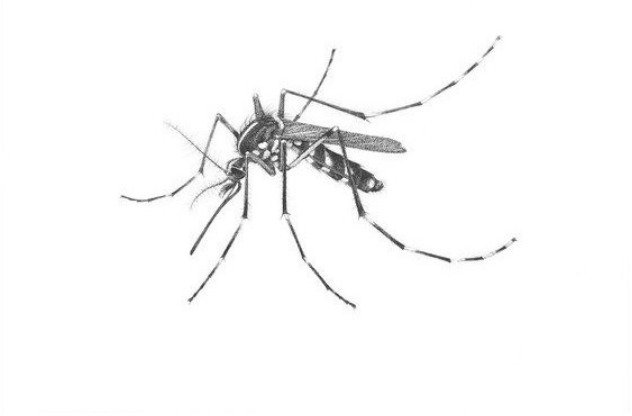
UNIVERSITÀ
DI PAVIA

Dipartimento di Biologia e Biotecnologie "L. Spallanzani"

Effects of developmental temperature on the biology of *Aedes albopictus*: a strain comparison



Martina Carlassara

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXVI – A.A. 2020-2023.

UNIVERSITÀ
DI PAVIA

Dipartimento di Biologia e Biotecnologie "L. Spallanzani"

Effects of developmental temperature on the biology of *Aedes albopictus*: a strain comparison

Supervised by Prof. Mariangela Bonizzoni

Martina Carlassara

Dottorato di Ricerca in
Genetica, Biologia Molecolare e Cellulare
Ciclo XXXVI – A.A. 2020-2023.

Abstract

Mosquitoes are poikilothermic animals with a holometabolous life cycle. Thermal conditions they experience during development have a great impact on both juvenile and adult stages. For instance, cold thermal regimes are expected to cause mosquitoes to grow slower but larger and to live longer and to have a higher fecundity compared to mosquitoes developing at warmer temperatures. The potential transmission of pathogens is directly related to developmental speed, adult longevity and fecundity because a faster development, a longer lifespan and a higher fecundity increase the vector population density and the number of vectors which have been exposed to pathogens through a blood meal on an infected host and have become infectious. Despite the known importance of ambient temperature during the mosquito development, we lack data on the extent of its impact on both juvenile and adult stages of the mosquito life. Especially in the case of invasive species, unravelling the relationship between developmental temperature and the mosquito performance at the various life stages and identifying possible differences across populations might highlight the most vulnerable life stages for control strategies and identify the populations with the highest invasion potential in face of current climatic changes. The Asian tiger mosquito *Aedes albopictus* is a highly invasive species which vector health-threatening arboviruses such as Zika, Dengue and Chikungunya and is currently present in every continent of the World except Antarctica. For this species, we lack precise information on traits which are highly influenced by developmental temperature and are also important for pathogen transmission, such as developmental speed, longevity and fecundity.

Within this context, during my PhD I investigated the effect of different developmental temperatures on the performance of juvenile stages and their further effects on adults of *Ae. albopictus* mosquitoes of a tropical (Foshan, China) and a temperate (Crema, Italy) origin. Furthermore, I extended my study to the transcriptome, the physiology, thermal traits and the density of *Wolbachia*, a thermosensitive bacterium of great importance for mosquito fitness. The temperatures I decided to investigate include 18°C, which represents the average daytime temperature registered during spring 2021 in Northern Italy, 32°C, which was the peak of warm temperatures registered in the summer 2021 in the same region, and 28°C, which is the standard rearing temperature for *Ae. albopictus*. My results show strong strain-specific effects on both fitness and energy reserve in mosquitoes reared under different thermal regimes, whereas I measured few thermal-related changes in adult thermal preference and knock-down temperature (i.e. heat resistance). Overall, the thermal condition of 32°C was the one mostly reducing the mosquito fitness at the adult level, whereas the temperature of 18°C drastically slowed down development of early stages. At both 18°C and 32°C I recorded responses linked to stress (such as cytochrome p450 and heat shock proteins) and the lowest densities of *Wolbachia.* I further proved that mosquitoes' wing length and body mass follow the Bergmann's rule and found that only the tropical Foshan strain follows developmental

isomorphy, suggesting that depending on their geographical origin different strains might have different adaptability potential of early life stages in response to climate change.

Abbreviations

CHIKV = Chikungunya Virus

CI = Cytoplasmic Incompatibility

Cr = Crema

CTmax = Critical Thermal maximum

CTmin = Critical Thermal minimum

d0 = day 0, day of emergence

d12-14 = day12-14

d7 = day 7

DE = differential expression

DENV = Dengue virus

df = Degrees of Freedom

dpi= day(s) post-infection

DR = developmental rate

EIP = extrinsic incubation period

F = F ratio

FE = Fold enrichment

Fo = Foshan

FPKM = Fragments per kilobase per Million

gDNA = genomic DNA

GO = gene ontology

HR = Hazard Ratio

Hsf = heat-shock factor

Hsp = heat-shock protein

IIT = Incompatible Insect Technique

IPCC = Intergovernmental Panel on Climate Change

KDT = knock-down temperature

LDR = larval developmental rate

LDT = larval developmental time

MAYV = Anti-Mayaro Virus

MS = mean squares

PCA = Principal Component Analysis

PDR = pupal developmental rate

PDT = pupal developmental time

Pmax = maximal performance capacity

qPCR = quantitative polymerase chain reaction

R = Pearson's correlation coefficient

RNAi = RNA interference

ROS = reactive oxygen species

RT = room temperature

Ta = ambient temperature

Tbr = thermal breadth

Topt = otpimal temperature

Tp = Thermal preference

TPC = Thermal performance curve

Tt = thermotolerance

VBD = vector-born disease

VC = vector capacity

WHO = World Health Organization

WNV = West Nile Virus

Y0 = year 0

Y2 = year 2

YFV = Yellow Fever Virus

Tables of Contents

1.Introduction

1.1 Climate change

During the last half-century, the globe had experienced an unprecedented temperature increase [1]. In 2021 the Sixth Assessment on climate change of the Intergovernmental Panel on Climate Change (IPCC) reported the indisputable influence of human activities on global warming [2]. The anthropogenic increase of global temperature was assessed to be 1.07°C, calculated as the difference between the average temperatures of 2010-2019 and the baseline period (1850-1900) [3]. The following year, this estimated temperature increased by 0.18°C [4] and is expected to increase further (+0.25°C) by the next decade [5]. Predictions for 2021-25 thermal anomalies compared to the baseline period between 1981 and 2010 show enhanced warming at high northern latitudes and the Arctic resulting in anomalies more than twice as large as the global mean [6] (Figure 1).

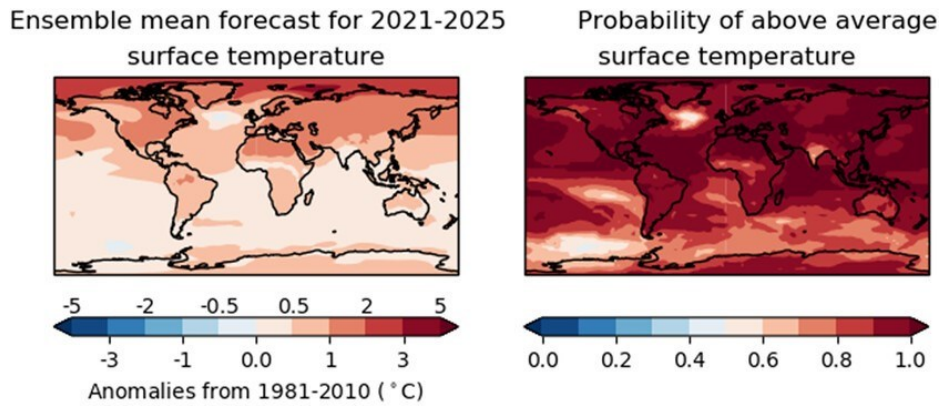


Figure 1. Predictions for 2021–25 anomalies relative to 1981-2010. Ensemble mean for temperature (left); probability of above average for temperature (right) [6].

The Mediterranean basin is considered a climate change hotspot and Italy has shown a general warming tendency with an increase of about 1.54ºC in the average ambient temperature (Ta) since 1980, together with an increase in the minimum and maximum temperature extremes [7]. This translates into a net increase of about 14% of the number of summer days, with also an increase in the number of tropical nights (defined as nights with a minimum temperature exceeding 20ºC) and hot days (defined as days with a maximum temperature above 35ºC) and in the frequency of heat waves [8]. Climate change also includes extreme weather events such as hot and cold snaps, which occur not only in winter and summer, but also in spring and fall. Lower than average temperatures in spring are known to affect survival, behaviour, physiology and reproduction of insects [9].

1.2 Insects and temperature

Climate change-driven thermal escalation is expected to produce deleterious effects on many animal species, especially on ectotherms such as reptiles, fish, amphibians and arthropods, in which body temperature, hence their overall fitness, physiology and symbiotic relationships, is dependent on Ta [10]. It has become critical to predict the alterations in ectotherms' activity and distribution in response to climate change to prevent extinction of vulnerable species and epidemiological or economic consequences in case of pathogen vectors or pest species [10-13].

Insects can live within a range of Ta, restricted by the critical thermal limits, which defines their thermal tolerance. In case of Ta not included in this favourable range, either above or below the critical thermal limits of a taxon, temperature can hamper insects' activity and lead to their death [14]. This concern has been mostly associated with tropical populations, as they are already experiencing conditions close to their upper thermal limits [10] and would therefore perish with further Ta increases. Nevertheless, since climate change in tropical areas may increase precipitation and cloud cover, tropical taxa may be less threatened by global warming than originally predicted [15]. On the other hand, higher Ta together with a decrease in precipitation and cloud cover are expected to be a relevant issue for taxa in regions adjoining the tropics [15] and for mid-latitude taxa [16].

1.2.1 Coping mechanisms in insects

Insects and other ectotherms can cope with unfavourable thermal conditions mainly through three mechanisms, which are not mutually exclusive [17]:

(1) range shift, namely the movement to new areas characterised by more suitable thermal regimes. A consequence of range shift is the concurrent presence in the same area (sympatry) of recently diverged species, which can possibly increase their interaction and thus hybridisation [18];
(2) phenotypic plasticity, the ability of a genotype to produce varying phenotypes based on perceived environmental stimuli [19]. Phenotypic plasticity includes a variety of responses to the surrounding environment and is obtained through modulation of gene expression of either single genes [20] or of larger parts of the transcriptome [21-23]. These responses take less time than evolutionary adaptation to occur and allow the individual to cope with unfavourable environmental conditions [13], especially when there are genetic constraints to the evolution of the specific traits or such traits are low or not heritable [22]. There are different types of plastic responses, such as acclimation, dormancy [13] and change in energy metabolites [24];

(3) evolutionary adaptation, with genetic shifts driven by selection [25]. Genetic changes can result in phenotypic changes as the insect population adapts to the environment across generations [26].

Thermal plasticity (or acclimation) is characterised by reversible changes in physiological phenotypes because of exposure to new environmental conditions, occurring in the time range of days to months [27;28]. Through acclimation an organism can increase its thermal tolerance, for instance by adjusting its body temperature to new settings [13;29]. Acclimation to higher Ta can occur, for instance, via heat-shock response [29;30] and through a faster development followed by a smaller size in adults [31;32]. However, thermal tolerance modulation is usually limited at high temperatures (as reviewed by Couper et al., 2021) [13].

Heat-shock response is an example of phenotypic plasticity in insects, which can occur within minutes or hours [27]. When experiencing a stress condition, such as a sudden change in temperature, an infection, hypoxia or osmotic stress, and in the state of dormancy [33;34], heat-shock proteins (Hsp) have a protective role in insects [35], since they bind aberrant proteins to obtain the correct refolding, avoid denaturation in polypeptides or lead to ubiquitin-dependent degradation of those proteins which are irreversibly damaged [34]. Heat-shock response was discovered in 1962 in *Drosophila melanogaster*, whose transcriptional activity increased at 37°C [36]. High temperatures activate the heat-shock factor (Hsf), a transcriptional factor which induces transcription of genes coding for Hsp [34]. However, the production of Hsp is expensive and may be toxic for the cell. Thus, the overexpression of Hsp may represent a poor adaptation to heat, as suggested in a study of Ware-Gilmore et al. (2022) [37], in which *Ae. aegypti* families with higher thermal tolerance showed less expression of Hsp genes than families with low resistance to heat.

The decrease in size involved in acclimation is due to the high cost of the increased growth speed, which exploits resources normally used during development [38;39]. Growing at an increased speed can result also in lifespan shortening due to a higher mitochondrial activity, followed by a higher production of reactive oxygen species (ROS) and thus oxidative damage in the cell, that ultimately causes organism ageing [32;40]. Furthermore, a shorter lifespan can also be related to a faster telomere shortening due to oxidative stress. However, this effect needs to be further investigated, since the damage might be partially recovered by a higher telomerase expression, predicted to take place at warmer temperatures [41].

Dormancy is among the most important plastic traits that allow overwintering in insects and consists in the decrease or suppression of the metabolic activity and development suspension (as reviewed by Diniz et al., 2017) [42]. Dormancy may happen in different life stages (embryonic, juvenile and adult stages) and can be a response to climatic signals, such as photoperiod and humidity [42]. Two types of dormancies are known depending on the triggering signal: diapause and quiescence. In diapause, the main signals are determined by photoperiodic changes and gradual decreases in thermal

regimes, as in winter, which trigger endogenous and programmed pathways in the individual. Quiescence is instead a completely exogenous-dependent phenomenon and thus, as the signal disappears, the effect ends [43;44].

To cope with thermal variations, insects can utilise energy reserves stored in the fat body, such as glycogen and lipids, which is represented by 90% of triglycerides [45]. During flight, dormancy or temperature variations, the organism is in need of energy and thus the fat body releases metabolites through the haemolymph [45;46]. Glycogen can be mobilised in response to thermal stress [47] and is used to produce trehalose and sugar alcohols (polyols) that prevent cellular damage at low temperatures [48] by protecting membranes and maintain protein stability [49]. Stored lipids are processed and utilised as cryoprotectants, especially glycerol [50].

1.2.2 Developmental isomorphy

Developmental rate (DR) of ectotherms is known to be highly influenced by Ta, with cool thermal regimes resulting in low DR and warm thermal regimes in high DR [51]. Considered a general trait shared among ectotherms [52], the hypothesis of developmental isomorphy states that, if only basic processes (for instance, cell division) regulate DR, a life stage should not differ in proportion due to temperature variation [53]. Therefore, in ectotherms showing developmental isomorphy there would be constraints in developmental adaptation to different environments [52]. To test isomorphy, data such as larval developmental rate (LDR) and pupal developmental rate (PDR) must be collected in insects reared at different thermal conditions and the ratio between LDR and PDR calculated. Through regression analyses the equation relative to the resulting line can be obtained, which includes the slope value (b). A species is isomorphic if the slope of the ratio LDR/PDR is equal to 0 ($b=0$). If the slope is either $b<0$ or $b>0$ there is a negative or a positive relation between LDR/PDR and temperature, indicating that temperature has a different effect on the speed of development of larvae and pupae (Figure 2).

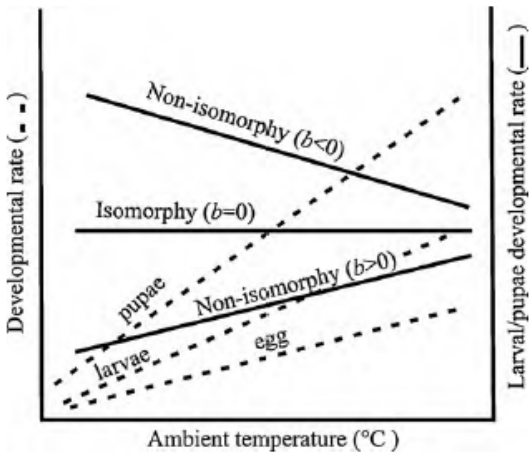


Figure 2. Hypothetical developmental rate regressions at different ambient temperatures. Non-isomorphic and isomorphy cases are described [53].

Up to date, the majority of studies on insect species support the developmental rate isomorphy as a general phenomenon [52]. Nevertheless, exceptions to this hypothesis can be found in literature, for instance Folguera et al. (2010) found a negative (b<0) and therefore non-isomorphic response in *Drosophila buzzatii* [53].

1.3 How to study thermal biology in insects?

Thermal biology is defined as "the study of physiological and ecological consequences of body temperature and of the biophysical, morphological, and behavioural determinants of organism temperature" [54]. In insects that are vectors for pathogens, we need to consider that temperature affects not only the vector life history traits, behaviour and ecology, but also pathogen replication and dissemination rates [55].

The thermal biology of a species can be investigated through a longitudinal approach by looking at the existence of correlations between phenotypes of a species and its distribution across ecological gradients (i.e. elevation, latitude and climate) and/or through common garden experiments whereby a species performance is studied under specific thermal conditions [56]. Climate change research has recently started investigating species' responses to global warming by studying their thermal biology to predict response to ongoing environmental changes [57-59] and experimental data from both approaches are currently available only for a handful number of insect species, *in primis Drosophila spp*. [60-67].

The effect of temperature on a specific trait can be established by quantifying that trait at different Ta and plotting these values in a graph, which is called Thermal Performance Curve (TPC, shown in Figure 3) [22;64;68-70].

From the TPC, several parameters can be extrapolated, namely:

(1) Optimal Temperature (*Topt*): the temperature at which the curve reaches its maximum (peak);
(2) Maximal Performance Capacity (*Pmax*): the height of the peak associated with *Topt*;
(3) Thermal Breadth (*Tbr*): the range of temperatures in which the performance of a given species is at least 80% of the maximum performance [68];
(4) The Critical Thermal Limits: the Critical Maximum Temperature (*CTmax*) and the Critical Minimum Temperature (*CTmin*), temperatures at which the performance value is equal to zero [22;70].

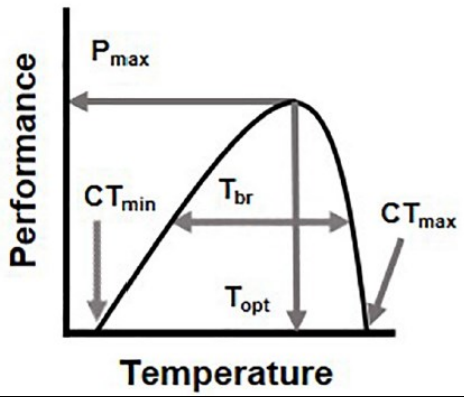


Figure 3.

Example of Thermal Performance Curve (TPC) showing each parameter [22].

Therefore, a TPC starts from a specific *CTmin*, increases as temperature becomes warmer, reaches a *Topt*, and drops until the *CTmax* for a given species is reached [10]. TPC parameters may vary across populations of a given species and can be influenced differently by acclimation [71]. For instance, *Tbr* could increase in size in acclimated taxa [72] and in general temperate ectotherms are thought to be more plastic and to show a larger *Tbr* than tropical species, since they experience climates characterised by a larger Ta range during the year [56;64;73]. However, the origin from either temperate or tropical regions did not affect *Topt* or *Tbr* across 22 *Drosophila* species [64], which suggests that tropical taxa may have a larger *Tbr*, due to large daily thermal variations, but smaller acclimation potential, due to smaller seasonal thermal variations, when compared to temperate ectotherms [74].

Furthermore, another important parameter to consider while investigating the effect of Ta on ectotherms is Thermal Preference (*Tp*), which is the temperature preferred by individuals of a species. *Tp* often agrees with the *Topt* of different performance traits in a species, such as locomotion and fecundity [75]. However, empirical data show that *Topt* can also exceed *Tp* [76]. In *Drosophila Tp* is stable, particularly in early life stages, despite the different species and geographical origins [64].

1.4 Vector-borne diseases

Vector-borne diseases (VBDs) are a class of diseases caused by viruses (such as DENV, YFV and CHIKV), bacteria (for instance *Xylella* and *Spiroplasma*) or parasites (such as *Plasmodium*) transmitted by a vector [77-79]. According to the World Health Organization (WHO), 80% of the population worldwide is at risk for at least one of these diseases, which are the cause of 700,000 deaths per year [80]. Mosquitoes, aquatic snails, ticks, blackflies, tsetse flies, fleas, lice and sandflies are the main vectors of VBDs as reported by the WHO [80]. The main strategies applied to control VBDs are focused on vector control, which is so far the most effective at contrasting the spread of VBDs compared to drugs and vaccines [81]. Vector control strategies aim at reducing or eliminating the contact between humans and vectors, thus reducing or eliminating pathogen transmission [81]. However, adults' insecticide resistance and change in larval development sites are some of the obstacles for current control strategies [82;83]. Table 1 reports some examples of VBDs and the relative pathogen type vectored by mosquitoes.

A key parameter to investigate VBDs dynamics is vectorial capacity (VC), which measures the likelihood of a vector to transmit a pathogen. VC is defined by the following formula:

$$VC = ma^2bp^n/-\log_e p$$

where "n" is the pathogen's extrinsic incubation period (EIP) in days, "m" is vector density in relation to humans, "p" is vector survival through one day, "b" is the transmission rate among exposed vectors and "a" are human biting rates [84].

VC is influenced by intrinsic and extrinsic factors. The first are traits having a genetic component such as host preference, susceptibility to the infection [85], and vector competence, i.e. the vector's intrinsic capacity to support pathogen replication, dissemination and transmission [86;87]. Among extrinsic factors, Ta has a major impact on VBDs transmission, because of its effect on both pathogen and vector's fitness and on the EIP [88]. Permissive temperatures fall into a specific range, and within this range the magnitude of the transmission varies [89]. Outside this range, when temperature hinders the survival, the reproductive capacity, the development or the functionality of the metabolism of either the vector or the pathogen, transmission does not occur [55].

Table 1. Incomplete list of mosquito vectors with type of pathogen and disease caused. WHO[80].

Vector	Disease	Pathogen
Aedes	Chikungunya	Virus
	Dengue	Virus
	Lymphatic filariasis	Parasite
	Rift Valley fever	Virus
	Yellow fever	Virus
	Zika	Virus
Anopheles	Lymphatic filariasis	Parasite
	Malaria	Parasite
Culex	Japanese encephalitis	Virus
	Lymphatic filariasis	Parasite
	West Nile fever	Virus

1.5 Mosquitoes

Mosquitoes are insects belonging to the Culicidae family (from Latin culex, meaning "gnat") [90], order Diptera, and include more than 3,574 species [91] classified into two subfamilies: Anophelinae and Culicinae [92]. Anophelinae include mosquitoes of the genus *Anopheles*, which are an important threat for human health and economy since they vector malaria parasites [93]. The Culicinae subfamily is divided into 11 tribes [92], and includes two medically important genera, *Culex* (tribe Culicini) and *Aedes* (tribe Aedini) and the genus *Toxorhynchites*, previously assigned to the subfamily Toxorhynchitinae [94]. Insects of the *Toxorhynchites* genus do not feed on blood, but larvae feed on juvenile stages of pests, such as other mosquito's species, and therefore could be used as biological control agents [95]. The *Culex* genus includes vector species as the northern house mosquito *Culex pipiens* and the southern house mosquito *Culex quinquefasciatus*, while the *Aedes* genus includes the yellow fever mosquito *Aedes aegypti* and the Asian tiger mosquito *Aedes albopictus* [90]. *Aedes* mosquitoes vector arboviruses (arthropod-borne viruses), such as Dengue virus (DENV), Chikungunya virus (CHIKV) and yellow fever virus (YFV) [96].

1.5.1 *Aedes albopictus*: general characteristics

Aedes albopictus is a holometabolous mosquito, whose development includes four life-stages, namely: egg, larva (four instars), pupa and adult. The adult body is divided into head, thorax and abdomen. Adults are medium-sized mosquitoes (ranging from 2 to 10 mm long) and are characterised by the silver scales forming a median longitudinal line on the thorax and those located on palpus and tarsi. Sexual dimorphism is present, and males can be recognized due to their smaller size (on average 20% smaller than females), by their plumose antennae and their mouthpart specialised to feed on nectar (Figure 4).

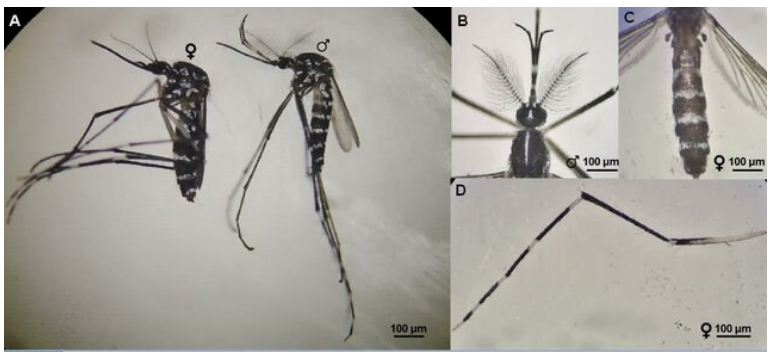


Figure 4. *Ae. albopictus* adults of both sexes (A), thorax (B), abdomen (C) and third leg (D) [97].

Only females require a blood meal, which is necessary for egg development. Females can feed on many vertebrates, both cold and warm-blooded animals, such as birds, reptiles, and amphibians [98], even though the preference is towards mammalian hosts [99]. Usually host-seeking and biting occurs outdoors during the daytime, mainly in the morning and in the first hours of the evening [99-101]. Almost 100 eggs are laid by a female at every gonadotropic cycle, for a total number of about 350 eggs laid by a female on average [102]. Females lay eggs above the waterline and, as the water level reaches the eggs, the latter hatch during the following 48 hours after soaking. Larvae develop into pupae passing through four stages - namely first, second, third and fourth instars - while feeding on microorganisms in the water. After two to three days larvae become pupae, which is the only life stage without feeding activity. After metamorphosis pupae emerge into adults, usually within 24 to 48 hours. The longevity of an adult mosquito ranges from 3 to 4 weeks. *Aedes* mosquitoes' life cycle usually lasts 15 days from egg to adult, but the length of every developmental stage varies depending on external environmental conditions, such as temperature [99].

As previously stated, *Aedes* mosquitoes are vectors for arboviruses. The life cycle of these viruses requires a host as a viral reservoir (such as vertebrates) and a vector to be acquired and then be transmitted to the next host [103]. As shown in Figure 5, mosquito females acquire viral particles when blood-feeding on an infectious host. In the mosquito midgut the virus replicates and spreads in the whole body through the mosquito circulatory system (haemocoel) and, if its dissemination is successful, the arbovirus reaches the salivary glands, from which it will be transmitted to the next host during the following blood-meal [104]. The EIP is the time between the acquisition of the viral particles from an infected host through blood-feeding to the moment in which the virus reaches the salivary glands and the mosquito becomes able to transmit it. EIP varies in length according to different factors, such as the viral titer in the host and the environmental temperature [105]. *Aedes albopictus* is able to vector many arboviruses, such as Flaviviridae (WNV, YFV, DENV, ZIKV), Togaviridae (CHIKV, MAYV) and Bunyaviridae (La Crosse virus) [100;106].

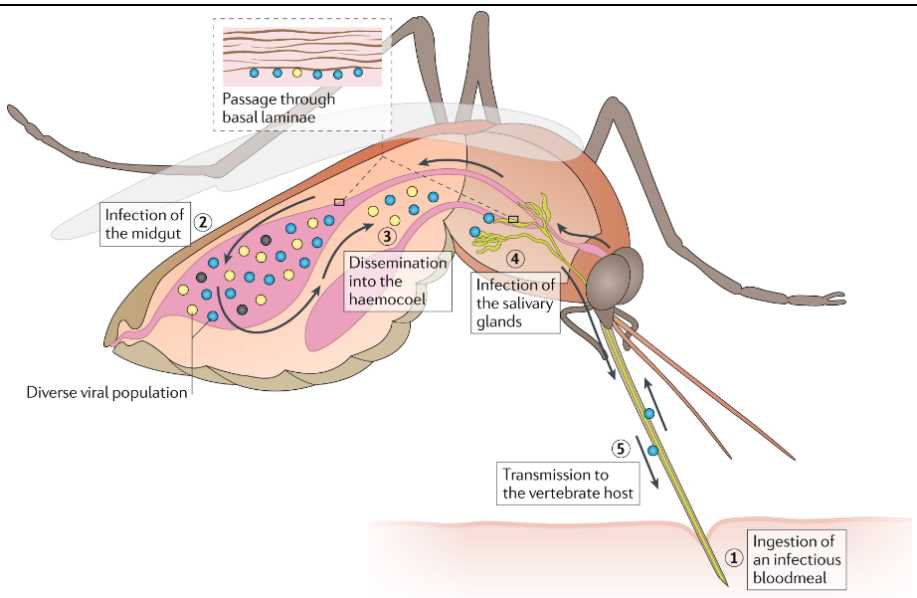


Figure 5. Acquisition and transmission of arboviruses by a mosquito female [104].

1.5.2 Geographical distribution of *Aedes albopictus*

Aedes albopictus is a highly invasive species which originated from South-East Asia, where it represents a zoophilic species found in the tropical forests. From these environments, *Ae. albopictus* moved to urban areas, in which conditions for its spreading were favourable (shelter and host abundance, thus higher chances for blood-feeding). In fact, urbanisation provides the ecological conditions favouring the increased developmental speed and survival rate in *Ae. albopictus* mosquitoes compared to rural areas [107;108]. The spreading of *Ae. albopictus* from the native Asian regions to the rest of the World was fueled by the increased movement of humans and goods, such as used tyres and the lucky bamboo *Dracaena sanderiana* [109;110] and its adaptation to new environments was favoured by its great ecological plasticity [111;112].

Aedes albopictus expansions occurred in two stages: the first stage took place in the early 1900s, when this species reached both Indian and Pacific Oceans' islands [113]. The second stage started the 1950s and continued throughout the following decades, when *Ae. albopictus* invaded all continents of the World, both tropical and temperate regions, except for Antarctica [114-116]. The first recordings of *Ae. albopictus* in Europe occurred in Albania in 1979 [112]. In 1990 this species was detected in the port of Genoa [117] from which it spread to all Mediterranean areas [111;118]. In July of 2005, the species was found in the Netherlands [119] and, according to Oliveira et al. (2021) [120] in the next three decades around 70% of Europe including British Isles, Ireland and southern

countries of Scandinavia are expected to become suitable for *Ae. albopictus*. In the continental USA *Ae. albopictus* was recorded in 1985 (Texas), but it was first found at the end of the 18th century in Hawaii [121;122]. From the colonised North American states and from Asia *Ae. albopictus* spread south and reached Brazil in 1986 and Mexico two years later [123;124]. In Africa its presence was first recorded during the 1980s in Madagascar island [125] and then in continental Africa: first in Cape Town, South Africa in 1991 [126] and then in western countries such as Nigeria in 1991 [127], Cameroon in 2000 [128] and Gabon in 2007 [129] and Equatorial Guinea, central Africa, in 2001 [130]. Figure 6 shows the global distribution of *Ae. albopictus* by integrating data of 2013 [114] and lastly reported in 2020 [131].

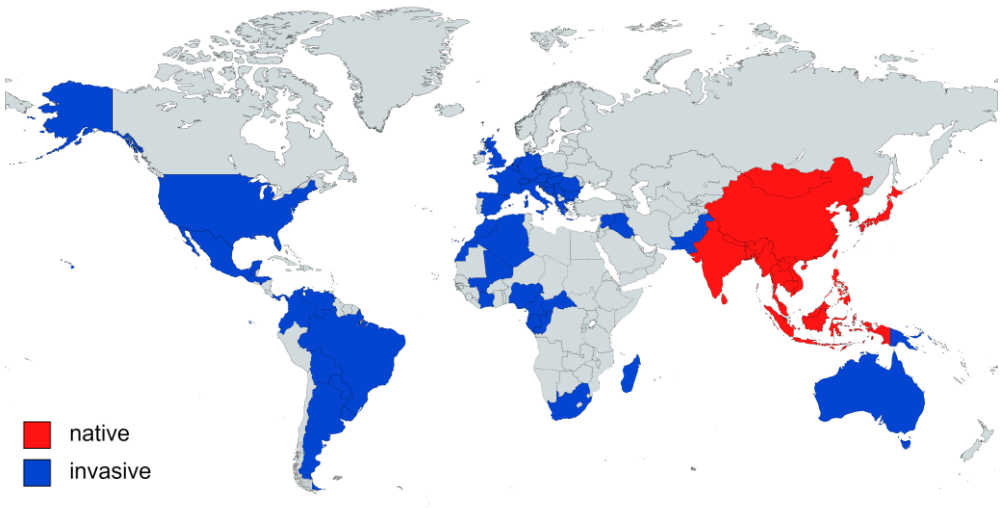


Figure 6. Global distribution of *Ae. albopictus* [114;131]. Regions in red represent the native home range, in blue are regions that have been invaded (invasive) by this species and in grey are regions of unknown colonisation.

