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University of South Bohemia in České Budějovice

Faculty of Science

Reproductive diapause in males of the linden bug, *Pyrrhocoris apterus*

Ph.D. Thesis

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Annotation

This thesis focuses on the male reproductive diapause in the linden bug, *Pyrrhocoris apterus*. We examined in detail the response of males to changing photoperiodic conditions, the change in levels of juvenile hormone, and its role in male diapause, as well as the effect of low temperature on diapause termination. We compared the physiology of males and females and pointed out important sexual dimorphism in the control of diapause. Finally, we analyzed a completely novel insect neuropeptide to and explored its possible link in the chain of control of reproductive diapause.

Declaration

I hereby declare that I am the author of this dissertation and that I have used only those sources and literature detailed in the list of references

České Budějovice, datum

..... jméno a příjmení

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List of papers and author's contribution

The thesis is based on the following papers (listed chronologically):

I. **Hejníková M.**, Paroulek M., Hodková M. (2016) Decrease in Methoprene tolerant and Taiman expression reduces juvenile hormone effects and enhances the levels of juvenile hormone circulating in males of the linden Pyrrhocoris apterus. Journal of Insect Physiology 93-94: 72-80. DOI: 10.1016/j.jinsphys.2016.08.009
M. Hejníková and M. Hodková together designated the study; M. Hejníková performed and analyzed all experiments, except liquid chromatography–mass spectrometry performed and analyzed by M.Paroulek; M. Hejníková and M. Hodková wrote the manuscript.

II. **Hejníková M.**, Nouzova M., Ramireze C.E., Fernandez-Limae F., Noriegad F. G., Doležel D. (2022) Sexual dimorphism of diapause regulation in the hemipteran bug *Pyrrhocoris apterus*. Insect Biochemistry and Molecular Biology 142: 103721. DOI: 10.1016/j.ibmb.2022.103721
M. Hejníková performed all *P. apterus* experiments; D. Doležel and M. Hejníková designed the study and interpreted results; the rest of the co-authors performed and analyzed JH measurement; D. Doležel wrote the manuscript with input of all co-authors.

III. Kotwica-Rolinska J., Krištofová L., Chvalová D., Pauchová L., Provazník J., **Hejníková M.**, Sehadová H., Lichý M., Vaněčková H., Doležel D. (2020) Functional analysis and localisation of a thyrotropin-releasing hormone-type neuropeptide (EFLa) in hemipteran insects. Insect Biochemistry and Molecular Biology 122: 103376. DOI: 10.1016/j.ibmb.2020.103376
M. Hejníková performed initial RNAi experiments against EFLa. Injected males and observed locomotor activity, mating behavior and reproduction, and measured ACPs in testes and MAG. M. Hejníková quantified EFLa RNAi efficiency in adult males. J. Kotwica-Rolinska created EFLa null mutants and performed experiments on females and males. D. Doležel performed the phylogenetic part in the study and wrote the manuscript.

Papers not included in this thesis:

I. Ibrahim E., **Hejníková M.**, Shaik A., Doležel D., Kodrík D. (2017) Adipokinetic hormone activities in insect body infected by entomopathogenic nematode. Journal of Insect Physiology 98: 347-355. DOI: 0.1016/j.jinsphys.2017.02.009

II. Dvořáček J., Sehadová H., Weyda F., Tomčala A., **Hejníková M.**, Kodrík D. (2020) First Comprehensive Study of a Giant Among the Insects, Titanus giganteus: Basic Facts from Its Biochemistry, Physiology, and Anatomy. Insects 11(2): 120. DOI: 10.3390/insects11020120

III. Kotwica-Rolinska J., ChodákováL., Smýkal V., Damulewicz M., Provazník J., Wu B. CH., **Hejníková M.**, Chvalová D., Doležel D. (2022) Loss of Timeless underlies an evolutionary transition within the circadian clock. Molecular Biology and Evolution 39(1) DOI: 10.1093/molbev/msab346

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David Doležel, Ph.D.

