

How single and combined stressor tolerance influences stressor sensitivity in *Daphnia magna* and its microbiome

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Summary

With increasing temperatures and increasing pesticide use, aquatic organisms are exposed to a range of different stressors, which moreover can interact with each other. However, organisms can also evolve tolerance to one or multiple stressors. Yet, this tolerance can be associated with costs and can also alter and possibly magnify stressor interactions. More research is needed to correctly predict the effect of combined stressors for tolerant organisms. In this thesis, we studied the single and combined effects of the pesticide malathion and a heatwave on life-history traits (survival, maturation time and reproductive performance) of different tolerance clone types of the water flea *Daphnia magna* and its microbiome. A full-factorial design was set up, including four different tolerance types (control, malathion-tolerant, heat-tolerant and combined-tolerant clones) sequentially exposed to two two-days pesticide treatments (solvent control or malathion exposure) crossed with two four-days temperature treatments (20 °C or a 32 °C heatwave).

For the life-history traits, it was shown that all three tolerance types were indeed tolerant to the stressor against which they evolved tolerance. All four tolerance types also had distinct microbiome compositions under stressor-free conditions, except for the malathion- and combined-tolerant clones, suggesting that the microbiome composition of the combined-tolerant clones was less driven by heat-tolerant bacteria and more by malathion-degrading bacteria. In the absence of both stressors, no costs of tolerance were observed for all three tolerance types, indicating it is not disadvantageous to be tolerant when not exposed to any stressors. When exposed to only malathion, also no costs of tolerance were observed for all three tolerance types. After malathion exposure (not followed by the heatwave), the microbiome composition of all tolerance types shifted only slightly. However, this shift seemed to be stronger for the heat-tolerant clones, possibly indicating a trade-off between heat-tolerant and malathion-tolerant bacteria. For malathion-tolerant clones exposed to the heatwave, there was a lethal cost of tolerance as they showed lower heat tolerance. The heatwave also induced a big shift in microbiome composition for all four tolerance types. The cost of tolerance observed for malathion-tolerant clones might be partly explained by the shift in microbiome composition that the heatwave imposed. Under combined stressor exposure, for all four tolerance types, only additive interactions were observed for survival and reproductive performance. For the control clones, however, combined stressor exposure resulted in an antagonistic interaction, with prior exposure to malathion nullifying the negative effects of the heatwave. The microbiome composition of the four tolerance types also shifted after exposure to both malathion and the heatwave, and for all three tolerant clone types, trade-offs did occur between tolerant bacteria, as the relative abundance certain bacteria increased, it decreased for others.

In conclusion, while there were no costs of tolerance when not exposed to any stressors or when exposed to malathion, there was a lethal cost of tolerance for the malathion-tolerant clones

exposed to the heatwave. Especially after heatwave exposure, the microbiome composition of non-heat adapted clones shifted to a more heat-tolerant community. In a multi-stressor world, clones tolerant to only one stressor did not carry any costs of tolerance, yet combined-tolerant clones performed best.

Introduction

1. Stressors

1.1 Increasing temperatures

In recent decades, the climate on earth has undergone significant changes. Over the course of the 19th century, scientists have identified the major natural factors that influence the climate system, like volcanic activity and incoming solar radiation (IPCC CH1, 2021). However, since the 1970's, when scientific assessments began, it became clear that human activity was the main source of global warming (IPCC CH1, 2021). The most important climate system drivers altering the earth's energy balance and leading to climate change are aerosols and greenhouse gasses (IPCC CH2, 2021). Greenhouse gasses cause global warming because they trap heat and re-emit this heat into the earth's atmosphere. While action is undertaken to reduce climate change, the IPCC still predicts a temperature increase of 1.5 °C by 2040 and, in a high emission scenario, a temperature rise of between 2.4 °C and 4.8 °C by 2100 (IPCC CH4, 2021). Even relatively small increases (e.g. +0.5 °C) in warming can already lead to more severe weather extremes, especially in the case of droughts, heavy precipitation, and temperature extremes (IPCC CH11, 2021). These changes in climate will have detrimental effects on human life, as weather events like temperature extremes and heatwaves will become more severe and will occur more frequently (IPCC CH11, 2021). However, not only humans have to undergo climate change, but also animals will face challenges to cope with it.

Global warming will have a significant impact on freshwater organisms as it will affect water availability and temperature (Woodward et al., 2010). For example, for cold-water fish, like salmon and trout, increasing temperatures will especially impact hatching and spawning of eggs (Jain et al., 2013). As salmon eggs already suffer from mortality at 18 °C and trout eggs do not hatch over 14.4 °C, increasing temperatures will lead to incompleteness of the life cycle and thus losses in fish abundance (Jain et al., 2013). Warmer temperatures will also cause a decrease in available oxygen in the water, which can lead to mortality (Jain et al., 2013). Not only large aquatic organisms, but also smaller organisms are affected by the rising temperatures. For example, as the temperature optima of cyanobacteria and green algae lies above 25 °C, there will be harmful algal blooms at higher temperatures (Visser et al., 2016). These harmful algal blooms not only cause the environment to become anoxic and therefore kill fish, but are also toxic to humans and other animals (Carmichael & Boyer, 2016). Increasing temperatures can thus affect all the different levels of the ecosystem and as all levels are connected through top-down or bottom up, even one level affected can have disastrous consequences for the other trophic levels.

Also at the individual level, different life history traits can get affected by warming. Increasing mean temperatures can have a negative effect on both development time and reproduction

(Giebelhausen & Lampert, 2001). Giebelhausen and Lampert (2001) researched how age at first reproduction and first egg clutch of *Daphnia* were altered at four temperatures (15 °C, 20 °C, 25 °C and 30 °C). Increasing temperatures led to a reduction in age at first reproduction, as it declined from 15 °C to 20 °C, however at higher temperatures, no further decrease was observed and over all the conditions, a minimum age at first reproduction of five days was detected (Giebelhausen & Lampert, 2001). Temperature had a negative effect on first egg clutch as clutch size halved when comparing 30 °C to 15 °C (Giebelhausen & Lampert, 2001). In addition to these sublethal effects, warming can also be lethal. For *Daphnia* exposed to either 17.5 °C or 21 °C, survival decreased at the higher temperature (Ramos-Rodríguez et al., 2022). Not only increasing mean temperatures but also temperature extremes like heatwaves can affect life history traits. When *Daphnia* were exposed to a heat spike (30 °C), their survival and clutch size was reduced (Van de Maele et al., 2025).

Many aquatic organisms will increase their metabolic rate to cope with increasing temperatures. Brown et al. (2004) describe the metabolic rate as “the rate of energy uptake, transformation and translocation”. As indicated by the Boltzmann factor, $e^{-E/kt}$, metabolic rate and temperature have an exponential relationship (Brown et al., 2004). When temperature increases, more food and oxygen is needed in order to survive (Jain et al., 2013). When *Daphnia* with a habitat temperature of 16 °C are exposed to increases of 2 °C and 12 °C over the course of six months, their metabolic rate, estimated by heartbeat- and respiration rates, increased (Khan & Khan, 2008). This increase in activity was paired with an increase in activity of ATPases and synthesis of cellular ATP, meaning that there was an increased energy demand and production (Khan & Khan, 2008).

1.2 Pesticide pollution

Organisms do not only need to deal with increasing temperatures, but also with a lot of other stressors, like microplastics, metals and pesticides. The growing world population of humans is one of the main causes of increased pesticide use (Tudi et al., 2021). According to the United Nations (2024), the world's population will keep growing for another 50-60 years, reaching 10.3 billion by 2080. To be able to provide enough food for this increasing population, food productivity needs to improve (Tudi et al., 2021). There are two main options to increase food productivity: 1) increase productivity on existing farmlands, and 2) increase the production area (Edgerton, 2009). Both options lead to increased pesticide use as crops need to be protected from pests. Without pesticides, losses would reach 80% for cotton production, 50% for wheat production and an annual food loss of 45% due to pest infestation (Oerke, 2006; Sharma et al., 2019). In addition, climate change is also a key factor as to why pesticide use increases. Due to higher temperatures in the north, longer growing seasons will occur, and due to more intense precipitation, applied pesticides will suffer a faster degradation and volatility (Noyes et al., 2009). Crop-pest synchrony might also be affected as climate change can influence the

population growth rates and generational numbers of the crop pest species (Noyes et al., 2009). All these factors lead to a more intense and more frequent use of pesticides.

When pesticides are applied to their target crop or organism, they can also affect non-target organisms through surface runoff, adsorption, spray-drift, and leaching (Tudi et al., 2021). Spray-drift occurs during pesticide application due to spray droplets moving airborne from treatment to non-treatment sites, while runoff occurs due to excess water which moves pesticide particles down a sloping surface (Tudi et al., 2021). Climate change also affects run-off as pesticide transport is driven by temperature increases and rainfall intensity (Delcour et al., 2015; Noyes et al., 2009). Run-off especially leads to pesticide contamination of water bodies like lakes, streams, and ponds, where the pesticides can affect the inhabiting aquatic organisms (Tudi et al., 2021). The most toxic pesticides are insecticides, followed by fungicides and herbicides, due to their lipophilic characteristics and their slow degradation in the environment (Ansari et al., 2014; Mahmood et al., 2016).

As well as increasing temperatures, pesticide exposure also affects different trophic levels. Predators can be affected directly through pesticide exposure and accumulation of pesticides in the food chain, but pesticides can also reduce the abundance of for example insects and shrubs which serve as food for higher trophic levels, thereby indirectly affecting the top predators and consumers (Mahmood et al., 2016). As pesticides can also accumulate on nectar and pollen, pollinators are also often exposed (Serrão et al., 2022). In honeybees, the foragers pick up the polluted nectar and pollen and afterwards store these in the hive, which can lead to the collapse of the whole colony (Serrão et al., 2022). In aquatic habitats, insect larvae and zooplankton crustaceans show great sensitivity to neurotoxic chemicals like pesticides, resulting in increased mortality (Sánchez-Bayo, 2021). For example, *Daphnia* exposed to paraoxon-methyl suffer a reduced survival in a concentration- and exposure time-dependent way, with a decrease in survival when concentrations above 2.2 µg/L were applied (Duquesne, 2006). Also other life history traits can be affected by pollution exposure. *Daphnia* exposed to paraoxon-methyl showed a delayed reproduction and a decline in both offspring per surviving individual and total number of offspring as a result of declined survival, especially at concentrations above 1.5 µg/L paraoxon-methyl (Duquesne, 2006). When *Daphnia* were exposed to fenvalerate concentration of 0.6 mg/L or more, maturation also delayed (Reynaldi et al., 2006). Even antipredator behaviour may get affected by pesticide exposure. Research by Pestana et al. (2009) showed that *C. riparius* and *S. vittatum* exposed to imidacloprid weakened their antipredator behaviour, leading to higher mortality.

To deal with the diverse types of pest species, many different insecticide classes exist. Some examples are organochlorines, organophosphates, carbamates, pyrethroids, neonicotinoids and

benzoylureas (Ware & Whitacre, 2004). Carbamates and organophosphates are widely used classes of insecticides and are popular because resistance is less quickly developed than to organochlorines and residues are less persistent in the environment (Gupta et al., 2019). Currently, 50% of the worldwide market of insecticides is represented by organophosphates (Gupta et al., 2019). Their severe neurotoxicity introduces threats to aquatic organisms and wildlife, as they inhibit acetylcholinesterase (AChE) (Fukuto, 1990; Gupta et al., 2019). This enzyme acts as a catalyst for hydrolysis of the neurotransmitter acetylcholine (ACh). The organophosphate-induced inhibition of AChE causes a build-up of the neurotransmitter ACh at the neuromuscular junction, which leads to constant stimulation of the nerve and muscle fibres resulting in exhaustion, paralysis and eventually death (Fukuto, 1990).

One example of an organophosphate insecticide is malathion (NCIB malathion, 2024). Malathion is mainly used as a plant protection product for both fruit- and vegetable crops as well as field crops and is used as a control against pest species (Jensen & Whatling, 2010). Next to its function as a plant protection product, malathion is also used for the control of vector-borne diseases like malaria and is even deployed as a pharmaceutical product for head lice control in humans (Jensen & Whatling, 2010). As mentioned before, malathion can end up in aquatic bodies like streams and ponds due to run-off and spray drift. In the San Joaquin river system traces of malathion ranged from 0.01 µg/L to 2.8 µg/L in 1992-1993 (Siepmann & Slater, 1998). In *D. magna*, as well as other organisms like humans, malathion is biotransformed to the more toxic and active compound malaoxon (Trac et al., 2016). Malaoxon is formed by oxidative desulfuration of malathion, mediated by the enzyme cytochrome P-450 (A. Zhang et al., 2011).

The rate of formation of this toxic oxon metabolite can be the reason why a latency in death is observed when performing toxicity tests on *D. magna* (Trac et al., 2016). For *D. magna*, the lethal concentration of malathion is 7.28 µg/L after 48 h (Wei et al., 2023).

2. Interactions between increasing temperatures and pesticides

Exposure to increasing temperatures or pesticides can have adverse effects on the life of many organisms, but what happens if those two stressors occur at the same time and interact with each other? The interactions between pesticides and climate change are encapsulated in two concepts: 1) the climate induced toxicant sensitivity (CITS) concept and 2) the toxicant induced climate change sensitivity (TICS) concept (Hooper et al., 2013).

First of all, global climate change can affect (decrease or enhance) the toxicity of pesticides (CITS). This concept is based on the fact that when temperatures increase, aquatic ectotherms increase their metabolic rate (Hallman & Brooks, 2015). This in turn goes paired with increased food uptake and respiration rates in order to counterbalance metabolic costs (Hallman & Brooks, 2015). Because of this, contaminant uptake will increase and will surpass the capacity

to mitigate toxicity via elimination and biotransformation, leading to an increased toxicity of the toxicant (Hallman & Brooks, 2015). The increased toxicity at higher temperatures is thus related to the higher energy metabolism at increased temperatures (Sokolova & Lannig, 2008). When the environment is altered by climate change, it can also change the toxicokinetics of chemicals, which affect the concentration, duration, and exposure sites of the toxicant (Hooper et al., 2013). Absorption and elimination of chemicals are elevated with increasing temperatures and thus increasing toxicant uptake, but due to increased depuration, it also reduces pesticide half-lives (Hooper et al., 2013; Noyes & Lema, 2015). In most cases, temperature also enhances metabolism to more toxic metabolites (Hooper et al., 2013). In the midge *Chironomus dilutes*, increasing temperatures are followed by increased toxicant uptake and increased transformation to oxon metabolites that are more toxic (Hooper et al., 2013). While in type 2 pyrethroids, increasing ambient temperatures also show increased toxicity, for other pyrethroid pesticides, lower temperatures went paired with decreased elimination rates and thus also causing greater toxicity (Hooper et al., 2013).

Second, exposure to toxic pesticides can also change the way an animal can cope with stress introduced by climate change (TICS) (Hooper et al., 2013). The TICS concept encapsulates how exposure to toxicants can increase the susceptibility of organisms to climate change (Noyes & Lema, 2015). Under pesticide exposure, more energy for repair and detoxification is needed which may lead to an increased oxygen demand (Verheyen et al., 2022). However, under pesticide exposure, oxygen supply may be reduced due to respiratory functioning impairment (Verheyen et al., 2022). As a result, when exposed to a toxicant, there will be an imbalance between oxygen demand and oxygen supply at lower temperatures, which results in a decreased heat tolerance (Verheyen et al., 2022). The TICS concept is, for example, demonstrated by Op De Beeck et al. (2017), who found that *Ischnura elegans* larvae experienced a decreased upper thermal tolerance when exposed to multiple chlorpyrifos pulses.

The CITS and TICS concepts can also be coupled and have been shown to directly affect one another (Op de Beeck et al., 2017). For damselflies exposed to chlorpyrifos in a warmer environment, the reduction in upper thermal limit was less big compared to damselflies exposed to chlorpyrifos in a colder environment (Op de Beeck et al., 2017). Under warming, the accumulation of chlorpyrifos was lower due to higher breakdown rates leading to a decrease in pesticide toxicity (CITS), which resulted in a lowered TICS effect (Op de Beeck et al., 2017).

As mentioned before, increasing temperatures can make pesticides more or less toxic to an organism, as the result of synergistic or antagonistic stressor interactions, respectively. A synergistic interaction occurs when the observed effect of two combined stressors is greater than the predicted additive effect of both stressors (based on the sum of their individual effects), while an antagonistic interaction occurs when the combined effect is smaller than the predicted

additive effect of both stressors (Coors & De Meester, 2008). The identification of interaction types between stressors is crucial for risk assessment (Coors & De Meester, 2008). To correctly assess the interaction type for a combination of two stressors, the correct null model needs to be implemented (Delnat et al., 2019). In some cases, the observed stressor interaction can also be concentration dependent (Delnat et al., 2019). For example, Delnat et al. (2019) exposed mosquito larvae (*Culex pipiens*) to three chlorpyrifos concentrations combined with 4 °C warming treatment or no warming treatment. For survival, fat content, AChE and the activity of the electron transport system they found a synergistic interaction between warming and chlorpyrifos, making chlorpyrifos more toxic at increasing temperatures. For all these variables, except the activity of the electron transport system, the interaction was dependent on the chlorpyrifos concentration (Delnat et al., 2019). For AChE activity, an additive interaction between low chlorpyrifos concentration and warming occurred, while at the high chlorpyrifos concentration, a synergistic interaction with warming occurred (Delnat et al., 2019). This synergistic interaction can be explained by a faster biotransformation into the chlorpyrifos-oxon at higher temperatures, in combination with higher concentrations of chlorpyrifos and thus more material that can be biotransformed (Delnat et al., 2019).

3. Tolerance

3.1 Thermal tolerance

Evolving tolerance to environmental cues is important as it can rescue a population from extinction when facing the stressor (Janssens et al., 2022). As freshwater species often have few dispersal mechanisms, they have difficulties reaching new habitats when the current one gets too hot, and thus have to find a way to become tolerant (Woodward et al., 2010). In *D. magna*, the main mechanism of evolving thermal tolerance is increasing their critical thermal maximum or CTmax. The CTmax describes the upper temperature at which loss of motor function is observed because of the mismatch in increasing oxygen demand and decreasing oxygen supply with increasing temperatures (Geerts et al., 2015). By performing resurrection ecology, it is observed that more recent *Daphnia* populations showed a higher CTmax and higher thermal plasticity than older populations (Cuenca Cambronero et al., 2018a). This positive correlation between induced and consecutive thermal tolerance of the recent population is likely due to an increase in heatwave and ambient temperature over the studied decades (Cuenca Cambronero et al., 2018a).

