Physiology of thermal phenotypic plasticity
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Evolutionary and ecological physiology aims to understand the physiological mechanisms of adaptation in organisms interacting with environmental conditions, by incorporating evolutionary processes. An important objective is to describe and understand how organisms cope with changing environments, and the fitness effects of variation in these mechanisms. The focus of this thesis was the underlying physiology and fitness consequences of thermal phenotypic plasticity in springtail. The aim was to get a better understanding of (1) the basic thermal fatty acid response, (2) the body lipids in which this thermal response occurred, and (3) the differences in physiological plasticity between species and the effects of variation on the thermal sensitivity.

In this study we found that the thermal response of the membrane fatty acids was not as straightforward as predicted by the HVA theory (Q1): the thermal fatty acid response was variable between experiments and highly variable compared to the prediction by the HVA theory. Notwithstanding this high variability, the thermal fatty acids response showed high physiological plasticity. Biological membranes are however immensely complex and simple conclusions can therefore not easily be drawn from analysis of only (some) membrane components. Storage lipids have fewer avenues available to homeoviscously adept to thermal change, which resulted in less variability in response to temperature than compared to membrane lipids. Consequently, the thermal fatty acid response of storage lipids did respond according to the predictions by the HVA theory (Q2), more so than membrane lipids did. The
main observed deviations in storage lipids agreed with those we found in membrane lipids. There are many potential causes of high variability in the thermal lipids response for both membrane and storage lipids that hamper straightforward HVA and simple conclusions. Membrane and storage lipid restructuring was highly plastic and is probably essential for changing the physical properties of biological membranes and storage lipids.

Surface dwelling Collembolan species differed in their physiology plasticity of the thermal fatty acid response from soil dwelling Collembolan species. Although correlative, some soil dwelling species showed lower extreme temperature tolerance in relation with lower physiological plasticity compared to surface dwelling species (Q3). None of our observations are direct proof of homeoviscous adaptation, but collectively they point towards adaptive changes in the membrane and storage lipids that may putatively help to increase their resistance to the changing thermal environment these animals are exposed to. Unfortunately, for some species extinction will be lurking more than for others. For the future, we suggest more research on the effects of the environment/context on HVA. We would like to stress the use of multiple HVA mechanisms and/or overall membrane and storage fluidity by e.g. Fourier transform infrared spectroscopy (Oldenhof et al., 2010), when possible in combination with molecular ecology (Angiletta et al., 2006). Although future studies should ideally include direct assessments of the physical properties of body lipids, the results from the present study do report the major changes during thermal acclimation of Collembolan fatty acids and therefore serve as a starting point for such future investigations.

To avoid detrimental thermal effects, ectotherms commonly compensate for changes in membrane lipid fluidity by homeoviscous adaptation (HVA), which involves changing the structure and composition of membrane lipids (Sinensky, 1974; Hazel and Landrey, 1988b). Although not the only change involved in HVA, a major effect of changes in ambient temperature is that lipids (fatty acids) become more unsaturated at low temperature to compensate solidification and become more saturated at high temperatures to compensate liquification (Sinensky, 1974; Hazel and Williams, 1990; Hazel, 1995; Hochachka and Somero, 2002). Empirical evidence in ectotherms has shown that the unsaturation index (UI: average number of double bonds per fatty acid) or the unsaturation to saturation ratio (U/S ratio) increases in response to cold (Cossins et al., 1977; Bly et al., 1986; Hazel and Landrey, 1988b; Suutari and Laakso, 1993; Bennett et al., 1997; Ohtsu et al., 1993; Kostal and Simek, 1998; Logue et al., 2000; Bayley et al., 2001; Overgaard et al., 2005, 2008; Michaud and Denlinger, 2006; Brodte et al., 2008; Upchurch, 2008). In contrast to the expectations, we found mostly variable responses for the overall unsaturation (UI or U/S ratio) of membrane fatty acids of the springtail Orchesella cincta in relation to temperature (Chapter, 2-5, and Table 1). Although the thermal response was highly variable and not as straightforward as
predicted from the HVA theory, overall, our springtails did show very high thermal plasticity of their membrane fatty acid profiles, resulting in highly plastic thermal phenotypes.

Although there is no causal evidence yet as to why storage lipids should homeoviscously adapt to temperature, it has been suggested that the need for HVA is caused by lipid-protein interaction, where lipids become worse substrates for lipases at low temperatures (Kostal and Simek, 1998). Maintenance of lipid fluidity is thus expected to be adaptive in storage lipids as well. Several environmental conditions are known to affect the storage fatty acid composition, e.g. drought (Holmstrup et al., 2002) and diet (Canavoso et al., 1998; Haubert et al., 2004; Chamberlain et al., 2005), but the temperature response of storage lipids has received relatively little consideration (Haubert et al., 2008). The few studies that have considered the thermal response of storage lipids have shown that under cold conditions ectotherms indeed have increased unsaturation of their storage lipid fatty acids (Kostal and Simek, 1998; Brodte et al., 2008; Haubert et al., 2008). In our experiments we found similar deviations from the HVA prediction in membrane and storage lipid responses to temperature (Chapter, 2-5, and Table 2). The distribution of the storage fatty acid response compared matched more to the prediction by the HVA theory, than it would based on a random distribution (35 matching responses, 11 opposite responses, 20 no responses; \( \chi^2 = 13.36, P=0.001 \)).