Due to the changing climate, *Aedes* mosquitoes' distribution is expected to shift both geographically and seasonally, expanding and decreasing in different regions of the World [132;133]. Importantly, the different origin of the populations (native or invasive) could result in different thermal responses within the same species due to the high genetic variability and the different level of plasticity among different populations [117;134-137]. *Aedes* borne diseases will be impacted severely by climate change, with intensification and expansion of human health threats [138;139], including both the widespread of Dengue and yellow fever and emerging threats like Chikungunya, Zika, West Nile, and Japanese encephalitis [133;140-142]. Predicting the climate change effect on *Aedes* and *Aedes*-borne viruses distributions is a key human health concern [143].

1.5.3 *Aedes albopictus* and its microbiota, focus on *Wolbachia*

Most of the microbial taxa are acquired from the environment in which the mosquito spends each life stage: for *Aedes* larvae the water of the breeding site is the main source of microbiota, which is acquired through feeding, whereas there can be bacterial exchanging among adults and the breeding site water during emergence and egg laying [144]. Depending on the tissue localisation within the adult mosquito body, the microbiota composition varies, as shown in Figure 7. In *Ae. albopictus*'s salivary glands Gammaproteobacteria represent the dominant class, whereas this class is equally present with Alpha- and Betaproteobacteria in the gut. In the reproductive tissue, Alphaproteobacteria is the class dominating the microbiota of both sexes (97% in females and 73% in males). See Scolari et al., 2019 for a review [144].

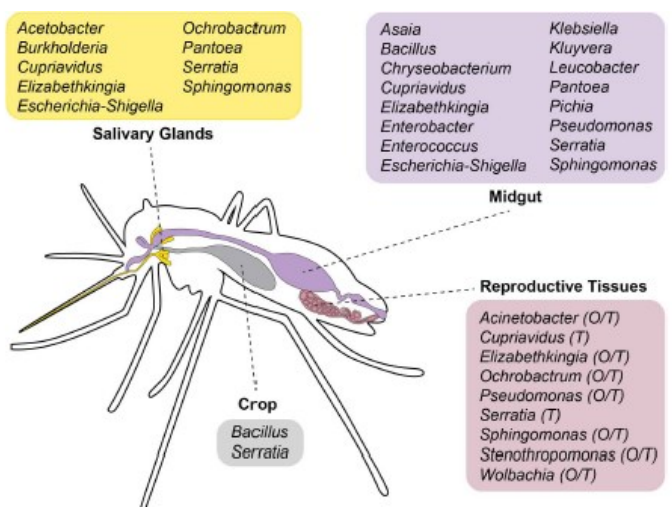


Figure 7. Localisation of bacterial genera identified in *Aedes spp*. mosquitoes in salivary glands, midgut, crop and reproductive tissues (O for ovaries; T for testes) [144].

The most prevalent bacterium of the *Ae. albopictus* ovarian microbiota is *Wolbachia pipiens*, a maternally inherited endocellular alphaproteobacterium of the order Rickettsiales [145]. *Wolbachia* consists of 17 supergroups (A-H) based on molecular phylogenesis, the host range and type of symbiosis [146]. Supergroups are further divided into groups according to a reference strain within each group [147]. *Wolbachia* is found in around 70% of all insect species [148;149]. *Aedes albopictus* is superinfected with two *Wolbachia* strains, *wAlbA* and *wAlbB,* which belong to the supergroups A and B, respectively [150], whereas *Ae. aegypti* is not naturally infected with *Wolbachia* [151]. Despite the original lack of *Wolbachia*, transinfections with different strains were successful in *Ae. aegypti* [152;153], especially with *wMel* and *wAlbB*, which are established in the field [154-156].

Wolbachia is not only present in the germline, but also has tropism towards somatic tissues, as metabolic, digestive, and nervous systems [149;157]. For instance, in *Ae. albopictus, Wolbachia* was detected also in the salivary glands and the midgut [157-159], even if ovaries are the tissues with the highest *Wolbachia* abundance (94%) [144].

Wolbachia density in insect hosts is known to vary according to season and location [160], thus it is considered a thermal sensitive bacterium. Studies carried out specifically in stable transinfected *Ae. aegypti* mosquitoes have shown that in adults resulting from larvae that had experienced extreme hot temperatures, the density of some *Wolbachia* strains was reduced [160-163]: these findings suggest a decrease of *Wolbachia* in the natural populations belonging to hot climates. In a recent study by Mancini et al. (2021) [160] *wAlbB* density was measured in heat-challenged and control *Ae. aegypti* mosquitoes' ovaries, resulting in high densities in both groups, suggesting that this might be a stable *Wolbachia* strain, even when exposed to thermal stress. Another recent study from Lau et al. (2020) [164] investigated *Wolbachia* densities in *Ae. aegypti* exposed to low temperatures. Results showed that *wAlbB* density was reduced at low temperatures in both males and females. Interestingly, in females *wAlbB* density increased as they grew older. These findings suggest that *wAlbB* is more stable in warm environments and more sensitive to cold. Nevertheless, the host's age might rescue its density. Since *Wolbachia* density has a huge influence on the host overall fitness, thermal-related changes in density may be associated with modification in the host-phenotypes, for instance affecting the maternal transmission of the bacterium itself [149].

As other heritable symbionts, *Wolbachia* was proved to influence ecologically important host traits, such as thermal preference. For instance, an investigation carried out on *Drosophila spp* infected with different strains of *Wolbachia*, infection was seen to alter the host thermal preference towards colder temperatures [149]. Only in the case of *Drosophila mauritiana* infected with the *Wolbachia B* strain *wMau*, the host preferred a warmer thermal regime [149]. Another study in *D. melanogaster* infected with *wMel*, *wMelCS* and *wMelPop* had a preferred temperature of 23.2°C, 20.6°C and 20.5°C, respectively, thus shifting towards cold thermal regimes [165]. This effect is suggested to regulate the bacterial density and maintenance inside the host [149;165]. Up to date, there is no information relative to *Wolbachia* modulation of thermal preference in *Aedes* mosquitoes.

The most important impact of *Wolbachia* on the host is at the level of reproduction. *Wolbachia* can manipulate the capacity of the host to reproduce through parthenogenesis, feminization of males, male killing and cytoplasmic incompatibility (CI) [145]. CI happens when an infected male mates with an uninfected female or when the two mating individuals harbour incompatible *Wolbachia* strains, resulting in no fertile embryo and therefore no viable progeny [166]. Thanks to such manipulations, *Wolbachia* increases the relative fitness of its hosts, favouring its spread [160]. Modern vector control strategies take advantage of CI caused by *Wolbachia*, such as the Incompatible Insect Technique (ITT), which is based on the release of incompatible

mosquito males in the field [167]. IIT has been already used in *Ae. albopictus* by generating a triple-infection with *wPip* [168].

A fortuitous discovery was made when *Ae. aegypti* mosquitoes permanently infected with *Wolbachia* were generated: *wMel* infection resulted in reduction of the mosquito vector competence, blocking transmission of viruses such as DENV [154;169;170]. This phenotype was further observed when *Ae. aegypti* mosquitoes stably infected with *wAlbA*, *wAu* and *wAlbB* were generated [153]. Furthermore, co-infection of ZIKV and DENV did not impact the effect of *Wolbachia* on viral transmission in *Ae. aegypti wMel*-infected [171]. A similar investigation was carried out also in *Ae. albopictus*, naturally free of *wMel*, in which infection with this bacterial strain could prevent DENV transmission [172]. The effect of *Wolbachia* is not restricted to viruses, but also other pathogens, for instance *Plasmodium* [173] and parasitic worms such as the filarial nematode *Brugia pahangi* [174]. Despite the intimate relationship between *Wolbachia* and its host, the mechanisms through which *Wolbachia* alters vector competence are still unclear. Caragata et al. (2019) [171] efficiently outlined the proposed mechanisms of action of *Wolbachia* up to date, which constitutes a broad-spectrum host immune system stimulation: the differential expression of immunity genes, such as those encoding antimicrobial peptides like cecropin [174], as well as genes related to ROS production [175], together with RNAi pathway [176;177], and the competition for host resources, such as cholesterol [178]. Although the mechanisms of *Wolbachia*-dependent antiviral activity have not been clarified yet, this phenotype has been used to implement transmission blocking control strategies worldwide [179].

1.5.4 Effect of temperature on *Ae. albopictus*

As previously stated, mosquitoes are ectotherms and thus can perform their life activities within a range of Ta, between their critical minimum and maximum limits. In the specific case of *Ae. albopictus*, a study of Delatte et al. (2009) [180] proved that this species is able to develop and survive within a wide range of Ta, with the immature stages developing from the minimal temperature of 10.4°C, having 29.7°C as optimal developmental temperature and reaching full development up to 35°C, temperature at which no complete development occurred, with a mortality of 91.7% at the pupal stage [180]. Depending on the origin of *Ae. albopictus* populations, different thresholds for development were found [181]. Not only development, but also *Ae. albopictus* gonotrophic cycle is affected by temperature. At 30°C the shortest gonotrophic cycle was recorded for this species, counting of 3.5 days, but females had the highest number of cycles (3.9) [182].

Furthermore, longevity and egg hatchability were traits investigated at different rearing temperatures, resulting in a longer lifespan at cold temperatures (15°C) compared to warm (35°C) and higher hatchability at 20°C (66.9%) compared to 25°C (49.2%) [180].

However, as for temperature effect on mosquito development, different survival values arise depending on the geographical origin of *Ae. albopictus* population studied [180].

Up to date, thermal performance variations at the strain or population level are poorly investigated [183], especially in *Aedes* mosquitoes [63]. In general, less information is available on the impact of Ta on *Ae. albopictus* than *Ae. aegypti* (see Reinhold et al., 2018 for a review) [184]. For instance, regarding the flight activity, in *Ae. aegypti* the *Topt*, both in terms of time flown and distance covered, was measured at 21°C [185], whereas this is not known for *Ae. albopictus*. Also host-seeking behaviour counts of more research activities for *Ae. aegypti* than *Ae. albopictus*, showing a loss of biting activity at 15°C [186] and 36°C [187], a peak of activities when females are kept at 28°C [186] and a faster feeding between 26°C and 35°C [188]. Lastly, in mosquitoes in general, the magnitude of the effect of temperature on the different life-stages has not been thoroughly assessed.

It is critical to improve knowledge on thermal biology of this species to build models for *Ae. albopictus* population dynamics and distributions in the context of global climate.

2.Aim of the work

The increase of environmental temperature due to current global warming is not only favouring the expansion of the distribution range of many insect species, but it is also changing their phenology. Insect phenology is tightly linked to developmental timing, which is regulated by Ta. The degree to which effects of developmental temperature extend across developmental stages and the inter stage relationships have not been thoroughly quantified in mosquitoes. In my PhD program, I aimed at assessing the impact of developmental temperature on *Ae. albopictus*, which is a highly aggressive and invasive species which moved globally from South-East Asia in the past 60 years. This mosquito species is of public health relevance since it is the primary vector of arboviruses in temperate areas of the World. Importantly I addressed this question comparatively in a native tropical and an invasive temperate strain to understand whether the relationship between developmental temperature and mosquito biology (in terms of fitness traits, physiological responses and transcriptome) may differ across geographic populations. Lastly, I investigated the effect of temperature on the density of the endosymbiont *Wolbachia* since it plays a major role in mosquito embryo viability.

Chosen developmental temperatures include 18°C, 28°C and 32°C: 18°C represents the average daytime temperature registered in northern Italy between April-May 2021 [189], when mosquitoes started emerging from the winter season; 32°C is the average peak of heat that occurred in the same region during August 2021 [190], while 28°C is the standard *Ae. albopictus* rearing condition [191].

3. Materials and Methods

3.1 Mosquito strains

In this study I used two *Aedes albopictus* strains: the tropical Foshan (Fo) and temperate Crema (Cr) strain. The Fo strain, for which the *Ae. albopictus* reference genome assembly has been derived [191;192], was established in the early 1980s from Foshan (China). This strain has been maintained at the University of Pavia since 2013, as previously described [193]. The Cr strain was derived from larvae collected in the city of Crema (Italy) in September 2017. Since establishment, the two strains have been maintained in parallel at 28°C and 70-80% relative humidity, with a light/dark cycle of 12 h. Larvae are reared in plastic containers at a controlled density to avoid competition for food, which is provided daily in the form of fish food (Tetra Goldfish Gold Colour). Adults are fed with cotton soaked in 20% sugar as a carbohydrate source. Adult females are offered commercial defibrinated mutton blood (Biolife Italiana) using a Hemotek feeding apparatus.

3.2 Thermal regimes, assessment of life-history traits and thermal traits

For both the Fo and Cr strains, a total of twenty-four groups, consisting each of 100 eggs, were placed in plastic containers (17x6.5x12 cm), with 200 ml autoclaved water, and subjected to different thermal regimes. A set of eight groups of eggs was hatched and reared until adult emergence at 18°C; a set of eight groups of eggs was hatched and reared until adult emergence at 32°C; an additional set was kept at standard 28°C. To avoid confounding effects of humidity and photoperiod cycles, these parameters were maintained equal across thermal regimes. While in nature mosquitoes are exposed to fluctuating temperatures, having a constant developmental temperature allows to isolate the effect of a unique experimental variable and generate baseline data for future, more complex analyses [194]. In each tray, the number of eggs hatching was checked to assess the percentage of larvae emerging (egg hatchability rate) and the hatching time, also larval and pupal viability were measured, together with the developmental time necessary for each juvenile stage, i.e. larval developmental time (LDT) and pupal developmental time (PDT). Developmental speed considers the time between egg hatching and adult emergence. From emerging adults I calculated pupal viability and adults were sexed to evaluate the sex ratio for each group, calculated as percentage of females. A sample of 30 females was used to determine wing length as a proxy for adult size [195]. The right wing was dissected and measured from the axial incision to the apical margin, excluding the fringe of the scales (Appendix 1). Measurements were carried out under the inverted microscope (Olympus CKX53) using the software cellSens Standard (Olympus). Taking into account the hyper allometric relationship between wing length and fecundity, I derived fecundity from my wing length data based on the function ln(egg number)=0.79+1.4*WL [196;197].

For each thermal regime, I also measured mosquito longevity and assessed their thermal preference (*Tp*), namely the temperature at which mosquitoes prefer to lay, and heat resistance as knock-down temperature (*KDT*), namely the temperature at which the mosquito ceased to move [198]. Briefly, for each strain, 500 eggs were hatched at 18°C, 28°C or 32°C and adult survival was monitored daily and individually until death. To measure *Tp,* 5-9 day-old mosquitoes were collected and isolated by cooling down individuals on the day of emergence in a 4°C fridge. Mosquitoes were then released at the centre of a custom-built thermal gradient connected to a cool water bath on one side and to a warm water bath on the other, as in Reinhold *et al.* (2022) [199]. These water baths were set at a specific temperature to create a continuous gradient along the aluminium plate. The gradient of temperatures between 15.3°C-41.5°C had increments of 0.79°C±0.285 from one side to the other, with the centre of the gradient having a temperature of 27.9°C±0.85. After a 5 minutes adaptation time, mosquitoes were monitored for 30 minutes until they settled on a resting spot corresponding to a specific temperature, which was defined as the mosquito *Tp*. A maximum of ten sugar-fed individuals of the same sex, strain and thermal regime were released on the thermal gradient each time. A total of eight replicates of ten mosquitoes each were conducted for sex and strain for mosquitoes reared at each of the tested thermal regimes. *KDT* was determined for individual mosquitoes using a custom-made device. Briefly, mosquitoes were collected as previously described in the *Tp* experiment set-up. An aluminium plate with nine wells, each holding a single 5-9 day-old mosquito, was set at 25°C using a Peltier. Temperature was then increased with increments of 0.5°C/minute, until 50°C was reached. A camera (Logitech C922 Pro) connected to a computer was placed above the device to monitor mosquito behaviour and determine mosquito *KDT*. A total of five replicates of nine mosquitoes each for sex, strain and thermal regime were run. For these experiments, mosquitoes were fed exclusively with 20% sugar, to avoid any bias in longevity, *Tp* and *KDT* measurements due to blood feeding and digestion [200-202].

3.3 Colorimetric assay

I used a colorimetry protocol modified after Foray et. al (2012) [203] to assess protein, glycogen, lipids and triglyceride contents. A total of 50 mosquitoes were processed for each strain, temperature and sex condition. Mosquitoes were collected and isolated by cooling down individuals on the day of emergence in a 4°C fridge. Individuals were then weighed and stored in individual tubes at -70°C until processing. 180 µL of aqueous lysis buffer (pH 7.4) composed of 100 mM potassium dihydrogen phosphate (Sigma-Aldrich, #7778-77-0), 1 mM dithiothreitol (Sigma-Aldrich, #3483-12-3), 1 mM ethylenediaminetetraacetic acid (Sigma-Aldrich, #60-00-4) in water, was added to each sample which was then grinded using polypropylene pellet pestles (KIMBLE, #749521-0500) for approximately 30 seconds. The homogenates were vortexed and then centrifuged at 4°C for 15 minutes at 2000 rpm.

For the quantification of total proteins, 5 µL of each bovine serum albuminutes (Fisher Scientific, #9048-46-8) standard (0, 0.2, 0.4, 0.6, 0.8, 1.0 µg/µL) was transferred to a 96-well borosilicate plate. Then, 2.5 µL of each sample's supernatant and 2.5 µL of aqueous lysis buffer were added to the individual wells. 250 µL of Bradford micro-assay reagent (Sigma-Aldrich, #B6916) was added to each well and the plate was incubated in the dark at room temperature for 5 minutes. The plate was then placed in the microplate reader (Accuris SmartReader 96) and shaken at low speed for 5 seconds within the instrument and then read at 595 nm. Absorbance values were recorded and used to calculate protein concentration in mosquito samples. After quantification of protein, the samples were then divided into two tubes: a stock solution and a glycogen pellet. To accomplish this, 2.5 µL of aqueous lysis buffer, 20 µL of sodium sulphate solution (20% sodium sulphate (Sigma-Aldrich, #7757-82-6) in water), and 1500 µL of chloroform-methanol solution (1:2 ratio of chloroform (Sigma-Aldrich, #67-66-3) to methanol (Sigma-Aldrich, #67-56-1) were added into each sample tube. The tubes were vortexed and then centrifuged at 4°C for 15 minutes at 2000 rpm. Following centrifugation, the supernatant was transferred into a separate 2 mL centrifuge tube and will be referred to as the stock solution. The pellet left in the original tube was then used for glycogen quantification.

For the quantification of glycogen, the pellet was initially washed with 400 µL of 80% methanol diluted in water, vortexed, and then centrifuged at 4°C for 5 minutes at 15000 rpm. The supernatant was removed, and the washing step was repeated once more. Glucose (Sigma-Aldrich, #50-99-7) standards were made by adding 25 µL of each standard (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 µg/µL) into individual tubes and allowing the solvent to fully evaporate (approximately 36 hours at room temperature). After discarding the supernatant, 1 mL of anthrone solution (0.142% w/v anthrone (Sigma Aldrich, #90-44-8) in 70% sulfuric acid (Sigma-Aldrich, #7664-93-9) diluted in water) was added to each sample and standard and then vortexed. The tubes were then incubated at 90°C for 15 minutes and vortexed halfway through the incubation period. The tubes were then placed on ice for at least 5 minutes and then 250 µL of each sample and standard was transferred into a 96-well borosilicate plate. The plate was then read at 625 nm. Absorbance values were recorded and used to calculate glycogen concentration in mosquito samples.

For the quantification of total lipids, the stock solution of each sample was vortexed and then 150 µL of each sample was transferred to a 96-well plate. 5 µL of each triolein (Sigma-Aldrich, #122-32-7) standard (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 µg/µL) was also transferred to the plate. The plate was incubated at 90°C until the solvent fully evaporated. 10 µL of 98% sulfuric acid was then added to each sample and standard and the plate was once again incubated at 90°C for two minutes. The plate was then placed on ice for 5 minutes and 190 µL of vanillin solution (0.120% w/v vanillin (Fisher Scientific 121-33-5) in 68% orthophosphoric acid (Sigma-Aldrich 7664-38-2) diluted in water was added to each well. The plate was then shaken at low speed in the plate reader for 15 minutes and read at 525 nm. Absorbance values were recorded and used to calculate total lipid concentration in mosquito samples.

For the quantification of triglycerides, each sample's stock solution was vortexed and then 500 µL of stock solution was transferred to a new 2 mL centrifuge tube. Each tube was incubated at 90°C until the solvent completely evaporated. The tubes were removed from the heat and 1 mL of chloroform and 200 mg of anhydrous silicic acid (Sigma-Aldrich 10279-57-9) was added to each tube. The tubes were centrifuged at 4°C for 10 minutes at 2000 rpm. 400 µL of each sample's supernatant was transferred into individual wells of a 96-well plate. 5 µL of each triolein standard (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 µg/µL) was also transferred to the plate. The plate was incubated at 90°C until the solvent fully evaporated. 10 µL of 98% sulfuric acid was then added to each sample and standard and the plate was once again incubated at 90°C for two minutes. The plate was placed on ice for 5 minutes after the incubation period and 190 µL of vanillin solution was added to each well. The plate was then shaken slowly in the plate reader for 15 minutes and then read at 525 nm. Absorbance values were recorded and used to calculate triglyceride concentration in mosquito samples.

3.4 Water content

I used the protocol described in Benoit *et al.* (2007) [204] to determine water content in mosquitoes. Briefly, mosquitoes were collected on the day of their emergence, weighed at their initial mass, and then placed in a -20°C freezer for 6 hours to be killed. The samples were then transferred to an incubator (FisherBiotech Hybridization Incubator) set to 70°C. Daily measurements of each individual's weight were made until the values became constant, indicating that there was no more water left in the samples. This final mass was then used to calculate the percentage of water within each individual.

3.5 Chitin content assay

For both strains, three pools of 15 larvae (4th instar) were generated from eggs hatched at both 18°C and 28°C to perform a chitin colorimetric assay as described by Lehmann and White (1975) [205].

Briefly, each pool was weighted and homogenised in 0.5 ml of distilled water using a pestle. The pestle was then rinsed with an additional 0.5 ml of distilled water. After centrifugation at 1800g for 15 minutes at room temperature (RT), pellet was resuspended in 0.4 ml of 3% sodium dodecyl sulfate and incubated at 100 °C for 15 minutes. After centrifugation for 10 minutes, samples were cooled 10 minutes in ice and again centrifuged at 1800g for 10 minutes at RT. Pellet was then washed with 0.5 ml water and centrifuged as before and then resuspended in 0.3 ml of 14 M potassium hydroxide and incubated at 130°C for 1h. After 5 minutes in ice to cool, 0.8 ml of ice-cold 75% ethanol were added to each sample. After an incubation of 15 minutes on ice, 30 µl of Celite 545

suspension were added to each sample. Celite 545 suspension was prepared by adding 1g of Celite® 545 (Acros organics) to 12.5 ml of 75% ethanol. Samples were then centrifuged at 1800g for 5 minutes at 4°C and the pellet was washed with 0.5 ml of 40% cold ethanol, followed by centrifugation at 1800g for 5 minutes at 4°C. Pellet was further washed with 0.5 ml of distilled water and centrifuged again at 1800g for 5 minutes at 4°C before being resuspended in 0.5 ml of water. A total of 60 µl of each sample was added to 20 µl of ammonium sulfamate and vortexed for 5 minutes at RT. 20 µl of freshly prepared 3-methyl-2-benzothiazolone hydrazone hydrochloride hydrate were further added to each sample, which were incubated at 100 °C for 5 minutes. After cooling for 5 minutes at RT, 50 µl of 0.83% (w/v) Iron(III) chloride hexahydrate was added to each tube and incubated at RT for 25 minutes. 100µl of each sample was transferred to a well of a 96-well microplate and absorbance was determined at 650 nm in a Clariostar plate reader. Absorbance was also measured for chitosan standards of 200 mg/ml, 150 mg/ml, 100 mg/ml, 50 mg/ml, 25 mg/ml, 10 mg/ml and blank samples (water only).

3.6 Transcriptome analyses

I generated three larval pools, each consisting of ten 4th instar larvae randomly collected from each of the eight trays/thermal regime. For each thermal regime I also collected females at the day of their emergence for a total of three pools of 10 mosquitoes each. Larvae and adult females were sampled at the same time of the day. Both adult and larval samples were homogenised in 50 microliters of Trizol (Life Technologies, Madrid, Spain) and stored at -80°C until RNA extraction. Total RNA was extracted using the standard Trizol protocol and re-suspended in 20 µl of nuclease-free water. Total RNA was sent to Macrogen Europe BV for quality control, TruSeq Stranded mRNA library preparation and sequencing on Illumina platform. Each library was sequenced pair-end (2x100bp) at a depth of 30 million reads.

I used the nf-core/rnaseq bioinformatics pipeline (https://nf-co.re/rnaseq), which includes quality check and trimming of the reads for RNA-seq analysis, using the AalbF2 genome assembly [191]. Fragments per kilobase of exon per Million mapped reads (FPKM) was used as a proxy for gene expression [206]. Analysis of gene differential expression (DE) among conditions was conducted with DeSeq2 in RStudio (2022.02.3) using the function "DESeqDataSetFromMatrix" [207]. I selected as DE genes, those genes resulting with a Log2FC value $\geq |2|$ and a p-value <0.01 from each comparison. I assessed DE genes between Fo and Cr across the same developmental stages and thermal regime (e.g., Fo larvae 18°C vs Cr larvae 18°C). For comparisons between strains, genes that were DE between Fo and Cr at 28°C were not considered to derive the list of DE genes between strains at both 18°C and 32°C to avoid accounting for strain-specific differences. Relative fold changes in gene expression between samples were determined as a ratio of each FPKM; among strain comparisons were always Fo vs Cr unless otherwise stated. To obtain the Gene Ontology (GO) functional assignment of the 26,843 protein-coding

genes in *Aedes albopictus* genome version (AALFPA) and gene enrichment of differentially expressed genes of AALFPA, the the strategy of Lozada-Chavez et al., (2023) [208] was followed. Briefly, a custom annotation database was created (i.e. org.albopictus.eg.db R package) from merged results with Blast2GO [209] of three functional approaches: (1) Gene Ontology annotations covering ~63% of the AALFPA-proteome, as retrieved from VectorBase v55 [210]; (2) a homologs BLAST search of the AALFPA-proteome against the NCBI Diptera nr database v5; (3) a functional homologs search with InterProScan v5 [211] against four protein-domain databases: Pfam v33.1 [212], ProSiteProfiles v20.2 [213], SUPERFAMILY v2.0 [214], and TIGRFAM v15.0 [215]. After this, 80% of the AALFPA-proteome was annotated. A GO enrichment analysis for major GO categories was performed with our in-house org.Aalbopictus.eg.db and clusterProfiler v4.2.2 [216] to identify functional groups that were enriched in our sets of DE genes. P-values ($p \leq 0.05$) obtained with clusterProfiler were corrected for multiple tests with the Benjamini-Hochberg procedure, and the redundancy of enriched GO terms for each major GO classification was removed with simplify, both from clusterProfiler.

3.7 Estimates of *Wolbachia* density by qPCR

In both Cr and Fo mosquitoes, I determined the presence and density of *wAlbA* and *wAlbB* [150]. Briefly, genomic DNA (gDNA) was extracted using the Wizard® Genomic DNA Purification Kit (Promega) from 16 larvae and 16 ovaries the day of emergence, 7 and 12-14 days post emergence for each thermal regime. Abundance of *wAlbA* and *wAlbB* was assessed in each sample by qPCR using the *Ae. albopictus* homothorax gene (AALC636_001297) as reference [160]. PCR reactions were performed in a total volume of 20 µl, containing 10µl SYBR Green (ThermoFisher), 4 µl of gDNA, 4 µl of H2O and 1 µM of each primer: qwAlbA F (GGGTTGATGTTGAAGGAG) and R (CACCAGCTTTTACTTGACC); qwAlbB F (AAGGAACCGAAGTTCATG) and R (AGTTGTGAGTAAAGTCCC); and qHTH F (TGGTCCTATATTGGCGAGCTA) and R (TCGTTTTTGCAAGAAGGTCA), for wAlbA-wsp, wAlbB-wsp, and *Aedes albopictus* homothorax gene (AALC636_001297), respectively [160;217]. The temperature was cycled at 95°C for 2 minutes, then 40 cycles at 95 °C for 5 s and 60 °C for 30 s, followed by the melting curve generation.

3.8 Data Analyses

I used Prism 8 (GraphPad) for statistical analyses unless otherwise stated. The Shapiro-Wilk test was used for assessing normality distribution of data, and

parametric (ANOVA) and nonparametric (Kolmogorov-Smirnov, Mann Whitney or Kruskal-Wallis) tests were selected accordingly. Differences between strains in egg hatchability rate, egg hatching time, larval viability, larval and pupal developmental time, developmental speed, wing length, adult sex ratio, and body mass were tested through two-way ANOVA with Tukey's multiple comparisons test. Pupal viability, *Tp*, *KDT*, energy reserves and *Wolbachia* density data were analysed within strain with the Kruskal-Wallis test (followed by Dunn's multiple comparison test) and between strain with Mann Whitney test. *Tp* assay data were further analysed with the Kolmogorov–Smirnov test. Longevity data were used to extrapolate the median survival time and the hazard ratio, which is a measure of how rapidly each mosquito died, with the Mantel-Haenszel method. Comparisons of mosquito longevity were carried out with the Kaplan-Meier survival analysis, Log-rank (Mantel-Cox) test. Using the R package ggpubr [218] (R version 4.2.2), I tested Pearson's correlation on wing length data and body mass of males and females to investigate the effect of the different temperatures tested on said strains. I considered significant Pearson's correlation with a p-value<0.05 and evaluated as very strong correlations those analyses resulting in a correlation coefficient (R) between ±0.9 and ±1, strong between ±0.7 and ±0.89, medium ±0.4 and ±0.69, weak ±0.1 and ±0.39 and negligible when below 0.1 [219]. To further quantify the effect of developmental temperature, strain or sex, and their combinations on studied fitness traits (except for longevity and pupal viability), I used two-way ANOVA and obtained F-ratio (F) and the relative p-value, together with the percentage of variation explained by each source. I further used Principal component analysis (PCA) on life history traits and energy reserves, to explore the traits most influenced by temperature and strain differences, using the function "fviz_pca_biplot" from the factoextra package [220] (R version 4.2.2).

For each strain and temperature tested, I also calculated LDR and PDR as inverse of larval and pupal developmental time (LDT and PDT) and further derived their ratio (LDR/PDR) to test isomorphy through regression analyses (as previously shown) [53].

To compare the overall thermal performance of tested strains, I draw a Thermal Performance Curve (TPC) including data of egg hatchability, egg hatching time, larval and pupal viability, larval and pupal developmental time, developmental speed, sex ratio, wing length, body mass and longevity following a previously reported standardisation procedure of MacLean et al. (2019) [64], in which TPCs were drawn based on developmental viability, development speed and adult fecundity. Briefly, a value of 1 is assigned to the highest value for each trait, and the rest of the values are processed as ratio. Curves were designed using the cubic spline method, setting as extreme values 10.4 °C, the lowest developmental temperature registered for *Ae. albopictus* [180], and the mean *KDT* resulted from my study (46°C).

Lastly, chitin content data were analysed with the parametric Unpaired t test, comparing the chitin ratio 28°C/18°C between the two strains.

4.Results

4.1 Life history traits

For both the native tropical Fo and the invasive temperate Cr strains, eggs were hatched at 18°C, 28°C and 32°C and mosquito development was followed, including adult longevity. Appendix 2 reports means and standard deviations for each tested parameter, including thermal traits and energy reserves. Appendix 3 reports results of ANOVA, Kruskal-Wallis and Mann Whiteny tests, Appendix 4 shows Mantel-Haenszel tests. Figure 8 displays life history traits and the relative statistics, both for within and between strain comparisons; Figure 9 shows longevity results as survival curves, within (9A) and between strain (9B).