One way to increase the CTmax and thus increase thermal tolerance is by becoming smaller. Indeed, the CTmax is related to body size: smaller individuals have higher CTmax values (Geerts et al., 2015). Body size is linked to CTmax because larger bodies require more oxygen, and at higher temperatures, the demand for oxygen increases, which can thus create limitations for larger individuals (Geerts et al., 2015). As a plastic response, many animals thus become smaller when exposed to increasing temperatures, and this is described as the ‘temperature-size

rule' (Verheyen & Stoks, 2019). The lower body size at higher temperatures is caused by several mechanisms, including more allocation of resources to development instead than to growth, leading to a lower growth efficiency at higher temperatures (Verberk et al., 2021).

The underlying genetic mechanisms contributing to thermal tolerance are not yet well understood, but Jansen et al. (2017) did find that under heat exposure some genes are downregulated, and others are upregulated. The five candidate genes related to thermal tolerance that Jansen et al. (2017) found are GPX, DamVTG1, Cyp, Lip, and Sema-1a. Other genes being closely related to temperature stress are heat shock proteins (HSP) (Chen et al., 2021). When *Daphnia* embryos are exposed to heat stress, two HSP70 genes (*Dmhsp70-A* and *Dmhsp70-B*) showed an augmented transcription (Chen et al., 2021). HSP70 has a specific structure, which leads to a quick expression when subjected to stress but also leads to a quick degradation when the stressor is no longer present (Chen et al., 2021). The five candidate genes as well as HSP genes are likely also related to tolerance to other stressors as they also support responses to different stressors beside increasing temperatures (Chen et al., 2021; Jansen et al., 2017). Haemoglobin (Hb) levels are also related to thermal tolerance (Cuenca Cambronerio et al., 2018b). Hb is a respiratory protein, and when expression is increased, the higher Hb levels can counteract the reduction in oxygen supply correlated to increasing temperatures (Cuenca Cambronerio et al., 2018b). Both changes in Hb affinity and concentration can improve activity and supply to tissue in hypoxic conditions (Cuenca Cambronerio et al., 2018b). When comparing *Daphnia* subpopulations exposed to either hyper-thermal stress with subpopulations exposed to no stress, a higher Hb content was detected (Cuenca Cambronerio et al., 2018b). The plasticity in Hb content does not only provide better tolerance to higher temperatures, but Hb-rich *Daphnia* also exhibited better competitive abilities under thermal stress (Cuenca Cambronerio et al., 2018b).

3.2 Pesticide tolerance

Because of the increasing applications of pesticides, organisms must evolve tolerance. There are two main ways in which an individual can build up tolerance to a pesticide. The first way is tolerance due to less sensitive or insensitive pesticide target-sites (i.e. target-site tolerance) (Stenersen, 2004). This can occur due to specific mutations in the genome which result in the (over)expression of mutated target-sites that have a lower binding affinity to the pesticide than the original target-site (Stenersen, 2004). For example, in the mosquitos *Culex pipiens* and *Anopheles gambiae*, tolerance to organophosphates and carbamates has been observed due to a single mutation (G119S) in the *ace-1* gene, which created a restriction site leading to the expression of insensitive AChE (Weill et al., 2004). The second possibility to achieve pesticide tolerance is via increased detoxification of the pesticide (i.e. metabolic tolerance) (Stenersen, 2004). Esterases, glutathione transferases and cytochrome P450 (CYP) enzymes are some of the most important enzymes and isozymes related to detoxification of pesticides (Stenersen,

2004). For CYP enzymes, increased tolerance is not so much due to new enzymes, but due to a difference in the relative amount of detoxification enzymes (Stenersen, 2004). When *Daphnia* are exposed to nanoparticles of cerium oxide, this leads to increased expression of various CYP genes (Kang et al., 2024). While the expression of several CYP genes, like CYP4C1.4 and CYP4c3.6, was upregulated in response to cerium oxide, the expression of other genes, like CYP4C1.3 and CYP4c3.9, was downregulated (Kang et al., 2024). The change in CYP gene regulation thus indicates their role in the detoxification of xenobiotics (Kang et al., 2024). In *Bactrocera dorsalis* exposed to malathion, the glutathione S-transferases genes BdGSTd10 and BdGSTd1 were upregulated after malathion exposure, indicating their function in malathion detoxification (Meng et al., 2019).

3.3 Cost of tolerance

While evolving tolerance could save a species from extinction, it could also pose as a problem and have associated costs (Moe et al., 2013). A possible downside of genetic adaptation to a stressor is that the resulting tolerance may lead to reduced fitness levels in environments where the stressor is absent (Moe et al., 2013). Indeed, tolerance can entail energetic costs, as maintaining tolerance to one stressor can lead to reduced energetic reserves (Rivero et al., 2011). If the cost of tolerance is too great in the absence of the stressor, tolerance will likely be lost after several generations. This was seen in an oligochaete (*Limnodrilus hoffmeisteri*) population which evolved resistance to cadmium, and after a wash-out, loss of resistance over the course of 9-18 generations was observed (Levinton et al., 2003). If resistance to a no longer present stressor is too costly, it can thus be lost and rapid ecological restoration can occur (Levinton et al., 2003).

At population level, genetic adaptation often results in the decrease of genetic diversity, which in turn increases the extinction risk (Moe et al., 2013). This decrease in diversity is especially an issue, because it can lead to a higher susceptibility to other stressors, caused by the reduced gene pool (Moe et al., 2013). For example, freshwater snails (*Biomphalaria glabrata*) exposed to cadmium for four generations, thereby evolving tolerance to the pollutant, experienced a decreased cadmium tolerance when temperature stress was imposed (Salice & Anderson, 2010). Important to note is that the snails were exposed to cadmium for three generations, not exposed in the fourth generation, and again in the fifth generation (Salice & Anderson, 2010). As the cadmium-exposed snails were still tolerant to cadmium in the fifth generation, after removal of cadmium exposure in the fourth generation, this indicates genetic adaptation of the cadmium-exposed snails (Salice & Anderson, 2010). According to Salice & Anderson (2010), the decreased tolerance to cadmium was not related to costs due to changes in fitness but rather due to the disappearance of certain genotypes linked to resistance against a different stressor like heat. Furthermore, for *Daphnia* populations, which were tolerant to increased temperatures, a trade-off between thermal tolerance and pollutant tolerance occurred once exposed to the

pollutant zinc: resurrected clones which evolved heat tolerance were more susceptible to zinc exposure than resurrected clones which were not tolerant to heat, as zinc exposure only reduced sugar content and CTmax in the recent subpopulation (Dong et al., 2024).

The cost of tolerance does not only occur at the individual level or at the population level but can also occur at the community level. In communities exposed to a pollutant, the most sensitive species will be erased from the community, leading to an increased overall tolerance of the community (Moe et al., 2013). This however, may come with a cost as species diversity is reduced due to the loss of the sensitive species, resulting in a lower probability of species containing tolerance for new and different stressors (Moe et al., 2013). Being adapted to a certain stressor may therefore thus involve a trade-off when new stressors occur. Communities that eliminated drought-sensitive species can in turn become more susceptible to new pollutants, while communities adapted to chronic pollution might become more susceptible to future climate change (Moe et al., 2013). This was observed in communities which evolved heavy metal tolerance, but in turn were more susceptible to UVB-radiation (Moe et al., 2013).

4. Effect of evolution of tolerance on interactions between stressors

Evolution of tolerance to a stressor can also change the interaction strength or even the interaction type between that stressor and another stressor. For example, evolution of tolerance to one stressor can decrease the magnitude of synergistic interactions with a second stressor. In thermal tolerant *Daphnia* simultaneously exposed to warming and zinc, a positive growth rate was observed, while for the non-thermal tolerant *Daphnia*, a synergistic interaction between both stressors with a negative effect on the *Daphnia* was observed (C. Zhang et al., 2018). This indicates that evolving tolerance can offset the occurrence of synergistic stressor interactions (C. Zhang et al., 2018). As evolving thermal tolerance is expected to decrease the energetic cost needed to deal with warming, there is more energy left to cope with other stressors, like toxicants (C. Zhang et al., 2018). Nevertheless, the opposite can also occur. In some cases, evolving tolerance to one stressor can be accompanied by increased sensitivity to that stressor in combination with another stressor. Adaptation can thus come with a cost in a multi-stressor environment, as a result of a decreased fitness and plasticity due to a trade-off in energy (Siddique et al., 2021; Van de Maele et al., 2025). For example, pesticide adapted *Gammarus pulex* individuals showed a stronger synergistic interaction between the pesticide clothianidin and increasing temperatures than the reference non-adapted population (Siddique et al., 2021). In such cases, the general stress capacity of individuals which evolved pesticide tolerance was reduced and as a consequence, evolution of tolerance was disadvantageous in a multi-stressor environment (Siddique et al., 2021). In another study, *G. pulex* from agricultural streams (tolerant to clothianidin) and reference streams were exposed to both clothianidin and prochloraz together with suboptimal temperatures of 19 °C and 22 °C, and a control temperature of 16 °C (Shahid et al., 2024). The three different stressors interacted synergistically with each

other, as temperature elevation increased the metabolic rate, which imposed stress on the individuals (Shahid et al., 2024). In the agricultural pesticide-adapted population, the synergistic effect was even larger due to their lack of plastic response, which makes it harder to retain pesticide tolerance in a multi-stressor environment (Shahid et al., 2024). Animals tolerant to two stressors like temperature and pesticides can also occur, however there has not yet been much research performed on possible fitness consequences of combined tolerance. The thesis research of El Moussaoui however, is about multi-generational exposure. This study showed that *Daphnia* cultured in esfenvalerate and 25 °C for 19 generations, were less sensitive when exposed to 25 °C than when exposed to 20 °C without esfenvalerate exposure (El Moussaoui, University of Padova, 2021).

5. Microbiome

5.1 Microbiome and its host

As defined by Whipps et al., (1988), the microbiome is a “characteristic microbial community in a reasonably well-defined habitat, which has distinct physio-chemical properties” (Berg et al., 2020, p. 4). Bacteria, protozoa, fungi, and archaea are all part of the microbial community, and they carry out key roles within organisms, such as contributing to host adaptation, fitness, and evolution (Akbar et al., 2022). Hosts can physiologically change their microbiome to the microbial pool of the environment, this means that they can alter their microbiome to match to the microbial composition of their environment (Akbar et al., 2022). Because of this they are able to react to environmental feedback and cues (Akbar et al., 2022). The interactions that *Daphnia* have with their microbiome can be either long-lasting or transient and can vary between full symbiosis to obligate interactions (Akbar et al., 2022). The three most essential functions that gut microbiota perform in *Daphnia* are (1) producing essential nutrients that the host itself cannot produce, (2) metabolizing algae, and (3) outcompeting pathogens by recycling waste (Akbar et al., 2022). Resistance against the invasion of pathogens can also be established through microbiome diversity, as a more diverse resident community leads to the lower survival of alien species through niche competition (Mallon et al., 2015). A more diverse microbial community can thus better resist pathogen invasion than communities which are less diverse (Mallon et al., 2015). Next to these crucial functions, recent research from Janssens et al. (2022) shows that the microbiome can even play a key role in its host’s tolerance to pesticides (see 5.3.).

5.2 Effect of stressors on the microbiome

Pollutants do not only affect the life history traits and physiology of organisms, but they can also affect their microbiome. When changes and imbalances in the microbial composition occur, which is called dysbiosis, this can negatively impact the host’s health (Stock et al., 2021). A high coevolution between host and microbiome or a less diverse community can also make the host more sensitive to other stressors (Stock et al., 2021). This poses a problem as the

microbiome plays many key roles in the host's physiology and health, and therefore, changes in the microbiome (either changes in the taxa present or changes in the abundance of taxa) caused by human impacts can have adverse effects on the host (Stock et al., 2021).

Pesticide exposure can impact the function and dynamics of the microbiome (Suppa et al., 2020). Rather than changes in the core gut bacteria, pesticide exposure may lead to changes in the low-abundant bacteria (Suppa et al., 2020). This can be explained by that core taxa are maintained throughout synergistic interactions and low competition which makes them less vulnerable to stressors than low-abundant, non-core taxa (Suppa et al., 2020). Yet, despite this, the non-core taxa are the key influencers of community composition and thus microbiome functioning (Suppa et al., 2020). In addition, these non-core taxa might enhance detoxification, and lipid and carbon metabolism (Suppa et al., 2020). Suppa et al., (2020) examined the effect of glyphosate and the pesticide Roundup on the microbiome of *D. magna* and found an increase in low-abundant taxa like *Flavobacteriaceae* and *Rhizobiaceae*. By cleaving the C-P bond in organophosphate compounds, the families *Flavobacteriaceae* and *Rhizobiaceae* can use these compounds as a carbon source (Suppa et al., 2020).

Also temperature stress can change the microbiome composition by affecting the rare taxa (Frankel-Bricker et al., 2019). The fact that the most abundant taxa are not affected by temperature, might be explained by that these taxa are likely most resilient to changes in temperature (Frankel-Bricker et al., 2019). On the other hand, the change in abundance of rare taxa can indicate that temperature is having only a little impact on the functioning of the microbiome (Frankel-Bricker et al., 2019). Frankel-Bricker et al., (2019) tested how small temperature differences (16 °C, 18 °C and 20 °C) affect the microbiome composition of *D. magna*. Both microbiome structure and composition were affected by temperature, and based on four families, under which *Nocardioidaceae* and *Crocinitomicaceae*, a distinction could be made between temperatures (Frankel-Bricker et al., 2019).

5.3 Microbiome conferring tolerance to its host

The microbiome plays an important role in the acquirement of tolerance of its host to pesticides, because it can consist of bacteria that are involved in detoxification of pesticides. Accidental exposure to pesticides can lead to the bacteria acquiring mechanisms that can degrade the pesticide (R. J. Russell et al., 2011). The bacteria utilize these new nutrients as a source to increase their proliferation and reproduction rate, meaning that bacterial species capable of degrading pesticides will also increase in abundance when exposed to the pesticide (Janssens et al., 2022; R. J. Russell et al., 2011). Phosphotriesters, which are a substrate from organophosphates, can be degraded by several bacteria like for example *Pseudomonas* and *Arthrobacter* (Kumar et al., 2018; R. J. Russell et al., 2011). There are three classes of bacterial enzymes that degrade organophosphates: the organophosphorus acid anhydrolases (OPAA), the

phosphotriesterases (PTEs) and the methyl parathion hydrolases (MPHs) (R. J. Russell et al., 2011). All of these enzymes function by hydrolysing the R3 phosphoester bond of organophosphates (R. J. Russell et al., 2011).

To demonstrate a causal role of the microbiome in host tolerance to stressors, a microbiome transplant experiment can be performed. Macke *et al.*, (2017) showed a causal role of the microbiome of *Daphnia* in tolerance to toxic cyanobacteria. The gut microbiota of the tolerant clones consisted mainly of Flavobacteria, which are often accumulating in the presence of *Microcystis* blooms, as they can degrade and cause lysis to *Microcystis* cells (Macke et al., 2017). In this experiment, there was no change in bacterial composition observed, which could mean that the bacteria present in the gut are so versatile that they could genetically adapt to the change to cyanobacterial diet (Macke et al., 2017). Both host-genotype induced and environmentally induced shifts in microbiota functionality thus help mitigate tolerance in *Daphnia* to toxic cyanobacteria (Macke et al., 2017). Macke et al. provide three reasons as to how the gut microbiome of *Daphnia* can help with tolerance against the toxic cyanobacteria: 1) the microbiota can aid in digestion of *Microcystis*, as experiments have demonstrated the capabilities of bacteria to use these toxicants as nutrient supply, 2) the gut bacteria can aid in detoxification of toxins, and 3) via indirect epigenetic effects, as the produced metabolites can alter the host's genes expression (Macke et al., 2017). These options could also potentially aid in the tolerance of *Daphnia* against pesticides. That pesticide degrading bacteria will increase in abundance and can confer tolerance to its host is also demonstrated by research of Wang et al. (2020). In two microbial strains, metabolization of high atrazine concentrations and an increase in growth was indicating that they can utilize atrazine as their nitrogen source (Wang et al., 2020). In wasp populations fed with these two strains, they observed resistance to atrazine in the wasps, meaning that the microbial bacteria can confer resistance to atrazine towards their host (Wang et al., 2020). This phenomenon is called symbiont-mediated resistance and is also observed in exposure of trichlorphon to the pest fruit fly *Bactocera dorsalis* (Cheng et al., 2017; Wang et al., 2020). Trichlorphon is an organophosphate and can be degraded by *Citrobacter sp.*, a gut symbiont bacteria, as they contain genes for phosphate hydrolysis (Cheng et al., 2017). Just like in the experiment of Wang et al. (2020), the individuals inoculated with the bacteria acquired a higher trichlorphon resistance (Cheng et al., 2017). Microbiome adaptation can however also be affected by exposure to another stressor. When *Nilaparvata lugens* (brown planthopper) were pre-exposed to a high temperature (38 °C), imidacloprid sensitivity increased as they showed higher mortality to the pesticide than when exposed to 28 °C (Y. Zhang et al., 2021). This was due to a reduction in bacterial content caused by the exposure to 38 °C, and especially a reduction in relative abundance of *Wolbachia*, which is an important detoxifying bacteria (Y. Zhang et al., 2021).

The microbiome may not only play a key role in conferring pesticide tolerance to its host, but also in conferring thermal tolerance. Being exposed to higher temperatures can lead to the gain of taxa which are thermal tolerant and to the loss of taxa which are thermally sensitive et al., 2023). This is beneficial as according to Stock et al. (2021), negative effects coupled with global warming can be mitigated by the evolutionary changes in the microbiome when exposed to increasing temperatures. An experiment, performed by Theys et al. (2023a), showed that the microbiome indeed plays an important role in conferring thermal tolerance to its host. When high-latitude damselfly *Ischnura elegans* larvae were exposed to a heatwave, their microbial community shifted in the direction of that of thermally evolved low-latitude larvae (Theys et al., 2023a). Especially the relative abundance of bacteria that increased the energy budget, like Burkholderiaceae and Aeromonadaceae increased (Theys et al., 2023a). The important bacteria aiding in thermal tolerance are thus often bacteria that help the host cope with increasing temperatures by providing more energy, and in this study were all Proteobacteria (Theys et al., 2023a). The microbiome responses of different hosts to thermal stress are often host specific, however across host species, the bacterial lineage of Proteobacteria show a consistent increase when exposed to thermal stress, and thus indicating that they play a key role in a response against thermal stress (Sepulveda & Moeller, 2020).

6. Problem definition

Increasing temperatures and pesticide exposure both have negative effects on organisms and when these stressors occur simultaneously, they can strengthen each others effect. Luckily, populations can evolve tolerance to stressors. However, it remains unclear whether such tolerance comes with a cost, especially when organisms face multiple stressors at once. To what extent is it thus possible that organisms become multi-tolerant and how is this affected by the costs of tolerance. Additionally, while the microbiome has been proven to aid in pesticide and heat tolerance, its role in influencing the costs of tolerance and interactions between multiple stressors is not well understood. Understanding these dynamics is especially important for organisms like *Daphnia magna*, a keystone species as it is both a grazer on phytoplankton and a food source for higher trophic levels. Therefore, more research is needed to explore the costs of tolerance, the potential for multi-tolerance, and the role of the microbiome in shaping these outcomes under combined environmental stressors.

