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From environmental cue to phenotypic variation – a functional investigation of wing polyphenism in an emerging model species

ERIK GUDMUNDS



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2023

ISSN 1651-6214
ISBN 978-91-513-1697-0
URN urn:nbn:se:uu:diva-495377

Dissertation presented at Uppsala University to be publicly examined in Ekmanssalen 15:00024, EBC, Norbyvägen 16, Uppsala, Friday, 17 March 2023 at 13:30 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Professor Laura C. Lavine (Department of Entomology, Washington State University, Pullman, WA 99164-6382).

Abstract

Gudmunds, E. 2023. From environmental cue to phenotypic variation – a functional investigation of wing polyphenism in an emerging model species. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology* 2231. 57 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1697-0.

Phenotypic plasticity is a ubiquitous feature of living organisms and enable individuals to adapt to changing environments. A particularly prominent example of plasticity is found in polyphenisms, where environmental cues received during development leads to the generation of discrete variation in populations. In this thesis, I have studied the mechanisms underlying wing polyphenism in the water strider *Gerris buenoi* in order to contribute to the understanding how environmental factors can act through growth regulatory systems to induce adaptive variation. Specifically, in Paper I, I investigated which environmental factors control wing morph determination in *G. buenoi* and found that this species most strongly responds to variation in photoperiod conditions, but also to crowding during the juvenile stages. Exposure to challenging nutritional conditions had no effect on wing morph frequencies. Further, I found that the nutrient sensitive insulin/insulin-like growth factor signaling pathway, which have been found to regulate wing polyphenism in species where nutrition is a determinant cue for wing morph induction, has no role in regulating *G. buenoi* wing polyphenism, an observation in line with data showing that wing morph determination is robust to variation in nutrient conditions. In Paper II, I explored a role for the developmentally important hormones ecdysone and juvenile hormone in *G. buenoi* wing polyphenism. Here, I used microinjections of 20-hydroxyecdysone and topical application of methoprene, as well as RNAi against hormone receptors for ecdysone and juvenile hormone. In these experiments, I found a small but significant effect of RNAi against the ecdysone receptor, indicating that ecdysone may play a role in wing morph induction. In Paper III, I used RNA sequencing to identify candidate growth regulatory pathways for wing morph induction by photoperiod and found a significant role for the conserved Fat/Hippo pathway in *G. buenoi* wing morph determination. Taken together, the results presented in this thesis suggest that evolution of genetic mechanisms underlying wing polyphenism may be constrained with regard to the particular environmental cue that is used to predict the future adaptive landscape. Further, the work presented in this thesis demonstrates the power in combining sequencing methods with functional genetic tools in order to more deeply characterize the causal basis to adaptive variation, an approach to ecological and evolutionary studies which I reviewed in Paper IV.

Keywords: *Gerris buenoi*, water strider, wing polyphenism, photoperiodism, Fat/Hippo signaling

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ISSN 1651-6214

ISBN 978-91-513-1697-0

URN urn:nbn:se:uu:diva-495377 (<http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-495377>)

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List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

- I. Gudmunds, E., Narayanan, S., Lachiver, E., Duchemin, M., Khila, A., Husby, A. (2022) Photoperiod controls wing polyphenism in a water strider independently of insulin receptor signalling. *Proceedings of the Royal Society B: Biological Sciences*, 289(1973):20212764
- II. Gudmunds, E., Armisen, D., Mesilaakso, L., Scofield, D., Khila, A., Husby, A. (2023) Contribution of ecdysone signaling to wing polyphenism in the water strider *Gerris buenoi*. *Unpublished manuscript*.
- III. Gudmunds, E., Armisen, D., Eriksen, F., Khila, A., Husby, A. (2023) Wing polyphenism in a water strider is caused by photo-period-induced expression of the Fat/Hippo pathway. *Unpublished manuscript*.
- IV. Gudmunds, E., Wheat, CW., Khila, A., Husby, A. (2022) Functional genomic tools for emerging model species. *Trends in Ecology & Evolution*, 37(12):1104-1115

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Introduction

Synopsis

In a broad perspective, this thesis addresses a fundamental question in evolutionary biology – What are the sources of phenotypic variation? This is a question which can be studied in many dimensions; spanning from how species come to vary at macroevolutionary time scales to how populations adapt to short-term environmental fluctuation. The particular dimension that I have been interested in is that of mechanism and function. The reason why this dimension is important to study in the context of evolutionary biology originates from the fact that selection acts on phenotypes and phenotypes stem from the functional properties of genes and their interaction with the environment. Simply put, if we do not understand the mechanisms that generate phenotypic variation, we will not be able to identify exactly what selection is or has been acting upon. Therefore, by elucidating the mechanisms that generate phenotypes and variation in them, we can be more precise in describing how evolution takes place, and frame research questions with higher accuracy. Through this line of thinking, I hope that the work presented in this thesis can provide insight into how phenotypic variation originates. In the coming sections I describe the reasoning behind this hope with more contextual details.

The organism that I have studied, *Gerris buenoi*, belongs to the semi-aquatic insect group referred to as water striders, or in systematic terms as Gerridae. The water striders, like most insect groups, display phenotypic diversity in a wide range of traits (Andersen, 1982; Armisen et al., 2022, 2018) and one of these traits is the capacity to disperse by flight which is the trait I have studied in this thesis.

The evolution of flight is a factor that is commonly brought up to explain the evolutionary success of insects (Condamine et al., 2016; Labandeira et al., 1994; Linz and Tomoyasu, 2018; Rainford et al., 2014; Roff, 1990). It may thus appear strange that the capability to fly has been lost in many insect lineages, including in many water strider species (Roff, 1990; Wagner and Liebherr, 1992). However, the development, maintenance and use of the insect flight apparatus is costly in terms of energy expenditure, and in conditions where flight might not be of importance for survival and reproduction, it may be advantageous to direct the available energy to production of offspring instead. This within-individual competition for resources is a well-known life history trade-off between dispersal and reproduction (Zera and Denno, 1997)

and has been demonstrated in many insect species and is in many cases a likely explanation for loss of flight (Guerra, 2011).

The expression of a functional flight apparatus, i.e. fully sized and patterned wings and wing muscles, is a variable trait within some insect species and populations. Here, induction of flight capability, or incapability, can occur either through genetic variation encoding the respective phenotype (Braendle et al., 2005; Li et al., 2020), or it can occur through exposure to certain environmental factors, such as nutrition, temperature or photoperiod (Fairbairn and King, 2009; Fawcett et al., 2018; Hardie, 1980; Lin et al., 2018). Additionally, genetic and environmental factors can interact to determine whether wings and wing muscles should be developed or not (Fairbairn and King, 2009). As a result, discrete variation in flight capability can be observed within populations and species, commonly taking the outward appearance of wing length variation or winged and wingless forms (Zera and Denno, 1997). This phenomenon is called wing dimorphism and has evolved in a diversity of insect lineages (Roff, 1986).

A common way to distinguish between the modes of induction in wing dimorphisms, i.e. whether the wing phenotype is induced through genetic or environmental factors, is to use the terms wing polymorphism and wing polyphenism, respectively (Zera, 2009). I will use this terminology throughout this thesis, however, in cases where it may not yet be known how wing morphs are determined or to refer to the capacity of a species or population to express alternative wing morphs regardless of induction mode, I will use the term wing dimorphism.

Wing dimorphism is a variable trait within the water strider phylogeny. Some species are wing dimorphic, while others are obligately monomorphic, with only winged or wingless individuals (Andersen, 1982; Armisen et al., 2022). Both wing polyphenism and wing polymorphism are found among the wing dimorphic species, such as in *Gerris odontogaster* where wing morph is determined by photoperiod (Vepsäläinen, 1971), or as in *Gerris lacustris*, in which morph induction depends on genetic factors (Vepsäläinen, 1974). The water strider that I have studied is wing polyphenic, as earlier studies (Spence, 1989) as well as my own (**Paper I**) have demonstrated.

The variability in wing dimorphism in water striders provides the opportunity to explore several aspects regarding the evolutionary dynamics of genetic versus environmental trait determination systems, or phrased differently, how species evolve to become either more or less phenotypically plastic. One of these aspects is whether the genetic and environmental factors that cause wing length variation act through the same or through different regulatory systems. For example, since there are a number of possible ways to reduce the size of an insect wing (López-Varea et al., 2021), or abolish a wing completely (Abouheif and Wray, 2002), it is conceivable that genetic and environmental factors can act through separate regulatory systems to cause variation. It is also conceivable, however, that the evolutionary history of the trait as well as

developmental genetic factors, have led to constraints in how wing dimorphism is regulated, and that genetic and environmental regulation work by the same or through similar mechanisms (Nijhout and Davidowitz, 2009). By providing answers to questions of this nature we may gain insight into evolutionary potential or limitations of phenotypic plasticity.

To go back to the reasoning in the first paragraph in this section, I view my work presented in this thesis as an entry point into exploring aspects of evolution of phenotypic plasticity, such as those outlined above. Specifically, the purpose in identifying the mechanisms underlying wing morph determination in *G. buenoi* has not only been to gain knowledge about how insect polyphenisms operate, which is an interesting topic in itself. But it has also been to provide the basis for comparative studies directed towards identifying the genetic variation underlying wing dimorphism in water striders in a macroevolutionary and microevolutionary perspective. For example, by identifying which mechanism(s) act in one species to environmentally induce development of a short-winged individual, one gains a testable hypothesis on whether the same mechanism act in other species where induction of short wings is genetically determined. Testing such a hypothesis with ample methodology can have two equally interesting outcomes; that evolution of genetic respectively environmental modes of short-wing induction have or have not converged on the same regulatory system. Systematically asking this question in several species may then reveal patterns of constraints or flexibility in the evolution of wing dimorphism, thereby allowing inference on the evolutionary dynamics of phenotypic plasticity.

In the coming introductory sections, I outline important processes and literature that are of high relevance to the work presented in later parts of this thesis.