4.1.1 Within strain comparison

In Fo egg hatchability ranged from 52.3% to 81.67% in mosquitoes reared at 18°C or 28°C, respectively; values measured across the three tested developmental temperatures were not significantly different. The same trend was observed for larval viability, which ranged from a minimum of 68.8% in mosquitoes reared at 18°C to a maximum of 78.21% for mosquitoes reared at 28°C, for pupal viability, which had a range from 82.41% at 18°C to 89.52% at 28°C, for sex ratio, which ranged from 44.27% of females in mosquitoes reared at 32°C to 47.04% of females in mosquitoes reared at 18°C and for male body mass, which ranged from a minimum of 0.78 mg in mosquitoes reared at 32°C to a maximum of 0.87 mg in those reared at 18°C. On the contrary, egg hatching time, larval and pupal developmental time, developmental speed, wing length and female body mass significantly differed in mosquitoes reared at the different developmental temperatures tested. Egg hatching time was the longest (14.01 days) in mosquitoes reared at 18°C and shortest (3.93 days) in mosquitoes reared at 28°C, with differences being significant between values of mosquitoes reared at 18°C and both 28°C (p-value<0.0001) and 32°C (4.83 days; p-value<0.0001), but not between 28°C and 32°C. Both larvae and pupae took the longest to develop when reared at 18°C (17.01 and 4.67 days for larval and pupal development, respectively), and the shortest at 32°C (5.42 and 1.54 days for larval and pupal development, respectively); differences were significant for both traits for mosquitoes reared 18°C in comparison to those reared both at 32°C (p-value<0.0001 for both comparisons) and 28°C (6.75 days for larvae and 1.83 days for pupae; p-value<0.0001 for both comparisons). Both developmental times were not significantly different in mosquitoes reared at 28°C and 32°C. Developmental speed of mosquitoes reared at 18°C was 35.69 days, which decreased significantly to 12.52 and 11.78 days in mosquitoes reared at 28°C (p-value<0.0001) and 32°C (p value<0.0001), respectively. Wing length of Fo females reared at 18°C was significantly longer (3.04 mm) compared to that of mosquitoes reared at 28°C (2.51 mm; p-value <0.0001) and 32°C (2.53 mm; p-value <0.0001). Fo had a higher fecundity when reared at 18°C (153.67 eggs) compared

to both 28°C (74.65 eggs; p-value <0.0001) and 32°C (75.62 eggs; p-value<0.0001), whereas there was no difference in fecundity in mosquitoes reared at 28°C or 32°C. Females reared at 28°C had a higher body mass (1.81 mg) than those reared at 32°C (0.98 mg; p-value<0.0001) and 18°C (1.43 mg; p-value=0.0003). Furthermore, both males and females had a shorter longevity when reared at 32°C (17.58 days for males, 12.72 days for females) than when reared at both 28°C (25.39 days for males, 26.84 for females; p-value<0.0001 for both comparisons) and 18°C (23.59 days for males, 24.78 days for females; p-value <0.0001 for both comparisons). Longevity was further analysed in terms of Hazard-Ratio (Mantel-Haenszel), calculating the rate of death in each comparison. Fo males died twice or 3 times faster when reared at 32°C than when reared at 18°C (p-value<0.0001) or 28°C (p-value<0.0001), respectively. Fo females reared at 32°C died 4 or 5 times faster than those reared at 18°C (p-value<0.0001) or 28°C (p-value<0.0001), respectively. I also compared body mass and longevity between sexes. As expected, females had a higher body mass than males at all tested temperatures (p-value<0.0001 in all comparisons). When comparing male and female longevity in mosquitoes reared at 18°C, 28°C or 32°C, the latter was the only condition in which there was a significant difference between sexes, with males living longer than females, which died at rate almost double than that of males (p-value<0.0001).

In Cr mosquitoes, egg hatchability rate and sex ratio were the only traits not significantly different in mosquitoes reared at the three tested developmental temperatures. Egg hatchability rate ranged from 51.75% in mosquitoes reared at 32°C to 71.25% in mosquitoes reared at 28°C. The percentage of females ranged from 31.17% in mosquitoes reared at 32°C to 42.71% in mosquitoes reared at 18°C. In Cr egg hatching time was shorter in mosquitoes reared at 28°C (3.44 days) compared to values of mosquitoes reared at both 18°C (9.77 days; p-value<0.0001) and 32°C (7.74 days; p-value=0.0017). Differently than in Fo, larval viability was significantly different in Cr mosquitoes reared at 32°C (47.75%) than both 18°C (70.99%; p-value=0.0002) and 28°C (66.63%; p-value=0.0034); pupal viability differed across thermal regimes, being lower at 28°C (87.93%) compared to mosquitoes developing at 32°C (96.51%; p-value=0.0192); male body mass differed among developmental temperatures, with males emerging from eggs hatched at 28°C being lighter (0.53 mg) than those from eggs hatched at both 18°C (1.21 mg; p-value<0.0001) and 32°C (1.12 mg; p-value<0.0001). As in Fo, both larval and pupal developmental time were the longest in mosquitoes reared at 18°C (17.18 and 5.35 days for larval and pupal developmental time, respectively), but the shortest in mosquitoes reared at 28°C (6.97 and 1.7 days for larval and pupal developmental time, respectively), with significant differences between values of mosquitoes reared at 18°C and both 28°C (p-value<0.0001 for both comparisons) and 32°C (7.91 and 1.8 day for larval and pupal developmental time, respectively; p-value<0.0001 for both comparisons), but not between 28°C and 32°C. Like Fo, developmental speed, wing length and female body mass differed in mosquitoes reared at 18°C, 28°C and 32°C. Mosquitos reared at 28°C had a shorter developmental speed (12.2 days), compared to

mosquitoes reared at both 18°C (32.3 days; p-value<0.0001) and 32°C (17.46 days; p-value=0.0007). Females reared at 18°C had longer wings (3.32 mm) compared to those reared at both 28°C (2.89 mm; p-value<0.0001) and 32°C (2.6 mm; p-value<0.0001). Also wing length of females reared at 28°C and 32°C showed a significant difference (p-value<0.0001). Fecundity was higher in females reared at 18°C (232.74 eggs) compared to values of mosquitoes reared at both 28°C (124.25 eggs; p-value<0.0001) and 32°C (83.65; p-value<0.0001); differently than Fo, there was a significant difference in fecundity in mosquitoes reared at 28°C and 32°C (p-value<0.0001). Regarding longevity, males had a shorter lifespan when reared at 32°C (11.44 days) with respect to males reared at 18°C (26.64 days; p-value<0.0001) and 28°C (24.51 days; p-value<0.0001); the comparison in longevity of males reared at 28°C or 18°C showed a significant difference (p-value=0.0067). Like males, Cr females had a lower longevity value when reared at 32°C (12.19 days) than females reared at both 28°C (31.21 days; p-value<0.0001) and 18°C (36.76 days; p-value<0.0001), and at 28°C females lived less than females reared at 18°C (p-value<0.0001). Hazard-Ratio (Mantel-Haenszel) analysis of longevity showed that males died 6 times or 9 times faster when reared at 32°C compared to those kept at 18°C (p-value<0.0001) or 28°C (p-value<0.0001), respectively; males reared at 28°C died 1.4 times faster than those reared at 18°C (p-value=0.0067). Females reared at 32°C died 20 times or 12 times faster than those reared at 18°C (p-value<0.0001) or 28°C (p-value<0.0001), respectively; females reared at 28°C had a mortality rate 1.7 times higher than those reared at 18°C (p-value <0.0001). I also compared body mass and longevity between sexes. As expected, females had a higher body mass than males at all tested temperatures (p-value<0.0001 in all comparisons), as shown for Fo. Contrary to Fo, longevity was significantly different between sexes at 18°C and 28°C, with males dying twice and 1.2 times faster than females at 18°C (p-value<0.0001) and 28°C (p-value<0.0001), respectively.

4.1.2 Among strain comparison

Egg hatchability rate, pupal developmental time and sex ratio were the only traits with no significant differences between the two strains at any developmental temperature tested.

In mosquitoes reared at 18°C, significant differences between strains were observed in 1) egg hatching time, with Cr hatching faster than Fo (9.77 and 14.01 days, respectively; p-value=0.0031); 2) wing length with Cr having larger wings (3.32 mm) than Fo (3.04 mm; p-value <0.0001); 3) fecundity, with Cr having a higher fecundity (232.74 eggs) compared to Fo (153.67 eggs; p-value<0.0001), 4) body mass of both sexes, with Cr having heavier males (1.21 mg) and females (2.23 mg) than Fo (0.87 mg and 1.43 mg, respectively; p-value=0.0001 in males and p-value<0.0001 in females) and 5) female median longevity, with Cr females living on average 36.76 days and Fo 24.8 and, based on the Hazard-Ratio, Fo females died 2.8 faster than Cr ones (p-value <0.0001).

In mosquitoes reared at 28°C, statistically significant differences between strains were detected in 1) wing length, with Cr females having larger wings (2.89 mm) than Fo ones (2.51 mm; p-value <0.0001); 2) fecundity, with Cr having more eggs (124.25 eggs) than Fo (74.65 eggs; p-value<0.0001); 3) body mass of both sexes, with Fo males (0.81 mg) and females (1.81 mg) being heavier than Cr (0.53 mg and 1.12 mg, respectively for males and females; p-value<0.0001 for both comparisons) and 4) female longevity, with Cr mosquitoes living on average 31.2 days and Fo 26.84 days which, based on the Hazard-Ratio, died 1.4 times faster (p-value=0.0073).

In mosquitoes reared at 32°C, significant differences between strains were observed in 1) larval viability, with Cr having a lower value (47.75%) than to Fo (76.6%; p-value <0.0001); 2) pupal viability, with Cr (96.51%) giving rise to more adults than Fo (83.97%; p-value=0.0017); 3) larval developmental time, with Fo (5.42 days) having a shorter larval developmental time than Cr (7.91 days; p-value=0.0267); 4) developmental speed, with Cr having a longer developmental speed (17.46 days) than Fo (11.78 days; p-value=0.0002); 5) female body mass, with Cr being heavier (1.55 mg) than Fo (0.98 mg; p-value<0.0001) and 6) male longevity, with Cr males living a shorter life (11.4 days) than Fo ones (17.6 days; p-value<0.0001), dying almost 3 times faster.

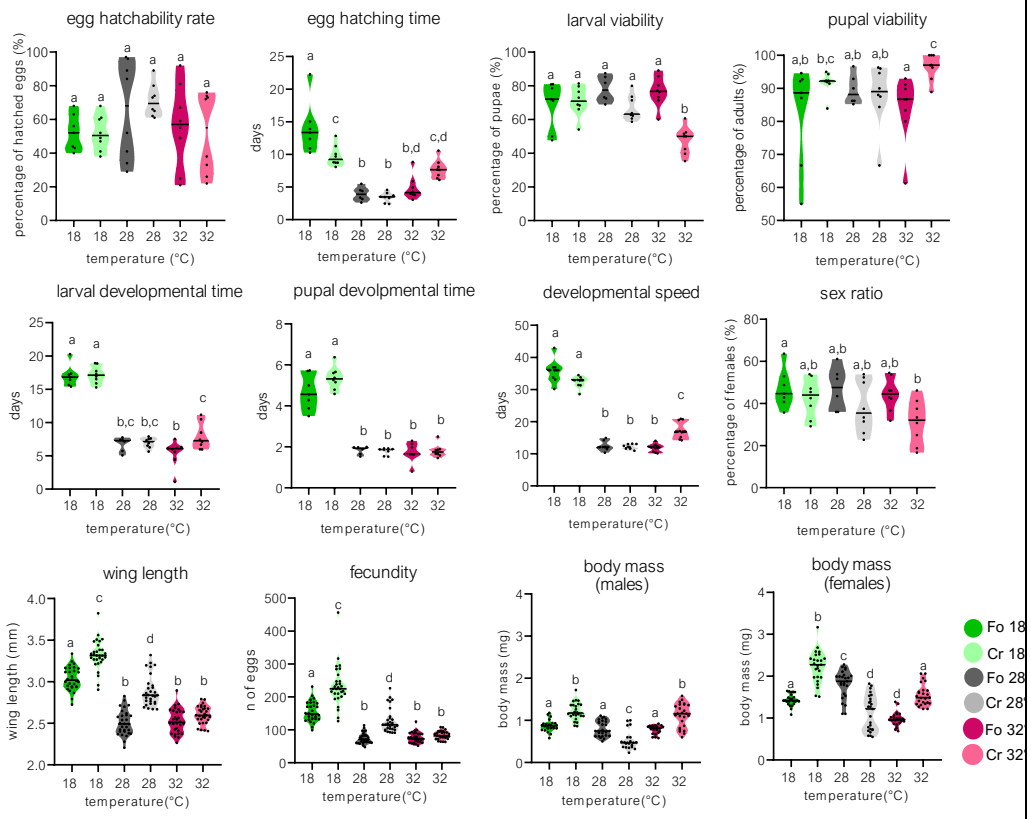


Figure 8. Violin plots showing life history traits in Fo and Cr reared at 18°C (green), 28°C (grey) and 32°C (pink). Traits analysed include egg hatchability rate, egg hatching time, larval and pupal viability, larval and pupal developmental time, developmental speed, sex ratio, wing length, fecundity, body mass in males and in females. Letters ("a" to "d") refer to statistical comparisons: same letter denotes no significant difference.

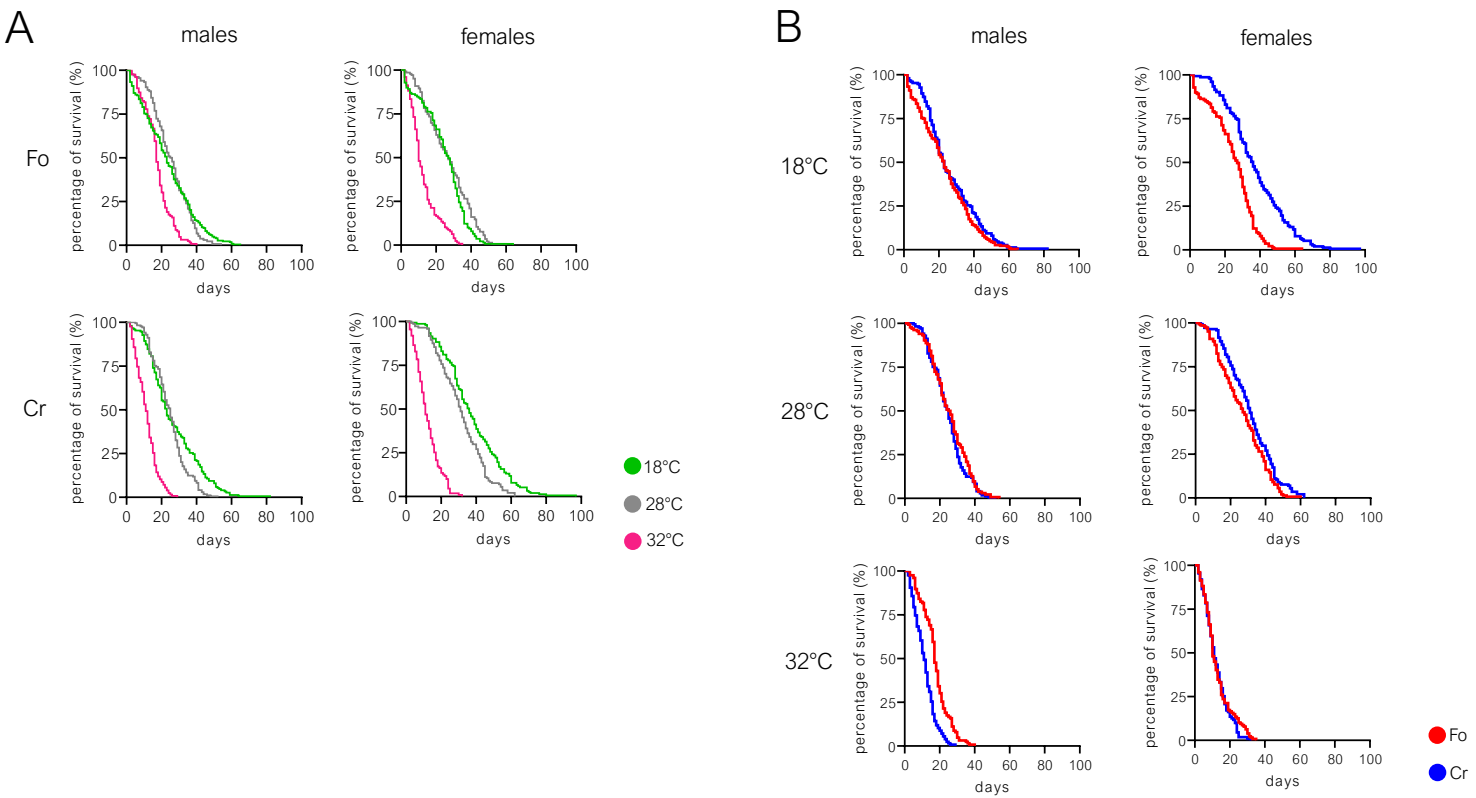


Figure 9. Longevity data represented as survival curves **(A)** within strain at 18°C (green), 28°C (grey) and 32°C (pink) and **(B)** between Fo (red) and Cr (blue) strains. In both panels, males are placed on the left, females on the right.

To verify that my life-history traits results were not stochastic and to further validate the differences that I found, data assessment on egg hatchability rate, larval viability, larval developmental time and sex ratio was re-performed after 2 years in mosquitoes of the Fo strain reared at both 28°C and 32°C, and in Cr mosquitoes reared at 28°C. No statistical difference was observed after a one-way ANOVA in any of the measured traits in either Fo or Cr, as shown in Figure 10.

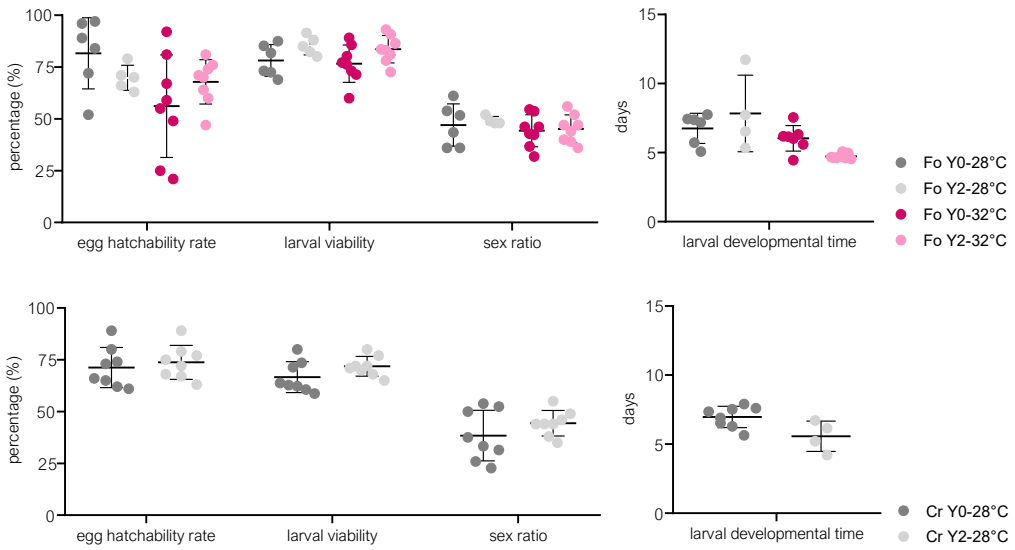


Figure 10. Comparison of life history traits values as estimated in Fo (top) mosquitoes after a two-year span (Y0 vs Y2); Data assessment was performed with mosquitoes reared both at 28°C (grey dots) and 32°C (pink dots). Comparison of fitness trait values as estimated Cr (bottom) mosquitoes reared at 28°C after a two-year span (Y0 vs Y2). Mean and standard deviations are reported.

I further draw TPCs for both strains (Figure 11). *Tbr* did not differ between strains, but Fo showed a slight shift towards warmer thermal regimes, with a peak at 25.78°C, while Cr showed a peak at 23.28°C. Among the tested developmental temperatures, both Cr and Fo performed the best at 28°C. These results suggest an overall similar plasticity in the two strains, with Cr having a better performance at colder temperatures and Fo at warmer temperatures.

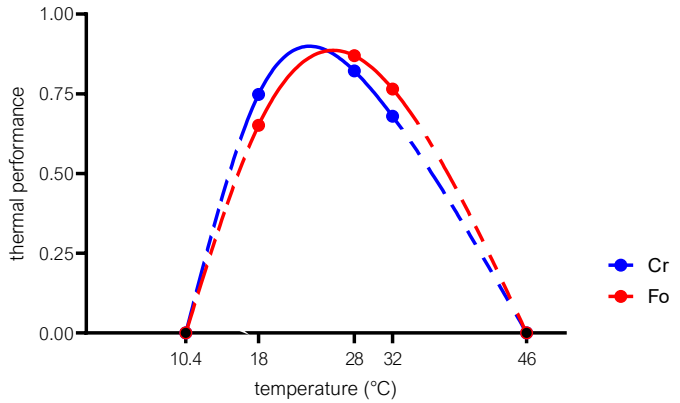


Figure 11. Thermal performance curves (TPC) for both Fo (red) and Cr (blue) strains. Continue lines represent the curves obtained through life history traits at 18°C, 28°C and 32°C, whereas dotted lines are derived from said measurements using Prism (GraphPad 8).

4.2 Thermal traits

For each strain and developmental temperature, I also collected *Tp* and *KDT* data, which are shown in Figure 12. Appendix 5 reports Kolmogorov-Smirnov (*Tp*) and Mann Whiteny and Kruskal-Wallis (*Tp* and *KDT*) tests results.

4.2.1 Within strain comparison

In Fo mosquitoes males had a stable mean *Tp* from 25.6°C to 25.94°C across all tested developmental temperatures. On the contrary, females reared at different developmental temperatures showed significantly different *Tp*. Females reared at 28°C preferred cooler temperatures (24.09°C) on the thermal gradient than mosquitoes reared at 32°C (26.1°C; p-value=0.0125). No difference in *Tp* was found in mosquitoes reared at 18°C and either 28°C or 32°C. *Tp* varied between sexes only in mosquitoes reared at 28°C, with males preferring on average warmer temperatures than females, both in terms of mean temperature (p-value=0.0169) and distribution (p-value=0.0303).

Regarding *KDT*, Fo males reared at 32°C stopped to move at a higher temperature (45.92°C) than those reared at 18°C (45.31°C; p-value=0.0037) and 28°C (45.21°C; p-value=0.0127). There was no difference between *KDT* values of males reared at 28°C and 18°C. Females reared at 28°C had a lower *KDT* value (45.5°C) than females reared

both at 18°C (46.38° C; p-value<0.0001) and 32°C (46.32°C; p-value<0.0001). *KDT* varied significantly between sexes only in mosquitoes reared at 18°C, with females having a mean value of 46.38°C and males 45.31°C (p-value<0.0001).

In Cr both males and females reared at different developmental temperatures showed significantly different *Tp*. Cr males reared at 28°C preferred 28.1°C, a value significantly higher than that chosen by males reared at 18°C (25.48°C; p-value= 0.0170) and 32°C (25.05°C; p-value=0.0043); there were also significant differences in terms of distribution (28°C vs 18°C p-value=0.0172 and 28°C vs 32°C p-value=0.008). Females reared at 18°C preferred 26.93°C, a temperature significantly warmer than that chosen by females reared at 32°C (24.21°C; p-value=0.0139); there was also a significant difference in terms of distribution with mosquitoes reared 18°C having an even spread of *Tp* than those reared at 32°C (p-value=0.0027). As in Fo, Cr males reared at 28°C preferred a warmer temperature (28.1°C) compared to females (26.31°C), both in terms of mean temperature and distribution (p-value=0.0292 and p-value=0.0054, respectively).

Regarding *KDT*, both males and females of Cr reared at 18°C stopped moving at a higher temperature (46.1°C and 46.61°C, respectively) than those reared at 28°C (45.45°C and 46.21°C, respectively; in males p-value=0.0029 and in females p-value=0.0421). There was no difference in *KDT* values of mosquitoes reared at 28°C and 32°C in either male or females. Interestingly, females stopped moving at a higher temperature than males across all rearing temperatures (at 18°C 46.1°C and 46.61°C, in males and females, respectively, p-value=0.0068; at 28°C 45.45°C and 46.21°C, in males and females, respectively, p-value<0.0001; at 32°C 45.68 and KDT=46.59°C, for males and females, respectively, p-value< 0.0001).

4.2.2 Among strain comparison

Most mosquitoes had a *Tp* of 25.77°C±6.62, independently of their developmental temperature, sex and strain.

In males reared at 28°C, I observed strain differences with Cr males having a *Tp* of 28.1°C, value significantly higher than that of Fo males (25.71°C); differences were both in terms of distribution (p-value=0.0272) and mean (p-value=0.0198): Cr males preferred temperatures in the middle of the gradient, whereas Fo males were evenly distributed. Similarly, when reared at 28°C, Fo females preferred on average colder temperatures (24.09°C) than Cr females (26.31°C; p-value=0.0455).

When reared at 32°C, Cr females had significantly lower *Tp* (24.21°C) and that of Fo (26.1°C; p-value 0.038). This difference extended to the distribution of *Tp*, with Cr females being distributed across the low and middle temperatures, while Fo was showing a peak in the middle of the thermal gradient (p-value=0.0103).

Regarding *KDT*, most mosquitoes were knocked down at 46.5°C±1.04, independently of their developmental temperature, sex and strain. I observed strain differences in male mosquitoes reared at 18°C and in females reared at 28°C, with Fo having a lower *KDT* than Cr (in males at 18°C Fo=45.31°C and Cr=46.1°C, p-value=0.0004; in females at 28°C Fo= 45.5°C and Cr=46.21°C, p-value<0.0001). From these data, the *CTmax* of *Ae. albopictus* was extrapolated: 45.31°C-46.61°C.

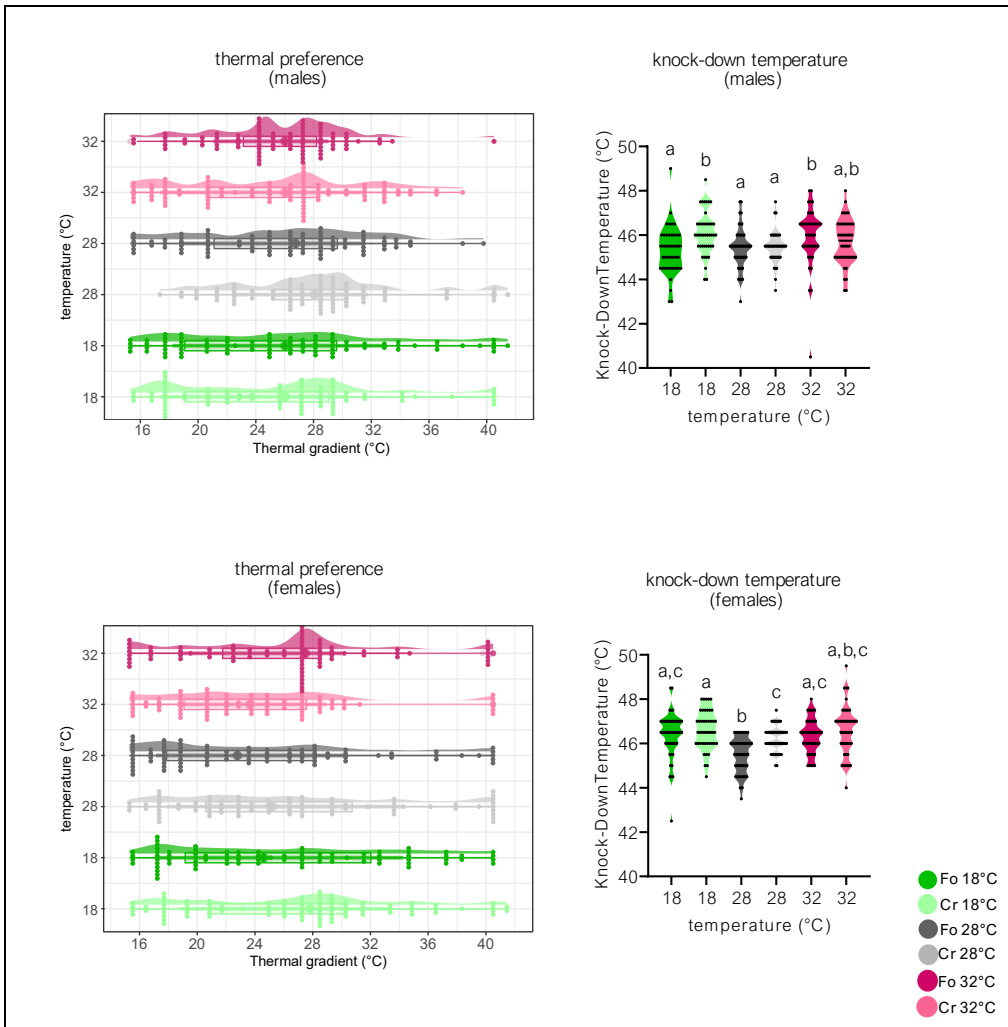


Figure 12. Rain plots showing thermal preference results (*Tp*; on the left) in males (top) and females (bottom) and violin plots showing knock down temperature results (*KDT*; on the right) in males (top) and females (bottom) when reared at 18°C (green), 28°C (green) and 32°C (pink). In violin plots letters ("a" to "c") refer to statistical comparisons: same letter denotes no significant difference.

4.3 Water content and energy reserves

Comparisons of water content, protein, glycogen, total lipids and triglyceride for each strain and developmental temperature are shown in Figure 13; statistical analyses are shown in Appendix 6.

4.3.1 Within strain comparison

Fo males had a lower percentage of body water when reared at 32°C (22.97%) compared to values of those reared at both 28°C (75.94%; p-value<0.0001) and 18°C (78.48%; p-value<0.0001). In Fo females a lower percentage of water was found in mosquitoes reared at 32°C (21.49%) compared to both 18°C (77.3%; p value <0.0001) and 28°C (69.82%; p value<0.0001). Females reared at 28°C had a significantly lower water content than those reared at 18°C (p-value=0.0012). Males and females had a different water content only when reared at 28°C, with males having a higher water content than females (p-value=0.0023).

Fo males and females had their highest protein content when reared at 28°C (80.89 μg/mg and 69.21 μg/mg for male and females, respectively) and their minimum when reared at 18°C (40.14 μg/mg and 40.85 μg/mg, respectively). In males all comparisons showed significant differences (28°C vs 18°C p-value <0.0001, 28°C vs 32°C p-value=0.0023 and 18°C vs 32°C p-value=0.0098). In females reared at 18°C the protein content was lower than that of mosquitoes reared at 28°C (p-value=0.0002) and 32°C (p-value=0.0044), while there was no significant difference in protein content of mosquitoes reared at 28°C and 32°C. By comparing the protein content of males vs females, the only statistically significant difference was recorded in mosquitoes reared at 28°C, with males having more proteins than females (p-value=0.0328).

Fo males had the highest content of glycogen when reared at 32°C (72.99 μg/mg) and the lowest when reared at 18°C (29.79 μg/mg); males reared at 18°C had a significantly lower glycogen content than those reared at 32°C (p-value<0.0001). There was no difference in glycogen content in females at any temperatures. By comparing the glycogen content of males vs females, males had a higher content than females both at 28°C (p-value=0.003) and 32°C (p-value=0.0127).

Both sexes had their lowest total lipid content when reared at 28°C (5.52 μg/mg and 3.2 μg/mg in males and females, respectively), and these values were significantly different than those recorded in mosquitoes reared at 32°C (in males 8.51 μg/mg, p-value=0.0128; in females 7.08 μg/mg, p-value<0.0001) and at 18°C in females (7.62 μg/mg, p-value<0.0001). Comparison of total lipid content between sexes was significant only in mosquitoes reared at 28°C, with males having more lipids than females (p-value=0.0001).

Fo males had no significantly different content of triglyceride when reared at the different developmental temperatures. In females, triglyceride content was lower when reared at 18°C (4.81 μg/mg) than at 28°C (8.81 μg/mg; p-value=0.0002) and 32°C (7.31 μg/mg; p-value<0.0001). Furthermore, when I compared males and females, they differed in triglyceride content at both 18°C (p-value<0.0001) and 32°C (p-value=0.0065), with males being richer in triglyceride than females. At the control temperature of 28°C, there was no difference between sexes.

Cr mosquitoes had a lower content of body water when reared at 32°C for both sexes (22.12% and 27.13% in males and females, respectively) than mosquitoes reared at both 28°C (76.83% and 77.08% in males and females, respectively; p-value<0.0001 for both comparisons) and 18°C (80.02% and 74.82% in males and females, respectively; p-value<0.0001 for both comparisons). Furthermore, when reared at 32°C females had more water than males (p-value=0.0126), whereas there were no sex differences in water content of mosquitoes reared at 18°C or 28°C.

As in Fo, in Cr both sexes had a higher protein content when reared at 28°C (67.44 μg/mg and 49.51 μg/mg for males and females, respectively) than 18°C (in males 52.62 μg/mg, p=0.0108; in females 33.43 μg/mg, p-value= 0.0024); females displayed differences in protein content also when reared at 18°C and 32°C (46.51 μg/mg; p-value<0.0001). Males were richer in proteins than females when development occurred at both 18°C (p-value=0.0014) and 28°C (p-value=0.0044), while there was no difference between sexes in mosquitoes reared at 32°C.

Cr females had a higher content of glycogen when reared at 28°C (67.31 μg/mg) than both at 18°C (48.03 μg/mg; p value=0.0151), and 32°C (48.79 μg/mg; p-value=0.0104). No difference was recorded in males and between sexes at any tested temperature.

Similar to Fo, in both sexes of Cr mosquitoes, lowest content of total lipids was observed in mosquitoes reared at 28°C (5.93 μg/mg and 4.37 μg/mg for males and females, respectively), with significant differences with respect to mosquitoes reared at 32°C (11.41 μg/mg and 8.93 μg/mg for males and females respectively; in males p-value=0.0001, in females p-value<0.0001) and for females also to mosquitoes reared at 18°C (8.38 μg/mg, p-value<0.0001). When reared at 18°C, females had more lipids than males (p-value=0.0021), the opposite at 28°C (p-value=0.0302).

Like in Fo, content of triglyceride was higher in Cr females reared at 28°C (11.07 μg/mg) compared to 18°C (5.48 μg/mg; p-value=0.0004), while there was no difference in triglyceride content of females reared at 28°C and 32°C. When comparing males and females, at 18°C and 32°C males had more triglyceride than females (p-value<0.0001 for both comparisons), while at 28°C there was no difference.

