Stockholm University

Unraveling the regulatory mechanisms of pupal diapause termination

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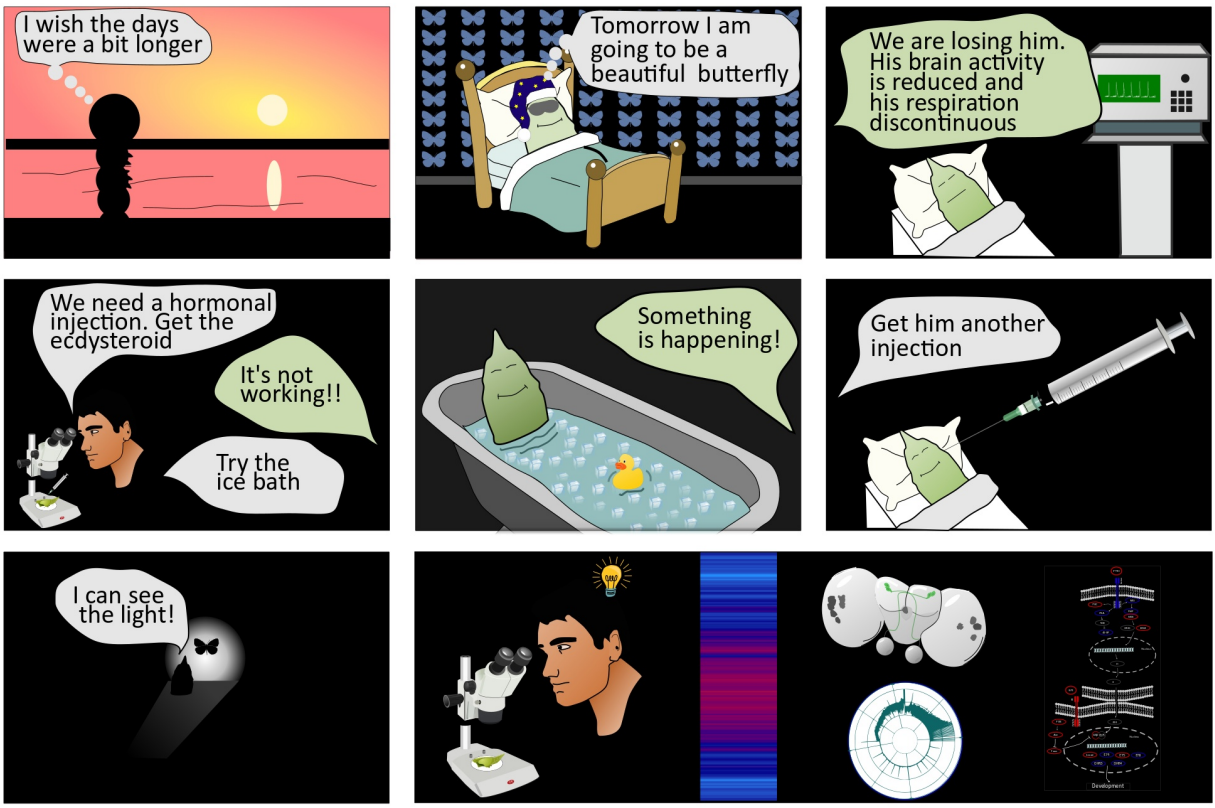

Doctoral Thesis in Functional Zoomorphology at Stockholm University, Sweden 2023

Unraveling the regulatory mechanisms of pupal diapause termination

Philip Süess

Academic dissertation for the Degree of Doctor of Philosophy in Functional Zoomorphology at Stockholm University to be publicly defended on Friday 27 October 2023 at 10.00 in Vivi Täckholmsalen (Q-salen), NPQ-huset, Svante Arrhenius väg 20.

Abstract

Diapause is an essential part of many insect's life cycle and is a developmental halt induced by environmental cues in advance of deteriorating conditions. Insects typically enter diapause to avoid unfavorable environmental conditions like low temperatures, poor food quality and the absence of conspecifics. The aim of my PhD thesis was to describe how diapause is regulated and what determines its termination timing in the green-veined white butterfly Pieris napi. Answers to these questions deepens the knowledge about species distribution and population dynamics in times of a rapidly changing climate.

In ectothermic insects, developmental rate generally follows a thermal performance curve (TPC) with higher temperatures leading to a faster development. However, in the diapause of P. napi low temperatures result in a higher proportion of terminations. In Chapter I, I assessed the thermal performance of diapause termination rate in P. napi by exposing them to several different temperature treatments. The diapause termination rate follows a left-shifted TPC with an optimum termination rate at 0°C and slower termination rates toward higher temperatures. This left-shifted TPC prevents the precocious termination of diapause in autumn while at the same time enabling populations to remain synchronized over a long period.

Development in insects is regulated by the major developmental hormones, insulins, juvenile hormones, ecdysone, and the prothoracicotropic hormone (PTTH). The pupal diapause is regulated by the neuropeptide PTTH produced in the brain and ecdysone produced in the prothoracic glands. In Chapter II I studied this hormonal axis by assessing PTTH levels and injections with 20-hydroxyecdysone (20E), the active form of the ecdysone pathway. The PTTH neuropeptide shows diapause stage dependent changes to haemolymph levels and intracellular structure and the sensitivity to 20E returns in a time- and temperature dependent manner correlating with diapause termination progression. This indicates that diapause termination timing is regulated by the PTTH ecdysone axis and that the ecdysteroid receptor has a central part in the regulation.

Diapause is a prolonged period without the ability to replenish energy resources, insects therefore accumulate resources before diapause and reduce the metabolic rate to a minimum. In Chapter III, I investigated how the diapause physiology as well as the reduced metabolic rate affect the mode of respiration. Respiratory patterns change with diapause stages and in diapause, discontinuous gas exchange is defended even at high temperatures, supporting the finding that the diapause physiology affects the metabolic rate and with it the respiratory patterns.

In Chapter IV I followed up the lead from Chapter II and tested a hypothesis on the hormonal regulation of diapause termination in which the transcription factor Foxo, the ecdysteroid receptor as well as insulin interact to regulate ecdysone sensitivity. While many findings in the transcriptome and the protein levels support the hypothesis we need further investigations into this mechanism. Furthermore, the protein levels of most proteins studied do not correlate with the mRNA levels, and while this has been described under direct developing conditions too it would be interesting to study where those different levels stem from.

These findings show that the three levels, temperature, metabolic rate and the hormonal pathways are tightly linked to the diapause physiology and are most likely involved in the regulation of diapause termination timing.

Keywords: *Diapause, diapause termination, prothoracicotropic hormone, ecdysone, thermal performance.*

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To my parents

The thesis is based on the following articles, which are referred to in the text by their Roman numerals:

I **Philip Süess***, Loke von Schmalensee*, Karl Gotthard, Philipp Lehmann. Diapause termination and post-diapause development are predictable successive temperature rate processes allowing for phenological synchronization in the green-veined white butterfly *Pieris napi*. *In preparation.*

II **Philip Süess**, Heinrich Dircksen, Kevin T. Roberts, Karl Gotthard, Dick R. Nässel, Christopher W. Wheat, Mikael A. Carlsson, Philipp Lehmann (2022). Time-and temperature-dependent dynamics of prothoracicotropic hormone and ecdysone sensitivity co-regulate pupal diapause in the green veined white butterfly *Pieris napi*. *Insect Biochemistry and Molecular Biology* 149:103833.

III **Philip Süess**, Kevin T. Roberts, Philipp Lehmann (2023). Temperature dependence of gas exchange patterns changes predictably with diapause progression in the butterfly *Pieris napi*. *Resubmitted after revision.*

IV **Philip Süess**, Sabine Ziesemer, Rachel Steward, Kevin T. Roberts, Christopher W. Wheat, Philipp Lehmann. Transcriptional signature of diapause in the hormonal pathways of the green-veined white butterfly *Pieris napi*. *In preparation.*

*Shared first authorship.

