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

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Citation

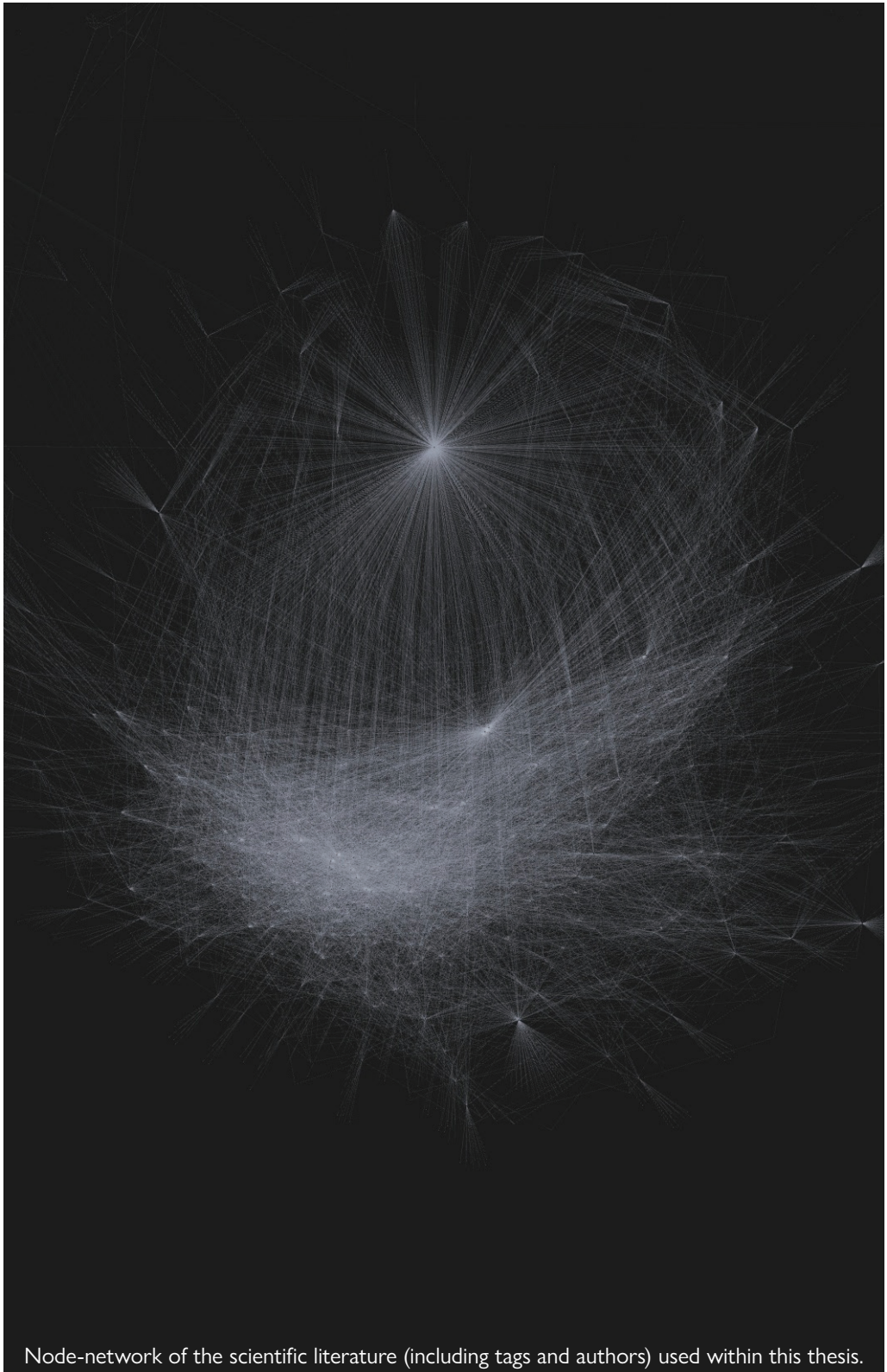
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Chapter 2

Biting the hand that feeds: anthropogenic drivers interactively make mosquitoes thrive



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Highlights

- 1) Impacts of interlinked anthropogenic stressors are relatively unknown.
- 2) Such stressors include land use change, freshwater pollution, and extreme weather.
- 3) Full-factorial experiments assessed stressor impacts on disease vector *Culex pipiens*.
- 4) The stressors and their interactions had major impacts on key mosquito life-history traits.
- 5) Results show important ramifications for mosquito populations and the pathogenic landscape.

Abstract

Anthropogenic stressors on the environment are increasing at unprecedented rates and include urbanization, nutrient pollution, water management, altered land use and climate change. Their effects on disease vectors are poorly understood. A series of full factorial experiments investigated how key human induced abiotic pressures, and interactions between these, affect population parameters of the cosmopolitan disease vector, *Culex pipiens s.l.* Selected pressures include eutrophication, salinity, mean temperature, and temperature fluctuation. Data were collected for each individual pressure and for potential interactions between eutrophication, salinization and temperature. All experiments assessed survival, time to pupation, time to emergence, sex-ratio and ovipositioning behavior. The results show that stressors affect vector survival, may speed up development and alter female to male ratio, although large differences between stressors exist to quite different extents. While positive effects of increasing levels of eutrophication on survival were consistent, negative effects of salinity on survival were only apparent at higher temperatures, thus indicating a strong interaction effect between salinization and temperature. Temperature had no independent effect on larval survival. Overall, increasing eutrophication and temperatures, and the fluctuations thereof, lowered development rate, time to pupation and time to emergence while increasing levels of salinity increased development time. Higher levels of eutrophication positively impacted egg-laying behavior; the reverse was found for salinity while no effects of temperature on egg-laying behavior were observed. Results suggest large and positive impacts of anthropogenically induced habitat alterations on mosquito population dynamics. Many of these effects are exacerbated by increasing temperatures and fluctuations therein. In a world where eutrophication and salinization are increasingly abundant, mosquitoes are likely important benefactors. Ultimately, this study illustrates the importance of including multiple and combined stressors in predictive models as well as in prevention and mitigation strategies, particularly because they resonate with possible, but yet underdeveloped action plans.

Keywords: Interactive effects, Habitat alteration, Mosquito, Planetary boundary framework, Salinization, Temperature fluctuations

2.1. Introduction

2 Anthropogenic pressures play a pivotal role in shaping ecosystems on a global and local scale (Foley et al., 2005; Nelson et al., 2006), and subsequently affect species abundance and composition (Barausse et al., 2011; Cecco et al., 2022; Eremeeva and Sushchev, 2005). While most insect species are declining due to anthropogenic pressures, such as urbanization, agricultural practices, water management and climate change (Hallmann et al., 2017; van der Sluijs, 2020), recent evidence shows that many mosquito species thrive under these conditions (Colón-González et al., 2021; Messina et al., 2019; Roche et al., 2015), thus showing a contrasting pattern, particularly in areas of high anthropogenic pressures (Kamdem et al., 2012; Nilsson et al., 2018; Ramasamy and Surendran, 2016; Schrama et al., 2020; Steiger et al., 2012). This suggests that human-induced environmental changes might have a positive impact on the availability of disease transmitting organisms (hereafter vectors), which might also influence the incidence of mosquito transmitted pathogens, such as protozoa and arthropod-borne viruses (arboviruses) (Franklinos et al., 2019; Kamdem et al., 2012; Neiderud, 2015).

Research on understanding impacts of human-induced changes on infectious diseases has until now focused on the large-scale influence of climatic variables such as mean temperature for mosquito occurrence and precipitation for mosquito abundance (Loetti et al., 2011; Mottram et al., 1994; Petrić et al., 2014; Reisen et al., 1984), while the effects of anthropogenic pressures that operate mainly on a local scale have been poorly addressed. Such local stressors include effects of changes in land use, freshwater use, biogeochemical flows, local temperature fluctuations, as well as the interplay between these drivers on microclimatic conditions (Rejmánková et al., 2013). While observational studies carried out at the landscape scale hint at the importance of these factors (Ferraguti et al., 2016; Krol et al., 2022; Schrama et al., 2020; Versteirt et al., 2013), a comprehensive understanding of the causal relationships between mosquito population size and structure and specific landscape features is lacking (Franklinos et al., 2019; Schrama et al., 2020). Moreover, the interactions of the different pressures, which often co-occur, have not been investigated in depth (Figure 2.1, supplementary Table 2.1). To understand the importance of these key anthropogenic pressures on mosquito population dynamics, which might lead to increased vulnerability to changes in (combinations of) environmental factors, in-depth experimental work is needed.

