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An egg is always an adventure: anthropogenic impacts on *Culex pipiens* population dynamics

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Citation

Boerlijst, S. P. (2025, March 18). *An egg is always an adventure: anthropogenic impacts on *Culex pipiens* population dynamics*. Retrieved from <https://hdl.handle.net/1887/4198436>

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Fourth instar larvae of the house mosquito

Chapter 3

In the heat of the moment:

including realistic thermal fluctuations using an open-source thermoregulator results in dramatically altered key population parameters



Ecology & Evolution (2024)
DOI: 10.1002/ece3.70124

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Abstract

Temperature is commonly acknowledged as one of the primary forces driving ectotherm vector populations, most notably by influencing metabolic rates and survival. Although numerous experiments have shown this for a wide variety of organisms, the vast majority has been conducted at constant temperatures and changes therein, while temperature is far from constant in nature, and includes seasonal and diurnal cycles. As fluctuating temperatures have been described to affect metabolic processes at (sub)cellular level, this calls for studies evaluating the relative importance of temperature fluctuations and the changes therein. To gain insight in the effects of temperature fluctuations on ectotherm development, survival, and sex-ratio, we developed an inexpensive, easily reproducible, and open-source, Arduino-based temperature control system, which emulates natural sinusoidal fluctuations around the average temperature. We used this novel setup to compare the effects of constant (mean) temperatures, most commonly used in experiments, block schemes and natural sinusoidal fluctuations as well as an extreme variant with twice its amplitude using the cosmopolitan mosquito species *Culex pipiens* s.l. as a study organism. Our system accurately replicated the preprogrammed temperature treatments under outdoor conditions, even more accurately than traditional methods. While no effects were detected on survival and sex-ratio within the ranges of variation evaluated, development was sped up considerably by including temperature fluctuations, especially during pupation, where development under constant temperatures took almost a week (30%) longer than under natural fluctuations. Doubling the amplitude further decreased development time by 1.5 days. These results highlight the importance of including (natural) oscillations in experiments on ectotherm organisms – both aquatic and terrestrial – that use temperature as a variable. Ultimately, these results have major repercussions for downstream effects at larger scales that may be studied with applications such as ecological niche models, disease risk models and assessing ecosystem services that rely on ectotherm organisms.

Keywords: Arduino, *Culex pipiens*, heat wave, larval development, mesocosm, microcontroller, mosquito, temperature fluctuations.

3.1 Introduction

Temperature is commonly acknowledged as one of the primary forces driving ectotherm populations (Mellanby and Gardiner 1997; Newell 1966). Temperature influences metabolic rates at cellular and subsequently organismic level (Kuznetsov et al. 2016) as a result of differences in optimal temperatures for different enzymatic reactions (Rao and Bullock 1954). In extreme cases, survival might be affected, as a result of denaturation and impaired membrane function (Bowler 2018). Experiments using temperature are of major importance to predict the effects of large-scale disturbances like climate change on these organisms.

Global warming affects not only daily mean temperature, but also the daily interval in temperature (Colinet et al. 2015; Easterling et al. 1997; Paaijmans et al. 2013). Additionally, effects on a local scale exist, wherein effects like urban heat islands affect diurnal and seasonal temperature fluctuations. Various studies suggest that indeed the diurnal amplitude also might affect the development of ectotherm organisms (Kern, Cramp, and Franklin 2015; Kingsolver, Higgins, and Augustine 2015; Kuznetsov et al. 2016; Ludwig and Cable 1933; Waqas et al. 2020), possibly via temperature dependent processes such as growth and cell differentiation (van der Have and de Jong 1996; Ratte 1984). Similar, and possibly sex-specific effects (Agnew, Haussy, and Michalakis 2000; Alcalay et al. 2018), have been detected for mosquito vectors (Colinet et al. 2015; De Majo et al. 2019; Headlee 1941; Huffaker 1944; Ratte 1984), which undergo their subadult development in shallow (often temporary) water bodies where drastic temperature changes are common. To understand how to manage such anthropogenic impacts, it is thus crucial to understand the exact effects of temperature fluctuations on arthropod vector development. However, a small-scale and inexpensive experimental system to do so was until recently unavailable (Hagstrum and Hagstrum 1970; Hermann et al. 2022).

Most ecological experimental studies including temperature as a variable (i.e., micro- and mesocosms) have hitherto been dependent on decentralized temperature regimes in climate cabinets (Greenspan et al. 2016; Hagstrum and Hagstrum 1970) or with heating elements set to a constant temperature (Bayoh and Lindsay 2004; Brust and Kalpage 1967; Impoinvil et al. 2007; Shapiro, Whitehead, and Thomas 2017; Shelton 1973). The latter system occasionally has been adapted to a block scheme, where temperatures fluctuate between two levels that are fixed over a set amount of time (Alcalay et al. 2018; Spanoudis et al. 2019) or by physically moving the study organism between climate chambers

(Niederegger, Pastuschek, and Mall 2010). However, the widespread availability of micro-controllers (Bolanakis 2019) allow for a well replicated assessment of the relative importance of thermal variation.

To better understand the precise effects of temperature on ectotherm development, we developed an inexpensive, easily reproducible, and open-source Arduino-based temperature control system. This setup allows for emulation of natural sinusoidal fluctuations above ambient temperatures whilst keeping the number of degree-days over all treatments the same. Here, as a case study to validate the metabolic effects, we compared the effects of commonly used constant (mean) temperature and block schemes with natural sinusoidal fluctuations, as well as an extreme regime with twice its amplitude. We used the mosquito species *Culex pipiens* (hereafter *Cx. pipiens*), a cosmopolitan vector for a range of viral pathogens including West Nile virus, Sindbis virus and Usutu, as a model species. As the subadult stages of this species are aquatic, this allows for easy implementation of temperature regimes via immersible heating elements.

3.2 Materials and methods

To study the effects of natural temperature oscillations on metabolic rates in aquatic systems, we used the following procedure.

3.2.1 Temperature control system

Our novel temperature control system (holistic intermittent heatwave instrument; hereafter HIHI) for investigating the effects of temperature fluctuations is comprised of a closed container to hold the electronics, a power supply, the internal electronics, and relay-controlled power strips. The setup allows for up to eight groups of heaters to be connected per HIHI, for a total of 10 Ampère per group. Using 200W heaters, this translates to 80 mesocosms in total.

3.2.1.1 Container

As the HIHI may be used outdoors, care was taken to protect the electronic components and their connections from rain/humidity whilst preventing build-up of heat. Two polycarbonate storage boxes were used to house the electronic components, one for the microcontroller and relay board, and one for the power strips and their connection to the heaters. Holes were cut into the bottom of both boxes for ventilation, and into the overhang of the lid to allow wires to pass through. The cables were glued in place with hot glue to prevent moisture from entering. To allow for sufficient air supply, holes were

cut on the bottom of the containers and were placed on a layer of stones to allow for aeration. Placement of the containers was limited by the length of the power cables and cables of the temperature sensors. In our case we placed the containers in the middle of the experimental setup.

3.2.1.2 Power supply

HIHI is operated by one 5v micro-USB power supply, which may be connected to a laptop to allow for logging the temperatures from the serial logger included in the Arduino IDE or may be connected to a 1A phone charger. The relay board operates on grid power (240V).

3.2.1.3 Control box

The control box internal electronics are shown in Table 3.1 and Figure 3.1. The costs for the electronics are estimated at 49 euro. The programmed temperature is compared to the current surface water temperature, measured by DS18B20 (i-button) sensors (Maxim integrated, San Jose). Based on this information, each treatment is heated or left to (passively) cool via activation or deactivation of the heaters connected via an optocoupled relay with a specified interval.

Table 3.1 Components for the temperature controller

Component	Quantity	Price (€)	Specifications	Use
Arduino uno	1	24,95		Controls temperature
Temperature sensor	4	4x2,95	DS18B20; minimum of 2 per treatment	Measures temperature
Optocoupler relay module	1	10,00	2ph109375a or equivalent; 240V/10A per relay; 2 channel or more	Turns heaters on or off
Resistors	4	4x0,03	470Ω	Limits current to sensors
perfboard	1	0,70	Size approximately 4x6 cm	To assemble circuit onto
Insulated conductor cable	1	0,60	1m	Connects components
Female header pin	1	0,18	1x6 pins	Connector to relay module
	Total price	48,35		

3.2.1.4 Relay-controlled power strips

The power strips are driven by opening the live wire and connecting these to the COM and NO connections of their respective relay-group. Heaters were plugged into the power strips. Care was taken to adequately distribute the heaters over multiple relays as not to overload the relay and/or power strip (commonly rated for 10A and 16A, respectively).