Candidate contributions to thesis articles*

	I	II	III	IV
Conceived the study	Substantial	Significant	Significant	Substantial
Designed the study	Substantial	Substantial	Substantial	Significant
Collected the data	Substantial	Substantial	Significant	Minor
Analysed the data	Minor	Substantial	Substantial	Substantial
Manuscript preparation	Significant	Substantial	Substantial	Substantial

*** Contribution Explanation**

Minor: contributed in some way, but contribution was limited.

Significant: provided a significant contribution to the work.

Substantial: took the lead role and performed the majority of the work.

Contents

Introduction

Seasonality of insect life

Insects in the temperate zones of our world are faced with many challenges throughout the year due to pronounced seasonality (Mellanby, 1939; Wolda, 1988). In spring, they have to synchronize their emergence from the dormancy state, with the return of their food resources and their conspecifics for mating (Andrewartha, 1952; Denlinger, 2022a; Lees, 1956). In summer, the temperatures are generally favorable for development and resources are plentiful; at the same time, the insects face increased competition for mates and food as well as increased predation. Many insects are already preparing for adverse conditions ahead of the unfavorable conditions, by accumulating energy resources and entering an alternative developmental pathway (Denlinger, 2022b). In autumn, the temperatures start to drop and the food resources start to disappear. At this time, the insects need to have accumulated enough energy resources to overwinter and a place to spend the winter months (Hahn and Denlinger, 2011). They furthermore have to prepare their physiology, accumulation of energy resources and cryoprotectants, for the stressfull low temperatures as well as long periods of inactivity exerting a toll on their energy reserves (Asahina, 1970; Nielsen et al., 2022). In winter, resources are generally unavailable and the insects lay dormant until spring (Andrewartha, 1952; Mellanby, 1939). Still in winter, the overwintering stage is terminated, while the physiology still needs to withstand adverse environmental conditions until the warm temperature of the spring days return.

Diapause

Insect dormancy in winter is called diapause and is generally the absence of development and the suppression of the body's physiological functions (Andrewartha, 1952; Koštál et al., 2009; Schneiderman and Williams, 1953). Insects with one generation per year have an obligate diapause and enter diapause independent of environmental conditions, while insects that have multiple generations each year have a facultative diapause and environmental conditions like photoperiod, temperature or humidity trigger diapause (Beck, 1962; Cullen and Browning, 1978; Schebeck et al., 2022; Takagi and Miyashita, 2008). The most common trigger for diapause in the temperate region is a short photoperiod (Denlinger, 2022b). During late summer, as days become shorter, insects enter diapause an alternative developmental pathway characterized by a physiological change to accommodate the unfavorable conditions (Aalberg Haugen and Gotthard, 2015). Other environmental conditions leading insects into diapause are food quality, temperature, and humidity (Cullen

and Browning, 1978; Morris, 1967; Takagi and Miyashita, 2008). Once diapause has been triggered, insects accumulate resources in their fat body and generally accumulate more sugars, fat and amino acids than their conspecifics that develop directly, although with some exceptions (Kivelä et al., 2019; Lehmann et al., 2017a; Short and Hahn, 2023). Furthermore, they accumulate sugars and cryoprotectants in their haemolymph to protect themselves from the damage caused by low temperatures in the winter months (Koštál et al., 2004). This phase is called diapause induction and the change to diapause initiation generally takes place with a life stage change (Koštál, 2006). In diapause initiation, the insects change their physiology to accommodate for a long period of limited resources (Hahn and Denlinger, 2011; Short and Hahn, 2023). They reduce the metabolic rate to a minimum, shut down brain activity and stop further development (Highnam, 1958; Schneiderman and Williams, 1953). The initiation phase is followed by endogenous diapause, in which the insects cannot leave the diapause stage without certain environmental conditions being met (Koštál, 2006; Lehmann et al., 2017b).

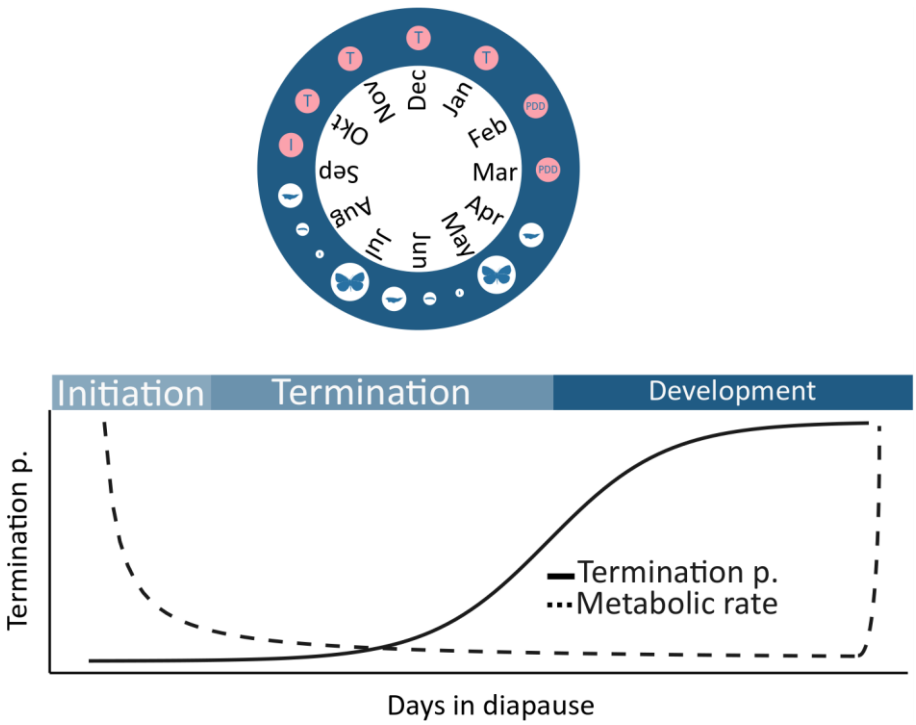

Figure 1: Schematic of* P. napi *life cycle and diapause progression. *Top: the life cycle of the green-veined white butterfly* Pieris napi *in Stockholm. The adults eclose in late spring, mate and lay eggs. The first generation grows*

through the early summer and the adults mate in the middle of summer. The second generation grows into early autumn and once they pupate, they enter diapause. In early autumn the insects progress through diapause initiation (I). When temperatures are low enough the pupae are going through diapause termination (T). Diapause is generally terminated around January. The insects then enter post-diapause development in late winter and early spring. Bottom: Pupae reduce their metabolic rate to a fraction of the metabolic rate in the active state in diapause initiation. During endogenous diapause and post-diapause development at low temperatures the metabolic rate remains suppressed and only rises once temperatures increase and development resumes. Pieris napi *needs to accumulate around 120 days of low temperatures to terminate diapauses, indicated by the solid line for termination propensity. Diapause stages after Košťál et al.* (2006).

Some insects terminate the endogenous diapause when the photoperiod returns to longer days in spring, but various insects have to accumulate a certain amount of low temperatures to terminate diapause (Tauber and Tauber, 1976). In general, the time in endogenous diapause can vary widely between different species and even within the same species at different latitudes (Denlinger, 2022c). In *Mamestra brassicae* the diapause duration at 28° N is 130 days and at 43° N 30 days and in the pitcher plant mosquito *Wyeomyia smithii* there is a fivefold difference between the diapause duration from 60° to 40° N (Bradshaw and Lounibos, 1977; Denlinger, 2022c).
Once the insects have experienced the prerequisite environmental conditions, the insects terminate diapause and enter the post-diapause phase (Koštál, 2006). If the temperatures are too low for development in post-diapause development, the insects progression is stalled, the metabolic rate remains suppressed, and the cold hardiness is maintained but insects are still responsive to high temperatures (Lehmann et al., 2016). When temperatures rise to levels permitting development, the insects start progression immediately and the metabolic rate suppression is lifted (Ragland et al., 2009). The insects return to the developmental trajectory they interrupted for winter and continue development (Riemer et al., 2021).