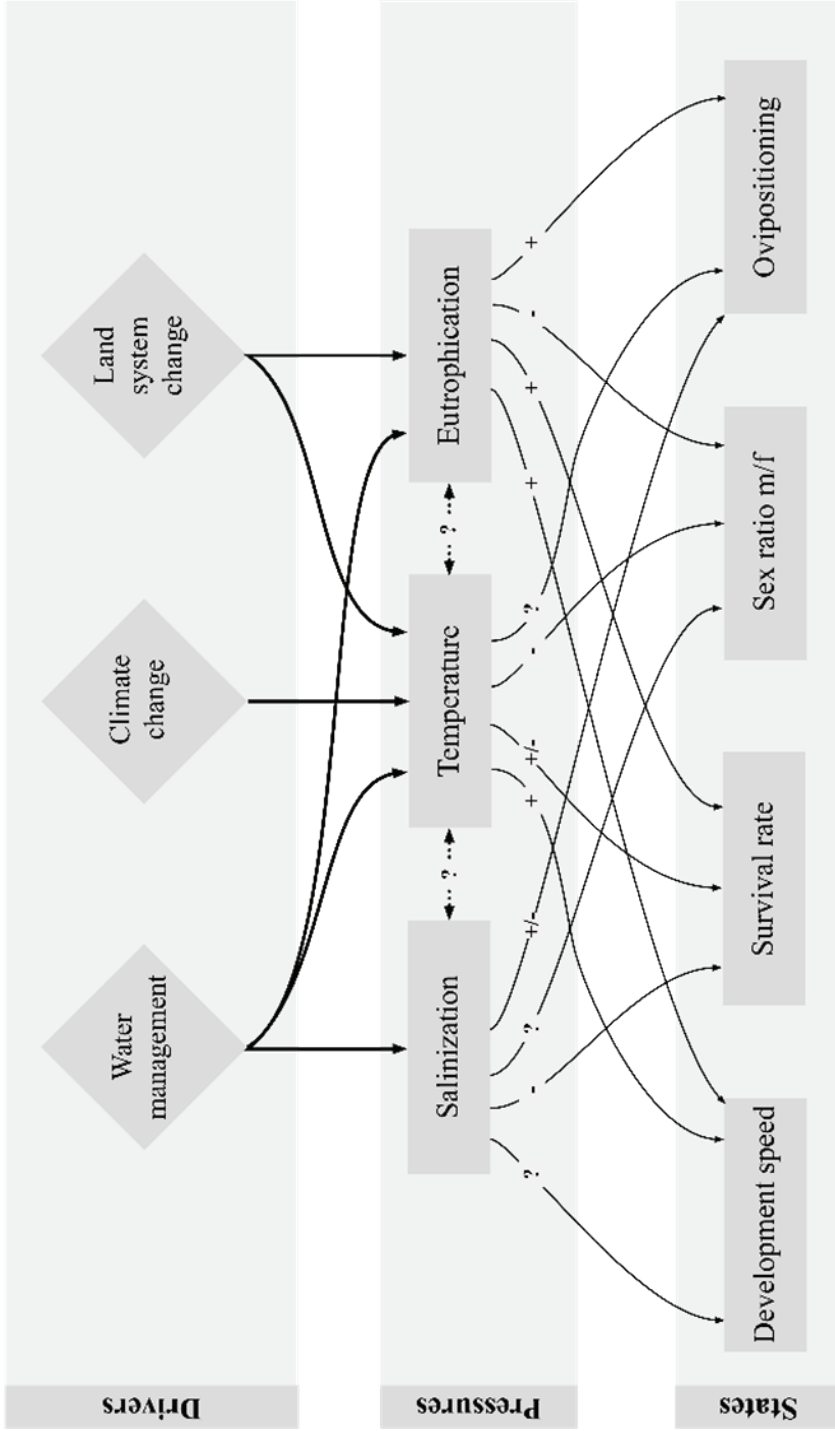


Figure 2.1 Overview of anthropogenic pressures as drivers on abiotic pressures and its impacts on downstream population parameters of mosquitoes. For each of the established impacts between pressures and states, it is indicated whether the association is positive (+), negative (-) or both (+/-) (based on literature summarized in Supplementary Table 1). Question marks indicate hypothesized links and interactions of pressures where further work is needed.

Here, we experimentally explore the impact of salinization, temperature and eutrophication on mosquito populations (Figure 2.1). Freshwater use – more broadly defined as water management, thus including biogeochemical flow boundaries – mainly impacts eutrophication, temperature, and salinity by a variety of pathways, such as dredging of inland water bodies (Lohrer and Wetz, 2003; Smith and Huang, 2010), water retention (Ceccatelli et al., 2021; Modoni et al., 2013; Zhu et al., 2015) and freshwater extraction (de Louw et al., 2013; Schmork and Mercado, 1969). Land use change, in which humans transform the natural landscape impacts water bodies with respect to dissolved nutrient concentrations (potentially leading to eutrophication) and temperature via changes in sunlight in cleared areas (Franklinos et al., 2019) and albedo (Adegoke et al., 2007), respectively. Climate change, caused by greenhouse gas emissions leads to increasing mean temperatures and changes in diurnal temperature oscillations due to increasing nightly temperatures (Braganza et al., 2004).

Each of these large-scale environmental impacts can be expected to directly affect mosquito populations. Salinization, which occurs naturally in coastal areas (Don et al., 2006; Lassiter, 2021), is strengthened by climate induced sea-level rise, draining of fresh water, management of waterways and exploitation of groundwater for agriculture (Pauw et al., 2012; Ramasamy and Surendran, 2012). It is known to have species specific impacts on mosquito mortality rates (Jude et al., 2012; Kengne et al., 2019; Mottram et al., 1994) and egg laying (hereafter ovipositioning) behavior (De Brito Arduino et al., 2015; Navarro et al., 2003; Silberbush et al., 2014), but landscape wide responses to changes in salinity levels are poorly characterized. Eutrophication has previously been shown to influence development speed, survival, sex-ratio (Krol et al., 2019; Merritt et al., 1992; Reisen et al., 1984; Rejmánková et al., 2013; Schrama et al., 2018) and ovipositioning behavior (Rejmánková et al., 2013), but interactions with other stressors, e.g. larval densities of the same species (hereafter conspecific density), are not well represented.

While the effects of temperature on mosquitoes have been studied extensively, the effects of an increases in temperature fluctuations, which is expected because of climate change (Masson-Delmotte et al., 2021), are yet unknown. Temperature is known to affect mosquito development speed and mortality levels as well as sex-ratio with sex-specific effects (Loetti et al., 2011; Mottram et al., 1994; Petrić et al., 2014; Reisen et al., 1984), where increasing temperature generally leads to shortened development times and higher mortality. However, the vast majority

of experiments focusing on temperature have been performed by manipulating (continuous) mean temperatures (Ciota et al., 2014; Kiarie-Makara, Ngumbi, and Lee n.d.; Loetti et al., 2011), even though temperature fluctuations have previously been hypothesized to influence mosquito development (Alcalay et al., 2018; Couret and Benedict, 2014; Kiarie-Makara et al., 2015; Mottram et al., 1994; Spanoudis et al., 2019). As yet, its impact remains severely understudied (Alcalay et al., 2018; Beck-Johnson et al., 2017).

Aside from a comprehensive understanding of the isolated effects of the selected stressors, there is also a large potential for the influence of non-additive interactive effects (Tran et al., 2018). Indeed, a growing number of studies point to the ubiquity of interactive non-additive effects between stressors on population parameters (Agnew et al., 2000; Alcalay et al., 2018; Couret and Benedict, 2014; Schrama et al., 2018), but little is currently known about interactive effects. Interaction between eutrophication, larval density and temperature may manifest itself as changes in food intake per capita; interaction between salinization and temperature might increase energy expenditure for active osmoregulation via increased competition (Bradley, 1987; Kengne et al., 2019). The current lack of information on solitary and interactive effects of the selected stressors calls for a coherent and comprehensive assessment.

This study aims to evaluate and quantify the effects of eutrophication, salinization, temperature fluctuations including the interactions between eutrophication and conspecific density, temperature and eutrophication, and temperature and salinity on multiple mosquito population parameters in a series of mesocosm experiments. The cosmopolitan mosquito species *Culex pipiens* s.l. (hereafter denoted as *Cx. pipiens*) is used as a model species. *Cx. pipiens* is a known vector, e.g., for West Nile virus, Usutu virus, Sindbis virus, and parasites Filarioidea, Plasmodium and Hemoproteus (Bravo-Barriga et al., 2016; Gutiérrez-López et al., 2016; Hubálek, 2008; Kazlauskienė et al., 2013). It has a wide habitat tolerance ranging from clean rainwater-filled containers to strongly polluted temporal waterbodies such as ground puddles and even manure tanks (Becker et al., 2013; Rejmánková et al., 2013). Wild caught individuals were used – unless mention otherwise – without distinguishing between the different (sub)species of the species complex. This allowed for a more accurate estimate of the pressures on a mixed natural community.

2.2. Methods

2.2.1. General experimental design

All experiments were carried out in polypropylene mesocosm containers under outdoor conditions at the Living Lab field station or at the botanical garden of Leiden University, The Netherlands. The experiments focused on semi-realistic field conditions, as the temperatures, eutrophication levels and salinities are representative of a range of larval habitats (Alcalay et al., 2019; Ikeshoji and Mulla, 1970; Loeb and Verdonschot, 2008; Oude Essink et al., 2010; Roberts and Irving-Bell, 1997; Rockett, 1987; Wallis, 1954). The treatments were - unless mentioned otherwise - applied to 65 l black polypropylene mesocosms filled up to 30 l and placed in a second, fully buried identical mesocosm thus providing an air-filled layer of insulation to buffer temperature fluctuations (Krol et al., 2019).