Objectives and hypotheses

In this thesis, the aim is to investigate how exposure to a pesticide and/or warming affects the life history traits (survival rate, age at maturity and reproductive performance) of four clone types (control, pesticide-tolerant, heat-tolerant, combined-tolerant). In addition, it will also be examined how exposure to pesticide and/or warming shapes the microbiome of *Daphnia magna* and whether this differs in function of their tolerance type as the microbiome of tolerant clones might be adapted to the stressor(s).

1. When not exposed to any stressors

Since control clones do not put energy in maintaining tolerance against a stressor, it is expected they will have a high fitness, expressed as high survival, fast maturation and high reproductive performance, when not exposed to any stressor. As evolving tolerance costs energy, it is hypothesized that when pesticide-tolerant clones are not exposed to any stressor, they will show a lower fitness (lower survival, delayed maturation and lower reproductive performance) compared to control clones (Rivero et al., 2011). Similarly, heat-tolerant clones investing energy in tolerance maintenance might also have a lower fitness than control clones when not exposed to any stressor (Rivero et al., 2011). Assuming a reduced energy budget when tolerant to a single stressor, it is thought that especially the combined-tolerant clones are to show a decreased fitness when exposed to no stressors, because they invest energy in maintaining tolerance to two stressors.

In the absence of stressors, there is no need for the microbiome to change in composition and therefore, the four clones are expected to maintain their original microbiome. Therefore, it is hypothesized that the tolerant clones will also maintain their adapted microbiome in the control conditions and all four clones will thus have different microbiome compositions in the control condition (Macke et al., 2017).

2. When only exposed to malathion

When only exposed to the pesticide malathion, the control clones are expected to show lower survival, delayed maturation and lower reproductive performance (Duquesne, 2006). In contrast, the pesticide-tolerant clones will thrive (high survival, fast maturation, high reproductive performance) when exposed to the pesticide compared to the other clone types, as the pesticide-tolerant clones evolved adaptations to deal with the pesticide (Kang et al., 2024; Weill et al., 2004). For heat-tolerant clones exposed to the pesticide, it is hypothesised that they will show a lower fitness than the control clones and the pesticide-tolerant clones in case of the expected trade-off between their heat tolerance and pesticide tolerance (Dong et al., 2024; Moe et al., 2013). As not much research has been performed on combined-tolerant clones, it is difficult to predict how they will react when exposed to a pesticide. The combined-tolerant clones might have a higher fitness than the heat-tolerant clones but not as high as pesticide-

tolerant clones as they also direct energy towards maintaining heat tolerance. How the combined-tolerant clones will react in comparison to control clones is difficult to predict. Combined-tolerant clones direct energy toward thermal tolerance maintenance whereas control clones do not, but control clones possess no tolerance. If the energy demand for control-clones to deal with pesticide exposure is the same as the amount of energy combined-tolerant clones lose by also maintaining thermal tolerance, those two clone types might show the same fitness patterns.

As the pesticide-tolerant clones are expected to already possess the right bacteria to deal with pesticide stress (Janssens et al., 2022), no change in microbiome composition is expected when again exposed to the pesticide. Yet, it is expected that pesticide exposure will induce a change of the control and heat-tolerant clones (Suppa et al., 2020). As heat-tolerant and control clones do not possess the bacteria aiding in pesticide tolerance, a change in microbiome composition towards pesticide-degrading bacteria is expected in response to pesticide exposure (Russell et al., 2011). The microbiome of the combined-tolerant clones already possesses pesticide-degrading bacteria, however the abundance of these maybe be less large than the abundance of pesticide-degrading bacteria of the pesticide-tolerant *Daphnia*. As the combined-tolerant *Daphnia* also possess heat-tolerant bacteria, a trade-off might occur between both types of tolerant bacteria. Therefore, upon malathion exposure, the abundance of pesticide-degrading bacteria in the microbiome of the combined-tolerant clones might increase.

3. When only exposed to heat stress

As control clones will be sensitive to heat stress, it is expected that they will show a lower fitness than when not exposed to any stressor (Giebelhausen & Lampert, 2001; Ramos-Rodríguez et al., 2022; Van de Maele et al., 2025). For the pesticide-tolerant clones exposed to warming, it is hypothesized that they will be even more sensitive than the control clones exposed to warming as a cost of their tolerance, as maintenance of pesticide tolerance requires a lot of energy and hence, less energy is left to cope with other stressors, like increasing temperatures (Rivero et al., 2011). When exposed to heat stress, the heat-tolerant clones are expected to show the highest fitness due to their heat tolerance mechanisms (Cuenca Cambronero et al., 2018a; Geerts et al., 2015). Combined-tolerant clones might show a higher fitness than pesticide-tolerant clones, a lower fitness than heat-tolerant clones and the same fitness pattern as control clones when exposed to heat stress. The results of El Moussaoui's PhD thesis (2021) might also indicate that combined-tolerant clones exposed to a single stressor show a higher fitness than when not exposed to any stressor.

It is hypothesized that thermal stress will induce a shift in the microbiome composition of control clones, with the microbiome composition changing towards the microbiome composition of heat-tolerant clones (Theys et al., 2023a). As the heat-tolerant clones already

possess heat-tolerant bacteria, their microbiome composition is not expected to change. For the pesticide-tolerant clones, it is expected that they lose some pesticide-degrading bacteria after temperature stress because of tolerance trade-offs for the bacteria (Y. Zhang et al., 2021). Similarly to the control clones, the microbiome of pesticide-tolerant clones might shift from pesticide-degrading bacteria towards bacteria aiding in thermal tolerance. Combined-tolerant clones already possess heat-tolerant bacteria but probably not as much as the single heat-tolerant *Daphnia*. Therefore, upon heat exposure, the abundance of heat-tolerant bacteria in the microbiome of the combined-tolerant clones might increase.

4. When exposed to both malathion and heat stress

It is hypothesized that when the control clones are sequentially exposed to both stressors, they will show a very low fitness as they have not evolved any defence mechanisms to either stressor, and a synergistic interaction is expected between the pesticide exposure and the heat spike according to the TICS concept (Hooper et al., 2013; Op de Beeck et al., 2017). In contrast, as the combined-tolerant clones have evolved adaptations to both stressors, it is hypothesized that they will show no change in fitness when exposed to both stressors compared to the control conditions. For the heat-tolerant clones exposed to the combination of malathion and warming, it is hypothesized that their fitness will not be affected as much as the control clones, as heat tolerance might offset the synergistic interaction between multiple stressors (C. Zhang et al., 2018). In contrast, research has shown that pesticide tolerance lowers the general stress capacity, creating a trade-off condition when exposed to multiple stressors (Siddique et al., 2021; Van de Maele et al., 2025). That is why it is hypothesized for pesticide-tolerant clones to perform similarly to control clones when exposed to both stressors.

When combined-tolerant clones are exposed to both a pesticide and heat stress, no change in microbiome composition is expected as their microbiome composition already consists of bacteria aiding in pesticide tolerance as well as bacteria aiding in thermal tolerance. However, a change in abundance can still occur as all bacteria within the microbiome will need to be heat-tolerant, but only a few need to be able to detoxify malathion to shield the other bacteria (and its host) from the toxic effects of malathion. On the other hand, the microbiome of control clones is expected to shift towards a combination of both bacteria aiding in thermal tolerance as well as pesticide tolerance. The microbiome of heat-tolerant and pesticide-tolerant clones already possess bacteria aiding in tolerance against one of both stressors. However, it is still expected for heat-tolerant clones to also gain some pesticide-degrading bacteria and for malathion-tolerant clones to gain some heat-tolerant bacteria.

Materials and methods

1. *Daphnia* as model organism

In this thesis, the model organism *Daphnia magna* is used due to the suitability of its characteristics in relation to the planned experiment. *Daphnia* have already been used in research for more than hundred years, and are still widely used today regarding many different topics like phenotypic plasticity, vertical migration and climate change ecology (Ebert, 2022). *D. magna* are aquatic arthropods, belonging to the class of Branchiopodia and play a key role in the aquatic ecosystem (Ebert, 2022). They are primary consumers, mainly feeding on algae and are consumed themselves by invertebrates and fish (Ebert, 2022). Through asexual parthenogenesis, *Daphnia* develop diploid eggs without fertilisation leading to offspring that is genetically identical to the mother (Ebert, 2022). This clonal reproduction is advantageous in toxicity experiments for comparing results between different clones (Ebert, 2022). When unsuitable conditions arise (e.g. drying out or freezing of the pond), *Daphnia* can also reproduce sexually (Ebert, 2022). The males that are reproduced asexually can then fertilise haploid eggs, which results in dormant resting embryos that will continue their development when a suitable environment appears (Ebert, 2022). The asexual reproduction cycle of *Daphnia* is very short, and fecundity is high which results in the production of many neonates, which is beneficial for an experiment where many replicates and individuals are needed (Tkaczyk et al., 2021). Another practical reason to use *Daphnia* as test organism is that *Daphnia* are transparent, which makes it easy to see if they are sexually mature or not, as their eggs are visible through the carapace (Tkaczyk et al., 2021). For toxicity testing, acute parameters like immobilisation and death rate can be observed (Tkaczyk et al., 2021). As both immature and mature *Daphnia* are easily visible by eye, endpoints like survival and maturation are simple to assess. *Daphnia* also have a high evolutionary potential, as they can evolve local adaptation quickly and resurrected clones can be used in common garden experiments (Ebert, 2022). The microbiome of *Daphnia* is also well studied and extensive research on microbiome interactions and changes has been performed (Akbar et al., 2022; Frankel-Bricker et al., 2019). All previously mentioned characteristics thus make *D. magna* a perfect research organism for this thesis.

2. Experimental set-up

To investigate how exposure to a pesticide and/or warming affects the life history traits and the microbiome composition of *D. magna*, a life history experiment with four *D. magna* clone types was performed. The four clone types had different tolerance levels: control clones sensitive to both stressors, malathion-tolerant clones, heat-tolerant clones, and combined-tolerant clones (tolerant to both malathion and heat). Of each of the four tolerance types, three clones were studied. This resulted in a total of twelve clones. Please note that I performed the life history experiment and exposure experiment for four clones (one clone of each tolerance type: control clone (C2), pesticide-tolerant clone (M6), heat-tolerant clone (T9) and combined-tolerant clone

(MT11)), while the experimental work with the other eight clones (two clones of each tolerance type) was performed last year (see thesis Sarrazijn Kelsey, 2023-2024). The analysis executed in this thesis was done on the data of all twelve clones together.

2.1 Origin of the *Daphnia magna* clones

The clones used in this thesis were obtained from a selection experiment performed in 2022. The *D. magna* clones used in the selection experiment were sampled from a natural population of Langerodevijver in Neerijse, Belgium. In the selection experiment, a full factorial design was used in which five experimental populations were exposed to two pesticide treatments (control exposure or malathion exposure of 2.5 µg/L) and sequentially to two temperature treatments (20 °C constant or with a 32 °C heatwave). The pesticide treatment started two days after the start of the experiment and the temperature treatment was imposed four days after the start of the experiment and lasted four days. After a total of ten days, the selection experiment stopped and the surviving mature individuals were collected and maintained under standard laboratory conditions. In this thesis, three control clones from the control selection treatment, three malathion-tolerant clones from the malathion selection treatment, three heat-tolerant clones from the heatwave selection treatment, and three combined-tolerant clones from the combined selection treatment were used. A more in dept description of the selection experiment can be found in the thesis of Slegers Seppe and Van Buggenhout Helena (2022-2023).

2.2 Cultivation of the *Daphnia magna* clones

The clones were kept in mono-cultures under standard conditions (20 °C ± 1 °C, 16:8 L:D photoperiod) and reared for two generations prior to the experiment to remove maternal effects. To start a new generation, 6 *Daphnia* neonates of maximum 24 hours old, were placed in a clean glass jar of 210 mL. This jar was filled with 1/3 of water coming from the mother's jar and 2/3 of clean dechlorinated tap water. The first three days, neonates were fed daily with 0.1 mL of 1×10^8 cells/mL of the algae *Acutodesmus obliquus*, and *Daphnia* older than 3 days were given 0.2 mL daily. Once the *Daphnia* were mature, the amount of *Daphnia* per jar was reduced to three. The second until fifth brood of these *Daphnia* were used for the experiments. Twice a week, the jars of the *Daphnia* were replaced by new clean jars, containing 1/2 of the old jar's water and 1/2 of clean water.

2.3 Life history experiment

To investigate the effect of malathion and/or a heat wave on the life history traits of the four tolerance clone types, a full factorial life history experiment was performed with two pesticide treatments (solvent control of 1.5 µL/L ethanol or malathion exposure of 1.5 µg/L) and two sequential temperature treatments (20 °C constant or with a 32 °C heatwave) (Figure.1). This resulted in following treatment combinations: 1) a solvent control condition and no stressor exposure, 2) a malathion exposure condition, 3) a heatwave exposure condition, and 4) a

malathion and heatwave combined exposure condition. A malathion concentration of 1.5 µg/L was chosen based on a previously performed range finder where 1.5 µg/L induced moderate mortality of the control clones while not causing complete mortality of the heat-tolerant clones. This malathion concentration has also been detected in waterbodies and is thus environmentally realistic (Siepmann & Slater, 1998). For the heatwave, a four-day heatwave of 32 °C was chosen. This temperature was chosen as research has shown that during the heatwave of 2018 in Europe, maximum surface water temperatures of mostly 25 °C to 30 °C were reached (Woolway et al., 2020). Per clone (in the experimental part of this thesis: four clones), all four conditions were tested with five replicates, resulting in a total of 80 experimental jars.

In each experimental jar of 500 mL filled with test medium (either solvent medium or malathion medium), ten *Daphnia* neonates (<24 h old) were placed. For the first two days, all the jars were placed in an incubator of 20 °C. After two days, half of the solvent control jars and half of the pesticide jars were placed in a different incubator that would mimic a heatwave, reaching 32 °C as the highest temperature. At the start of the heatwave, temperature gradually increased from 20 °C to 32 °C with 2 °C per hour. At the end of the heatwave, temperature gradually decreased from 32 °C to 20 °C with 2 °C per hour. The other half of the experimental jars stayed in the 20 °C incubator. From the third day on, survival, maturation and release of brood (timing and clutch sizes) of all the jars were checked daily. After 6 days, all the jars, regardless of their treatment, were placed in an incubator with a temperature of 20 °C. In total, these jars were followed up for 16 days. The first 6 days (during stressor exposure), the *Daphnia* were fed 0.25 mL of 1×10^8 cells/mL of the algae and for the remaining 10 days, 0.5 mL. Every 6th, 10th and 14th day, half of the medium of the *Daphnia* was refreshed.

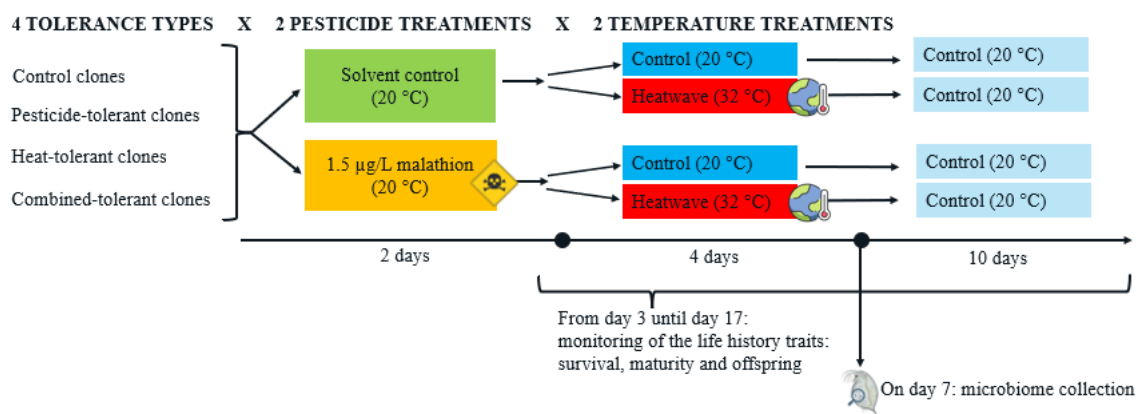


Figure 1: Full factorial design of the life history experiment. The four different tolerance types were exposed to two different pesticide treatments and subsequently exposed to two temperature treatments. After the 6 days of stressor exposure, all clones were maintained at 20 °C to monitor the different life history traits.

2.4 Microbiome collection

Extra experimental jars were set up for microbiome collection. These jars had the exact same set-up as the life history experiment, but instead of following them up for 10 additional days

after stressor exposure, they were collected immediately after the end of the temperature treatments on day 6 (Figure 1). Instead of starting up one jar per treatment-type combination, multiple jars were set up to obtain enough surviving *Daphnia* in the end to freeze. Also 5 replicates of each treatment-type combination were set up. In total, 382 jars were set up, with the minimum and maximum amount of jars needed depending on the type-treatment combination (Table 1). After the exposure experiment was finished, the *Daphnia* were prepared for microbiome analysis. Per clone, for each condition, 10 *Daphnia* were washed twice in autoclaved water, and afterwards were placed in an Eppendorf tube and placed in a freezer of - 80 °C. These samples were sent to KU Leuven Gasthuisberg where the microbiome composition analysis was performed.

Table 1: The minimum and maximum amount of jars set up for microbiome collection per type-treatment combination and per replicate.

		Control clones	Malathion-tolerant clones	Heat-tolerant clones	Combined-tolerant clones
Solvent control	Min	3	4	3	4
	Max	3	6	3	6
Malathion	Min	3	3	3	3
	Max	3	3	3	3
32 °C	Min	2	5	2	5
	Max	3	8	3	8
Malathion + 32°C	Min	3	4	3	4
	Max	3	4	3	4
Total amount of jars per type		106	60	146	70

2.5 Preparation of the test concentrations and the actual concentrations

To obtain the final solution with a nominal test concentration of 1.5 µg/L malathion, an initial stock solution of 1 mg/mL of malathion in ethanol was made. This stock solution was kept in a dark bottle and in a dark room at 4 °C. Then, of this stock solution, a dilution of 10 µg/mL was made using MilliQ water. This intermediate solution was eventually used to make the test concentrations of 1.5 µg/L malathion in dechlorinated tap water. The same steps were followed to make the solvent control solution with a concentration of 1.5 µL/L ethanol. Starting from a stock of 100% ethanol, a dilution to 10 µL/mL in MilliQ water was made to prepare the final test solution of 1.5 µL/L in dechlorinated tap water.