Thermal regulation of diapause

Insects are ectothermic animals, their body temperature is dependent on the environmental temperatures and they can influence their body temperature minimally by movement, sun exposur or aggregating into big groups (Heinrich, 1995). Therefore, developmental rate is innately linked to the environment (Sinclair et al., 2012). In warm summer temperatures insects are able to develop at a fast pace and generation times are short (Huey and Kingsolver,

1989; Ratkowsky and Reddy, 2017). In spring and autumn, temperatures are lower and developmental times are longer (Gilbert and Raworth, 1996). In winter, temperatures are generally too low for insect development and is generally halted (Mellanby, 1939). The rate of development in insects follows a thermal performance curve (TPC), in which higher temperatures generally result in faster development (Kingsolver and Woods, 2016). The shape of the TPC function is non-linear and given by a minimum temperature (T_{min}), an optimal temperature (T_{opt}) and the maximum temperature (T_{max}) and varies with populations and species (Ratkowsky and Reddy, 2017; Sinclair et al., 2012). T_{min} and T_{max} constitute the lower and upper limits at which development can occur and is likely restricted by enzymatic reactions (Teitz et al., 2023). T_{opt} constitutes the temperature at which development progresses at the highest rate.

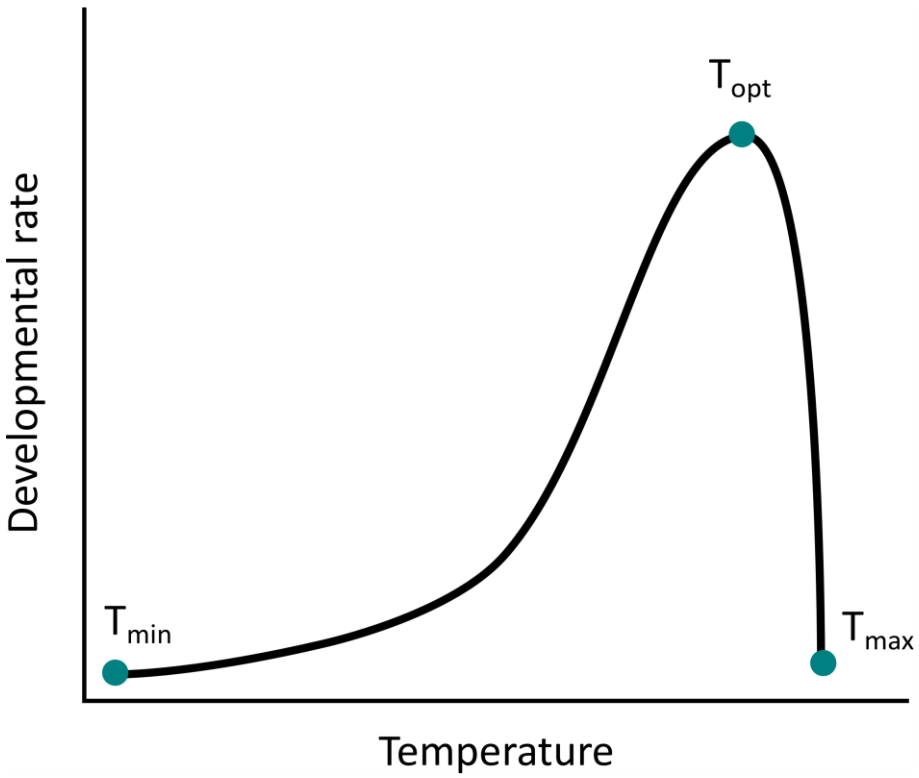

Figure 2: A thermal performance curve for development in an ectothermic animal*. Development progresses fastest at high temperatures before it drops towards the maximal temperature T_{max}. The TPC is left skewed with an exponential decrease in developmental rates towards the minimum temperature T_{min}.*

In diapause, this connection between high temperatures and faster developmental progression is not existing as in many insect species lower temperatures terminate diapause, while high temperatures lead to an unsuccessful diapause (Dong et al., 2013; Jiang et al., 2010). This disconnection between high temperature and faster progression is observed in species as diverse as the silk worm *Hyalophora cecropia*, the lady beetle *Coccinella septempunctata*, the linden bug *Pyrrhocoris apterus*, the green-veined white butterfly *Pieris napi* and the fruit fly *Rhagoletis cerasi* (Lehmann et al., 2017b; Moraiti et al., 2014; Takeuchi et al., 1999; Tauber and Tauber, 1976; Williams, 1956). In *P. napi* low temperatures of around 4°C lead to an increased number of insects that successfully terminate diapause when compared to insects overwintering at higher temperatures of 18°C (Lehmann et al., 2017b, 2016). This indicates that there is an inverse relation between temperature and the rate of progression through diapause in winter in which lower temperatures progress diapause termination at a higher rate than at higher temperatures (Nguyen and Hong, 2023; Toxopeus et al., 2023). A model for diapause termination rates at different temperatures will be a tool to predict population performances in winter conditions and will enable us to predict the thermal performance over the course of the whole year. Creating a model for the diapause termination rate will inform us about the timing of populations and will be a supporting tool in agriculture and conservation biology.

Hormonal regulation of diapause in holometabolous insects

Development in insects is intricately regulated by the major developmental hormones like ecdysone (E), juvenile hormone (JH), Insulin, and the prothoracicotropic hormone (PTTH, Henrich et al., 1998; Jindra et al., 2013; Nijhout, 1998; Smith and Rybczynski, 2012). Insulins connect the life stage changes with the nutritional status of the animal and sufficient nutritional stores lead to the secretion of insulins that trigger the secretion of PTTH and subsequently the production of ecdysone (Antonova et al., 2012). Ecdysone is the main driver of development and its secretion leads to life stage changes and metamorphosis (Henrich et al., 1998). In the presence of JH, the life stage change occurs within the juvenile form (n^{th} instar to $n^{th}+1$ instar), while the absence of JH causes the insects to undergo pupation and metamorphosis (Nijhout, 1998). Ecdysone production from cholesterol and its secretion from the prothoracic gland (PG) itself is triggered by the neuropeptide PTTH (PG, Smith and Rybczynski, 2012). PTTH is produced in two pairs of neurosecretory cells (NSC) in the dorsolateral part of the brain and is connected to the circadian clock and secreted during the scotophase (no light) (Selcho et al., 2017; Steel and Vafopoulou, 2006; Vafopoulou and Steel, 2001, 1996). It then moves through the haemolymph to the PG where it binds to torso, a receptor tyrosine

kinase that interacts with the ras/raf pathway. The ras/raf pathway interacts with the mitogen-activated protein kinases MAPKs ERK1 and ERK2, which in turn interact with the ecdysone production cascade (Fujiwara et al., 2006). Ecdysone is produced from cholesterol through several cytochrome P450 enzymes in the Halloween pathway (Lafont et al., 2012). Ecdysone is secreted and travels to the target organs where it is turned into 20-hydroxy-ecdysone (20E), the active form of the ecdysone pathway. Ecdysone binds to the ecdysteroid receptor dimer formed by ultraspiracle (usp) and ecdysone receptor (EcR, Henrich, 2012). The interaction of 20E with the ecdysteroid receptor activates the transcription factors broad, E74 and E75 (Ou and King-Jones, 2013). Further downstream DHR3 and DHR4 are activated, which trigger the developmental cascades involved in life stage change and metamorphosis (Lam et al., 1999).

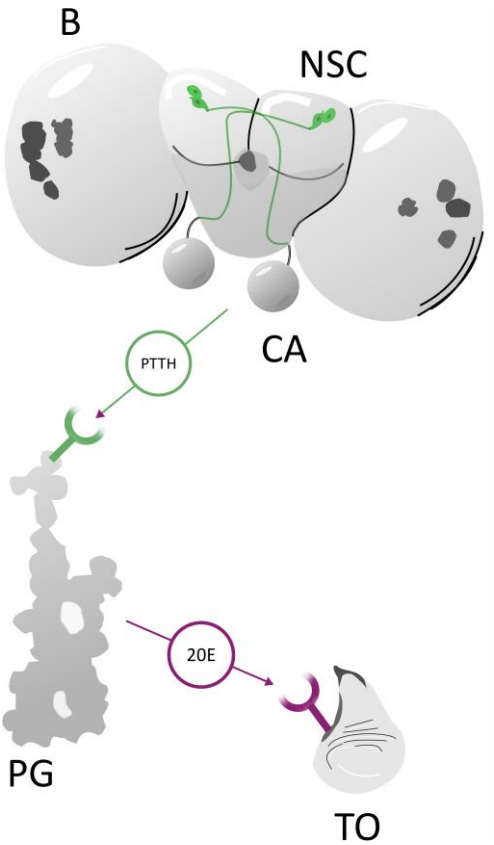

Figure 3: Schematic of a* P. napi *brain and a simplified ecdysteroid signaling pathway. *PTTH is produced in the brain (B) within two pairs of neurosecretory cells (NSC). Those cells protrude through axons into the corpora allata (CA) from which PTTH is secreted into the haemolymph. The PTTH targets the prothoracic gland (PG) where it binds to Torso, the PTTH receptor.*

This triggers a cascade leading to the synthesis of ecdysone from cholesterol by the Halloween genes. Ecdysone is secreted into the haemolymph where to reach the target organs (TO) where it is turned into 20-hydroxy-ecdysone (20E), the active form of the ecdysteroid pathway. 20E binds to the ecdysteroid receptor dimer usp/EcR and starts a transcription cascade that leads to development.