Each mesocosm was filled with tap water and then spiked with a microbial community acquired by filtering a standardized amount of water from an adjacent lake through a Φ 200 nm plankton net with Φ 53 μ m collector, so that each volume of water in the mesocosms contained a similar microbial community to that found in the same volume of water in the lake. To simulate the different levels of eutrophication standardized dried cow manure (2.4 % N; 1.5 % P₂O₅; 3.1 % K₂O) was added. The mesocosms were then left to incubate for a minimum of one week so that the microbial communities could stabilize. During this period, the mesocosms were covered with shading cloth to prevent Chironomidae and Culicidae colonization. After the acclimation period, all water within the mesocosms was filtered with a Φ 300 μ m sieve, to remove large particles and any remaining macro-invertebrates. Unless mentioned otherwise, *Culex pipiens* s.l. egg rafts were collected during a four-day period prior to the start of an experiment from naturally colonized black plastic buckets at the Living lab field station. Larvae were randomly selected and then added in random block design.

During the experiments all containers were covered with emergence traps (Cadmus et al., 2016) to prevent i) colonization by *Culex pipiens* s.l. mosquitoes and any other species that might compete with the experimental population, ii) mosquito predators from entering and iii) trap emerged adults. The water level was kept stable by daily replenishment of the evaporated volume with dechlorinated tap water after measurements were taken. The temperature in each mesocosm was recorded every thirty minutes for the duration of each experiment by a temperature logger (iButton DS1921G#F5D) near the water surface. Abiotic

conditions were measured weekly using a HACH HD40 for dissolved oxygen, pH and conductivity measurements, an Aquafluor 8000-010 for chlorophyll *a* and turbidity measurements and a Vernier Labquest2 for ammonium, chloride, nitrate and phosphor concentrations.

2.2.2. Experiments

2.2.2.1. Eutrophication-density interaction

The experiment on interactive effects of eutrophication and larval densities took place between August and October of 2020. Eutrophication levels were chosen so as to span the entirety of the oligotrophic-hypertrophic range with levels representing a low-eutrophic water body (5 mg/l N-total), a high-eutrophic water body (20 mg/l N-total) and a hypertrophic land puddle (100 mg/l N-total) (Loeb and Verdonschot, 2008). To simulate the different levels of eutrophication standardized dry cow manure (2.4 % N; 1.5 % P₂O₅; 3.1 % K₂O) was added. Larval densities representative of 0.1, 1 and 10 egg rafts (20, 200 and 2000 larvae, respectively) were used to study the effects of increasing conspecific competition (Agnew et al., 2000). The treatments were applied with four replicates in a random block design.

Egg laying behavior was not measured within this experiment as the effects of eutrophication could be determined from experiment 2.2.2 and the effects of larval presence had already been studied (Mwingira et al., 2020).

2.2.2.2. Eutrophication-temperature interaction

The experiment on the interactive effects of eutrophication and temperature levels was conducted in 65 l black polypropylene mesocosms filled up to 30 l between April and June of 2020. Eutrophication levels were chosen as described in Section 2.2.1, additionally including a treatment representing rainwater (0 mg/l N-total). Temperature levels were selected around the optimal rearing temperature (Loetti et al., 2011) and set at 20, 25 and 30 degrees Celsius. Temperature treatments were regulated using 200 W heaters (HSaqua).

The treatments were applied with four replicates in a random block design. Twenty first instar larvae were added to each of the mesocosms. The low larval density served to exclude potential effects of density dependence (Alcalay et al., 2018). After the experiment, each mesocosm was filtered with a Φ 300 μ m sieve to remove any remaining (dead) larvae and/or pupae. The emergence traps were then removed and ovipositioning choice was scored for five consecutive days.

2.2.2.3. *Salinity and temperature interaction*

The experiment on interactive effects of salinity and temperature was conducted in white 12 l polypropylene mesocosms filled to 10 l during the months of May and June of 2021. The selected salinity levels were representative for the current and expected salinity levels encountered at the Dutch coastal areas, freshwater (0 g/l chloride), brackish (0.3 g/l chloride) and saline (1 g/l chloride) conditions (Oude Essink et al., 2010).

Salinity levels were applied using a sodium chloride stock solution. Temperature levels were selected near the optimal rearing temperature (Loetti et al., 2011) and set at 27 and 30 degrees Celsius. A eutrophication level of 20 mg N-total was applied to minimize competition over resources, as determined by the experiment described in Section 2.2.1. The salinity-temperature treatments were applied and 200 first instar larvae were added with three replicates in a random block design. Any pupa encountered during the experiment was counted and removed as the pupae do not eat and have a hardened exoskeleton (Becker et al., 2010), which limits their interaction with the environment (Balasubramanian et al., 2019; Mottram et al., 1994; Patrick and Bradley, 2000). Consequently, data on time to emergence and sex-ratio were not collected for this experiment.

To test the effect of salinity on ovipositioning choice, 5 blocks of five 12 l black mesocosms were prepared with a concentration that is representative of the range between freshwater and saltwater (0, 0.4, 1.6, 16 and 32 g/l sodium chloride). The blocks were distributed across the botanical gardens (80 m apart) in the shade. Egg rafts were collected for five consecutive days.

2.2.2.4. *Temperature fluctuations*

The experiment on the effects of increasing levels of temperature fluctuation was executed in white 12 l polypropylene mesocosms filled to 10 l between the end of March and beginning of May 2021. This experiment took place during the overwintering of the natural populations, so that the ambient temperature would not overtake the experimental temperatures. During this experiment, the water temperature conditions of an average day in June were mimicked. June is commonly regarded as the optimal month for mosquito development in NW Europe as the amount of sunlight energy is maximal (Becker et al., 2010).

To determine appropriate temperatures, water surface temperatures were monitored in three white 8 l containers with an interval of 30 min between the months of May and July 2020. From these data a mean, minimum and maximum temperature, as well as the temporal interval between these, were derived and used to set four treatments of increasing fluctuation with the same amount of energy applied per 24 h. These included a constant, block and curve scheme as well as a treatment with twice the amplitude of daily fluctuations, hereafter referred to as curve2 (Supplementary Figure 2.1). The treatments were temperature-controlled with thermostats and an Arduino UNO microcontroller. For this experiment *Culex pipiens pipiens* first instar larvae were obtained from culture from Wageningen University & Research, The Netherlands. The treatments were applied and 200 first instar larvae were added with five replicates in a random block design. A eutrophication level of 20 mg N-total was applied to minimize competition over resources, as determined during the experiment on interactive effects of eutrophication and larval density (see Section 2.2.1). Egg laying behavior was not measured within this experiment as temperature was determined to not affect larval habitat site selection during the experiment on interactive effects of temperature and eutrophication (see Section 2.2.2).

2.2.3. Rearing of larvae

For the experiment on temperature fluctuations *Culex pipiens pipiens* first instar larvae were obtained from culture from Wageningen University & Research, The Netherlands.

For the remaining experiments, *Culex pipiens* s.l. egg rafts were collected during the four days prior to the start of an experiment from naturally colonized black plastic buckets at the Living lab field station. For this, the buckets were filled with 6 l hypertrophic water (100 mg N-total). The larvae were subsequently allowed to hatch in white 12 l mesocosms containing 10 l of lake water where they were kept at ambient temperature until the start of the experiment. Previous pilot studies indicated that this type of experiments attracts *Culex pipiens* only. The collected egg rafts were distinguished from those of *Culiseta annulata* by their difference in size (Chapman et al., 2020; Sames et al., 2005).

2.2.4. Measurements and life stage identification

Larval development was measured five days a week by stirring clockwise once with a 400 mm wide Φ 200 μ m sieve to create a circular water flow to prevent the larvae from diving. The sieve was subsequently used to collect the larvae by

2 fully submerging the sieve and moving anti-clockwise twice. All collected larvae were morphologically characterized to developmental stage by using the size of the head capsule as a morphological indicator (Becker et al., 2010). The identifications were compared daily with a previously reared reference collection of *Cx. pipiens* developmental stages. The procedure was repeated up to five times until at least five, twenty or a hundred larvae were sampled for the densities of 20, 200 and 2000, respectively.

Each day, emerged mosquitoes were collected with an aspirator and sex was determined based on characteristics including, but not limited to, plumose/pilose antennae, and the length of the palps (Becker et al., 2010). The survival rate was determined by dividing the number of adults that had emerged by the applied density. The median time to pupation was defined as the interval between the start of the experiment and the first day upon which at least 50 % of the subsampled larvae had turned/developed into pupae. The median time to emergence was determined by calculating the interval between the start of the experiment and capture of 50 % of the emerged adults. When no more pupae and adult mosquitoes were found for two subsequent days in a mesocosm, it was assumed that there were no living mosquitoes left and the mesocosm was closed off.