To check how the actual pesticide concentrations changed over the time course of the experiment, pesticide samples at four different time points were taken: 1) at the start of the experiment, 2) after 48 hours (right before the heatwave exposure), 3) after 96 hours (from both jars on 20 °C and jars on 32 °C) and 4) at day 7 at the end of the temperature treatment (from

both the jars that were not exposed to the heatwave and jars that were exposed to the heatwave). These pesticide concentrations were measured with an ultraperformance liquid chromatography MS/MS with Triple Quadrupole Mass Spectrometry in triplicate (two replicates from Sarrazijn Kelsey in 2023-2024 and one replicate from me this year). The mean actual start concentration ($\pm$ SE) was 0.71 ($\pm$ 0.04) μ g/L malathion and thus considerably lower than the nominal concentration of 1.5 μ g/L. After 48 hours, malathion degraded substantially to a concentration of 0.23 ($\pm$ 0.04) μ g/L. After 96 hours, the malathion concentrations had dropped to 0 μ g/L. This was the case for both jars on 20 °C and 32 °C. These measurements might suggest that the heatwave did not affect the speed of pesticide degradation as after 96 hours, on both 20 °C and 32 °C, the actual pesticide concentration was 0 μ g/L malathion.

2.6 Microbiome composition determination

During the first step, the NucleoSpin Tissue Kit (Machery Nagel) was used for DNA extraction, following the manufacturer's protocol. To identify the microbiome composition, the V3-V4 region of the 16S rRNA was amplified, using the 16S-IllumTS-F and 16S-IllumTS-R primers (Klindworth et al., 2013). In a Biometra TOne Thermocycler (Westburg), PCR's were conducted and the commercial Quant-iT™ Picogreen kit (Thermo Fisher) was used to quantify the DNA. Using the MiSeq Reagent Kit v2 on the Illumina MiSeq platform at Genomics Core (KU Leuven, Leuven Belgium), the purified amplicons were pair-end sequenced. Afterwards, to process the data, the QIIME2 pipeline v2024.10 was used (Bolyen et al., 2019). The microbiome samples were split and sequenced on two different MiSeq runs. On the first run (eight *Daphnia* clones exposed to all the treatment combinations, resulting in 160 samples, excluding the blank samples), a total of 24541992 reads, with an average of 148739.3455 reads per sample (min. = 25258 and max. = 390826 reads) was obtained. On the second run (four *Daphnia* clones exposed to all treatment combinations, resulting in 80 samples, excluding the blank samples), a total of 12283830 reads, with an average of 153547.875 reads per sample (min. = 1384 and max. = 478494 reads) was obtained. Within QIIME2, the denoising function of DADA was used to trim at 14 bp and truncate at 240 bp, and chimeras were removed. The paired-end sequences were also filtered, denoised and dereplicated. After denoising, on the first run, on average 51.84% of the data per sample remained, and on the second run, on average 42.50% of the data per sample remained. After merging both datasets, a total of 1233 amplicon sequence variants (ASVs) across all samples was obtained with an average length of 427.18 bp (SE = 0.44). For the taxonomy assignment, the Silva 138 SSU Ref NR 99 database was used (Quast et al., 2013). To identify contaminated DNA features, the decontam package in R was used (version: 1.22.0, Davis et al., 2018) and 13 taxa were removed. After cleaning, removing of singletons and filtering, an additional 208 taxa were removed. Samples were rarefied at a cut-off level of 12183 reads (rngseed 711) which resulted in two samples being lost and 161

taxa being removed. The rarefaction curve is provided in the Appendix B. After rarefying, 238 samples and 819 ASVs remained.

3. IA model

To determine the interaction type (synergism, antagonism or additive) between the pesticide and heatwave exposure, the independent action (IA) model was used. This model is mainly used for stressors that act dissimilarly (Coors & De Meester, 2008). The IA model also recognizes that when one stressor is the cause of death for an individual, that individual cannot be killed by a second stressor (Delnat et al., 2019). The joint effect of both stressors (E_{mix}) on the life-history traits of *Daphnia magna* was predicted with the corresponding effects of the single stressors (E_i), by using following equation (Bliss, 1939; Coors & De Meester, 2008):

$$E_{\text{mix}} = \prod_{i=1}^i (1 - E_i)$$

The effect of the single stressors is calculated by this equation:

$$E_i = \frac{(e_i - e_{\text{control}})}{(e_{\text{max}} - e_{\text{control}})}$$

with e_i being the observed effect, e_{max} the maximum possible effect and e_{control} the value in the control condition (Coors & De Meester, 2008). For survival, reproductive performance and clutch size, e_{max} was set at zero. For reproduction time, e_{max} was set at 17 days and for maturation time at 16 days (respectively the longest reproduction and maturation times based on the experimental data). A synergistic interaction occurs when the observed effect is significantly stronger than the predicted combined effect of the single stressors, while for an antagonistic interaction, the opposite occurs (Coors & De Meester, 2008). With the use of the model deviation ratio (MDR), the strength of the interaction was quantified. MDR was calculated by dividing the observed by the predicted effects (Shahid et al., 2019; Van de Maele et al., 2025). If MDR is 1, the interaction between both stressors is additive (Van de Maele et al., 2025). For survival, reproductive performance and total offspring, an $\text{MDR} > 1$ indicates a synergism and an $\text{MDR} < 1$ an antagonism. For maturation time and reproduction time, an $\text{MDR} < 1$ indicates a synergism and > 1 an antagonism. The more the MDR value deviates from 1, the stronger the antagonistic or synergistic interaction (Van de Maele et al., 2025).

4. Statistical analyses

Survival was expressed as the percentage of surviving *Daphnia* per jar, and will be analysed overall but also on specific timepoints: at day 3 (after the malathion exposure period), at day 7 (after the heatwave exposure period) and at day 17 (at the end of the experiment after the follow-up period). Maturation time was defined by the day on which half of the *Daphnia* in the jar were mature. Reproduction time was defined by the first day on which the first *Daphnia* in the jar produced neonates. The total offspring was calculated and corrected for the amount of

Daphnia still alive after 17 days. To calculate the reproductive performance, the protocol of Delnat et al. (2022a) was followed using the Euler-Lotka equation: $1 = \int e^{-rx} l_x m_x dx$, with l_x the proportion of survivors and m_x the number of offspring at day x . Afterwards, using r , lambda was calculated: $\lambda = \exp(r)$. Lambda was used in the statistical analysis because even when there is a jar where no *Daphnia* survive, lambda can be calculated ($\lambda=0$), while r cannot be calculated in the case of no survival ($r=-\infty$) (Delnat, 2022a). Reproduction time and total offspring are described in Appendix B as these are components of reproductive performance. For the microbiome composition, the relative abundance values and the Unweighted UniFrac distance metric plots are also provided in Appendix B.

All the data were analysed using R version 4.5.0 (R Core Team, 2025). For all life-history traits, linear mixed effects models were fitted, including tolerance type, pesticide treatment and temperature treatment as independent variables, while taking into account all of their interactions, using following packages: lme4 (version: 1.1-37; Bates et al., 2015), afex (version: 1.4-1; Singmann et al., 2012), and car (version: 3.1-3; Fox & Weisberg, 2019). As random factors, start date and clone nested in tolerance type were included. For survival, also the pattern over the full 17 days was checked. In this model, day was added as extra independent variable. To meet the assumptions on normality of residuals, reproduction time and reproductive performance were transformed using a box cox transformation using the MASS package (version: 7.3-60.0.1; Ripley & Venables, 2002). For reproductive performance, the transformed results were plotted in the main text and the plot of the untransformed values is added in Appendix B. To further analyse significant interactions between all three treatments, pairwise comparisons were made using the emmeans package (version: 1.11.1; Lenth, 2025).

Analyses of the microbiome of the *Daphnia* was performed at the ASV level. To estimate the differences in α -diversity, three different indices were used: the observed number of ASVs, the inverse Simpson index and the Faith's phylogenetic diversity index. These were all calculated using the "estimate_richness" function in the phyloseq package (version: 1.52.0; McMurdie & Holmes, 2013). Afterwards, linear mixed effects models were fitted with temperature treatment, pesticide treatment and tolerance type, and their interactions, as independent variables and clone nested in tolerance type as random factor. For the β -diversity, a PERMANOVA was performed on the Bray-Curtis and the Unweighted UniFrac distance metrics, with the same independent variables as for α -diversity, using the adonis2 function of the vegan package (version: 2.6-10; Oksanen et al., 2001), PERMANOVAS with 100000 permutations and a set seed of 100000 were run. With non-metric multidimensional scaling (NMDS), these metrics were plotted using the ordinate function in the phyloseq package (Bray-Curtis: $k = 2$ with stress = 0.15; Unweighted Unifrac: $k = 3$ with stress = 0.22). To visualize all graphs for both the statistical analysis of life-history and the microbiome, the ggplot2 package was used (version: 3.5.2; Wickham et al., 2016).

Results

1.Life history

1.1 Survival

Both the effect of malathion exposure and the effect of heat exposure on the overall survival of the *Daphnia magna* clones over the 17 day test period was time-dependent and varied among the clones from the four tolerance types (Pesticide * Type * Day, Temperature * Type * Day, Table 2, Figure 2). In the following section, survival is discussed at three specific timepoints: immediately after the pesticide exposure (day 3), immediately after the combined stressor exposure period (day 7) and at the end of the experiment (day 17).

Table 2: Results of the linear mixed-effects model which tested for the effects of pesticide treatment (Pesticide), temperature treatment (Temperature) and tolerance type (Type) on the overall survival per day of the *Daphnia magna* clones. Significant p-values (p<0.05) are shown in bold.

	Overall survival		
	Df	Chisq	P-value
Pesticide	1	100.1	<0.001
Temperature	1	0.883	0.3474
Type	3	12.04	0.0072
Day	1	197.3	<0.001
Pesticide * Temperature	1	0.394	0.5302
Pesticide * Type	3	103.9	<0.001
Temperature * Type	3	0.928	0.8187
Pesticide * Day	1	31.83	<0.001
Temperature * Day	1	24.37	<0.001
Type * Day	3	32.75	<0.001
Pesticide * Temperature * Type	3	0.193	0.9787
Pesticide * Temperature * Day	1	0.249	0.6177
Pesticide * Type * Day	3	16.18	0.0010
Temperature * Type * Day	3	57.1	<0.001
Pesticide * Temperature * Type * Day	3	1.773	0.6208

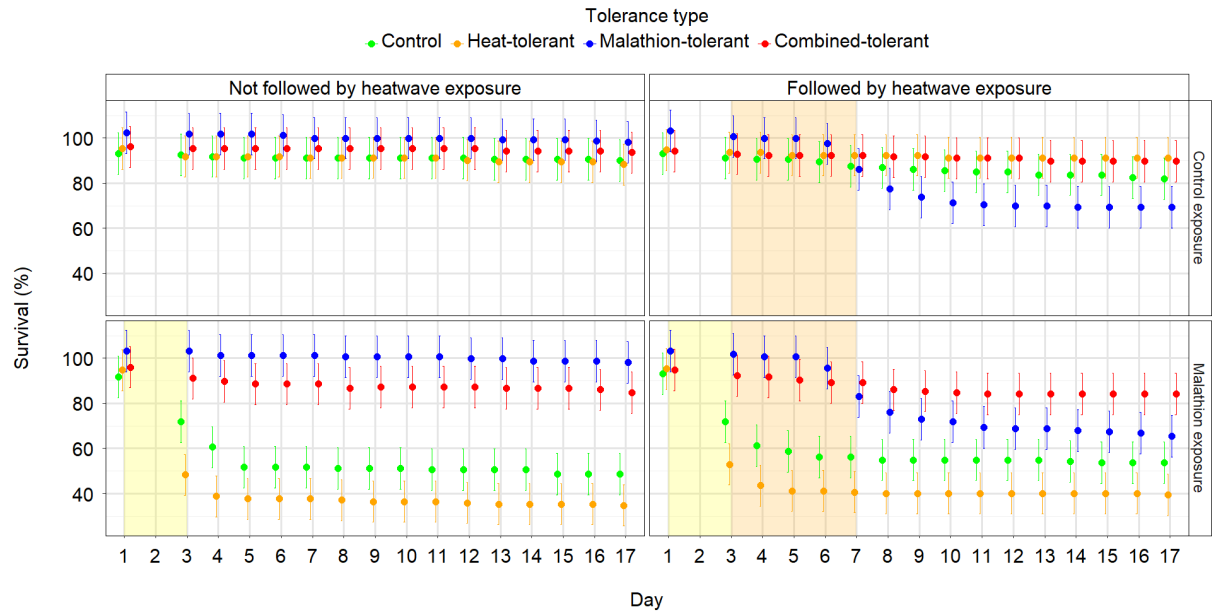


Figure 2: The effect of exposure to the pesticide malathion (two days) and a subsequent heatwave (four days) on the overall survival over the 17 day test period of *Daphnia magna* of the four different tolerance types. The yellow strokes indicates the 2-day malathion exposure and the orange strokes indicate the 4-day heatwave exposure. Mean survival percentages (± 1 SE) are given.

Table 3: Results of the linear mixed-effects model which tested for the effects of pesticide treatment (Pesticide), temperature treatment (Temperature) and tolerance type (Type) on the life history traits of the *Daphnia magna* clones: survival on day 7, survival on day 17, maturation time, reproduction time, reproductive performance. Significant p-values ($p < 0.05$) are shown in bold. P-values indicating trends ($0.05 < p < 0.10$) are indicated in italics.

		Survival on day 3		Survival on day 7		Survival on day 17		Maturation time		Reproductive performance	
		Df	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq	P-value	Chisq
Pesticide	1	63.925	<0.001	84.539	<0.001	72.209	<0.001	2.558	0.511	56.157	<0.001
Temperature	1	0.009	0.926	2.105	0.1468	7.253	0.007	0.433	0.052	117.018	<0.001
Type	3	12.614	0.006	25.778	<0.001	7.334	0.0620	3.774	0.465	13.015	0.005
Pesticide * Temperature	1	0.252	0.616	0.206	0.6501	0.395	0.5295	2.472	0.072	2.655	0.103
Pesticide * Type	3	76.506	< .001	71.528	<0.001	53.815	<0.001	11.095	0.480	45.298	0.006
Temperature * Type	3	0.833	0.842	7.825	0.0498	22.494	<0.001	3.238	0.011	27.938	<0.001
Pesticide * Temperature * Type	3	0.135	0.987	0.764	0.858	1.137	0.7681	5.115	0.164	9.550	0.023

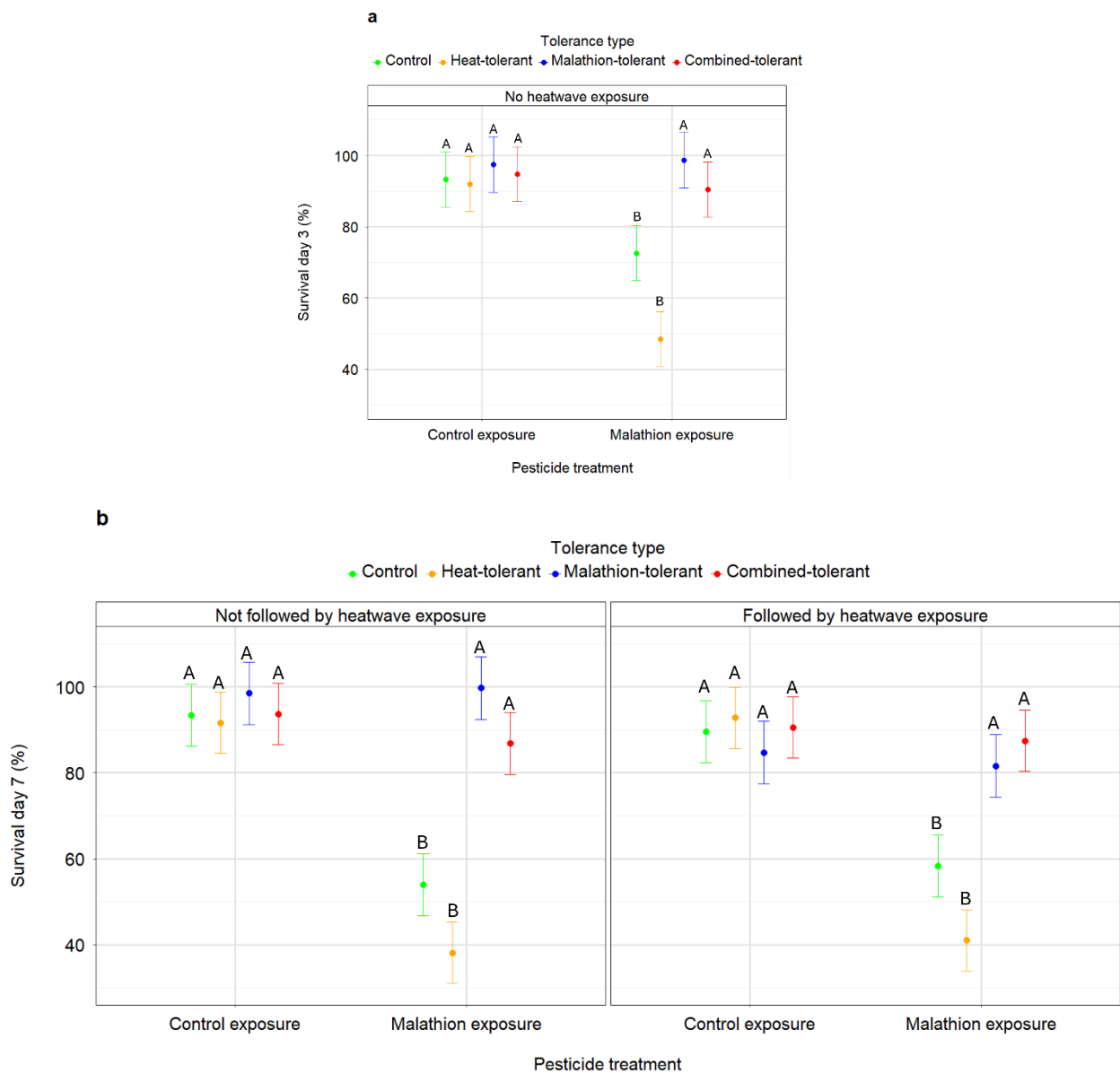
Table 4: Overview of the interaction types of malathion exposure and heatwave exposure as determined by the IA model for the four different tolerance types of *Daphnia magna* clones.

	Control	Heat-tolerant	Malathion- tolerant	Combined-tolerant
1) Survival day 7				
Interaction type	additive	additive	additive	additive
2) Survival day 17				
Interaction type	additive	additive	additive	additive
3) Maturation time				
Interaction type	antagonism	additive	additive	additive
MDR	1.24			
4) Reproductive performance				
Interaction type	additive	additive	additive	additive

The effect of malathion on survival on day 3 (after pesticide exposure), depended on the clone's tolerant type (Pesticide * Type, Table 3, Figure 3a). In the absence of malathion, no significant difference in survival of the four different tolerance types was observed (contrasts: all $p = 0.96$). After exposure to malathion, survival decreased of the control clones (-21.4%) and the heat-tolerant clones (-47.3%) (both $p < 0.002$) to the same extent ($p = 0.068$). However, this decrease tended to be stronger for the heat-tolerant clones as survival decreased twice as much than for the control clones. Malathion exposure did not affect the survival of the malathion- and combined-tolerant clones ($p = 0.95$) (Figure 3a).