Overall, both membrane and storage lipids showed much variation in the thermal phenotypic plasticity, when observing their fatty acids profiles. What can be the causes of this magnitude of variation in the thermal fatty acid response? Although the U/S ratio and the UI are commonly used as a correlate of the lipid viscosity (Hazel and Williams, 1990; Hochachka and Somero, 2002), this study shows that investigating fatty acid composition to get a full understanding of HVA is not fully sufficient. Besides other thermal adaptation mechanisms (c.f. Murray et al., 2007), there are also other physiological avenues of membrane modifications that can assist in HVA, such as phospholipid head group modifications, changes in the amount of sterols incorporated in the membrane bilayer, or the placement of the fatty acids on the sn1 or sn2 position of the glycerol backbone (Hochachka and Somero, 2002). Cold acclimation, for example, frequently leads to an increase in phosphatidylethanolamine (PE) and a decrease in phosphatidylcholine (PC) (Hazel and Carpenter, 1985; Hazel and Landrey, 1988a). One supporting observation from our study of potential interactions between the unsaturation of the fatty acids and additional/alternative HVA mechanisms, is storage fatty acids had relatively less mismatches with the prediction by the HVA theory compared to membrane fatty acids. Storage lipids have fewer avenues available to show homeoviscous adaptation: they lack the ability to influence membrane fluidity by changing the headgroup composition. The larger variation in membrane lipid responses can thus be caused by more alternative HVA mechanisms. We investigated none of these other mechanisms and thus for now, our ideas remain hypothetical. Hence, the
inconsistency between the different physiological responses does highlight the importance of measuring mechanical membrane properties to determine actual membrane fluidity.

The most notable response contradicting the HVA theory prediction is that of the C18 PUFAs. A possible explanation for the accumulation of C18 PUFAs at higher temperature is that omega 3 and omega 6 fatty acids can only be acquired through dietary intake and are therefore essential in most insects (De Renobales et al., 1987). The increase of C18:2n6 and C18:3n3 at high temperature may therefore possibly reflect a compromise between HVA and the preservation of essential fatty acids for future use (Chapter 4). We designed and performed an experiment (Box 1) to investigate whether food affects the thermal fatty acid response (unpublished data). We fed Orchesella cincta and Folsomia candida both algae and yeast, where yeast lacked one of the presumed essential fatty acids (C18:3n3). The fatty acid profile of F. candida fed yeast showed C18:2n6, C20:4n6, and C20:5n3, although no C18:3n3 (presumably all produced C18:3n3 is further converted to C20:5n3), suggesting that they potentially have a Δ15 front-end desaturases due to the lack of C18:3n3 in yeast. O. cincta, however, we were not even able to rear them on yeast, but they did grow and reproduced on algae, suggesting that at least C18:3n3 truly is an essential fatty acid for this springtail species. For future study it would thus be interesting to find out whether O. cincta truly lacks Δ15 front-end desaturases, while F. candida does not. In Chapter 7 we did not pick up gene fragments of Δ15 desaturases in O. cincta, but as these desaturases are mostly familiar from plants or bacteria, we do not know yet what they would look like in insects.

There are additional diet-related potential causes of the high variability in the thermal response. First, diet in general has been observed to affect the fatty acid profiles of species (Canavoso et al., 1998; Haubert et al., 2004; Chamberlain et al., 2005). Moreover, oocytes of ewes (yes, no ectotherms this time) fed with PUFA-diets compared to control diets, showed higher membrane PUFA concentrations and higher freeze-resistance (Zeron et al., 2002). From our own study, we did two experiments that showed a possible cause for variation. In our Box (1), we show that a different diet resulted in more unsaturated profiles and higher thermal plasticity for F. candida fed yeast compared to algae. In Chapter 6, the different species were starved to prevent diet to affect the thermal response. The lack of food affected the thermal fatty acid response. We found that with the increase of temperature the C20 PUFAS increased, possibly at the expense of their essential C18 PUFA precursors, which increased with temperature in all other studies including food. As we did not measure actual fluidity of the lipids or any consequences for lipid performance, these ideas remain potential causes for now.

Another potential cause of the high variability in the thermal response is that fatty acid composition shows extreme sensitivity to the experimental setups. Acclimation duration, magnitude of temperature change, and even the direction of temperature change, affected the thermal fatty acid response, although some fatty acids showed very straightforward linear
thermal responses (C_{16:0} and C_{20:5\alpha3} in storage lipids). Also, repeated adaptations might be costly and therefore hampering HVA under fluctuating thermal regimes (Williams and Somero, 1996, Bennett et al., 1997; Pernet et al., 2007; Chapter 5). In Chapter 5, we showed that the fatty acid response was not repeatedly adapted to a two-day fluctuating thermal regime, but adapted to the warm temperature, although Williams and Somero (1996) and Pernet et al. (2007) showed acclimation to the average temperature in response to tidal and daily thermal changes, respectively.

Age of the ectotherms also seemed to affect the thermal response. In Chapter 3, we showed a much larger variation in the thermal lipids response for juvenile springtail than for adults, and additionally the fatty acids were more saturated. Age-specific differences in lipid composition are known for several species, although the similarity in the specific changes is low among species (Bychek and Gushchina, 1999; Cakmak et al., 2007; Haubert et al., 2008). Additional functions of fatty acids, such as precursors in biosynthesis of waxes, pheromones, defensive secretions (Stanley-Samuelson et al., 1988), or reproductive eicosanoids (Stanley, 2006), may constrain insects in adjusting fatty acid composition to temperature, or may cause changes in fatty acid composition over the lifetime, due to distinct requirements of different life stages.