Ovipositioning behavior was recorded by daily counts of egg rafts per mesocosm per day. Encountered egg rafts were removed to minimize the positive feedback caused by their presence (Bruno and Laurence, 1979).

2.2.5. Statistical analysis

All data were analyzed in R version 4.04 (R Core Team, 2022). Differences in survival, development, sex-ratio and ovipositioning behavior between and within eutrophication and density levels, between and within eutrophication and temperature levels and between levels of temperature fluctuation, were tested with general linear models comparing the number of emerged mosquitos, average development stage, the number of emerged mosquitoes per sex and the number of egg-rafts respectively. Dissolved oxygen, nitrate, ammonium, and chloride levels as well as chlorophyll α concentrations were included as main effects. The effects of survival, development and ovipositioning behavior between and within salinity and temperature levels, were tested with general linear models comparing the number of emerged mosquitos, average development stage and the number of egg-rafts respectively. For this, chlorophyll α and dissolved oxygen

concentrations were included as main effects. Lastly, the effects of survival and development between and within salinity and temperature levels, were tested with general linear models comparing the number of emerged mosquitos and average development stage. For this, chlorophyll a and dissolved oxygen concentrations were included as main effects. All (16) corresponding full models are presented in Supplementary Table 2. 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.

2.3. Results

As the aim of this paper is to shed light on the effect that anthropogenic pressures have on each of the population parameters, the results have been grouped per dependent variable. This facilitated the comparison of effects between the different pressures. Estimated regression coefficients and standard errors for each of the tests are listed in Supplementary Table 2.

2.3.1. Impact of anthropogenic pressures on absolute survival

Absolute survival rate was not impacted by different levels of temperature, temperature fluctuations, or combined effects of temperature and eutrophication within the ranges tested ($p > 0.1$). Survival did however decrease under increasing temperatures within the saline treatment ($F(22,178) = 1.983$, $p < 0.01$, partial $\omega^2 = 0.073$, power = 0.964). Post hoc analysis revealed significant differences in survival between the highest salinity at 30 degrees and lowest salinity at 27 degrees for day 7 of the experiment and between the two temperatures at the highest salinity for days 7 and 10 ($p < 0.05$ after Bonferroni correction) (Figure 2.2a). Survival also decreased with increased conspecific density ($F(2, 25) = 11.613$, $p < 0.001$, partial $\omega^2 = 0.384$, power = 0.994) and decreasing amounts of food per capita ($F(4, 25) = 5.745$, $p = 0.002$, partial $\omega^2 = 0.358$, power = 0.981). Post hoc analysis revealed significant differences between the oligotrophic treatment with 2000 larvae and all other treatments ($p < 0.001$, Bonferroni correction) as well as among the hypertrophic treatments with 20 and 200 larvae ($p < 0.05$, Bonferroni correction; Figure 2.2d).

2.3.2. *Impact of anthropogenic pressures on time to pupation*

Development time up to pupation decreased significantly with increasing temperatures in both the salinity experiment ($F(1,15) = 4.868$, $p < 0.05$, partial $\omega^2 = 0.156$, power = 0.594; Figure 2.3a) and the experiment on interactive effects of eutrophication and temperature ($F(2,40) = 38.57$, $p < 0.001$, partial $\omega^2 = 0.620$, power = 1; Figure 2.3c) by 7 and 3 days, respectively. Additionally a trend was found for the interaction between temperature and salinity ($F(2,15) = 2.709$, $p < 0.1$, partial $\omega^2 = 0.140$, power = 0.529). Post hoc analysis revealed that this was caused by a trend in contrasts between the temperatures within highest salinity level ($p < 0.06$, Bonferroni correction) (Figure 2.3a). Increasing levels of temperature fluctuation also decreased development time up to pupation. Differences were detected between the constant and curve treatments ($\chi^2 = 2.017$, $p = 0.022$) and the constant and curve 2 treatments ($\chi^2 = 2.711$, $Df = 3$, $p = 0.003$) (Figure 2.3b). No solitary or interactive effects of eutrophication level were found (Figure 2.3c) except at higher densities, where decreasing amounts of food per capita resulted in longer development times. This was true when manipulating the eutrophication level ($F(2, 25) = 22.286$, $p < 0.001$, partial $\omega^2 = 0.556$, power = 1), density ($F(2, 25) = 5.924$, $p = 0.008$, partial $\omega^2 = 0.225$, power = 0.876) and the combination of the two ($F(4, 25) = 3.012$, $p = 0.037$, partial $\omega^2 = 0.191$, power = 0.802). Post hoc analysis revealed significant contrasts between the oligotrophic treatment with 2000 larvae (a) and all other treatments (b) ($p < 0.001$, Bonferroni correction; Figure 2.3d).

2.3.3. *Impact of anthropogenic pressures on time to emergence*

Development up to emergence was similarly impacted as development times decreased with increasing temperatures ($F(3, 41) = 230.7$, $p < 0.001$, partial $\omega^2 = 0.936$, power = 1; Figure 2.4b). Increasing levels of temperature fluctuations lowered development time ($F(3, 14) = 230.7$, $p < 0.001$, partial $\omega^2 = 0.833$, power = 1). Post hoc analysis indicated differences between the constant treatment (a) and all other treatments (b, c) ($P < 0.001$, Bonferroni correction) and between the block (b) and curve treatments (c) ($p < 0.05$, Bonferroni correction; Figure 2.4a). Eutrophication did not impact development under low densities but did so under higher densities (Figure 2.4c). Decreasing amounts of food per capita increased time to emergence when manipulating eutrophication levels ($F(2, 25) = 19.429$, $p < 0.001$, partial $\omega^2 = 0.520$, power = 1), density ($F(2, 25) = 4.246$, $p = 0.026$, partial $\omega^2 = 0.160$, power = 0.741) and a trend was detected when altering both ($F(4, 25) = 2.297$, $p = 0.087$, partial $\omega^2 = 0.132$, power = 0.672). Post hoc

analysis revealed significant contrasts between the oligotrophic treatment with 2000 larvae (b) and all other treatments (a) ($p < 0.001$, Bonferroni correction; Figure 2.4c).

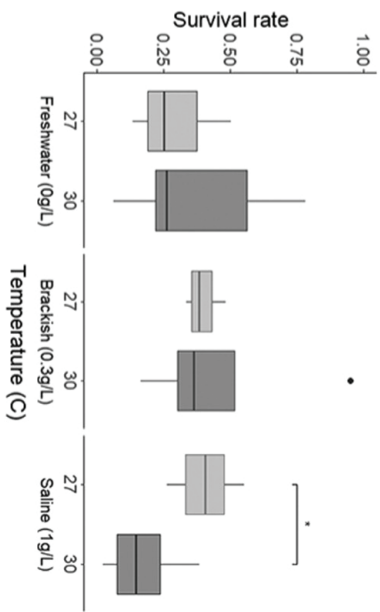
2.3.4. *Impact of anthropogenic pressures on sex ratio*

The proportion of females increased with increasing temperatures ($F(2, 40) = 38.57$, $p < 0.001$, partial $\omega^2 = 0.620$, power = 1; Figure 2.5b) and between the eutrophic and hypertrophic treatments ($H(2) = 9.5126$, $p = 0.015$; Figure 2.5c) within the highest density. The sex ratio was not impacted by temperature fluctuations and density within the ranges tested ($p > 0.05$). No other effects on sex ratio were detected.

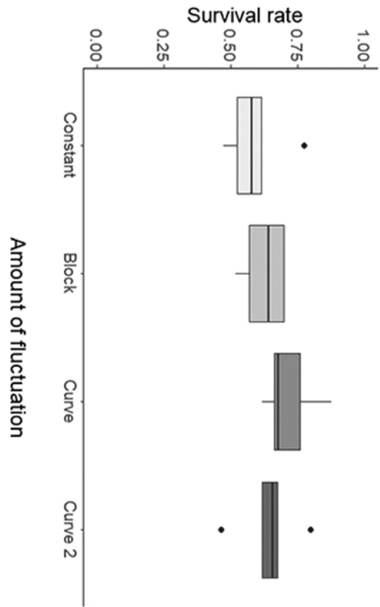
2.3.5. *Impact of anthropogenic pressures on ovipositioning behavior*

Egg laying behavior decreased with increasing salinity ($F(16,63) = 8.480$, $p < 0.001$, partial $\omega^2 = 0.491$, power = 1, Figure 2.6a). Post hoc analyses revealed a contrast between the treatments 16 g/l and 32 g/l and all lower salinities after day 1 of the experiment ($p < 0.05$, Bonferroni correction). Ovipositioning behavior also increased with increasing eutrophication ($\chi^2 = 32.857$, $df = 3$, $p < 0.001$, Figure 2.6b). Post-hoc analysis indicated a difference between the hypertrophic treatment (b) and all other eutrophication levels (a).

