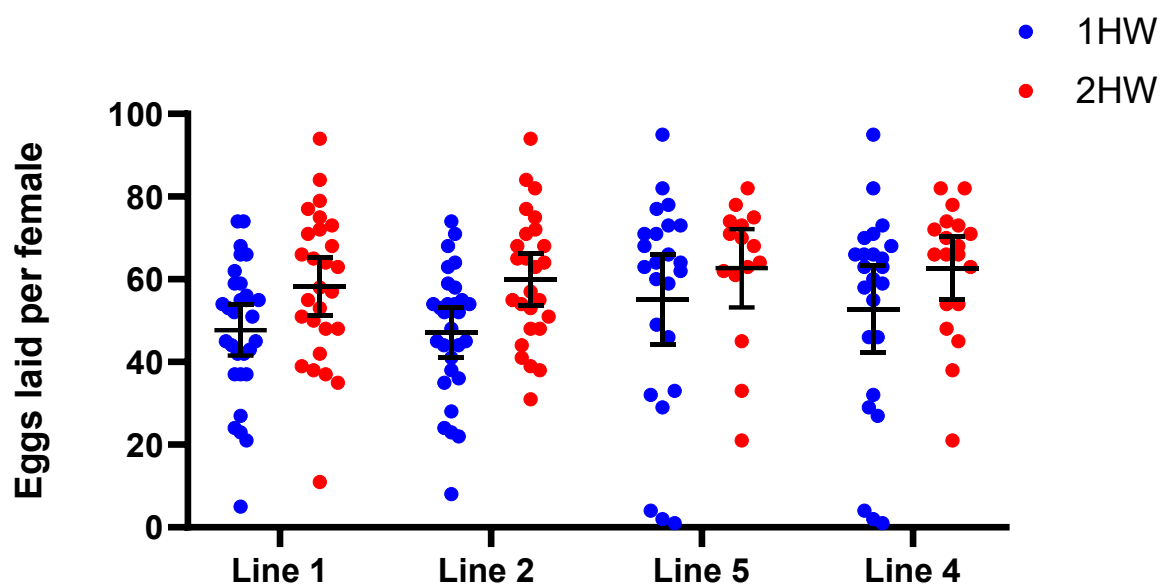


Figure 23. Comparison of fecundity. Fecundity was scored after oviposition in each line. Error bars show the standard error of the mean (SEM).

I observed, with 2way ANOVA and Sidak's multiple comparisons test, no statistical difference in the comparison of sterility between one HW exposure and double HW exposure, in Line 1 (p-value: 0.999) and in Line 2 (p-value: 0.992) for control lines, and in Line 5 (p-value: 0.922), Line 4 (p-value: 0.725) for warm-evolved lines.