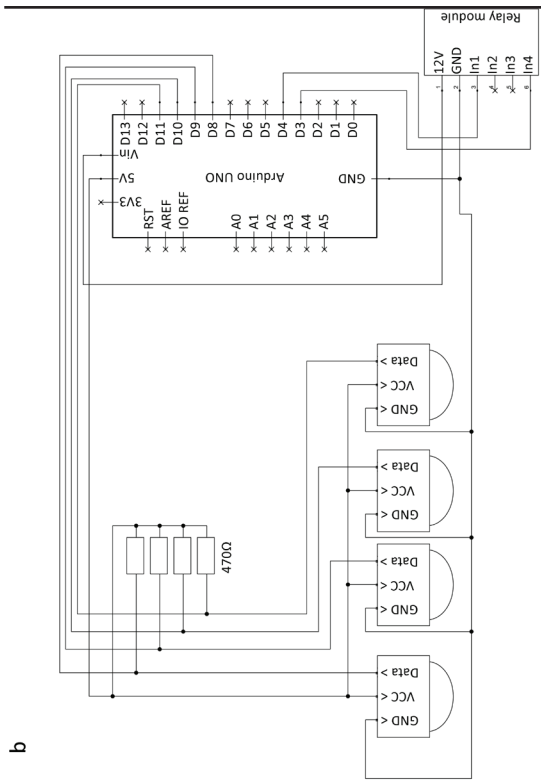
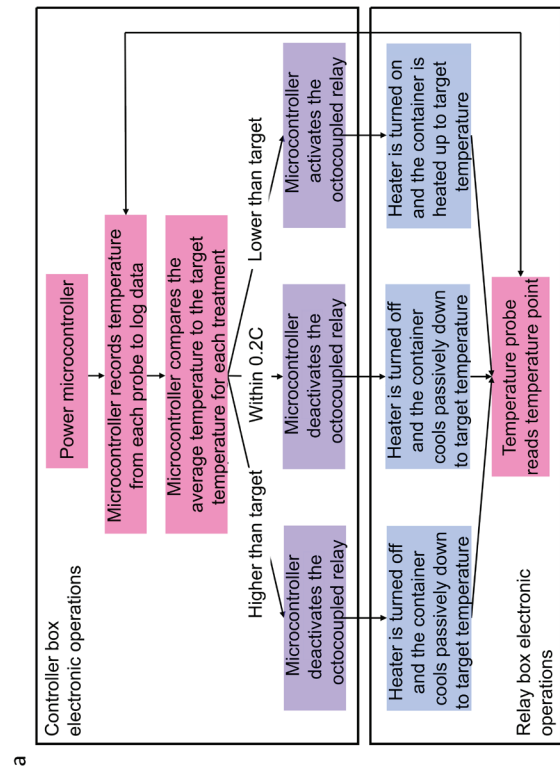


Figure 3.1 Schematic overview of a) the operational process of the temperature controller and b) the internal electronics and their connections.

3.2.1.5 Operational process

The operational procedure that the temperature controller undergoes is specified in Figure 3.1a. The microcontroller can be pre-programmed with user-specified temperature schemes for each time interval (e.g., 1, 2 or 5 minutes). The operational code needed to operate the temperature controller may be downloaded from GitHub and uploaded using the open-source Arduino integrated development environment (Wheat 2011; see data accessibility section). Temperature schemes can be altered to any temperature scheme, for instance real-world thermal regimes captured from environmental data loggers, by changing the temperature arrays. Diurnal temperature fluctuations can be programmed with the “curve calculation” Excel file provided in the dryad repository, by changing the interval, daylength, mean temperature and amplitude and subsequently copying the produced array to the code.

3.2.2 Validation tests

Two exploratory studies were performed as generic validation to 1) determine the interval with which the temperature controller should operate and 2) to determine and correct for any bias present in the programmed temperatures. These tests were performed in white polypropylene carbonate 12L buckets (31,6 x 32,5 x 25,5 cm), which were also used for the proof of principle experiment.

3.2.2.1 Interval calculation

The interval for the temperature controller was determined by recording the time needed to either warm or passively cool the 12L bucket by 0.1°C. This was done by taking the average time over 2°C heating/cooling. Using 200W HS-aqua heaters, a 2-minute interval proved sufficient, as this allowed the container to either cool by 0.1°C or to heat up by 0.08°C during the allotted time. From this, a heat transfer coefficient could be derived to predict intervals that may be used for similar setups (surface, volume and humidity) under a range of temperatures as compared to ambient air temperature (Table 3.2).

Table 3.2 Predicted intervals to cool 0.1°C derived from Newtons law of cooling, a heat transfer coefficient of $623587 \text{ W}/(\text{m}^2\text{K})$, a diameter of 32 cm, relative humidity of 68% and variable temperature interval as compared to ambient air temperature.

Temperature difference ($^{\circ}\text{C}$)	Interval (minutes)
20	0.69
18	0.77
16	0.87
14	1.00
12	1.16
10	1.40
8	1.75
6	2.34
4	3.52
2	7.13

3.2.2.2 Bias correction

Adherence to the programmed temperatures specified in section 2.3 was validated by use of a one-day pilot. The setup was allowed to run normally, and the number of degree-days, equal to the sum of the mean temperature per 15 minutes for a 24-hour period, was estimated for each treatment by calculating the approximation of the surface between each (2-minute interval) timepoint as a trapezoid. Using this data, a bias of $+0.1^{\circ}\text{C}$ was found, after which the formulas were corrected, and the pilot was run again to validate that the bias had been reduced (supplementary Table S3.1).

3.2.3 Temperature treatments

Recent insect development models propose that ectotherm metabolism and development do not respond additively to temperature fluctuations (Wu, Shiao, and Okuyama 2015), and are dependent on a variety of temperatures (Kuznetsov et al. 2016; Ludwig and Cable 1933; Newell 1966; Waqas et al. 2020; Wu et al. 2015) for optimal cell growth and differentiation (van der Have and de Jong 1996; Ratte 1984). This could result in variation across populations as a result of (local) adaptations (Sternberg and Thomas 2014). As to our knowledge an optimum diurnal temperature fluctuation has hitherto not been established for our model species, we chose to simulate as much realism as possible, and thus used temperatures associated with the peak of the mosquito season for our latitude. Because of this we simulate an average day at the peak of the Dutch

mosquito season. To ensure the number of degree days to be consistent across the four treatments of increasing fluctuation, we used the following procedure. Based on aquatic surface temperatures measured in May and July 2020, we determined mean, minimum, maximum temperature, and the temporal interval between these (Supplementary Figure S3.1). These temperature metrics were used to create four treatments with equal mean (Figure 3.2) using the methods described in the operational code (see data availability section).

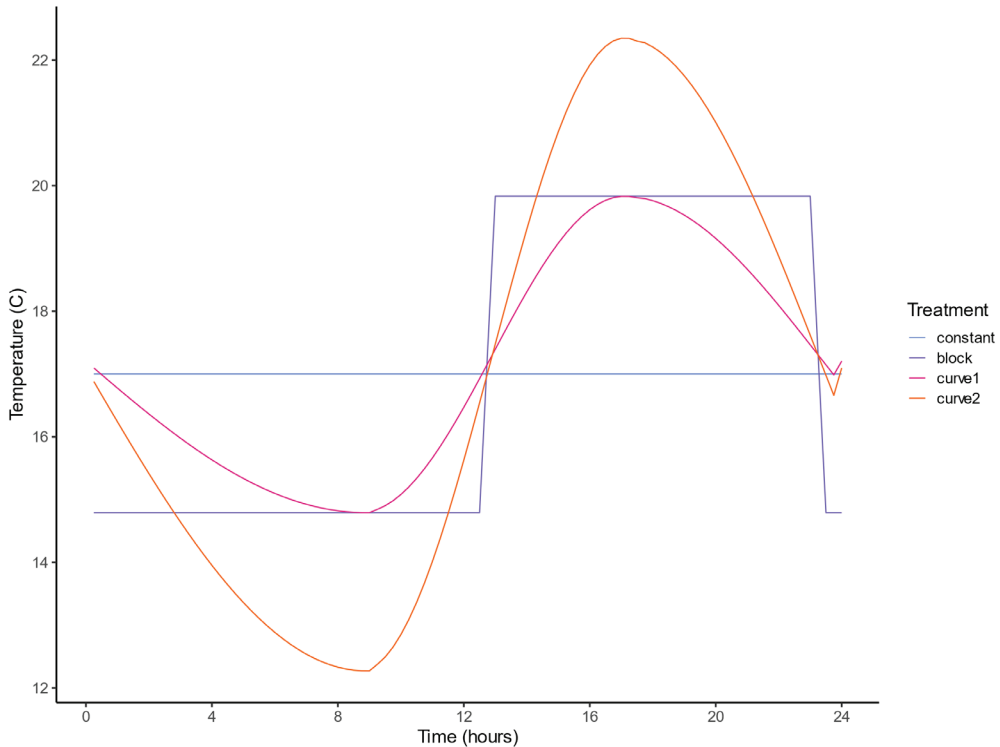


Figure 3.2 Visual representation of the programmed temperature regimes of increasing levels of temperature fluctuation. The area under the graph – indicative for the amount of energy in the aquatic system – is the same for all treatments. The temperatures mimic an average day in June (2020) in the Netherlands.