Phenotypic plasticity

Organisms live in heterogenous environments and are thus required to accommodate and respond to environmental variation in order to survive and reproduce. This environmental responsiveness of organisms occurs in countless ways reflecting the enormous diversity of life, and is broadly referred to as phenotypic plasticity and commonly defined as:

“The capacity of a single genotype to exhibit a range of phenotypes in response to variation in the environment.” (Whitman and Agrawal, 2009).

While this definition of phenotypic plasticity is generally accepted it is worthwhile to point out that variations exist (see Whitman and Agrawal 2009 and West-Eberhard 2003 for discussion of definitions). Additionally, it is important to note that this definition does not restrict phenomena of plasticity to

such examples which are considered as highly evolved and clearly adaptive, such as textbook examples like predator-induced morphological variation in *Daphnia* (Lüning, 1992) or coat color variation in sub-arctic mammals (Zimova et al., 2018). It also includes phenotypic variation expressed as reactions to potentially stressful environments, e.g. abiotic substances, extreme temperatures, low or high pH, lack of oxygen or poor nutrition. Additionally, it includes novel expression of phenotypes in response to new environments or environmental change (Zera, 2009). Generation of phenotypic variation through such scenarios might very well be, and definitively have been, considered mere susceptibilities that generate phenotypic anomalies (West-Eberhard, 2003b). However, the reason why they are important to include in the definition of phenotypic plasticity is because any expression of phenotypic variation has the potential to be selected, and such susceptibilities may have constituted the selectable starting material which have led to the evolution of the many adaptive plastic responses we know about today (West-Eberhard, 2003a).

The standard way to assess and visualize plasticity is to plot the phenotypic value of a trait against an environmental gradient, this is called the reaction norm (Whitman and Agrawal, 2009). The appearance of the reaction norm shows the degree of plasticity. For example, considering a basic scenario with a linear phenotypic response to environmental variation, steep slopes signify a higher degree of plasticity whereas less steep slopes indicate less or no plasticity (if the slope is zero). Genetic variation in plasticity is reflected in differences in reaction norms between individuals with different genotypes and forms the basis for evolution of plasticity (Lynch and Walsh, 1998). Research have shown that such variation, also called gene-by-environment effects, is common in natural populations (Goldstein and Ehrenreich, 2021). However, how selection act on reaction norms have been a subject of debate (Gotthard and Nylin, 1995) and can be difficult to demonstrate (Hendry, 2016). Several convincing examples have nevertheless been reported (Scheiner and Levis, 2021) and much conceptual work in the last three decades has been developed that make a strong case that phenotypic plasticity has a fundamental impact on evolution and generation of adaptive phenotypic diversity (Pfennig, 2021).

Plasticity in organisms occur in many distinguishable ways, for example, some plastic responses can be highly specific whereas other can be very general, such as the ubiquitous effects of nutrition and temperature on growth (Blanckenhorn, 2009), which represent a case of responsive plasticity. A contrasting term is anticipatory plasticity, where the phenotypic effects in individuals are induced before being exposed to the environment to which the plasticity is an adaptation to, for example induction of diapause as winter approaches (Zera, 2009). Another distinction between different modes of plasticity is whether it is reversible or not. Examples of reversible plasticity includes that of winter coat coloration in mammals, mentioned above, or changes in muscle mass in relation to physical activity. Here, the particular

phenotypic changes are reversed at the time when either the season change again or the stimuli of physical conditioning is removed. Examples of irreversible plasticity include temperature-dependent sex determination in reptiles (Rhen and Schroeder, 2016) and most kinds of morphological plasticity in insects, the latter of which is discussed below.

Morphological plasticity in insects

The insects display a remarkable diversity in shape, size and color, and plasticity has been documented in a wide variety of morphological traits; wing shape (Reis et al., 2021), color (van der Burg and Reed, 2021) and size (Hayes et al., 2019), leg length (Toubiana et al., 2021) and shape (Siddiqui et al., 2019), antennae (Greenwood and Chapman, 1984) and more. Virtually all levels of organismal regulatory systems can be involved in producing plastic responses, including chromatin organization, gene expression, protein synthesis rates, post-translational modifications, protein-protein interaction, hormone synthesis and release, metabolite accumulation and neuronal activity. Moreover, morphological variation generated by these processes can be expressed in various insect life stages. While plasticity expressed at any time in an insect's life can be of adaptive importance, I will hereon limit the discussion to only such traits that are manifested in the adult stage, which is the case for wing dimorphism in water striders and which also has been the focus of many of the studies on environmentally induced morphological variation in insects.

The morphology of adult insects is as a rule irreversible and plastic responses typically occurs as a result of environmental input to physiological and developmental processes acting during defined stages of embryonic or post-embryonic development (Nijhout and Davidowitz, 2009). While environmental influence on these processes can lead to broad phenotypic changes, such as the effect of nutrition on overall body size, which occur in virtually all insects (Koyama and Mirth, 2018), some insect species also display more specific environmentally induced morphologies. For example, the third leg pair in males of the semi-aquatic riffle bug species *Microvelia longipes* grows proportionally much larger compared to the first or second leg pair in response to nutritional conditions. Here, the third leg pair is used as a weapon to compete with other males for suitable egg-laying spots (Toubiana and Khila, 2019). A similar exaggeration of growth induced by nutritional conditions is found in horn size of males of *Ontophagus* beetles, where the horns are used as weapons in the competition for females (Moczek, 2009). Other examples of specific plastic responses generating adaptive morphologies are wing coloration in Lepidoptera (Brakefield and Frankino, 2009) and wing polyphenisms in Hemiptera (Hayes et al., 2019).

Insect polyphenism

The use of the term polyphenism has drifted extensively from its original definition by Ernst Mayr in 1963, who introduced the term to describe all kinds of non-genetic phenotypic variation (Canfield and Greene, 2009; Mayr, 1963). Today it is more commonly used to describe discrete or discontinuous phenotypic variation which is induced by environmental factors. As hinted above, I will use polyphenism to describe environmentally induced discrete variation whereas the term polymorphism will be used for discrete variation induced by genetic factors.

A commonality among many cases of insect polyphenisms is that the discrete variation observed in one trait, e.g. wing length in wing polyphenic insects, is accompanied by variation in a variety of other traits. For example, as covered above, long-winged individuals of wing polyphenic insects develop fully functional wing musculature in order to capacitate flight, whereas the wing musculature in short-winged individuals is underdeveloped (Zera, 2009). Furthermore, long-winged and short-winged individuals commonly differ in thorax volume, diapause incidence and time to reproduction (Andersen, 1982; Li et al., 2020; Zera and Denno, 1997). Similarly, wing coloration or presence/absence of eye spots is often the most obvious variable traits in seasonal polyphenisms of lepidopterans, but other traits co-occur with these, including diapause, metabolism, flight behavior and wing shape (Brakefield and Frankino, 2009). Therefore, the phenotypic variation generated by polyphenisms should in many cases not be viewed as discrete variation in isolated traits, but rather as highly integrated responses which generate individuals that differ in a suite of traits and thus constitutes different morphotypes, or morphs.

The occurrence of alternative morphs in a population is generally taken as a sign that polyphenisms are adaptive and that a particular morph is better suited than the other in a given environment. However, the adaptive significance of morphs can be difficult to study in natural settings and laboratory studies are limited in the environmental complexity that can be achieved. For some species convincing evidence is nevertheless available (Brakefield and Frankino, 2009). One example is the western white butterfly *Pointia occidentalis* which develop two alternative adult morphs with different melanic patterns on the wings (Kingsolver, 1995). The melanized morph, which is induced by short spring-time photoperiods, is more capable of flying in cool conditions because of the thermal effects of melanization and specific basking behavior, whereas adults emerging during the summer have reduced wing melanization and are less prone to overheating (Kingsolver, 1995, 1987; Watt, 1969). Through mark-release-recapture and color manipulation experiments it was demonstrated that individuals with darker wings survive to a significantly lower degree during summer conditions compare to individuals with less dark wings (Kingsolver, 1996).

Environmental and endocrine basis of morph determination

The example of *P. occidentalis* above demonstrates a common theme of polyphenic responses; the morph-inducing environmental factor(s) do not necessarily have to be the same environmental factor(s) that have implications on fitness, depending on which morph is expressed. In the *P. occidentalis* polyphenism, photoperiod is the morph-determining factor whereas the polyphenism is presumably an adaptation to environmental constraints in flight conditions. Similarly, wing length polyphenism induced by high population density during juvenile stages is presumably an adaptation to future competition for resources. Polyphenisms are thus characterized by the use of environmental factors as cues to predict future conditions, where the particular value of the cue, e.g. hours of daylight or darkness, degree of contact with conspecifics, high or low temperatures, leads to the expression of the alternative morphologies.

Since adult insect morphology is irreversible due to the inability of adult insects to grow, inductive cues must be sensed and acted upon during embryonic or post-embryonic development. The environmental sensing process is normally described to occur during a sensitive window, a defined time in development when environmental information can be integrated and stored in the physiological systems of an individual (Nijhout, 2003). Through internal signaling, this information is then released to affect the developmental processes which generate the adult morphology. The internal signaling processes are orchestrated by hormones and it is through variation in hormone release and the effects of hormones in tissues that development of different morphs is induced (Nijhout, 2003).

Hormone secretion occurs from a variety of tissues in insects and are under the control of internal factors, such as developmental progression, as well as factors of external origin, like the time of day or nutritional levels (Nijhout, 2013). For example, the hormone ecdysone (or 20-hydroxyecdysone) is released by the prothoracicotropic gland (PTG) when critical thresholds of size have been passed to induce moulting (Nijhout et al., 2014), but can also be released in photoperiodically entrained circadian intervals with peaks in scotophase and troughs in photophase (Steel and Vafopoulou, 2006). Physiological responses to varying nutrient levels are in part mediated by insulin-like peptides (ILPs), which are released from the central nervous system (Antonova et al., 2012). Another example of an important insect hormone is the juvenile hormones (JHs) which are released by the corpus allatum and determine whether a moulting event should generate a juvenile or an adult individual (Schiesari et al., 2011). High levels of JH keep the individual in the juvenile stages, whereas low levels of JH lead to a metamorphic moult generating an adult (Goodman and Cusson, 2012).