The interaction between hormones in the larval-pupal transition has been extensively studied and is well-documented (Mizoguchi et al., 2001; Riddiford and Truman, 1993; Sehnal and Meyer, 1968; Shen et al., 2021). In the last larval stage of *B. mori*, 2-4 days before pupation a small burst of PTTH secretion triggers a small amount of ecdysone secretion, which in turn initiates pupation (Mizoguchi et al., 2001). As soon as the larva has pupated, the PTTH levels, that have fallen sharply during pupation, increase for two consecutive days and thereby trigger a large secretion of ecdysone, initiating metamorphosis (Mizoguchi et al., 2001). In insects entering diapause due to the short photoperiod, the first peak of PTTH secretion is missing, leading to a suppressed secretion of ecdysone delying pupation (Ahmadi et al., 2021; Mizoguchi et al., 2013).
Insect diapause can take place in any of the life stages of an insect, however it is species specific and only a few insects undergo diapause in multiple life stages (Numata and Shintani, 2023). *B. mori* as well as the gypsy moth *Lymantria dispar* enter diapause in the embryonic stage, the wall brown *Lasiommata megera* and the European corn borer *Ostrinia nubilalis* enter diapause in the larval stage (Denlinger, 2022d). Pupal diapause is common in Lepidoptera and is studied more closely in *P. napi*, *M. sexta*, and *M. brassicae* (Hartfelder et al., 1994; Lehmann et al., 2017b; Ogihara et al., 2017). Adult diapause can be found in the common brimstone *Gonepteryx rhamni* and is the most common form in the order of Coleoptera, in which it occurs in about 90% of the species (Denlinger, 2022d; Lehmann et al., 2015). The wide variety of diapause stages also indicates a wide range of hormonal programs involved in the diapause trajectory. In *B. mori* embryonic diapause is induced by secretion of the diapause hormone in the mothers and is regulated heavily by maternal factors (Hasegawa, 1957). In *M. brassicae*, PTTH acts as an endocrine switch between diapause and adult development (Mizoguchi et al., 2013). In the absence of PTTH, or the inhibition of its receptor binding the insects enter diapause (Mizoguchi et al., 2013; Yamada et al., 2017). As a consequence the ecdysteroidogenic enzymes in the Halloween pathway have a reduced expression and levels of ecdysone in the haemolymph is reduced during diapause (Ogihara et al., 2017).
The hormonal regulation of diapause termination is less well studied and most experiments so far focused on the application and injection of major developmental hormones. Pupal diapause can be terminated by 20E in *P. napi*, *Bactrocera minax, Mamestra configurata, H. zea* and *M. sexta* (Bradfield and

Denlinger, 1980; Chen et al., 2016; Meola and Adkisson, 1977; Süess et al., 2022). The ecdysone 20E was able to break the embryonic diapause of *B. mori*, and the larval diapause of *Diatraea grandiosella* and *O. nubilalis* (Chippendale and Yin, 1979, 1976; Gharib et al., 1981). Other hormones that can terminate diapause are insulin that terminates the pupal diapause in *P. brassicae* (Arpagaus, 1987). While in adults the diapause can be terminated by the application of a JH analogue (Numata and Hidaka, 1984; Schooneveld et al., 1977). Even though those life stages are different and are regulated in slightly different parts of the hormonal pathway, all of them show a link to ecdysone.

Respiratory patterns in diapause

Due to the prolonged time without being able to replenish energy resources, insects in diapause reduce their metabolic rate to an absolut minimum (Schneiderman and Williams, 1953). This reduction of metabolic rate is accompanied by the shift from continuous gas exchange to discontinuous gas exchange with the complete closing of all spiracles for prolonged periods (Jõgar et al., 2011). While discontinuous gas exchange has primarly been described in active species like the seed-harvester ant *Pogonomyrmex barbatus* and *scarabaeus* dung beetles it has also been observed in the diapause of *P. brassicae* (Davis et al., 1999; Gibbs and Johnson, 2004; Jõgar et al., 2011). The evolutionary origin of discontinuous gas exchange remains debated, with the hypothesis being the prevention of water loss in dry environments, the avoidance of hypoxic conditions, the reduction of oxidative damage and the prevention of small particels entering the body through the tracheae (Chown et al., 2006; Contreras et al., 2014; Gibbs and Johnson, 2004; Lighton, 1996). Insects immobilized during a period of several months potentially run into multiple of those problems and the presence of discontinuous gas exchange during diapause might help to alleviate some of the problems.
In *P. napi* the metabolic rate decreases significantly during diapause and only increases once diapause has terminated and temperatures have risen to levels progressing development at a high rate (Lehmann et al., 2016). However, how the physiological changes throughout diapause influences the respirometric patterns has not been analyzed and needs further studying to improve metabolic rate measurements as well as to have a proxy for metabolic processes taking place.

Aims and Methods

Diapause has been studied excessively and many high quality studies have worked out the hormonal regulation of diapause induction (Chen et al., 2016; Gharib et al., 1981; Mizoguchi et al., 2013). However, the timing and energy management during diapause, and a comprehensive model of how diapause termination is endogenously regulated remains unidentified. With this PhD thesis, I aim to advance the understanding of diapause termination regulation with the following questions:

1. **What thermal dynamics underlie diapause termination timing?**

Diapause survival is increased at low temperatures as compared to high temperatures in *P. napi* (Lehmann et al., 2017b). However, how this translates into the thermal performance of diapause termination timing has not been shown. In **Chapter I** we addressed the thermal performance of diapause termination timing, by sampling insects from constant temperatures 0°C, 4°C, 8°C and 15°C throughout winter and assessing their ability to eclose to create a thermal performance curve as described for developmental rate in summer. Additionally we overwintered insects at fluctuating temperatures, more closely reflecting the environmental fluctuations experienced by the insects under natural conditions. The treatments were 0°C – 8°C, -2°C – 10°C, 0°C – 15°C and the reverse 15°C – 0°C. Those temperatures were used as a dataset to test the TPC model created for the constant treatment and assess the prediction accuracy of the model. Furthermore, insects were put outside to reflect semi-natural conditions and the model was tested against those conditions.
The TPC for diapause termination rate was created with an adapted Lobry-Rosso-Flandrois function, by Bayesian interference.