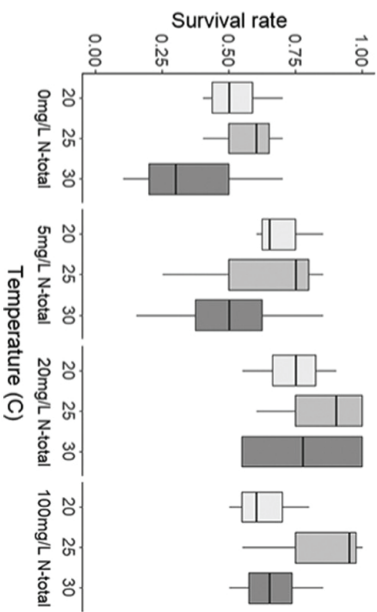
A



B



C



D

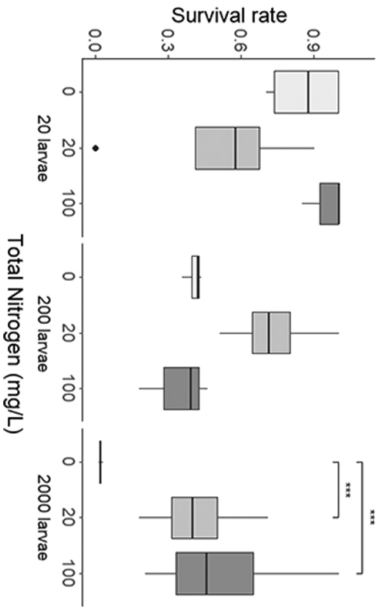


Figure 2.2 Absolute survival rate across different anthropogenic pressures. Absolute survival rate depicted as the number of emerged adults as a fraction of the initial number of larvae at the end of each experiment for A. effects of salinity at different temperatures, B. increasing levels of temperature fluctuation, C. effects of increasing eutrophication at different temperatures and D. effects of eutrophication at different densities.

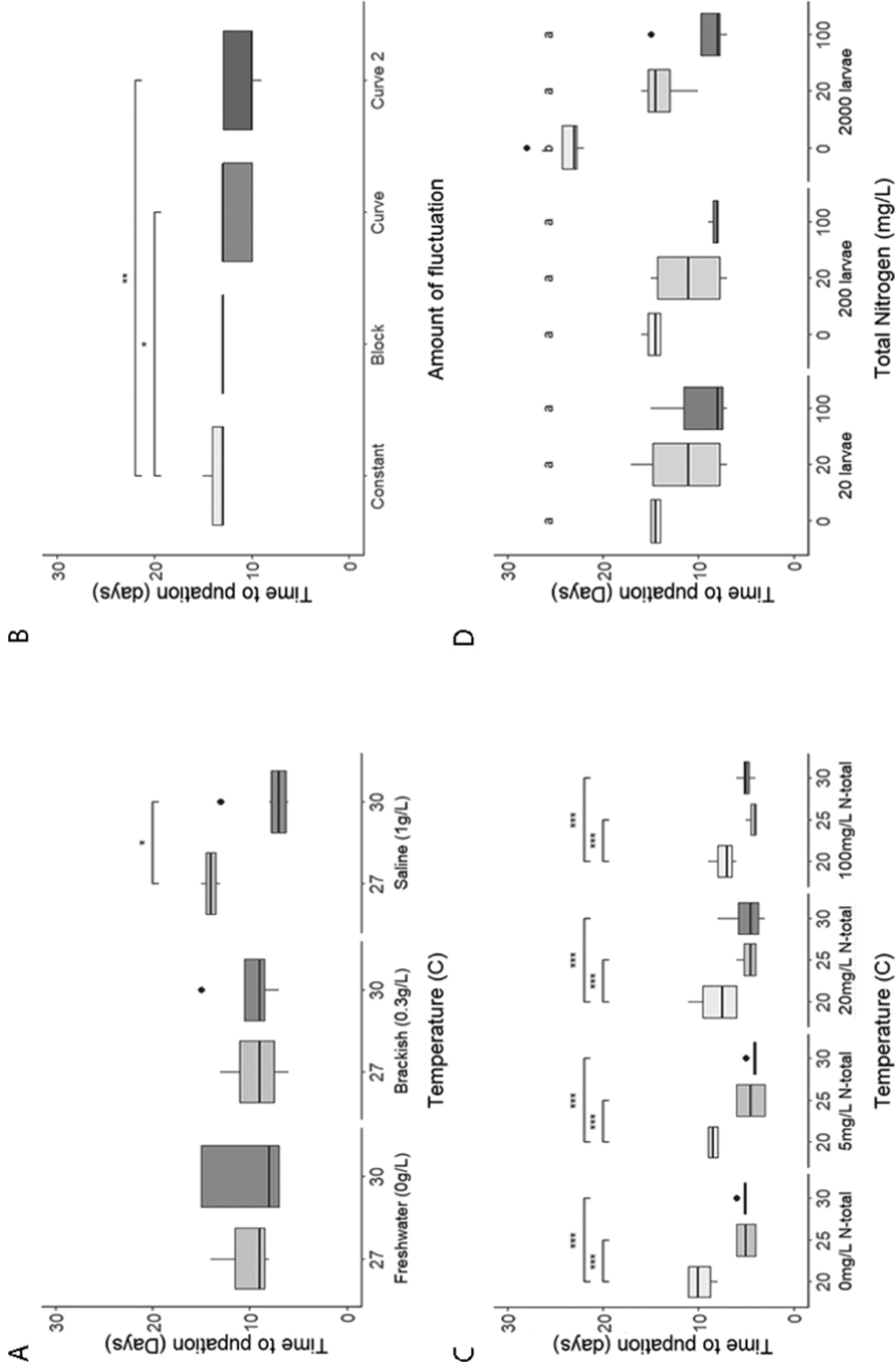


Figure 2.3 Median time to pupation across different anthropogenic pressures. Time to pupation in days at the end of each experiment for A. temperature grouped by salinity, B. increasing levels of temperature fluctuation, C. temperature grouped by eutrophication and D. eutrophication grouped by density. a, b Significantly contrasting groups.

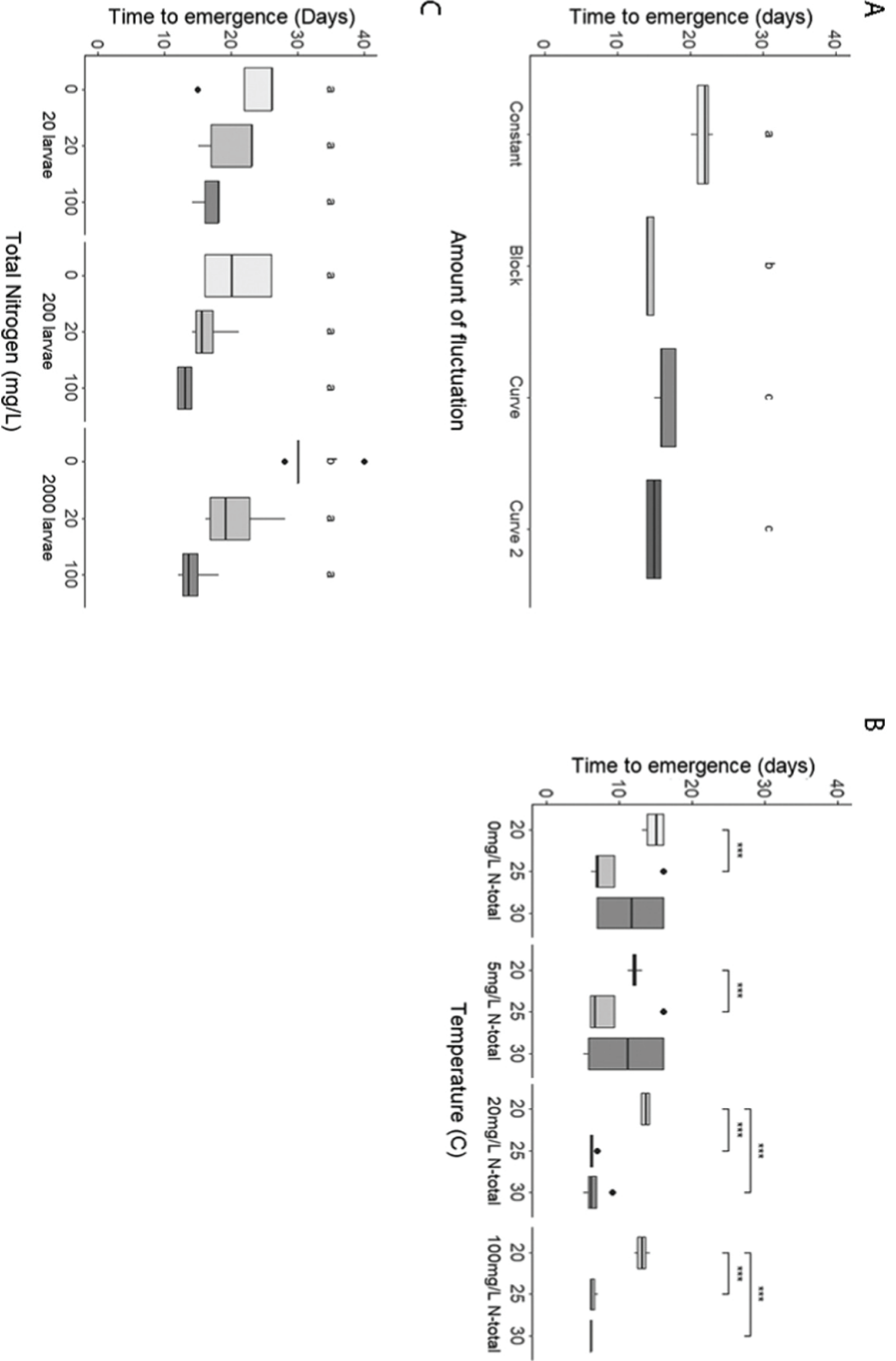


Figure 2.4 Median time to emergence across different anthropogenic pressures. Time to emergence in days at the end of each experiment for A. increasing levels of temperature fluctuation, B. temperature grouped by eutrophication and C. eutrophication grouped by density. a, b, c Significantly contrasting groups.