The signaling action of hormones is received by receptors expressed in peripheral tissues that transduce the circulatory signal to internal growth

regulatory pathways and developmental genetic programs (Antonova et al., 2012; Goodman and Cusson, 2012; Henrich, 2012). Importantly, tissues have different sensitivities to hormones, forming the basis of tissue-specific responses, where a secretion pulse of a hormone can have large effect in one tissue but a milder effect in others (Frederik Nijhout, 2013; Uyehara et al., 2022). Given that JH, 20E and ILPs are the most common hormones found to regulate polyphenisms, and that these hormones have widespread roles in regulation of growth and development apart from polyphenic responses, tissue specific hormone sensitivity may be a particularly important mechanism that enables polyphenisms to evolve.

The hormonal control of morph determination has been investigated in numerous species (Fawcett et al., 2018; Hardie, 1980; Iwanaga and Tojo, 1986; Vellichirammal et al., 2017; Xu et al., 2015; Zera, 2004) and empirical evidence of hormone titer measurements as well as results of hormone manipulation experiments have generated models for how hormones can regulate polyphenisms. Below I outline a few examples of these models in relation to development to alternative morphs.

Seasonal coloration polyphenism in Lepidoptera wings

The adaptive basis of light and dark morphs of butterflies was exemplified above for *P. occidentalis*, however, wing color polyphenisms are common in Lepidopterans and through research in several species a common pattern of hormonal regulation has emerged, even though the direction of environmental responses can differ between species (Brakefield and Frankino, 2009). In all known cases, sensitivity to photoperiod and temperature during the larval stages controls whether an individual develops to either the dark or the light-colored morph (Hartfelder and Emlen, 2012). The environmental information is transduced to the internal environment early in the pupa by the secretion of ecdysone. Here, the developmental program for wing coloration is sensitive to ecdysone signaling in a defined critical window and is therefore programmable by the timing of ecdysone release. Low levels of ecdysone during the critical window is permissive of melanin production whereas high levels of ecdysone instead inhibit melanization (Nijhout, 2009). Importantly, the amplitude and duration of the ecdysone peak is the same between morphs but differs in timing, where an earlier onset of ecdysone secretion occurs in those individuals that develop to the light-colored wing morph (Burg et al., 2020).

Wing dimorphism in Hemimetabolous insects

Investigations of the endocrine basis of wing dimorphism in Hemimetabolous insects has to a large extent concentrated on ecdysone and JH and have largely built on knowledge of how these hormones regulate of the processes of ecdysis and metamorphosis (Zera, 2009; Zera and Denno, 1997; Zhang et al., 2019). Here, experimental evidence from a handful of species led to the formulation of the so-called classical model of hormonal regulation of wing

dimorphism (Zera, 2004). Central to this model is the dynamics of decrease in JH titer and the surge of ecdysone occurring in the ultimate instar to induce the metamorphic moult. As briefly touched upon above, the role of JH in insect development is to direct the developmental program during juvenile stages so that the next moult either occurs to another juvenile (high JH titers), or to an adult (low JH titers). This is regulated in concert with surges in the titers of ecdysone. The metamorphic effect of ecdysone, i.e. induction of adult developmental programs, is inhibited when a moulting event is preceded by high JH titers whereas it becomes active when the JH titer drops in the last instar (Zera, 2009). In the classical model, it is hypothesized that wing morph determination occurs through variation in the relative decline in JH titers and the timing or amplitude of the ecdysone pulse, in relation to critical periods of tissue sensitivity in the last nymphal instar (Zera, 2004).

By far, most empirical support for the classical model comes from studies of the cricket *Gryllus rubens* (reviewed in (Zera, 2009)). Here, JH levels in nascent short-winged individuals of the last nymphal instar is higher compared to those of long-winged destined individuals, and exogenous application of JH can redirect development from a long-winged trajectory to a short-winged trajectory (Zera and Denno, 1997). The variation in JH titers was found to correlate with the concentrations and activities of the JH degrading enzyme juvenile hormone esterase (JHE), which was higher in the long-winged developmental trajectory. Furthermore, the ecdysone titer was found to be lower in individuals on a short-winged trajectory. Building upon these results it was suggested that the elevated JH titer together with low ecdysone levels in nascent short-winged individuals inhibits the metamorphic effect of ecdysone in developing wings and wing muscle tissue, leading to development of short-winged individuals (Zera, 2009).

While studies in crickets have provided most support for the classical model of endocrine regulation of wing dimorphism, evidence from other species have also contributed. For example, applications of the juvenile hormone analog methoprene increases the frequency of short-winged morphs in the soapberry bug *Jadera haemotoloma* (Dingle and Winchell, 1997) and the same effect has been found in JH application experiments in the brown planthopper *Nilaparvata lugens* (Ayoade et al., 1999; Iwanaga and Tojo, 1986; Ye et al., 2019). Similarly, JH treatment in the aphid *Aphis fabae* elevates the frequency of wingless individuals, which are otherwise induced by long photoperiods (Hardie, 1980). In yet another aphid, *Acyrtosiphon pisum*, manipulation of ecdysone signaling in mothers causes significant differences in the frequency of winged offspring (Vellichirammal et al., 2017)

Taken together, several lines of evidence points toward a role for JH and ecdysone in regulation of wing dimorphism in Hemimetabolous insects. However, details about how and when tissues are responsive to variable levels of these hormones is lacking and much is still unknown about how hormones

interact with growth regulatory and patterning pathways. More details on the molecular mechanisms of wing morph determination will be covered below.

Hormonal regulation of phase polyphenism in Locusts

Phase polyphenism in locusts is one of the most remarkable examples of phenotypic plasticity and has been studied in considerable detail from both a biological and agro-economic perspective (Lecoq and Cease, 2022). The species of grasshoppers that display phase polyphenism can exist in solitary, gregarious or intermediate phases, differing in morphology as well as behavior (Simpson and Sword, 2009). At low population densities individuals are found in the solitarious phase and display avoidance behavior to other individuals, are less active, and spend less time grooming (Simpson et al., 2001). Upon crowding, a noticeable change in behavior of solitarious individuals can occur within hours, where individuals aggregate and sometimes migrate in large numbers. This shift in behavior is referred to as gregarization (Simpson and Sword, 2009). Gregarization can be reversed if a solitarious individual is exposed to crowded conditions temporally, the longer time spent in crowded conditions, the more resistant individuals become to a reversal to solitarious behavior. This can lead to positive feedback of gregarization within a population. The molecular basis of the behavioral change has been linked to the neurotransmitters serotonin and catecholamine (Anstey et al., 2009; Ma et al., 2011) as well as the biogenic amine receptors tyramine receptor and octopamine receptor α (Ma et al., 2015).

While the behavioral changes can occur within hours, the morphological change induced by crowding takes longer time to be induced (Simpson and Sword, 2009). The most obvious difference between solitarious and gregarious individuals is coloration, where the solitarious phase nymphs generally are green and the gregarious phase nymphs are yellow/orange and black (Tanaka et al., 2016). At least two hormones have been linked to these color differences. A neuropeptide hormone called $[\text{His}]^7$ -corazonin has been shown to induce the black color of the gregarious phase (Sugahara et al., 2015), whereas JH is involved in induction of the green color of the solitarious phase (Breuer et al., 2003). The involvement of JH in coloration is corroborated by findings showing that the JH titer is higher in the solitarious morph during the fourth instar, and interestingly, ecdysone levels differs between the morphs as well (Hartfelder and Emlen, 2012). A recent study connected the action of JH to induce the green color to the protein yellow protein of the takeout family, taking steps toward an understanding of the genetic mechanisms underlying the color polyphenism (Sugahara and Tanaka, 2018).

Molecular mechanisms of photoperiod-to-endocrine signaling

While it is clear that hormones play important regulatory roles in insect polyphenisms, many aspects of how environmental cues are incorporated into endocrine regulatory systems remain unexplored (Nijhout and Davidowitz,

2009; Zera and Denno, 1997). Given the complexity of insect endocrine regulation this gap in knowledge is not entirely surprising, but for a number of reasons it is important to fill. Not the least because it will give deeper insights into how polyphenisms work, but also because the environment-to-endocrine signaling axis may be where selection is predisposed to act on genetic variation underlying variation in reaction norms (Suzuki and Nijhout, 2008, 2006). In this section I will discuss how environmental factors can control endocrine processes. Due to the broadness of this subject, I will limit the discussion to what is known about photoperiod-to-endocrine regulation. As is clear from the results presented in **Paper I**, photoperiod is the main environmental factor controlling wing polyphenism in *G. buenoi*, further warranting this restriction.

Sensing light versus sensing photoperiod

In polyphenisms reactive to photoperiod it is obvious that light-sensitive organs are of importance. However, it may be less obvious that these organs not necessarily have to be the compound eyes or ocelli which are the primary visual organs in insects (Goto et al., 2009). Two of the most compelling examples of how organs other than compound eyes and ocelli can be photoperiodically sensitive come from *Manduca sexta* and *Bombyx mori*. In both species, it was shown that brains cultivated *in vitro* at different photoperiods can alter photoperiodically regulated diapause patterns when re-implanted into larvae of the final instar (Bowen et al., 1984; Hasegawa and Shimizu, 1987). Many other creative experimental approaches involving e.g. tissue ablation, surgical removal of organs and spatially constrained light treatments have shown that compound eyes as well as various regions in the head can be involved in photoperiodic responses (Goto et al., 2009). The visual system for sensing light can thus be separate from the system that senses photoperiod.