The effect of both malathion and the heat wave on survival on day 7 (immediately after the stressor exposure period) and survival on day 17 (at the end of the experiment after the follow-up period) depended on the tolerance type of the clones (Pesticide * Type, Temperature * Type, Table 3, Figure 3b, Figure 3c). In the absence of both stressors, no significant difference in survival of the clones from the different tolerance types was observed for both day 7 (contrasts: all $p > 0.87$) and day 17 (contrasts: all $p > 0.85$) (Figure 3b, Figure 3c). Exposure to malathion (not followed by the heatwave) decreased survival of the control (-42.2% by day 7, -44.4% by day 17) and of the heat-tolerant clones (-58.4% by day 7, -59.4% by day 17) (all $p < 0.0001$) for both clone types to the same extent (both days: $p = 0.1713$ and $p = 0.3977$), while not affecting the survival of the malathion-tolerant and combined-tolerant clones (both $p > 0.77$) (Figure 3b, Figure 3c). Exposure to the heatwave (in the absence of malathion exposure) did not have an effect on survival by day 7 (all clones: $p > 0.16$), as all four clone types maintained high survival (Figure 3b). However by day 17, the heatwave did decrease survival but only of the malathion-tolerant clones (-29.8%, $p < 0.003$) indicating delayed negative effects of the heatwave (Figure 3c). When exposed to malathion followed by the heatwave, survival decreased of the control clones (-37.5% by day 7, -39% by day 17), heat-tolerant clones -55.2%

by day 7, -54.2% by day 17) and malathion-tolerant clones (-17.2% by day 7, -33.7% by day 17), while survival of the combined-tolerant clones remained high and thus did not differ from survival in the absence of both stressors (Figure 3). After combined stressor exposure, the combined-tolerant clones had the highest survival by day 17, followed by the malathion-tolerant and control clones that had an equally lower survival rate ($p = 0.83$) and the heat-tolerant clones that had the lowest survival rate (contrast combined-tolerant clones vs heat-tolerant clones: $p = 0.006$) (Figure 3c). For all four tolerance types, both after the combined exposure period and after the follow up period, both stressors interacted additively (Table 4).



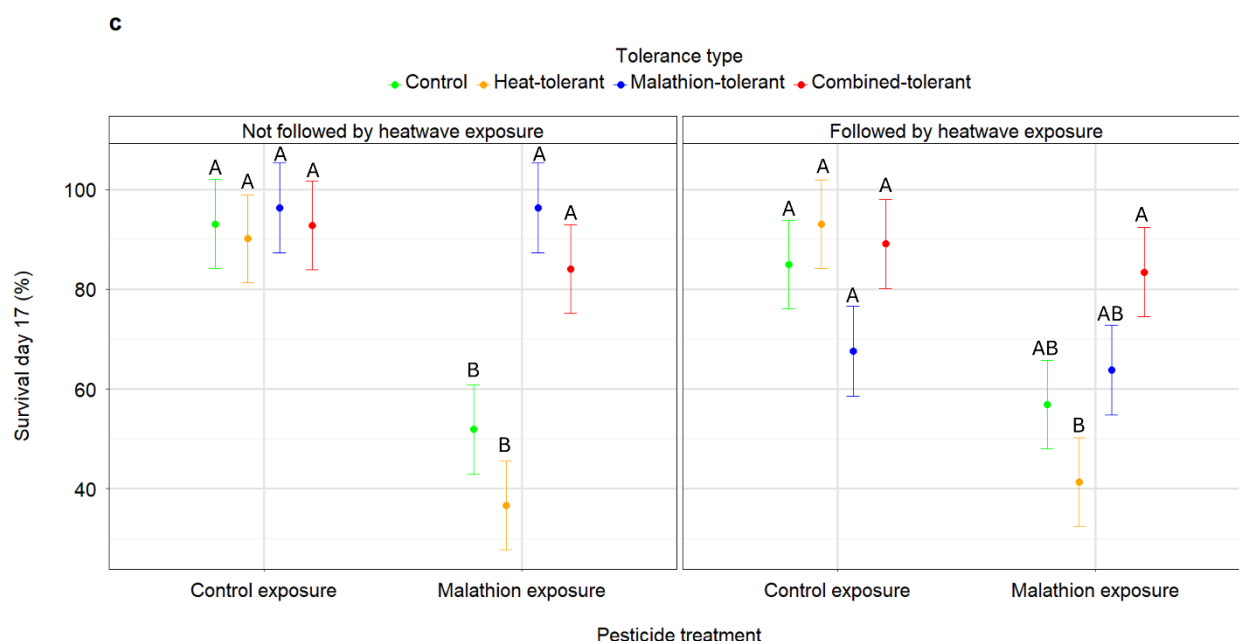


Figure 3: The effect of exposure to the pesticide malathion (two days) and a subsequent heatwave (four days) on survival of *Daphnia magna* of the four different tolerance types (a) on day 3, (b) on day 7 and (c) on day 17. Mean survival percentages (± 1 SE) are given. Different letter codes indicate significant differences or trends among the tolerance types within one of the four stressor combinations.

1.2 Maturation time

Of both stressors, only the heatwave exposure had a significant effect on maturation time and this effect depended on the tolerance type of the clones (Temperature * Type, Table 3, Figure 4). There also tended to be a pesticide and temperature interaction (Pesticide * Temperature, Table 3, Figure 4). When not exposed to any stressors, maturation time did not differ significantly between the different clone tolerance types (contrasts: all $p > 0.97$) (Figure 4). Exposure to malathion (not followed by the heatwave) did not affect maturation time (for all tolerance types: $p > 0.81$) and hence no differences in maturation time were observed after malathion exposure (all $p > 0.97$). After the heatwave exposure (in the absence of malathion exposure), maturation time of the control clones increased (+39.7%, delay of 2.8 days, $p < 0.0003$), while the maturation time of the other three clone types was not affected by the heatwave (all $p > 0.97$) (Figure 4). After the heatwave exposure, there tended to be a difference in maturation time between control and heat-tolerant clones and between control and combined-tolerant clones (both $p = 0.086$, Figure 4). When exposed to malathion followed by the heatwave, maturation time of all clones was not affected and all four tolerance types had the same maturation time (all $p > 0.97$) (Figure 4). For the heat-tolerant, malathion-tolerant and combined-tolerant clones, the two stressors interacted additively (Table 4). For the control clones however, an antagonistic interaction was identified between malathion and the heatwave (Table 4), indicating that the combined stressor exposure had a less big effect on maturation time than expected based on the sum of the effects of the single stressors. After combined

stressor exposure, malathion exposure seems to decrease maturation time in comparison to no malathion exposure at 32 °C, however this decrease was not significant ($p = 0.18$, Figure 4).

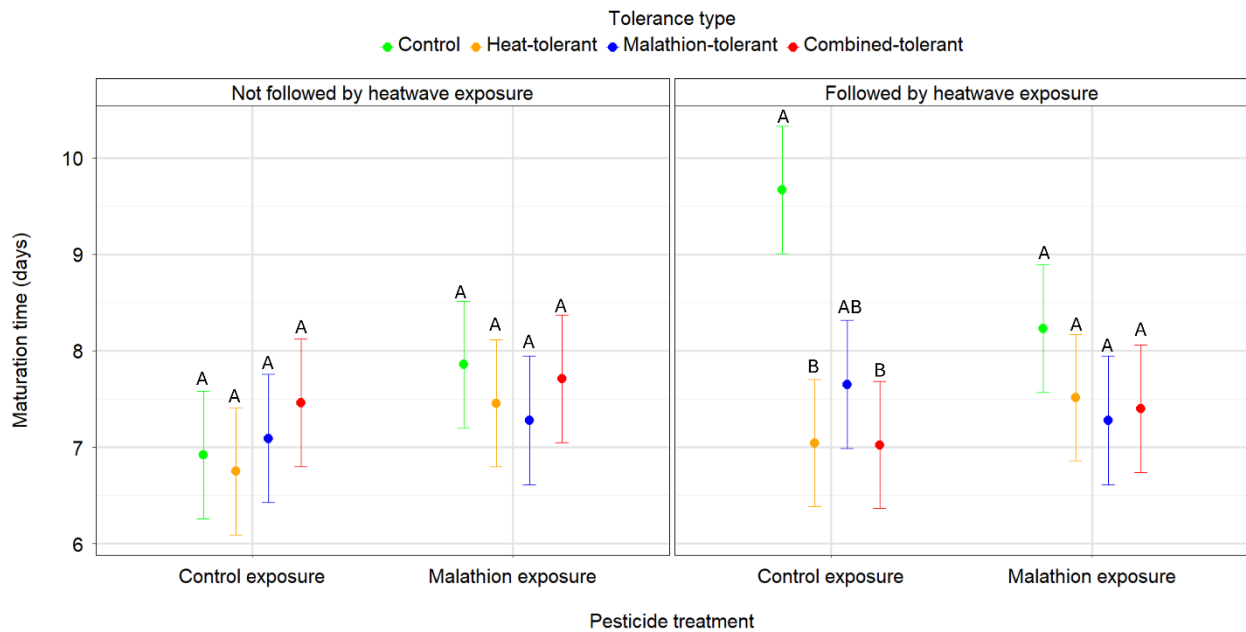


Figure 4: The effect of exposure to the pesticide malathion (two days) and a subsequent heatwave (four days) on maturation time of *Daphnia magna* of the four different tolerance types. Means (± 1 SE) are given. Different letter codes indicate significant differences or trends among the tolerance types within one of the four stressor combinations.

1.3 Reproductive performance

The effects of the malathion exposure and of the heatwave exposure on reproductive performance depended on each other and on the clone's tolerance type (Pesticide * Temperature * Type, Table 3, Figure 5, Figure B4). In the absence of both stressors, no difference in reproductive performance between the clones of the different tolerance types was detected (contrasts: all $p > 0.45$) (Figure 5, Figure B4). After exposure to malathion (not followed by the heatwave), the reproductive performance of the control clones (-40.0%) and the heat-tolerant clones (-49.8%) decreased (contrasts: both $p < 0.0001$) and this to the same extent ($p = 0.853$) (Figure 5, Figure B4). The reproductive performance of the malathion-tolerant and combined-tolerant clones was not affected by the pesticide exposure (both $p > 0.71$) and remained high (Figure 5, Figure B4). When exposed to the heatwave (in the absence of malathion exposure), the reproductive performance of all clones decreased significantly: control clones (-35.5%), heat-tolerant clones (-23.8%), malathion-tolerant clones (-43.5%) and combined-tolerant clones (-24.2%) (all $p < 0.0023$, Figure 5, Figure B4). The heat-tolerant clones tended to show a higher reproductive performance than the malathion-tolerant clones ($p = 0.064$) and control clones ($p = 0.086$) after the heatwave exposure, while combined-tolerant clones had an intermediate reproductive performance (Figure 5, Figure B4). Combined exposure to malathion and the heatwave decreased the reproductive performance of all clone types: control clones (-42.9%), heat-tolerant clones (-60.9%), malathion-tolerant (-55.7%) and combined-tolerant clones (-

25.9%) (Figure 5, Figure B4). After sequential exposure to both stressors, the reproductive performance of the combined-tolerant clones was significantly higher than the reproductive performance of both the heat-tolerant and malathion-tolerant clones (contrasts: both $p < 0.009$), and tended to be higher than the control clones ($p = 0.053$) (Figure 5, Figure B4). For all four tolerance types, both stressors interacted additively (Table 4).

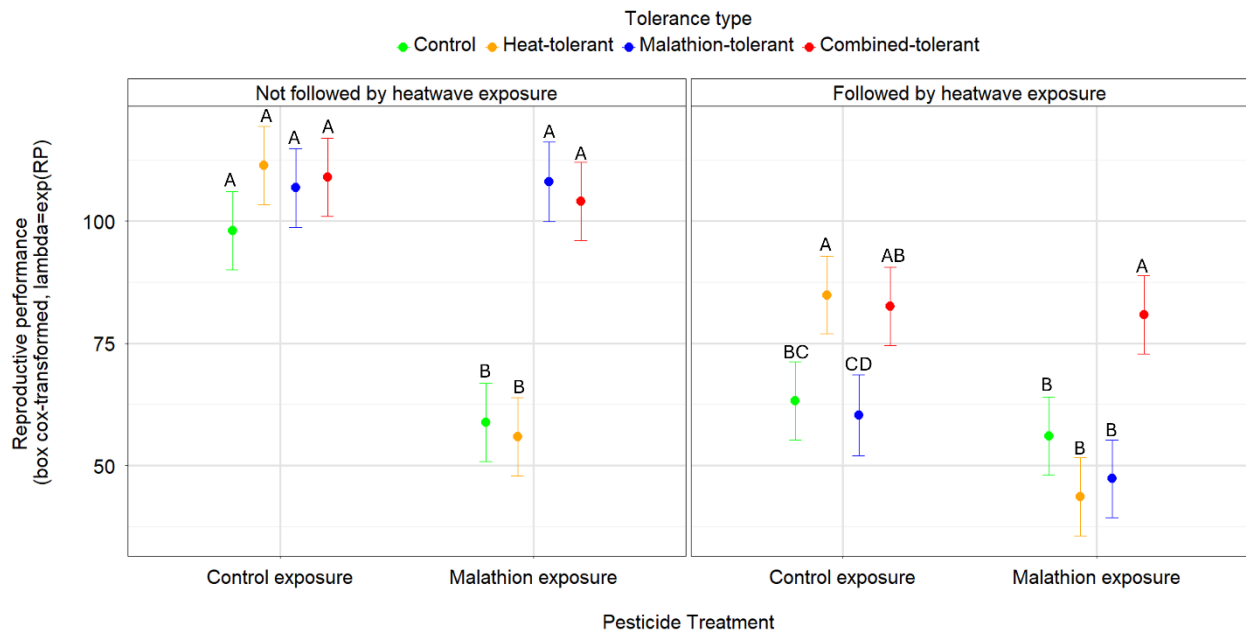


Figure 5: The effect of exposure to the pesticide malathion (two days) and a subsequent heatwave (four days) on reproductive performance of *Daphnia magna* of the four different tolerance types. Plotted are the results from the box cox transformed model. Means (± 1 SE) are given. Different letter codes indicate significant differences or trends among the tolerance types within one of the four stressor combinations.

2. Microbiome

2.1 Relative abundance

The comparison of the relative abundances is based on Figure 6, but was not statistically tested due to time constraints as we received the sequencing results only later on in the academic year.

When comparing the microbiome composition of *D. magna* clones per tolerance type when exposed to the four different stressor combination treatments, a visible effect of temperature treatment can be observed (Figure 6). For all four tolerance types, there seemed to be little to no effect of malathion exposure (not followed by the heatwave) on the relative abundance of most families in the microbiome, except for the control clones where malathion exposure (not followed by the heatwave) seemed to have a little effect on the relative abundance of Unidentified families (Figure 6).

When exposed to the heatwave, either preceded by malathion exposure or not, the relative abundance of three different families visibly decreased for all clone types (Figure 6). Based on Figure 6 and Table B3, after exposure to the heatwave, the relative abundance of T34, which is an unclassified family within the order of Burkholderiales, decreased substantially (between -93.7% and -99.1), starting from relative abundances between 1.12% and 11.55% (malathion-tolerant clones and heat-tolerant clones respectively). This decrease in relative abundance after the heatwave exposure was also observed for Flavobacteriaceae (-82.5% and -89.4%) starting from ranges between 2.17% and 5.75% (malathion-tolerant and heat-tolerant clones respectively, Table B3). For Spirosomaceae, the overall degree of decrease in relative abundance after heatwave exposure was larger for the malathion- and combined-tolerant clones (between -67.6% and -81.0%) than for the control and heat-tolerant clones, where the decrease was less large when also preceded by malathion exposure (in the absence of malathion exposure: -54.3% and -71.1% respectively; when preceded by malathion exposure: -15.5% and -21.2% respectively). The starting relative abundances of the Spirosomaceae ranged between 2.93% and 4.9% (combined-tolerant and malathion-tolerant clones respectively). Another family which also seemed to decreased after exposure to the heatwave, but not visibly, is the Crocinitomicaceae (starting from 1.57% and decreasing to 0.04%, Table B3).

There are also some families which seemed to increase in relative abundance after the heatwave (Table B3). For example, Microbacteriaceae, Sphingomonadaceae, Chitinophagaceae and Burkholderiaceae were not detected in the microbiome of *Daphnia* of all four tolerance types when exposed to 20 °C, for both control and malathion exposure, but were observed in the microbiomes of *Daphnia* exposed to the heatwave (in relative abundances up to 0.45%). Other families were also present when exposed to 20 °C but seemed to have higher relative abundances after exposure to the heatwave (either preceded by malathion exposure or not): Pseudomonaceae (all clones: increase with > 100%, starting from relative abundances between 0.02% and 0.04%), Rhodobacteriaceae (starting form 0.06% and ranging up to 1.75%) and Saprospiraceae (starting form 0.01% and ranging up to 0.93%). When looking at the rare families, for all four clone types, the relative abundance also increased after the heatwave (in relative abundance up to 0.75%). For all clone types the relative abundance of Alteromonadaceae increased after the heatwave exposure (in relative abundance up to 1.22%). However, after malathion exposure their relative abundance also increased but to a lesser extent (in relative abundance up to 0.04%). For the control, malathion- and combined-tolerant clones, the heatwave caused an increase in relative abundance of an unclassified family in the order of Candidatus Campbellbacteria (+ >100%, starting from ranges between 0% and 0.06% for the control and malathion-tolerant clones respectively), while for the heat-tolerant clones, only a very small increase was observed after heatwave exposure (+ 9%, starting from 0.11%) but a decrease when preceded by malathion exposure (from 0.11% to 0.06%).

For some families, the heatwave increased or decreased their relative abundance dependent on the clone's tolerance type (Table B3). For the Comamonadaceae, the relative abundance seemed to decrease after heatwave exposure for the malathion- and combined-tolerant clones (between -16.6% and -26.4%), while it increased for the control and heat-tolerant clones (between +14.4% and +36.0%). For the Cyclobacteriaceae, the relative abundance also decreased after heatwave exposure for the control, malathion- and combined-tolerant clones (between -6.58% and 61.0%), while it increased for the heat-tolerant clones (+36.2% and +147%). While for the control and heat-tolerant clones, the relative abundance of the Sphingobacteriaceae seemed to increase, while it decreased for the malathion- and combined-tolerant clones. Notably, the relative abundance of the unidentified families increased after the heatwave exposure (with or without prior malathion exposure), except for the control clones (Table B3).