The membrane lipid composition and its subsequent fluidity supports the functioning membrane-linked enzymes (Sandermann and Strominger, 1972; Sinensky, 1974; Heller and Hofer, 1975; McMurphie and Raison, 1979; Owicki and McConnell, 1979; Else and Wu, 1999; Wu et al., 2004). Both the functioning of enzymes and membranes are much affected by temperature (Hochachka and Somero, 2002) and the viscosity of the semi-fluid membrane lipid mosaic must be maintained to allow for proper function of transmembrane proteins while simultaneously maintaining proton motive force (Sinensky 1974; Hazel, 1995; van de Vossenberg et al. 1999; Hall et al., 2010). Membrane enzymes are mostly aggregated within membrane domains and the fatty acid composition of these domains is adapted to support optimal enzyme conformation (Somero, 2004). Hypothetically, the capability of the fatty acid composition to adapt can on the one hand help to broaden the thermal range in with a enzyme can function. On the other hand, the required fatty acid composition in the domains can potentially disturb the membrane integrity of the membrane matrix in response to temperature change.

Lastly, we did not talk about the humidity of the environment. Holmstrup et al. (2002) found that drought-acclimation increased the resistance to cold and acute drought but reduced the resistance to heat shock. The composition of membrane fatty acids changed during drought-acclimation resulting in a higher degree of unsaturation by the end of the acclimation period, which resembles typical membrane alterations seen in ectothermic animals exposed to cold. Barhndorff et al. (2007) exposed an artic springtail to both cold (-10 and -20°C) and desiccation at +1°C, which caused significant changes in the membrane fatty acid
composition with some similarities. These changes included a decrease in average chain length of the fatty acids due primarily to an increase in the phospholipid fatty acids C_{16:0} and a decrease in C_{18:3n3} and C_{20:4n6}. Although drought can potentially interact with the thermal fatty acid, we believe that more often both responses will go hand-in-hand. Several authors have even proposed that many of the adaptations in ectothermic animals promoting cold tolerance were in fact originally developed to tolerate desiccation (several authors cited in Holmstrup et al., 2002).

In contrast, what did not cause the variation in the thermal phenotypic plasticity was the method we used. Subtle differences in fatty acid composition are thus challenging to detect, because especially the long-chained poly-unsaturated fatty acids (C_{20} PUFAs) are particularly unstable and susceptible to peroxidation by initiators such as oxygen in combination with heat, free radicals, light, and/or metal ions (Christie, 1989; Ohman, 1996; Laguerre et al., 2007; Kaniuga, 2008). We started our study by investigating the effect of different sample preparation methods for GC analysis on the preservation of unstable PUFAs in (any) biomaterial (Chapter 2). We used the sample preparation method with the highest PUFA yield and the lowest variation in the experiments designed to answer our research questions. The high constituency of the thermal response in the C_{20} PUFA C_{20:5n3} over the different experiments (Table 1 and 2) shows that the variation in the thermal fatty acid response was not due to the method by with the fatty acids were measured in this study.

To investigate differences in physiological plasticity between species and subsequent thermal tolerance, we compared the physiological plasticity of below ground species to above ground species and investigated the effect of reduced plasticity on fitness? A strong gradient in thermal variability is found across soil profiles and Collembola exhibit a strong vertical stratification across these soil profiles (Berg et al. 1998; Krab et al. 2010). Soil dwelling species habituate relatively stable thermal environments (Willmer 1982) compared to surface dwelling or near surface dwelling species, which can experience an increased range of thermal fluctuations with much more rapid shifts between extremes. For a species to perform well over a broad range of temperatures, it either needs genotypes that each specialize on part of the temperature range, or it needs genotypes with high physiological capabilities (Huey and Kingsolver, 1989; Huey and Kingsolver, 1993; Stevens, 1989; Deere and Chown, 2006; Frazier et al., 2006; Terblanche et al., 2007; Chown and Terblanche, 2007). We found that surface dwelling Collembola indeed had much higher plasticity of fatty acids in response to temperature than soil dwelling species (Chapter 6). Moreover, surface dwelling Collembola had higher tolerance to extreme temperatures than at least one soil dwelling species. However, we did not measure how well this species performs, in terms of growth or reproduction, within its thermal niche. Future experiments addressing this issue could answer the question if soil dwelling species, although living in a thermally more stable environment, are at much
more risk to go extinct when under climatic change. We expect that a relatively small thermal change in the soil would have more severe fitness consequences for soil dwelling species due to their low thermal phenotypic plasticity and subsequent small thermal niche as for surface dwelling species with their high physiological capabilities.

**BOX 1: unpublished experiment**

Set up: 2 species (Folsomia candida and Orchesella cincta) x 2 temperatures (5°C and 20°C) x 2 diets (yeast and algae). Hypothesis: diet affects the thermal fatty acids response differently in different species. In our lab, Folsomia candida cultures are usually fed yeast, while Orchesella cincta is fed with algae. Fatty acid profile of algae contained, among other fatty acids, C\(_{18:2n6}\), C\(_{18:3n3}\), C\(_{20:4n6}\), and C\(_{20:5n3}\), while yeast lacked C\(_{18:3n3}\) and C\(_{20:5n3}\). We collected eggs and reared the animals from hatching on the appointed diet until adult (4 weeks), after which the animals were transferred to the opposite temperature for 2 weeks and subsequently measured for lipid composition.