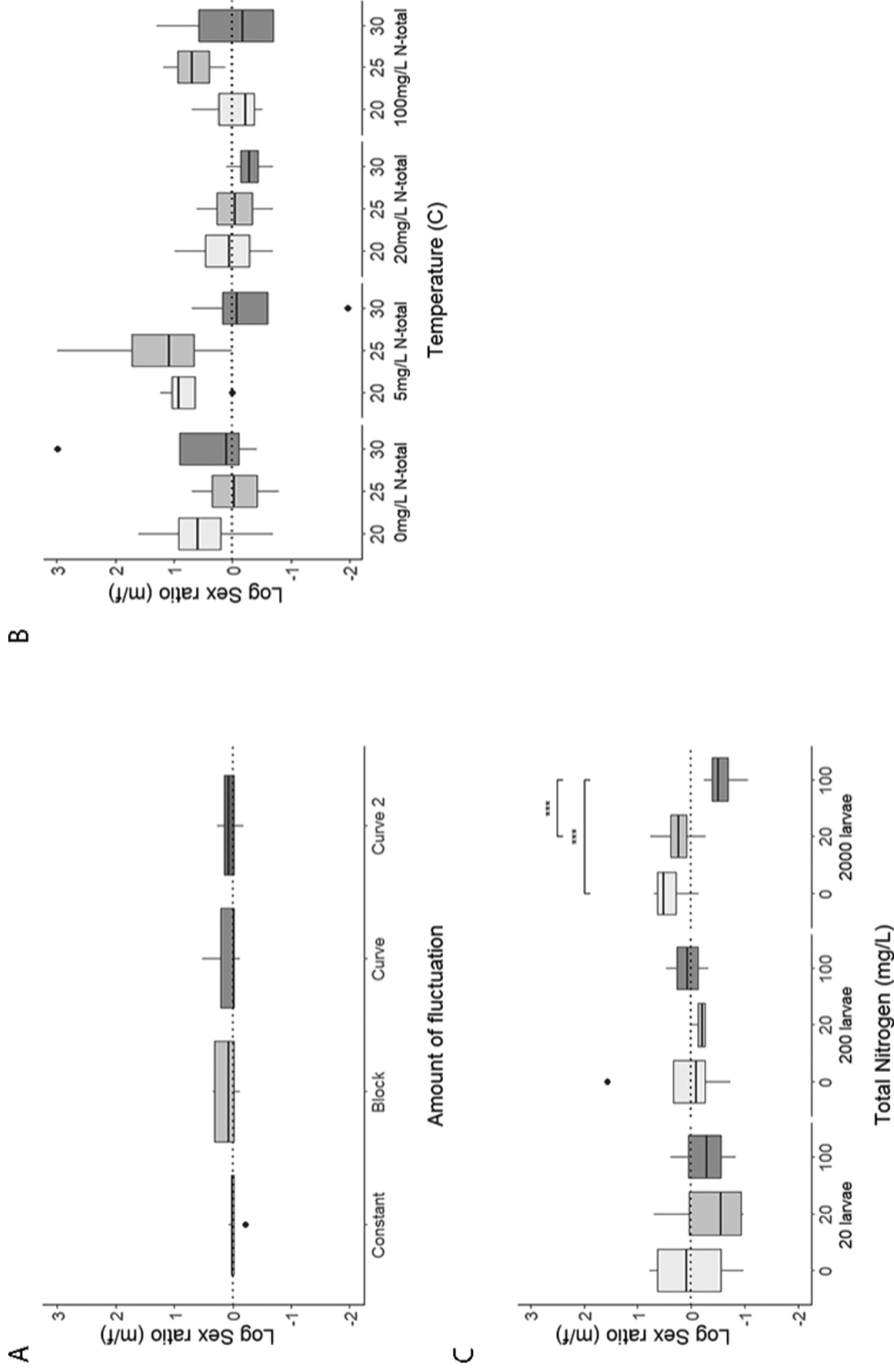


Figure 2.5 Sex-ratio (SR) across different anthropogenic pressures. Male to female sex ratio (transformed as natural logarithm) at the end of each experiment for A. increasing levels of temperature fluctuation, B. temperature grouped by eutrophication and C. eutrophication grouped by density.

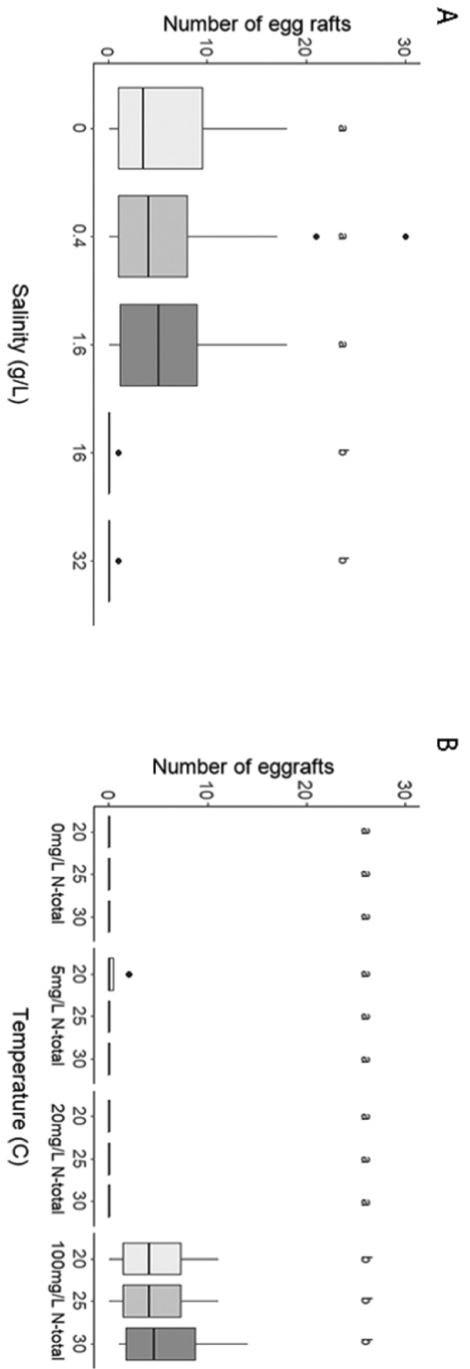


Figure 2.6 Ovipositioning behavior across different anthropogenic pressures. Number of egg rafts for A. temperature grouped by salinity, B. temperature grouped by eutrophication. a, b Significantly contrasting groups.

2.4. Discussion

Anthropogenic pressures are mounting in a fast-changing world. This study assessed the life-history responses of *Cx. pipiens* to the three most prominent anthropogenically induced pressures that act directly on the mosquito larval habitat: eutrophication, temperature, and salinization. A series of full-factorial mesocosm experiments demonstrated that each of these pressures, together shaping the larval habitat, are relevant during different immature life-stages. Overall, the results suggest large and previously underestimated impacts of multiple pressures – both single and interactive – on mosquito population dynamics, well beyond the impact of changes in temperatures. However, the direction and magnitude of the effect of stressors on the investigated mosquito life history parameters, differs profoundly between the investigated stressors. Also, while interactive effects between stressors were common, some interactions are more important than others. These are further discussed in the following paragraphs.