4.3.2 Among strain comparison

Water content in males was comparable between strains at all tested developmental temperatures, whereas Cr females reared at 28°C (77.08%) and 32°C (27.13%) had a higher body water content compared to Fo (at 28°C 69.82%, p-value=0.0034; at 32°C 21.49%, p-value=0.0264). Females reared at 18°C showed no difference in water content between strains.

Protein content significantly differed between females of two strains at all tested temperatures, with Fo displaying the higher protein concentrations than Cr (at 28°C Fo=69.21 μg/mg and Cr=49.51 μg/mg, p-value=0.0043; at 18°C Fo =40.850 μg/mg and Cr= 33.43 μg/mg, p-value=0.0226; at 32°C Fo= 53.63 μg/mg and Cr= 46.51 μg/mg, p-value=0.0318).

Glycogen content significantly differed between the two strains when reared at 18°C with Fo mosquitoes of both sexes having lower glycogen (29.79 μg/mg and 29.58 μg/mg, in males and females, respectively) compared to Cr (43 μg/mg and 48.03 μg/mg, for males and females respectively; p-values=0.0042 in males and p-value=0.0001 in females). In mosquitoes reared at 32°C and 28°C, differences in glycogen content between strains were limited to males or females, respectively.

Lipid content significantly differed between the two strains when reared at 32°C with Cr showing higher concentrations compared to Fo, in both sexes (11.41 μg/mg and 8.51μg/mg, in Cr and Fo males, respectively, p-value=0.0286; 8.93 μg/mg and 7.08 μg/mg, in Cr and Fo females, respectively, p-value=0.0075).

Lastly, analysis of triglyceride content between strains revealed no differences between strains, at any tested temperatures, neither in males nor in females.

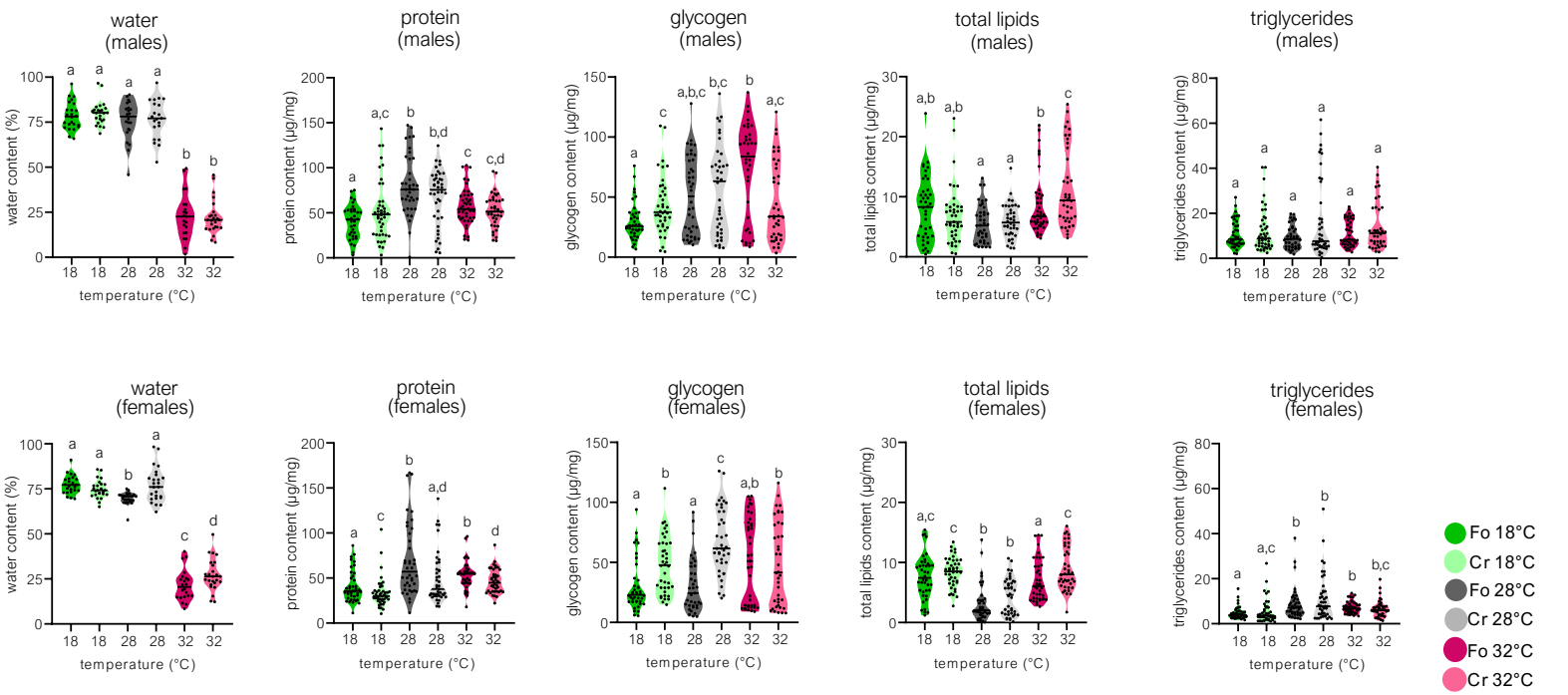


Figure 13. Data of Fo and Cr mosquitoes reared at 18°C(green), 28°C (grey) and 32°C (pink) are shown in violin plots, with solid lines representing median values and each dot an individual for water content (percentage of total body water) and protein, glycogen, total lipids and triglycerides content as µg of nutrient per mg of mosquito for males (top) and females (bottom). Letters ("a" to "d") refer to statistical comparisons: same letter denotes no significant difference.

4.4 Testing the effect of temperature, strain and sex on traits

I used the analysis of variance (ANOVA) to examine the effects of developmental temperature, strain, sex and their combination on egg hatchability, egg hatching time, larval viability, larval and pupal developmental time, developmental speed, sex ratio, wing length and body mass. With this analysis, I was able to assess the percentage of variation accountable for each source of variation (namely temperature, strain, sex or their combinations).

My results showed that "temperature" accounted for most of the variation in egg hatchability rate and egg hatching time, in larval and pupal developmental time, as well as in developmental speed and wing length. "Strain" and "sex" were the main sources of variation for sex ratio and body mass, respectively. Lastly, "temperature x strain" accounted for most of the variation in larval viability, followed by "strain". For each trait, Appendix 7 reports all sources of variation considering the relative degrees of freedom (df), Mean Squares (MS), F ratio (F), p-value and percentage of variation (% of total variation).

Through Pearson's correlation, I proved that wing length and body mass follow Bergmann's rule in both *Ae. albopictus* strains [31]. I found a negative correlation between temperature and both wing size and body mass in both sexes, with a strong relationship between temperature and wing size, moderate between temperature and body mass in Cr females, and weak between temperature and body mass in males of both strains and females of Fo (Figure 15). Table 2 reports correlation coefficients (R) and the relative p-value for each correlation analysis.

I also tested PCA to better understand the effect of temperature on the two strains. For a better resolution and understanding, I made a PCA on life history traits (Figure 16) and energy reserves of males (Figure 17) and females (Figure 18), separately.

In the first PCA (Figure 16), the life history traits which accounted for most of the contribution to the variance were egg hatchability rate, egg hatching time, LDT, PDT, larval and pupal viability, developmental speed, body mass, wing length and sex ratio. Longevity of both sexes had a low contribution to the PCA (around 3%). Data of the two strains of mosquitoes reared at 18°C clustered separately and were also separated from the rest of the data from mosquitoes reared at 28°C and 32°. Data of mosquitoes reared at 32°C clustered together in Fo, but not in Cr.

Protein and water content mostly contributed to variation shown in the PCA of males (Figure 17). Interestingly, in the 3rd quadrant I could appreciate the clustering of both Fo and Cr strains when reared at 18°C and 28°C due to the water content, whereas between the 3rd and 4th quadrant there was a clustering of data on triglycerides, total lipids and glycogen from mosquitoes reared at both 28°C and 32°C. Lastly data on protein content from mosquitoes reared at 18°C and 28°C clustered in the 4th quadrant.

Similar to the results in males, PCA of energy reserves of females (Figure 18) showed that content of protein and water mostly contributed to the variance of the data. I also observed data clustering into three groups: in the 2nd quadrant data of mosquitoes reared at 18°C clustered together independently of the strain due to the effect of protein content; in the 1st and the 2nd quadrants, clustering of data from mosquitoes reared at 28°C and 32°C was driven by content of total lipids, glycogen and triglyceride; in the first quadrant, data of the water content of mosquitoes reared at 18° and 28°C clustered together.

4.5 Testing isomorphy

I calculated LDR and PDR and their ratio (LDR/PDR) to test the hypothesis of developmental isomorphy, a generalised rule in ectothermic organisms [52]. Regression analyses revealed a positive relationship between LDR and temperature in both strains (Fo b=0.0138 and Cr b=0.006; p-value=0.0314 and p-value<0.0001, respectively). As in LDR, I found a positive relationship with developmental temperature for PDR (Fo b=0.003 and Cr b= 0.0027; p-value<0.0001 for both slopes). Comparing the regressions of the two developmental rates (LDR vs PDR), I saw a significant difference in the slopes (p-value<0.0001) in Cr, but not in Fo. When LDR and PDR were compared between strains, both regression analyses resulted in no differences for the slopes nor intercepts. Regarding the ratio LDR/PDR I found different slopes between the two strains (p-value=0.0043) and, while in Cr the regression of the LDR/PDR on temperature had a negative slope (b= -0.005, p-value=0.0029) disproving an isomorphy, in Fo the slope was not significantly different than 0 (Figure 14, Appendix 8).

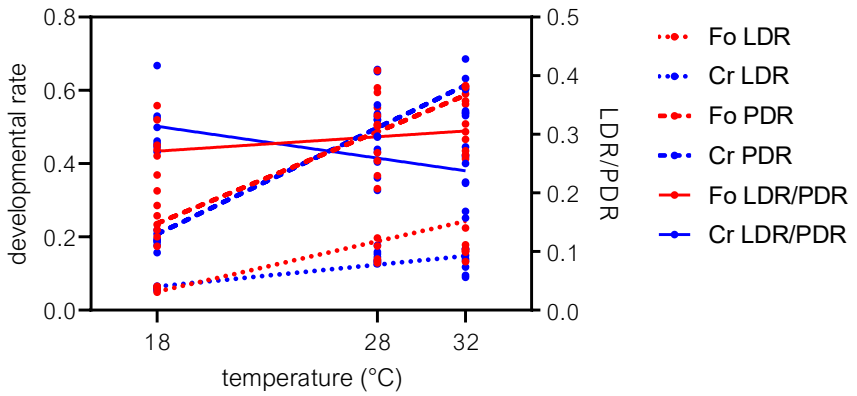


Figure 14. Developmental rate regressions at different ambient temperatures for Fo (red) and Cr (blue).

Table 2. Pearson's correlation results, reporting the traits being analysed, the strain and the eventual sex, Pearson's correlation coefficient (R) and the relative p-value.

Trait	**Strain**	**Sex**	**Pearson's R**	**p-value**
wing length	Fo	-	-0.8210974	< 2.2e-16
	Cr	-	-0.8764682	< 2.2e-16
body mass	Fo	Males	-0.2603079	0.02953
	Cr	Males	-0.2706239	0.02346
	Fo	Females	-0.2538844	0.0302
	Cr	Females	-0.6209003	2.803e-09

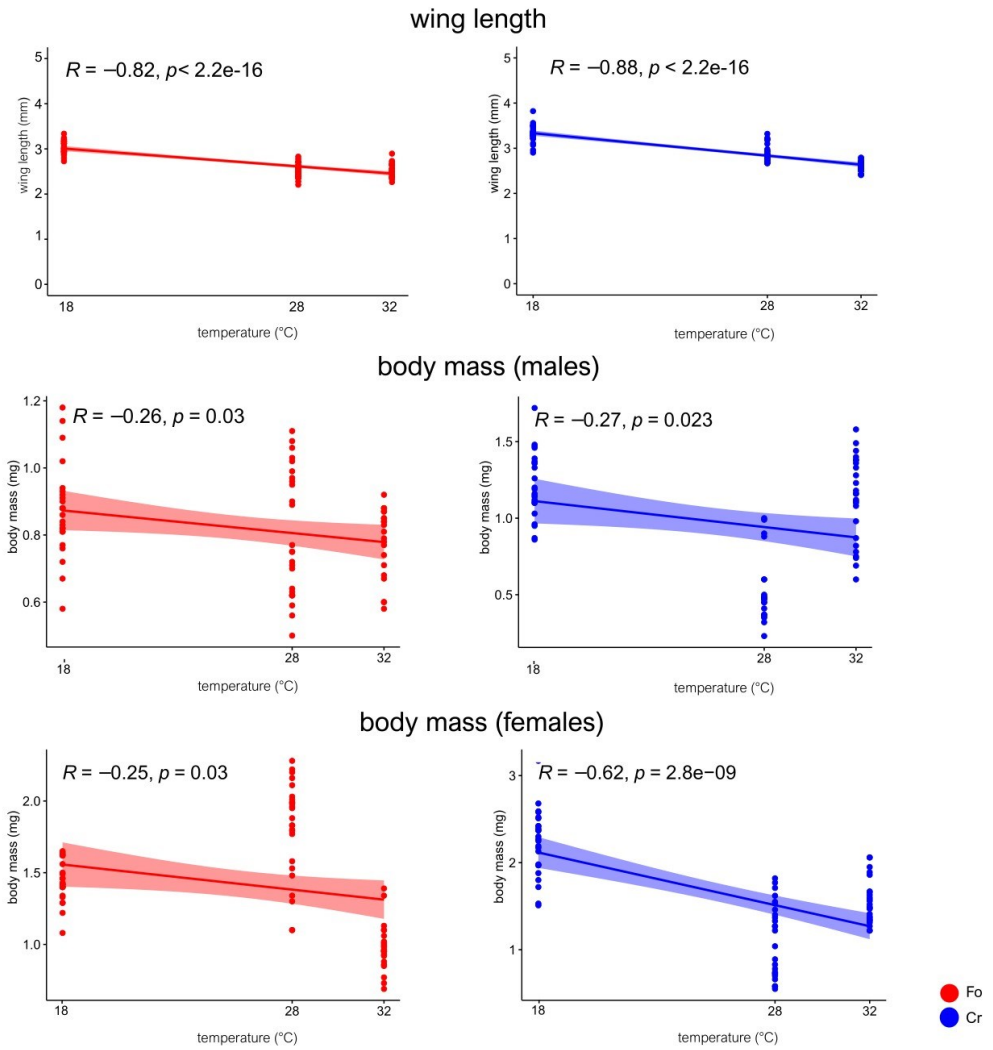


Figure 15. Pearson's correlations in Fo (red) and Cr (blue) calculated for wing length and body mass in males and females. Pearson's correlation coefficient (R) and the relative p-values are reported for each analysis.

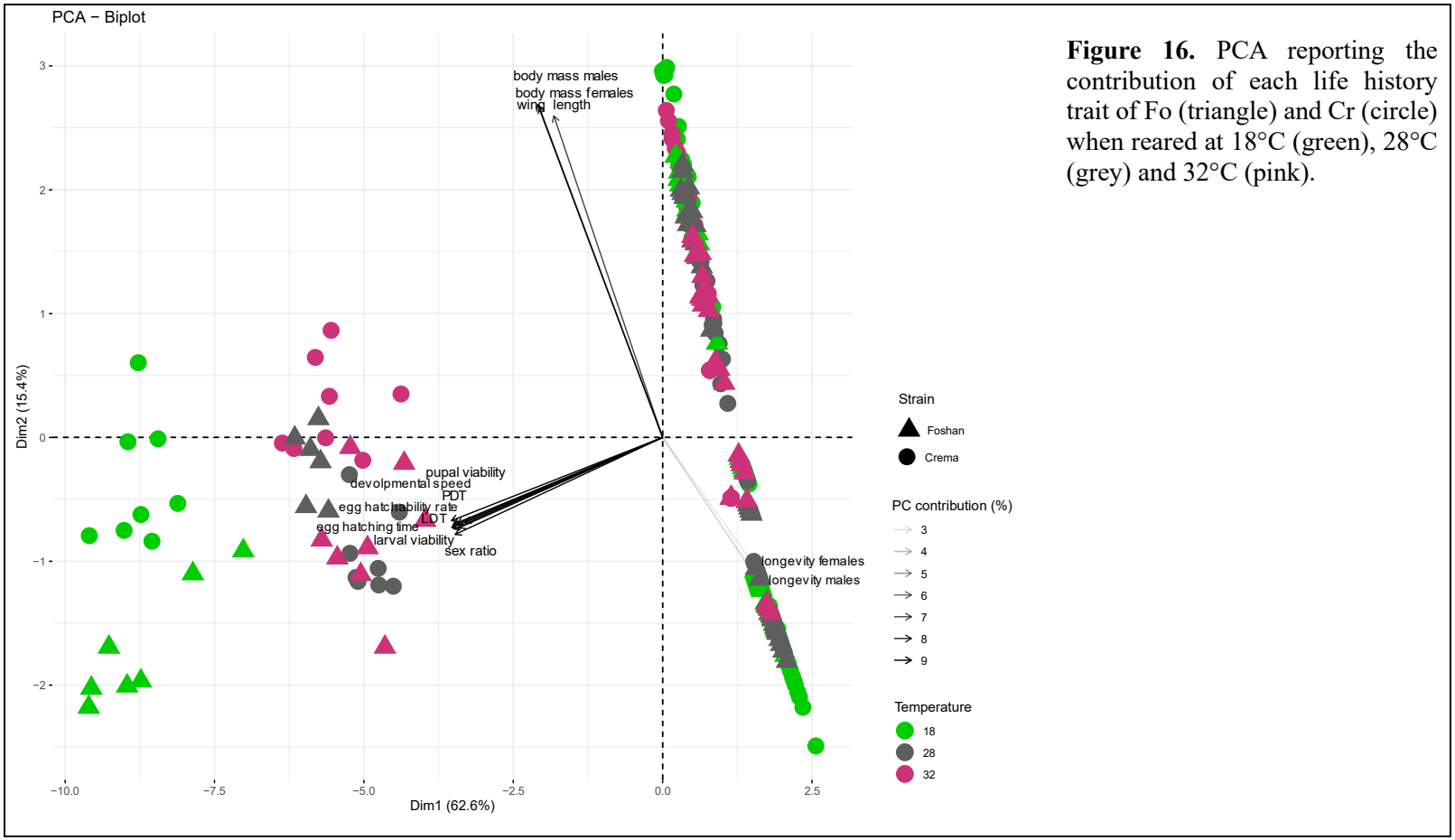


Figure 16. PCA reporting the contribution of each life history trait of Fo (triangle) and Cr (circle) when reared at 18°C (green), 28°C (grey) and 32°C (pink).

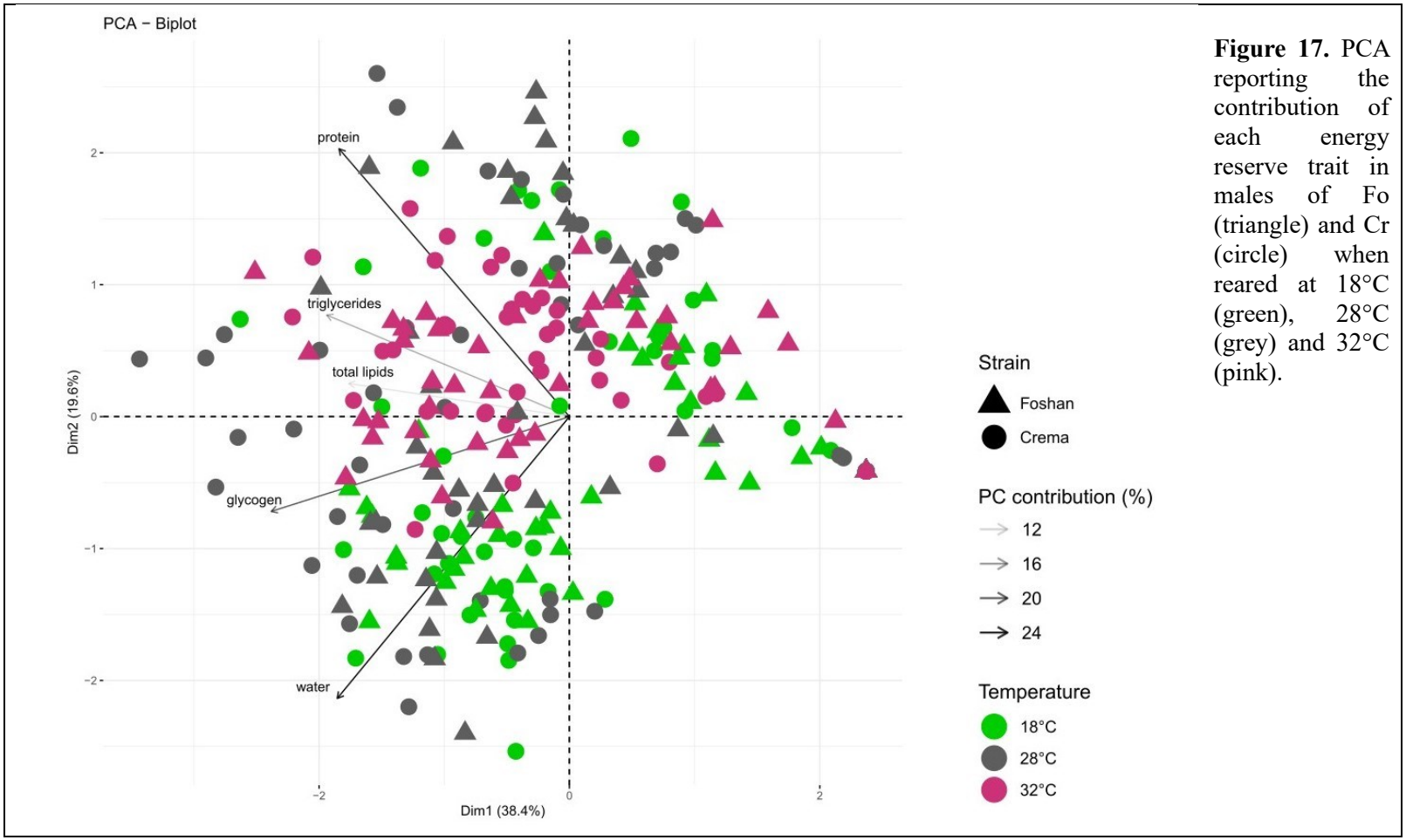


Figure 17. PCA reporting the contribution of each energy reserve trait in males of Fo (triangle) and Cr (circle) when reared at 18°C (green), 28°C (grey) and 32°C (pink).

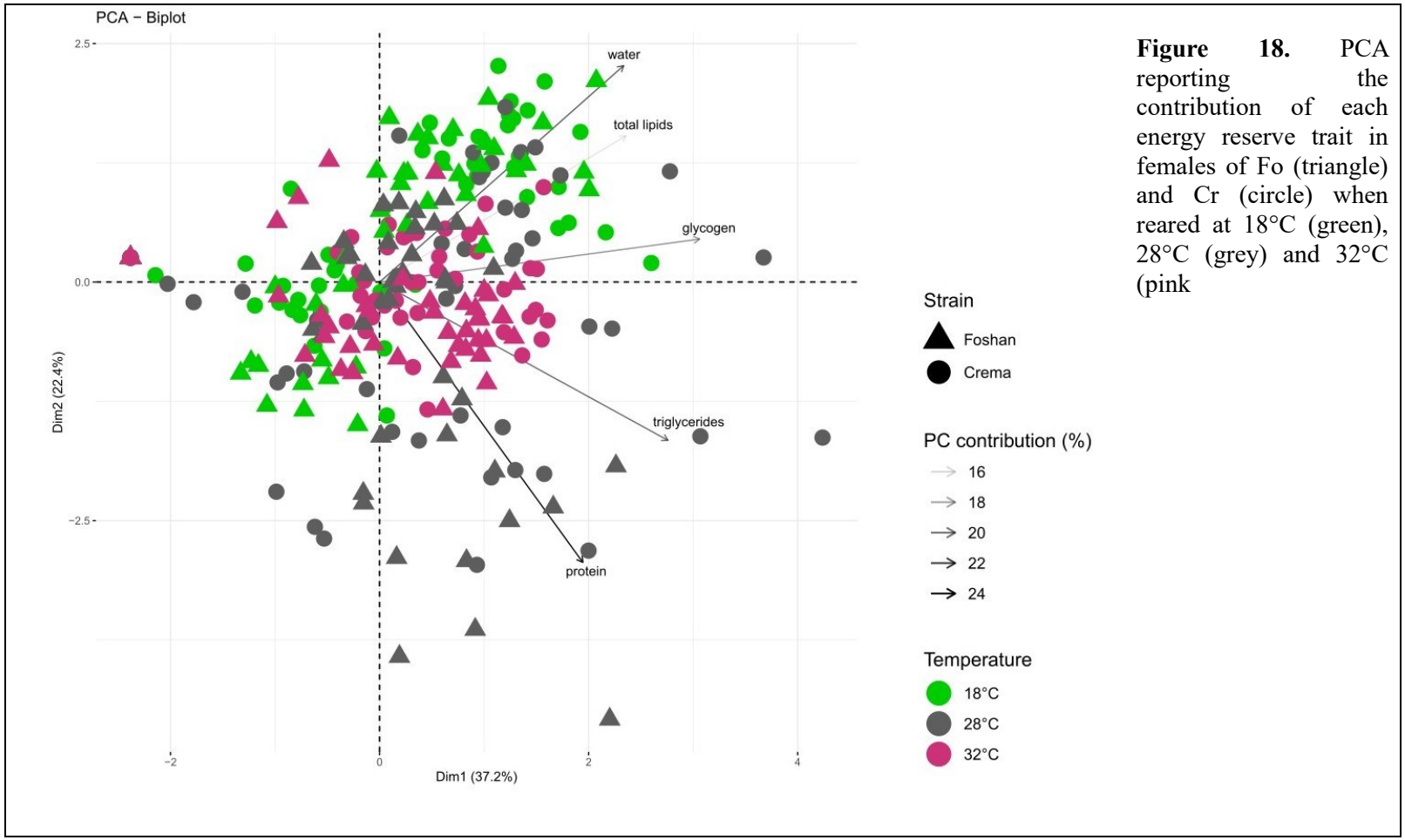


Figure 18. PCA reporting the contribution of each energy reserve trait in females of Fo (triangle) and Cr (circle) when reared at 18°C (green), 28°C (grey) and 32°C (pink

4.6 Transcriptome analysis

I performed transcriptomic analyses in both 4th instar larval and emerging female adults of mosquitoes of the Cr and Fo strains reared at 18°C, 28°C and 32°C. I identified DE genes between Fo and Cr across the same developmental stage and thermal regime (either 18°C or 32°C) by filtering out genes that are DE between the two strains at 28°C, to avoid accounting for strain-specific differences. I also compared the transcriptome of larvae and adults of the two strains and performed GO enrichment analysis to obtain a set of temperature-related candidate genes (Appendix 9A for DE genes found at 18°C compared to 28°C and Appendix 9B for DE genes found at 32°C compared to 28°C).

Overall, mosquitoes reared at 18°C had the highest number of DE genes, with respect to mosquitoes reared at 32°C, but levels of expression were lower. In larvae reared at 18°C I saw 155 DE genes in Cr and 143 in Fo; in larvae reared at 32°C I saw 39 DE genes in Cr and 65 in Fo. In adults reared at 18°C I saw 57 DE genes in Cr and 236 DE genes in Fo; in adults reared at 32°C I saw 26 DE genes in Cr and 33 in Fo (Figure 19).

By comparing the list of DE genes in larvae and adults, I found two genes that in Fo mosquitoes reared at 18°C were DE in both life stages (AALFPA_044093-*homocysteine S-methyltrasferase* and AALFPA_080605-*membrane glycoprotein*).

The main difference between strains was the opposite differential expression of several cuticle proteins which, in larvae reared at 18°C, were downregulated in Fo and upregulated in Cr when compared to larvae reared at 28°C. These results were subsequently confirmed by GO analysis, which showed an enrichment for *structural constituent of cuticle* among upregulated genes in Cr larvae reared at 18°C and downregulated in Fo (Figure 20, Appendix 10).

Gene ontology enrichment analysis showed more ontology categories in mosquitoes reared at 18°C than 32°C thermal regime. In both thermal conditions, Cr mosquitoes had more enrichment hits than Fo. Fo and Cr samples collected from adult individual reared at 18°C were commonly enriched in genes involved in *organonitrogen compound catabolic process*, *odorant binding*, *lipid transporter activity* and *serine hydrolase/endopeptidase activities*, while larvae at 18°C where commonly enriched in genes related to *extracellular region* and *chitin binding* functions. Fo adult mosquitoes reared at 18°C had the transcriptome further enriched in genes involved in the *tricarboxylic acid cycle*. Cr adults reared at 18°C had the transcriptome further enriched in genes involved in *lipid transport*, while the transcriptome of larvae reared at 18°C was further enriched in *DNA-binding transcription factor activity, RNA polymerase II-specific*, *peptidoglycan muralytic activity*, *glycosaminoglycan metabolic process* and *carboxylic ester hydrolase activity* (Figure 20, Appendix 10).

Lastly, in larvae of both strains reared at 32°C, transcriptome was enriched genes associated with *hydrolase activity on glycosyl bonds/compounds;* the same function was enriched in the transcriptome of Fo adults reared at 32°C with respect to 28°C. In the transcriptome of adults of Cr and larvae of Fo, there was an enrichment of *ATP hydrolysis activity*. The transcriptome of Cr mosquitoes reared at 32°C was further enriched in *UDP-glycosyltransferase activity* and *carbohydrate metabolic process* (Figure 21, Appendix 10).

I also looked at temperature-related variation in the expression of immunity genes [191]. Expression of immunity genes was regulated by temperature mostly at the larval stage, in both strains, with similar functions (i.e. antimicrobial peptides, CLIP domain-containing serine proteases and fibrinogen-related proteins), but involving different genes. Additionally, *toll-like receptors* (AALFPA_050924, AALFPA_052084, AALFPA_053911, AALFPA_053911 and AALFPA_063239) were upregulated (up to 97.86 folds more) in both adult and larvae reared at either 18°C or 32°C compared to 28°C in Cr mosquitoes, whereas in the Fo mosquitoes the highest differential expression was identified for the *antimicrobial peptide defensin-A* (AALFPA_065006), which was 8.87 times upregulated and downregulated in adults reared at 32°C and 18°C, respectively, when compared to mosquitoes reared at 28°C, and *protein spaetzle 4* (AALFPA_059265), which was downregulated 62.28 and 9.24 times in larvae reared at 32°C or 18°C, respectively. Appendix 11 reports the complete list of hits found comparing my dataset with immunity genes.

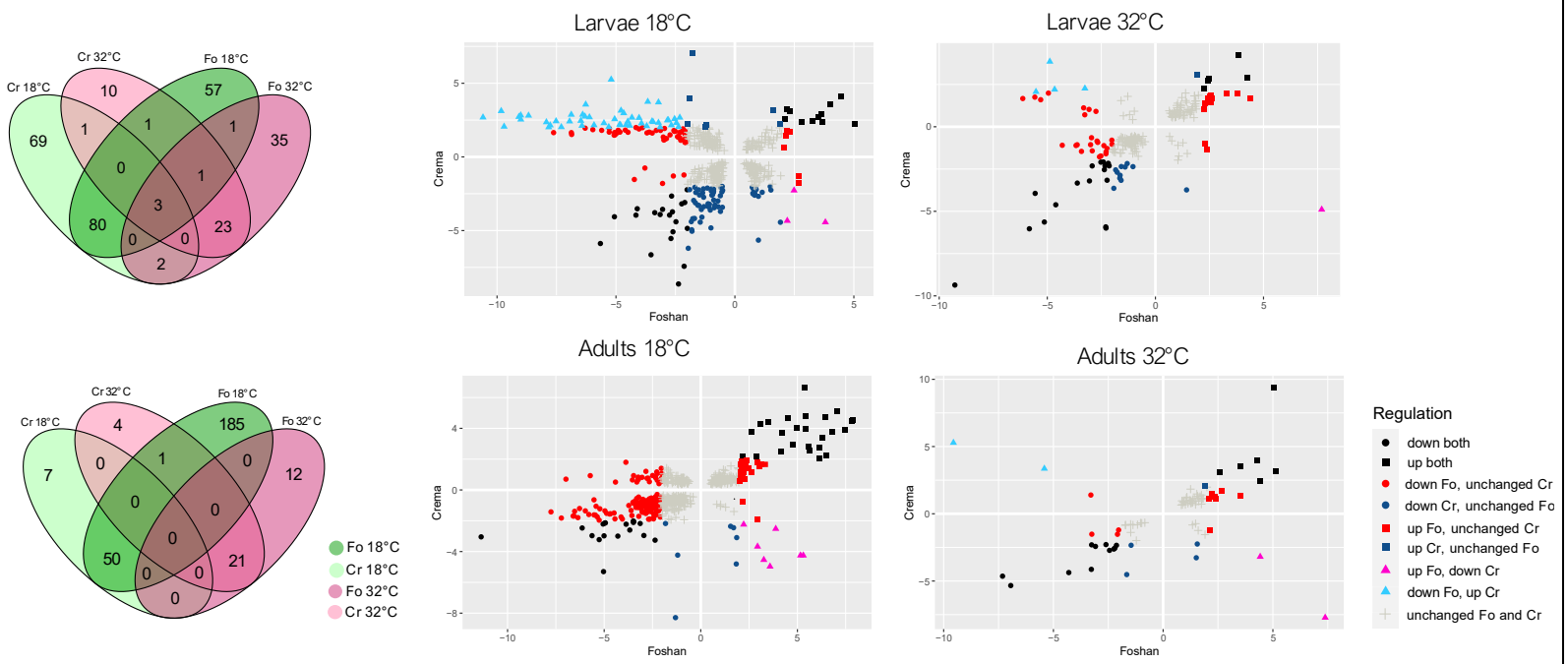


Figure 19. On the left differential expression of Fo (x-axis) and Cr (y-axis) mosquitoes at the larval (top) and adult (bottom) stages, when reared at either 18°C or 32°C. Each point represents a gene expression level (Log2FC) having as x-value the Log2FC of the comparison between Fo individuals at the tested temperature (18°C or 32°C) vs the control (28°C) and as y-value the Log2FC of the comparison between Cr individuals at the tested temperature (18°C or 32°C) vs the control (28°C); on the right Venn-diagrams showing the number of unique and shared genes in larvae (top) and adults (bottom) across the different temperatures and strains.