Content

List of abbreviations

ACPs	accessory gland proteins
bHLH-PAS	basic helix-loop-helix Per-ARNT-Sim
CA	*corpora allata*
CC	*corpora cardiaca*
CPP	critical photoperiod
ILPs	insulin-like peptides
ILS	insulin/insulin-like signaling
JH	juvenile hormone
$JHSB_3$	juvenile hormone III skipped bisepoxide
LD	long day
MAG	male accessory gland
Met	Methoprene-tolerant
PDF	Pigment-dispersing factor
PI	*Pars intercerebralis*
PL	*Pars lateralis*
RNAi	RNA interference
SD	short day
Tai	Taiman
TRH	thyrotropin-releasing hormone
TSH	thyroid-stimulating hormone

Introduction

Overview

In the life of many organisms, it is just a matter of time when such environmental conditions occur, that do not correspond to their living standards or represent a risk for future offspring. Choosing the right strategy to cope with adverse conditions, whether drought, lack of food, the presence of predators, or high or low temperatures, is often a matter of life and death. In an effort to predict and overcome the inhospitable periods, the organisms have developed various mechanisms and life strategies. When harsh environmental conditions occur, the choices for the organisms are as follow: succumb and die, tolerate the environmental stress through physiological adaptation and regulation, or avoid them. The avoidance strategy evokes simple movement through space, typically migration or dispersion. However, a large group of organisms are unable to undertake long-distance movement, and therefore they adapted by the means of escape through time – by the decision to undergo dormancy when facing a harsh environment. Some species rely on a single strategy while others use a range of options when faced with various environmental stressors (Danks, 2002; Ślusarczyk, 2004).

Dormancy (from Latin *dormans*, asleep) is a collective term for the temporary arrest or restriction of physiological processes in organisms. It is an active, highly controlled stage. It can be both – a program based on genetics or caused by external influences. The main purpose is to conserve energy to help the organism survive an adverse period. Dormancy can last for varying lengths of time until suitable conditions occur. Some organisms, such as bacteria or *Anostraca* are able to wait years before deciding to continue their active life (MacRae, 2016; van Vliet, 2015).

We can distinguish several categories of dormancy. Temperate mammals undergo hibernation – (winter sleep), which is usually induced either by external circumstances or by internal control. Similar to hibernation, estivation (summer sleep) occurs during the hot season when animals are threatened by lack of water and high temperatures, especially in tropical regions. Finally, in insects, there is diapause and quiescence. These two strategies can be easily confused because the phenotypic effects of the mechanisms are often similar. However, quiescence is a direct rapid response of organisms at any ontogenetic stage to changes in the

environment, such as temperature, oxygen, food resources, or water. The dynamics of the processes are subjected to the dynamics of external conditions (Koštál, 2006). Diapause, on the other hand, is a planned, hormonally controlled developmental arrest triggered by biotic and abiotic factors (photoperiod, pheromones, and food quality) that precede the arrival of hostile conditions (Denlinger et al., 2012).

Diapause phenomenon

Diapause can be described as a hormonally mediated, dynamic state of low activity that occurs during genetically determined stages of metamorphosis, specific for each species, in response to environmental "token stimuli" that precede unfavorable conditions (Lees, 1956; Taylor et al., 1987). Some animals enter diapause regardless of the signal received by token stimuli. In that case, we talk about obligatory diapause, which is a fixed, genetically determined component that occurs in each generation at the predetermined point in the life cycle. Obligatory diapause is mostly associated with univoltine animals (animals having only one generation per year). For example, in the domestic silk moth *Bombyx mori*, diapause occurs at the early embryonic stage (Sonobe et al., 1986) whereas in the gypsy moth *Lymantria dispar*, diapause occurs at the late embryonic stage when the first instar is ready to hatch (Bell et al., 1990). However, the majority of animals undergo a period of arrest optionally, only if the early environmental cues indicate that such arrest is necessary for survival. This is referred to as facultative diapause. Organisms do not have to exclusively utilize one strategy. To reflect the variability of the fluctuating environment as best as possible, the plasticity of the life cycle of some species allows the adoption of the most advantageous strategy. Moreover, both variants may even occur in the population during the same period (Fielding, 2006; Moraiti & Papadopoulos, 2017; Schebeck et al., 2022).