3.2.4 Proof of principle experiment

In order to validate the reliability of the proposed system, and the effect of temperature fluctuations on the development and (sex-specific) survival of an ectotherm organism, a proof-of-principle experiment was conducted at the living lab field station of Leiden University, the Netherlands (Boerlijst et al. 2023).

The current experiment used the mosquito *Cx. pipiens* as a model organism. The experiment took place between the end of March and beginning of May of 2021 and took 34 days. As the outside temperatures are relatively low in that period, this allowed us to mimic the natural temperature conditions of an average day in June 2020, because the ambient temperatures sufficiently low to cool down the buckets to the desired temperatures. June is commonly regarded as the optimal month for mosquito development in NW Europe as the amount of sunlight energy, a direct determinant of the water temperature, is at its maximum (Becker et al., 2010). The experiment, containing four temperature treatments (Figure 3.2), had 5 replicates each consisting of white polypropylene carbonate 12L buckets (31,6 x 32,5 x 25,5 cm; Figure 3.3; Supplementary Figure S3.1). The containers are representative of the artificial containers that *Cx. pipiens* is known to colonize (Koenraadt and Harrington 2008). Within these small, temporary water bodies, fluctuation of temperature is highest and there is little competition and predation (Kumar and Hwang 2006).



Figure 3.3 Overview of the experimental setup. Panel a shows the mesocosms covered by emergence traps and the HIHI and power strips in the middle. Panel b shows the HIHI, with the Arduino and circuit board to the left, and the relay board to the right.

Each bucket was filled with 10 liters of dechlorinated tap water and a standardized community of algae and bacteria, collected with a plankton net (250 μm with a 53 μm collector) from a neighboring lake (Dellar, Boerlijst, and Holmes 2022). The filtered algae and bacteria obtained were divided equally over all containers so that one liter of water in the set-up contained as much microbes as a liter of

ditch water. An eutrophic environment (20 mg/L N-total; Loeb and Verdonschot 2008) was created, using cow manure pellets (2,4% N; 1,5% P₂O₅; 3,1% K₂O) to minimize intraspecific competition (Boerlijst et al. 2023). The buckets were thereafter covered with a 0.1mm mesh to prevent natural colonization and left to acclimatize for one week. 200 first instar larvae were then added to each container and the four treatments were semi-randomly assigned within a Latin square design (Appendix 3). During the experiment, an emergence trap (Cadmus, Pomeranz, and Kraus 2016) was used to prevent colonization, protect the mosquitoes from predators and to prevent the emerged mosquitoes from flying out. Evaporated water was replenished daily using dechlorinated tap water stored at ambient temperature.

Life-stage specific development and sex-specific emergence rates were included as dependent variables. Additionally, dissolved oxygen concentration, turbidity and chlorophyll *a* concentration were measured as indicators for resource competition due to their relation with bacterial and algal metabolism (Ansa-Asare, Marr, and Cresser 2000; Coolidge 2017).

3.2.4.1 Study organism

Culex pipiens var. *pipiens* egg rafts were obtained from the rearing facility of Wageningen university, the Netherlands. The larvae were subsequently allowed to hatch in a white plastic bucket containing 10 liters of lake water where they were kept at ambient temperature until the start of the experiment.

3.2.4.2 Measurements

The temperature in each mesocosm was recorded every 15 minutes for the duration of the experiment by a temperature logger (iButton DS1921G#F5D) placed 5cm under the water surface to i) measure the temperature near the water surface where mosquito larvae spend most of their time (Becker et al. 2010) and ii) prevent the loggers from emerging due to evaporation. Larval development was measured 5 days a week and time to pupation, time to emergence, survival rate and sex-ratio were determined congruent with the methods of Boerlijst et al, 2022. Dissolved oxygen concentration (DOC), chlorophyll *a* and turbidity levels were measured weekly with a Hach HD40 and Aquafluor 8000-010, respectively, using manufacturers protocols.

3.2.5 Statistical analysis

All data were analyzed in R version 4.04 (R Core Team 2018). To compare the amount of energy per day between the different treatments, we calculated the degree days in unit of hour per day per mesocosm as approximated integral of the iButton measurements with trapezoidal integration from the pracma package (Borchers 2022). Adherence of the treatments to their respective programmed temperatures was analyzed using an ANOVA on a linear mixed effect models with the formula: $\text{Temperature} \sim \text{time} + \text{predicted temperature} + (1|\text{Cosm}) + (1|\text{Day})$ for the block, curve and curve 2 treatments. As all predicted values for the constant treatment are equal to its mean, we analyzed this treatment using a Wilcoxon rank sum test. The block treatment was analyzed using the (10) days prior to the short circuit of this treatment (Figure 3.4a). Effects on the life-history of the within day variation within the full experiment (Supplementary Figs. S3,4) are described in the discussion. Daily mean temperature for each of the respective treatments was assessed using a two-way ANOVA using the formula $\text{Degree days} \sim \text{Day} + \text{Treatment} + \text{Day:Treatment}$. Data from day eight was excluded as a blown fuse within the field facility had disrupted the block and constant treatments.

Differences in time to pupation, time to emergence, survival and sex-ratio were assessed with general linear models comparing the number of emerged mosquitos, average development stage, the number of emerged mosquitoes per sex respectively whilst using DOC, chlorophyll α and turbidity as main factors. Covariates and their interactions were stepwise removed from the full models during model optimization if not significant and if the Akaike information criterion indicated a worse fit of the data. All full models are presented in supplementary Table S3.2.

3.3 Results

To assess the effect of temperature fluctuations on culicid metabolic rate, data was collected on 1) temperature, to allow for comparison with target temperatures and thus HHI accuracy and 2) life-history traits to detect developmental differences across different levels of thermal fluctuation.

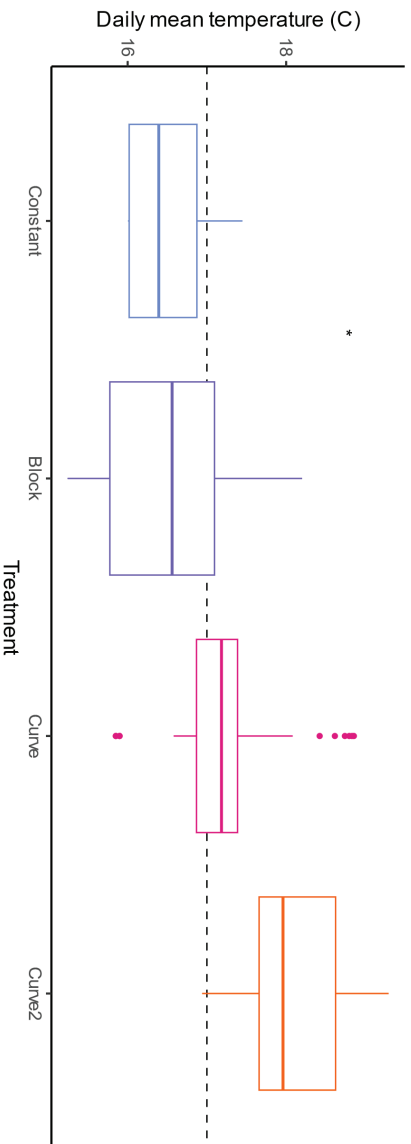
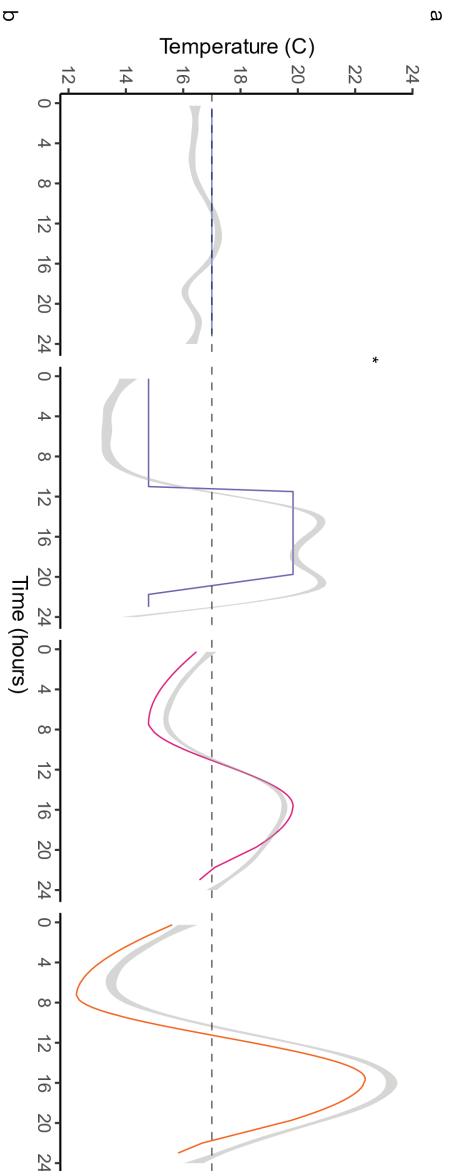