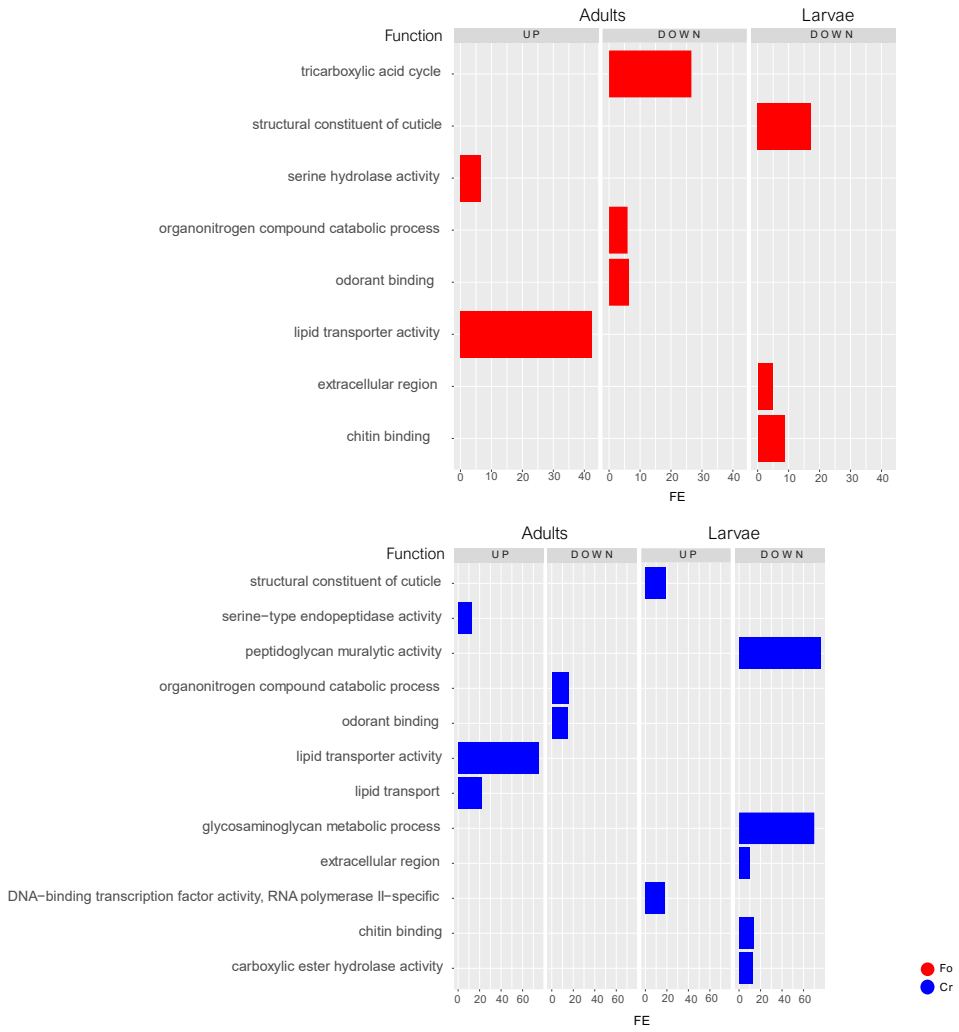


Figure 20. GO enrichment profiles are compared between strains (Fo in red, Cr in blue), in adults and larvae at 18°C compared to 28°C.

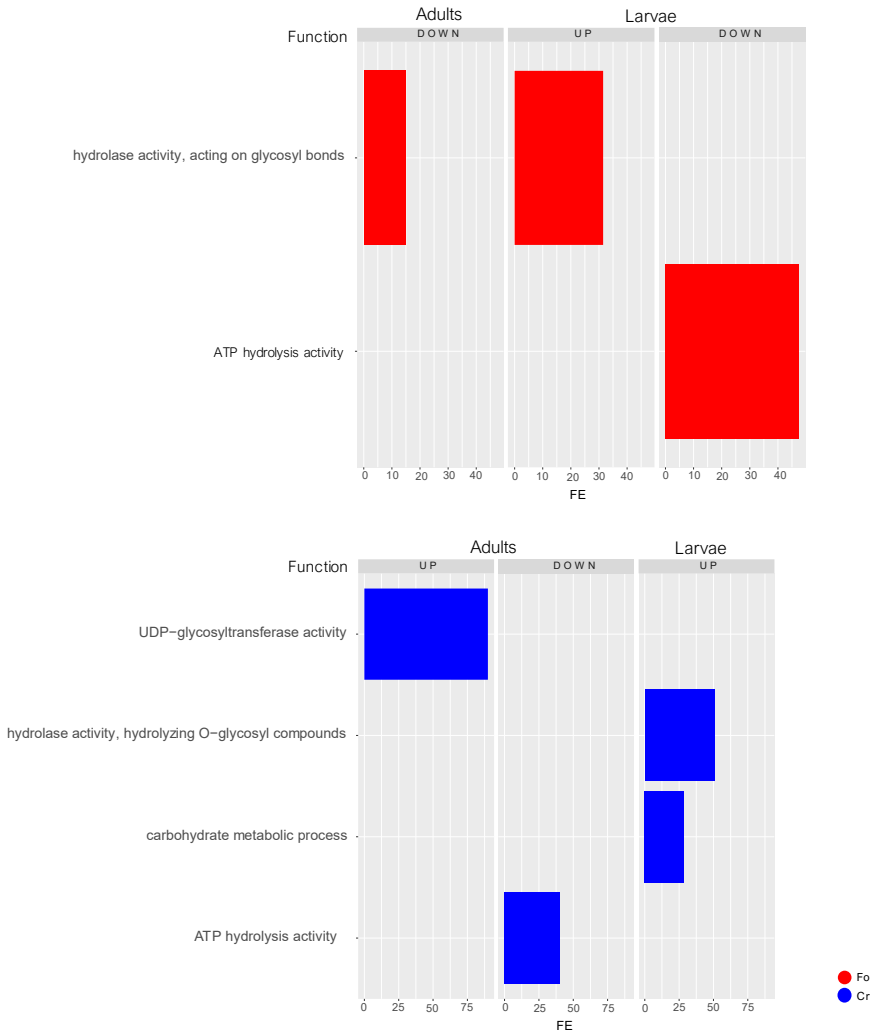


Figure 21. GO enrichment profiles are compared between strains (Fo in red, Cr in blue), in adults and larvae at 32°C compared to 28°C.

I further compared DE genes to candidate temperature-associated genes previously identified in *D. melanogaster* [221]. I found *heat shock protein 70* (AALFPA_060102, AALFPA_060249, AALFPA_041794, AALFPA_049001, AALFPA_067347, AALFPA_068981, AALFPA_051922, AALFPA_041503), *heat shock protein 83* (AALFPA_052248), *defensin* (AALFPA_065006), two *pickpocket proteins* (AALFPA_080544, AALFPA_059066), two *fork head-like* (AALFPA_045830, AALFPA_047110) genes and *cytochrome p450* (AALFPA_049004, AALFPA_074444, AALFPA_068814, AALFPA_046252, AALFPA_052377, AALFPA_080403) genes to be consistently modulated by temperature, in *D. melanogaster* and *Ae. albopictus*; modulation in expression consisted mostly in downregulation at 18 and/or 32°C (Table 3).

Table 3. *Drosophila* genes related to thermal stress [221] detected in this study.

Function	gene ID	Regulation	strain	temperature (°C)
heat shock protein 70	AALFPA_060102	Down	Cr	18 and 32
	AALFPA_060249	Down		
	AALFPA_041794	Down		
	AALFPA_049001	Down		
	AALFPA_067347	Down		
	AALFPA_068981	Down		
	AALFPA_051922	Down	Cr	32
	AALFPA_067347	Down	Fo	32
	AALFPA_041503	Down		
cytochrome P450	AALFPA_049004	Down	Cr	18
	AALFPA_074444	Down	Fo	18
	AALFPA_068814	Down		
	AALFPA_046252	Down		
	AALFPA_052377	Down		
	AALFPA_080403	Down		18 and 32
heat shock protein 83	AALFPA_052248	Down	Cr	18
Defensin	AALFPA_065006	Down		18
		Up		32
Pickpocket	AALFPA_059066	Up		18 and 32
	AALFPA_080544	Up		18 and 32
Forkhead	AALFPA_045830	Up		18
	AALFPA_047110	Up		18 and 32

4.7 Chitin quantification

On the basis of my results of Gene Ontology Enrichment, which highlighted an opposite regulation of the cuticle structure genes, I decided to investigate the content of chitin of larvae.

Briefly, for both strains three pools of 15 larvae (4th instar) were generated from eggs hatched at both 18°C and 28°C to perform a chitin colorimetric assay as described by Lehmann and White (1975) [205]. Starting from known concentrations of chitin, I draw a standard curve based on the absorbance at 650 nm (Figure 22) and obtain the formula y=0.0036x+0.0678.

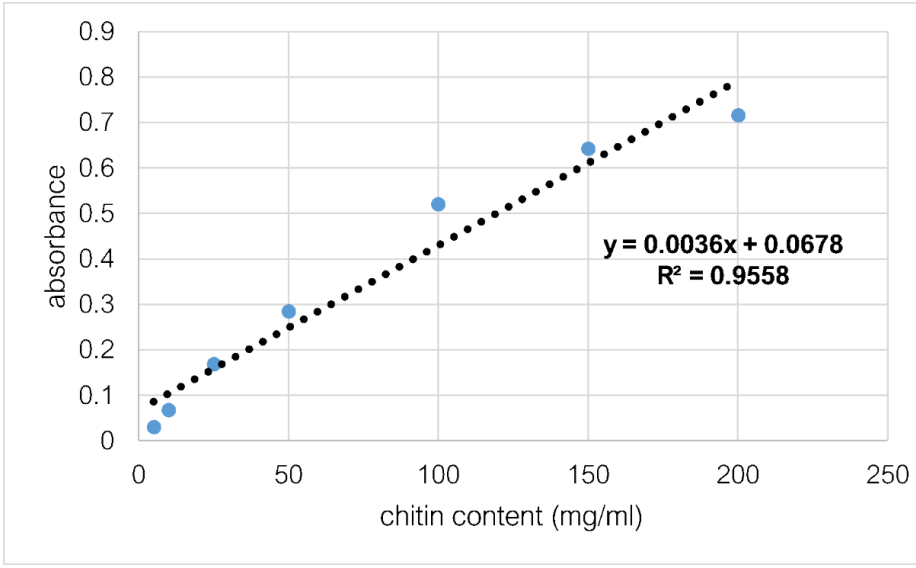


Figure 22. Chitin standard curve based on known chitin concentrations.

Given this formula, I calculated the amount of chitin present in my larval samples. Lastly to evaluate the change in chitin concentration from larvae reared at 28°C and at 18°C, I calculated their ratio.

Based on my results, chitin content was higher in larvae reared at 28°C with respect to 18°C in both strains and the difference between the two strains was significant (p-

value=0.0053; Figure 23). This difference may suggest a higher cuticle re-organization in Cr larvae in cold temperatures compared to Fo.

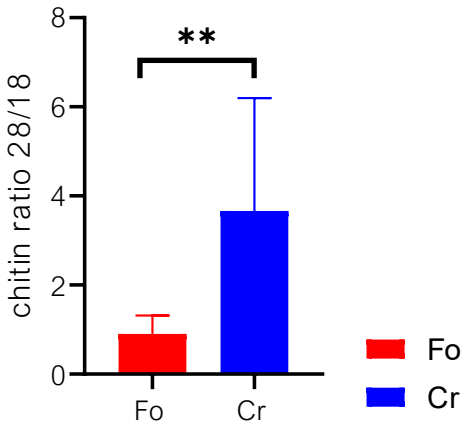


Figure 23. Chitin content ratio between larvae reared at 28°C and 18°C in Fo (red) and Cr (blue).

4.8 *Wolbachia* density

In both strains and across the three tested developmental temperatures, densities of *wAlbA* and *wAlbB* were determined in 4th instar-larvae and in ovaries collected at the day of emergence (d0), at 7 days post emergence (d7) and between 12 and 14 days post emergence (d12-14), by qPCR. Quantities of *Wolbachia* were compared within and between strains among temperatures and developmental stages and results are shown in Figure 24. Statistical analysis results are reported in Appendix 12.

4.8.1 Within strain comparison

In Fo, the quantity of *Wolbachia* was significantly different across developmental temperatures in all developmental stages.

Briefly, in 4th instars larvae there was no difference for *wAlbA* at any temperature, whereas larvae reared at 28°C had a higher *wAlbB* density than those reared at 18°C

(p-value<0.0001). There was a further difference in *wAlbB*, with larvae reared at 18°C having a lower density compared to those reared at 32°C (p-value=0.0305).

At d0, ovaries of Fo mosquitoes reared at 32°C had a lower bacterial density for both *wAlbA* and *wAlbB*, compared to mosquitoes reared at 18°C (p-value=0.0222 and p-value<0.0001 for *wAlbA* and *wAlbB*, respectively). There was no difference between 28°C and either 32°C or 18°C.

At d7 the only difference was recorded when *wAlbB* density was compared between ovaries of mosquitoes reared at 28°C and 18°C, with mosquitoes reared at 28°C having ovaries richer in *wAlbB* than those reared at 18°C (p-value=0.0097).

Finally, at d12-14 the only variation in density was in *wAlbB*, which was present in higher abundance in ovaries of mosquitoes reared at 18°C compared to 32°C (p-value=0.0211).

As in Fo, in Cr the quantity of *Wolbachia* was significantly different across developmental temperatures through all developmental stages, except for d12-14.

In 4th instars larvae, *wAlbB* density was higher in mosquitoes reared at 18°C with respect to values observed in mosquitoes reared at both 28°C (p-value<0.0001) and 32°C (p-value=0.0013).

At d0, ovaries of Cr reared at 18°C had higher *wAlbA* and *wAlB* quantities than those of mosquitoes reared at 28°C (p-value=0.0006 and p-value<0.0001 for *wAlbA* and *wAlB*, respectively) and at 32°C (p-value=0.001 and p-value=0.0021 for *wAlbA* and *wAlB*, respectively).

At d7, mosquitoes reared at 28°C had a higher *wAlbA* and *wAlbB* density than those reared at 32°C (p-value=0.0002 and p-value=0.0001 for *wAlbA* and *wAlB*, respectively). Furthermore, *wAlbB* was more abundant in ovaries of mosquitoes reared at 18°C than at 32°C (p-value=0.0032).

Lastly, at d12-14, there was no difference for either *Wolbachia* strain at any temperature.

4.8.2 Among strain comparison

Significant differences in the quantity of both *Wolbachia* strains were found between Cr and Fo mosquitoes through all life stages.

In larvae, Fo and Cr differed in *wAlbA* density at all tested temperatures; Cr mosquitoes always had higher density of *wAlbA* than Fo (18°C p-value=0.0189, 28°C p-value=0.0256, 32°C p-value=0.0184). *wAlbB* density was different in

mosquitoes of the two strains when reared at 18°C (p-value<0.0001) and 32°C (p-value=0.0185).

At d0, the ovaries of Fo and Cr differed in *wAlbA* density when reared at 28°C (p-value=0.0187) and in *wAlbB* when reared at 18°C (p-value=0.0014) and 32°C (p-value=0.0469).

At d7, only *wAlbB* showed differences in density between strains: ovaries of Cr had more *wAlbB* than Fo mosquitos when reared at 18°C (p-value=0.0005), the opposite when reared at 32°C (p-value=0.0435).

Finally, at 12-14 days ovaries of Fo and Cr varied in both *Wolbachia* strains density when reared at 18°C, with Fo being richer than Cr for both *wAlbA* and *wAlbB* (p-value=0.0019 and p-value=0.0003, respectively for *wAlbA* and *wAlbB*).

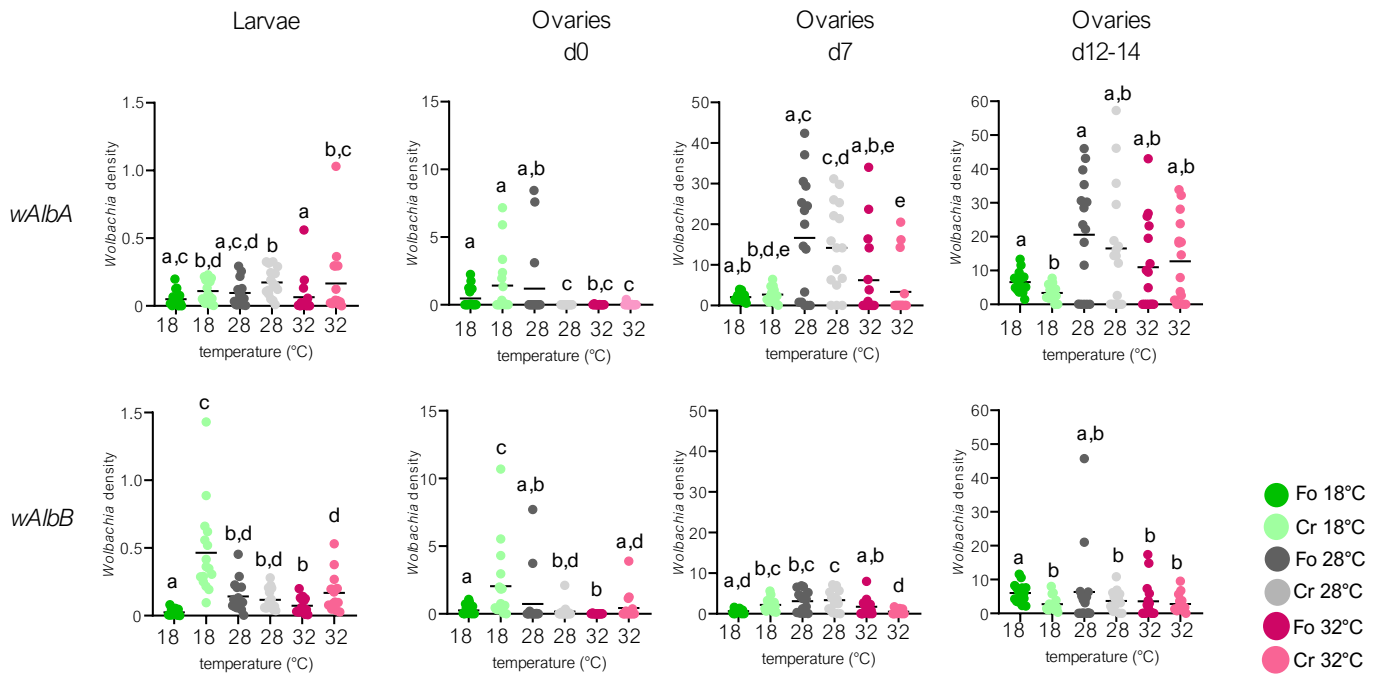


Figure 24. Dot-plots reporting densities of *wAlbA* (top) and *wAlbB* (bottom) detected in Fo and Cr larvae, ovaries collected at emergence (d0), after 7 days (d7) and after 12 to 14 days (d12-14) at a rearing temperature of 18°C (green), 28°C (grey) and 32°C (pink). The median is represented by a solid line. Each dot represents a single sample. Letters ("a" to "e") refer to statistical comparisons: same letter denotes no significant difference.

5. Discussion

In my project I compared the responses of the native Fo strain and the invasive Cr strain, by exposing them to three different thermal regimes (18°C, 28°C and 32°C). I analysed life-history traits, thermal traits, energy reserves and transcriptome. Lastly, I evaluated changes in *Wolbachia* density in 4th instar larvae and ovaries at different time points post eclosion.

5.1 Fo and Cr show similar thermal breath, but respond differently to Ta

My results show a clear effect of developmental temperature on key life history traits, such as egg hatching time, LDT and PDT, developmental speed and wing length. Moreover, both body mass and wing length had a negative correlation with developmental temperature, with mosquitoes decreasing in size and mass as ambient temperature increased indicating that *Ae. albopictus* follows Bergmann's rule, which states that in colder environments organisms are larger, allowing in this way heat conservation [222].

Temperate insects during the year experience a wider range of temperatures compared to tropical ones and thus are expected to have a larger thermal breath [56;64;73]. I did not observe a difference in the overall thermal breath of the two strains, but I saw the Cr strain having the peak of performance at 23.28°C, while Fo at 25.78°C. In general, fitness performance decreased in mosquitoes reared at 32°C due to a lower fecundity and shorter adult longevity. Even though less severe, fitness performance was also reduced in mosquitoes reared at 18°C, with respect to 28°C, due to the development delay of early life stages.

Despite the similar thermal breath, the two strains responded differently to the tested temperatures. When reared at 18°C Cr had a better performance than Fo, due to a faster hatching, a higher fecundity and an increased female longevity. Furthermore, at 18°C Cr accumulated more glycogen, which can be used in response to thermal stress to prevent cellular damage at low Ta [48]. On the other hand, Fo performed better at 32°C, with an increased larval viability and reduced LDT and developmental speed. These data suggest that both strains have retained similar levels of plasticity, but also suggest adaptation of the Cr strain to a temperate climate. These results were further enforced by the fact that fitness assessment showed no difference when repeated in Cr and Fo after rearing the two strains in parallel under laboratory conditions for two years.

5.2 Early stages are threatened mainly by cold developmental temperatures

My analyses show that juvenile stages were mostly affected by the cold thermal regime. A cold temperature clearly impaired early development, starting from egg hatching time, LDT and PDT and the overall developmental speed. All these traits lasted significantly longer at 18°C compared to the other temperatures, showing no differences between 28° and 32°C in most of the cases. The exceptions were relative to the Cr strain, which was particularly sensitive to the warm condition: it had a longer hatching time, a lower developmental speed and a lower larval viability when reared at 32°C than to 28°C. The successful development of the early stages is a key factor since it accounts for the density of adult populations. On this note, while comparing the developmental rate of both strains, I proved the absence of an isomorphic effect of temperature on Cr. This last result indicates that the juvenile stages of Cr, differently than Fo, might adapt without evolutionary constraints in response to thermal changes [52].

The severe effect of temperature on the larval stage was further proved in the transcriptome analysis: larvae had a higher number of DE genes compared to adults, especially in mosquitoes reared at 18°C. Fo larvae showed to be particularly affected by temperatures: they showed the greatest number of DE immunity genes when reared at 18°C and 32°C in comparison to 28°C. I also found that genes associated with cuticle were downregulated in Fo larvae reared at 18°C and upregulated in Cr. The insect cuticle is known to be a dynamic structure shaped by environmental conditions [223] and involved in the protection against water loss and mechanical damages [224]. Since chitin is one of the main components of the cuticle, I decided to investigate the chitin content in larvae of both strains by comparing the chitin ratio between 4th instar larvae reared at 28°C and 18°C. I saw that the content of chitin was higher in larvae reared at 28°C, with Cr mosquitoes having a significant higher 28°C/18°C chitin ratio (and therefore a larger chitin decrease at 18°C) when compared to Fo. This result does not clarify the relationship between the up-regulation of cuticle-related genes and the quicker development of Cr larvae at 18°C compared to Fo, but I hypothesise that Cr larvae might increase the mobility of chitin and other constituent of the cuticle, mitigating the stress of developing in cold conditions. Further investigations on larval cuticle formation and organisation at different thermal regimes are needed since this structure might play a major role in juvenile stages survival and development in the scenario of climate change.

5.3 Adult mosquitoes have developed different strategies to cope with temperature

I compared the list of DE genes identified in *Ae. albopictus* to the list of genes associated with temperature in *D. melanogaster* [221] to evaluate the existence of candidate thermal genes. My transcriptomic data indicated down-regulation of heat shock proteins (specifically of *heat shock protein 70* and *heat shock protein 83*) in both *Ae. albopictus* strains when mosquitoes were reared both at18°C and 32°C with respect to 28°C. In a previous study of Ware-Gilmore et al. (2022), *Ae. aegypti* mosquito families with higher

thermal tolerance demonstrated less expression for all three *Hsp* genes than families with low resistance to heat [37]. The production of heat-shock proteins is in fact highly expensive for the cell and may cause toxicity at the cellular level [225;226]. Since both temperate and tropical strains downregulated Hsp, this might suggest that *Ae. albopictus* evolved better responses to thermal stress, avoiding the damage caused by the high concentration of Hsp.

Moreover, I investigated the temperatures at which mosquitoes of both sexes reared at 18°C, 28°C and 32°C preferred to lay (*Tp)*. In general, spots around 26°C and 28°C were the most chosen (10.14% and 8.34%, respectively), and the average *Tp* ranged from 24.09°C to 28.01°C, with the latter being in accordance with the temperature used as standard in my research. Our results agree with previous research by Verhulst et al. (2020) [227], which reported that *Ae. aegpyti* and *Ae. japonicus* preferred temperatures colder than their *Topt* when inserted in a thermal gradient. In this study both strains of *Ae. albopictus* had a *Tp* lower than the *Topt* for this species (29.7°C) [180] despite the ambient temperature at which they were exposed during their whole development (from egg to adult). Choosing thermal regimes cooler than the *Topt* might be a strategy evolved in order to avoid the risk of stressful and damaging higher temperatures and to increase the longevity, while conserving energy [227]. When reared at 28°C, Fo mosquitoes were found in spots colder than Cr (Fo=25.7°C and Cr=28.1°C and Fo=24.09°C and Cr=26.3°C in males and females, respectively) and at 32°C Cr females were more evenly distributed than Fo, which had a peak in the middle segments of the gradient. This response shows that mosquitoes of the native strain are more likely to lay at cooler temperature than those of the temperate strain and therefore might have retained a stronger behavioural response to avoid excessive warm temperatures.

5.4 *Wolbachia* density declines at different temperatures depending on the host strain

Since *Wolbachia* is considered a thermal sensitive bacterium [160] the possible unbalance of *Wolbachia* in females due to exposure to cold and warm temperatures might induce alterations in CI, which in *Ae. albopictus* naturally occurs in crosses between *wAlbA* mono-infected females and bi-infected males, resulting in non-viable embryos [166]. My results show that most of the variations of *Wolbachia* density were related to *wAlbB* and the effect of temperature was specific for each mosquito strain. Since the age of reproductive maturity in *Ae. albopictus* females starts at 8 days post-emergence [228] different thermal regimes (either hot or cold temperatures) might cause the most deleterious effect on reproduction due to the possible alteration of CI in a strain specific manner: while in Cr after seven days *wAlbB* density was at its lowest in the warmest thermal regime, in Fo the coldest temperature was the condition with the lowest amount of *Wolbachia.* These results suggest that Cr females are be more likely to be mono-

infected with *wAlbA* or harbouring less *wAlbB* at warm ambient temperatures when they are seven days old, whereas the native strain females might have a decrease in *wAlbB* at cold conditions at the same age, and therefore their mating with bi-infected males would cause CI. However, studies using diurnal field relevant temperature cycles, and possibly other ecological parameters, should be considered using different host populations and *Wolbachia* variants.

5.5 Implications for *Ae. albopictus* distribution and disease transmission

While *Aedes* mosquitoes are expected to shift both in terms of geographical and seasonal distributions due to climate change [132;133], my results suggest that *Ae. albopictus* strains of distinct geographical origin could be affected differently by Ta: the temperate strain displayed a better vector fitness performance at a lower temperature than the tropical strain. However, since my study considers only to two strains, further analyses investigating more strains collected from multiple sites in the same countries (Italy and China) are needed to confirm the differences emerged between populations of the two localities.

My results also prove that Italian late-spring and summer conditions have a strong impact on *Ae. albopictus* life history traits; late-spring thermal condition might affect mostly young individuals, while in summer most of the effects could be on adults. The effect of ambient temperature on longevity of adult females is particularly important since the transmission of pathogens is directly correlated to the age of its vector [229]. Older females are more likely to get exposed and subsequently infected. After being infected, as females grow older, they are more likely to have overcome the EIP and therefore become infectious.

Moreover, by measuring *KDT* I could pose the critical thermal maximum (*CTmax*) of *Ae. albopictus* in the range between 45.31°C and 46.61°C. These results highlight the possibility that global warming might impact not only *Ae. albopictus* further invasion, but also its persistence in regions currently allowing its presence.

Within the Mediterranean Area, defined as climate change hotspot [230], Italy is currently experiencing the drastic effects of climate change, with 1499 extreme weather events being recorded in 2020 alone, almost four times those which occurred in 2010 [231]. Analysing thermal records in the period between 1961 and 2011, a warming trend resulted for both minimum and maximum temperatures in all year seasons, with summer having the biggest temperature increase, followed by spring [8]. In this context, my results suggest that *Ae. albopictus* vector fitness performance could be higher in spring than during summer months, if summer Ta exceeds 32°C. Temperature does not influence only vector fitness, but also its permissiveness to arbovirus. When *Ae. albopictus* was tested for vector competence to DENV and CHIKV at 18°C, only CHIKV was seen

reaching the salivary glands at 7 dpi [232]. When vector competence was tested at 32°C, both DENV-2 and CHIKV transmission were seen [233]. These data further suggest the higher risk of CHIKV transmission by *Ae. albopictu*s mosquitoes compared to DENV in Europe but particularly in Italy, especially in cool spring months.

6. References

[1] Gulev SK, Thorne PW, Ahn J, Dentener FJ, Domingues CM, Gerland S, et al. Changing State of the Climate System. *In* Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [Masson-Delmotte VP, Zhai A, Pirani SL, Connors C, Péan S, Berger N et al. (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA. 2021;87–422.

[2] Masson-Delmotte VP, Zhai A, Pirani SL, Connors C, Péan S, Berger N et al. (eds.). IPCC, 2021: Summary for Policymakers. *In* Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA. 2021;3−32.

[3] Eyring V, Gillett NP, Achuta Rao KM, Barimalala R, Barreiro Parrillo M, Bellouin N et al. Human Influence on the Climate System. *In* Climate Change 2021: The Physical Science Basis. Contribution of Working Group I to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change. [Masson-Delmotte VP, Zhai A, Pirani SL, Connors C, Péan S, Berger N et al. (eds.)]. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA. 2021;423–552.

[4] Haustein K., Allen MR, Forster PM, Otto FEL, Mitchell DM, Matthews HD and Frame DJ. A real-time Global Warming Index. Scientific Reports. 2017;7(15417).

[5] Matthews HD and Wynes S. Current global efforts are insufficient to limit warming to 1.5°C. Science. 2022;376(6600):1404-1409.

[6] Hermanson L, Smith D, Seabrook M, Bilbao R, Doblas-Reyes F, Tourigny E, Lapin V, Kharin VV, Merryfield WJ, and Sospedra-Alfonso R.WMO Global Annual to Decadal Climate Update A Prediction for 2021-25, Bulletin of the American Meteorological Society. 2022;103(4): E1117-E1129.

[7] Toreti A and Desiato F. Changes in temperature extremes over Italy in the last 44 years. International Journal of Climatology. 2008;28(6).

[8] Fioravanti G, Piervitali E and Desiato F. Recent changes of temperature extremes over Italy: an index-based analysis. Theoretical and Applied Climatology. 2016;123(3–4).

[9] Earls KN, Porter MS, Rinehart JP and Greenlee KJ. Thermal history of alfalfa leafcutting bees affects nesting and diapause incidence. The Journal of Experimental Biology. 2021;224(22).

[10] Deutsch CA, Tewksbury JJ, Huey RB, Sheldon KS, Ghalambor CK, Haak DC and Martin PR. Impacts of climate warming on terrestrial ectotherms across latitude. Proceedings of the National Academy of Sciences. 2006;105(18):6668-72.