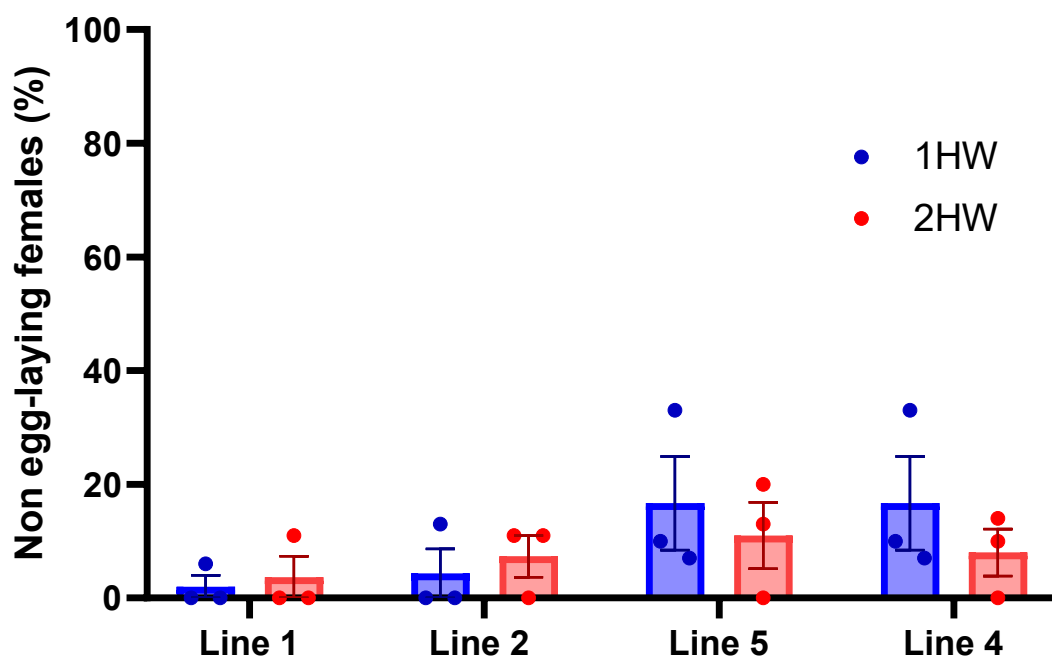


Figure 24. Comparison of sterility. Sterility was scored after oviposition in each line. Error bars show the standard error of the mean (SEM).

I observed, with Kruskal-Wallis test, that is no statistical difference in Line 1 (p-value: >0.999) and in Line 2 (p-value: >0.999) for fertility of control lines. Also I observed no statistical difference in warm-evolved lines, in Line 5 (p-value: >0.999) and in Line 4 (p-value: 0.550). But I also observed statistical difference between Line 1 and Line 5 both two HW exposure (p-value: 0.048) and between Line 1 and Line 4 both two HW exposure (p-value: 0.001).

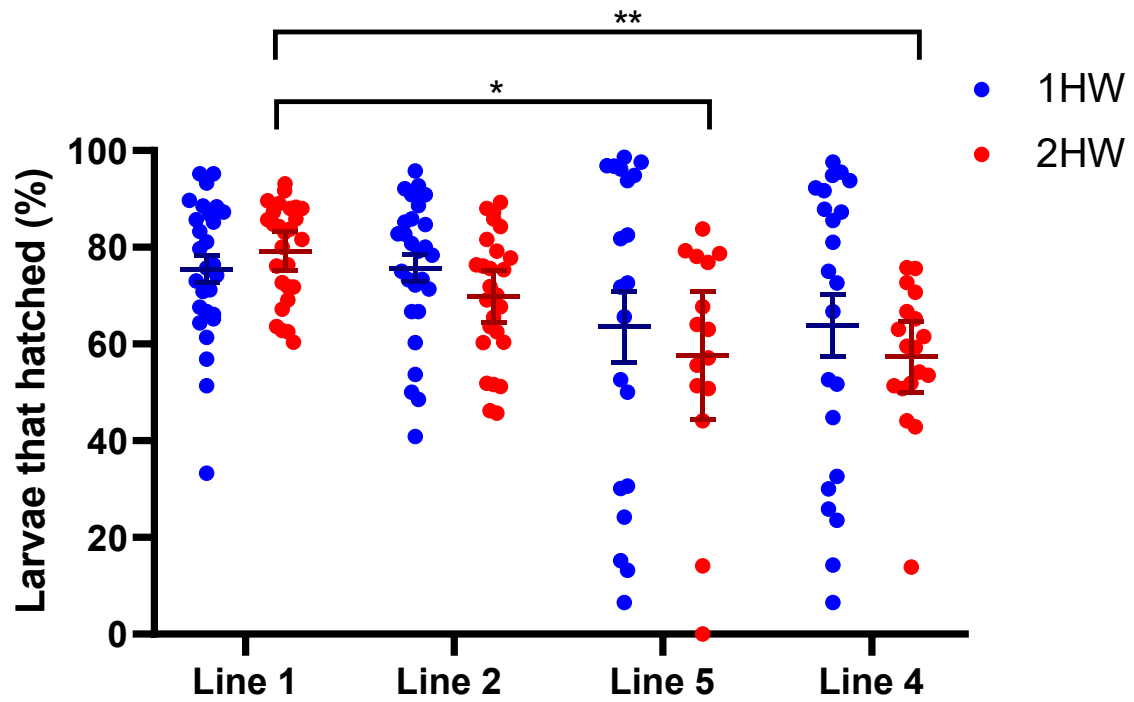


Figure 25. Comparison of fertility. Fertility was scored after all the eggs hatched. Error bars show the standard error of the mean (SEM). An * refers to a p-value <0.05, ** to a p-value <0.01.