Linking photoperiodism to endocrine regulation

Much of the information of how information about photoperiod can influence insect development and life-history through hormones comes from research on photoperiodic control of diapause (Denlinger et al., 2017) and studies of developmental timing mechanisms (Steel and Vafopoulou, 2006). One of the most comprehensive models of photoperiod-to-endocrine regulation have been developed through research on pupal diapause termination in the Chinese oak silkworm *Antheraea pernyi* (Takeda and Suzuki, 2022). Here, diapause termination occurs by ecdysone secretion from the PTG through stimulation by prothoracicotropic hormone (PTTH), a neurosecretory hormone. PTTH is released from cells in close proximity to circadian clock neurons (Sauman and Reppert, 1996a, 1996b). The circadian clock in these neurons is programmed by photoperiod and communicates to the PTTH secretory cells through secretion of melatonin, or the melatonin precursor, serotonin (Matsumoto and Takeda, 2002; Mohamed et al., 2014). An enzyme called

arylalkylamine *N*-acetyltransferase (aaNAT) catalyzes the conversion of serotonin to melatonin and is thought to be controlled transcriptionally by the circadian clock (Takeda and Suzuki, 2022). In long-day photoperiods, or during night, the model posits that aaNAT transcription is elevated, leading to synthesis of more melatonin which when secreted can bind to receptors expressed by the PTTH containing cells, which in turn activates PTTH secretion through an intracellular signaling pathway. During short-day photoperiods, less aaNAT is transcribed, which leads to a higher level of serotonin release which stimulates the PTTH secretory cells to retain PTTH, resulting in less ecdysone release from the PTG (Takeda and Suzuki, 2022). This model is interesting because it connects the activity of the circadian clock to ecdysone release and thus constitute a clear candidate pathway for how photoperiodically induced polyphenisms can be regulated.

While it has been firmly established that the release of ecdysone can occur with different dynamics depending on photoperiod conditions (Hayes et al., 2019; Nijhout, 2009), it is less known how tissues can respond differently to the same ecdysone pulse. On this matter, research in the bloodsucking bug *Rhodnius prolixus* has provided important information. In this species it has been shown that ecdysone titers fluctuates diurnally, with troughs during the photophase and peaks in the scotophase (Steel and Vafopoulou, 2006). Interestingly, EcR nuclear abundance was found to cycle in circadian patterns and in synchrony with the daily change in ecdysone titer in a tissue-specific manner (Vafopoulou and Steel, 2006). These results suggest a potential mechanism where tissue-specific ecdysone responses might occur through programming of EcR localization by photoperiod-controlled daily fluctuation in ecdysone titers (Steel and Vafopoulou, 2006).

The molecular mechanisms of wing polyphenism

In this section, I will discuss the available literature on how wing polyphenism is regulated at the molecular genetic level. Most studies have addressed this subject in species within the order Hemiptera, which includes water striders, but wing polyphenisms reminiscent of those commonly found in hemipterans have been characterized in other insect orders. For example, female-limited seasonal wing polyphenism in the tussock moth (Lepidoptera) has been shown to occur through a degenerative effect on growing wing tissue by ecdysone signaling (Lobbia et al., 2003; Yoshida and Kato, 2019). Comparing wing polyphenism in diverse insect orders will undoubtedly be useful for deeper insights of how this trait has recurrently evolved during insect evolution. However, I will here restrict the discussion to hemipteran wing polyphenisms.

A major step toward a molecular genetic understanding of morph determination processes were made in the middle of the last decade when two groups independently identified the conserved insulin/insulin-like growth factors

signaling (IIS) pathway to regulate alternative wing morph fates in the brown planthopper *N. lugens* (Lin et al., 2016b; Xu et al., 2015). In general, activation of this pathway occurs by the binding of insulin-like peptides (ILPs) to insulin receptors (InRs), which then initiates an intracellular signaling cascade through a number of kinases (Teleman, 2009). Firstly, the binding of ILPs to InR induces an activation of phosphoinositide 3-kinase (PI3K) which phosphorylates a cell membrane-associated second messenger protein. This in turn activates the Akt kinase to phosphorylate the forkhead box protein O (FOXO) transcription factor. When phosphorylated, FOXO is sequestered in the cytoplasm, while in its unphosphorylated state it can enter the nucleus where it induces gene expression programs which inhibits proliferation (Teleman, 2009).

In line with a growth-inhibitory role of FOXO, a consistently found result is that FOXO knockdown in the brown planthopper leads to development of long-winged individuals (Lin et al., 2016b, 2016c; Xu et al., 2015; Zhang et al., 2021) and evidence suggest that wing progenitor tissue in FOXO RNAi individuals is more mitotically active compared to control treatments (Lin et al., 2020). Therefore, the long-winged fate is promoted by high IIS pathway activity and the short-winged fate is promoted by low levels of activity.

The mechanism that regulates the level of IIS downstream signaling occurs through two InR homologs (Xu et al., 2015). Of these, InR1 is responsible for the canonical communication with the intracellular IIS signal transduction pathway, whereas InR2 can inhibit InR1 activity. This is thought to occur through heterodimerization between InR1 and InR2, where InR2 may makes InR1 less able to activate PI3K (Lin et al., 2016b), or reduce the affinity to ILPs, resulting in less IIS activity and thus activation of FOXO and a reduction in cellular proliferation.

Regulation of the brown planthopper wing polyphenism thus largely occurs through the relative activities of InR1 and InR2, but how the activity of these proteins is regulated by environmentally sensitive signaling mechanisms is not yet fully understood. One possibility would be through a threshold response to ILPs released after high glucose intake, where high enough ILP concentrations overcomes the inhibitory effects of InR2 on internal IIS activation (Lin et al., 2018). An alternative but not mutually exclusive mechanism could involve transcriptional regulation of both or either of the receptors to introduce ratios of InR dimer complexes which enables divergent tissue/cellular responses to ILP signaling. Evidence in line with such a mechanism comes from the observation that a micro RNA (miRNA) regulated by JH and ecdysone can inhibit expression of InR1 (Ye et al., 2019).

The discovery that the IIS pathway is important for regulation of wing polyphenism in the brown planthopper inspired other researchers to test a role for this pathway in other hemipteran insects. E.g., Fawcett *et al.* showed that RNAi against FOXO and injection of insulin modify the reaction norm to nutrition in the wing polyphenic soapberry bug (Fawcett et al., 2018). Similarly,

RNAi against insulin receptors in the fire bug *Pyrrhocoris apterus* produced shifts in wing morph frequencies (Smýkal et al., 2020). From these observations and those from other polyphenisms in which IIS has been implicated, it was suggested that IIS could be a hot-spot for the evolution of polyphenisms in general (Smýkal et al., 2020).

Research aims

The main aim of my PhD thesis has been to identify the proximate processes that underlie wing length polyphenism using *G. buenoi* as a model organism and has been conducted with the intent to provide a starting point for comparative studies of the genetic basis to variation in wing dimorphism between water strider species. While ecological factors underlying water strider wing dimorphism has been investigated in detail before, my thesis provides the first account on the genetic and hormonal basis for wing morph induction. In a broader phylogenetic perspective, my studies also aimed contribute to the general understanding of induction mechanisms of polyphenisms in insects, with a particular emphasis on providing a wider substrate for inferences on wing polyphenism within Hemiptera, for which much knowledge already exist. Finally, the aim has also been to conceptually highlight and summarize the utility of molecular biological methods in ecological and evolutionary biology research.

Paper I Previous research in *G. buenoi* indicated that wing morph determination may occur through photoperiod variation. In this paper, we aimed to explore whether wing morphology is sensitive to photoperiod variation in lab conditions. Further, we explored the role of nymphal density and nutrition as potential cues for wing morph induction. Lastly, we investigated whether the IIS pathway, which in other hemipterans have been shown to regulate wing morph fates, also play a role in *G. buenoi* wing polyphenism.

Paper II Hormones play a central role in regulation of polyphenisms and the two hormones ecdysone and JH have been linked to wing dimorphisms in various species. In this paper, we used RNAi, hormone manipulation and RNAseq to explore a potential role of ecdysone and JH in regulation of the *G. buenoi* wing polyphenism.

Paper III Much of the research on the genetic mechanisms underlying wing polyphenism have been conducted through candidate gene approaches, limiting the scope for identifying novel

mechanisms involved in wing morph induction. Through morph-specific RNAseq, our aim in **Paper III** was to explore the global changes in transcriptomes between nascent wing morphs and to identify new candidate growth regulatory pathway to functionally validate with RNAi.

Paper IV The aim of **Paper IV** was to review the existing literature on the limitations of sequencing methods to identify the genetic basis of ecologically and evolutionary relevant phenotypic variation, and to highlight the strength and benefits of using functional genetic methods like RNAi and CRISPR-Cas to validate candidate genes and genetic variation.

Methods

Study organism

G. buenoi is native to North America, occurring in the continental USA, Canada and Alaska. It is considered a generalist species with regard to aquatic habitats, but is mostly found in temporary ponds (Spence, 1989). As noted in other sections in this thesis, *G. buenoi* is wing polyphenic and are found with three distinct wing lengths, or morphologies (Armisen et al., 2018). The micropterous and macropterous, i.e. short-winged and long-winged, morphs are the most common, whereas a morph with intermediate wing length (mesopterous) can also occur. Functionally, the distinction of the mesopterous morph from the micropterous is superfluous, as neither can use their wings to fly. However, from a developmental stand point it can be argued that the mesopterous morph represent a distinct ontogeny, where the mechanisms for wing growth have been activated but not sufficiently to produce a full-length wing. Like most polyphenisms, the morphs of *G. buenoi* does not only differ in wing length, but also in thorax morphology, where the most pronounced differences are found in the pronotum. In macropterous individuals, the pronotum is distinctly bulkier in the wing hinge area.

Most research on *G. buenoi* has been performed in Canadian populations (Spence, 1989), and it is also from there that the founding individuals of the lab population used throughout my work was originally collected (described in **Paper I**). Where studied, it has been found that Canadian *G. buenoi* populations are partially bivoltine (Spence, 1989). Here, overwintering adults emerge during spring to give rise to a new generation, in which some individuals directly reproduce whereas others enter diapause when reaching adulthood. The decision to reproduce or go into diapause is associated with the age at the summer solstice, where the younger an individual is at the solstice, the more likely it is for it to diapause. Therefore, the overwintering diapausing population is a mix of individuals from the first and second summer generation (Spence, 1989). Wing morphology is partly associated with the voltinism pattern, where the diapausing population almost exclusively is macropterous, while the directly reproducing individuals is predominantly micropterous but can also be macropterous (Spence, 1989).