*F. candida* performed well on both diets: they grew up from egg, reproduced (although more juveniles were counted for the yeast diet adults), resisted cold shock (no effect of diet), and they resisted heat shock (algae diet resulted in longer heat resistance). The fatty acid profile of *F. candida* showed, among other fatty acids, C\(_{18:2n6}\), C\(_{20:4n6}\), and C\(_{20:5n3}\), but not C\(_{18:3n3}\), suggesting they are able to convert C\(_{18:2n6}\) into C\(_{20:5n3}\) using \(\Delta 15, \Delta 5, \Delta 6\) desaturase. In membrane lipids, the unsaturation to saturation ratio was higher at 5°C than at 20°C (\(F_{1,12}=61.85, P<0.001\)), higher in animals fed a yeast diet than algae (\(F_{1,12}=13.92, P<0.01\)), and yeast fed animals showed higher thermal plasticity (\(F_{1,12}=6.00, P<0.05\)). Storage lipids showed similar results: unsaturation to saturation ratio was higher at 5°C than at 20°C (\(F_{1,12}=257.94, P<0.001\)), higher in animals fed a yeast diet than algae (\(F_{1,12}=122.58, P<0.001\)), and yeast fed animals showed higher thermal plasticity (\(F_{1,12}=4.90, P<0.05\)) (Figure 1, Box). *O. cincta* being more dependent on the essential fatty acid, we expected them to show higher plasticity when fed an algae diet compared to a yeast diet. Although *O. cincta* hatched, they did not grow at all when fed yeast, suggesting at least that C\(_{18:3n3}\) truly is an essential fatty acid for this springtail species. Growth and survival of *O. cincta* on yeast was however inhibited to such extend that we were not able to collect enough material for analysis of lipid composition.

*Figure 1.* Unsaturation to saturation ratio (U/S ratio) for *Folsomia candida* fed on algae or yeast after two weeks of acclimation to 5°C or 20°C.
Table 1. Membrane lipids of *Orchesella cincta* in response to 5°C acclimation. The counter temperature was 25°C in chapter 2, 20°C in Chapter 5, and 15°C in Chapter 3. In Chapter 4, the response was based on an increase or decrease of the reaction norm (RN) after pre-acclimation to either 5°C or 25°C. Acclimation time was ten days in Chapter 5, two weeks in Chapter 2, and four weeks in Chapter 3 and 4. Based on the homeoviscous adaptation (HVA) theory, unsaturation was expected to increase. Thermal responses of Chapter 3 and 5 were based on the PCscores (see text).

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Data of Chapter 6 was not included in this table, because animals were starved in contrast to the other chapters.

* Increase (in proportion)

* Decrease (in proportion)

* No increase or decrease (in proportion)

* No symbol fatty acid was not observed during experiment (or excluded due to missing values)

UI unsaturation index or average number of double bonds per fatty acid

U/S ratio unsaturation to saturation ratio
Table 2. Storage lipids of *Orchesella cincta* in response to 5°C acclimation. The counter temperature was 25°C in chapter 2, 20°C in Chapter 5, and 15°C in Chapter 3. In Chapter 4, the response was based on an increase or decrease of the reaction norm (RN) after pre-acclimation to either 5°C or 25°C. Acclimation time was ten days in Chapter 5, two weeks in Chapter 2, and four weeks in Chapter 3 and 4. Based on the homeoviscous adaptation (HVA) theory, unsaturation was expected to increase. Thermal responses of Chapter 3 and 5 were based on the PCscores (see text).

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Uᵢ unsaturation index or average number of double bonds per fatty acid

U/S ratio unsaturation to saturation ratio


Storey KB, Storey JM (1988) Freeze tolerance in animals. Physiological Reviews 68: 27-84.


Summary

The world in which plants and animals live changes continuously. Day-night rhythm, daily or seasonal weather patterns, or climate change causes environmental conditions to vary on different time scales. In addition to abiotic factors, biotic factors change as well (e.g. community composition). To prevent extinction of a species, individuals must constantly adapt to this changing environment, but the question is whether they are able to? The question is important, because differences in adaptability between species can lead to disruption of the functioning of an ecosystem due to abolition of certain functions that organisms occupy within the ecosystem. Organisms have evolved short term (physiological) and long-term (genetic) mechanisms to cope with environment change. The phenotype of an organism is the result of interactions between its genotype and environment conditions. The capabilities and flexibility an organism has to adjust its phenotype to the environment, is called phenotypic plasticity.

Ectotherms are organisms whose body temperature depends on environmental heat sources. Because almost all biological processes depend on body temperature, environmental temperature is very important for the performance of ectotherms. In this thesis, I studied how ectotherms physiologically adjust to changing environmental temperatures. I have looked at their thermal phenotypic plasticity and I have chosen specifically to look at their (body) lipid adaptation. Cell membranes mainly consist of lipids: membranes keep the body together on a micro scale and protect it against influences of the outside. In addition, lipid storage is an important source of energy for the body, but in order to metabolize it, it must be in an accessible form for enzymes, which cannot cope with lipids that are too solid due to low temperature. Thermal adaptation of lipids is therefore very important for the functioning of the whole body.

Springtails were chosen as the model system, because they occur in a wide variety of habitats: they occur from the poles to the tropics and from forest to beach or even water. As a consequence, they have evolved strategies to cope with a large range of environmental conditions, and both genetic and physiological adaptations have been described. Springtails are small soil arthropods that graze especially on fungi and algae. The function of their grazing for the ecosystem is enhancement of the nutrient recycling.

The main research aim of this thesis was to find out how springtails adapt their lipid composition to altered temperature. What are the differences in thermal adaptation between species, and which fatty acids increase or decrease in response to temperature? Subsequently, I wanted to know whether differences in thermal phenotypic plasticity relate to variation in extreme temperature tolerance. The fatty acid response was not as straightforward as
predicted from literature. Theory assumes that when the temperature decreases, fatty acids become more unsaturated, and by increasing the temperature they become more saturated. Without adaptation, lipids solidify at low temperature and liquefy at high temperature. By adjusting the degree of the fatty acid saturation, the viscosity (fluidity) of body lipids remains in equilibrium (this is called homeoviscosity). This thesis showed that springtails indeed changed their lipid composition in response to temperature; hence, they showed a plastic response. For example, the proportion of the fatty acid $C_{16:0}$ often increased with temperature, while $C_{20:5\text{n3}}$ mostly decreased. There was however a difference in the response of the membrane lipids compared to the storage lipids: the response of the storage lipids fitted better with the expectation from the literature. The most likely cause of this difference was that storage lipids have fewer alternative avenues available for lipid adaptation. Membrane lipids can, besides adjusting the fatty acid saturation, change the shape of the phosphoheads and the position of the head on the molecule, while storage lipids can only adjust the fatty acid saturation.