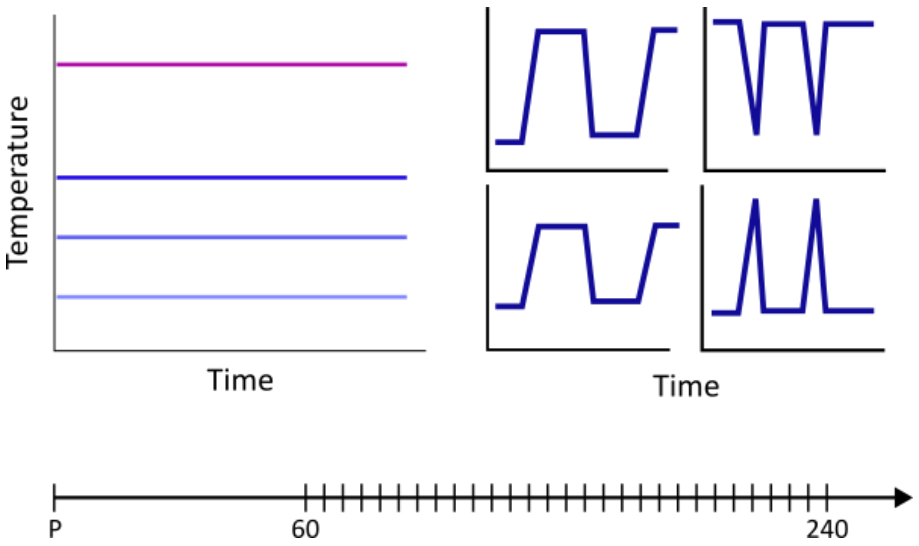

Figure 4: Temperature treatment and sampling regime. *Pupae were placed in the treatments 10 days after pupation. Left top: constant treatments at 0°, 4°, 8°, and 15°C. Top right: cyclic treatments 0-8°C, -2-10°C, 0-15°C, and 15-0°C. Bottom: sampling scheme, six pupae were taken every 4 days from each treatment from day 60 after pupation until day 240 after pupation and moved to a 20°C climate cabinet to check for eclosion (successful diapause termination).*

2. How do the major developmental hormones ecdysone and PTTH interact with the diapause and temperature and are they involved in the diapause regulation?

In **Chapter II** the presence of PTTH and the effect of ecdysone on diapause was assessed to put the diapause of *P. napi* in context with the hormonal regulation of diapause in other species. The presence of PTTH in the two dorsolateral NSC was measured using an immunohistochemical staining (IHC) protocol with an antibody against *P. napi* PTTH. Furthermore, through HPLC analysis and ELISA the size and amount of PTTH and the presence in the haemolymph in diapause and post-diapause was assessed. The effect of 20E, the active form of the ecdysone pathway on diapause termination, was evaluated by injections of different 1µg/µL, 0.1µg/µL, and 0.01µg/µL 20E at the initiation and termination of diapause and at 4°C and 18°C.

Figure 5 Immunofluorescence picture of PTTH and a schematic of the 20E injection. *Left: immunohistochemical staining of two PTTH producing cells in the brain. Right: injection of 20E into the proximity of the brain to assess the effect of 20E on diapause.*

3. **How are respiratory patterns correlated to diapause stages and metabolic rates and are the patterns temperature dependent?**

In **Chapter III** the respiratory patterns during diapause were studied with flow-through respirometry. The pupae were put in a single-channel set-up and their gas exchange was observed for several days during diapause initiation and during endogenous diapause at different temperatures and in post-diapause development.

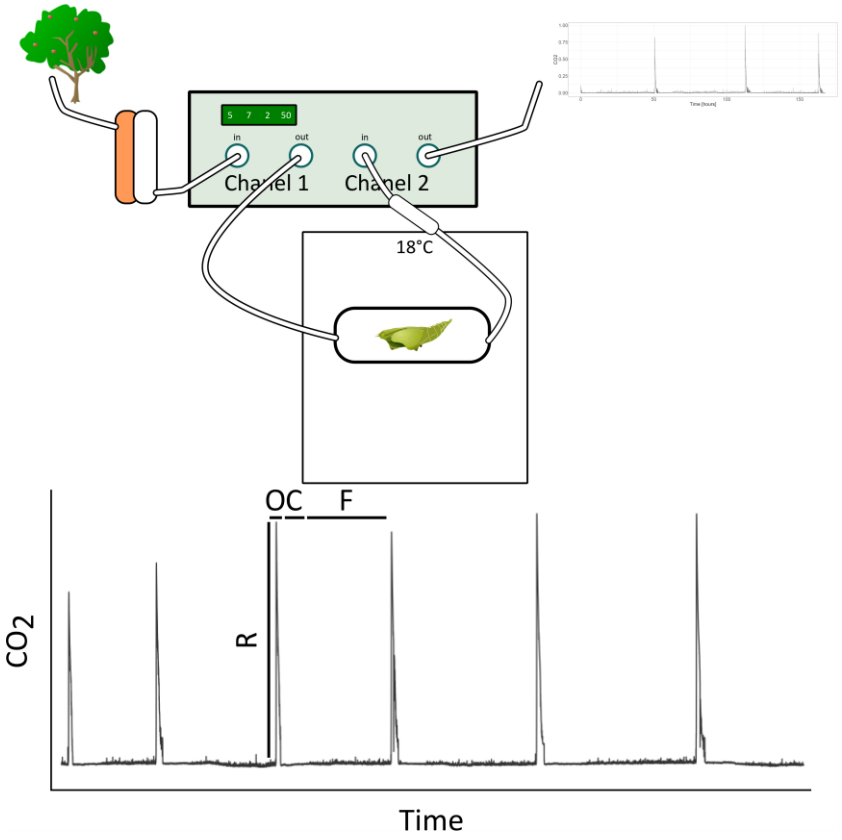

Figure 6 Schematic of a respirometer and a discontinuous gas exchange pattern. *Top: Respirometer: ambient air is scrubbed of CO_2 and water and pushed through Channel 1 as a control. The air then leaves the respirometer and is pushed through the chamber with the pupa. The air coming from the pupa is scrubbed of water vapor and entered into the respirometer which measures CO_2 every second for several days. Bottom: Discontinuouos gas exchange over several days. O = open phase, C = closed phase, F = flutter phase, R = maximum rate of discharge. 1 /(O + C + F) = frequency of DGE cycles.*

4. How is diapause termination hormonally regulated?

Based on the data from Chapter II we formulated a hypothesis on the interaction between temperature and the insulin pathway and their impact on the ecdysone pathway during diapause. In **Chapter IV** we test this hypothesis analyzing the transcriptomic data from Pruisscher et al. (2022). The transcriptomic data was subset for key developmental hormones and their immediate regulators (e.g. PTTH pathway, ecdysone pathway, insulin pathway, and signatures of development) as well as some indicators for development. Then

the mRNA levels of some regulators were compared with their protein levels found in the brain of the pupae. Furthermore, correlations between regulators interacting with each other were analyzed and put into the context of the hypothesis.

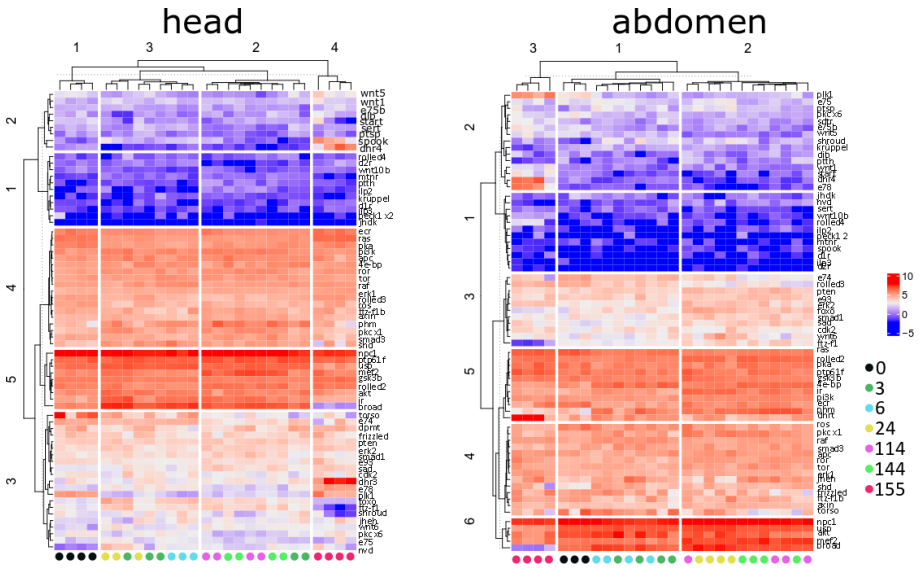

Figure 7: A heatmap of the major developmental pathways*. In the head, the day of pupation, day 0 combines into cluster 1, as does day 155, cluster 4, when the pupae have started to develop again. Early diapause groups into one cluster, 3, and late diapause groups into a separate cluster, 2, indicating 4 different stages of diapause in the brain. Day 3 of diapause is split between early and late diapause and this might be that there are still some pathways active at the beginning of diapause that are reactivated at the end of diapause. In the abdomen the subset analyzed genes group into 3 clusters. The first cluster is early diapause in which day 0, 3 and 6 are collated (cluster 1). Mid and late diapause group into the second cluster (cluster 2) and day 155 forms the last cluster (cluster 3). This indicates that there are little transcriptomic changes in the adomen during diapause initiation as well as during the endogenous diapause.*