Wings are formed gradually in *G. buenoi*, where wing progenitor structures called wing buds starts to develop during instar two but are firstly visually apparent in instar three. For each successive moult, the wing buds increase in

size and undergo distinct changes in shape. Importantly, no morphological differences between wing buds of nascent micropterous and macropterous individuals are visible to the eye during the nymphal stages.

G. buenoi has proven to be amenable to rearing in lab conditions, where continuous direct reproduction can be induced by continuous long photoperiods, without the need for a period of diapause. At 20-25°C nymphs are hatched from eggs after seven to eight days, and nymphal development is normally completed after three weeks. Depending on photoperiod conditions, time until reproduction for adults vary. Short photoperiods induce macropterous individuals which will not breed unless exposed to a long photoperiod. This process takes approximately two weeks. Individuals emerging as adults in a long photoperiod become predominantly micropterous and starts breeding after a week.

As food, any small to medium-sized arthropod works for water striders, but in my work, I have used commercially bred crickets. For two reasons this has been the most practically and experimentally sound alternative. The amount of food required for large experiments and keeping of a stock population is high, rearing arthropods to match this need would require too much space and too much time. Also, the crickets can be ordered in specific sizes/ages, giving the opportunity to standardize the amount of food given in experiments.

Experiments in controlled environments

Being able to expose individuals to controlled and standardized environments has been an absolute requirement for the work in this thesis. Here, I have to a very large degree relied on growth rooms designed for plant ecology studies with a thermostat and broad-spectrum light armatures which are programmable in both light intensity and periods. The rooms have been designed for the purpose of performing experiments, and conditions within the rooms are thus minimally variable. Although these rooms have been most useful for my studies, it is worthwhile to mention that they do not have the capacity to replicate the gradual increase and decrease of light conditions occurring during dawn and dusk in natural settings.

Water striders require water as habitat and for this purpose regular tap water is sufficient. To contain this water, I have used opaque plastic containers with transparent plastic lids of the same model within experiments or between experimental replicates with the intent to remove potential effects of different rearing environments.

One of the most challenging factors with regard to performing controlled experiments on water striders is keeping the water surface clean. Since the light sources in the growth rooms are designed for plants, the growth of green algae, which sometimes can affect the water surface tension, has been a prevalent problem. The best remedy for not letting this affect the outcome of

standardized experiments have been to regularly change the water in the boxes and to use air stones in the water, which keeps the algae from aggregating on the surface. Whereas algae can have a deteriorating effect on water strider survival when let out of control, I have not observed any effects of the presence of algae and patterns of wing morph induction.

RNA interference and microinjections

RNAi, or sequence-specific knockdown of gene expression through short non-coding RNAs, has since its discovery (Fire et al., 1998) become an essential tool for functional genetic research. Although most powerful when utilized in species like *D. melanogaster* where tissue-specific expression of the RNAi substrate is possible, it has also played essential roles in functionally characterizing genes in non-traditional model organisms (reviewed in **Paper IV**). Here, RNAi together with a well assembled and annotated genome sequence allows for, in theory, functional assessment of any gene in a species genome. However, in reality, it is not always straight-forward to conduct a gene knockdown experiment. First of all, if a gene is essential for the survival of the organism and tissue-specific knockdown techniques are not available, the systemic effect of RNAi will likely lead to death and thus often an inability to study the phenotype of interest. Our efforts to explore the role of the hormone receptors EcR and Met or the transcriptional co-activator Yki is an excellent example of that. To a certain degree, one can modulate the dosage of dsRNA (the most commonly used RNAi substrate) to decrease the knockdown effects and thus potentially get surviving individuals. Although with this approach there is a chance that the threshold dose for a potential RNAi response in a particular phenotype of interest may be above the threshold for mortality (e.g. EcR RNAi in **Paper II**), in which case a true role for the gene of interest would remain hidden.

Another limitation with systemic RNAi, which is the general approach for the studies of genetic mechanisms underlying wing polyphenisms cited in this thesis, is that if an effect on the phenotype of interest is found after knockdown of a candidate gene, one can never be sure that the effect comes from a gene knockdown in the tissue of focus. Instead, the effect could come from knockdown of the gene in another tissue that is involved in the regulation of other tissues. This is of particular importance for the interpretation of knockdown experiments targeting genes which are known to be involved in hormone regulations, such as hormone receptors. To a certain degree, this obstacle can be tackled with proper controls and additional complementary molecular studies, e.g. qPCR.

The standard procedure for performing an RNAi knockdown experiment in non-model insects involves the generation of a template for dsRNA synthesis through PCR and then utilizing microinjections to introduce the dsRNA to the

circulatory system. For studying wing polyphenism with RNAi, having microinjection protocols that minimize traumatic effects on injected individuals is important, since injection itself has proven to have an effect on wing morph frequencies (Lin et al., 2016c; Smýkal et al., 2020). In my experience, trauma during microinjection in *G. buenoi* can occur but is usually visible in the wings of adult individuals as scarred tissue, and not in a change in wing morph frequencies.

To draw reliable conclusions from an RNAi experiment, proper controls are important. In this respect, the lack of validation of the knockdown experiments presented in both **Paper II** and **Paper III** pose some limitations on the conclusions and this issue needs to be addressed in the future. The controls that are commonly used in RNAi experiments is to use qPCR to validate that knockdown of the targeted gene is sufficiently achieved, and to use a different dsRNA targeting another part of the gene of interest. The latter one controls for the potentiality of a dsRNA to induce phenotypic changes due to off-target effect, i.e., unintentional knockdown of another gene. Here, the use of a separate dsRNA reduces the chances that an observed phenotype after targeting a specific gene originates from off-target effects, as any two dsRNA:s are not likely to have the same potential off-target effects. When dealing with genes within a gene family with high sequence conservation, the dual dsRNA control can be difficult to employ as one might be constrained in which part of a transcript to target with RNAi. This aspect also highlights another limitation with RNAi which we observed in **Paper I**, where high sequence similarity between genes can lead to unintentional co-knockdowns, which make it difficult to deduce which gene has a potential effect on a phenotype of interest.

The use of RNAi to investigate the effects of gene expression differences identified in e.g. RNAseq experiments is perhaps when the methods comes to its most powerful use. Here, RNAi has the potential to recapitulate ecologically relevant variation underlying the generation of alternative phenotypic outcomes, and has in such a case high inferential capacity on the role of a particular gene in a phenotype of interest.

Hormone manipulations

Manipulation of hormones titers as well as using hormone analogs and antagonists is a common practice in studies of polyphenic insects and have in many cases been informative on how polyphenisms are regulated. (Fawcett et al., 2018; Hardie, 1980; Iwanaga and Tojo, 1986; Rountree and Nijhout, 1995; Vellichirammal et al., 2017; Zera, 2004). However, it has been argued that relying on hormone manipulation experiments as the only evidence for endocrine regulation of morph induction severely limits any conclusions, because injection of one hormone can lead to changes in endogenous titers of many other hormones, which in turn could be affect the phenotype (Zera,

2009). This is a valid argument and its implication should be weighed carefully when interpreting hormone manipulation experiments. Nevertheless, we performed methoprene (a juvenile hormone analog) topical applications and 20-hydroxyecdysone (20E, the active form of ecdysone) injections on *G. buenoi* nymphs without prior information about endogenous concentrations of these hormones.

We carried out two separate experiments for each of the two hormone treatments (see **Paper II** for details). In the first experiment, we injected or applied the compounds in instar three, four and five individuals which were staged, meaning that all individuals were of roughly the same age. In the second experiment, we carried out the treatments on groups of individuals of varying ages within each instar. The reasoning behind the former experiment was to have control of how much compound per mass unit was applied, and to see which concentrations had a physiological effect. The reasoning behind the latter experiment came from the evidence in other species that individuals are sensitive to hormones only in narrow intervals of development, and by allowing for variation in age among the treated individuals, those that were in this sensitive stage would potentially show a switch in wing morphology.

To summarize, hormone manipulation has proven useful in studying the endocrine regulation of polyphenisms, but needs to be accompanied with complementary methods for conclusions regarding a role for a hormone in regulation of the inductive process. Here, hormone titer measurements are most useful as a complementary analysis (Zera, 2009)

RNA sequencing

Protein coding genes are expressed at two levels, RNA and protein. Whereas the expression levels of proteins in most cases are more relevant to gene function than the expression level of mRNAs, the most feasible way to assess gene expression in the generation of phenotypes is to utilize RNA sequencing (RNAseq). The information gathered with this method generates a snapshot of the repertoire of RNAs expressed within cells at a given timepoint, and one can thus make inferences about the cellular processes occurring at that time.

RNAseq has been used in several studies to explore the gene expression differences that generate alternative wing morphs in polyphenic insects (Vellichirammal et al., 2016; Zhang et al., 2022, 2021) and has indeed been informative, despite the general challenge to confidently enough predict which morph an individual will develop to.

An important aspect of using RNAseq to study polyphenic development is to gather data with temporal/developmental resolution, where the aim should arguably be to capture the transcriptomes before, during and after the switch mechanism takes place (Nijhout, 2009). This is not an easy task, but can be

greatly informed by experiments with either inductive hormonal or inductive environmental factors.

As described in the introductory section, regulation of polyphenisms occur through the integration of environmental cues into physiological processes that lead to hormone secretion which in turn affects peripheral tissues. Therefore, in order to elucidate a complete picture of the underlying mechanisms of morph determination, it is necessary to investigate regulatory events occurring in several different tissues and organs within an insect during the sensitive developmental stages. In this regard, RNAseq is in principal a suitable method to use where it in an optimal case should be done with high-tissue resolution. However, for most tissues, such kind of experiments are practically difficult to accomplish because many of the model species for wing polyphenism are small which complicates tissue dissection procedures. Here, development of single-cell or low-input protocols would likely be of good use.