4.4 Set up and sperms visualization

I began investigating the setup and visualization of male spermatozoa. After dissecting individual males, visualization was performed using an Olympus CKX53 microscope equipped with an Olympus SC50 camera for image acquisition, using the imaging software "cellSens". The results show the spermatozoa of males and their morphology, revealing that they are considerably longer than human spermatozoa, and that the head and tail of individual cells are difficult to distinguish.



Figure 26. Single spermatozoon, illustrating the difficulty in precise morphological identification, with the head highlighted (red circle). Image captured using an Olympus CKX53 microscope with an Olympus SC50 camera.

Among the results, additional images provide a clear illustration of sperm visualization.

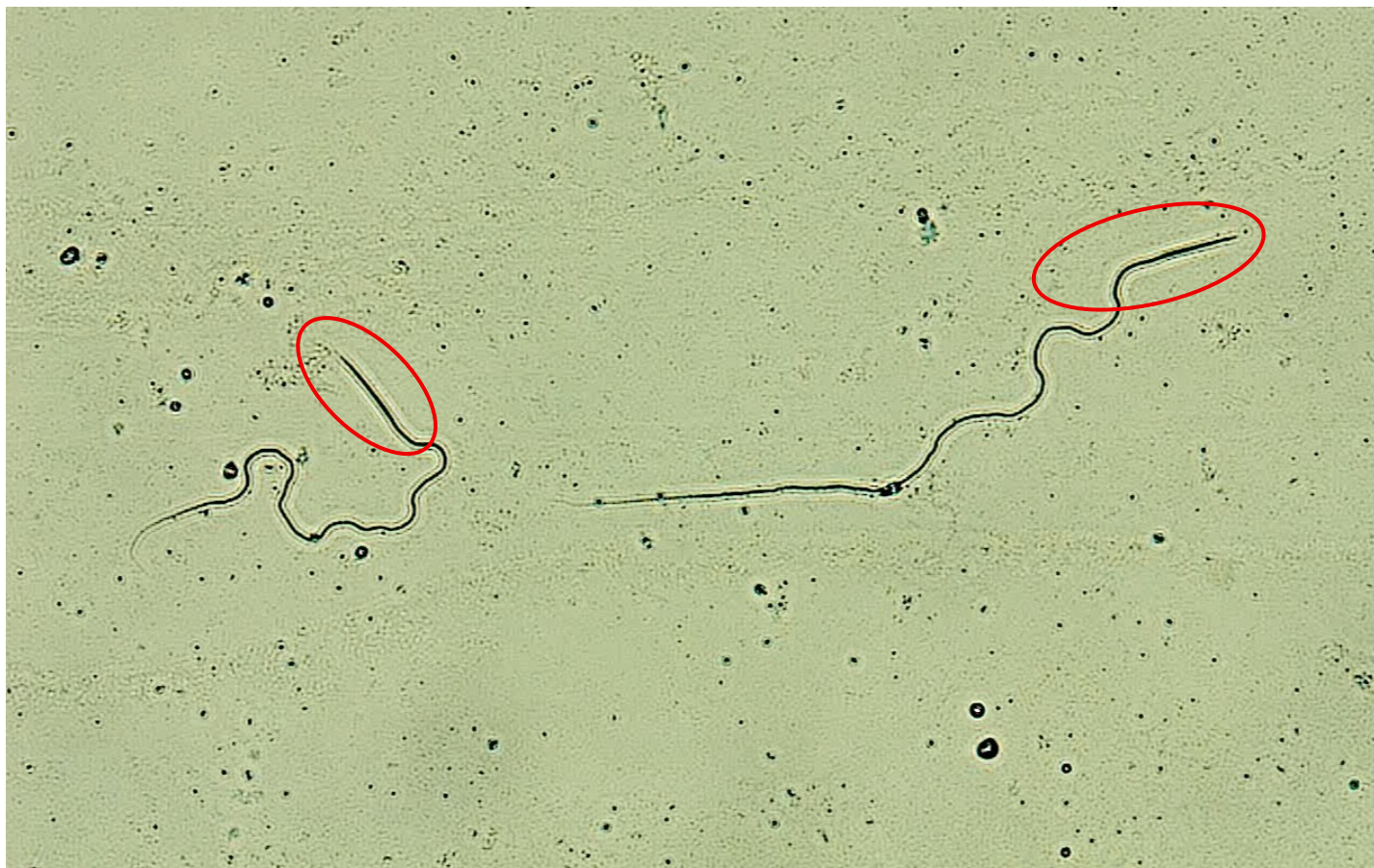


Figure 27. Two spermatozoa, with the head of each cell highlighted (red circle). Image captured using an Olympus CKX53 microscope with an Olympus SC50 camera.

One of the key aspects of spermatozoa visualization is the ability to distinguish individual cells from one another, which is also essential for counting spermatozoa per individual. The results show that it is difficult to perfectly distinguish each individual cell and to obtain a fully accurate sperm count.



Figure 28. Cluster of three spermatozoa. Image captured using an Olympus CKX53 microscope with an Olympus SC50 camera.



Figure 29. Cluster of spermatozoa, showing their distribution pattern. Image captured using an Olympus CKX53 microscope with an Olympus SC50 camera.