Major findings and interpretation

Thermal performance of diapause termination

Diapause termination rate in *P. napi* under laboratory conditions follow a left-shifted thermal performance curve and can be used for the prediction of diapause termination timing under fluctuating temperatures. The left-shifted TPC found in **Chapter I** shows a T_{opt} of 0°C, a T_{min} of -9°C and a T_{max} of 29°C. When applying the TPC model to predict diapause termination timing under fluctuating it remained within an error margin of 10 days while males remained within an error margin of 5 days, demonstrating the ability to predict diapause termination timing under fluctuating conditions with confidence. However, when applied to the pupae overwintering outside, under semi-natural conditions, the predictive power of the model does not sufficiently predict diapause termination as almost all of the pupae have already terminated diapause. This suggests a mechanism of diapause termination not accounted for in the laboratory. One possibility is the photoperiod which is shown to affect diapause termination timing in some species (Denlinger, 2022c; Yang et al., 2014). However, an additional experiment in *P. napi* with different photoperiods, 24:0 dark: light, 12:12 dark: light and 0:24 dark: light, yielded no significant difference between the treatments. In nature, the photoperiod is however much more varied and photoperiod changes on a gradual scale throughout the year. Another possibility is the presence of a positive TPC for diapause initiation. As in diapause initiation the physiology of the insects is changing rapidly, the changes are most likely brought about much more quickly in higher temperatures than at lower temperatures (von Schmalensee et al., 2021). One implication of this is that by moving the pupae to low temperatures after 10 days in 18°C the diapause initiation was prolonged by the low temperatures, while in nature the warm temperatures during late summer and early autumn still allowed the insects to progress through diapause initiation quickly. This would account for the divergence between the laboratory model and the semi-natural conditions.

The combination of the diapause termination rate TPC found in **Chapter I** with the developmental rate TPC of post-diapause development creates a model of how populations can synchronize their emergence in spring over the prolonged time from late summer to spring. The implication being that insects, which have pupated earlier than their conspecifics change to the diapause termination rate TPC and follow a slow progression through diapause due to the high temperatures. This is the first step of synchronization as well as a mechanism to prevent precocious development in autumn (Lindestad et al., 2020). The second synchronization takes place in winter when the pupae that have

terminated diapause early will move to the developmental rate TPC and progress through development at a low speed due to the low temperatures while the insects still in diapause terminate quickly. This mechanism is important for ephemeral species that only live in their adult form for a couple of weeks and that have to synchronize emergence with their mates and their food sources.

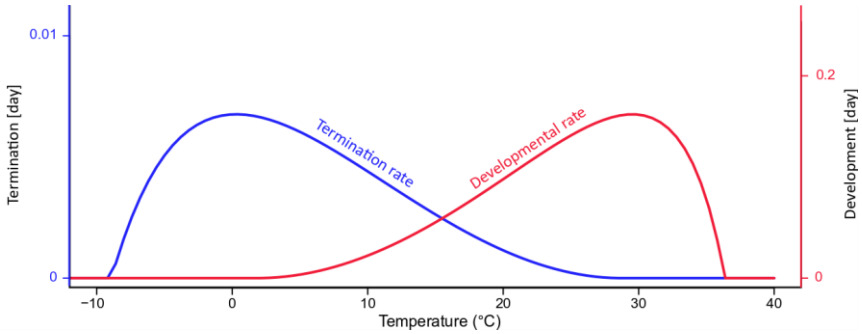

Figure 8: diapause termination rate TPC in light blue and post diapause developmental rate TPC in red. *Together acting to synchronize eclosion of adults in the spring.*

Hormonal regulation of diapause termination

The results obtained in **Chapter II** when investigating the hormonal regulation of diapause in *P. napi* supports the observation in other species like *H. virescens*, *S. ocellatella*, *H. armigera*, *O. nubilalis* and *H. zea* of how PTTH is involved in the regulation of diapause (Ahmadi et al., 2021; Gelman et al., 1992; Wei et al., 2005; Xu and Denlinger, 2003). The PTTH levels in the haemolymph are low during diapause and increase only after diapause has been terminated. While PTTH is still present in the two dorsolateral NCS throughout diapause, the intracellular patterns of the staining changes. In the beginning of diapause, staining of the PTTH peptide is smooth and the form of the cells can be clearly distinguished (Hartfelder et al., 1994). In diapause initiation, the staining changes to a granulated form with bright spots of staining and dark spots of almost no staining in the cell. In post-diapause development, the staining reverses to the smooth form as seen in the directly developing pupae and at the beginning of diapause, indicating the storage of PTTH in the NSC during diapause (Sedlak, 1981).
The effect of 20E on the diapause of *P. napi* is consistent with the effect on the diapause of other species, as 20E is able to terminate diapause (Bradfield and Denlinger, 1980; Chen et al., 2016; Meola and Adkisson, 1977). A high

concentration of 1µg 20E injected in the proximity of the brain terminated diapause in all diapause stages, and lead to the development of fully formed adult butterflies, however the insects were not able to eclose and survive. This is in line with the finding that high levels of ecdysone can delay or even prevent ecdysis in *S. crassipalpis* (Žďárek and Denlinger, 1987). The low level of 0.01µg 20E was ineffective in terminating diapause precociously. The injection of 0.1µg seems to approach levels of 20E relevant in nature, leading to diapause termination on day 1 after pupation, with the insects however being unable to eclose and survive. The sensitivity to 20E is then lost quickly throughout diapause initiation and 0.1µg of 20E has no effect on the pupa anymore at day 14 to day 30 after pupation. Thereafter diapause can be terminated precociously in a time dependent manner positively correlating with natural diapause termination progression. To test temperature dependence of the return of ecdysone sensitivity pupae overwintering at 18°C were injected with the 20E levels shown to be relevant under low temperatures. Pupae overwintering at 18°C generally do not terminate diapause and when injected with 20E those pupae were not able to terminate diapause and stayed in pupal form until they died (Lehmann et al., 2017b). This demonstrates that the sensitivity to 20E returns in a *time- and temperature dependent* manner correlating with diapause termination progression, implicating the ecdysteroid receptor in its regulation.

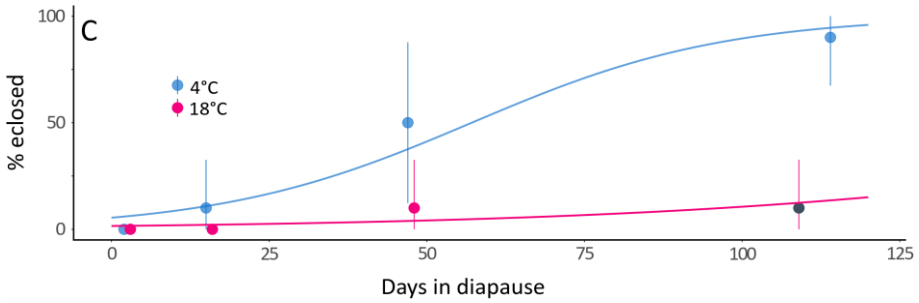

Figure 9: Diapause in* P. napi *can be terminated precociously by injecting pupae with 20E. *While this mechanism is effective for insects at lower temperatures, wherein sensitivity to 20E returns in a time-dependent manner, it does not recover at elevated temperatures.*