Figure 3.4 Measured temperatures as compared to the programmed t temperature regimes of increasing levels of temperature fluctuation. Panel a shows the measured temperatures over time, including standard error, in gray, and the programmed regimes in color. Panel b shows the mean daily temperature in color for the temperature sensors shown in gray. * subset of first 10 days of the experiment (up to the short circuit of the respective treatment).

3.3.1 Temperature series

To compare the reliability of our proposed system, we compared predicted and actual temperature measurements per 15 minutes for each treatment (Figure 3.4a). A 0.5°C difference was detected in temperature between the predicted and actual measurements ($W = 5963$, $p < 0.001$). No significant differences were detected for the block ($F_{1,1} = 0.01$, $p = 0.94$), constant ($F_{1,1} = 0.003$, $p = 0.97$), curve ($F_{1,1} = 0.43$, $p = 0.63$) and curve 2 ($F_{1,1} = 1.63$, $p = 0.42$) treatments.

The daily mean temperatures derived from the iButtons were subsequently compared per day across treatments with increasing temperature fluctuation to detect differences in energy input (Figure 3.4b). Differences were detected over time ($F_{20} = 8.972$; $p < 2e-16$; power = 1) across treatments ($F_3 = 144.66$; $p < 2e-16$; power = 1), also interactively ($F_{60} = 4.759$; $p < 2e-16$; power = 1). Post hoc pairwise t-tests indicated differences between Constant and the treatments curve and curve 2 at day 3 and 7 ($p < 0.05$). Further differences were detected between Block and the treatments Constant from day 11 onwards ($p < 0.05$), Curve at day 9-18, 20 and 22 ($p < 0.05$) and curve 2 at day 9 ($p < 0.05$).

3.3.2 Life-history effects

Absolute survival rate and sex-ratio were not impacted by different levels of temperature fluctuation within the ranges tested ($p > 0.1$). No differences in DOC, chlorophyll and turbidity were found between the treatments.

Increasing levels of temperature fluctuation decreased development time up to pupation (Figure 3.5). Differences were detected between the constant and curve treatments ($\chi^2 = 2.017$, $p = 0.022$) and the constant and curve 2 treatments ($\chi^2 = 2.711$, $Df = 3$, $p = 0.003$). Increasing levels of temperature fluctuations also lowered time to emergence ($F(3, 14) = 230.7$, $p < 0.001$, partial $\omega^2 = 0.833$, power = 1). Post hoc analysis indicated differences between the constant treatment and all other treatments ($P < 0.001$, Bonferroni correction) and between the block and curve treatments ($p < 0.05$, Bonferroni correction). Differences in development time exacerbated during pupation (Figure 3.5, Supplementary Figures. S3.5-6, Table 3.3).

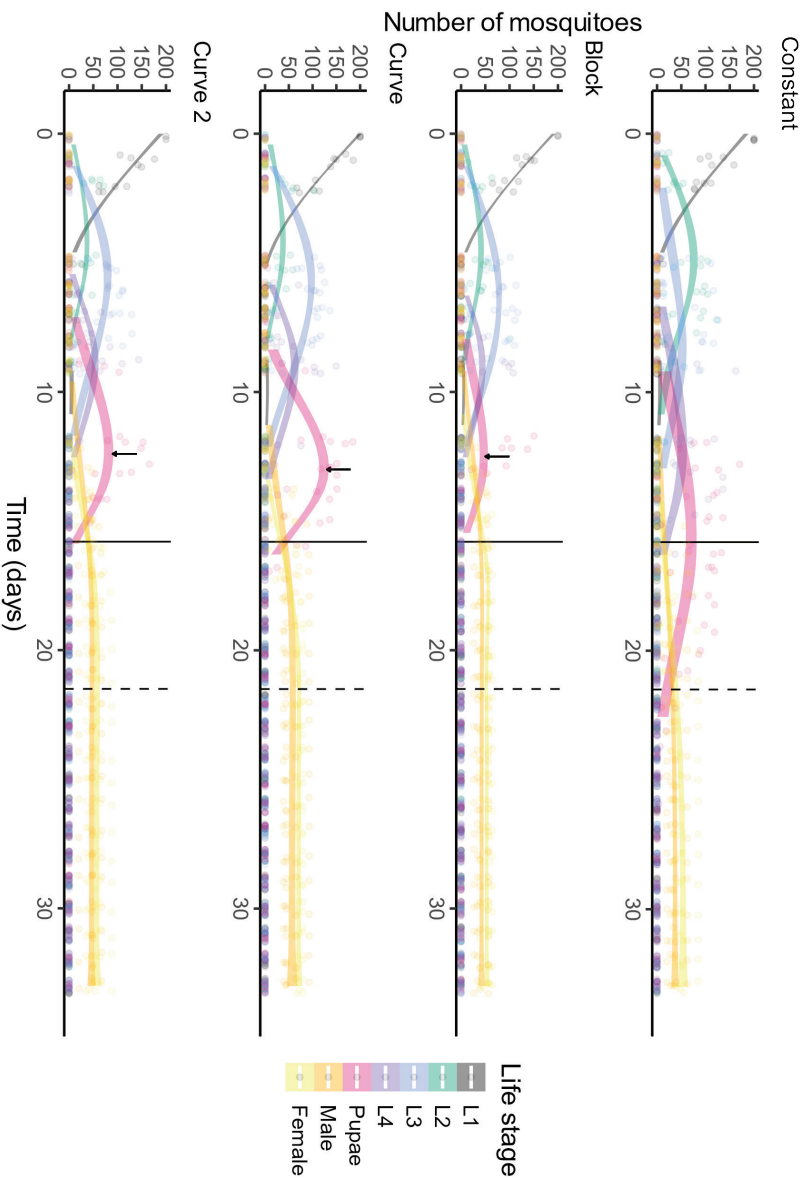


Figure 3.5 Counts per life stage for constant, block, curve and curve 2. As mortality over time was not measured, the sum of the life stages at each timepoint has been assumed to be equal to the total number of emerged adults for each respective container, except for day 0, which is equal to the starting density. Subsequently, for visualization purposes, all mortality is assumed to occur between day 0 and 1. Median time to pupation and 95% pupation in the control treatment are indicated by solid and dashed lines respectively. Median time to pupation in the other treatments are indicated by a purple arrow.

Table 3.3 Differences in median time to pupation (ΔTTP) and median time to emergence (ΔTTE) for each contrasting treatment. Increase/decrease indicates whether differences enlarged or reduced during pupation.

Treatment	ΔTTP (days)	ΔTTE (days)	$\Delta TTE - \Delta TTP$ (days)	$\Delta TTE / \Delta TTP$ (%)	Increase / decrease
Block – Constant	-2.4	-7.3	-4.8	291	Increase
Block – Curve	-0.5	-2.2	-1.7	440	Increase
Block – Curve 2	0.2	-0.6	-0.8	-300	Decrease
Constant – Curve	2.0	5.1	3.1	253	Increase
Constant – Curve 2	2.6	6.7	4.0	247	Increase
Curve – Curve 2	0.7	1.6	0.9	229	Increase

3.4 Discussion

Here we developed a system to assess the effect of thermal fluctuations on ectotherm metabolic rate. Our HIHI system accurately replicated the preprogrammed temperature treatments under outdoor conditions. Comparatively, it performs even more accurately than the traditional Constant and Block regimes, which tend to overshoot and overcompensate due to the thermostat its accuracy of approximately one degree Celsius (Figure 3.4a) as compared to the 0.5 degrees Celsius accuracy of the temperature sensor in the proposed setup. The new system was successfully applied to assess the impact of temperature fluctuations on the development of *Culex* mosquito larvae.