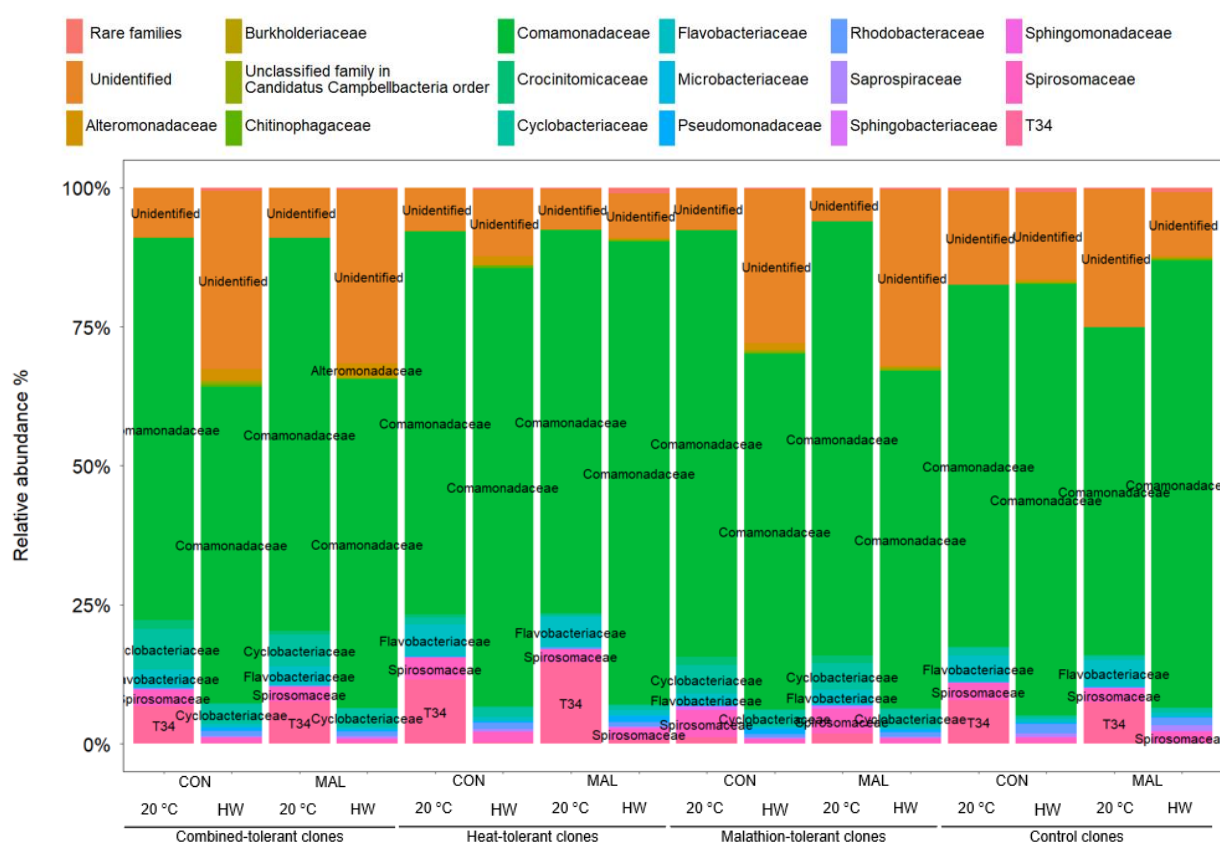


Figure 6: The effect of exposure to the pesticide malathion (CON = control, and MAL = malathion) and a subsequent heatwave (20 °C = no heatwave exposure, and HW = heatwave exposure) on the relative abundance of bacterial families in the microbiome of the different *Daphnia magna* tolerance types. Families that have an occurrence of <1% are included in “Rare families”.

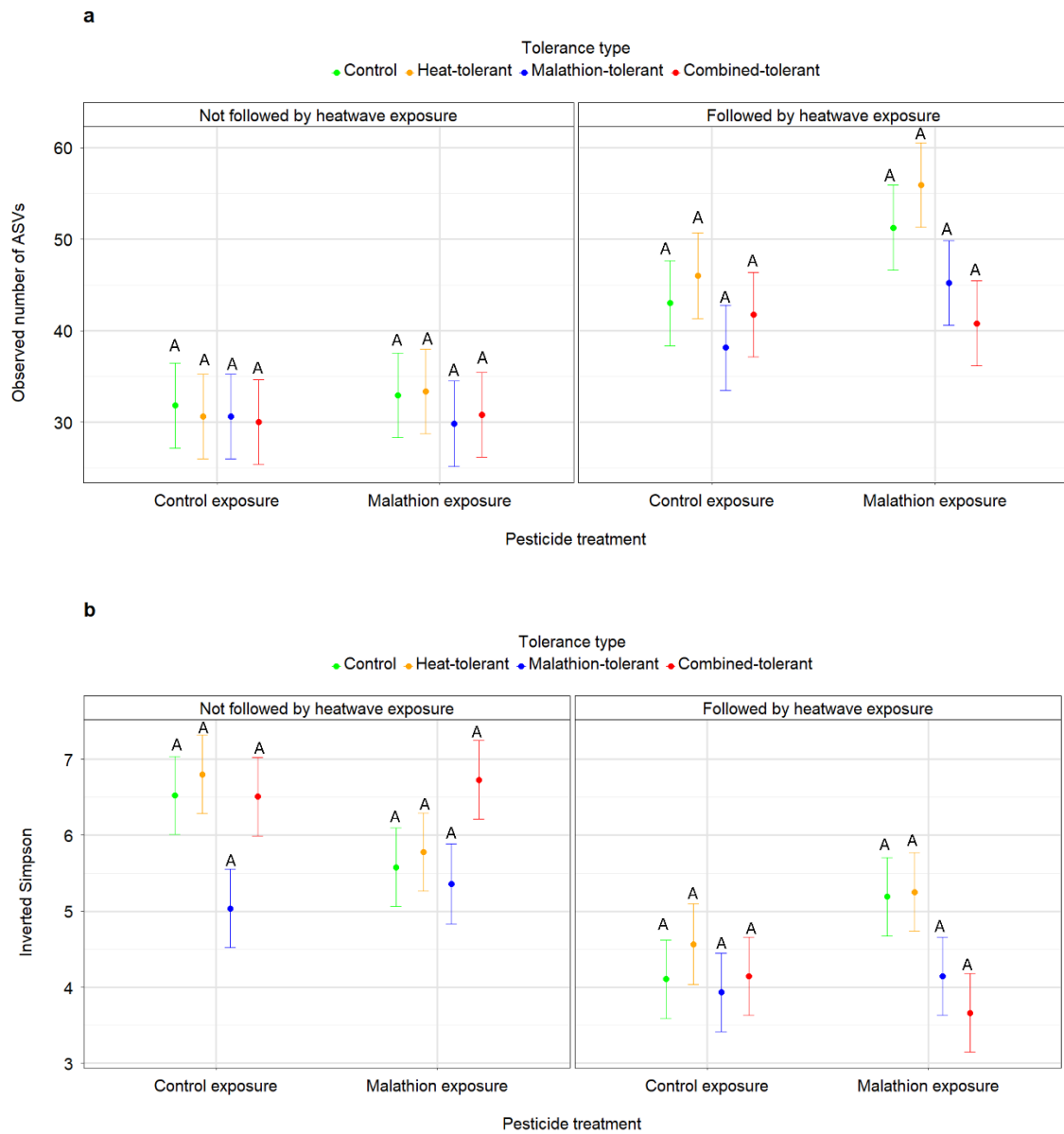
2.2 Bacterial α -diversity

Table 5: Results of the linear mixed-effects model which tested for the effects of pesticide treatment (Pesticide), temperature treatment (Temperature) and tolerance type (Type) on different α -diversity indices for the microbiome of the *Daphnia magna* clones: the observed number of ASVs, the inverse Simpson index and the Faith's phylogenetic diversity index. Significant p-values ($p < 0.05$) are shown in bold. P-values indicating trends ($0.05 < p < 0.10$) are indicated in italics.

	Df	Observed number of ASVs		Inverse Simpson		Faith's phylogenetic diversity	
		Chisq	P-value	Chisq	P-value	Chisq	P-value
Pesticide	1	6.990	0.008	0.002	0.965	8.874	0.003
Temperature	1	110.523	<0.001	63.246	<0.001	162.403	<0.001
Type	3	1.470	0.689	3.823	0.281	0.768	0.857
Pesticide * Temperature	1	3.657	<i>0.056</i>	3.061	<i>0.080</i>	1.931	0.165
Pesticide * Type	3	3.133	0.372	0.687	0.876	5.569	0.135
Temperature * Type	3	5.879	0.118	8.670	0.034	5.000	0.172
Pesticide * Temperature * Type	3	2.211	0.530	7.754	<i>0.051</i>	2.903	0.407

Overall, heatwave exposure rather than malathion exposure had the biggest effect on the α -diversity of the microbiome of the *Daphnia magna* clones (Figure 7). For all three α -diversity indices, there was no difference among the four clones types when not exposed to any stressors (Observed number of ASVs: all $p > 0.98$, Inverted Simpson: all $p > 0.178$ and Faith's: all $p > 0.97$). For the observed number of ASVs, the effect of the heatwave on species richness tended to depend on the preceding pesticide treatment (Pesticide * Temperature, Table 5, Figure 7a). While malathion had no effect on species richness by itself, the heatwave increased species richness and this effect was even stronger when preceded by malathion exposure (Figure 7a, Figure 8). For the inverted Simpson index, the effect of the heatwave on α -diversity tended to depend on both the preceding pesticide treatment and the clone tolerance type (Pesticide * Temperature * Type, Table 5, Figure 7b). Malathion exposure itself did not affect the inverse Simpson index for all clone types (Figure 7b). When not preceded by malathion exposure, the heatwave decreased the α -diversity of the control, heat-tolerant and combined-tolerant clones (all $p < 0.002$), while not affecting the α -diversity of the malathion-tolerant clones ($p = 0.22$) which was already the lowest under control conditions (Figure 7b). When preceded by malathion exposure, the heatwave only decreased the α -diversity of the combined-tolerant clones ($p < 0.001$) (Figure 7b). For Faith's phylogenetic diversity, next to the main effect of

temperature, also a main pesticide effect was observed (Table 5, Figure 7c and Figure 9). Both stressors increased the Faith's phylogenetic diversity (Figure 7c, Figure 9).



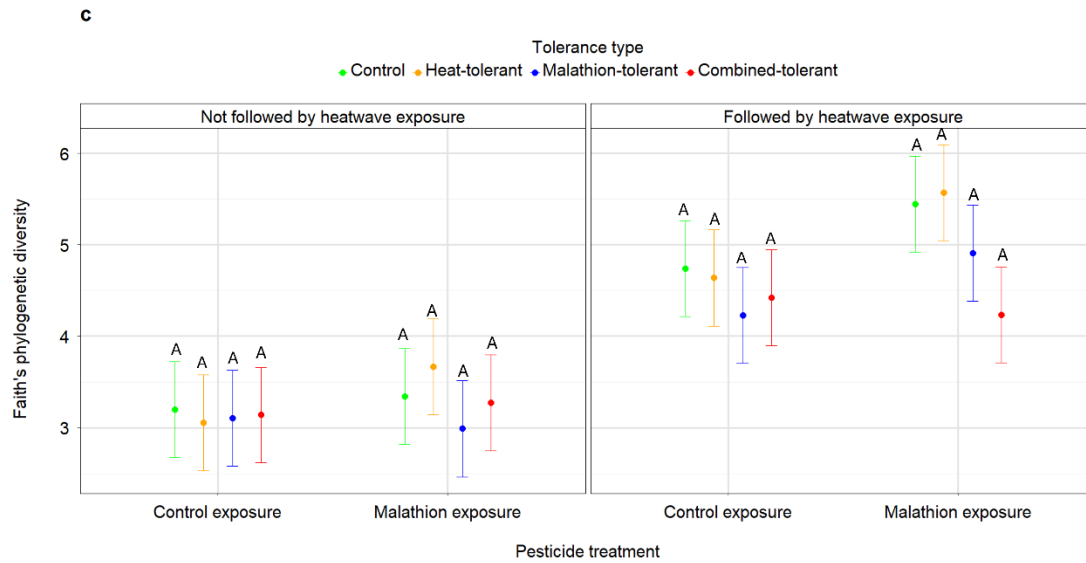


Figure 7: The effect of exposure to the pesticide malathion and a subsequent heat wave on the patterns in α -diversity of the microbiome of *Daphnia magna* of the four different tolerance types: (a) Observed number of ASVs, (b) Inverted Simpson index, and (c) Faith's phylogenetic diversity index. Means (± 1 SE) are given. Different letter codes indicate significant differences or trends among the tolerance types within one of the four stressor combinations.

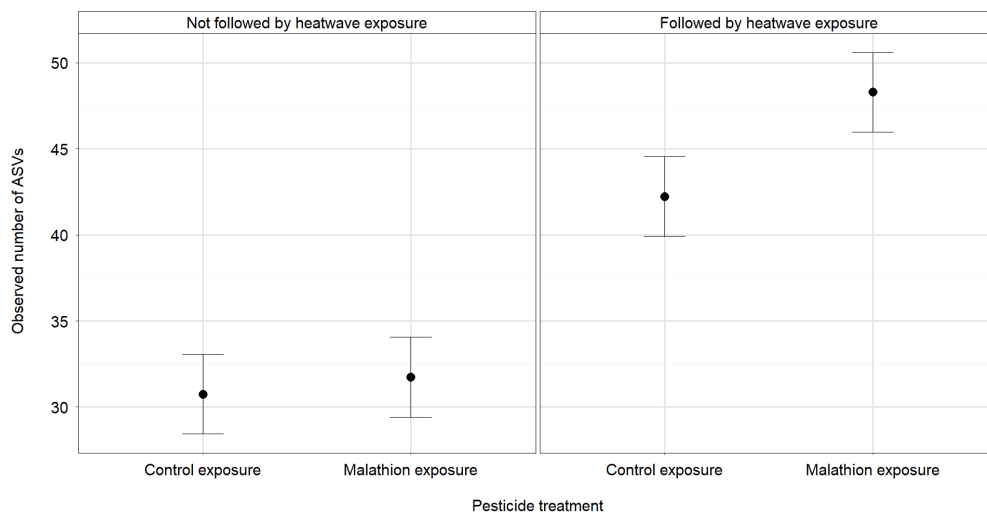


Figure 8: Two way interaction of the pesticide treatment (control exposure vs malathion exposure) and subsequent temperature treatment (no heatwave vs heatwave) on the Faith's phylogenetic diversity index. Means (± 1 SE) are given.

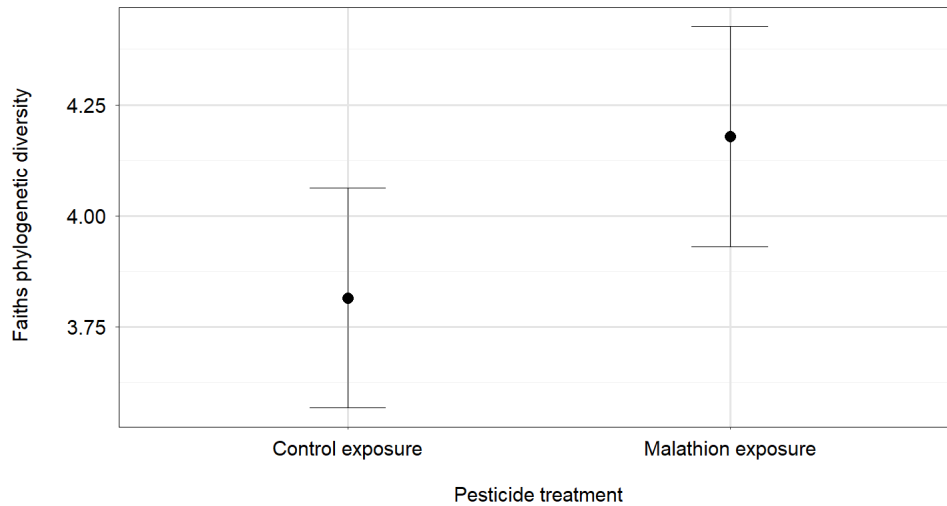


Figure 9: Main effect of the pesticide treatment (control exposure vs malathion exposure) on the Faith's phylogenetic diversity index. Means (± 1 SE) are given.

2.3 Bacterial β -diversity

Table 6: Results of PERMANOVA for the effects of pesticide treatment (Pesticide), temperature treatment (Temperature) and tolerance type (Type) on the different β -diversity of the microbiome of the *Daphnia magna* clones: Bray-Curtis dissimilarity. Significant p-values ($p < 0.05$) are shown in bold. P-values indicating trends ($0.05 < p < 0.10$) are indicated in italics. R^2 indicates the proportion of variance explained by each variable. F-values were calculated using numerator degrees of freedom (Df column) and denominator degrees of freedom from the residuals (Df = 222).

	Df	Bray-Curtis dissimilarity		
		R^2	F	P-value
Pesticide	1	0.0061	1.964	<i>0.064</i>
Temperature	1	0.1954	63.0094	<0.001
Type	3	0.0706	7.5844	<0.001
Pesticide * Temperature	1	0.0008	0.2584	0.976
Pesticide * Type	3	0.0059	0.6336	0.886
Temperature * Type	3	0.0301	3.2329	<0.001
Pesticide * Temperature * Type	3	0.0026	0.2834	0.999

Overall, the temperature treatment had the biggest effect on the β -diversity of the microbiome of the *Daphnia magna* clones, and the effect of the temperature treatment depended on the tolerance type of the clones for both the Bray-Curtis and unweighted UniFrac distance metrics (Temperature * Type, Table 6, Table B4, Figure 10, Figure B5). When not exposed to any

stressors, for the Bray-Curtis dissimilarity metric, the microbiome community composition of all clone types significantly differed from each other (all contrasts: $p < 0.043$), except for the malathion-tolerant and combined-tolerant clones that have a similar microbiome composition ($p = 0.273$) (Figure 10). For the Unweighted UniFrac distance metric, when not exposed to any stressors, only a significant difference in microbiome community composition between control and malathion-tolerant clones and between control and combined-tolerant clones was observed (both $p < 0.039$, Figure B5b).

After exposure to the heatwave, a shift in microbial composition occurred for all four clone types, but the shift is however slightly larger for the control and heat-tolerant clones in comparison to the malathion- and combined-tolerant clones (Figure 10, Figure B5). Indeed, for the Bray-Curtis dissimilarity metric, after the heatwave, all four clone types have a significantly different microbiome community compared to when exposed to 20 °C (all $p = 0.001$). After heatwave exposure, the microbiome of the control and heat-tolerant clones differed from each other without prior malathion exposure ($p = 0.004$), while it was similar with prior malathion exposure ($p = 0.429$). After combined exposure, the microbiome composition of the control and heat-tolerant clones thus differed from the microbiome of the malathion- and combined-tolerant clones that also have a similar microbiome (with prior malathion exposure: $p = 0.376$), meaning that the microbiome composition of those two sets of clone types shifted in the different directions in response to exposure to both malathion and the heatwave (Figure 10). For the Unweighted UniFrac distance, also a shift in microbiome composition occurred after the heatwave, however here, only the malathion- and combined-tolerant clones experienced a shift in the same direction ($p = 0.195$), while the other clones all differed significantly from each other (all: $p < 0.026$) (Figure B5).