Respiratory patterns in diapause

Insects show plasticity in their mode of respiration and can breath continuously during activities with a high metabolic demand, and discontinuously during activities with low metabolic demand (Lehmann, 2001). The pupae of *P. napi* have been shown in **Chapter III** to respire continuously just after pupation, but changed to discontinuous gas exchange (DGE) during diapause initiation. This shift in the respiratory pattern correlates to the decrease in the

metabolic rate and the change of the diapause stage and takes place over a prolonged time, indicating the shift to be guided by physiological modifications instead of the faster neuron control (Lehmann et al., 2016; Sláma, 1999). In diapause, the DGE is defended even under high temperature conditions that are detrimental to the overwintering success, even though the amount of CO_2 per hour increases exponentially with temperature. The amount of CO_2 produced in a single open phase remains constant. This alludes to the presence of physiological constraints for the internal pressure of CO_2 (ρCO_2) and the regulation of the respiratory patterns by CO_2 as shown in other insects (Levy and Schneiderman, 1966; Rowe et al., 2022). While the amount of CO_2 expelled in a single open phase remains constant, the length of the open phase and the maximum rate of discharge of CO_2 are temperature dependent. The duration of the open phase increases towards lower temperatures, potentially due to muscular constraints at low temperatures (MacMillan and Sinclair, 2011; Mellanby, 1939). At the same time, the maximum rate of CO_2 discharge in an open phase decreases towards lower temperatures. Those two findings suggest that while CO_2 is discharged actively at high temperatures, the discharge at low temperatures takes place through passive diffusion of air (MacMillan and Sinclair, 2011; Sláma, 1988). The main alleviation of the increased metabolic rate under high temperatures takes place however through the increase of the frequency of the DGE cycle, more open phases during the same time under high temperature conditions. This furthermore, is another indication that the discharge under high temperatures is taking place actively to shorten the duration of the open phase and therefore reduce the amount of exchange with the environment and the evaporation of water (Jõgar et al., 2004).

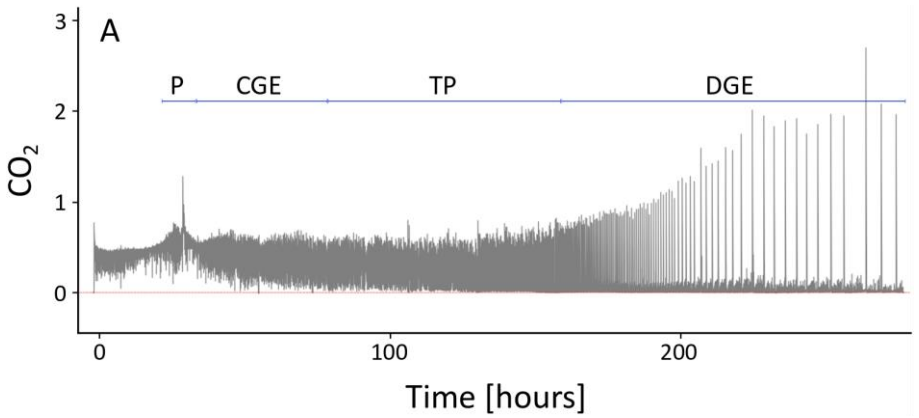

***Figure 10: Respiratory pattern in diapause initiation and the gradual change from continuous gas exchange (CGE) to discontinuous gas exchange (DGE) through a period of transition (TP)**. P = pupation.*

A hypothesis for the hormonal regulation of diapause termination

In **Chapter IV** we tested the hypothesis proposed in Chapter II, where we propose that during diapause initiation the ecdysteroid receptor is silenced by the binding of Foxo to ultraspiracle (Usp), binding partner in the heterodimeric ecdysteroid receptor (Koyama et al., 2014). This inhibition of the receptor is then removed in a *time- and temperature* dependent mechanism by the secretion of insulin-like peptide 2, an ILP receptive to environmental temperatures (Li and Gong, 2015). The insulin pathway is involved in the phosphorylation and the subsequent degradation of Foxo and therefore the potential release of the ecdysteroid receptor (Calnan and Brunet, 2008). After freeing the ecdysteroid receptor, low levels of ecdysone cause the upregulation of PTTH synthesis and secretion thereby causing the increase of ecdysone synthesis and secretion (Mizoguchi et al., 2015).

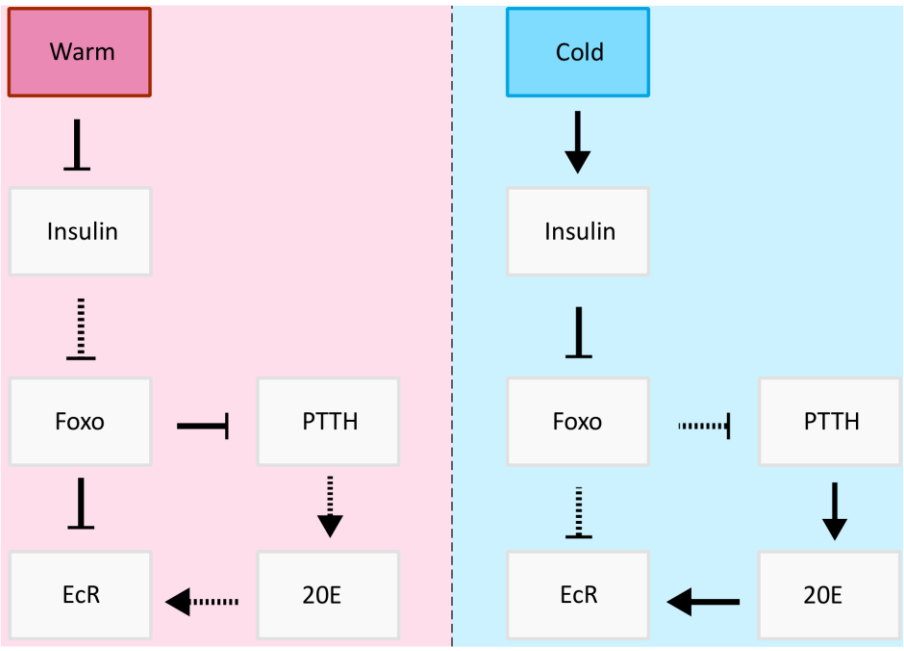

Figure 11: working model of the hormonal regulation of the diapause termination program. *The increase of Foxo at the beginning of diapause silences the ecdysteroid receptor preventing the signaling cascade of the PTTH ecdysone axis. In diapause low temperatures cause the secretion of ILP2 resulting in the phosphorylation of Foxo and the step-wise return of ecdysone sensitivity. Under high temperature conditions ILP2 is not secreted and Foxo remains bound to the ecdysteroid receptor.*

When sub-setting the transcriptomic data with a manually curated list of genes in the developmental hormone regulation, we found that the PCA of our data, shows a similar pattern as with the overall transcriptomic data found in Pruisscher et al. (2022). The PCA pattern in diapause follows a different trajectory on PC1 and only resembles the pattern in directly developing insects once they are in post-diapause development. The mRNA levels of PTTH as well as the protein levels corroborate the findings of Chapter II that showed low levels of PTTH protein in the haemolymph. The PTTH mRNA levels also correlate with the protein levels at the high temperatures during diapause initiation, however this correlation is lost in diapause, indicating changes in post-transcriptional and post-translational mechanisms (Dahan et al., 2011; Fortelny et al., 2017; Liu et al., 2016). Furthermore, the PTTH monomer is enzymatically inactive, the dimer is the active form, and with diapause progression, the ratio between monomers and dimers changes in favor of the active dimers (Kataoka et al., 1991). The immediate response factors to PTTH are downregulated, Raf/Ras, and Erk, corroborate a downregulation of the PTTH axis during diapause initiation and endogenous diapause (Smith and Rybczynski, 2012). The downstream factors of the ecdysone signaling are downregulated throughout diapause and support the idea of a silencing of the ecdysone sensitivity. This is further supported by the upregulation of the *foxo* gene. Further substantiating the hypothesis is the observed upregulation of the insulin pathway throughout diapause. However, while the protein Foxo negatively correlates with the abundance of *ilp2* mRNA, the Foxo protein levels do not correlate with the *foxo* mRNA levels implying a strong regulatory mechanism on the post-translational level. However, regardless of the difference between the protein levels and mRNA levels, the Foxo protein levels are highest at the initiation and beginning of endogenous diapause supporting the the idea that Foxo is involved in silencing the ecdysteroid receptor. The mismatch between mRNA and protein is potentially explained by the fact that Foxo is a common protein expressed in nearly every cell and by sampling the whole head instead of only the brain, the signals might be biased (Calnan and Brunet, 2008). Many of the findings in this chapter support the hypothesis and further advances in the proteomic analysis of diapause regulation will answer more of the still remaining questions. Furthermore, the mismatch between mRNA levels and protein levels should be analysed too as there might be some post-transcriptional or post-translational processes specific to diapause (Saito et al., 1985).