Sequencing of RNA isolated from growing wing tissue has now been performed in at least three studies, see **Paper III** and (Zhang et al., 2022, 2021). In our work, we exposed *G. buenoi* individuals to photoperiod conditions inductive to the alternative wing morphs (**Paper I**) and then sampled groups at four different developmental stages (**Paper III**), two during instar four and two during instar five. Here, the intent was to at least capture the gene expression occurring before and after but preferably also during the action of the switch mechanism. Analysis of the RNAseq data was made through comparisons between photoperiods within developmental stages. In **Paper II**, we sampled individuals at only one developmental time point, which was the same as the last time point in instar five for the experiment in **Paper III**.

When designing these experiments, we faced a challenge in how to most effectively stage individuals to reduce developmental variation generated by different growth rates between photoperiods. To overcome this challenge, we sampled individuals based on relative age within each instar, expressed as a percentage of development based the average duration of each instar within each photoperiod. Additionally, we wanted to reduce potential variation that could originate from different circadian rhythms induced by the different photoperiods. Therefore, we only sampled individuals within a time window which was midday in each photoperiod plus minus two hours.

Summary of the papers

Paper I - Photoperiod controls wing polyphenism in a water strider independently of insulin receptor signalling

Water striders display a significant amount of variation in wing dimorphism and have been intensively studied as a model system for dispersal variation in an ecological and evolutionary perspective (Andersen, 1993; Fairbairn, 1988, 1985; Fairbairn and King, 2009; Preziosi et al., 1996; Spence, 1989; Vepsäläinen, 1978). Several studies have identified environmental cues that affect wing morph determination in wing polyphenic species. For example, temperature influences wing morph frequency of *Aquarius remigis* (Fairbairn and King, 2009), whereas photoperiod and density regulate wing morph induction in *Gerris odontogaster* (Vepsäläinen, 1971) and *Tenagomeris euphrosyne* (Han, 2020), respectively. In this paper one aim was to identify the factors that determine wing morph in *G. buenoi*.

Previous research had shown that *G. buenoi* wing morph expression is highly seasonal where populations undergo a shift in production of wing morphs at the summer solstice (Spence, 1989). Here, individuals completing nymphal development before the summer solstice are exclusively short-winged whereas adults emerging after show a gradually increased tendency to emerge as long-winged individuals, depending on how many days they spent as nymphs after the summer solstice. These data strongly suggest that photoperiod or gradual change in photoperiod control wing morph determination in *G. buenoi*, and is to a large degree similar to the data on a natural population of the closely related species *G. odontogaster* (Vepsäläinen, 1971).

By exposing lab populations of *G. buenoi* to varying photoperiod conditions we corroborated the idea that photoperiod controls wing morph determination in this species (**Paper I, Figure 1**). Specifically, in a long-day (18L:6D) condition, we found a very strong bias for individuals to develop to the short-winged (or micropterous) morph. In short-day conditions (12L:12D or 14L:10D) individuals developed exclusively to the long-winged (macropterous) morph. In the intermediate photoperiods of 15L:9D and 16L:8D we found intermediate wing morph frequencies and also expression of the intermediate wing morph (mesopterous). Further experiments showed that nymphal crowding have a moderately macropterizing effect on individuals reared in 18L:6D, whereas wing morph determination was robust to variation in nutritional conditions (**Paper I, Figure 2**). Taken together, these results led to the conclusion

that wing morph determination in *G. buenoi* is mainly regulated by photoperiod with the exception that crowded conditions can induce macropterous development in long-day conditions.

To explore how the sensitivity to photoperiod changes over development, we carried out an experiment where we shifted photoperiod from either long-day to short-day conditions, or short-day to long-day conditions, at defined developmental time points (**Paper I, Figure 1**). Here we found that individuals are sensitive to the short- to long-day shift as late as the second day of the ultimate instar. At this time, about 30% of development in instar five is completed. In contrast, individuals experiencing the long- to short-day shift began losing the sensitivity to photoperiod change in instar three and lost it completely in instar four. The main conclusion from this experiment was that a long-day photoperiod is highly inductive of the short-winged morph and that this inductive effect can be initiated relatively late in development.

G. buenoi was the first water strider species for which the genome were sequenced and assembled (Armisen et al., 2018) making it a good model for functional genetic studies. Interestingly, comparative genomic analysis revealed that the *G. buenoi* genome contains a lineage-specific retro copy of the gene encoding insulin receptor 1 (InR1) in addition to having the conserved InR1 and InR2 (Armisen et al., 2018). This expansion of the insulin receptor repertoire, together with the numerous publications showing a role of insulin receptors in wing morph determination in other hemipteran species (Fawcett et al., 2018; Smýkal et al., 2020; Xu et al., 2015), prompted us to investigate whether the IIS pathway was involved in the *G. buenoi* wing morph determination regulated by photoperiod.

After considerable effort and employment of rigorous controls, we found no effect on wing morph frequencies after RNAi against the insulin receptors or the IIS pathway transcription factor FOXO, and thus concluded that the IIS pathway is not regulating wing morph determination in *G. buenoi* (**Paper I, Figure 3**). This conclusion is in line with the observation that *G. buenoi* wing morph determination is robust to nutritional conditions, and suggest that other growth regulatory pathways control the *G. buenoi* wing polyphenism.

Paper II - Contribution of ecdysone signaling to photoperiodic wing polyphenism in the water strider *Gerris buenoi*

Hormones play determinant roles in insect development and in regulation of life-history traits such as reproduction and dispersal (Zera, 2003). Studies of diverse insects have also shown that hormones are central in regulation of environmentally induced morphological variation, including wing polyphenisms (Hartfelder and Emlen, 2012). Two hormones in particular have been in the focus of endocrinological studies of wing morph determination; ecdysone and JH (Zera, 2009). Here, high levels of JH during the early phases of the last

juvenile stage are thought to have an inhibitory effect on the metamorphic action of ecdysone on wing and wing muscle tissue, leading to incomplete tissue responses and development of short wings and small wing musculature. When JH levels are low, however, tissues respond readily to the pulse of ecdysone and grow to achieve full size. This model of hormonal control has various degrees of support, depending on species under consideration (Zera, 2009, 2003), but of particular importance is the fact that exogenous treatments with JH (or its analogs) induce development of short-winged individuals in several different species (Dingle and Winchell, 1997; Hardie, 1980; Iwanaga and Tojo, 1986; Ye et al., 2019; Zera and Tiebel, 1988).

In **Paper II**, we used RNAi, hormone manipulations and RNAseq to explore the role of JH and ecdysone in *G. buenoi* wing polyphenism. Firstly, we performed RNAi against the ecdysone receptor (EcR) which resulted in high mortality even at low dsRNA doses, but amongst the few surviving individuals in 12L:12D we found a statistically significant difference in wing morph frequency compared to the negative control treatment (**Paper II, Figure 1**). Secondly, we targeted the putative JH receptor methoprene tolerant (Met) with RNAi where we hypothesized that a potential effect of JH in wing morph determination would occur in 18L:6D, the photoperiod where individuals almost exclusively develop to the micropterous or mesopterous morphs. By knock-down of the putative JH receptor Met, we would remove the inhibitory effect of JH on wing development, potentially generating macropterous individuals. In our results, we found no support for this hypothesis (**Paper II, Figure 1**).

In addition to the RNAi experiments, we performed microinjection of 20E and topical application of methoprene (a JH analogue). These experiments were carried out in both 12L:12D and 18L:6D at different developmental stages and doses. While we found no effects on wing morph frequencies after hormone treatments (**Paper II, Supplemental Figure 1**), we observed elevated mortality after 20E injection compared to negative controls, and development of juvenile-adult intermediates after methoprene applications, a phenotype characteristic of manipulation of JH signaling.

In an alternative approach to explore the potential role of ecdysone and JH in wing morph determination we sequenced RNA isolated from individuals reared in 12L:12D and 18L:6D with the aim to explore potential gene expression differences in hormone synthesis genes. Since EcR RNAi had a small but significant effect on wing morph frequencies, we focused the analysis particularly on genes in the 20E synthesis pathway. We found that none of the conserved 20E synthesis genes annotated in the *G. buenoi* genome were differentially expressed. However, two homologous genes associated with ecdysteroid metabolism in *D. melanogaster* were significantly differentially expressed, where one was upregulated in 12L:12D and the other in 18L:6D (**Paper II, Figure 2A**).

Finally, we utilized the RNAseq data set generated in **Paper III** to specifically explore the expression of ecdysone related genes in nascent wing tissue

of individuals reared in 12L:12D and 18L:6D. Here, we found that a large proportion of these genes were differentially expressed in late instar five, indicating that ecdysone signaling at this time point of development differs between individuals destined to become macropterous respectively micropterous (**Paper II, Figure 2B**).

Taken together, the results in this paper highlights a potential role for ecdysone signaling through EcR in *G. buenoi* wing morph determination. However, given the small sample sizes in the EcR RNAi experiment and the uncertainties why 20E injection did not affect wing morph frequencies and whether the endogenous level of 20E differ between morphs, we refrain from drawing any conclusion before more data is available. In particular, we identify a need to measure 20E titers in nascent wing morphs to get a better understanding of the potential way this hormone can regulate wing morph fates.

Paper III - Wing polyphenism in a water strider is caused by photoperiod-specific expression of the Fat/Hippo pathway

Much of what is known about the molecular genetic mechanisms of wing polyphenisms come from studies that have been published in the recent decade where RNAi have been used to knock down gene expression of candidate genes (Zhang et al., 2019). This work has been dominated by studies of the brown planthopper *N. lugens* and have provided insight into the molecular mechanisms that underlie development of alternative wing morphs (Lin et al., 2020, 2018, 2016c, 2016a, 2016b; Xu et al., 2015; Xue et al., 2021; Ye et al., 2019; Zhang et al., 2022, 2021; Zhuo et al., 2017). The discovery that the IIS pathway regulates wing morph fate (Lin et al., 2016b; Xu et al., 2015) not only guided further studies of how wing morphs are induced in the brown planthopper, but also generated informed hypothesis for studies in other hemipterans which found a conserved role for the IIS pathway in wing morph regulation (Fawcett et al., 2018; Smýkal et al., 2020). As described above, IIS is seemingly not involved in regulation of wing polyphenism in *G. buenoi*, suggesting that some other growth regulatory pathway play this role instead, and that the role of IIS is not ubiquitous in regulation of wing polyphenisms in Hemipterans.