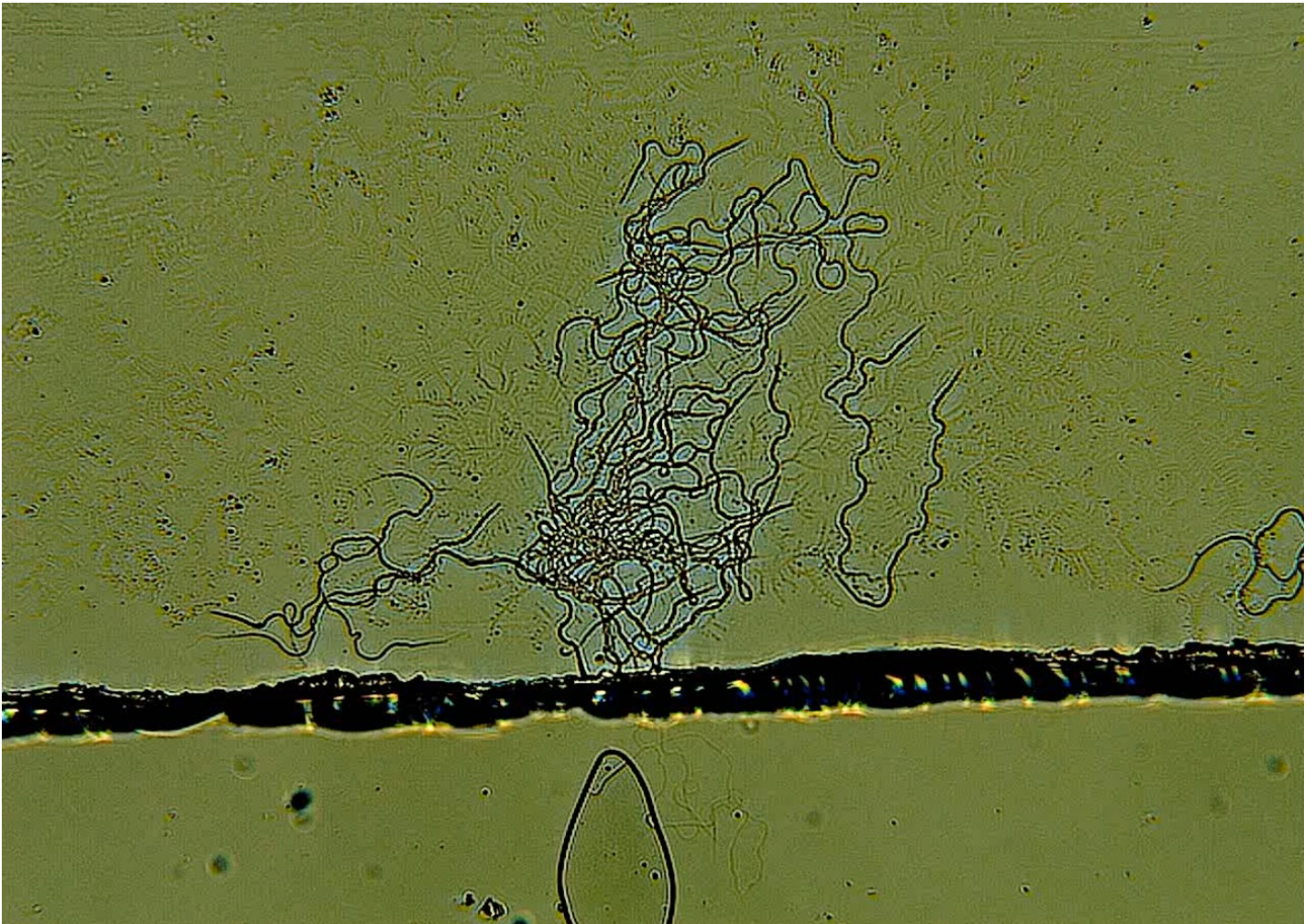


Figure 30. Dense group of spermatozoa captured within a single camera field. Image captured using an Olympus CKX53 microscope with an Olympus SC50 camera.

5. DISCUSSION.

In this work, I tested changes in mortality, longevity and male reproductive capacity in *Ae. albopictus* males exposed to either one or two HWs. Importantly, the HW regime used is ecologically relevant because the nightly drop of 11°C from a daytime peak of 37°C served as a recovery period (94–96). Italian meteorological records show the frequent occurrence of heat events over the last decade, typically consisting of 2 to 3 days with a thermal maximum in the range of 37–38 °C followed by 24–27 °C at night during the summer months (105). These thermal conditions closely resemble my HW regime, except that, in the laboratory, transition between daytime and nighttime temperatures occur more rapidly (approximately 40 minutes) than under natural day-night cycles.

Tested males derived from two different types of lines, a group of lines (lines 4 and 5) had been exposed to thermal evolution for 27–28 generations, the other had been maintained under control condition. This allows to test if warm-evolution prime for HW exposure.

First I observed that overall mortality rate of pupae was lower than that of adults. This indicates that the recovery period was considerably more effective at the pupal stage and that pupae are more resistant to HWs than adults.

At the adult stage mortality exceeded 50% after HW exposure, indicating that adults are highly sensitive to HWs.

Interestingly, in neither males exposed to a single or a double HW, I observed significant reduction of the fecundity and fertility of females. This results was unexpected because several studies previously substantial negative effects on the reproductive output of various insect species, including *Drosophila melanogaster* and other Drosophilidae, the red flour beetle *Tribolium castaneum*, the bed bug *Cimex lectularius*, the parasitoid *Aphidius avenae*, and the oriental fruit moth *Grapholita molesta* (97–103).