I started by determining the best way to measure lipids in springtails. It matters how the biomaterial is processed in the laboratory before the lipids are measured. Especially the polyunsaturated fatty acids are very sensitive to certain treatments: they oxidize easily after which they are no longer detectable. I found that slow saponification and flushing the headspace before saponification and methylation with nitrogen gas, maximize preservation of these polyunsaturated fatty acids, resulting in more reliably measurements of the lipid composition.

The fatty acid adaptation in a springtail species (*Orchesella cincta*) during lipid adaptation was determined in response to moderate temperature change in juvenile and adult springtails. Adult springtails showed more unsaturated fatty acids than juveniles, but both groups increased the proportion of unsaturated fatty acids in response to a temperature decrease. In storage lipids, this response was reversed during a temperature increase, but not in membrane lipids. A plausible cause for this difference between membrane and storage lipids could be the alternative avenues for adaptation, as explained above.

Subsequently, I looked at thermal reaction norms for specific fatty acids over five different temperatures. Which fatty acids change in proportion and which do not? The fatty acid composition of *O. cincta* after four weeks of acclimation changed largely in line with the previous experiment. Again the fatty acids showed a high degree of plasticity, but we found some striking deviations from expectations. Two specific fatty acids ($C_{18:2\text{n6}}$ and $C_{18:3\text{n3}}$) increased in quantity at a higher temperature, while an overall decline of these polyunsaturated fatty acids would be expected. These fatty acids are probably essential and can therefore only be obtained through the diet. Only two insect species have been found to produce these fatty acids themselves, other species probably do not. If these fatty acids are essential for *O. cincta*, then preservation of these fatty acids for future processes can have a
higher priority than the thermal response. There is then a trade-off between optimal temperature adaptability and future reproduction, for which these essential fatty acids are needed, which can lead to a sub-optimal temperature adaptation.

In addition to the physiological aspects of thermal adaptation of lipid composition, I looked at the RNA expression of several genes encoding desaturase enzymes in *O. cincta*. An organism gets a large proportion of the fatty acids from its diet or produces them *de novo*. If the ingested fatty acids do not have the required (un)saturation, organisms can reduce the saturation by use of specific desaturase enzymes. In *O. cincta*, indeed no homologues of Δ12 or Δ15 desaturases were found, confirming that C_{18:2n6} and C_{18:3n3} for *O. cincta* probably are essential fatty acids. In this experiment, I also observed that most of the desaturase genes showed much higher expression in animals that were adapted to heat than to cold; the opposite of what I expected. Increased activity due to warmer temperatures possibly resulted in other priorities than temperature adaptation, e.g. increased feed intake and reproduction.

Laboratory experiments investigating thermal adaptation often simulate only a single temperature change, but in a natural environment, the temperature fluctuates more often. To examine how *O. cincta* responds to repeated temperature changes, I exposed the animals to a repeated two-day temperature fluctuation, and a warm and cold constant temperature. In the early cycles of this regime, *O. cincta* adapted its fatty acids to the new temperature, but later in the experiment there was an attenuation of this response. Instead, their fatty acid composition was stable and similar to the fatty acid composition of animals that were adapted to constantly warm temperature, even during the cold part of the temperature cycle. To relate adjustment of lipid composition to functioning of *O. cincta*, I also determined extreme temperature tolerance after exposure to the three temperature regimes. *O. cincta* exposed to constant cold was better able to withstand cold shock, while individuals exposed to constant warmth were better able to withstand a heat shock. *O. cincta* exposed to fluctuating temperature resisted a heat shock just as well and cold shock a little better than individuals adapted to constant warmth. Although the fatty acid composition was consistent with that of individuals exposed to constant warmth, the animals that were exposed to fluctuating temperature had a higher thermal tolerance.

Differences in temperature fluctuations in the soil are mostly found along a vertical gradient. Springtail species that live deep in the soil differ in microhabitat with species living more on the soil surface, because the environmental conditions are very constant deeper in the soil. Soil dwelling species have had less (evolutionary) selection pressure on adaptation to changing temperatures than surface dwelling species. Consequently, I expected surface dwelling species (e.g. *O. cincta*) to be physiologically better able to adapt to changing temperatures than soil dwelling species. Surface dwelling species indeed showed larger physiological plasticity than soil dwelling species. Also, their temperature tolerance was higher: surface dwelling species could better withstand extreme heat than soil dwelling
species. Unless soil dwelling species are able to behaviorally protect themselves to temperature, this group of springtails renders more vulnerable to climate change, despite the fact that surface dwelling species will be exposed to larger temperature changes.