2.4.1. Impacts of anthropogenic pressures on mosquito survival and development time

While eutrophication and conspecific density had large, consistent effects on larval survival, exposure to different temperature regimes and differences in salinity levels only showed effect on development time. These results likely reflect different physiological processes. Eutrophication acts directly on resource competition, temperature acts on metabolic rate and salt has a toxic though sublethal effect (Balasubramanian et al., 2019; Emidi et al., 2017). Indeed, under decreasing levels of eutrophication, larval survival was markedly lower as a result of fierce competition over resources (El Hussein et al., 2018; Merritt et al., 1992; Reisen et al., 1984). This is abundantly clear when comparing the survival under the hypertrophic treatment (45 %) to that in the oligotrophic treatment (<1 %). Similar effects were found when comparing the survival at the highest larval densities (40 %) to the lowest densities (55 %) at average eutrophication levels. This effect has been reported to be even stronger when several developmental stages are present, as mosquito larvae have repeatedly been observed to cannibalize on lower instars (El Hussein et al., 2018; Koenraadt and Takken, 2003). The effect of salinity had a negatively and stepwise effect on the larval energy budget, likely because the toxic chloride needs to be expelled actively within *Culex* larvae (Bradley, 1987; Donini et al., 2007; Bradley and Phillips, 1976; Patrick and Bradley, 2000), thus slowing larval development (Clark et al., 2004). The higher instars seemed disproportionately more sensitive to this effect, which can be explained by the lower surface-to-volume ratio of higher instars. Within

the investigated temperature ranges, which mimic optimal rearing conditions, a marked effect of temperature on development time was observed, which is in line with previously reported effects that relate this to an increase in metabolic rate. Overall, the results suggest that single effects of the investigated stressors on mosquito population parameters are present in each of the mosquito life history parameters and follow directly from the physiological processes they act on.

Although the results of the interactive experiments are far more complex, in general terms they follow a very similar pattern. An illustration of this is the interactive effect of temperature with salinity and eutrophication. As temperature increases the metabolic rate, higher temperatures can be expected to lead to speeding up the single effects of both stressors. Indeed, the experimental results show that survival rate at oligotrophic conditions was lower when larvae were exposed to higher temperatures. Likewise, larval survival at higher levels of salinity was lower when larvae were exposed to higher temperatures. As these results were all collected within a relevant parameter range for the current northern European situation, it is difficult to make any inferences outside this range. For example to delta regions in tropical areas that struggle with salinization such as the region around the Mekong delta (Bauer et al., 2022). Likewise, higher temperature ranges may alter the effect of other stressors. Indeed, several experiments have reported lethal effects of temperatures above 30 degrees Celsius (Ciota et al., 2014; Loetti et al., 2011), but it is unknown whether this might change when interacting with other stressors. One effect that might be accentuated is the masking effect, where eutrophic conditions could mask temperature stress by facilitating rapid emergence, similar to what has been observed with its effect on pesticide toxicity (Barmantlo et al., 2018). A comparable exacerbation of stressor effects by interaction with mean temperature might be present for increasing temperature fluctuations, as these seem to further increase metabolic rates which are discussed below. Although the experiments took place during different periods of the year, no confounding effects of photoperiod on development time were expected, as most experiments were conducted before mid-summer and diapause generally sets on after August (Robich and Denlinger, 2005). Additionally, diapause-associated developmental alterations take place during adulthood (Robich and Denlinger, 2005; Zhou and Miesfeld, 2009), whereas these experiments were limited to sub-adult development.

4.2. *Impact of fluctuating temperature regimes*

The impacts of higher constant temperature on larval development rate are clear and widely acknowledged (van der Have and de Jong, 1996). The experiments demonstrated similar effects both prior to and during metamorphosis, irrespective of nutrient availability, but strengthened by increased salinity. Moreover, the results show that fluctuations in temperature have large effects on larval development time, well beyond what was expected. Larval development time, at natural temperature fluctuations, was on average 7 days shorter than under constant temperature regime. Several authors have previously hypothesized that temperature fluctuations might have a large effect on insect growth rate (Alcalay et al., 2018; Hagstrum and Milliken, 1991; Loetti et al., 2011). The experimental results also show that most of these differences arise during pupation (Figure 2.3). This is probably caused by temperature sensitivity of a multitude of enzymatic reactions around different optimum temperatures impacting enzymatic activity and thereby metabolic rate, possibly as an evolutionary adaptation in cold-blooded organisms, as has been found in insects in general (Behrens et al., 1983; Hagstrum and Milliken, 1991).

2.4.3. *Impacts of anthropogenic pressures on sex-ratio of mosquitoes*

Anthropogenic pressures may have different effects on male and female mosquitoes, thus leading to sex-specific life history trajectories (Alcalay et al., 2018). For instance, although bigger males and females both live longer (Reisen et al., 1984), comparably lower natural selection for size might push males towards early maturation, leading to smaller sizes and wing length (Alcalay et al., 2018; Loetti et al., 2011; Reisen et al., 1984; Virginio et al., 2015). The longer average development time of female mosquitoes may also result in prolonged exposure to stress, potentially making them more vulnerable (Hamaidia and Soltani, n.d.; Schrama et al., 2018; Virginio et al., 2015). These experiments show that significantly fewer females survived under the highest density where food-stress was most prominent. Female abundance was higher at higher temperatures, irrespective of eutrophication level, which might be due to higher development rates allowing for earlier emergence and subsequent lower exposure to competition and chemical stressors such as secondary metabolites produced by cyanobacteria. Sex ratio was not affected by temperature fluctuations or density, the latter of which indicates that cannibalism is opportunistic and does not select for certain sexes, which is in line with Loetti et al. (2011)'s notion that sexual dimorphism in growth rate only exists from the fourth larval development stage onwards. Overall, our findings indicate that expected rising temperatures and nutrient pollution will lead

to a higher female survival. As females represent the sex transmitting pathogens, this may have implications for disease transmission.

2.4.4. *Impacts of anthropogenic pressure on egg-laying behavior of mosquitoes*

The results show a pronounced impact on ovipositioning behavior for chemical cues (eutrophication and salinization), but no impact of temperature. These results are in line with the notion that *Cx. pipiens* is widely known to actively seek and evaluate temporal water bodies by sensing gaseous substances (Becker et al., 2013), bacteria (Buxton et al., 2020; Dhileepan, 1997; Hasselschwert and Rockett, 1988), pheromones (Day, 2016; Ko and Eliel, 1986; Michaelakis et al., 2005) and visual cues (Bentley, 1989; Ortiz Perea and Callaghan, 2017). Thus, increasing levels of eutrophication, and the higher levels of bacterial activity that resulted from this, were indeed preferred during egg laying, as females oviposited exclusively in the hypertrophic treatment. Likewise, increasing salinity levels impacted ovipositioning behavior, though it is unclear whether ovipositioning responded directly to high salt levels or whether it occurred through salt-induced changes in bacterial composition. No measurable effect of temperature on ovipositioning was observed, which is unexpected as higher water temperatures in larval habitats have very large impact on larval development rate, suggesting that the response is primarily chemically induced rather than physically. Thus, changes in water pollution or salt content may have far ranging consequences for ovipositioning, reinforcing some of the previously observed effects on development rate and survival.

2.4.5. *Implications*

The planetary boundary framework (Steffen et al., 2015) is commonly acknowledged as a concept defining the global drivers of environmental change and the safe operating space for humanity within them. Crossing the boundaries increases the risk of large-scale and irreversible environmental changes. There is widespread consensus that, for the vast majority of species, crossing the planetary boundaries leads to long term negative effects at the individual or population level (Wu et al., 2021). So far, evidence for the effects on infectious diseases had remained fragmented (Butler, 2012). Our results indicate that mosquito species may be suffering far less or even benefiting from large scale environmental change. Furthermore, the fact that the effect of most of these stressors was exacerbated by increasing temperatures illustrates how ongoing crossing of planetary boundaries may lead to even more beneficial habitat conditions. As such, these results underscore the importance of changes in environmental conditions

(Lambin et al., 2010; Schrama et al., 2020) by identifying its consistent pattern across a range of important stressors and climatic conditions. Given the global extent and intensity of the investigated anthropogenic pressures, these results are likely relevant for a wide array of disease vectors and provides a mechanism for the association between ecosystem degradation and disease. Overall, these results strongly suggest that the planetary boundary concept has important consequences for changes to mosquito abundances and likely feeds through to changes in disease risk, thus highlighting the need to further include these in models and interventions. The crucial question is, however, whether the mosquitoes are better adapted to these changes than their predators and (invasive) competitors, whose ranges are expanded due to human activities (Costanzo et al., 2005). As such, extended work is needed on interactions with(in) biotic pressures, including interspecific competition and predation.

CRedit authorship contribution statement

SB and MS conceived the general idea for the experiments. SB set up the experiments and carried out the measurements together with EJ, AU and LK. SB carried out all statistical analysis, with feedback provided by PB and MS. EB, EJ, MS and PB contributed to the writing of the manuscript. All authors read and approved the final manuscript.