Fluctuations in temperature in our proof-of-principle study had large effects on larval development time, in line with recent models of insect development (Colinet et al. 2015; Kuznetsov et al. 2016; Vajedsamiei et al. 2021; Waqas et al. 2020; Wu et al. 2015), but we did not detect any effect on survival or sex-ratio. Median time to pupation decreased with increasing levels of fluctuation from 16 to 13 days. This effect became even larger during pupation itself with median time to emergence ranging from 22 to 14 days. This is in line with the notion of Kingsolver et al. (2015), that the effect of temperature fluctuations varies across developmental stages. Natural (curve) temperature fluctuations resulted in an average decrease of 7 days (or a third of the development time), as compared to constant temperatures. For both time to pupation and time to emergence, most of the differences in development appear when comparing the constant to natural levels of fluctuation. When comparing Constant to Curve2, thus doubling the amplitude, development decreased by another 1.5 days. Development under the block treatment resembled the natural fluctuation remarkably well, which might partially be explained by the deviation from the preprogrammed mean temperature and occasionally higher amplitude as a result of the short circuit for this treatment after the first 10 days (Supplementary Figs. 3,4). However, the effects of the treatment surpass the change in development rate that might be explained by just a 2°C increase alone (Loetti, Schweigmann, and Burrioni 2011), which suggests that there are additional biological processes at play, with life-stage specific effects.

The remarkable difference during the pupation phase sheds light on the required additional biological explanation. Pupae solely metamorphose, as their pupal enclosure only allows for respiration, not feeding (Becker et al. 2010). This indicates

that interaction with their environment is limited during this stage. As such, the difference in development time with different temperature fluctuations during this life stage is largely internally regulated. Although temperature fluctuations have been described to affect metamorphosis disproportionately (Banahene et al. 2018; Niederegger et al. 2010), we hypothesize that larval stages might be more severely affected than currently visible. During the stages prior to metamorphosis, by other interactions with the environment (competition, food availability, etc.), because of which could mask the effect of thermal fluctuations is less noticeable. Below we elaborate on the possible biological processes underlying the effects of temperature fluctuations on metabolic rate.

Plastic responses to thermal stress, as a part of environmentally induced phenotypic change (plasticity), have been previously described in other ectotherm organisms to be correlated to gene activation, sometimes leading to life-stage-specific tolerances (Belén Arias, Josefina Poupin, and Lardies 2011). Although some gene-specific responses and adaptations are documented (Clark and Worland 2008), there is a very poor understanding of system-wide responses (Gracey et al. 2004). It might be assumed that combined with enzymatic activity (Rao and Bullock 1954), adaptations like gene-activation and its effect on metabolic rate might make development under (natural) variable temperatures more favorable. The exact *modus operandi* behind this pattern, and the relative importance of temperature and genetic dependence on temperature fluctuations, however, remains unknown and requires further study.

Overall, our results strongly suggest that including thermal oscillations in experiments likely results in substantial differences in estimations of key life history parameters (e.g. development time), in our case for mosquitoes. Based on a large body of historical (pre-1970's) as well as more recent literature, there are good reasons to believe that our results are highly similar to those of a large range of ectotherm organisms (De Majo et al. 2019; Hall and Warner 2020; Kuznetsov et al. 2016; Newell 1966; Spanoudis et al. 2019; Waqas et al. 2020; Wu et al. 2015). We speculate that the reason for this is that developmental mechanisms are highly conserved. As such, temperature fluctuations, and systematic impacts thereon – like climate change and urban heat islands – should be considered in experimental work determining the effects of temperature and its interactions. Given that development time of multiple other ectotherm organisms has been shown to be affected by fluctuating temperatures, these findings may have implications ranging far beyond those for mosquitoes.

Our novel temperature control system (HIHI) allows for a crucial step, when aiming to include ecological realism in experimental setups. Our system provides an economic means to simulate natural fluctuations under field-like conditions above ambient temperature and provides a major improvement as compared to currently used systems. A remaining question is whether further steps need be taken when emulating climatic conditions. Fluctuations at different mean temperatures have been described to affect ectotherm organisms non-linearly (De Majo et al. 2019; Kingsolver et al. 2015; Wu et al. 2015), with species specific optimal means (Niederegger et al. 2010). As such there is a need to implement thermal fluctuations in experiments on for instance the effects of heat spikes and more complex fluctuations (Greenspan et al. 2016) on (potentially sex-specific) mortality and assessing severeness of metabolic effects in other organisms. These alterations could be implemented by simply adapting the temperatures within the code. When doing this, we urge future users to verify the interval using a similar pilot as in section 2.2.1 as passive cooling is dependent on a multitude of variables including, but not limited to, ambient temperature, humidity and volume/surface ratio. The current setup does not allow for active cooling as such equipment is costly and likely introduces significant additional disturbance. Therefore, without adaptations, the current setup is limited to temperature regimes above ambient temperatures, or experiments using phenological forcing. Regions with distinct seasonal temperature variations may thus be better suited for the proposed equipment in its current form. As such, alterations would be necessary if cooling below ambient temperature is desired. For instance, by circulating or cooling water via heat exchangers or using a jacketed mesocosm (Potter 2023; Silverberg, Gagnon, and Lee 1995). Alternatively, ambient heating could be minimized by using shading cloth (Schrama et al. 2018; Sukiato et al. 2019). Adaptation to terrestrial setups might be preferable, which can be done similar to the works of Cheng et al. (2011) and Greenspan et al. (2016) i.e. by introducing a humidity sensor and ultrasonic transducer. Similarly, more complex systems should be considered to assess, incorporating interactive effects related to water flow of e.g., presence of organic matter, salinity, vegetation, etc. As such our tool provides a reliable and cost-effective means for a broad range of applications.

Acknowledgements

Pieter Rouweler & Sander Koenraadt are gratefully acknowledged for supplying mosquitoes from the Wageningen University rearing facility. Many thanks to Anne Ummels for her help with the data collection. Martha Dellar, Gertjan Geerling and Louie Krol are thanked for their help during internal review.

Authors' contributions

SB and MS conceived the general idea for the equipment and experiment. Design and development were done by SB. Data collection was performed by SB assisted by MS. EB, MS and PB assisted with the writing of the manuscript. All authors read and approved the final manuscript.

Data Availability

The data supporting the findings of this study, R-script and the operational code are available from the Zenodo repository: <https://zenodo.org/doi/10.5281/zenodo.10724529>. Build instructions are available within the article its supplementary materials.

Funding

This publication is part of the project 'Preparing for vector-borne virus outbreaks in a changing world: a One Health Approach' (NWA.1160.1S.210) which is (partly) financed by the Dutch Research Council (NWO).

Conflict of Interest statement

The authors declare that they have no competing interests.

Supplementary information

Section 1 Supplementary figures and tables

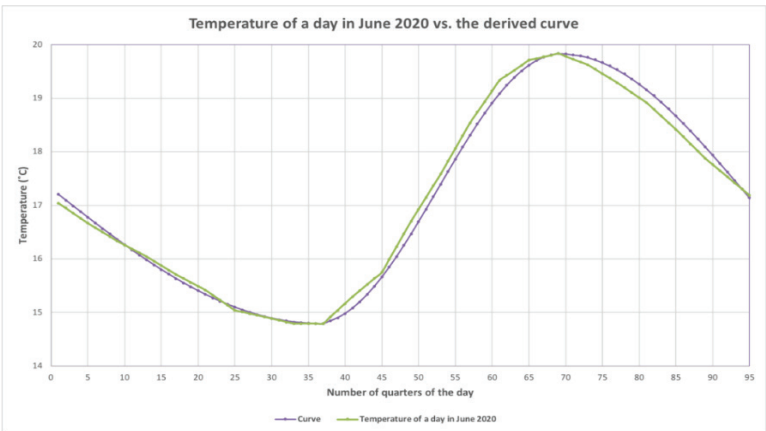


Figure S3.1 Average surface water temperature of a day in June 2020 at the experimental site (green). From this data a sinusoidal curve was constructed using the day-length, mean, minimum, maximum temperature, and interval (purple).