There are many factors that can explain observed variation in the responses to temperature. This thesis shows that age of the individual, species, period of adaptation, rate of temperature change, and direction of temperature change affected the degree of body lipid adaptation. From the literature possible effects of diet, air or soil moisture, and the interaction with enzymes were added to the list of possible causes of variation in the thermal lipid response. Still membrane and storage lipids showed a high degree of plasticity in response to temperature, from which I conclude that fatty acid modifications are probably essential for the physical characteristics of body lipids under different environmental temperatures. My results have highlighted adaptive changes in body lipids that can increase the thermal resistance during the temperature changes to which these animals are exposed.
De omgeving van planten en dieren verandert continu, op verschillende tijdschalen en met variërende voorspelbaarheid, zoals dag-nacht ritme, weersveranderingen van dag tot dag, seizoenen, of klimaatverandering met langzaam verhoogde gemiddelde temperaturen en grotere temperatuurschommelingen. Niet alleen de abiotische factoren veranderen continu, ook de biotische factoren zoals beschikbaar leefgebied en/of de samenstelling van de levensgemeenschap. Om uitsterven te voorkomen, moeten individuen van die soort zich continu aanpassen aan een veranderende omgeving. De vraag is in welke mate organismen dit kunnen? Het is belangrijk om inzicht te krijgen in deze kwestie, omdat verschillen in aanpassingsvermogen tussen soorten kunnen leiden tot verstoring van het functioneren van een ecosysteem, door het wegvallen van specifieke functies die een organisme binnen een ecosysteem bekleedt. Organismen kunnen zich aanpassen aan een veranderende omgeving op de korte termijn (fysiologisch) en langere termijn (genetisch). Het fenotype van een organisme is de som van zijn genotyp (zijn genen) en het effect van de omgeving (fysiologische status). De mogelijkheden en flexibiliteit die een organisme heeft om zijn fenotype aan te passen aan de omgeving heet fenotypische plasticiteit.

Ectothermen zijn organismen die voor hun lichaamstemperatuur afhankelijk zijn van de omgevingstemperatuur. De belangrijkste omgevingsfactor voor ectothermen is temperatuur, omdat vrijwel alle biologische processen afhankelijk zijn van temperatuur. In dit proefschrift heb ik onderzocht hoe ectothermen zich op de korte termijn fysiologisch aanpassen aan een veranderde temperatuur. Ik heb gekeken naar hun thermische fenotypische plasticiteit en ik heb specifiek gekozen om te kijken naar de aanpassing van hun lichaamsvetten (lipiden). Celmembranen bestaan voornamelijk uit vet, houden het lichaam bij elkaar op microschaal, en beschermen het lichaam tegen invloeden van buitenaf. Opslagvetten zijn een belangrijke energiebron voor het lichaam, maar dan moet het wel in bruikbare vorm zijn en bijvoorbeeld niet te zeer gestold als gevolg van de lage temperatuur. Vetaanpassing is daarom erg belangrijk voor het functioneren van het hele lichaam.

Ik heb gekozen om springstaarten als modelsysteem te gebruiken, omdat ze een zeer wijde verspreiding hebben: ze komen voor van pool tot tropen en van bos tot bloempot op het balkon. Hierdoor zijn ze aangepast aan een breed scala aan omgevingsomstandigheden, zowel genetisch als fysiologisch. Springstaarten zijn kleine bodemectothermen die in het ecosysteem de nutriënten kringloop bevorderen door het grazen van vooral schimmels en algen.

Ik heb eerst onderzocht wat de beste manier is om vetten in springstaarten te meten. Het maakt uit op welke manier het biomateriaal bewerkt wordt in het laboratorium voordat de vetten hieruit worden gemeten. Vooral de meervoudig onverzadigde vetten zijn erg gevoelig.
voor bepaalde behandelingen; zij raken bijvoorbeeld snel geoxideerd, waarna ze niet meer te detecteren zijn. Ik weet nu dat een methode met langzame saponificatie en het spoelen van de ‘headspace’ met stikstofgas voor saponificeren en methyleren zorgt voor zo veel mogelijk conservering van deze meervoudige onverzadigde vetten, zodat ik de vetzuursamenstelling betrouwbaarder kan meten.

Het hoofddoel van deze thesis was echter om te onderzoeken hoe springstaarten hun vetzuursamenstelling veranderen onder invloed van temperatuur, welke vetzuren toe- of afnemen en hoe sterk ze reageren, en of er verschillen tussen soorten zijn in hun aanpassingen. Vervolgens heb ik gekeken of verschillen in thermische fenotypische plasticiteit geassocieerd zijn met verschillen die optreden in temperatuurtolerantie. In eerste instantie bleek de respons van de vetten helemaal niet zo rechtlijnig als werd voorspeld in de literatuur. De theorie veronderstelt dat wanneer de temperatuur omlaag gaat, vetzuren meer onverzadigd worden, en bij een verhoging van de temperatuur juist meer verzadigd. Zonder aanpassing wordt vet hard bij lage temperatuur en zacht bij hoge temperatuur. Met aanpassing van de mate van verzadiging van de vetzuren blijft de viscositeit (vloeibaarheid) van de lichaamsvetten in evenwicht (homeoviscositeit). Deze thesis liet zien dat springstaarten hun vetzuren inderdaad met temperatuur veranderen (plastische response). Zo nam de proportie van het verzuur $C_{16:0}$ vaak toe met temperatuur en nam $C_{20:5\text{n3}}$ bijna altijd af. Er was verschil in de respons van de membraanvetzuren ten opzicht van de vetzuren in het opslagvet van de springstaarten: de respons van de opslagvetten paste beter bij de verwachtingen op basis van wat we vonden in de literatuur. De meest waarschijnlijke oorzaak van dit verschil in respons is de kleinere hoeveelheid alternatieve aanpassingsmogelijkheden die het opslagvet heeft. Membraanvet kan naast het aanpassen van de vetzuurverzadiging, ook de vorm van de molecuulkop ook de positie van de kop op het molecuul aanpassen, terwijl opslagvet alleen de vetzuurverzadiging kan aanpassen.