Since diapause is a complex dynamic process requiring thorough preparation, it can be described in several phases, as proposed by Košťál in his work (Koštál, 2006; Koštál et al., 2017) (Fig. 1). (1) Pre-diapause phase including *induction* and *preparation* – takes place in a species-specific sensitive period of ontogeny when signals from the environment are received and converted to a decision to either continue direct development or enter diapause, after token stimuli reach a critical

point. (2) Diapause starts with the *initiation phase* – reaching an ontogenetic stage where development is temporarily interrupted. Proliferation and differentiation are halted, and the metabolic rate decreases. The organism may continue to feed in order to accumulate energy reserves and search for a suitable habitat. The *maintenance phase* that follows is considered the true diapause. Despite the fact that the surrounding environment is usually still adequate for direct development, the individuals remain locked in developmental arrest by endogenous factors. The metabolic rate drops to a minimum level, or to a certain stable level. A change in external conditions leads to the last *termination phase*, which causes a decrease in the intensity of the diapause to a minimum level or triggers it back to maximum intensity. At the end of this phase, the full potential for a resumption of direct development is reached, but this is possible only under favorable conditions. If, after the end of diapause, external conditions are not suitable for the continuation of direct development, the organism remains in the dormant (3) post-diapause phase, which is basically quiescence. Only the changes in the limiting factors (temperature, humidity, water presence, etc.) allow the organism to continue its life cycle.