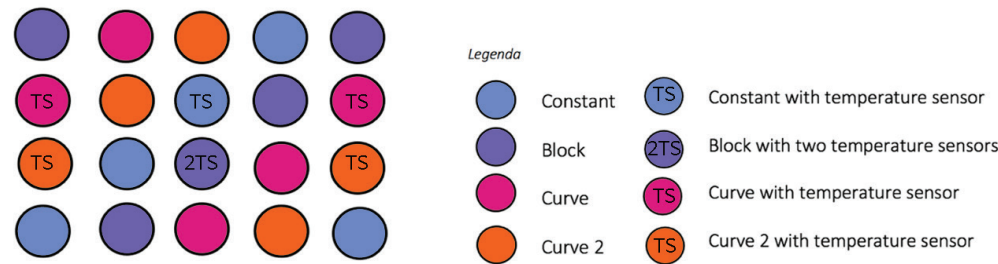


Figure S3.2 Overview of the experimental design. The circles show the containers with treatments with increasing temperature fluctuation. There was one-meter distance between all containers. The temperature sensors indicate DS18B20 sensors used in real time by the HIHI (for Curve and Curve2) or thermostat (for Constant and Block) to activate/deactivate the heaters per treatment.

Table S3.1 Proportion of degree days in each of the treatments during the calibration pilot

Treatment	Prior to calibration		After calibration	
	Degree-days	Proportion respective to constant (%)	Degree-days	Proportion respective to constant (%)
Constant	741.77	100.0	894.05	100.0
Curve 1	753.58	101.6	896.03	100.2
Curve 2	766.45	103.3	885.20	99.0

Table S3.2 Estimated regression coefficients and standard errors for each of the tests

Population Parameter	Formula	Treatment	Estimate	Std. Error	t value	Pr(> t)
Survival rate	survival_rate ~ Treatment	(Intercept)	118.4	9.486569	12.4808025	1.16E-09
		Block	6.6	13.416035	0.4919486	6.29E-01
		Curve	25.2	13.416035	1.8783493	7.87E-02
		Curve2	10.2	13.416035	0.7602842	4.58E-01
Sex ratio	sex ratio ~ Treatment	(Intercept)	0.97788	0.1040257	9.40E+00	1.12E-07
		Block	0.16664	0.1471145	1.13E+00	2.75E-01
		Curve	0.17644	0.1471145	1.20E+00	2.49E-01
		Curve2	0.090245	1.56E-01	0.5783508	5.72E-01
Time to pupation	TTP ~ Treatment	(Intercept)	13.6	5.92E-01	22.988	1.11E-13
		Block	-0.6	8.37E-01	-0.717	4.84E-01
		Curve	-1.8	8.37E-01	-2.151	4.71E-02
		Curve2	-2.6	0.8367	-3.11E+00	0.00677
Time to emergence	TTE ~Treatment	(Intercept)	21.666667	0.6374553	33.989314	7.43E-15
		Block	-7.266667	0.8063242	-9.01209	3.33E-07
		Curve	-5.066667	0.8063242	-6.283659	2.01E-05
		Curve2	-6.666667	0.8063242	-8.267973	9.31E-07

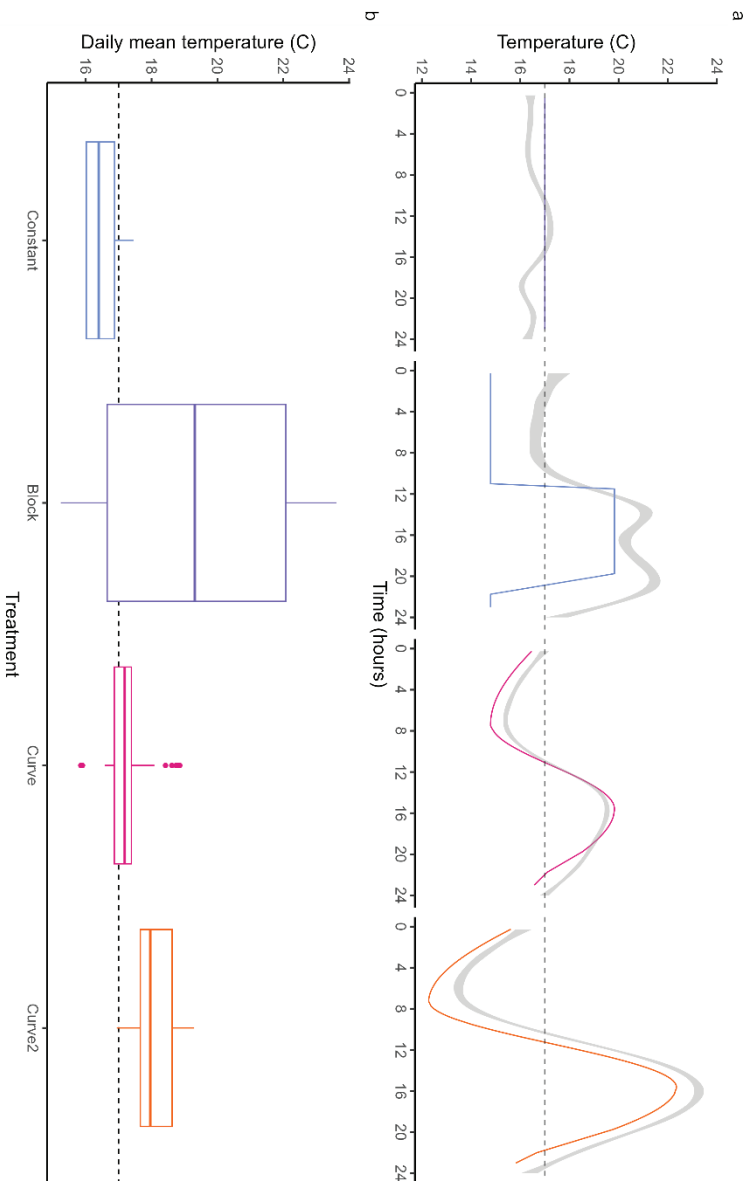


Figure S3.3 Measured temperatures as compared to the programmed temperatures over time for daily fluctuation regimes of increasing levels of fluctuation for the entire duration of the experiment. Panel a shows the measured temperatures over time with standard error in gray and the programmed regimes in color. Panel b shows the mean daily temperature in color with the standard error (± 0.5) for the temperature sensors shown in gray.

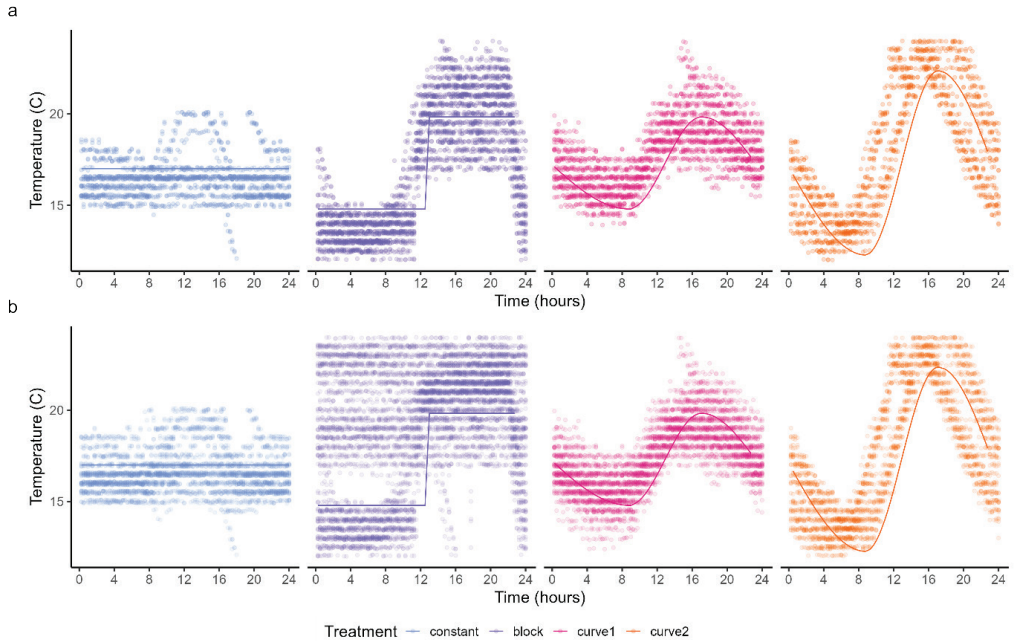


Figure S3.4 Raw data of measured temperatures as compared to the programmed temperatures over time for the daily fluctuation regimes of increasing levels of fluctuation. Panel a shows the measured temperatures over time and the programmed regimes in as lines up to the short circuit. Panel b shows the measured temperatures over time and the programmed regimes as lines for the entire duration of the experiment.