In meer detail keken we naar de vetzuurverandering in een springstaartsoort *Orchesella cincta* gedurende het aanpassen van vetten in reactie op een temperatuursverandering in jonge en volwassen springstaarten. Volwassen springstaarten hadden meer onverzadigde vetzuren dan jonge springstaarten, maar voor beide groepen werden de vetzuren meer onverzadigd tijdens aanpassing aan een temperatuurdaling. Vice versa werden opslagvetten meer verzadigd na een temperatuurstijging, maar de membraanvetten reageerden niet op temperatuurstijging, waarschijnlijk door die alternatieve aanpassingsmogelijkheden zoals hierboven genoemd.

Hoe zit het nou precies met die vetzuurveranderingen in reactie op temperatuur? Welke vetzuren reageren nu op temperatuur en welke niet? De vetzuursamenstelling van *O. cincta* na vier weken aanpassen aan vijf verschillende temperaturen veranderde grotendeels in overeenstemming met het vorige experiment. Weer lieten de vetzuren een hoge mate van plasticiteit zien, maar we vonden een aantal opvallende afwijkingen van de verwachtingen.
Twee specifieke vetzuren (C\textsubscript{18:2n6} en C\textsubscript{18:3n3}) namen toe in hoeveelheid bij een hogere temperatuur, terwijl men juist een afname van deze meervoudig onverzadigde vetzuren zou verwachten bij hogere temperatuur. Een mogelijk verklaring hiervoor is dat in sommige soorten deze vetzuren essentieel zijn en dus alleen via het dieet te verkrijgen. Er is slechts voor twee insectensoorten gevonden dat ze deze vetzuren zelf kunnen produceren, andere dieren kunnen dit waarschijnlijk niet. Als deze vetzuren essentieel zijn voor springstaarten dan kan het reserveren van deze vetzuren voor toekomstige processen een hogere prioriteit hebben dan de temperatuurrespons. Er is dan een trade-off tussen temperatuuraanpassing en toekomstige reproductie, die kan leiden tot een suboptimale temperatuuraanpassing.

Naast vetzuuraanpassingen, heb ik gekeken naar de RNA expressie van genen die verschillende desaturase enzymen coderen in \textit{O. cincta}. Een organisme haalt een groot deel van de vetzuren uit het voer of produceert ze zelf. Indien de opgenomen vetzuren niet de goede verzadiging hebben, kan een organisme ze zelf minder verzadigd maken door gebruik van specifieke desaturase enzymen. Tijdens het bekijken van de RNA expressie in \textit{O. cincta}, is inderdaad geen expressie van Δ12 of Δ15 desaturases gevonden. Dit bevestigt dat C\textsubscript{18:2n6} en C\textsubscript{18:3n3} voor \textit{O. cincta} waarschijnlijk essentiële vetzuren zijn. In deze proef zag ik ook dat de meeste desaturase genen veel hoger tot expressie kwamen bij dieren die aangepast waren aan warmte dan aan koude, tegenovergesteld aan wat we verwachten. Mogelijk zorgde verhoogde activiteit door warmere temperatuur voor andere prioriteiten dan die van temperatuur aanpassing, e.g. verhoogde voeropname en voortplanting.

Laboratoriumexperimenten aan temperatuuraanpassing simuleren vaak een enkele temperatuurwisseling maar in een natuurlijke omgeving fluctueert de temperatuur veel vaker dan eens. Om te onderzoeken hoe \textit{O. cincta} reageert op herhaaldelijk wisselende temperatuur heb ik ze blootgesteld aan een tweedaagse temperatuurwisseling. In het begin paste \textit{O. cincta} de vetzuren nog wel aan de nieuwe temperatuur aan, maar al snel vond er een uitdoving van die respons plaats. In plaats daarvan was hun vetzuursamenstelling continu gelijk aan de vetzuursamenstelling van dieren die aan constant warme temperatuur waren aangepast. Om te kijken hoe goed deze springstaarten functioneren op basis van hun temperatuuraanpassing, bepaalde ik hun extreme temperatuurtoerantie. \textit{O. cincta} aangepast aan constant koude temperatuur was beter instaat om een koude schok te weerstaan, terwijl individuen aangepast aan continue warmte beter in staat waren een hitschok te weerstaan. Als \textit{O. cincta} hun vetten aanpaste aan wisselende temperatuur, weerstonden individuen een hitschok net zo goed, en een koude schok net iets beter dan individuen aangepast aan constante warmte. Hoewel de vetzuursamenstelling in overeenstemming was met die van individuen aangepast aan constante warmte, hadden de dieren die hun vetten aanpaste aan wisselende temperatuur een hogere temperatuurtoerantie.

Verschillen in temperatuurwisselingen in de bodemhabitat worden vooral gevonden langs een verticale gradiënt. Springstaartsoorten die diep in de bodem leven verschillen in
microhabitat met soorten die meer oppervlakkig leven, omdat dieper in de bodem de omgevingsomstandigheden constant zijn. Dieplevende soorten hebben hierdoor minder (evolutionaire) selectie druk gehad op het aanpassen aan wisselende temperatuur dan dieren die meer aan de oppervlakte van de bodem leven. Als gevolg hiervan verwachtte ik dat dieren die meer aan de oppervlakte leven (bijvoorbeeld *O. cincta*) zich fysiologisch gezien beter aan kunnen passen aan wisselende temperatuur dan dieren die dieper in de bodem leven. Oppervlakkig levende springstaartsoorten lieten inderdaad grotere mate van fysiologische flexibiliteit zien dan dieplevende soorten. Ook hun temperatuurtolerantie was hoger: zij konden beter tegen extreme hitte dan dieplevende soorten. Dieplevende springstaartsoorten hadden fysiologisch gezien dus een minder plastisch fenotype dan oppervlakkig levende soorten. Tenzij dieplevende soorten zich gedragsmatig kunnen beschermen tegen temperatuur, is deze groep springstaarten daardoor kwetsbaarder voor klimaatverandering, ondanks dat oppervlakkig levende soorten aan grotere temperatuursveranderingen worden blootgesteld.