In **Paper III**, we utilized the strong effect of photoperiod on *G. buenoi* wing morph induction to investigate wing tissue transcriptomic differences between nascent wing morphs with the aim to identify candidate genetic pathways for wing morph determination. This experimental approach has not been feasible in many other wing polyphenic species because induction of wing morphs by environmental factors are often partial, and in order to investigate the gene expression differences between alternative developmental trajectories, one must be confident of what morph a particular sampled nymph will develop into (Lin et al., 2016c). In two recent studies, RNAi with complete

penetrance to bias wing morph trajectory has been used to sample transcriptomes with high confidence (Zhang et al., 2022, 2021) and provided novel details of the regulatory networks of wing morph determination. However, the manipulation of individuals onto alternative developmental trajectories with RNAi may invoke artefactual gene expression and provide limited knowledge about the effect of environmental factors on tissue gene expression responses.

We found that most transcriptomic differences between nascent wing morphs occurred during late instar five, where a considerable number (~2300) of genes were differentially expressed (**Paper III, Figure 2**). Gene ontology enrichment analysis indicated significant enrichment for a plethora of biological processes, including GO terms associated with juvenile hormone signaling, insulin receptor signaling, ecdysone receptor signaling and Hippo signaling. Among the differentially expressed genes underlying enrichment for Hippo signaling we found *Dachsous*, *Fat* and *Yorkie*, all of which play an important role in wing growth, patterning and size determination in *D. melanogaster* (Irvine and Harvey 2015; Gridnev and Misra 2022). These genes are core components of the Fat/Hippo pathway (regulation of Hippo signaling occur through several branches, of which *Fat* and *Dachsous* constitutes one) which have been highlighted before as a missing link between circulatory components and local tissue responses of growth and patterning (Gotoh et al., 2015). Intriguingly, RNAi against *Dachsous*, *Fat* and *Yki* in nascent macropterous individuals produced a small but significant shift to development of micropterous or mesopterous individuals (**Paper III, Figure 3**). Additionally, RNAi against these genes produced aberrations in the pronotum.

Taken together, these results suggest that expression of genes in the Fat/Hippo pathway is down-regulated stage-specifically by exposure to long photoperiods to inhibit wing growth, leading to the stunted wings seen in micropterous individuals. Future work is needed to connect the variable expression of these genes to variation in upstream factors, for which we hold ecdysone as the main candidate given that this hormone has been linked to Fat/Hippo signaling in *D. melanogaster* (Parker and Struhl, 2020; Zhang et al., 2015). Furthermore, rigorous controls are currently lacking and need to be employed to firmly establish that knockdown of these genes recapitulate gene expression levels found in nascent micropterous individuals.

The idea that the Fat/Hippo but not the IIS pathway play a causal role in photoperiod induced wing polyphenism makes it tempting to speculate that the evolutionary routes of induction systems are biased with regard to the environmental cue that is used to predict the future adaptive landscape. For example, induction in the brown planthopper occurs at least in part through variation in host plant quality, where feeding on host plants with high glucose content predict the onset of senescence in rice plants and induces development of long-winged individuals which in turn facilitates migration (Lin et al., 2018). Thus, regulation through the nutrient sensitive IIS pathway provides a seemingly direct connection between the inductive cue of high glucose

concentration and regulation of tissue growth. Since IIS is sensitive to nutritive conditions in the first place, the modifications required to co-opt this pathway to regulate the brown planthopper wing polyphenism may occur with less constraints compared to modifications of other growth regulatory pathways. With the same logic, evolution of wing polyphenism in *G. buenoi* may have faced less constraints going through the Fat/Hippo pathway, which may be connected to photoperiod through ecdysone (see above), compared to other growth regulatory pathways. Future functional and comparative investigations, within and across groups of wing polyphenic insects, will be necessary to provide empirical support for this idea.

Paper IV - Functional genomic tools for emerging model species

The high feasibility and low cost of sequencing methods in the post-genomic era has enabled researchers to characterize nucleotide and transcriptomic variation in an ever-increasing number of species and populations in the pursuit of understanding the genomic basis of evolutionary and ecologically interesting phenomena. Although powerful, these methods are limited in their scope of inference, where methods for functional validation are needed to make causal connections between variation at the genetic, genomic or transcriptomic level, to phenotypic variation. In **Paper IV** we review how functional genetic methods like RNAi and CRISPR/Cas can and have been employed to functionally characterize phenotypes of evolutionary and ecological relevance. We highlight both the technical advantages and disadvantages of these methods, and give examples of when they have successfully been employed to validate the role of candidate genes in generation of phenotypic variation.

In a more conceptual perspective of the review, we argue that research within the field of ecology and evolution should strive to, when possible, functionally test candidate genes in an ecologically relevant manner. This argumentation stems from the proliferation of studies which have used knockout studies to characterize genes with putative relevance for population level phenotypic variation. While genetic knockouts can be informative to the understanding of which genes are important for a phenotype of interest, they rarely recapitulate the genetic variation that may be of interest to understand the genetic basis to population or species level phenotypic variation. We therefore emphasize the need to develop techniques for gene editing through the homology directed repair pathway, which have the potential to replace one natural genetic variant for another, and thus recapitulate ecologically and evolutionary relevant variation. This kind of approach gives inferences of the genetic basis to phenotypic variation much more power and will therefore be

important for a better understanding of how evolutionary and ecological processes modulate phenotypic variation.

Conclusions and future prospects

***G. buenoi* as a model for photoperiodically induced variation**

In a book chapter about photoperiodic effects on insect morphology (Nijhout, 2009), Frederik Nijhout pointed out important future directions in the pursuit to understand the mechanisms that act downstream of hormones to induce alternative developmental pathways. Of these, I believe that at least two are of high relevance to the findings in this thesis. Firstly, he identified a need for:

“...a good model organism for photoperiodically cued morphological change. Like other model systems it should be easy to rear in large numbers and have a short generation time. Obviously, it should have simple but unambiguous forms, and preferably be associated with diapause to give it the broadest possible appeal for research.” (Nijhout, 2009).

Arguably, the robust role of photoperiod on morph induction that we found in **Paper I** make *G. buenoi* to fit well into this description, and it may be of particular convenience that it is the wings that display the most striking polyphenic response, as wing development in insects is one of the most well-understood developmental processes in biology through research on *D. melanogaster* wing discs (Tripathi and Irvine, 2022). As a testament to the usefulness of *G. buenoi* as a model for photoperiodically induced morphological variation, we managed in **Paper III** to accommodate another pressing need that Nijhout called out for, namely, to obtain tissue-specific gene expression data over multiple developmental time points before and after hormonal induction (Nijhout, 2009). Whereas our results in **Paper II** does not allow for reliable conclusions with regard to the endocrine basis of wing polyphenism in *G. buenoi*, it is reasonable to believe that hormones play an important and decisive role in this species as has been documented in numerous others (see above). Taken together, one of the conclusions of this thesis is that *G. buenoi* is a suitable model to study photoperiodically induced morphological variation.

The genetic mechanism of wing morph determination in *G. buenoi* and its implication for comparative studies

The work presented in **Paper I, II and III** have provided important steps towards understanding the mechanism that underlie wing morph determination in *G. buenoi* and perhaps also other water striders. The two most

important conclusions are that the IIS pathway is not ubiquitous in regulating wing polyphenism in hemipteran insects, and that expression of the Fat/Hippo pathway is induced by photoperiod in *G. buenoi* and is likely causally involved in wing morph determination.

To obtain further insights into the process of wing morph determination in *G. buenoi* there is a need to establish a link between hormonal signals and the expression of Fat/Hippo pathway genes, presuming that this pathway indeed plays a downstream regulatory role in nascent wing tissue. Here, I suggest that ecdysone is a likely candidate hormone, an idea which is supported by the observation that a large number of ecdysone related genes were differentially expressed between nascent wing morphs in developing wing tissue and that EcR RNAi had a small but significant effect on wing morph frequencies. Important first steps towards exploring this hypothesis would be to measure titers of ecdysone during development in 12L:12D and 18L:6D and to measure expression levels of e.g. Dachous, Fat and Yorkie with qPCR in a time series of high developmental resolution. The ecdysone titer and qPCR expression data can then be correlated to infer whether potential variation in ecdysone co-occur with the gene expression changes in the Fat/Hippo components.

Additionally, it would also be interesting to explore molecular and cellular processes acting downstream of Dachous, Fat and Yorkie signaling. Here, further analysis of the RNAseq data presented in **Paper III** would be a good starting point. As it has been found that the wing length variation in the brown planthopper is dependent on numerous cell cycle regulators (Lin et al., 2020), it would be interesting to investigate whether such a signature can be seen in the transcriptomic data for *G. buenoi*. If so, it would suggest that regulation of the cell cycle is an important and conserved feature of wing polyphenisms, but that it can be achieved through different regulatory pathways.

While I find it likely that the generation of macropterous wings involves positive regulation of cell cycle components to sustain growth of tissue size, I suggest that generation of micropterous wings solely through lack of cell cycle progression is more of an open question. The reason being that programmed cell death (PCD) might be an important factor for remodeling tissues, and has been shown to act in the tussock moth female wing polyphenism (Yoshida and Kato, 2019). In line with such a scenario, the low expression of Yorkie in nascent micropterous individuals might de-repress genes involved in PCD (Verghese et al., 2012). In line with a role for PCD in micropterous wing development, I have observed that nascent micropterous wing development includes ontogenetic stages where the full volume of the instar five wing bud is completely filled with tissue, whereas the final size of adult micropterous wing is markedly shorter than the wing bud itself, suggesting that wing tissue at some point undergoes a decrease in size (unpublished observations). A closer documentation on the progression of growth and change in shape and size of wing tissue using microscopy methods would provide information on how wing size differentiation occurs at a tissue level.