The biological effects of a heat event depend on a delicate balance between heat-stress-related damage and the possibility of recovery during fluctuations toward milder temperatures [96]. The nightly recovery period in our HW regime may therefore have mitigated heat-related damage resulting from exposure to 37°C, a temperature close to the physiological threshold of *Ae. albopictus* (104).

Alternatively, damage in male sperm may have been subtle, with still enough sperm to fertilize all eggs from females, which had not been exposed to HW, resulting in no significant overall progeny reduction. My experimental design with forced single mating pairs may have also masked potential behavioral changes in sexual performance of HW-exposed males. My protocol for sperm visualization is expected to aid in testing for changes in sperm number and quality in HW-exposed males.

Reduction in male mortality after HW is an important result, which underscores the importance of scheduling releases of sterile or Wolbachia-infected males, in coordination with weather forecasts, to avoid, where possible, heat events that could reduce the effectiveness of male release.

6. CONCLUSION

In my thesis, I show that *Ae. albopictus* mosquitoes respond differently to an ecologically relevant HW depending on their developmental stage. My results indicate that thermal vulnerability is closely linked to the underlying biological response. For instance, males showed a limited stress response that correlated with their elevated mortality following HW exposure, underscoring the importance of investigating male biology when predicting mosquito population dynamics and designing control strategies. As ecological models and vector control programs increasingly incorporate climate variability, a stage-specific understanding of HW responses will be essential for accurate predictions and effective interventions.

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