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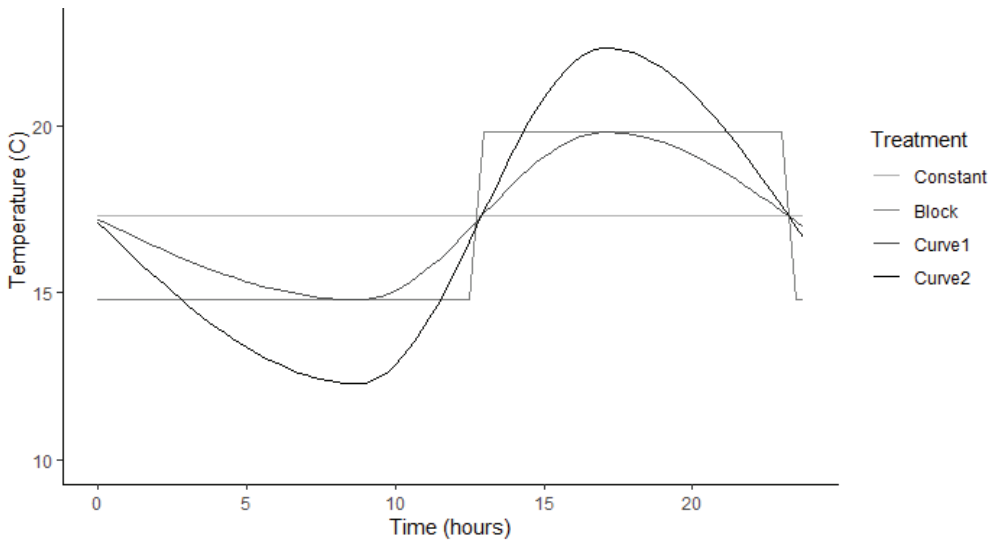


Figure S2.1 Treatments of increasing temperature fluctuation. Overview of the four levels of temperature fluctuation used during the experiment. All four treatments result in the same amount of degree days. The maximum, minimum and mean temperatures as well as the daylength were derived by averaging aquatic surface temperatures from May and July 2020.

Table S2.1 Non-exhaustive overview of relevant literature for each of the established relationships between pressure and states.

Pressure	State	References ¹
Temperature	Development speed	(Loetti et al. 2011; Mottram et al. 1994; Petrić et al. 2014; Reisen et al. 1984; Spanoudis et al. 2019)
	Survival rate	(Loetti et al. 2011; Mottram et al. 1994; Reisen et al. 1984; Spanoudis et al. 2019)
	Sex ratio (m/f)	(Loetti et al. 2011; Reisen et al. 1984; Spanoudis et al. 2019)
Salinization	Survival rate	(Balasubramanian et al. 2019; Bradley and Phillips 1976; Clark, Flis, and Remold 2004; Donini et al. 2007; Mottram et al. 1994; Silberbush et al. 2014)
	Ovipositioning	(De Brito Arduino et al. 2015; Hudson 1956; Navarro et al. 2003; Ramasamy and Surendran 2012, 2016; Roberts and Irving-Bell 1997)
Eutrophication	Development speed	(Buxton et al. 2020; Krol et al. 2019; Merritt et al. 1992; Reisen et al. 1984; Rejmánková et al. 2013; Schrama et al. 2018)
	Survival rate	(Krol et al. 2019; Merritt et al. 1992; Reisen et al. 1984; Rejmánková et al. 2013; Schrama et al. 2018)
	Sex ratio (m/f)	(Reisen et al. 1984)
	Ovipositioning	(Bentley 1989; Bond et al. 2005; Buxton et al. 2020; Day 2016; Dhileepan 1997; Ikeshoji and Mulla 1970; Navarro et al. 2003; Rockett 1987)

¹ Studies on direct (experimental) links between pressures and states were collected by use of the query “‘*Culex pipiens*’ AND populations AND (immature OR larva*) AND (survival OR development OR “sex ratio” OR “ovipositioning behavior”) AND (eutrophication OR salinization OR temperature OR “temperature fluctuation”) AND interacti** -insecticide’. When no literature was found for this species, additional searches were performed for other species within its genus or else for the genera *Aedes* or *Anopheles*.

Chapter 2

Table S2.2. Model coefficients. Estimated regression coefficients, standard errors and other values for the various statistical analyses of the results of experimental tests on *C. pipiens*.

Stressor	Population Parameter	Formula	Variable	Estimate	Std. Error	t value	Pr(> t)
Temperature	Survival rate	survival_rate ~ Treatment	(Intercept)	118.4	9.486569	12.4808025	1.16E-09
			Treatment-Block	6.6	13.416035	0.4919486	6.29E-01
			Treatment-Curve	25.2	13.416035	1.8783493	7.87E-02
			Treatment-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	
		Treatment-Block	0.16664	0.1471145	1.13E+00	2.75E-01	
		Treatment-Curve	0.17644	0.1471145	1.20E+00	2.49E-01	
		Treatment-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	
		Treatment-Block	-0.6	8.37E-01	-0.717	4.84E-01	
		Treatment-Curve	-1.8	8.37E-01	-2.151	4.71E-02	
		Treatment-Curve2	-2.6	0.8367	-3.11E+00	0.00677	
Time to emergence	TTE ~Treatment	(Intercept)	21.66667	0.6374553	33.989314	7.43E-15	
		Treatment-Block	-7.26667	0.8063242	-9.01209	3.33E-07	
		Treatment-Curve	-5.06667	0.8063242	-6.283659	2.01E-05	
		Treatment-Curve2	-6.66667	0.8063242	-8.267973	9.31E-07	
Eutro:Temp	Time to pupation	TTP ~ O2 + Eutrophication + Temperature	(Intercept)	4.148	1.53879	2.696	1.03E-02
			O2	0.02741	0.02276	1.204	0.2357
			Eutrophication1	-0.09498	0.72558	-0.131	0.8965
			Eutrophication2	-0.77008	0.55532	-1.387	0.1734

Biting the hand that feeds

		Eutrophication3	0.12179	0.37016	0.329	0.7439	
		Temperature1	2.34313	0.29348	7.984	9.95E-10	
		Temperature2	-1.25789	0.28737	-4.377	8.73E-05	
Time to emergence	TTE ~ Temperature	(Intercept)	8.1661	0.1195	68.35	2.00E-16	
		Temperature1	4.2339	0.1688	25.08	2.00E-16	
		Temperature2	-1.8804	0.1718	-10.94	7.09E-14	
Ovipositioning	Rafts ~ Eutrophication	(Intercept)	1.3333	0.3477	3.835	0.0004	
		Eutrophication1	-1.3333	0.6022	-2.214	0.03206	
		Eutrophication2	-1.1667	0.6022	-1.937	0.05916	
		Eutrophication3	-1.3333	0.6022	-2.214	0.03206	
Salinity	Survival rate	survival_rate ~ Treatment * Temperature * Day + Error(Cosm/(Treatment*Day))	Control:Brackish	0.2407	0.138	1.747	0.2172
			Control:Saline	-0.0224	0.143	-0.157	0.9866
			Brackish:Saline	-0.263	0.146	-1.804	0.198
Time to pupation	TTP ~ Treatment * Day * Temperature + Error(Cosm/(Treatment*Day))	Control:Brackish	8.12	16.2	0.503	0.8711	
		Control:Saline	6.48	18.5	0.35	0.9349	
		Brackish:Saline	-1.65	18.7	-0.088	0.9958	

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<i>Stressor</i>	<i>Population Parameter</i>	<i>Formula</i>	<i>Variable</i>	<i>Sum Sq</i>	<i>Mean Sq</i>	<i>F value</i>	<i>Pr(>F)</i>
Salinity	Ovipositioning	Rafts ~Location * Day + Treatment * Day + Error(Cosm/(Treatment * Day))	Day	4211	1052.8	46.883	2.00E-16
			Location:- Day	842	52.6	2.344	0.00861
			Day:Treatment	3047	190.4	8.48	2.07E-10
			Residuals	1415	22.5		
Eutro:Temp	Survival rate	survival_rate ~ Eutrophication + Temperature	Eutrophication	0.3115	0.10382	1.902	0.145
			Temperature	0.1329	0.06646	1.218	0.307
			Residuals	2.1286	0.05458		
	Sex ratio	Sex_ratio ~ Eutrophication + Temperature	Eutrophication	43.9	14.636	0.896	0.452
			Temperature	7.4	3.692	0.226	0.799
			Residuals	653.6	16.34		

Biting the hand that feeds

Eutro:Density	Survival rate	Survival_rate ~ Eutrophication * Density	Density	152.7	76.3	4.246	0.0259
			Eutrophication	698.7	349.3	19.429	8.11E-06
			Density:Eutrophication	165.2	41.3	2.297	0.0872
			Residuals	449.5	18		
Time to pupation	TTP ~ Eutrophication * Density	Density	116.2	58.09	5.924	0.00784	
		Eutrophication	437.1	218.55	22.286	2.78E-06	
		Density:Eutrophication	118.2	29.54	3.012	0.03708	
		Residuals	245.2	9.81			
Sex ratio	Sex_ratio ~ Eutrophication + Density	Density	0.31	0.1551	0.204	0.817	
		Eutrophication	2.875	1.4375	1.888	0.172	
		Density:Eutrophication	1.379	0.3447	0.453	0.77	
		Residuals	19.038	0.7615			
Time to emergence	TTE ~ Eutrophication * Density	Density	152.7	76.3	4.246	0.0259	
		Eutrophication	698.7	349.3	19.429	8.11E-06	
		Density:Eutrophication	165.2	41.3	2.297	0.0872	
		Residuals	449.5	18			