For the unweighted UniFrac distance metric, the pesticide treatment had a significant effect, while for the Bray-Curtis metric, the pesticide treatment only tended to affect the β -diversity (main effect Pesticide, Table B4, Figure B5). However, the changes in microbiome community composition in response to malathion exposure are minor compared to when exposed to the heatwave (Figure B5). Although there is no significant interaction effect between the pesticide treatment and the clone's tolerant type (Table B4), Figure B5a and B5b suggest that for the heat-tolerant clones, exposure to malathion (in the absence of the heatwave) affected the microbiome composition more compared to the other clone types (contrast heat-tolerant clones: $p = 0.329$, contrast other clones: $p > 0.788$).

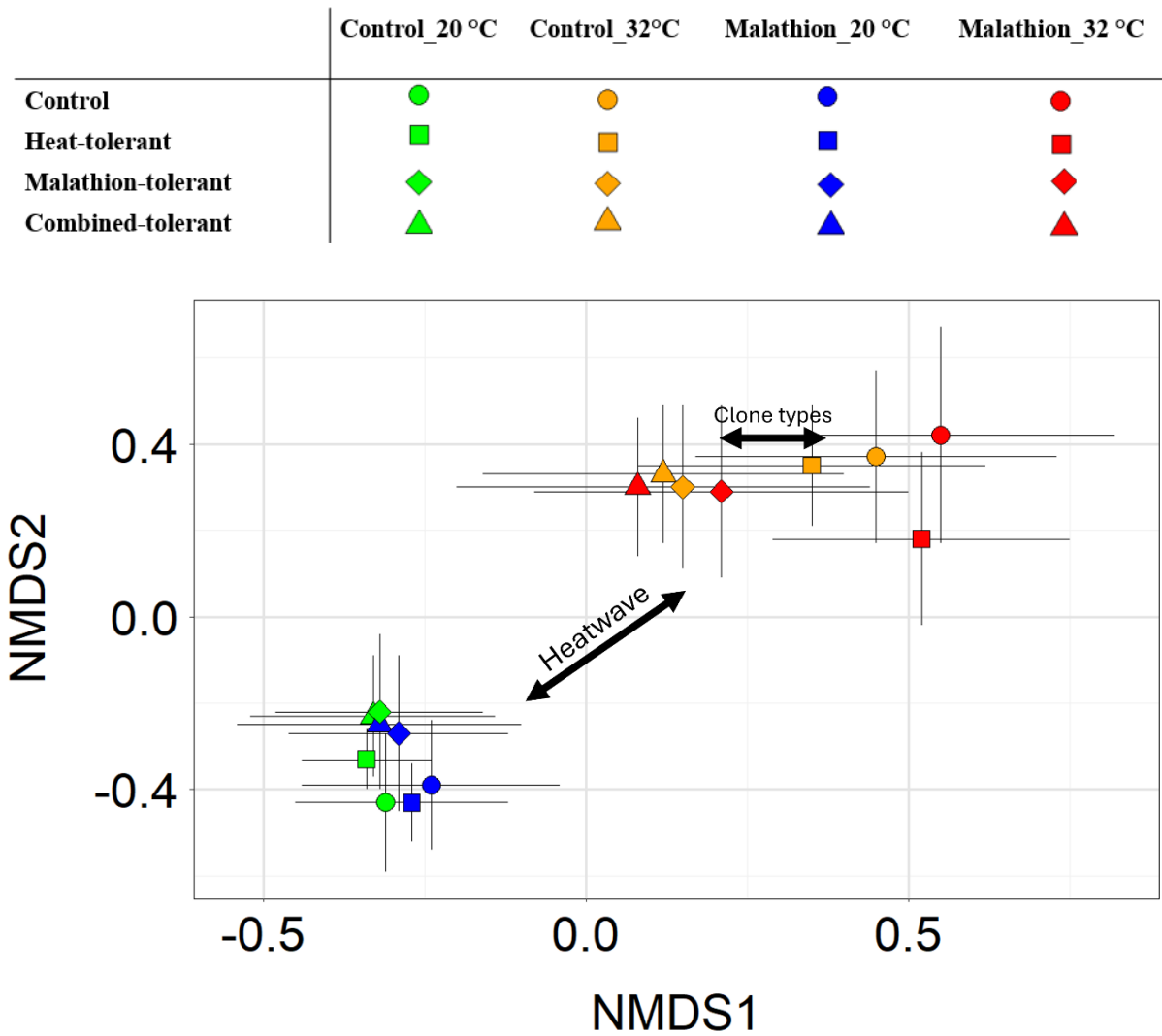


Figure 10: Patterns in β -diversity of the bacterial communities of *Daphnia magna* of the four different tolerance types exposed to the two pesticide treatments (control exposure vs malathion exposure) sequentially crossed with the two temperature treatments (not followed by heatwave exposure vs followed by heatwave exposure): the Bray-Curtis distance metrics. Means (± 1 SE) are given.

Discussion

The aims of this thesis were to investigate how exposure to a pesticide and/or warming affects the life history traits (survival rate, age at maturity and reproductive performance) of four *Daphnia magna* clones types (control, pesticide-tolerant, heat-tolerant, combined-tolerant) and how exposure to these stressors shapes their microbiome. In the first section of the discussion, the differences among the clones types when not exposed to any stressors are discussed. The second section focuses on differences among the clone types when exposed to only the pesticide malathion, while the third section zooms in on differences when only exposed to the heatwave. Finally, the last section is on the differences among the clone types when exposed to the combination of both stressors. For every stressor exposure scenario of pesticide and/or heatwave exposure, first the effects on the life history traits of the clones will be discussed and then the effects on microbiome community composition.

1.1 When not exposed to any stressors

1.1 Life history

When not exposed to any stressors, it was expected for the heat-tolerant, pesticide-tolerant and combined-tolerant to show a lower survival, faster maturation and lower reproductive performance than control clones due to expected costs of tolerance (Rivero et al., 2011). However, no significant differences in survival, maturation or reproductive performance were detected among the four clone types. This indicates that the malathion-, heat-, and combined-tolerant clones did not have any costs of tolerance in the stressor-free environment, for the studied traits. For these clones, maintaining tolerance to a stressor thus did not affect the energy budget to the extent of having a negative impact when not exposed to any stressors. This is in line with the findings of Van de Maele et al. (2025), who also found no costs of tolerance in chlorpyrifos-tolerant clones in a stressor-free environment. It is possible that there is thus truly no cost of tolerance or it could be that costs of tolerance only occurs after a longer period in the absence of stressors, as for example research on clothianidin-tolerant *Gammarus pulex* only found a significant cost of tolerance after three months of cultivation in a stressor-free environment (Siddique et al., 2020).

1.2 Microbiome

When not exposed to any stressors, it was expected for all four clone types to maintain their original adapted microbiome and thus to differ in microbiome composition. This hypothesis was partly confirmed as all clone types had a different microbiome community composition except for the malathion-tolerant and combined-tolerant clones that had a similar β -diversity. That microbiome composition differs between tolerance types is in line with previous research performed by Macke et al (2017). In their study, the microbiome composition of two susceptible genotypes (to toxic cyanobacteria) did differ from two tolerant genotypes when given a non-

toxic diet (Macke et al., 2017). This clear variation in β -diversity was explained by tolerance type as diet did not significantly impact the microbiome composition (Macke et al., 2017).

Malathion-tolerant, heat-tolerant and combined-tolerant clones had a different microbiome community composition than the control clones, suggesting that they have an adapted microbiome to the stressors they have been selected by and therefore are tolerant for. As the microbiome community composition of the heat-tolerant clones differed the most from the other three clones types, this could suggest that becoming heat-tolerant requires a more distinct set of adapted bacteria. As malathion- and combined-tolerant clones did not differ significantly in microbiome composition, and as the combined-tolerant clones did have a different microbiome composition than the heat-tolerant clones, this could suggest that the microbiome composition of the combined-tolerant clones is more driven by malathion-tolerant bacteria than by heat-tolerant bacteria in a stressor free environment.

Low abundant, non-core taxa are known to play an important role in pesticide degradation and conferring tolerance to pesticides (Suppa et al., 2020). One example is the Alteromonadaceae family, that is important in pesticide degradation as *Alteromonas* contains the enzyme organophosphorus acid anhydrolase (OPAA), which is shown to be effective in organophosphorus degradation (Singh & Walker, 2006). Alteromonadaceae had an overall low abundance and had the highest relative abundance in the microbiome of the malathion-tolerant clones, was still present in the combined-tolerant clones but with a lower relative abundance, and was almost not present in the heat-tolerant clones and not detected at all in the control clones. This may suggest that the microbiome of the malathion-tolerant and combined-tolerant clones was indeed adapted to malathion degradation.

Previous research has shown that several *Candidatus* species help in conferring heat tolerance to aphids (*Acyrtosiphon pisum*) (J. A. Russell & Moran, 2006). In line with this, when looking at the unclassified family in the order Candidatus Campbellbacteria, their abundance is the highest in the microbiome of the heat-tolerant clones, lower in the combined-tolerant clones, almost not present in the malathion-tolerant clones and not detected at all in the control clones, which may suggest that the microbiome of the heat-tolerant and combined-tolerant clones is adapted to heat tolerance.

The α -diversity, however, did not differ significantly among all four tolerance types. This is not in line with previous research that found a more diverse microbiome in chlorpyrifos-selected *Daphnia* than in control-selected *Daphnia* (Janssens et al., 2022) and research that also found a more diverse microbiome in low-latitude heat-adapted damselfly larvae than in high-latitude larvae (Theys et al., 2023a) .

2. When only exposed to malathion

2.1 Life history

As expected, malathion reduced the fitness of the control clones, as the pesticide reduced their survival and reduced reproductive performance by reducing clutch sizes (Appendix B, Figure B3). For the control clones, these reduced fitness values after malathion exposure can be explained by increased oxidative damage or the inhibiting effect organophosphates have on AChE, as AChE inhibition induces mortality and negatively affects reproduction and population growth rate (Duquesne, 2006). Our findings correspond with findings of Duquesne (2006), who also found that the organophosphate paraoxon-methyl negatively affected survival and reproduction in *Daphnia magna*, which was also linked to reductions in total offspring per surviving individual. However, in contrast to our prediction of a delayed maturation time after pesticide exposure, malathion had no effect on the maturation time of the control clones. This hypothesis was based on research by Reynaldi et al (2009), who found that fenvalerate exposure lead to delayed maturation in *Daphnia magna*. Yet, a delayed maturation after pesticide exposure is indeed not always observed as also Barry (1998) found that age at maturity of *Daphnia longicephala* was not affected by carbaryl exposure. The fact that maturation time was not affected while clutch size was, might be due to energy allocation (Glazier & Calow, 1992). According to Glazier and Calow (1992), the energy is first allocated towards maintenance and then towards either reproduction or somatic growth. For the here studied clones, it could thus be that energy was allocated towards growth and maturation instead of clutch size, resulting in decreased clutch sizes.

When only exposed to the pesticide malathion, it was expected for the heat-tolerant clones to show lower fitness values than the control clones due to an energetic trade-off between temperature tolerance and pesticide tolerance (Moe et al., 2013). However, the control clones and heat-tolerant clones had an equally low fitness for all three life-history traits. This means that, for the tested malathion concentration and for the here measured endpoints, there was no cost of heat tolerance when exposed to malathion. For the heat-tolerant clones, the negative effect of malathion exposure may also be partly explained by the inhibition of AChE. Any loss in energy budget of the heat-tolerant clones due to their heat tolerance was likely not that high to result in a cost of tolerance when exposed to malathion, as no difference in fitness values was found between the heat-tolerant and control clones. This is in contrast with what was found by Dong et al. (2024), who found that heat-tolerant *Daphnia* were more susceptible to zinc exposure than non-heat-tolerant *Daphnia* clones.

After malathion exposure, it was expected for the malathion-tolerant clones to thrive (high survival, fast maturation and high reproductive performance) and for the combined-tolerant clones to perform less well than the pesticide-tolerant clones but nevertheless better than the control clones, as both the malathion-tolerant and combined-tolerant clones would have

evolved adaptations to deal with malathion exposure. Indeed, this was the case for both survival and reproductive performance. The high reproductive performance found for both the malathion- and combined-tolerant clones was due to a higher total offspring number compared to the control clones (Appendix 2, Figure B3). The malathion- and combined-tolerant clones likely evolved adaptations to deal with malathion exposure like for example insensitive pesticide target-sites (for malathion: AChE that is insensitive to inhibition by malathion) and increased pesticide detoxification (e.g. CYP enzymes and glutathione transferases) (Stenersen, 2004). This corresponds to research that found no effect on survival of chlorpyrifos exposure on chlorpyrifos-tolerant *Daphnia*, but did find a decrease in survival for chlorpyrifos-sensitive *Daphnia* (Van de Maele et al., 2025). Notably, the combined-tolerant clones showed an equally high survival and reproductive performance as the malathion-tolerant clones. This indicates that the combined-tolerant clones do not carry any cost of heat tolerance under malathion exposure and that the energy they constitutively direct towards their heat tolerance might be low.

When comparing the effect of malathion on maturation time for the four tolerance types compared with their respective control treatments, no differences were found. This thus means that malathion did not negatively impact maturation time of the control and heat-tolerant clones, but it also did not have a positive effect on maturation time of the malathion- and combined-tolerant clones. For the heat-tolerant clones, this is in line with research that found no effect of zinc on maturation time of both control and heat-tolerant *Daphnia* (C. Zhang et al., 2018). For the malathion- and combined-tolerant clones, it looks like they did not invest energy into decreasing their maturation time but rather into increasing clutch size in response to malathion exposure (Appendix B, Figure B3). For the control and heat-tolerant clones, it might be more important to mature early instead of producing more offspring. Malathion is a stressor for those two tolerance types, and for these clones becoming mature early and producing some offspring before they could possibly die, may have been selected for instead of delaying maturation and producing larger clutch sizes at a later age. As malathion is no stressor for the malathion-tolerant and combined-tolerant clones, there was no need to accelerate maturation as they would survive the malathion exposure. For these clones it was thus likely better to invest energy in larger clutch sizes

2.2 Microbiome

When only exposed to malathion, a change in the microbiome composition was expected for the control and heat-tolerant clones towards more pesticide-degrading bacteria. As the microbiome of the malathion-tolerant clones was expected to be adapted to malathion, here no shift was expected after malathion exposure. For the combined-tolerant clones, who were expected to have a partly adapted microbiome, malathion exposure was expected to increase their abundance of pesticide-degrading bacteria. However, for all clone types, the α -diversity of their microbiome upon malathion exposure did not differ significantly from the α -diversity

in the absence of any stressors. Yet, there was a minor shift in microbiome community composition in response to malathion exposure for all clone types, and it appeared slightly that this shift in β -diversity was stronger for the heat-tolerant clones exposed to malathion than for the other three clone types exposed to malathion (yet this was not proven to be significant). The minor shift in microbiome community observed for the control clones exposed to malathion is in line with previous research that found a change in microbiome composition in *Daphnia* after exposure to roundup and glyphosate (Suppa et al., 2020). The shift in microbiome composition upon malathion exposure being so small might be due to the short exposure duration as after 48 hours of exposure, malathion had degraded to a concentration of 0.23 $\mu\text{g/L}$ and after 96 hours to 0 $\mu\text{g/L}$. Moreover, microbiome samples were only collected after 144 hours after the start of exposure, of which the last hours, there were no longer malathion residues present in the medium, and therefore, the microbiome might have largely “recovered” to its original composition.

For the heat-tolerant clones, malathion exposure seemed to have a bigger effect on its microbiome composition compared to the other clone types due to a possible cost of tolerance for the heat-tolerant bacteria: heat-tolerant bacteria being more sensitive to malathion. While there is no research yet performed on this topic, other research did find that *E. coli* bacteria resistant to heat, showed a decrease in virulence-expressing cells (Berdejo et al., 2024). Due to malathion exposure, a loss of heat-tolerant bacteria could have occurred together with a gain of some malathion-degrading bacteria, resulting in the same α -diversity with and without malathion exposure, but a difference in β -diversity. Indeed, for the microbiome of the heat-tolerant clones exposed to malathion, a decrease in relative abundance of the unclassified family in the order Candidatus Campbellbacteria, which have been associated with heat tolerance (J. A. Russell & Moran, 2006), occurred, as well as an increase in Alteromonadaceae, which have been linked to malathion degradation (Singh & Walker, 2006).

For the combined-tolerant clones exposed to malathion, also a decrease in relative abundance of the unclassified family in the order Candidatus Campbellbacteria as well as an increase in relative abundance of Alteromonadaceae were observed. This suggests that after malathion exposure, they also lost heat-tolerant bacteria (e.g. Candidatus Campbellbacteria) but gained pesticide-degrading bacteria (e.g. Alteromonadaceae).

While research has shown that Alteromonadaceae aid in pesticide degradation, the relative abundance of this family decreased in the microbiome of malathion-tolerant clones after malathion exposure. This is contradictory to what was expected. In fact, for the malathion-tolerant clones, only the Comamonadaceae and T34 families increased in relative abundance after malathion exposure. According to Singh et al. (2006), several Comamonadaceae species can degrade organophosphates and use certain compounds as a carbon source. While there is

no research yet on the role of T34 bacteria in pesticide degradation in *Daphnia*, other species belonging to families within the Burkholderiales have shown to be involved in pesticide degradation. As the Comamonadaceae are also belonging to the Burkholderiales and aid in pesticide degradation, this could mean that the T34 family also exhibits pesticide degrading properties. The Burkholderiaceae however also belong to the Burkholderiales, but showcase heat-tolerant properties. The reduction in relative abundance of the low-abundant Alteromonadaceae was thus possibly counterbalanced by the increased relative abundance of other pesticide-degrading families in response to malathion exposure.

3. When only exposed to heat stress

3.1 Life history

As expected for the control clones, the heatwave exposure increased maturation time and decreased reproductive performance, caused by both delayed reproduction (Appendix B, Figure B2) and smaller clutch sizes (Appendix B, Figure B3). This matches a previous study showing that *Daphnia magna* reduced clutch size and increased age at first reproduction after exposure to 30 °C (Giebelhausen & Lampert, 2001). However, contradictory to what was expected and to other research (e.g. Van de Maele et al., 2025), the heatwave did not affect survival of the control clones in this study. The high survival of control clones exposed to this level of temperature stress might be explained by a plastic defense response. Indeed, research has shown that temperature stress of 33 °C and 30 °C induces higher haemoglobin levels and increased transcription of certain HSP genes (Chen et al., 2021; Cuenca Cambronero et al., 2018b) that can help *Daphnia* to survive under temperature stress.