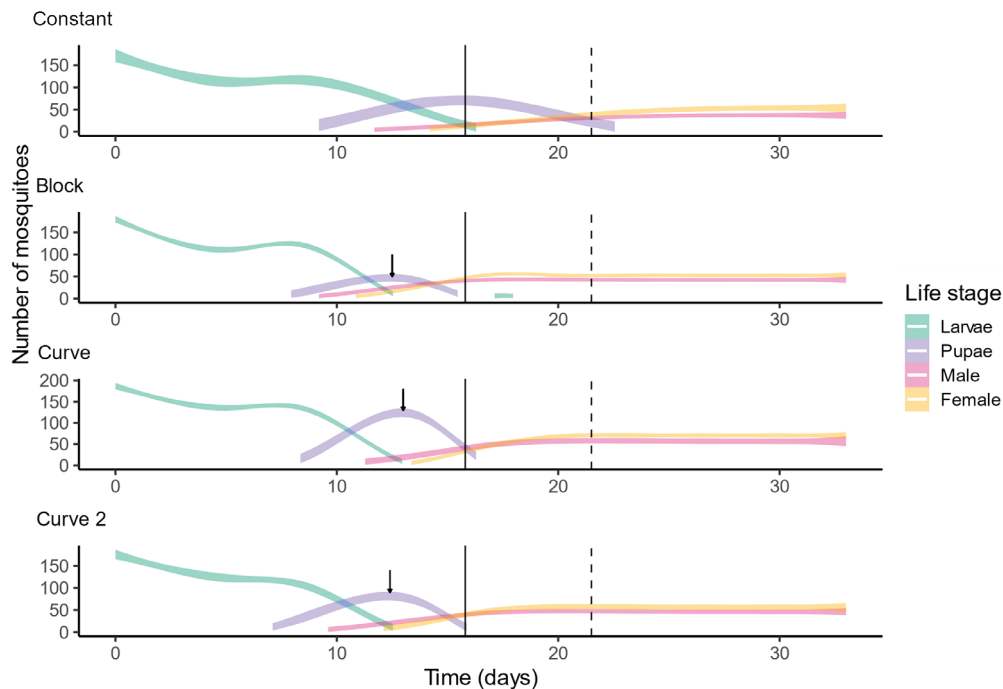


Figure S3.5 Counts per life stage for constant, block, curve 1 and curve 2. The proportion of the life stages at each timepoint has been extrapolated to the total number of emerged adults for each respective container. As mortality over time was not measured, the sum of the life stages at each timepoint has been assumed to be equal to the total number of emerged adults for each respective container, except for day 0, which is equal to the starting density. Subsequently, for visualization purposes, all mortality is assumed to occur between day 0 and 1. Median time to pupation and 95% pupation in the control treatment are indicated by solid and dashed lines respectively. Median time to pupation in the other treatments are indicated by a red arrow.

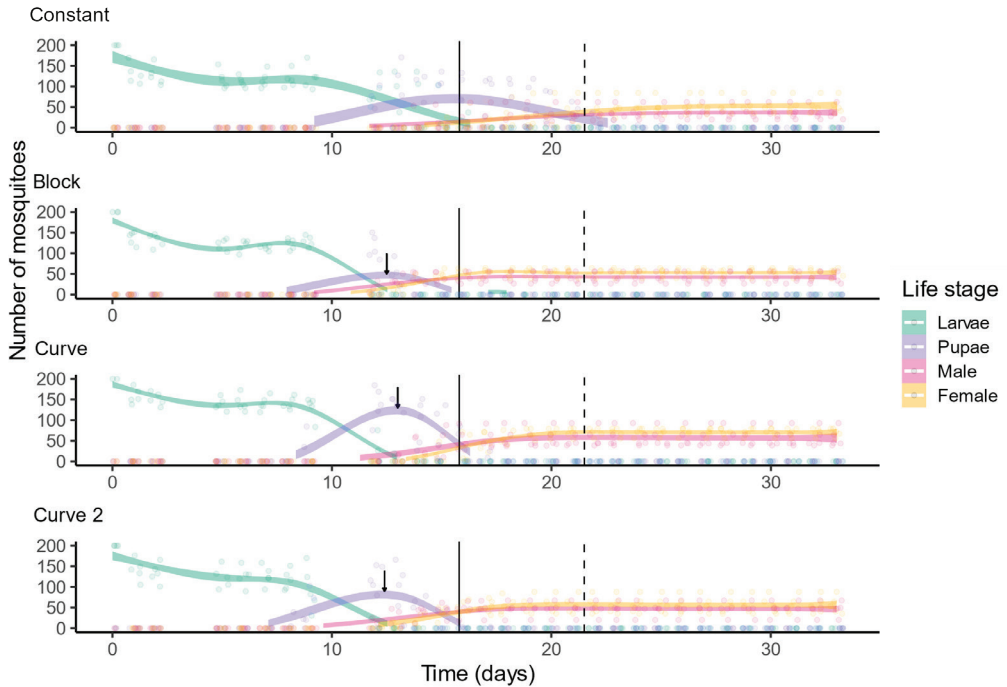


Figure S3.6 Counts per life stage for constant block, curve 1 and curve 2. As mortality over time was not measured, the sum of the life stages at each timepoint has been assumed to be equal to the total number of emerged adults for each respective container. As mortality over time was not measured, the sum of the life stages at each timepoint has been assumed to be equal to the total number of emerged adults for each respective container, except for day 0, which is equal to the starting density. Subsequently, for visualization purposes, all mortality is assumed to occur between day 0 and 1. Median time to pupation and 95% pupation in the control treatment are indicated by solid and dashed lines respectively. Median time to pupation in the other treatments are indicated by a purple arrow.

Section 2 *Build instructions H1H1*

The following are building instructions for the temperature control module. The module consists of two groups of connections. First, the incoming signals, from the temperature sensors, connected to a common ground (black) and 5V (red), and each a separate digital pin (D8-12) for their signal (yellow). Secondly, the outgoing signal, going to the relay board. The relay board is powered by the Vin (red) and ground (black) and is driven by two data lines from digital pin 3 and 4 activating and deactivating the relays. The relay board is connected using female header pins for easy storage. Optionally, the control module can be mounted on the Arduino like a shield using male header pins.

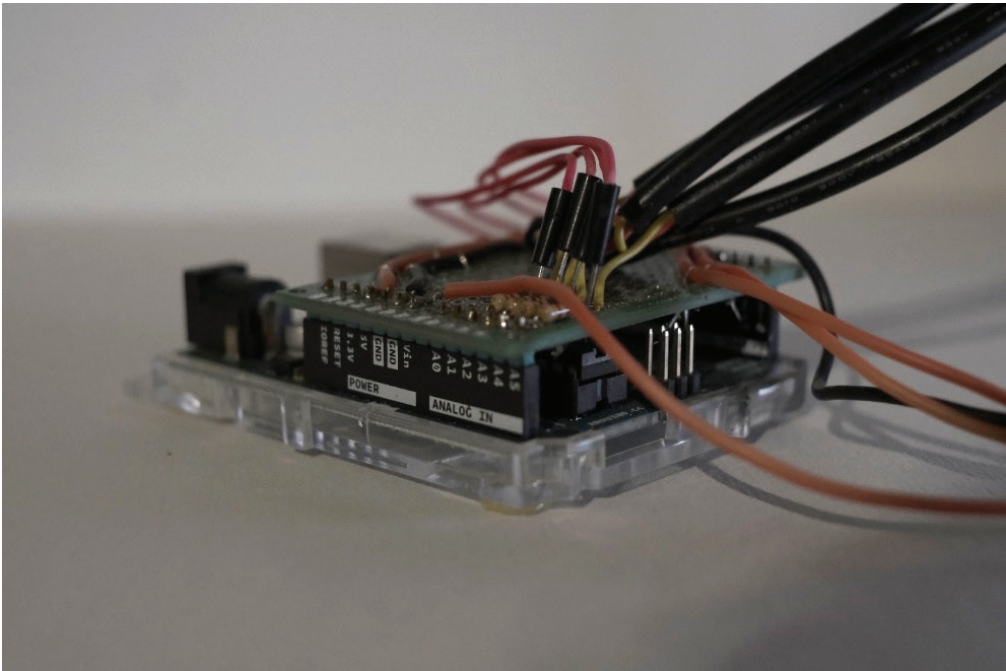


Figure S3.8 Overview of the temperature control module

Step 1. Components list

- Arduino uno
- DS18B20 temperature sensor (4x)
- 4 Channel octocoupler relay interface board (e.g. 2ph109375a)
- Resistors 470 Ω (4x)
- perfboard
- Red and black insulated wire (approx. 50cm each)
- Soldering iron and solder
- Female header pin 1x6; as connector to the relay board
- Optional: male header pin (1x14 and 1x8 as shown or 1x1 for each of the four corners; to mount the PCB to the Arduino)
- Optional: 20cm metal wire (for cable management and to secure the wires to the relay module)