Er zijn vele factoren die de gevonden variatie in temperatuurresponsen kunnen verklaren. In deze thesis kwam naar voren dat de leeftijd van het individu, soort, periode van aanpassing, mate van de temperatuursverandering, en richting van de temperatuursverandering effect hadden op de mate van lichaamsvetaanpassing. Vanuit de literatuur kwamen daar nog mogelijke effecten bij van dieet, lucht- of bodemvochtigheid, en de interactie met enzymen. Toch toonden membraan- en opslagvetten een hoge mate van plasticiteit in reactie op temperatuur, waaruit ik concludeer dat vetzuurveranderingen waarschijnlijk essentieel zijn voor de fysieke eigenschappen van lichaamsvet onder verschillende omgevingstemperaturen. Hoewel mijn resultaten geen direct bewijs vormen voor het behoud van de homeoviscositeit van lichaamsvet in reactie op temperatuur, wijzen ze samen in de richting van adaptieve veranderingen in lichaamsvetten die het weerstandsvermogen kunnen verhogen tijdens de temperatuursveranderingen waaraan deze dieren worden blootgesteld.
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Acknowledgements

With the completion of this thesis, my playtime comes to an end (so they say... I’m not really convinced yet). Science is mostly fun, but can be very difficult at times as well. Not only do we need to know our topic, the context, the back ground, the causes, the effects, we also have to do the thinking, the designing, the piloting, the implementing, the data collecting, the analysing, the writing, the presenting, the collaborating, the networking, and the acquisitioning for new projects. A diverse challenge when one comes to think about it, but never boring! Due to this diversity and the experimental design, in fact the most constant factor during my thesis was ‘change’ itself, whether related to personal development or experimentally applied.

Jacintha, I think we worked very well as a team. I very much appreciated your ultimate trust in me to make this thesis a success and the enormous amount of freedom you gave me. I hope I proved you right. In my first year, you immediately noticed my shortcomings and started to reframe them into potential qualities. Today, giving presentations and making new contacts are not half as scary as they were back then. Thank you for that! Although I started to work on the thermal physiology of springtails using techniques rather new for the animal ecology group, we managed to get things going (for this many thanks to Roel as well). We did some very fine experiments, although sometimes very basic. We started out with very ambitions plans, but soon realised basic research was much more needed first. Unfortunately this resulted in much less ecology than we aimed for. After four years, I think much more fundamental research is still needed, but in the future definitely much more ecology needs to be implemented. During my thesis, you were always there for me to answer my questions, for some discussion or comments on manuscripts (and comments they needed a lot!). VU Colleagues, although diverse in research interest, you match well together. I will not name people in particular, because I want to avoid any ranking. It was very nice working with you all. I know where to find you when collaboration seems fruitful in the future. You know where to find me as well. Family and friends, thanks for your support. I know the topic I worked on was often hard to grasp. Still you always tried to understand. Frank, you are the best! Although sometimes annoyingly critical, you were mostly right. You were always willing to read and comment on my manuscripts, which I much appreciated. Many thanks also for trying to prevent me from falling into the usual PhD pitfalls. Although I mostly walked into them anyway, due to my stubbornness or impertinence, at least I could agree with you afterwards that indeed they actually proved to be a pitfall.

Now, it is time to work....
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Curriculum Vitae

Jacoba (Coby) Annemaria van Dooremalen was born on May 5, 1980 in Hengelo (O), the Netherlands. In 1998, she obtained her high school degree at Strabrecht College in Geldrop, after which she started a BSc Animal Farming and Animal Health at HAS Ben Bosch. After graduation in 2002, she went to Australia and New Zealand for a year. In 2003, she started an MSc Animal Science at Wageningen University. In her first MSc thesis, supervised at the Animal Production Systems Group, she investigated the feasibility of integrating nature conservation and domestic pigs on a care farm. In her second MSc thesis, supervised at the Adaptation Physiology Group, she investigated the follicle development and its endocrine background during interrupted suckling in sow lactation. In her third MSc thesis, supervised at the Centre of Terrestrial Ecology of the NIOO-KNAW in Heteren and the Resource Ecology Group at Wageningen University, she investigated the energetic mechanism underlying reproductive success in the Pied Flycatcher. After obtaining her MSc degree at Wageningen University in 2006, she continued to work for a few months at the Centre of Terrestrial Ecology as a research assistant in eco-physiological experiments. At December 2006, she started her PhD thesis at VU University, Amsterdam. Her research focused on the physiology of lipids in thermal phenotypic plasticity and thermal tolerance in springtails. Presently, she is appointed as a scientific researcher at Bees@wur, Biointeractions and Plant Health, Plant Research International in Wageningen, working on bee health, bee physiology, and sustainable bee keeping practices.

List of publications

Van Dooremalen, C., W. Suring, J. Ellers (Provisionally Accepted) Changes in lipid composition and extreme temperature tolerance following fluctuating temperatures in Orchesella cincta. Journal of Insect Physiology.


Submitted and in preparation
Van Dooremalen, C., M. Berg, J. Ellers (Submitted) Thermal response of lipid composition is stronger for surface dwelling than for soil dwelling springtail species.