[11] Harvey JA, Tougeron K, Gols R, Heinen R, Abarca M, Abram PK et al. Scientists' warning on climate change and insects. Ecological monographs. 2023;93(1): e1553.

[12] Rohr JR, Cohen JM. Understanding how temperature shifts could impact infectious disease. PLoS Biology. 2020;18(11): e3000938.

[13] Couper LI, Farner JE, Caldwell JM, Childs ML, Harris MJ, Kirk DG et al. How will mosquitoes adapt to climate warming? eLife. 2021; 10: e69630.

[14] Bale JS. Insects at low temperature: A predictable relationship? Functional Ecology. 1991; 5:291.

[15] Chown SL, Hoffmann AA, Kristensen TN, Angilletta MJ Jr, Stenseth NC and Pertoldi C. Adapting to climate change: a perspective from evolutionary physiology. Climate Research.2010;43:3-15.

[16] Kingsolver JG, Diamond SE and Buckley LB. Heat stress and the fitness consequences of climate change for terrestrial ectotherms. Functional Ecology. 2013; 27(6):1415–1423.

[17] Valladares F, Matesanz S, Guilhaumon F, Araújo MB, Balaguer L, Benito-Garzón M., et al. The effects of phenotypic plasticity and local adaptation on forecasts of species range shifts under climate change. Ecology Letters. 2014; 17:1351–1364.

[18] Sánchez-Guillén RA, Muñoz J, Rodríguez-Tapia G, Feria Arroyo TP and Córdoba-Aguilar A. Climate-Induced Range Shifts and Possible Hybridisation Consequences in Insects. PLoS ONE. 2013; 8(11): e80531.

[19] West-Eberhard MJ. Encyclopedia of Ecology. 2008.

[20] Hamdoun AM, Cheney DP, and Cherr GN. Phenotypic Plasticity of HSP70 and HSP70 Gene Expression in the Pacific Oyster (*Crassostrea gigas*): Implications for Thermal Limits and Induction of Thermal Tolerance. Biological Bulletin.2003;205(2):160–169.

[21] Bay AR and Palumbi SR. Rapid Acclimation Ability Mediated by Transcriptome Changes in Reef-Building Corals Genome Biology and Evolution.2015;7(6):1602–1612.

[22] Logan ML and Cox CL. Genetic Constraints, Transcriptome Plasticity, and the Evolutionary Response to Climate Change. Frontiers in Genetics. 2020;11.

[23] Chen J, Nolte V and Schlötterer C. Temperature-Related Reaction Norms of Gene Expression: Regulatory Architecture and Functional Implications. Molecular Biology and Evolution. 2015;32(9):2393–2402.

[24] Parkash R, Lambhod C and Pathak A. Developmental and adult acclimation impact cold and drought survival of invasive tropical *Drosophila kikkawai*. Biology Open. 2021;10(6): bio058527.

[25] Balanyá J, Oller JM, Huey RB, Gildchrist GW and Serra L. Global Genetic Change Tracks Global Climate Warming in *Drosophila subobscura*. Science. 2006; 313:1773-1775.

[26] Rodrigues, Y.K., Beldade, P. Thermal Plasticity in Insects' Response to Climate Change and to Multifactorial Environments. Frontiers in Ecology and Evolution. 2020;8.

[27] Schulte PM, Healy TM and Fangue NA. Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. Integrative and Comparative Biology. 2011;51(5).

[28] Sørensen JG, Nielsen MM, Kruhøffer M, Justesen J and Loeschcke V. Full genome gene expression analysis of the heat stress response in *Drosophila melanogaster*. Cell Stress and Chaperones. 2005;10(4).

[29] Barley JM, Cheng BS, Sasaki M, Gignoux-Wolfsohn S, Hays C G, Putnam AB, Sheth S, Villeneuve AR and Kelly M. Limited plasticity in thermally tolerant ectotherm populations: Evidence for a trade-off. Proceedings of the Royal Society B: Biological Science. 2021;288(1958).

[30] Helms Cahan S, Nguyen AD, Stanton-Geddes J, Penick CA, Hernáiz-Hernández Y, DeMarco BB and Gotelli NJ. Modulation of the heat shock response is associated with acclimation to novel temperatures but not adaptation to climatic variation in the ants *Aphaenogaster picea* and *A. rudis*. Comparative Biochemistry and Physiology -Part A: Molecular and Integrative Physiology. 2017;204.

[31] Angilletta MJ and Dunham AE. The Temperature-Size Rule in Ectotherms: Simple Evolutionary Explanations May Not Be General. American Naturalist. 2003;162(3).

[32] Burraco P, Orizaola G, Monaghan P and Metcalfe NB. Climate change and ageing in ectotherms. Global Change Biology. 2020;26(10).

[33] Bodlah MA, Iqbal J, Ashiq A, Bodlah I, Jiang S, Mudassir MA, Rasheed MT and Fareen AGE. Insect behavioral restraint and adaptation strategies under heat stress: An inclusive review. Journal of the Saudi Society of Agricultural Sciences. 2023;22(6):327-350.

[34] Wojda I. Temperature stress and insect immunity. Journal of Thermal Biology. 2017;68.

[35] King AM and MacRae TH. Insect heat shock proteins during stress and diapause. Annual Review of Entomology. 2015;60.

[36] Ritossa F. A new puffing patter induced by temperature shock and DNP in *Drosphila.* Experientia. 1962; 18:571-573.

[37] Ware-Gilmore F, Novelo M, Sgrò CM, Hall MD and McGraw EA. Assessing the role of family level variation and heat shock gene expression in the thermal stress response of the mosquito *Aedes aegypti*. Philosophical Transactions of the Royal Society B: Biological Sciences. 2023;378(1873).

[38] Dmitriew CM. The evolution of growth trajectories: What limits growth rate? Biological Reviews. 2011;86(1).

[39] Metcalfe NB and Monaghan P. Compensation for a bad start: Grow now, pay later? Trends in Ecology and Evolution. 2001;16(5).

[40] Halliwell B and Gutteridge JMC. Free Radicals and Medicine. Biomedical EPR, Part A: Free Radicals, Metals, Medicine, and Physiology. 2015; 23: 25–74.

[41] Olsson M, Wapstra E and Friesen CR. Evolutionary ecology of telomeres: a review. Annals of the New York Academy of Sciences. 2018;1422(1).

[42] Diniz DFA, De Albuquerque CMR, Oliva LO, De Melo-Santos MAV and Ayres CFJ. Diapause and quiescence: Dormancy mechanisms that contribute to the geographical expansion of mosquitoes and their evolutionary success. Parasites and Vectors. 2017;10(1).

[43] Tauber MJ and Tauber CA: Insect seasonality: Diapause maintenance, termination, and post diapause development. Annual Review of Entomology. 1976; 21:81-107.

[44] Denlinger DL and Armbruster PA. Mosquito diapause. Annual Review of Entomology. 2014; 59: 73-93.

[45] Arrese EL and Soulages JL. Insect fat body: Energy, metabolism, and regulation. Annual Review of Entomology 2010; 55.

[46] Sinclair BJ and Marshall KE. The many roles of fats in overwintering insects. Journal of Experimental Biology. 2018;121.

[47] Storey KB. Organic solutes in freezing tolerance. Comparative Biochemistry and Physiology - A Physiology. 1997;117(3).

[48] Denlinger DL. Relationship between cold hardiness and diapause. In: Lee RE and Denlinger DL editors. Insects at Low Temperature. New York: Chapman & Hall. 1991;174–198.

[49] Salvucci ME. Effect of the α-glucosidase inhibitor, bromoconduritol, on carbohydrate metabolism in the silverleaf whitefly, *Bemisia argentifolii.* Archives of Insect Biochemistry and Physiology. 2000;45(3).

[50] Chowanski S, Lubawy J, Spochacz M, Ewelina P, Grzegorz S, Rosinski G and Slocinska M. Cold induced changes in lipid, protein and carbohydrate levels in the tropical insect *Gromphadorhina coquereliana.* Comparative Biochemistry and Physiology -Part A: Molecular and Integrative Physiology. 2015;183.

[51] Rombough P. Modelling developmental time and temperature. Nature. 2003;424(6946).

[52] Jarošík V, Kratochvíl L, Honěk A and Dixon AFG. A general rule for the dependence of developmental rate on temperature in ectothermic animals. Proceedings of the Royal Society B: Biological Sciences. 2004;271(SUPPL. 4).

[53] Folguera G, Mensch J, Muñoz JL, Ceballos SG, Hasson E and Bozinovic F. Ontogenetic stage-dependent effect of temperature on developmental and metabolic rates in a holometabolous insect. Journal of Insect Physiology. 2010;56(11).

[54] Colvard NB and Helmuth B. Thermal Biology. *In*: Kennish, M.J. (eds) Encyclopedia of Estuaries. Encyclopedia of Earth Sciences Series. Springer, Dordrecht. 2016;661-664.

[55] Mordecai EA, Caldwell JM, Grossman, MK, Lippi CA, Johnson LR, Neira M, Rohr JR, Ryan SJ, Savage V, Shocket MS, Sippy R, Stewart Ibarra AM, Thomas MB and Villena O. Thermal biology of mosquito-borne disease. Ecology Letters. 2019;22(10).

[56] Angilletta MJ. Thermal Adaptation: A Theoretical and Empirical Synthesis. In Thermal Adaptation: A Theoretical and Empirical Synthesis. Oxford University Press. 2009.

[57] Buckley LB. Linking traits to energetics and population dynamics to predict lizard ranges in changing environments. American Naturalist. 2008;171(1).

[58] Guerra-Correa ES, Merino-Viteri A, Andrango MB and Torres-Carvajal O. Thermal biology of two tropical lizards from the Ecuadorian Andes and their vulnerability to climate change. PLoS ONE. 2020;5(1).

[59] Tao S, Cheng K, Li X, Han X, Wang J, Zheng R and Sun B. The Thermal Biology of *Takydromus kuehnei* Indicates Tropical Lizards from High Elevation Have Not Been Severely Threatened by Climate Change. Frontiers in Ecology and Evolution. 2021;9.

[60] Pawar S, Huxley PJ, Smallwood TRC, Nesbit ML, Chan AHH, Shocket MS, Johnson LR, Kontopoulos D-G and Cator L. Variation in temperature of peak trait performance constrains adaptation of arthropod populations to climatic warming. bioRxiv. 2023;2023.01.18.524448.

[61] Hangartner SB, Hoffmann AA, Smith A and Griffin PC. A collection of Australian *Drosophila* datasets on climate adaptation and species distributions. Scientific Data. 2015;2.

[62] Hansen MM, Olivieri I, Waller DM and Nielsen EE. Monitoring adaptive genetic responses to environmental change. Molecular Ecology. 2012;21(6).

[63] Lahondère C and Bonizzoni M. Thermal biology of invasive *Aedes* mosquitoes in the context of climate change. Current Opinion in Insect Science. 2022;51.

[64] MacLean HJ, Sørensen JG, Kristensen TN, Loeschcke V, Beedholm K, Kellermann V and Overgaard J. Evolution and plasticity of thermal performance: An analysis of variation in thermal tolerance and fitness in 22 *Drosophila* species. Philosophical Transactions of the Royal Society B: Biological Sciences. 2019;374(1778).

[65] Mayekar HV, Ramkumar DK, Garg D, Nair A, Khandelwal A, Joshi K, and Rajpurohit S. Clinal variation as a tool to understand climate change. Frontiers in Physiology. 2022;13.

[66] Rodrigues MF and Cogni R. Genomic Responses to Climate Change: Making the Most of the *Drosophila* Model. Frontiers in Genetics. 2021;12.

[67] Sørensen JG, Kristensen TN and Overgaard J. Evolutionary and ecological patterns of thermal acclimation capacity in *Drosophila*: is it important for keeping up with climate change? In Current Opinion in Insect Science. 2016;17.

[68] Huey RB and Stevenson RD. Integrating thermal physiology and ecology of ectotherms: A discussion of approaches. Integrative and Comparative Biology.1979;19(1).

[69] Huey RB and Hertz PE. Is a Jack-of-All-Temperatures a Master of None? Evolution.1984;38(2).

[70] Paaijmans KP, Heinig RL, Seliga RA., Blanford JI, Blanford S, Murdock CC and Thomas MB. Temperature variation makes ectotherms more sensitive to climate change. Global Change Biology.2013;19(8).

[71] Deere JA and Chown SL. Testing the beneficial acclimation hypothesis and its alternatives for locomotor performance. American Naturalist.2006;168(5).

[72] Morgan R, Andreassen AH, Åsheim ER, Finnøen MH, Dresler G, Brembu T, Loh A, Miest JJ and Jutfelt F. Reduced physiological plasticity in a fish adapted to stable temperatures. Proceedings of the National Academy of Sciences of the United States of America. 2022;119(22).

[73] Huey RB and Kingsolver JG. Evolution of thermal sensitivity of ectotherm performance. Trends in Ecology and Evolution. 1989;4(5).

[74] da Silva CRB, Riginos C and Wilson RS. An intertidal fish shows thermal acclimation despite living in a rapidly fluctuating environment. Journal of Comparative Physiology B: Biochemical, Systemic, and Environmental Physiology. 2019;189(3–4).

[75] Angilletta MJ, Niewiarowski PH and Navas CA. The evolution of thermal physiology in ectotherms. Journal of Thermal Biology. 2022;27(4).

[76] Dillon ME, Liu R, Wang G and Huey RB. Disentangling thermal preference and the thermal dependence of movement in ectotherms. Journal of Thermal Biology.2012;37(8).

[77] Socha W, Kwasnik M, Larsk, M, Rola J and Rozek W. Vector-Borne Viral Diseases as a Current Threat for Human and Animal Health—One Health Perspective. In Journal of Clinical Medicine. 2022;11(11).

[78] Huang W, Reyes-Caldas P, Mann M, Seifbarghi S, Kahn A, Almeida RPP, Béven L, Heck M, Hogenhout SA and Coaker. Bacterial Vector-Borne Plant Diseases: Unanswered Questions and Future Directions. In Molecular Plant. 2020; 13(10).

[79] Marselle MR, Stadler J, Korn H, Irvine KN and Bonn A. Biodiversity and Health in the Face of Climate Change. Springer Open. 2019.

[80] World Health Organization https://www.who.int/news-room/fact-sheets/detail/vector-borne-diseases, accessed July 14th, 2023.

[81] Wilson AL, Courtenay O, Kelly-Hope LA, Scott TW, Takken W, Torr SJ and Lindsay SW. The importance of vector control for the control and elimination of vector-borne diseases. In PLoS Neglected Tropical Diseases. 2020;14(1).

[82] Kraemer MU, Reiner RC, Brady OJ, Messina JP, Gilbert M, Pigott DM, Yi D, Johnson K, Earl L, Carvalho RG, et al. Past and future spread of the arbovirus vectors *Aedes aegypti* and *Aedes albopictus*. Nature Microbiology. 2019; 4:854-863.

[83] Chadee DD, Martinez R: *Aedes aegypti* (L.) in Latin American and Caribbean region: with growing evidence for vector adaptation to climate change? Acta Tropica.2016;156:137-143.

[84] Azar SR and Weaver SC. Vector Competence: What Has Zika Virus Taught Us? Viruses. 2019; 11(867).

[85] Beerntsen BT, James AA and Christensen BM. Genetics of mosquito vector competence. Microbiology and Molecular Biology Reviews. 2000;64(1):115-37.

[86] Kramer LD and Ciota AT. Dissecting vectorial capacity for mosquito-borne viruses. Current Opinion in Virology. 2015; 15:112-8.

[87] Bellone R and Failloux AB. The Role of Temperature in Shaping Mosquito-Borne Viruses Transmission. Frontiers in Microbiology. 2020; 11:584846.

[88] Kamiya T, Greischar MA, Wadhawan K, Gilbert B, Paaijmans K and Mideo N. Temperature-dependent variation in the extrinsic incubation period elevates the risk of vector-borne disease emergence. Epidemics. 2019; 30:100382.

[89] Thomas MB and Blanford S. Thermal biology in insect-parasite interactions. Trends in Ecology & Evolution. 2003; 18:344–350.

[90] Woodbridge AF and Walker ED. MOSQUITOES (Culicidae) In Medical and Veterinary Entomology. Mullen G and Durden L (eds.). 2002; Chapter 12, 203-262.

[91] Harbach R. E. 2020. Mosquito taxonomic inventory. http://mosquito-taxonomic-inventory.info/, accessed July 10th, 2023.

[92] Debboun M, Reyna Nava M and Rueda LM. Mosquitoes, Communities, and Public Health in Texas. Academic Press. 2020.

[93] Loaiza JR, Bermingham E, Sanjur OI, Scott M, Bickersmith SA et al. Review of genetic diversity in malaria vectors (Culicidae: Anophilinae). Infection, Genetics and Evolution.2012;12: 1–12.

[94] Ribeiro H. Three new species and one new subspecies of *Toxorhynchites* (Diptera: Culicidae) of the afrotropical region. Journal of Medical Entomology.2005;42(3):224-32.

[95] Donald CL, Siriyasatien P and Kohl A. *Toxorhynchites* Species: A Review of Current Knowledge. Insects. 2020;11(11):747.

[96] Agarwal A, Parida M and Dash PK. Impact of transmission cycles and vector competence on global expansion and emergence of arboviruses. Reviews in Medical Virology.2017;27: e1941.

[97] Hamaidia K and Soltani N. New report of *Aedes albopictus* in Souk Ahras, Northeast Algeria. Biodiversitas Journal of Biological Diversity. 2021;22(7):2901-2906.

[98] Scholte EJ and Schaffner F: Waiting the tiger: establishment and spread of the *Aedes albopictus* mosquito in Europe. *In*: Emerging Pests and VectorBorne Diseases in Europe. Takken W, Knols BGJ (eds.). Wageningen: Wageningen Academic Publishers. 2007;241-260.

[99] Hawley AH. The biology of *Aedes albopictus*. Journal of the American Mosquito Control Association. 1988; 4:2e39.

[100] Paupy C, Delatte H, Bagny L, Corbel V and Fontenille D. *Aedes albopictus*, an arbovirus vector: from the darkness to the light. Microbes and Infection. 2009; 11:1177-85.

[101] Delatte H, Desvars A, Bouétard A, Bord S, Gimonneau G, Vourc'h G and Fontenille D. Blood-feeding behavior of *Aedes albopictus*, a vector of Chikungunya on La Réunion. Vector-Borne and Zoonotic Diseases. 2010; 10(3):249-58.

[102] Gubler DJ. Comparison of reproductive potentials of *Aedes* (Stegimyia) *albopictus* Skuse and *Aedes* (Stegomyia) *polynesiensis* Marks. Mosquito News. 1970; 30:201-209.

[103] Weaver SC and Barrett AD. Transmission cycles, host range, evolution and emergence of arboviral disease. Nature Reviews Microbiology. 2004;2(10):789-801.

[104] Weaver SC, Forrester NL, Liu J. et al. Population bottlenecks and founder effects: implications for mosquito-borne arboviral emergence. Nature Reviews Microbiology. 2021;19, 184–195.

[105] Focks DA, Daniels E, Haile DG and Keesling JE. A Simulation Model of the Epidemiology of Urban Dengue Fever: Literature Analysis, Model Development, Preliminary Validation, and Samples of Simulation Results. The American Journal of Tropical Medicine and Hygiene. 1995;53(5):489-506.

[106] McKenzie BA, Wilson AE and Zohdy S. *Aedes albopictus* is a competent vector of Zika virus: A meta-analysis. PLoS ONE. 2019; 14(5): e0216794.

[107] Li Y, Kamara F, Zhou G, Puthiyakunnon S, Li C, et al. Urbanization Increases *Aedes albopictus* Larval Habitats and Accelerates Mosquito Development and Survivorship. PLoS Neglected Tropical Diseases.2014;8(11): e3301.

[108] Westby KM, Adalsteinsson SA, Biro EG, Beckermann AJ and Medley KA. *Aedes albopictus* Populations and Larval Habitat Characteristics across the Landscape: Significant Differences Exist between Urban and Rural Land Use Types. Insects. 2021;12(3):196.

[109] Adhami J and Reiter P. Introduction and establishment of *Aedes* (Stegomyia) *albopictus* Skuse (Diptera: Culicidae) in Albania. Journal of the American Mosquito Control Association. 1998; 14:340–3.

[110] Qualls WA, Xue RD, Beier JC and Müller GC. Survivorship of adult *Aedes albopictus* (Diptera: Culicidae) feeding on indoor ornamental plants with no inflorescence. Parasitology Research.2013;112(6):2313-8.

[111] Caminade C, Medlock JM, Ducheyne E, McIntyre KM, Leach S, Baylis M and Morse AP. Suitability of European climate for the Asian tiger mosquito *Aedes albopictus*: recent trends and future scenarios. Journal of the Royal Society Interface. 2012; 9:2708–2717.

[112] Benedict MQ, Levine RS, Hawley WA and Lounibos LP. Spread of the tiger: global risk of invasion by the mosquito *Aedes albopictus*. Vector-Borne and Zoonotic Diseases. 2007; 7:76–85.

[113] Gimonneau G, Delatte H, Triboire A and Fontenille D. Influence of Temperature on Immature Development, Survival, Longevity, Fecundity, and Gonotrophic Cycles of *Aedes albopictus*, Vector of Chikungunya and Dengue in the Indian Ocean. Journal of Medical Entomology. 2009; 46:33–41.

[114] Bonizzoni M, Gasperi G, Chen X and James AA. The invasive mosquito species *Aedes albopictus*: current knowledge and future perspectives. Trends in Parasitology. 2013;29(9):460-8.

[115] Kraemer MUG, Reiner RC, Brady OJ, Messina JP, Gilbert M, Pigott DM, et al. Past and future spread of the arbovirus vectors *Aedes aegypti* and *Aedes albopictus*. Nature Microbiology. 2019; 4:854–63.

[116] Kraemer MUG, Sinka ME, Duda KA, Mylne AQN, Shearer FM, Barker CM, et al. The global distribution of the arbovirus vectors *Aedes aegypti* and *Ae. albopictus*. Elife. 2015;4: e08347–e08347.

[117] Manni M, Guglielmino CR, Scolari F, Vega-Rua A, Failloux A-B, Somboon P, et al. Genetic evidence for a worldwide chaotic dispersion pattern of the arbovirus vector, *Aedes albopictus*. PLoS Neglected Tropical Diseases. 2017;11: e0005332.

[118] Medlock JM, Hansford KM, Schaffner F, Versteirt V, Hendrickx G, Zeller H and Van Bortel W. A review of the invasive mosquitoes in Europe: ecology, public health risks, and control options. Vector-Borne Zoonotic Diseases. 2012;12(6):435-47.

[119] Scholte E, Jacobs F, Linton Y, Dijkstra E, Fransen J and Takken W. First record of *Aedes* (Stegomyia) *albopictus* in the Netherlands. European Mosquito Bulletin.2007;22:5–9.

[120] Oliveira S, Rocha J, Sousa CA et al. Wide and increasing suitability for *Aedes albopictus* in Europe is congruent across distribution models. Scientific Reports. 2021;11, 9916.

[121] Hahn MB, Eisen RJ, Eisen L, Boegler KA, Moore CG, McAllister J, et al. Reported Distribution of *Aedes* (Stegomyia) *aegypti* and *Aedes* (Stegomyia) *albopictus* in the United States, 1995-2016 (Diptera: Culicidae). Journal of Medical Entomology. 2016; 53:1169–75.

[122] Rai KS. *Aedes albopictus* in the Americas. Annual Review of Entomology. 1991; 36:459–484.

[123] Kotsakiozi P, Richardson JB, Pichler V, Favia G, Martins AJ, Urbanelli S, et al. Population genomics of the Asian tiger mosquito, *Aedes albopictus*: insights into the recent worldwide invasion. Ecology and Evolution. 2017; 7:10143–57.

[124] Vega-Rúa A, Marconcini M, Madec Y, Manni M, Carraretto D, Gomulski LM, et al. Vector competence of *Aedes albopictus* populations for chikungunya virus is shaped by their demographic history. Communications Biology. 2020; 3:326.

[125] Fontenille D and Rodhain F. Biology and distribution of *Aedes albopictus* and Aedes aegypti in Madagascar. Journal of the American Mosquito Control Association. 1989;5(2):219-25.

[126] Cornel AJ, Hunt RH. *Aedes albopictus* in Africa? First records of live specimens in imported tires in Cape Town. Journal of the American Mosquito Control Association. 1991;7(1):107-8.

[127] Savage H M, Ezike VI, Nwankwo A, Spiegel RC and Miller BR. First record of breeding populations of *Aedes albopictus* in continental Africa: implications for arboviral transmission. Journal of the American Mosquito Control Association.1992; 8(1):101-3.

[128] Fontenille D and Toto JC. *Aedes* (Stegomyia) *albopictus* (Skuse), a potential new Dengue vector in southern Cameroon. Emerging Infectious Diseases. 2001;7(6):1066-7.

[129] Coffinet T, Mourou JR, Pradines B, Toto JC, Jarjaval F, Amalvict R, Kombila M, Carnevale P and Pages F. First record of *Aedes albopictus* in Gabon. Journal of the American Mosquito Control Association. 2007;23(4):471-2.

[130] Toto JC, Abaga S, Carnevale P and Simard F. First report of the oriental mosquito *Aedes albopictus* on the west African island of Bioko, Equatorial Guinea. Medical and Veterinary Entomology. 2003; 17(3):343–346.

[131] Benelli G, Wilke ABB and Beier JC. *Aedes albopictus* (Asian Tiger Mosquito). Trends in Parasitology. 2020;36(11):942-943.

[132] Escobar LE, Romero-Alvarez D, Leon R, Lepe-Lopez MA, Craft ME, Borbor-Cordova MJ, et al. Declining Prevalence of Disease Vectors Under Climate Change. Scientific Report. 2016;6.

[133] Campbell LP, Luther C, Moo-Llanes D, Ramsey JM, Danis-Lozano R and Peterson AT. Climate change influences on global distributions of dengue and chikungunya virus vectors. Philosophical Transactions of the Royal Society B: Biological Sciences. 2015; 370:20140135.

[134] Stone CM, Zuo Z, Li B, Ruiz M, Swanson J, Hunt J, Kim C-H and Smith RL. Spatial, Temporal, and Genetic Invasion Dynamics of *Aedes albopictus* (Diptera: Culicidae) in Illinois. Journal of medical entomology. 2020;57(5), 1488-1500.

[135] Battaglia V, Agostini V, Moroni E, Colombo G, Lombardo G, Rambaldi Migliore N, Gabrieli P, Garofalo M, Gagliardi S, Gomulski LM, Ferretti L, Semino O, Malacrida AR, Gasperi G, Achilli A, Torroni A and Olivieri A. The worldwide spread of *Aedes albopictus*: new insights from mitogenomes. Frontiers in Genetics. 2022;13(931163).

[136] Pichler V, Kotsakiozi P, Caputo B, Serini P, Caccone A and Della Torre A. Complex interplay of evolutionary forces shaping population genomic structure of invasive *Aedes albopictus* in southern Europe. PLoS Neglected Tropical Disease. 2019;13(8).

[137] Vavassori L, Honnen A-C, Saarman N, Caccone A and Müller P. Multiple introductions and overwintering shape the progressive invasion of *Aedes albopictus* beyond the Alps. Ecology and Evolution. 2022;29(9),460-468.

[138] Lafferty KD. The ecology of climate change and infectious diseases. Ecology. 2009; 90: 888–900.

[139] Githeko AK, Lindsay SW, Confalonieri UE and Patz JA. Climate change and vector-borne diseases: a regional analysis. Bull World Health Organ. 2000; 78: 1136–1147.

[140] Messina JP, Brady OJ, Pigott DM, Golding N, Kraemer MU, Scott TW, et al. The many projected futures of dengue. Nature Reviews Microbiology. 2015; 13: 230–239.

[141] Paz S. Climate change impacts on West Nile virus transmission in a global context. Philosophical Transactions of the Royal Society B: Biological Sciences. 2015;370(1665):20130561.

[142] Carlson CJ, Dougherty ER and Getz W. An ecological assessment of the pandemic threat of Zika virus. PLoS Neglected Tropical Diseases. 2016;10: e0004968.

[143] Ryan SJ, Carlson CJ, Mordecai EA and Johnson LR. Global expansion and redistribution of *Aedes*-borne virus transmission risk with climate change. PLoS Neglected Tropical Diseases. 2019;13(3): e0007213.

[144] Scolari F, Casiraghi M and Bonizzoni M. *Aedes spp*. and their microbiota: a review. Frontiers in Microbiology. 2019; 10:2036.

[145] Chen H, Zhang M and Hochstrasser M. The Biochemistry of Cytoplasmic Incompatibility Caused by Endosymbiotic Bacteria. Genes (Basel). 2020;11(8):852.

[146] Gharabigloozare Y and Bleidorn C. Effect of high temperature on *Wolbachia* density and impact on cytoplasmic incompatibility in confused flour beetle, *Tribolium confusum* (Coleoptera: Tenebrionidae). BMC Research Notes. 2022;15: 240.

[147] Zhou W, Rousset F and O'Neil S. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. Proceedings of the Royal Society B: Biological Sciences. 1998;265(1395):509-15.

[148] Pereira TN, Rocha MN, Sucupira PHF et al. *Wolbachia* significantly impacts the vector competence of *Aedes aegypti* for Mayaro virus. Scientific Reports. 2018; 8:6889.

[149] Hague MTJ, Caldwell CN and Cooper BS. Pervasive effects of *Wolbachia* on host temperature preference. mBio. 2020; 11: e01768-20.

[150] Dutton TJ and Sinkins SP. Strain-specific quantification of *Wolbachia* density in *Aedes albopictus* and effects of larval rearing conditions. Insect Molecular Biology. 2004;13(3):317–322.

[151] Sinkins SP. *Wolbachia* and cytoplasmic incompatibility in mosquitoes. Insect Biochemistry and Molecular Biology. 2004;34(7):723-9.

[152] Fraser JE, De Bruyne JT, Iturbe-Ormaetxe I, Stepnell J, Burns RL, Flores HA and O'Neill SL. Novel Wolbachia-transinfected *Aedes aegypti* mosquitoes possess diverse fitness and vector competence phenotypes. PLoS Pathogens. 2017;13: e1006751.

[153] Ant TH, Herd CS, Geoghegan V, Hoffmann AA and Sinkins SP. The *Wolbachia* strain *wAu* provides highly efficient virus transmission blocking in *Aedes aegypti*. PLoS Pathogens. 2018;14: e1006815.

[154] Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, Greenfield M, Durkan M, Leong YS, Dong Y, Cook H, Axford J, Callahan AG, Kenny N, Omodei C, McGraw EA, Ryan PA, Ritchie SA, Turelli M and O'Neill SL. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. Nature. 2011; 476:454–457.

[155] Garcia GDA, Sylvestre G, Aguiar R, da Costa GB, Martins AJ, Lima JBP, Petersen MT, Lourenço-de-Oliveira R, Shadbolt MF, Rašić G, Hoffmann AA, Villela DAM, Dias FBS, Dong Y, O'Neill SL, Moreira LA and Maciel-de-Freitas R. Matching the genetics of released and local *Aedes aegypti* populations is critical to assure *Wolbachia* invasion. PLoS Neglected Tropical Diseases. 2019;13: e0007023.

[156] Nazni WA, Hoffmann AA, NoorAfizah A, Cheong YL, Mancini MV, Golding N, Kamarul GMR, Arif MAK, Thohir H, NurSyamimi H, ZatilAqmar MZ, NurRuqqayah M, NorSyazwani A, Faiz A, Irfan FMN, Rubaaini S, Nuradila N, Nizam NMN, Irwan SM, Endersby-Harshman NM, White VL, Ant TH, Herd CS, Hasnor AH, AbuBakar R, Hapsah DM, Khadijah K, Kamilan D, Lee SC, Paid YM, Fadzilah K, Topek O, Gill BS, Lee HL and Sinkins SP. Establishment of *Wolbachia* strain *wAlbB* in Malaysian populations of *Aedes aegypti* for dengue control. Current Biology 2019; 29:4241–4248.e5.

[157] Pietri JE, DeBruhl H and Sullivan W. The rich somatic life of *Wolbachia*. Microbiologyopen. 2016;5(6):923-936.

[158] Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F and O'Neill SL. *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochemistry and Molecular Biology. 1999;29(2):153-60.

[159] Zouache K, Voronin D, Tran-Van V, Mousson L, Failloux A-B, et al. Persistent *Wolbachia* and Cultivable Bacteria Infection in the Reproductive and Somatic Tissues of the Mosquito Vector *Aedes albopictus*. PLoS ONE. 2009; 4(7): e6388.

[160] Mancini MV, Ant TH, Herd CS, Martinez J, Murdochy SM, Gingell DD, Mararo E, Johnson PCD and Sinkins SP. High Temperature Cycles Result in Maternal Transmission and Dengue Infection Differences Between *Wolbachia* Strains in *Aedes aegypti*. mBio. 2021;12(6): e0025021.

[161] Ulrich JN, Beier JC, Devine GJ and Hugo LE. Heat Sensitivity of *wMel Wolbachia* during *Aedes aegypti* Development. PLoS Neglected Tropical Diseases. 2016; 10(7): e0004873.