Step 2. Circuit diagram

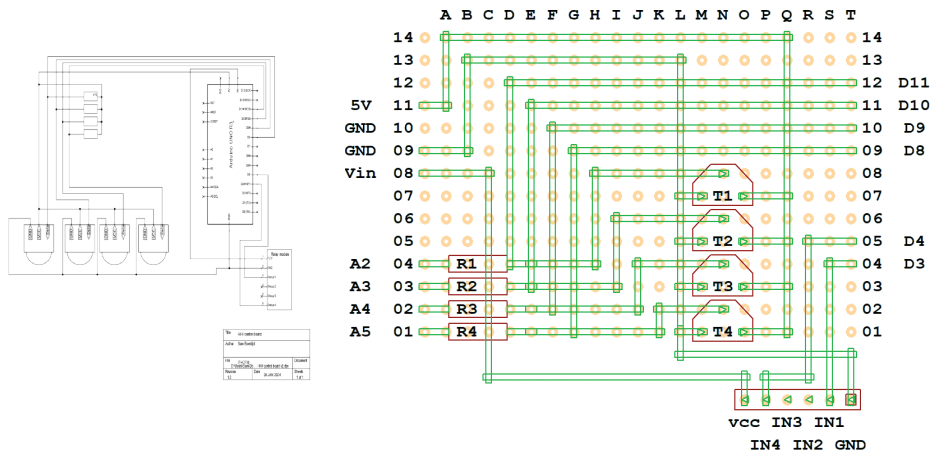


Figure S3.9 Schematic overview of the temperature control module, with a circuit diagram (left) and an example of the corresponding physical layout (right).

Step 3. Building process

- Insert all components into the perf board in a similar arrangement to the circuit diagram (Figure S8) and bend the legs to prevent them from falling out.
- Optionally, insert the header pins into unused connections on the Arduino. Lay the PCB on top so that it rests flat on the header pins.
- Connect all wires:
 - o Relay module: Vin - 12V, GND – GND, D3 – Relay 1, D4 – Relay 2
 - o Sensor1: GND – GND, 5V – VCC, D8 & R1 – Data
 - o Sensor2: GND – GND, 5V – VCC, D9 & R2 – Data
 - o Sensor3: GND – GND, 5V – VCC, D10 & R3 – Data
 - o Sensor4: GND – GND, 5V – VCC, D11 & R4 – Data
 - o Resistors: 5V – R1 & R2 & R3 & R4
- Solder the connections alternating between components, so that they can cool sufficiently.
- Optionally, cut the metal wire into 4cm strands and secure the wires leading to the relay module at the bottom of the control unit, so that stress on the wires will not damage the solder joint. You may use any remaining wire for cable management.
- Insert the female header pins into the relay module (Figure S3.11) and solder the respective wires in place. Take care not to heat each connection for too long as this may cause the plastic to melt.

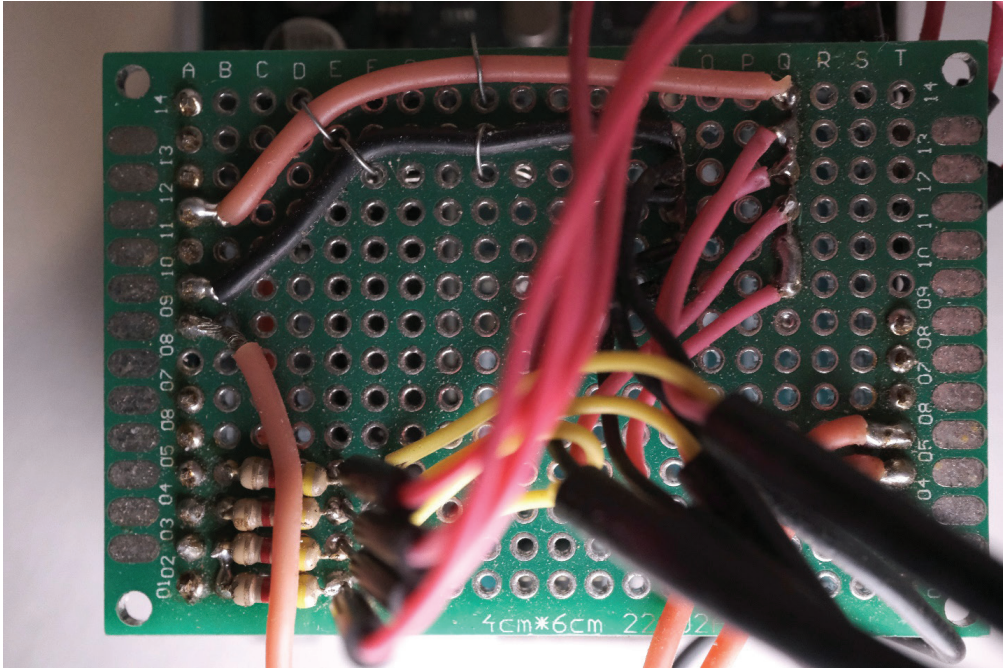


Figure S3.10 Overview of the control module.

Connections are indicated using the perf board its indices from A-T (left-right) and 1-14 (bottom-top). The ground (A9) and 5v (A11) connections are split across the temperature sensors at the top (N9-13, Q9-14). Their data lines in yellow at the bottom left (E1-5) are connected directly to digital pins 8-12 at the top right (T11-14), and via resistors at the bottom left (B1-5) to the 5v connector (via the underside of the board; B11). The relay module is powered by the Vin (B8) and ground (N9) and driven by digital pins 3 and 4 of the Arduino (T4,5).

Step 4 Connect the power strips

- Using a set of pliers, remove 3cm of the outer insulation of each power strip, at roughly 10cm from the plug.
- Cut the blue wire and strip 1cm of insulation of each end.
- Screw open the common and normally closed ports of the respective relays, insert one end of the blue wire into each of the ports and screw the ports shut to secure the wire.

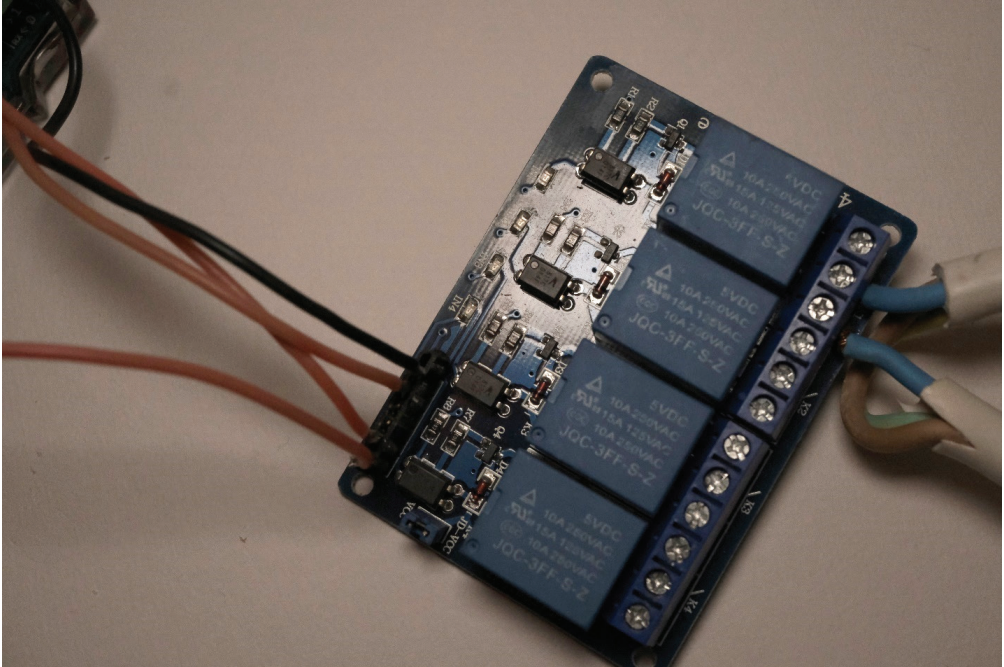


Figure S3.11 Overview of the relay module.

The input from the control board (left) consists of (from top to bottom) the ground in black, data line relay 1 in red, data line relay 4 in red and 5v in red. The output to the power strip (right), here shown for relay 1, is connected to the common (middle) and normally closed (NC; bottom) port of the relay.

Step 5. Upload the script

Connect the Arduino using the supplied USB connector to your computer and upload the script using Arduino IDE.

Step 6. Check functionality

- Prepare a glass of cold water
- Power the Arduino.
- Take the temperature sensors into your hand and heat them up to body temperature.
- Confirm that the relays open by auditory (hearing the relay 'click') and visual cues (using the led indicator).
- Drop the sensors into the cold water
- Confirm that the relays close by auditory (hearing the relay 'click') and visual cues (using the led indicator).