After the heatwave exposure, it was expected for the heat-tolerant clones to show high fitness values, possibly due to obtained heat tolerance mechanisms like increased haemoglobin content and increased thermal plasticity (Cuenca Cambronero et al., 2018a). Indeed, the heat-tolerant clones showed high survival after the heatwave. No negative effect was observed on the maturation time of the heat-tolerant clones after the heatwave, which indicated heat tolerance. However, the heatwave also did not positively affect maturation time of these clones as maturation time was similar to when not exposed to any stressors. Furthermore, the heatwave did have a slightly negative effect on the reproductive performance of the heat-tolerant clones (mainly caused by a decrease in total offspring, Appendix B, Figure B3) but their reproductive performance was still higher than the reproductive performance of the other clone types after heat exposure, still indicating a certain level of heat tolerance of the heat-tolerant clones. This is in line with other research where heat-tolerant larvae of the damselfly *Ischnura pumilio* showed higher growth rates and survival than *Ischnura elegans*, however after exposure to a heat spike the heat-tolerant *I. pumilio* showed lower values than when they were not exposed to the heat spike (Theys et al., 2023b).

The malathion-tolerant clones were expected to show lower fitness values when exposed to the heatwave compared to the control clones. This was however only the case for survival, indicating a lethal cost of tolerance of the malathion-tolerant clones when exposed to the heatwave. This can be explained by the energetic costs that come with maintenance of insecticide tolerance (Rivero et al., 2011). While the malathion-tolerant clones did not show a cost of tolerance in a stressor-free environment, the energy needed for their tolerance to malathion can no longer be used to deal with the increasing temperatures, hence resulting in a cost of tolerance under heat stress. This cost of tolerance was not detected for the other life history traits, as the heatwave negatively affected reproductive performance of both the malathion-tolerant and control clones to the same extent, and as heatwave exposure did not affect maturation time of the malathion-tolerant clones. This might be explained by that malathion-tolerant clones invested their remaining energy in maturation instead of clutch size and survival after maturation (Glazier & Calow, 1992).

After exposure to only the heatwave, it was expected for combined-tolerant clones to show lower fitness values than the heat-tolerant clones but nevertheless higher values than the control clones. However, for all life-history traits, combined-tolerant clones showed similar fitness values as the heat-tolerant clones. After heatwave exposure, just like the heat-tolerant clones, combined-tolerant clones showed a high survival, unaffected maturation time and a relatively high reproductive performance (but however not as high as in the absence of any stressors). The low energy budget needed for tolerance maintenance might explain why combined-tolerant clones still show high fitness values. While the energy needed for malathion tolerance maintenance might be high, the low energy needed for heat tolerance might still lead to combined-tolerant clones having enough energy left to show high fitness values.

3.2 Microbiome

When only exposed to the heatwave, it was expected for the heat-tolerant clones to keep their adapted microbiome composition, and for the composition of control, pesticide-tolerant and combined-tolerant clones to shift towards a composition with more heat-tolerant bacteria. However, the heatwave induced a major shift in microbiome composition for all four clone types. For the observed number of ASVs and the Faith's phylogenetic diversity, α -diversity increased for all four clone types when exposed to the heatwave. For the Inverted Simpson index, which mainly takes into account the common species and is less affected by changes in rare species, α -diversity decreased after the heatwave and this for all four clone types except for the malathion-tolerant clones. This indicates that after exposure to the heatwave, the richness and evenness of common species decreased (as shown by the Inverted Simpson index), while rare species appeared (as seen in the increase in observed number of ASVs).

The shift in microbiome composition observed for the control clones was expected and in line with previous research that showed a shift in microbiome composition of high-latitude *I. elegans* larvae towards that of heat-adapted low-latitude larvae after heatwave exposure (Theys et al., 2023a). For the heat-tolerant clones, the shift in both α - and β -diversity after heatwave exposure was not expected as an already adapted microbiome was expected. It could thus be that maintaining the heat-tolerant bacteria in a stressor-free environment was too costly and that the heat-tolerant clones hence lost some heat-tolerant bacteria. It might however also be that the heat-tolerant bacteria just disappear when their optimal temperatures are not reached. The shift in microbiome composition observed in the malathion-tolerant clones when exposed to the heatwave is also in line with previous research which showed a decrease in pesticide-degrading bacteria when *Nilaparvata lugens* were pre-exposed to 38° C (Y. Zhang et al., 2021).

After exposure to the heatwave, the relative abundance of the unclassified family in the order Candidatus Campbellbacteria increased for all three clone types except for the heat-tolerant clones. This further indicates the role Candidatus Campbellbacteria plays in conferring heat tolerance to its host and also confirms the hypothesis that the other three clone types would gain more bacteria which are heat-tolerant upon heat exposure. Notably, for the Alteromonadaceae, there was an increase in relative abundance in the microbiome of all clone types after heatwave exposure (in the absence of malathion exposure), although also known to degrade malathion (Singh & Walker, 2006). In *Daphnia*, there is no evidence yet of Alteromonadaceae aiding in heat tolerance, but in the coral, *Acropora cervicornis*, their relative abundance has been shown to increase in response to heat stress (Palacio-Castro et al., 2022). The heat stress could cause conditions to become more favourable for the growth of Alteromonadaceae, leading to an increase in their abundance. Other bacterial families like Burkholderiaceae and Chitinophagaceae were not detected when not exposed to the heatwave but were detected in the microbiome after exposure to the heatwave, and this for all clone types, thus indicating a possible role in heat tolerance. The role in heat tolerance has already been suggested for Burkholderiaceae by Theys et al. (2023b) which showed an increase in their relative abundance after heatwave exposure of *I. elegans* and for Chitinophagaceae by Wang et al. (2019) which showed the presence of a bacterial strain of this family in a hot spring, thus likely indicating its adaptation to high temperatures.

After exposure to the heatwave, for all clone types, the relative abundance of T34 decreased drastically. It could be that this family is heat-sensitive, which would explain its general decrease after exposure to the heatwave. Also the relative abundance of Flavobacteriaceae, which might play a role in food digestion (Qi et al., 2009) and in pesticide degradation (Janssens et al., 2022) decreased after the heatwave and this for all clone types.

Notably, for malathion- and combined-tolerant clones, a decrease in the relative abundance of Comamonadaceae was observed, while an increase was observed for the control and heat-tolerant clones. As several Comamonadaceae species can degrade organophosphates (Singh et al., 2006), there may be a trade-off between their malathion degradation capacities and their heat tolerance abilities, resulting in the malathion- and combined-tolerant clones experiencing a decrease in relative abundance of this family when exposed to the heatwave. As these clones already contain other pesticide-degrading bacteria but no or some heat-tolerant bacteria, the loss of relative abundance could possibly facilitate gain of more heat-tolerant bacteria. This increase in relative abundance for the control and heat-tolerant clones is however not in line with previous research that found a decrease in Comamonadaceae in damselfly larvae after exposure to a heatwave (Theys et al., 2023b).

4. When exposed to both malathion and heat stress

4.1 Life history

As expected, sequential exposure to both stressors decreased survival and reproductive performance of the control clones and more than when exposed to the single stressors. Here, the driving factor for decreased reproductive performance was a reduction in clutch size (Appendix B, Figure B3). This is in line with previous research showing chlorpyrifos-sensitive (control) *Daphnia* having a decreased survival after pesticide exposure followed by a heat spike (Van de Maele et al., 2025). Based on the TICS concept (Hooper et al., 2013), a synergistic interaction between malathion and the heatwave was expected for the control clones. However, for survival and reproductive performance, additive interactions between both stressors were observed, which was also found by Van de Maele et al. (2025). For both survival and reproductive performance, mainly the effect of malathion exposure and not heatwave exposure contributed to the total effect. In contrast, for maturation time, an antagonistic interaction was observed between both stressors, meaning that the combined effect observed when exposed to both stressors was less big than expected based on the sum of the single effects of the stressors. Indeed, the delaying effect of the heatwave (under single stressor exposure) on maturation time of the control clones disappeared when first exposed to malathion exposure. This resulted in the control clones experiencing no effect on their maturation time when sequentially exposed to both stressors. The observed antagonistic effect might be explained by survival selection (Delnat et al., 2022b). Malathion exposure could lead to the weakest individuals being removed, resulting in the stronger individuals remaining and maybe being more robust against the second stressor (Delnat et al., 2022b). The antagonistic interaction found for the control clones was also observed for reproduction time and total offspring (Appendix B, Table B2). While for reproduction time, the heatwave had a negative effect, it was nullified when first exposed to malathion. When first exposed to malathion subsequently followed by the heatwave, no delay

in reproduction time was observed anymore. For total offspring, the exposure of malathion before the heatwave did not totally nullify but did decrease the negative effect of the heatwave.

For the heat-tolerant clones, it was expected that they would show higher fitness values than the control clones under combined stressor exposure, as they have heat tolerance mechanisms to deal with the heat stress. However, in contrast to the hypothesis, the fitness values of heat-tolerant clones did not differ significantly from control clones: they had an equally low survival, equal maturation time and equally low reproductive performance. This is in line with research that found no difference in heat tolerance (measured by CT_{max}) between heat-adapted low-latitude and high-latitude *I. elegans* larvae when exposed to both chlorpyrifos and an increased temperature of 24 °C (Op de Beeck et al., 2017). For all life-history traits, additive interactions were observed between malathion exposure and the heatwave. Malathion exposure had the biggest impact on the heat-tolerant clones. Since malathion exposure was the first stressor the *Daphnia* were exposed to, the heat-tolerant individuals likely experienced negative effects of malathion due to a trade-off between heat tolerance and malathion tolerance. A lot of energy went towards repair and detoxification of malathion. The surviving *Daphnia* were still tolerant to the subsequent heatwave, but the life-history traits of the heat-tolerant clones were already negatively affected by the preceding malathion exposure.

After exposure to both stressors, it was expected for the malathion-tolerant clones to show similar fitness values as the control clones. Indeed, all three life-history traits did not differ significantly between control and malathion-tolerant clones. For the malathion-tolerant clones, only additive stressor interactions were observed after sequential exposure to both stressors. Here, for both survival and reproductive performance, especially the heatwave contributed to the observed results of combined stressor exposure, as initial exposure to malathion did not affect the malathion-tolerant clones. As the fitness values of the malathion-tolerant clones were equally low as the ones of the control clones under combined exposure, suggests there was no cost of pesticide tolerance when sequentially exposed to both malathion and the heatwave. This is contradictory to what was expected as previous research had shown pesticide and heat stress interacted synergistically for chlorpyrifos-adapted *Daphnia* and that their pesticide tolerance was overruled by exposure to multiple stressors (Van de Maele et al., 2025).

As expected, the combined-tolerant clones showed the highest fitness values for all the life-history traits after sequential exposure to both malathion and the heatwave due to their evolved heat tolerance as well as pesticide tolerance. Their survival and maturation time was unaffected by the sequential stressor exposure. For reproductive performance, there was an additive stressor interaction effect resulting in a slight decrease in reproductive performance. Out of both stressors, the heatwave mainly contributed to the observed interaction effect as exposure to the heatwave alone had a bigger impact than exposure to malathion alone. Although sequential

exposure to both stressors decreased the reproductive performance of the combined-tolerant clones, they are still proven to be tolerant to both stressors because of the higher fitness values of the combined-tolerant clones than the other three clone types, but this indicates that their tolerance also has its limits.

4.2 Microbiome

When exposed to both stressors, it was expected for control clones to gain both heat-tolerant and pesticide-degrading bacteria and thus experience a shift in microbiome composition. Indeed, the microbiome composition of the control clones changed significantly. For both the observed number of ASVs and Faith's phylogenetic diversity, α -diversity of the control clones increased, while for the Inverted Simpson index, α -diversity decreased after combined stressor exposure. This means that for the control clones, after exposure to malathion, followed by the heatwave, the evenness and richness of common species decreased (as shown by the Inverted Simpson index), while the occurrence and abundance of rare species increased (as shown by the increase in observed number of ASVs). Overall, it was mainly the heatwave that shifted the community composition of the microbiome of the control clones. Both the relative abundance of Alteromonadaceae (linked to pesticide degradation by Singh & Walker, 2006) and Candidatus Campbellbacteria (linked to heat tolerance by J. A. Russell & Moran, 2006) increased, but T34 decreased.

For the heat-tolerant clones, it was expected they would obtain pesticide-degrading bacteria in addition to their heat-tolerant bacteria. Indeed, their species richness increased after combined stressor exposure and there was a shift in their community composition, but the observed effect in change was mainly due to the heatwave, and not to malathion exposure. For the heat-tolerant clones, the relative abundance of the unclassified family in the order Candidatus Campbellbacteria decreased after combined stressor exposure, although linked to heat tolerance (J. A. Russell & Moran, 2006). In contrast, the relative abundance of this family increased for the malathion-tolerant clones, supporting the idea of a shift in the microbiome community of malathion-tolerant clones to a more heat-tolerant community). For all four clone types, the relative abundance of Alteromonadaceae (linked to pesticide degradation by Singh & Walker, 2006) increased after combined stressor exposure, but not as much as when only exposed to heat stress. This could indicate the cost of tolerance for bacteria and a trade-off between which bacteria are acquired. A consideration has to be made whether to gain bacterial taxa tolerant to one stressor but then lose bacterial taxa tolerant to another stressor.

For the malathion-tolerant clones, a gain in heat-tolerant bacteria was expected, and as mentioned before, this was indeed the case. With the gain of heat-tolerant bacteria, also the loss of some pesticide-degrading bacteria was expected, which for the malathion-tolerant clones was the family of the Comamonadaceae. For the combined-tolerant clones, no changes in

microbiome composition were expected under combined stress exposure. However, also a change in microbiome composition was observed when comparing to the composition in the absence of both stressors, and this change was mainly driven by the heatwave with also a decrease in the relative abundance of Comamonadaceae. As well as for the heat-tolerant clones exposed to the heatwave, either microbiome maintenance in the no stressor environment could be too costly or a trade-off in tolerance within the bacteria itself, leading to a loss of some tolerant bacteria. However, after combined exposure, for the control and heat-tolerant clones the relative abundance of the Comamonadaceae (linked to pesticide degradation by Singh & Walker, 2006) increased. This increase for the control and heat-tolerant clones could aid in the degradation of malathion.

Conclusions and future perspectives

This thesis aimed to investigate how exposure to a pesticide and/or warming affected the life history traits and microbiome community composition of four *Daphnia magna* tolerance types (control clones, malathion-tolerant clones, heat-tolerant clones and combined-tolerant clones). This thesis confirmed some already known effects of both stressors, but also brought new insights into the effects of both stressors on *Daphnia* with different tolerance levels.

For all studied life history traits (survival, time to maturation and reproductive performance), it was confirmed that the malathion-tolerant clones were indeed tolerant to malathion, that the heat-tolerant clones were indeed tolerant to the heatwave, and that the combined-tolerant clones were indeed tolerant to the combination of both stressors. All clones had a distinct microbiome community composition, except for the malathion-tolerant and combined-tolerant clones which had a similar microbiome composition, suggesting that the composition of the microbiome of the combined-tolerant clones was more driven by malathion-degrading bacteria than by heat-tolerant bacteria. Overall, this suggests that the microbiome of the different clone types was adapted to the stressors the clones have been selected by and are tolerant for. In the absence of both stressors, no costs of tolerance were observed for all three tolerance clone types. It is thus not disadvantageous to be tolerant to a stressor (combination) when in an environment without any stressors, at least not for the here studied time frame and life history traits.

While no cost of tolerance was observed for the heat-tolerant clones when exposed to malathion, a lethal cost of tolerance was observed for the malathion-tolerant clones when exposed to the heatwave, which is in line with the cost-of-tolerance concept of Moe et al. (2013). For the combined-tolerant clones, no costs of tolerance were observed when exposed to one of the two stressors. After malathion exposure, the composition of the microbiome of all four tolerance types slightly shifted, and this shift seemed to be stronger for the heat-tolerant clones. The latter hints at a possible tolerance trade-off at the level of the microbiome, although this was not conferred to the level of the host as no trade-offs were observed for the life-history

traits. When exposed to the heatwave, there was a major impact on the microbiome composition of all clone types. For the malathion-tolerant clones, this heatwave-induced disturbance of the microbiome might also confer to the observed cost of tolerance at the host level. Notably, when exposed to the heatwave, the community composition of the microbiome of malathion-tolerant and combined-tolerant clones shifted in a slightly different direction than that of the heat-tolerant and control clones.

When sequentially exposed to malathion and the heatwave, no support for the TICS concept was found. For all clone types, both stressors interacted additively for survival and reproductive performance. For time to maturation, there was even an antagonistic interaction between both stressors for the control clones, where prior malathion exposure nullified the delaying effect of the heatwave, and this antagonistic interaction shifted to an additive interaction when the clone was tolerant to malathion and/or heat. Under combined stressor exposure, combined-tolerant clones had the highest fitness, followed by the malathion-tolerant and heat-tolerant clones which had an equally low fitness as the control clones. This shows that in a multi-stressor world, being adapted and being tolerant to one stressor is not enough, and that organisms need to acquire tolerance to combinations of stressors. When sequentially exposed to malathion and the heatwave, the microbiome for all four clones shifted, mainly driven by the heatwave. For all three tolerance clone types, there were trade-offs between heat- and malathion-tolerant bacteria at the microbiome level, however these were not conferred to the host level.

Although this research is a step in the direction of more accurate risk assessments in a multi-stressor world where organisms also evolve tolerance to stressors, some logical steps can be taken for further research. Malathion is not the only widely used pesticide, so other studies could research the effect of other and new pesticides. As heatwaves and pesticides are not the only two anthropogenic stressors with which *Daphnia* are confronted, the implementation of a third stressor, like microplastics and metals, could be made. Next to these anthropogenic stressors, organisms also have to deal with already present natural stressors like food limitation and predation. Much research has already been performed on one of these stressors in combination with either heat stress or pesticide exposure, but not on the combination of three or more stressors. Increasing the levels of stress can however be challenging as more factors need to be taken into account and therefore result in big experimental designs.

The obtained pesticide concentration over the different time points can also be used to inspect degradation rates, which can be used in further research, to provide the use of even more realistic concentrations.

For the microbiome, especially exposure to the heatwave, and not malathion induced shifts in the microbiome composition of the clones. Possible explanations could be the short exposure time to malathion, as after 48 h, the malathion concentration had already decreased

substantially, and the fact that the microbiome samples were only taken after 144 h when no malathion residues were left in the medium. As it is not clear by this experiment whether the microbiome of the different clones had the time to ‘recover’ after malathion exposure and before microbiome collection, or whether the malathion exposure was just too short or too low to induce a shift in microbiome community composition, further research can elucidate this. For example, microbiome samples can be collected directly after the end of the malathion exposure period. As there is still few research on combined-tolerant clones and their microbiome, further research on this can also be performed to better understand the mechanisms, trade-offs and costs.

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