[162] Ross PA, Wiwatanaratanabutr I, Axford JK, White VL, Endersby-Harshman NM and Hoffmann AA. *Wolbachia* Infections in *Aedes aegypti* Differ Markedly in Their Response to Cyclical Heat Stress. PLoS Pathogens. 2017;13(1): e1006006.

[163] Ross PA, Axford JK, Yang Q, Staunton KM, Ritchie SA, Richardson KM, et al. Heatwaves cause fluctuations in *wMel Wolbachia* densities and frequencies in *Aedes aegypti*. PLoS Neglected Tropical Diseases. 2020; 14(1): e0007958.

[164] Lau MJ, Ross PA, Endersby-Harshman NM and Hoffmann AA. Impacts of Low Temperatures on *Wolbachia* (Rickettsiales: Rickettsiaceae)-Infected *Aedes aegypti* (Diptera: Culicidae). Journal of Medical Entomology. 2020;57(5):1567-1574.

[165] Truitt AM, Kapun M, Kaur R and Miller WJ. *Wolbachia* modifies thermal preference in *Drosophila melanogaster*. Environmental Microbiology. 2018;21(9):3259–68.

[166] Sicard M, Bonneau M and Weill M. *Wolbachia* prevalence, diversity, and ability to induce cytoplasmic incompatibility in mosquitoes. Current Opinion in Insect Science. 2019; 34:12-20.

[167] Alphey L, Benedict M, Bellini R, Clark GG, Dame DA, Service MW, et al. Sterile insect methods for control of mosquito-borne disease: an analysis. Vector- Borne Zoonotic Diseases. 2010; 10:295–311.

[168] Zhang D, Lees RS, Xi Z, Gilles JRL and Bourtzis K. Combining the Sterile Insect Technique with *Wolbachia*-Based Approaches: II- A Safer Approach to *Aedes albopictus* Population Suppression Programmes, Designed to Minimize the Consequences of Inadvertent Female Release. PLoS ONE. 2015; 10(8): e0135194.

[169] Walker T, Johnson PH, Moreira LA et al. The *wMel Wolbachia* strain blocks dengue and invades caged *Aedes aegypti* populations. Nature.2011;476:450–3.

[170] Hedges LM, Brownlie JC, O'Neill SL and Johnson KN. *Wolbachia* and virus protection in insects. Science 2008; 322:702.

[171] Caragata EP, Rocha MN, Pereira TN, Mansur SB, Dutra HLC and Moreira LA. Pathogen blocking in *Wolbachia*-infected *Aedes aegypti* is not affected by Zika and dengue virus coinfection. PLoS Neglected Tropical Diseases. 2019;13(5): e0007443.

[172] Blagrove MS, Arias-Goeta C, Failloux AB and Sinkins SP. *Wolbachia* strain *wMel* induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. Proceedings of the National Academy of Sciences. 2012;109(1):255-60.

[173] Bian G, Joshi D, Dong Y, Lu P, Zhou G, Pan X, Xu Y, Dimopoulos G and Xi Z. *Wolbachia* invades *Anopheles stephensi* populations and induces refractoriness to Plasmodium infection. Science. 2013;10:340(6133):748-51.

[174] Kambris Z, Cook PE, Phuc HK and Sinkins SP. Immune activation by life shortening *Wolbachia* and reduced filarial competence in mosquitoes. Science. 2009;326(5949):134-6.

[175] Pan X, Zhou G, Wu J, Bian G, Lu P, Raikhel AS and Xi Z. *Wolbachia* induces reactive oxygen species (ROS)-dependent activation of the Toll pathway to control

dengue virus in the mosquito *Aedes aegypti*. Proceedings of the National Academy of Sciences. 2012;109(1): E23-31.

[176] Terradas G, Joubert DA and McGraw EA. The RNAi pathway plays a small part in *Wolbachia*-mediated blocking of dengue virus in mosquito cells. Scientific Reports. 2017; 7:43847.

[177] Hussain M, O'Neill SL and Asgari S. *Wolbachia* interferes with the intracellular distribution of Argonaute 1 in the dengue vector *Aedes aegypti* by manipulating the host microRNAs. RNA Biology. 2013;10(12):1868-75.

[178] Caragata EP, Rance`s E, Hedges LM, Gofton AW, Johnson KN, et al. Dietary Cholesterol Modulates Pathogen Blocking by *Wolbachia*. PLoS Pathogens. 2013;9(6): e1003459.

[179] World Mosquito Program https://www.worldmosquitoprogram.org/en/work/wolbachia-method/how-it-works, accessed July 12th, 2023.

[180] Delatte H, Gimonneau G, Triboire A and Fontenille D. Influence of temperature on immature development, survival, longevity, fecundity, and gonotrophic cycles of *Aedes albopictus,* vector of chikungunya and dengue in the Indian Ocean. Journal of Medical Entomology. 2009; 46:33–41.

[181] Teng HJ and Apperson CS. Development and survival of immature *Aedes albopictus* and *Aedes triseriatus* (Diptera: Culicidae) in the laboratory: Effects of density, food, and competition on response to temperature. Journal of Medical Entomology. 2000; 37:40–52.

[182] Lowenberg Neto P and Navarro-Silva MA. Development, longevity, gonotrophic cycle and oviposition of *Aedes albopictus* Skuse (Diptera: Culicidae) under cyclic temperatures. Neotropical Entomology. 2004; 33:29–33.

[183] Sinclair BJ, Williams CM and Terblanche JS. Variation in thermal performance among insect populations. Physiological and Biochemical Zoology. 2012;85(6):594-606.

[184] Reinhold JM, Lazzari CR and Lahondère C. Effects of the Environmental Temperature on *Aedes aegypti* and *Aedes albopictus* Mosquitoes: A Review. Insects. 2018;9(4):158.

[185] Rowley WA and Graham CL. The effect of temperature and relative humidity on the flight performance of female *Aedes aegypti*. Journal of Insect Physiology. 1968; 14:1251–1257.

[186] Connor ME. Suggestions for Developing a Campaign to Control Yellow Fever. American Journal of Tropical Medicine and Hygiene. 1924;1: 277–307.

[187] Christophers SR *Aedes aegypti* (L.) the Yellow Fever Mosquito; Cambridge University Press: London, UK. 1960.

[188] Marchoux E, Salimbeni AT and Simond PL. La Fièvre Jaune: Rapport de la Mission Française; Annales de l'Institut Pasteur: Paris, France. 1903.

[189] World-weather https://world-weather.info/forecast/italy/crema/may-2021/, accessed December 1st, 2022.

[190] World-weather https://world-weather.info/forecast/italy/crema/august-2021/, accessed December 2nd, 2022.

[191] Paltini U, Masri RA, Cosme LV, Koren S, Thibaud-Nissen F, Biedler JK et al. Improved reference genome of the arboviral vector *Aedes albopictus*. Genome Biology.2020; 21(1).

[192] Chen XG, Jiang X, Gu J, Xu M, Wu Y, Deng et al. Genome sequence of the Asian tiger mosquito, *Aedes albopictus*, reveals insights into its biology, genetics, and evolution. Proceedings of the National Academy of Sciences of the United States of America. 2015; 112(44).

[193] Palatini U, Miesen P, Carballar-Lejarazu R, Ometto L, Rizzo E, Tu Z, van Rij R and Bonizzoni, M. Comparative genomics shows that viral integrations are abundant and express piRNAs in the arboviral vectors *Aedes aegypti* and *Aedes albopictus*. BMC Genomics. 2017; 18(1).

[194] Sinclair BJ, Marshall KE, Sewell MA, Levesque DL, Willett CS, Slotsbo S, Dong Y, Harley CD, Marshall DJ, Helmuth BS and Huey RB. Can we predict ectotherm responses to climate change using thermal performance curves and body temperatures? Ecology Letters. 2016;19(11):1372-1385.

[195] Robert LL and Olson JK. Effects of sublethal dosages of insecticides on *Culex quinquefasciatus*. Journal of the American Mosquito Control Association. 1989; 5:239-46.

[196] Armbruster P and Hutchinson RA. Pupal mass and wing length as indicators of fecundity in *Aedes albopictus* and *Aedes geniculatus* (Diptera: Culicidae). Journal of Medical Entomology. 2002;39(4):699-704.

[197] Nørgaard LS, Álvarez-Noriega M, McGraw E, White CR and Marshall DJ. Predicting the response of disease vectors to global change: The importance of allometric scaling. Global Change Biology. 2022;28(2):390-402.

[198] van Heerwaarden B and Sgrò CM. The effect of developmental temperature on the genetic architecture underlying size and thermal clines in *Drosophila melanogaster* and *D. simulans* from the east coast of Australia. Evolution. 2011;65(4):1048-67.

[199] Reinhold JM, Chandrasegaran K, Oker H, Crespo JE, Vinauger C and Lahondère C. Species-Specificity in Thermopreference and CO2-Gated Heat-Seeking in *Culex* Mosquitoes. Insects. 2022;13(1):92.

[200] Muturi EJ, Njoroge TM, Dunlap C and Cáceres CE. Blood meal source and mixed blood-feeding influence gut bacterial community composition in *Aedes aegypti*. Parasites and Vectors.2021; 14(1).

[201] Nag DK, Dieme C, Lapierre P, Lasek-Nesselquist E and Kramer LD. RNA-Seq analysis of blood meal induced gene-expression changes in *Aedes aegypti* ovaries. BMC Genomics. 2021;22(1).

[202] Camargo C, Ahmed-Braimah YH, Amaro IA, Harrington LC, Wolfner MF and Avila FW. Mating and blood-feeding induce transcriptome changes in the spermathecae of the yellow fever mosquito *Aedes aegypti*. Scientific Reports. 2020;10(1).

[203] Foray V, Pelisson PF, Bel-Venner MC, Desouhant E, Venner S, Menu F, Giron D and Rey B. A handbook for uncovering the complete energetic budget in insects: The van Handel's method (1985) revisited. Physiological Entomology. 2012; 37(3).

[204] Benoit JB and Denlinger DL. Suppression of water loss during adult diapause in the northern house mosquito, *Culex pipiens*. The Journal of Experimental Biology. 2007; 210(Pt 2):217-26.

[205] Lehmann PF and White LO. Chitin assay used to demonstrate renal localization and cortisone enhanced growth of *Aspergillus fumigatus* mycelium in mice. Immunity and Infection. 1975; 12:987–992.

[206] Zhao S, Ye Z and Stanton R. Misuse of RPKM or TPM normalization when comparing across samples and sequencing protocols. RNA. 202; 26(8).

[207] Love M, Huber W and Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology. 2015; 15, 550.

[208] Lozada-Chávez AN, Lozada-Chávez I, Alfano N, Palatini U, Sogliani D, Elfekih S, Degefa T, Sharakhova MV, Badolo A, Patchara S, Casas-Martinez M, Carlos BC, Carballar-Lejarazú R, Lambrechts L, Souza-Neto JA and Bonizzoni M. Molecular signature of domestication in the arboviral vector *Aedes aegypti*. bioRxiv. 2023;2023.03.13.532092.

[209] Götz S, García-Gómez. JM, Terol J, Williams TD, Nagaraj SH, Nueda MJ, Robles M, Talón M, Dopazo J and Conesa A. High-throughput functional annotation and data mining with the Blast2GO suite. Nucleic Acids Research. 2008;36(10):3420-35.

[210] Amos B, Aurrecoechea C, Barba M, Barreto A, Basenko EY, Bażant W et al. VEuPathDB: the eukaryotic pathogen, vector and host bioinformatics resource center. Nucleic Acids Research. 2022;50(D1): D898-D911.

[211] Jones P, Binns D, Chang HY, Fraser M, Li W, McAnulla C et al. InterProScan 5: genome-scale protein function classification. Bioinformatics. 2014;30(9):1236-40.

[212] Mistry J, Chuguransky S, Williams L, Qureshi M, Salazar GA, Sonnhammer ELL, Tosatto SCE, Paladin L, Raj S, Richardson LJ, Finn RD and Bateman A. Pfam: The protein families database in 2021. Nucleic Acids Research. 2021;49(D1): D412-D419.

[213] Sigrist CJ, de Castro E, Cerutti L, Cuche BA, Hulo N, Bridge A, Bougueleret L and Xenarios I. New and continuing developments at PROSITE. Nucleic Acids Research. 2013;41(Database issue): D344-7.

[214] Wilson D, Pethica R, Zhou Y, Talbot C, Vogel C, Madera M, Chothia C and Gough J. SUPERFAMILY--sophisticated comparative genomics, data mining, visualization and phylogeny. Nucleic Acids Research. 2009;37(Database issue): D380-6.

[215] Haft DH, Selengut JD and White O. The TIGRFAMs database of protein families. Nucleic Acids Research. 2003;31(1):371-3.

[216] Wu T, Hu E, Xu S, Chen M, Guo P, Dai Z et al. clusterProfiler 4.0: A universal enrichment tool for interpreting omics data. The Innovation. 2021; 2(3):100141.

[217] Martinez J, Ant TH, Murdochy SM, Tong L, Da Silva Filipe A and Sinkins S P. Genome sequencing and comparative analysis of *Wolbachia* strain *wAlbA* reveals *Wolbachia*-associated plasmids are common. PLoS Genetics. 2022;18(9).

[218] Kassambara A. ggpubr: 'ggplot2' Based Publication Ready Plots. https://CRAN.R-project.org/package=ggpubr. 2018.

[219] Schober P, Boer C and Schwarte LA. Correlation Coefficients: Appropriate Use and Interpretation. Anesthesia & Analgesia. 2018;126(5):1763-1768.

[220] Kassambara A and Mundt F. factoextra: Extract and Visualize the Results of Multivariate Data Analyses. https://CRAN.R-project.org/package=factoextra. 2020.

[221] Herrmann M and Yampolsky LY. False and true positives in arthropod thermal adaptation candidate gene lists. Genetica. 2021;149(3):143-153.

[222] Zamora-Camacho FJ, Reguera S and Moreno-Rueda G. Bergmann's Rule rules body size in an ectotherm: heat conservation in a lizard along a 2200-metre elevational gradient. Journal of Evolutionary Biology. 2014;27(12):2820-8.

[223] Rajpurohit S, Vrkoslav V, Hanus R, Gibbs AG, Cvačka J and Schmidt PS. Post-eclosion temperature effects on insect cuticular hydrocarbon profiles. Ecology and Evolution. 2020;11(1):352-364.

[224] Muthukrishnan S, Mun S, Noh MY, Geisbrecht ER and Arakane Y. Insect Cuticular Chitin Contributes to Form and Function. Current Pharmaceutical Design. 2020;26(29):3530-3545.

[225] Feder ME and Hofmann GE. Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. Annual Review of Physiology. 1999; 61:243-282.

[226] Krebs RA and Loeschcke V. Costs and benefits of activation of the heat-shock response in *Drosophila melanogaster*. Functional Ecology. 1994; 8:730-737.

[227] Verhulst NO, Brendle A, Blanckenhorn WU and Mathis A. Thermal preferences of subtropical *Aedes aegypti* and temperate *Ae. japonicus* mosquitoes. Journal of Thermal Biology. 2020; 91:102637.

[228] Hartman K. *Aedes albopictus* (On-line), Animal Diversity Web. 2021. https://animaldiversity.org/accounts/Aedes_albopictus/, accessed September 11th, 2023.

[229] Cator LJ, Johnson LR, Mordecai EA, Moustaid FE, Smallwood TRC, LaDeau SL, Johansson MA, Hudson PJ, Boots M, Thomas MB, Power AG and Pawar S. The Role of Vector Trait Variation in Vector-Borne Disease Dynamics. Frontiers in Ecology and Evolution. 2020; 8:189.

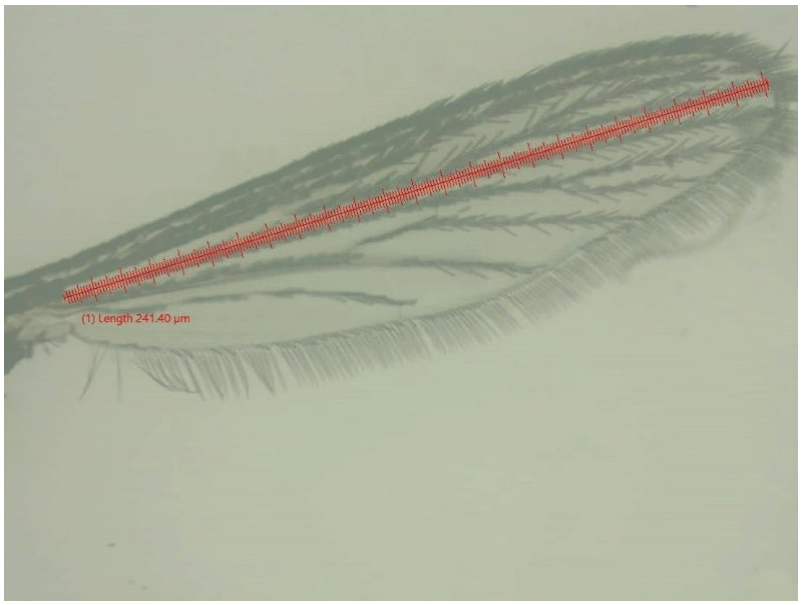
[230] Giorgi F. Climate change hot-spots. Geophysical Research Letters. 2006; 33: L08707.

[231] Levantesi S. Assessing Italy's climate risk. Nature Italy. 2021.

[232] Wimalasiri-Yapa BMCR, Stassen L, Hu W, Yakob L, McGraw EA, Pyke AT, Jansen CC, Devine GJ and Frentiu FD. Chikungunya Virus Transmission at Low Temperature by *Aedes albopictus* Mosquitoes. Pathogens. 2019;8(3):149.

[233] Westbrook CJ, Reiskind MH, Pesko KN, Greene KE and Lounibos LP. Larval environmental temperature and the susceptibility of *Aedes albopictus* Skuse (Diptera: Culicidae) to Chikungunya virus. Vector-Borne Zoonotic Diseases. 2010;10(3):241-7.

7. Appendix



Appendix 1. Dissected right wing which was measured from the axial incision to the apical margin, excluding the fringe of the scales (magnification 20x) as shown by the red scale.

Appendix 2. Means, standard deviations and number of individuals in brackets for each life-history trait, thermal trait and energy reserve tested.

Strains	**Fo**			**Cr**		
Temperature / **Traits**	**18°C**	**28°C**	**32°C**	**18°C**	**28°C**	**32°C**
egg hatchability rate (%)	52.3±10.7 (700)	81.67±17.17 (600)	56.1±24.7 (800)	52±10.36 (800)	71.25±9.71 (800)	51.75±24 (800)
egg hatching time (days)	14.012±3.7 (366)	3.93±1.06 (193)	4.83±1.83 (449)	9.77±1.59 (416)	3.44±0.73 (326)	7.74±1.45 (414)
larval viability (%)	68.8±14.2 (366)	78.21±7.65 (193)	76.6±9 (449)	70.99±8.8 (416)	66.63±7.47 (326)	47.75±8.12 (414)
pupal viability (%)	82.41±15.33 (224)	89.52±4.54 (133)	83.97±10.1 (294)	91.66±3.38 (251)	87.93±9.63 (205)	96.51±3.94 (165)
larval developmental time (days)	17.01±1.62 (259)	6.75±1.09 (149)	5.42±1.95 (351)	17.18±1.31 (274)	6.97±0.77 (223)	7.91±1.98 (170)

pupal developmental time (days)	4.67±0.86 (224)	1.83±0.2 (133)	1.54±0.71 (294)	5.35±0.55 (251)	1.70±0.17 (205)	1.8±0.32 (165)
developmental speed (days)	35.69±4.0 (224)	12.52±1.74 (133)	11.78±1.34 (294)	32.3±1.86 (251)	12.2±0.83 (205)	17.46±2.85 (165)
sex ratio (%)	47.04±9.17 (224)	47.03±10.23 (133)	44.27±7.74 (294)	42.71±9.11 (251)	38.39±12.22 (205)	31.17±10.5 (165)
body mass males (mg)	0.87±0.13 (24)	0.81±0.19 (22)	0.78±0.1 (21)	1.21±0.21 (24)	0.53±0.22 (21)	1.12±0.28 (25)
body mass females (mg)	1.43±0.14 (25)	1.81±0.34 (25)	0.98±0.16 (22)	2.23±0.38 (25)	1.12±0.42 (25)	1.55±0.25 (25)
wing length (mm)	3.036±0.140 (30)	2.51±0.15 (30)	2.528±0.145 (30)	3.320±0.178 (30)	2.886±0.183 (30)	2.601±0.108 (30)
fecundity (number of eggs)	153.67±30.35 (30)	74.65±16.06 (30)	75.62±16.04 (30)	232.74±61.04 (30)	124.25±36.48 (30)	83.65±12.42 (30)
longevity males (days)	23.59±14.85 (223)	25.39±10.82 (134)	17.58±7.72 (126)	26.64±4.5 (150)	24.51±9.64 (169)	11.44±6.05 (126)
longevity females (days)	24.78±12.45 (139)	26.84±13.05 (143)	12.72±8 (81)	36.76±16.52 (153)	31.21±13.20 (144)	12.19±6.84 (111)
thermal preference mlaes (°C)	25.94±7.13 (85)	25.71±5.38 (86)	25.6±4.39 (81)	25.48±6.68 (83)	28.1±5.27 (82)	25.05±6 (91)

thermal preference femlaes (°C)	25.82±7.51 (80)	24.09±7.47 (84)	26.1±6.78 (79)	26.93±6.99 (88)	26.31±7.71 (80)	24.21±6.42 (80)
knock-down temperature males (°C)	45.31±1.09 (42)	45.41±0.91 (47)	45.92±1.33 (42)	46.1±0.96 (43)	45.45±0.72 (43)	45.68±1.01 (47)
knock-down temperature females (°C)	46.38±1.02 (50)	45.5±0.8 (45)	46.32±0.77 (45)	46.61±0.88 (44)	46.21±0.56 (48)	46.59±1.1 (46)
protein content males (µg/mg)	40.136 ± 18.614 (35)	80.892±33.632 (37)	56.863±20.285 (44)	52.625±32.939 (42)	67.443±30.990 (43)	52.353±18.134 (40)
protein content females (µg/mg)	40.850±17.345 (40)	69.214±40.108 (40)	53.629±15.698 (36)	33.435±16.331 (43)	49.514±28.524 (43)	46.514±13.454 (40)
glycogen content males (µg/mg)	29.789±15.486 (38)	51.493±32.121 (40)	72.993±39.542 (36)	43.001±24.414 (40)	56.579± 36.479 (40)	45.965±32.823 (40)
glycogen content females (µg/mg)	29.577±20.632 (40)	30.840±22.365 (37)	51.225±35.787 (39)	48.032± 23.551 (40)	67.315±27.452 (40)	48.787±34.077 (40)
total lipid content males (µg/mg)	8.027±5.295 (40)	5.520±3.152 (40)	8.508±4.874 (40)	6.897±4.80 (41)	5.926 ±2.787 (40)	11.406±6.458 (40)

total lipid content females (µg/mg)	7.617±3.693 (40)	3.197±3.066 (39)	7.078±3.490 (40)	8.381±2.429 (44)	4.373±3.050 (40)	8.933±3.574 (40)
triglyceride content males (µg/mg)	10.722±6.129 (39)	9.360±5.010 (40)	11.166±5.908 (40)	12.197±9.610 (45)	16.555±17.128 (44)	14.100±10.386 (39)
triglyceride content females (µg/mg)	4.809±2.820 (40)	8.812±7.182 (40)	7.311±2.654 (40)	5.484±5.183 (45)	11.071±10.08 (45)	6.594±3.780 (40)
water content males (%)	78.482±7.674 (24)	75.939±10.950 (22)	22.972±12.732 (21)	80.022±6.465 (24)	76.829±10.748 (21)	22.122±9.393 (25)
water content females (%)	77.296±5.015 (25)	69.820±3.335 (25)	21.488±8.583 (22)	74.822±5.049 (25)	77.084±9.313 (25)	27.128±8.750 (25)

Appendix 3. P-values relative to the statistical analysis of egg hatchability rate, egg hatching time, larval and pupal viability, larval and pupal developmental time, developmental speed, sex ratio and wing length, fecundity and body mass. A) Within strain comparison; B) Between strain comparison; C) Between sex comparison of body mass. Two-way ANOVA with Tukey' multiple comparison test was used for all traits, except for pupal viability, which was analysed via Kruskal-Wallis test within strain and Mann Whitney test between strain.

A) Within strain comparison

Trait	Comparison	Fo	Cr
egg hatchability rate	28 vs 18	ns	ns
	28 vs 32	ns	ns
	32 vs 18	ns	ns
egg hatching time	28 vs 18	<0.0001	<0.0001
	28 vs 32	ns	0.0017
	32 vs 18	<0.0001	ns
larval viability	28 vs 18	ns	ns
	28 vs 32	ns	0.0034
	32 vs 18	ns	0.0002
pupal viability	28 vs 18	ns	ns
	28 vs 32	ns	0.0192
	32 vs 18	ns	ns
	28 vs 18	<0.0001	<0.0001

larval developmental time	28 vs 32	ns	ns
	32 vs 18	<0.0001	<0.0001
pupal developmental time	28 vs 18	<0.0001	<0.0001
	28 vs 32	ns	ns
	32 vs 18	<0.0001	<0.0001
developmental speed	28 vs 18	<0.0001	<0.0001
	28 vs 32	ns	0.0007
	32 vs 18	<0.0001	<0.0001
sex ratio	28 vs 18	ns	ns
	28 vs 32	ns	ns
	32 vs 18	ns	ns
wing length	28 vs 18	<0.0001	<0.0001
	28 vs 32	ns	<0.0001
	32 vs 18	<0.0001	<0.0001
Fecundity	28 vs 18	<0.0001	<0.0001
	28 vs 32	ns	<0.0001
	32 vs 18	<0.0001	<0.0001
	28 vs 18	ns	<0.0001

body mass males	28 vs 32	ns	<0.0001
	32 vs 18	ns	ns
body mass females	28 vs 18	0.0003	<0.0001
	28 vs 32	<0.0001	<0.0001
	32 vs 18	<0.0001	<0.0001

B) Between strain comparison

Trait	**18°C**	**28°C**	**32°C**
egg hatchability rate	ns	ns	ns
egg hatching time	0.0031	ns	ns
larval viability	ns	ns	<0.0001
pupal viability	ns	ns	0.0017
larval developmental time	ns	ns	0.0267
pupal developmental time	ns	ns	ns
developmental speed	ns	ns	0.0002

sex ratio	ns	ns	ns
wing length	<0.0001	<0.0001	ns
fecundity	<0.0001	<0.0001	ns
body mass males	0.0001	<0.0001	ns
body mass females	<0.0001	<0.0001	<0.0001

C) Between sex comparison of body mass

Temperature (°C)	**Fo**	**Cr**
18	<0.0001	<0.0001
28	<0.0001	<0.0001
32	<0.0001	<0.0001

Appendix 4. Statistical analysis of adult longevity between strains and developmental temperatures. Hazard ratios and long-rank p-values are reported for each comparison in brackets. A) Within strain comparison; B) Between strain comparison; C) Between sex comparison, Hazard Ratio (HR) is reported for males (M) and females (F).

A) Within strain comparison

Foshan Females			
Developmental Temperature	**18°C**	**28°C**	**32°C**
18°C	-	1.28 (ns)	0.2335 (<0.0001)
28°C	0.7811 (ns)	-	0.1984 (<0.0001)
32°C	4.283(<0.0001)	5.041 (<0.0001)	-
Foshan Males			
Developmental Temperature	**18°C**	**28°C**	**32°C**
18°C	-	0.9592 (ns)	0.4517 (<0.0001)
28°C	1.043 (ns)	-	0.3627 (<0.0001)
32°C	2.214 (<0.0001)	2.757 (<0.0001)	-

Crema Females			
Developmental Temperature	**18°C**	**28°C**	**32°C**
18°C	-	0.6036 (<0.0001)	0.04928 (<0.0001)
28°C	1.657 (<0.0001)	-	0.08267 (<0.0001)
32°C	20.29 (<0.0001)	12.10 (<0.0001)	-
Crema Males			
Developmental Temperature	**18°C**	**28°C**	**32°C**
18°C	-	0.7200 (0.0067)	0.1560(<0.0001)
28°C	1.389 (0.0067)	-	0.1111 (<0.0001)
32°C	6.411 (<0.0001)	9.002 (<0.0001)	-

B) Between strain comparison

Females Fo vs Cr		
Developmental Temperature	**Fo**	**Cr**
18°C	2.822 (<0.0001)	0.3543 (<0.0001)
28°C	1.399 (0.0073)	0.7147 (0.0073)
32°C	0.8837 (ns)	1.132 (ns)
Males Fo vs Cr		
Developmental Temperature	**Fo**	**Cr**
18°C	1.183 (ns)	0.8451(ns)
28°C	0.8791(ns)	1.137 (ns)
32°C	0.3584 (<0.0001)	2.790 0.0001)

C) Between sex comparison

Developmental Temperature	**Fo**	**Cr**
18°C	ns	HR F: 0.5071 HR M: 1.972 (<0.0001)
28°C	ns	HR F: 0.8065 HR M: 1.240 (<0.0001)
32°C	HR F: 1.741 HR M: 0.5744 (<0.0001)	ns

Appendix 5. P-values relative to the statistical analysis of thermal preference and knock-down temperature data analysed within strain (A), between strain (B) and between sex (C). In all the tables numbers correspond to the statistical test: (1) corresponds to Kolmogorov-Smirnov test, whether (2) in A is Kruskal-Wallis test, and in B and C Mann Whitney test.

A) Within strain comparison

Trait	comparison	Fo	Cr
thermal preference (male)	28 vs 18	ns (1,2)	0.0172 (1). 0.0170 (2)
	28 vs 32	ns (1,2)	0.008 (1), 0.0043 (2)
	32 vs 18	ns (1,2)	ns (1,2)
thermal preference (female)	28 vs 18	ns (1,2)	ns (1,2)
	28 vs 32	0.0125 (1)	ns (1,2)
	32 vs 18	ns (1,2)	0.0027 (1), 0.0139 (2)
knock-down temperature (male)	28 vs 18	ns (2)	0.0029 (2)
	28 vs 32	0.0127 (2)	ns (2)
	32 vs 18	0.0037 (2)	ns (2)
knock-down temperature (female)	28 vs 18	<0.0001 (2)	0.0421 (2)
	28 vs 32	<0.0001 (2)	ns (2)
	32 vs 18	ns (2)	ns (2)

B) Between strain comparison

Trait	18°C	28°C	32°C
thermal preference (male)	ns	0.0272 (1) 0.0198 (2)	ns
thermal preference (female)	ns	0.0455 (2)	0.0103 (1) -0.038 (2)
knock-down temperature (male)	0.0004 (2)	ns	ns
knock-down temperature (female)	ns	<0.0001 (2)	ns

C) Between sex comparison

Trait	18°C	28°C	32°C
thermal preference (Fo)	ns (1,2)	0.0303(1) 0.0169 (2)	ns (1,2)
thremal preference (Cr)	ns (1,2)	0.0054 (1) 0.0292 (2)	ns (1,2)
knock-down temperature (Fo)	<0.0001 (2)	ns (2)	ns (2)
knock-down temperature (Cr)	0.0068 (2)	<0.0001 (2)	<0.0001 (2)

Appendix 6. P-values relative to the statistical analysis of water content and energy reserves. A) Within strain comparison with Kruskal-Wallis test; B) Between strain comparison with Mann Whitney test. C) Comparison between sexes with Mann Whitney test.