The proximate aim of the work presented in this thesis has been to understand how alternative wing morphs are generated in the wing polyphenic water strider *G. buenoi*. But, seen from a broader perspective, the work has been conducted with the ultimate aim to identify the genetic variation that underlie variation in environmental and genetic modes of wing morph determination in water striders. While my work falls short of explicitly addressing this ultimate aim, I am confident that it will be helpful for future studies directed towards this subject. For example, evaluations of gene expression differences between wing morphs of other species could specifically target Fat/Hippo genes through qPCR instead of using RNAseq. Also, RNAi against Fat/Hippo components in dimorphic and monomorphic species can yield insights on whether the mechanism of wing size reduction is conserved, even in species which have lost environmental sensitivity. Finally, through knowledge about the functional properties of genes, i.e. whether they are casually involved in e.g. wing development or wing polyphenism, information of population level genetic variation becomes much more informative and give deeper insight into how genetic variation causes phenotypic diversity.

Svensk sammanfattning

Att en individs egenskaper bestäms av arv och miljö är sedan länge ett välkänt faktum. Till exempel, en individ som utsätts för fysiska påfrestningar kan bli både starkare och mer uthållig efteråt. Inom biologin kallas fenomenen som gör det möjligt för miljövariabler att påverka en individs egenskaper för fenotypisk plasticitet. Fenotyp är ett ord som beskriver en egenskap eller karaktär hos en eller flera individer och ordet plasticitet syftar till att egenskapen är böjbar eller föränderlig, och är därmed inte förutbestämd av en individs arv, eller mer specifikt, arvs massa.

I mitt avhandlingsarbete har jag studerat fenotypisk plasticitet i vingutveckling hos skräddare med syftet att förstå hur miljöfaktorer interagerar med genetiska faktorer för att generera fenotyper som kan vara av relevans för en individs överlevnad och förmåga till reproduktion. Vingar hos skräddare kan vara av olika längd, vilket gör att man i naturen kan hitta individer som är långvingade, kortvingade eller inte har några vingar alls. I många fall så avgörs vinglängden av de miljöfaktorer som en individ utsätts för under dess utveckling och är således ett exempel på fenotypisk plasticitet.

I mina studier har jag visat att fotoperiod, d.v.s. antalet timmar med mörker respektive ljus, en individ utsätts för är helt avgörande för beslutet att skaffa långa eller korta vingar i skräddararten *Gerris buenoi*. Då en individ växer upp i en kort fotoperiod (få timmar med ljus) blir den med mycket stor sannolikhet långvingad. Om en individ istället utsätts för en lång fotoperiod så utvecklar den med lika hög sannolikhet korta vingar.

Varför sker då detta och varför blir de just kortvingade vid långa fotoperioder? Mycket forskning har ägnats åt att svara på just den frågan, och det har att göra med att det är dyrt ur ett energiperspektiv att både utveckla en fungerande vingapparat, d.v.s. vingar och vingmuskulatur, och att använda den till att flyga med. I förhållanden där flygförmåga inte behövs så är det därmed bättre ur ett evolutionärt perspektiv att lägga energin på reproduktion istället. Den här förklaringen till inom- och mellanartsvariation i flygförmåga har genom åren fått mycket stöd. Att individer blir kortvingade vid långa fotoperioder tros ha att göra med att långa fotoperioder indikerar att de goda förhållanden som råder under sommaren, då dagarna är långa, kommer vara kvar tillräckligt länge för att det ska vara värt att satsa sin energi på reproduktion, som kan ske innan de mer besvärligare förhållanden anländer på senare på hösten, då vingarna behövs.

För många insektsarter som uppvisar liknande förmåga som skräddare att utveckla långa eller korta vingar beroende på miljöbetingelser, har det varit känt sedan länge att hormoner är viktiga komponenter för beslutsprocessen. I en del av mitt arbete har jag genom laboratorieexperiment karakteriserat två viktiga insektskshormoners roll i hur *Gerris buenoi* reglerar sin vinglängd. Här fann jag data som tyder på att ecdyson, som är viktigt hormon för skinnömsning hos insekter, kan ha en viktig roll i beslutsprocessen, men för att kunna dra den slutsatsen entydigt behövs det mer data.

I ett annat experiment som syftade till att förstå vilka gener som är involverade i den process som gör skräddarnas vingutveckling känslig för variationer i fotoperiod så sekvenserade jag RNA från individer i olika utvecklingsstadier. RNA är den biologiska molekylen som bland annat förmedlar informationen som finns sparad i arvsmassans gener till cellernas proteinsyntesmaskineri. Genom att sekvensera RNA kan man därför få reda på vad som händer i en cell vid ett specifikt tillfälle och se vilka gener som uttrycks under specifika förhållanden. Medan det är proteinerna som utför de flesta funktionerna i en cell eller i en vävnad så sker en stor del av regleringen av genernas uttryck på RNA-nivå.

I resultaten från RNA-sekvenseringen hittade jag en stark association mellan uttrycket av en grupp av gener som verkar i en specifik molekylär signaleringsväg och utvecklandet av långa vingar. När jag sedan manipulerade uttrycket av dem här specifika generna i individer som hölls i en kort fotoperiod, och var alltså på väg att utveckla långa vingar, så ändrades mönstret hos vissa individer som istället utvecklade korta vingar. Det här resultatet tyder på att de gener som jag upptäckte vara associerade med utveckling av långa vingar verkligen är viktiga för beslutsprocessen som sker via fotoperiod.

En av de större slutsatserna i avhandlingen kommer från det här experimentet, och är extra viktig därför att i många andra insektsarter så har det visat sig att en annan signaleringsväg är viktig för vingutvecklingsbeslutet. Mina resultat tyder alltså på att plasticitet i vingutveckling kan ske på flera alternativa sätt i insekter, vilket har betydelse för vår förståelse hur olika fenotyper kan påverkas av miljöfaktorer.

Vetskapen om vilka gener som styr förmågan att vara plastisk i vingutvecklingen gör det lättare att i ett bredare perspektiv studera evolutionen av den här egenskapen. Därför kan mina resultat i förlängningen komma att vara viktiga för att jämföra plasticitet i vingutveckling hos många andra skräddararter och också för att studera hur olika populationer av samma art kan skilja sig i förmåga att vara plastisk eller inte.

Acknowledgements

Arild: I am most grateful that you entrusted in me to venture into the, for us both, unknown territory of water strider functional genetics. I believe that I can safely conclude that over these four and a half years we have explored much and covered a lot of ground, given that we started with an empty room with not a single water strider in sight. It will be interesting to see what comes out of all the projects in the future. Thank you also for all the interesting discussions over the years, it has been most rewarding and I've learned more than I could imagined about evolution and population genetics.

Abdou: Thank you for sharing your enthusiasm about water striders, and for all the advice and collaboration. Without you, Arild and I would truly be out in the dark in many aspects!

Mattias: I have enjoyed your company in the strider lab a lot over the years. Without your hard work and seemingly endless stamina in caring for water striders, the studies I have conducted during my PhD would not have been what they are. Thank you for all the illuminating discussions on matters high and low.

David: Your bioinformatic skills have been like a gift from above during the last year and truly saved the RNAseq data from my embarrassingly blunt coding capabilities. It has been great collaborating with you and I wish you all the best in your future career and life.

Aleix: It has been a joy being part of introducing you to the world of water strider wing dimorphisms. I'm sure that you with your sharp mind, high ambition and impeccable working morale will reach far. I hope that my work presented in this thesis can be of help to your experiments in the future. Best of luck with all the interesting projects!

McKenna: It has been a joy getting to know you and I'm sure that you will become a great PI someday, and thank you for bringing all your fascinating animal physiology facts to the lab!

Shrinath, Elise, Marion, Freya, Lauri, Hanna, Elin, Kevin, Sofia: Much of the work presented in this thesis I owe to you. Whatever you do now and in the future, you should know that I am grateful for your contribution and hard work!

Gabriel: Thank you for all the stories from far and wide, and for always being enthusiastic about my strider work!

Jesper: Even though your humor may not always be as brilliant as your input on the matters of evolutionary biology and population genetics, you always bring a little positive spiciness and action when you enter a room, even on the dullest of days. Never stop stirring the social pot, Jesper, and thank you for your curiousness and enthusiasm, it has really helped me appreciate my own work!

Karin, Madee, Philipp: Thank you for your support and friendship, and for sharing some memorable adventures with me over the years. I will always remember our stays in Cesky Krumlov and Portugal with a lot of joy and the taste of beer.

Lars, Linnea, David, Milena, Shadi, Merce, Venkat, Thadd, Dasha, Martyna: It has been great working along-side you all, I wish you all the best in your current and future work!

Niclas, Anna, Elina, Vaishali, Veera: Thank you all for being great PIs and colleagues!

Yvonne, Christoffer, Gunilla, Johanna: Lab work at IEG would not be possible without you, and since I enjoy lab work, thank you!

Annette, Frida, Doug: Thank you for your kind help over the years!

Johan, Stefan, Börje, Fredrik m. fl: IEG along with the whole of EBC would fall apart if it was not for you. Thank you for all the help with instruments and other equipment essential for research.

Dan, David, Ante, Axel A, Axel Å: Det är svårt att undgå att ställa in hästarna när man doktorerar, det sker gärna för ofta, för länge. Därför vill jag tacka er, som hjälper till att släppa ut dem ibland.

Adrian, Louise, Joel, Elli, Anna, Olle, Adam, Alex: Tack för våra studieår, jag kommer alltid att minnas alla våra galenskaper, jag är glad att jag delade dem med er.

Kerstin, Håkan, Johanna: Ni är en stor inspiration och jag tror inte att jag hade kommit ens hälften så långt utan ert stöd. Tack, ni är bäst!

Lina: Tack för ditt outtröttliga stöd, Lina. Du är den största anledningen till att jag klarade av att slutföra det här. Ingen är värd ett större tack än du!

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