All the chapters combined give a new insight into the mechanisms of diapause regulation and open up new avenues of research. Chapter I and Chapter II combine the finding that diapause termination progression is a left shifted performance curve with an optimum at 0°C with the finding that sensitivity to ecdysone only returns when insects have experienced low temperatures

throughout diapause. Chapter II and Chapter IV are connected by the hormonal regulation of diapause termination and we produced a testable hypothesis on the hormonal regulation of diapause. Chapter III deepens our understandin in the respirometric patterns and connects to Chapter I where we showed that high metabolic rates at high temperatures lead to a decrease in successful termination of diapause. All those findings are aimed at the goal to further our understanding of insect diapause a major period in the life of most insect populations in the temperate regions. Understanding the regulation of diapause will allow us to get new insights into insect population development important in agriculture and conservation biology and can add novel mechanisms in the hormonal pathways not observed in developing insects.

Future projects

The aim of my PhD thesis was the description of the hormonal regulation of diapause termination through the four chapters of my thesis. I have found several lines of evidence that contribute to the understanding of diapause termination regulation and towards the completion of a definitive singalling model (Dong et al., 2013; Lehmann et al., 2017b; Ragland and Keep, 2017; Toxopeus et al., 2023). Diapause is a central life history trait of insects in the temperate regions and has evolved multiple times in numerous families of insects (Tauber et al., 1986). In my PhD thesis I focused on the diapause regulation in *P. napi* that undergoes pupal diapause, however larval and embryonic diapause appear to be regulated through similar hormonal pathways (Chippendale and Yin, 1976; Kidokoro et al., 2006; Singtripop et al., 2002; Tawfik et al., 2002). Even adult diapause that has been shown to be regulated by the juvenile hormones (JH), is linked to ecdysteroids by the interaction of JH, insulins and ecdysteroids (Liu et al., 2018; Numata and Hidaka, 1984). Future studies should focus on the e hormonal regulation of diapause termination in insects with a pupal diapause, and analyze the ability of Foxo to bind to usp and thereby silencing the ecdysteroid receptor. This interaction could be of interest in the agricultural sciences as a means to regulate pest populations. An experiment in *P. napi* showed that vertebrate insulin was able to terminate diapause, however further work has to be conducted on the interaction of insulin with time and temperature (Arpagaus, 1987; Li and Gong, 2015). Once this mechanism has been established it would be imminent to study further insects to see whether they follow the same mechanism or if there is a multitude of mechanisms that evolved in the phylogenetic tree of insects in temperate regions. The first step should be to compare the cabbage white *Pieris rapae*, a close relative to *P. napi*, which shows higher mortality in winter than *P. napi* (von Schmalensee et al., 2023). Analyzing the two closely related species in their approach to overwintering and the hormonal regulation will not only lead to a better understanding of diapause but can also improve our knowledge about the major hormonal pathways and their interaction with environmental factors like temperature and photoperiod. To expand the knowledge about the regulation of diapause termination, the frame work from this thesis should be taken as a basis and then applied to other insects. The hypothesis is that insects that overwinter in the larval and embryonic stage and have been shown to have diapause terminated by the injection of 20E showing a similar hormonel regulation (Singtripop et al., 2002; Tawfik et al., 2002). The diapause in the adult stage has been revealed to respond to JH and is mainly affecting the sexual organs of insects (Hodek, 2011; Numata and Hidaka, 1984). The comparison of similarities and differences in the hormonal regulation between the different life stages can reveal major convergence in

the hormonal pathway and reveal targets that respond quickly to environmental conditions (Numata and Hidaka, 1984; Schooneveld et al., 1977). Finally, it would be paramount to connect the hormonal regulation of diapause induction with the regulation of diapause termination to have a holistic understanding of the hormonal regulation of diapause. For this, assessing the levels of PTTH and ecdysone in diapause induction and diapause initiation in association with the photoperiod will deepen the knowledge of the interactions of the major developmental hormones with the environment.

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Sammanfattning

Diapaus är en viktig del av de flesta insekters livscykel och är ett utvecklingsstopp som induceras av miljösignaler före försämrade förhållanden. Insekter undviker aktivitet under perioder då miljöförhållandena är ogynnsamma för fysiologiska funktioner som kalla temperaturer, dålig matkvalitet eller frånvaro av arter. I de tempererade områdena på vår planet inträffar diapaus vanligtvis under de kalla och matfattiga vintermånaderna och utlöses av en kortare fotoperiod på sensommaren jämfört med försommaren. Medan utvecklingen hos insekter i allmänhet följer en termisk prestandakurva (TPC) av varmare temperaturer som leder till snabbare utveckling, är insekternas diapauseöverlevnad hos många arter högre när övervintring sker under kalla förhållanden. Därför bedömde vi i kapitel I den termiska prestandan för diapausavslutningshastigheten hos den rapsfjärilen *Pieris napi*. Diapausavslutningshastigheten för *P. napi* följde en vänsterförskjuten TPC med en optimal avslutningshastighet vid 0°C och lägre avslutningshastigheter mot högre temperaturer. Denna vänsterförskjutna TPC förhindrar att diapausen avbryts för tidigt på hösten samtidigt som den gör det möjligt för populationer att förbli synkroniserade under en lång period. I kapitel II studerade vi närvaron och frånvaron av PTTH i hjärnan samt dess förekomst i hemolymfan under och efter diapaus. Vidare, genom att injicera diapauserande insekter med 20-hydroxi-ecdyson (20E), den aktiva formen av ecdyson-vägen, analyserade vi effekten av ecdyson på diapaus. Neuropeptiden PTTH är närvarande under hela diapausen i två par neurosekretoriska celler. Vid diapausinitiering ändrades färgningen för PTTH från slät till granulat, vilket indikerar förändringen i den intracellulära strukturen hos PTTH. I hemolymfen finns det lite PTTH under diapaus och högre nivåer efter att diapaus har avslutats. Injektionen av 20E visade en tids- och temperaturberoende återgång av känslighet för ecdysonvägen, vilket implicerade receptorn i regleringen av diapausavslutning. I kapitel IV följde vi upp denna ledning och testade en hypotes om den hormonella regleringen av diapausavbrott genom interaktionen av Foxo, ecdysteroidreceptorn såväl som insulin i puppornas transkriptom under diapausen. De transkriptomiska data kompletterades med några proteinnivåer av nyckelregulatorer. Flera av de viktigaste hormonella regulatorerna visade det förväntade mönstret i transkriptomet som stöder hypotesen och kräver ytterligare undersökning. Proteinnivåerna av Foxo korrelerade dock inte med den transkriptomiska profilen och medan PTTH-profilerna korrelerar i början av diapausen går denna korrelation förlorad under diapausen, potentiellt på grund av förändringen i fysiologi såväl som de låga temperaturerna. I kapitel III undersökte vi de respirometriska mönstren under diapausen för att bedöma hur diapausfysiologin såväl som den reducerade ämnesomsättningen påverkar andningssättet. Vi fann att under diapausinitiering ändras det respirometriska

mönstret långsamt från kontinuerligt gasutbyte till diskontinuerligt gasutbyte (DGE). Denna DGE bibehålls under hela diapausen även under höga temperaturer. Efter att diapausen har avslutats förblir puppor i låga temperaturer i DGE medan övergången till kontinuerligt gasutbyte under höga temperaturer sker snabbt. Detta indikerar att DGE är associerat med diapausstadiet på en fysiologisk nivå. Dessa fynd visar att de tre nivåerna av temperatur, metabolisk hastighet och de hormonella vägarna är tätt kopplade till diapausfysiologin och är potentiellt involverade i regleringen av diapausavslutningstidpunkten.

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