A) Within strain comparison

		Males		**Females**	
reserve type	**comparison**	**Fo**	**Cr**	**Fo**	**Cr**
water content	28 vs 18	ns	ns	0.0012	ns
	28 vs 32	<0.0001	<0.0001	<0.0001	<0.0001
	32 vs 18	<0.0001	<0.0001	<0.0001	<0.0001
protein content	28 vs 18	<0.0001	0.0108	0.0002	0.0024
	28 vs 32	0.0023	ns	ns	ns
	32 vs 18	0.0098	ns	0.0044	<0.0001

glycogen content	28 vs 18	ns	ns	ns	0.0151
	28 vs 32	ns	ns	ns	0.0104
	32 vs 18	<0.0001	ns	ns	ns
total lipids content	28 vs 18	ns	ns	<0.0001	<0.0001
	28 vs 32	0.0128	0.0001	<0.0001	<0.0001
	32 vs 18	ns	0.0013	ns	ns
triglyceride content	28 vs 18	ns	ns	0.0002	0.0004
	28 vs 32	ns	ns	<0.0001	ns
	32 vs 18	ns	ns	ns	ns

B) Between strain comparison

Males	**18°C**	**28°C**	**32°C**
water content	ns	ns	ns
protein content	ns	ns	ns
glycogen content	0.0042	ns	0.0035
total lipids content	ns	ns	0.0286
triglyceride content	ns	ns	ns
Females	**18°C**	**28°C**	**32°C**
water content	ns	0.0034	0.0264
protein content	0.0226	0.0043	0.0318
glycogen content	0.0001	0.0001	ns
total lipids content	ns	ns	0.0075
triglyceride content	ns	ns	ns

C) Between sex comparison

Foshan	**18°C**	**28°C**	**32°C**
Water content	ns	0.0023	ns
Protein content	ns	0.0328	ns
Glycogen	ns	0.003	0.0127
Total Lipids	ns	0.0001	ns
Triglycerides	<0.0001	ns	0.0065
Crema	**18°C**	**28°C**	**32°C**
Water content	ns	ns	0.0126
Protein	0.0014	0.0044	ns
Glycogen	ns	ns	ns
Total Lipids	0.0021	0.0302	ns
Triglycerides	<0.0001	ns	<0.0001

Appendix 7. ANOVA analysis. Source of variation, degrees of freedom (df), Mean Squares (MS), F ratio (F), p-value (p) and percentage of variation (% of total variation) are reported.

egg hatchability rate						
source of variation	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**% of total variation**
temperature x strain	318.9	2	159.4	F (2, 39) = 0.5918	P=0.5582	2.392
temperature	2647	2	1324	F (2, 39) = 4.912	P=0.0125	19.86
strain	19.01	1	19.01	F (1, 39) = 0.07054	P=0.7919	0.1426
Residual	10508	39	269.4			
egg hatching time						
source of variation	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**% of total variation**
temperature x strain	99.06	2	49.53	F (2, 39) = 12.01	P<0.0001	12.98
temperature	510.7	2	255.3	F (2, 39) = 61.94	P<0.0001	66.91
strain	4.079	1	4.079	F (1, 39) = 0.9894	P=0.3260	0.5344
Residual	160.8	39	4.122			
larval viability						
source of variation	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**% of total variation**

temperature x strain	1880	2	940	F (2, 39) = 10.61	P=0.0002	23.38
temperature	859.6	2	429.8	F (2, 39) = 4.851	P=0.0131	10.69
strain	1806	1	1806	F (1, 39) = 20.38	P<0.0001	22.45
Residual	3456	39	88.6			
larval developmental time						
source of variation	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**% of total variation**
temperature x strain	13.64	2	6.821	F (2, 39) = 2.898	P=0.0671	1.151
temperature	1063	2	531.3	F (2, 39) = 225.8	P<0.0001	89.63
strain	10.19	1	10.19	F (1, 39) = 4.332	P=0.0440	0.8598
Residual	91.78	39	2.353			
pupal developmental time						
source of variation	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**% of total variation**
temperature x strain	0.9577	2	0.4788	F (2, 39) = 1.661	P=0.2031	0.7849
temperature	106.5	2	53.27	F (2, 39) = 184.8	P<0.0001	87.31
strain	1.021	1	1.021	F (1, 39) = 3.543	P=0.0673	0.837
Residual	11.24	39	0.2883			

developmental speed						
source of variation	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**% of total variation**
temperature x strain	165.1	2	82.57	F (2, 39) = 15.28	P<0.0001	3,626
temperature	4182	2	2091	F (2, 39) = 387.1	P<0.0001	91,82
strain	4.769	1	4.769	F (1, 39) = 0.8828	P=0.3532	0,1047
Residual	210.7	39	5.402			
sex ratio						
source of variation	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**% of total variation**
temperature x strain	148.7	2	74.34	F (2, 39) = 0.7542	P=0.4771	2.834
temperature	418	2	209	F (2, 39) = 2.120	P=0.1336	7.967
strain	839.4	1	839.4	F (1, 39) = 8.515	P=0.0058	16
Residual	3844	39	98.57			
wing length						
source of variation	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**% of total variation**
temperature x strain	0.7339	2	0.367	F (2, 174) = 15.76	P<0.0001	3.667

temperature	12.52	2	6.261	F (2, 174) = 268.9	P<0.0001	62.56
strain	2.708	1	2.708	F (1, 174) = 116.3	P<0.0001	13.53
Residual	4.051	174	0.02328			
body mass						
source of variation	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**	**% of total variation**
temperature	8.906	2	4.453	F (2, 288) = 50.86	P<0.0001	9.242
sex	33.58	1	33.58	F (1, 288) = 383.5	P<0.0001	34.85
strain	2.938	1	2.938	F (1, 288) = 33.56	P<0.0001	3.049
temperature x sex	4.123	2	2.062	F (2, 288) = 23.55	P<0.0001	4.279
temperature x strain	19.39	2	9.697	F (2, 288) = 110.8	P<0.0001	20.12
sex x strain	0.2117	1	0.2117	F (1, 288) = 2.418	P=0.1210	0.2197
temperature x sex x strain	1.999	2	0.9995	F (2, 288) = 11.42	P<0.0001	2.074
Residual	25.22	288	0.08755			

Appendix 8. Regression analysis of larval and pupal developmental rate to test developmental isomorphy. In table 8A the slope (b) is reported for each line with the reative p-value. On table 8B the p-values relative to the comparison of slopes (b) and elevations (p-value) within and between strains are reported.

A) Regression analysis

Strain	DR	b	p-value
Fo	LDR	0.01376	0.0314
Cr	LDR	0.005954	<0.0001
Fo	PDR	0.002975	<0.0001
Cr	PDR	0.002683	<0.0001
Fo	LDR/PDR	0.002462	ns
Cr	LDR/PDR	-0.005419	0.0029

B) Comparisons

Comparison		b (p-value)	elevation (p-value)
Fo LDR	Fo PDR	ns	<0.0001
Cr LDR	Cr PDR	<0.0001	-
Fo LDR	Cr LDR	ns	ns
Fo PDR	Cr PDR	ns	ns
Fo LDR/PDR	Cr LDR/PDR	0.0043	-

Appendix 9. List of top candidate genes. A) Genes with a value of differential expression (shown in Log2FC) higher or lower than 5 in mosquitoes reared at 18°C with respect to mosquitoes reared at standard conditions. B) Genes with a value of differential expression (shown in Log2FC) higher or lower than 5 in mosquitoes reared at 32°C with respect to mosquitoes reared at standard conditions.

A) List of top candidate genes at 18°C

Larvae

Gene ID	Function	Fo	Cr
AALFPA_080355	cuticle protein-like	-10.590	2.685
AALFPA_065121	unspecified product	-9.826	3.119
AALFPA_062399	cuticle protein 16.5-like	-9.704	2.059
AALFPA_074501	larval cuticle protein A2B-like	-9.082	2.722
AALFPA_071917	uncharacterized	-9.006	2.827
AALFPA_044756	larval cuticle protein A2B-like	-9.002	2.542
AALFPA_052150	pupal cuticle protein-like	-8.676	2.810
AALFPA_045746	cuticle protein 16.5-like	-7.926	2.361
AALFPA_067881	unspecified product	-7.827	2.017
AALFPA_079249	uncharacterized	-7.766	2.148
AALFPA_055643	paternally-expressed gene 3 protein-like	-7.623	1.648
AALFPA_070917	unspecified product	-7.520	2.426

Adults

Gene ID	Function	Fo	Cr
AALFPA_062552	serine protease SP24D-like	-11.368	-3.046
AALFPA_044453	Inositol oxygenase	-7.753	-1.425
AALFPA_080186	trypsin 3A1-like	-7.216	-1.824
AALFPA_065751	farnesol dehydrogenase-like	-6.978	0.701
AALFPA_044000	carbonic anhydrase 1-like	-6.606	-1.693
AALFPA_067529	uncharacterized	-6.558	-1.692
AALFPA_051416	uncharacterized	-6.529	-1.386
AALFPA_063370	Gly-rich secreted peptide	-6.359	-1.797
AALFPA_060167	unspecified product	-6.275	-1.206
AALFPA_041080	uncharacterized	-6.139	-2.470
AALFPA_041513	unspecified product	-5.975	-1.358
AALFPA_079107	pancreatic triacylglycerol lipase-like	-5.794	-1.593

AALFPA_066896	cuticle protein 16.5-like	-7.298	2.027
AALFPA_055471	cuticle protein 63-like	-6.950	2.415
AALFPA_056086	cuticle protein-like	-6.879	2.871
AALFPA_044519	zinc finger protein 512B-like	-6.876	1.649
AALFPA_063605	proline-rich protein 4-like	-6.863	1.524
AALFPA_073053	cuticle protein 38-like	-6.477	2.454
AALFPA_044846	unspecified product	-6.430	2.117
AALFPA_060891	cuticle protein-like	-6.400	2.873
AALFPA_075258	uncharacterized	-6.305	2.744
AALFPA_067435	cuticle protein 21.3-like	-6.295	1.951
AALFPA_049555	cuticle protein 65-like	-6.273	3.561
AALFPA_052832	cuticle protein 16.5-like	-6.116	2.697
AALFPA_062182	cuticle protein-like	-5.974	1.850
AALFPA_054459	cuticle protein 16.5-like	-5.923	2.159
AALFPA_073284	flocculation protein FLO11-like	-5.655	-5.879
AALFPA	unspecified product	-5.601	1.804

AALFPA_045141	Dynein light chain roadblock	-5.721	0.929
AALFPA_049482	uncharacterized	-5.629	-2.969
AALFPA_055005	farnesol dehydrogenase-like	-5.527	-0.642
AALFPA_055680	laminutes Dm0	-5.525	-1.620
AALFPA_066967	37 kDa salivary gland allergen Aed a 2-like	-5.459	-1.632
AALFPA_054623	uncharacterized	-5.419	-1.059
AALFPA_072741	Obp56	-5.359	-1.746
AALFPA_071576	E3 ubiquitin-protein ligase	-5.260	-1.960
AALFPA_046183	serine protease SP24D-like	-5.258	-3.226
AALFPA_052320	L-xylulose reductase-like	-5.225	-1.225
AALFPA_075088	uncharacterized	-5.055	-2.211
AALFPA_059047	uncharacterized	-5.034	-5.302
AALFPA_042658	survival motor neuron protein-like	-5.007	-2.980
AALFPA_072393	Putative mitochondrial fe-s cluster biosynthesis protein isa2	-1.309	-8.290
AALFPA_044057	aminopeptidase N-like	5.162	-4.249
AALFPA	uncharacterized	5.305	-4.251

_051090			
AALFPA_048337	cuticle protein CP14.6-like	-5.507	2.297
AALFPA_077368	cuticle protein 16.5-like	-5.303	2.030
AALFPA_073745	Putative cuticular protein 62bb	-5.285	1.682
AALFPA_064564	uncharacterized	-5.279	1.929
AALFPA_059328	cuticle protein 64-like	-5.202	5.256
AALFPA_048782	mantle protein-like	-5.078	1.477
AALFPA_070466	uncharacterized	-5.066	-4.062
AALFPA_079952	LIM domain-containing protein A-like	-5.060	1.653
AALFPA_051922	uncharacterized	-5.056	2.567
AALFPA_077829	pro-resilin	-5.037	1.504
AALFPA_054347	leucine-rich repeat extensin-like protein 5	-3.527	-6.644
AALFPA_075262	leucine-rich repeat extensin-like protein 5	-2.689	-5.537
AALFPA_041237	uncharacterized	-2.372	-8.625
AALFPA_044172	uncharacterized	-2.136	-7.424
AALFPA_065443	uncharacterized	-1.962	-6.201

_073757			
AALFPA_073049	hexamerin-1.1-like	5.382	6.670
AALFPA_057174	hexamerin-1.1	5.434	3.934
AALFPA_075990	trypsin-like	5.450	4.825
AALFPA_045267	phospholipase A1 VesT1.02	5.590	2.807
AALFPA_048823	lachesin-like	5.620	2.563
AALFPA_053598	chymotrypsin-2-like	6.119	2.766
AALFPA_070269	unspecified product	6.162	2.077
AALFPA_069626	uncharacterized	6.317	3.360
AALFPA_054839	unspecified product	6.425	4.701
AALFPA_045163	trypsin alpha-3-like	6.510	2.266
AALFPA_066112	trypsin-1-like	6.797	3.791
AALFPA_058409	trypsin-1-like	7.046	5.098
AALFPA_065448	unspecified product	7.467	3.888
AALFPA_066631	putative GPI-anchored protein pfl2	7.830	4.488
AALFPA_059094	transmembrane protease serine 11D-like	7.873	4.514

AALFPA_046919	zinc carboxypeptidase-like	-1.806	-5.044
AALFPA_067970	unspecified product	-1.764	7.016
AALFPA_049278	O-acyltransferase like protein-like	0.987	-5.650

B) List of top candidate genes st 32°C

Larvae

Gene ID	Function	Fo	Cr
AALFPA_072959	U1 small nuclear ribonucleoprotein C	-9.276	-9.36
AALFPA_041193	zinc finger and BTB domain-containing protein 24-like	-5.831	-6.032
AALFPA_059841	uncharacterized protein F12A10.7-like	-2.285	-5.991
AALFPA_066268	U1 small nuclear ribonucleoprotein C	-2.296	-5.921
AALFPA_066686	phenoloxidase-activating factor 2-like	-5.136	-5.634

Adults

Gene ID	Function	Fo	Cr
AALFPA_069919	SHC-transforming protein 4-like	7.384	-7.72
AALFPA_080800	heavy metal-associated isoprenylated plant protein 32-like	-6.944	-5.338
AALFPA_059756	heat shock protein 70 A1	-7.32	-4.64
AALFPA_069379	uncharacterized	5.156	3.16
AALFPA_053309	unspecified product	-9.554	5.284

AALFPA_063231	uncharacterized	7.69	-4.895
AALFPA_047351	cuticle protein 5-like	-5.561	-3.94
AALFPA_075119	uncharacterized	-5.305	1.598
AALFPA_041319	uncharacterized	-6.135	1.669
AALFPA_062937	cytadherence high molecular weight protein 1-like	-5.575	1.752
AALFPA_064909	histidine-rich glycoprotein-like	-5.523	2.07
AALFPA_070951	small subunit ribosomal RNA	5.053	9.415

Appendix 10. GO enrichment of genes differentially expressed in each strain at either 18°C or 32°C with respect to expression at 28°C; genes differentially expressed between the two strains at 28 were excluded. Molecular Function/Biological Process/Cellular Component, GO ID, GO description, adjust p-value (p.adjust), Fold Enrichment (FE) and Regulation are reported for each strain, life stage and temperature.

Strain	Life stage	Temperature	MF/BP/CC	GO ID	Description	p.adjust	FE	Regulation
Fo	adults	18	MF	GO:0017171	serine hydrolase activity	0.023	6.668	UP
			MF	GO:0005319	lipid transporter activity	0.036	42.365	UP
			BP	GO:1901565	organonitrogen compound catabolic process	0.019	5.981	DOWN
			BP	GO:0006099	tricarboxylic acid cycle	0.025	26.595	DOWN
			MF	GO:0005549	odorant binding	0.009	6.446	DOWN
		32	MF	GO:0016798	hydrolase activity, acting on glycosyl bonds	0.016	31.471	UP
			MF	GO:0016887	ATP hydrolysis activity	0.0002	47.376	DOWN

	larvae	18	CC	GO:0005576	extracellular region	0.008	4.7542	DOWN
			MF	GO:0042302	structural constituent of cuticle	3.0891e-11	17.278	DOWN
			MF	GO:0008061	chitin binding	0.032	8.7032	DOWN
		32	MF	GO:0016798	hydrolase activity, acting on glycosyl bonds	0.020	14.752	DOWN
Cr	adults	18	BP	GO:0006869	lipid transport	0.021	22.004	UP
			MF	GO:0004252	serine-type endopeptidase activity	0.0002	12.457	UP
			MF	GO:0005319	lipid transporter activity	0.003	75.316	UP
			BP	GO:1901565	organonitrogen compound catabolic process	0.019	15.807	DOWN
			MF	GO:0005549	odorant binding	0.011	14.565	DOWN

		32	MF	GO:0008194	UDP-glycosyltransferase activity	0.044	89.918	UP
			MF	GO:0016887	ATP hydrolysis activity	0.0005	40.608	DOWN
	larvae	18	MF	GO:0042302	structural constituent of cuticle	6.194e-08	19.198	UP
			MF	GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific	0.013	18.132	UP
			BP	GO:0030203	glycosaminoglycan metabolic process	0.014	69.973	DOWN
			CC	GO:0005576	extracellular region	0.0004	9.508	DOWN
			MF	GO:0061783	peptidoglycan muralytic activity	0.0002	75.531	DOWN
			MF	GO:0008061	chitin binding	0.0007	13.055	DOWN

			MF	GO:0052689	carboxylic ester hydrolase activity	0.036	12.440	DOWN
		32	BP	GO:0005975	carbohydrate metabolic process	0.035	28.678	UP
			MF	GO:0004553	hydrolase activity, hydrolyzing O-glycosyl compounds	0.039	50.644	UP

Appendix 11. List of immunity genes [191] that were differentially expressed in each strain at either 18°C or 32°C with respect to expression at 28°C; genes differentially expressed between the two strains at 28°C were excluded. Expression is reported as FC.

Strain	gene_ID	Gene family	Function	stage of DE	FC 32°C	FC 18°C	Regulation
Cr	AALFPA_055424	SRPN	leukocyte elastase inhibitor-like	Adult	-3.669236764	-11.76190632	down 18
Cr	AALFPA_051777	CLIP	phenoloxidase-activating factor 3-like	Adult	-2.709149484	-4.408873245	down 18
Cr	AALFPA_046680	SRPN	serpin B11-like	Adult	1.705403355	-5.29327277	down 18
Cr	AALFPA_075590	CLIP	CLIP domain-containing serine protease 2-like	Adult	-4.28471724	-2.559088428	down 32
Cr	AALFPA_044164	CTL	lectin subunit alpha-like	Adult	-9.028585627	-2.515368902	down 32
Cr	AALFPA_041070	CLIP	phenoloxidase-activating factor 3-like	Adult	-4.514730904	-2.651630955	down 32
Cr	AALFPA_055479	FREP	microfibril-associated glycoprotein 4-like	Adult	-4.163220583	8.664827329	down 32, up 18
Cr	AALFPA_068519	FREP	microfibril-associated glycoprotein 4-like	Adult	-2.989242626	4.553708683	up 18

Cr	AALFPA_053911	TOLL	toll-like receptor Tollo	Adult	41.27653608	87.9071739	up both
Fo	AALFPA_065006	AMP	defensin-A	Adult	8.866981097	-8.646522384	up 32, down 18
Fo	AALFPA_053911	TOLL	toll-like receptor Tollo	Adult	-2.121169633	4.158130394	up 18
Cr	AALFPA_064920	CTL	perlucin-like	Larvae	-3.906103021	-21.53482956	down 18
Cr	AALFPA_048664	AMP	attacin-B-like	Larvae	-5.112220274	2.707428052	down 32
Cr	AALFPA_072775	CLIP	serine protease grass-like	Larvae	-4.858496737	-3.136618352	down 32
Cr	AALFPA_054823	AMP	attacin-B-like	Larvae	-18.02940649	5.98434355	down 32, up 18
Cr	AALFPA_068519	FREP	microfibril-associated glycoprotein 4-like	Larvae	-2.745406922	4.796060973	up 18
Cr	AALFPA_050924	TOLL	toll-like receptor Tollo	Larvae	3.197051535	4.644717977	up 18
Cr	AALFPA_052084	TOLL	toll-like receptor Tollo	Larvae	5.871837325	7.42361041	up both

Cr	AALFPA_053911	TOLL	toll-like receptor Tollo	Larvae	55.83899226	97.85597241	up both
Cr	AALFPA_063239	TOLL	toll-like receptor Tollo	Larvae	37.94258325	37.96889216	up both
Fo	AALFPA_055050	ML	NPC intracellular cholesterol transporter 2 homolog a-like	Larvae	-2.074192798	-4.732622994	down 18
Fo	AALFPA_065926	CLIP	uncharacterized LOC109420638	Larvae	1.936116815	-6.376876761	down 18
Fo	AALFPA_058307	LYS	lysozyme-like	Larvae	3.494829322	-10.34004099	down 18
Fo	AALFPA_068519	CLIP	microfibril-associated glycoprotein 4-like	Larvae	-10.70208509	3.736040255	down 32
Fo	AALFPA_057926	ML	NPC intracellular cholesterol transporter 2 homolog a	Larvae	-10.03403521	-3.252163974	down 32
Fo	AALFPA_067720	CTL	uncharacterized LOC109427308	Larvae	-10.0185374	-3.234135221	down 32
Fo	AALFPA_077120	CTL	C-type lectin 37Da-like	Larvae	-6.558927827	1.890202761	down 32
Fo	AALFPA_058943	LYS	Lysozyme	Larvae	-6.088386727	3.861718492	down 32

Fo	AALFPA_076898	SPZ	neurotrophin 1	Larvae	-4.967327955	-3.734849217	down 32
Fo	AALFPA_044859	AMP	defensin-C	Larvae	-4.950176617	2.850302422	down 32
Fo	AALFPA_055479	FREP	microfibril-associated glycoprotein 4-like	Larvae	-7.267504199	4.550351522	down 32, up 18
Fo	AALFPA_059265	SPZ	protein spaetzle 4	Larvae	-62.27883275	-9.242736154	down both
Fo	AALFPA_068846	CTL	uncharacterized LOC109422059	Larvae	-34.81651749	-11.16537654	down both
Fo	AALFPA_070769	PRDX	uncharacterized LOC109622683	Larvae	-24.43894327	-5.525595677	down both
Fo	AALFPA_062412	PRDX	uncharacterized LOC109398465	Larvae	-16.59503768	-8.6704688	down both
Fo	AALFPA_062736	PRDX	Peroxidase	Larvae	-9.509701124	-9.581357012	down both
Fo	AALFPA_044965	CASP	caspase-1-like	Larvae	-8.498746085	-4.57645343	down both
Fo	AALFPA_049308	GALE	galectin-8-like	Larvae	-8.170645165	-12.6619702	down both

Fo	AALFPA_055241	ML	NPC intracellular cholesterol transporter 2 homolog a-like	Larvae	-5.030145042	-6.24659502	down both
Fo	AALFPA_078160	ML	NPC intracellular cholesterol transporter 2 homolog a-like	Larvae	-4.815381164	-6.077255723	down both
Fo	AALFPA_079815	CLIP	protein masquerade	Larvae	-4.510539491	-4.627650789	down both
Fo	AALFPA_055449	PRDX	Peroxidase	Larvae	-4.460977897	-9.982215157	down both
Fo	AALFPA_048664	AMP	attacin-B-like	Larvae	-2.986260456	4.368406161	up 18

Appendix 12. *Wolbachia* relative abundance (qHTH/qwAlb) statistics. A) Densities and standard deviations; B) Within strain (Kruskal-Wallis test); C) Between strain (Mann Whitney test); P-values are reported for each comparison

A) Relative abundance

Stage	**Temperature (°C)**	**Density *wAlbA* Fo**	**Density *wAlbA* Cr**	**Density *wAlbB* Fo**	**Density *wAlbB* Cr**
Larva	18	0.0495±0.057	0.110±0.083	0.0243±0.023	0.465± 0.329
	28	0.0952±0.090	0.174±0.106	0.142±0.112	0.1168±0.075
	32	0.0061±0.020	0.1653±0.263	0.0735±0.057	0.1676±0.135
ovaries d0	18	0.468±0.750	1.42±2.262	0.251±0.359	2.05±2.806
	28	1.20±2.779	0.0012±0.003	0.732±2.077	0.1668±02.525
	32	0.0646±0.143	0.0265±0.099	0.0049±0.017	0.4370±1.003
ovaries d7	18	2.08±1.006	2.70±1.686	0.640±0.511	2.17±1.449
	28	16.6±14.495	14.1804±10.784	3.120±2.554	3.3665±2.457

	32	6.2391±10.395	3.3712±6.9027	1.7103±2.0382	0.5083±0.589
ovaries d12-14	18	6.57±3.074	3.37±2.141	6.04±2.766	2.72±2.140
	28	20.6±16.747	16.5296±17.639	6.30±11.739	3.6933±2.832
	32	10.9541±13.182	12.7021±12.298	3.5686±5.416	2.7816±2.547

B) Within strain

Strain	**Stage**	**Comparison (°C)**	**p-value – *wAlbA***	**p-value - *wAlbB***
Fo	larva	28 vs 18	ns	<0.0001
		28 vs 32	ns	ns
		32 vs 18	ns	0.0305
Cr		28 vs 18	ns	<0.0001
		28 vs 32	ns	ns
		32 vs 18	ns	0.0013
Fo	ovaries d0	28 vs 18	ns	ns
		28 vs 32	ns	ns
		32 vs 18	0.0222	<0.0001
Cr		28 vs 18	0.0006	<0.0001
		28 vs 32	ns	ns
		32 vs 18	0.001	0.0021
Fo	ovaries d7	28 vs 18	ns	0.0097
		28 vs 32	ns	ns
		32 vs 18	ns	ns

Cr		28 vs 18	ns	ns
		28 vs 32	0.0002	0.0001
		32 vs 18	ns	0.0032
Fo	ovaries d12-14	28 vs 18	ns	ns
		28 vs 32	ns	ns
		32 vs 18	ns	0.0211
Cr		28 vs 18	ns	ns
		28 vs 32	ns	ns
		32 vs 18	ns	ns

C) Between strain

Stage	**Temperature (°C)**	**p-value - *wAlbA***	**p-value – *wAlbB***
Larva	18	0.0189	<0.0001
	28	0.0256	ns
	32	0.0184	0.0185
ovaries d0	18	ns	0.0014
	28	0.0187	ns
	32	ns	0.0469
ovaries d7	18	ns	0.0005
	28	ns	ns
	32	ns	0.0435
ovaries d12-14	18	0.0019	0.0003
	28	ns	ns
	32	ns	ns

Appendix 13. R scripts

A) Deseq2 script

```
library("DESeq2")
library("dplyr")
count <- read.csv('count.csv', header = TRUE, sep = ",")
metadata <-  read.csv('metadata.csv', header = TRUE, sep = ",")
df1 <- mutate_if(count, is.numeric, round)
rownames(df1)<- df1$gene_id
df1<- as.matrix(df1[,-1])
df2<- metadata[-1]
rownames(df2)<- metadata$Sample_id
df2[,colnames(df2)]<- lapply(df2[,colnames(df2)], factor)
dds <- DESeqDataSetFromMatrix(countData = df1, colData = df2, design =
~Strain)
ddsDESeq <- DESeq(dds)
res <- results(ddsDESeq)
write.csv(res, "DESeq.csv", row.names=TRUE)
```

B) Raincloud plots script for Figure 12, example females

```
library(ggplot2)
library(ggdist)
ggplot(FEMALES_Fo_Cr, aes(females,Tp, fill=females, color=females))+
scale_y_continuous(n.breaks=9)+
scale_x_discrete(expand=c(0,-5))+
stat_halfeye(alpha=0.7, justification=-0.2, adjust=0.4,
position=position_dodge(0.5), width=.9)+
scale_color_manual(values=c("#99FF99",
"#0CB702","#CCCCCC","#666666", "palevioletred1","violetred3"))+
geom_boxplot(width=0.4, alpha=0.2, position = "identity") +
scale_fill_manual(values=c("#99FF99", "#0CB702","#CCCCCC","#666666",
"palevioletred1","violetred3"))+
geom_dotplot(binaxis="y", dotsize=0.3, stackdir="center") +
theme_bw()+
coord_flip()+
labs(y= "Thermal gradient (°C)", x = "temperature (°C)")+
theme(legend.position = "none")
```

C) Pearson's correlation test script for Figure 15, example wing length Fo

```
if(!require(devtools)) install.packages("devtools")
devtools::install_github("kassambara/ggpubr")
library("ggpubr")
x<-correlation$temp
y<-correlation$wing_fo
cor.test(x, y, method=c("pearson"))
ggscatter(correlation, x = "temp", y = "wing__fo",
add = "reg.line", conf.int = TRUE,
cor.coef = TRUE, cor.method = "pearson",
xlab = "temperature", ylab = "wing length (mm)"),
color = "red",
palette = "jco", ylim=c(0,5))
```

D) PCA script for Figures 16-17-18, example Fig.16

```
library(factoextra)
library(ggpubr)
library(tidyverse)
table_full_data  <- read.csv("aalbo.traits.table.txt", sep="\t",
comment.char="#", header=TRUE);
table_1<- table_full_data %>% select(3,4,5,6,7,8,23,24,25);
table_1[is.na(table_1)] <- 0;
pca_table_1  <- prcomp(table_1, scale = TRUE);
pdf("pca.1.pdf", width  = 11, height = 9, useDingbats = FALSE )
fviz_pca_biplot(pca_table_1, obs.scale = 1, var.scale = 1,
alpha.var ="contrib", col.var = "black", geom="point", addEllipses = FALSE) +
geom_point(aes(shape=factor(table_full_data$strain),colour=factor(table_full_
data$temp)), size=6) +
scale_color_manual(values=c("green3","grey37","violetred3")) +
guides(shape = guide_legend(title = "Strain"), colour = guide_legend(title =
"Temperature"), alpha = guide_legend(title = "PC contribution (%)"))
dev.off()
```

E) Venn diagram script for Figure 19

```
if (!require(devtools)) install.packages("devtools")
devtools::install_github("yanlinlin82/ggvenn")
library(ggvenn)
Y <- list('cra18' =cra18, 'cra32'=cra32, 'foa18'=foa18, 'foa32'=foa32)
ggvenn(Y, fill_color = c("#99ff99", "palevioletred1", "#009900", "violetred3"),
stroke_size = 0.5, set_name_size = 4)
```

F) Ggplot script for Figure 19, examples of adults at 18°C and adults at 32°C

```
library(tidyverse)
library(ggplot2)
library(tidyverse)
library(ggplot2)


#adults 18
ggplot(data = A18 , aes(x =FoLog2FC, y =CrLog2FC,color
  =Regulation,shape=Regulation)) +
geom_point(size=2)+scale_shape_manual(values=c(16,16,16,3,15,15,17))+
scale_color_manual(values=c("#000000","#104E8B","#FF0000","#CDCDC1",
"#000000", "#FF0000", "#FF00CC" ))+ xlab("Foshan") +ylab("Crema")+
geom_hline(yintercept=0,color="white",size=1.2)+
geom_vline(xintercept=0,color="white",size=1.2)

#adults32
ggplot(data = A32 , aes(x =FoLog2FC, y =CrLog2FC,color
=Regulation,shape=Regulation)) +
geom_point(size=2)+scale_shape_manual(values=c(16,16,16,17,3,15,15,15,17)
)+scale_color_manual(values=c("#000000","#104E8B",
"#FF0000","#33CCFF" , "#CDCDC1","#000000", "#104E8B","#FF0000",
"#FF00CC" ))+ xlab("Foshan") + ylab("Crema")+
geom_hline(yintercept=0,color="white",size=1.2)+
geom_vline(xintercept=0,color="white",size=1.2)
```

G) Ggplot for Figures 20 and 21

```
library(ggplot2)
library(tidyr)
library(scales)
df <- read.table(file='C:\\Users\\...\\...\\tab1.txt', header=TRUE, sep = "\t")
ggplot(df, aes(x= Description)) +
geom_bar(aes(y =FE, fill = ONTOLOGY), stat="identity") +
scale_fill_manual(values=c("#990033", "#FFFF00"))+
labs(y ="FE", fill="Description") +
facet_grid(~DEG_TYPE) +
coord_flip()
```

Appendix 14. NextFlow (nf-core/rnaseq) pipeline

```
nextflow run nf-core/rnaseq  \
        --input /mnt/das/users_data/carlassara/nextflow/samples.csv \
        --fasta /ngs-
data/share_ref/bundle/current/Aedes_albopictus/AlbCanu/Nextflow_genome/V
ectorBase-55_AalbopictusFoshanFPA_Genome.fasta \
        --gff /ngs-
data/share_ref/bundle/current/Aedes_albopictus/AlbCanu/Nextflow_genome/V
ectorBase-55_AalbopictusFoshanFPA.gff \
        --outdir /mnt/das/users_data/carlassara/nextflow/ \
        -profile singularity \
        --pseudo_aligner salmon  \
        --max_cpus 4 \
        --max_memory 64.GB | tee
/mnt/das/users_data/carlassara/nextflow/rnaseq_nextflow_pipeline.myJob.LIV
E_REPORT.log
```

8. Acknowledgements

I would like to thank the Bonizzoni Lab, former and present members, but especially Ayda Khorramnejad, who supported me from the very first day.

My sincere gratitude goes to the Lahondère Lab members for having me during Fall 2021 and especially to Joanna M. Reinhold, for giving me some of my most precious memories.

I am grateful for The Company of Biologists, who funded part of my stay at Virginia Tech with their Travelling Fellowship.

Lastly, I would like to thank my loved ones, who followed my PhD journey with love, sympathy and support.

9. Publications

Carlassara M, Khorramnejad A, Oker H, Lahondère C, Bonizzoni M. Comparing thermal performance between tropical and temperate strains of the arboviral vector *Aedes albopictus*. Poster session UZI Congress 2022, Trieste.

[Unpublished manuscript under review, Global Change Biology] **Carlassara M**, Khorramnejad A, Oker H, Bahrami R, Lozada-Chavez AN, Mancini MV, Body MJA, Lahondère C, Bonizzoni M. Population-specific responses to developmental temperature in the arboviral vector *Aedes albopictus*: implications for climate change. doi.org/10.1101/2023.10.06.561151