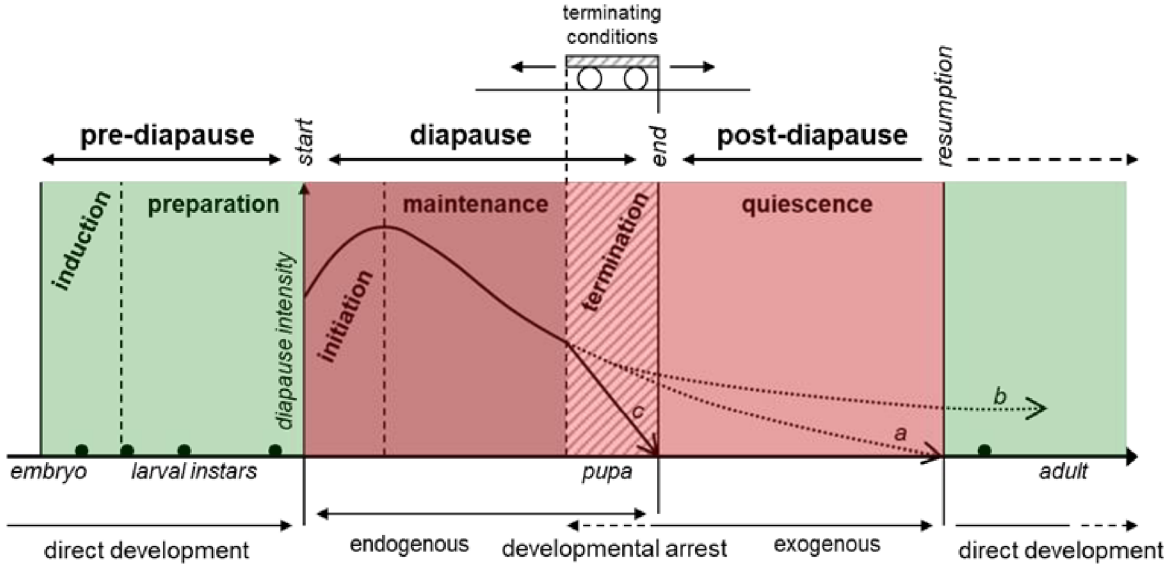

Fig. 1. Phases of diapause as proposed by Košťál 2006. The points on the line represent major ontogenetic stages in which diapause may occur. Division to phases is marked by vertical lines (not all phases must necessarily be present in all species and situations). Changes in diapause intensity are displayed: dotted branches (a and b) refer to the constant conditions, while solid branch (c) refers to the change of specific terminating conditions. (Adapted from Košťál V. (2006)

Diapause and insect - making the right decision

Insects are no different from other organisms in their efforts to survive. Likewise, they apply all available survival strategies, including the seasonal long-distance migration, typically known in monarch butterflies *Danaus plexippus* or desert locusts *Schistocerca gregaria*. However, most insects are unable to cross hundreds or thousands of kilometers to safety, therefore must cope with inhospitable living conditions by adapting physiological processes.

Insects are ectothermic organisms, meaning that the regulation of their body temperature depends on external sources. Therefore, they are vulnerable to extreme temperature fluctuations, which are naturally accompanied by food shortages. These temperature variations are most pronounced in the temperate and polar zones, where they are periodically repeated on an annual basis (changing seasons). Due to the tilt of the Earth's axis by 23.58° and its orientation to the Sun, the temperature changes are accompanied by changes in the length of the day and night (Koštál, 2011). With the gradual coming of winter, days become shorter and nights longer – the ratio of day to night, known as the photoperiod, changes.

For insects, it is existentially important to recognize accurately when the decision to enter diapause needs to be made. Thus, they evolved a special mechanism (biological photoperiodic calendar), which allows them to count photoperiod and by this recognize the season (Nelson et al., 2010). This precise system can readily distinguish daylength differences as small as 15 minutes (Denlinger, 1986). Despite long efforts to elucidate the nature of this photoperiodic timer, the molecular and genetic mechanism of action remains a mystery. Currently, three different theories are suggested for the possible operation of photoperiodism. The first theory, proposed as early as 1936 by Erwin Bünning, considers the circadian clock (endogenous regulator of the correct timing of daily events) as the main basis of photoperiodism and speculates it is a single mechanism with dual function. The second hypothesis – the so-called hourglass model – expects the existence of an independent system separate from the circadian clock. In this model, the photoperiodic response is triggered when the product of a sequence of biochemical reactions reaches a critical threshold. Finally, a very popular third theory suggests some level of correlation between the

photoperiodic system and the circadian clock (Dolezel, 2015; Emerson et al., 2009; Goto, 2013, 2022; Koštál, 2011; Kotwica-Rolinska et al., 2017, 2022).

Indeed, photoperiod is a truly important signal used by most insects living in temperate zones to predict seasonal changes. Since the same geographic location has the same length of a day on a given calendar date of the year, photoperiod is a much more consistent seasonal signal than temperature or precipitation, which varies greatly over time. Nevertheless, temperature still act as an additional factor modulating response to photoperiod (Saunders, 2014). Insects are only able to perceive the photoperiodic signal during a species-specific photosensitive phase, usually long before unsuitable conditions occur. The photosensitive phase can vary in duration and appear at any point in the life cycle. For example, in the insidious flower bug *Orius insidiosus*, larvae are highly photosensitive at the fifth instar stage and remain so for 14 days after adult ecdysis (Ruberson et al., 2000); for the flesh fly *Sarcophaga crassipalpis*, only the first instar larvae have been noted to be photosensitive (Joplin et al., 1990); or in the extreme example of the silkworm *Bombyx mori* and *Nasonia vitripennis* (Saunders, 1965), the mother is the one that passes on information from the photosensitive stage to the next generation (Denlinger et al., 2012).

Token stimuli do not directly alter the developmental process. The environmental cues are recognized by receptors and processed in the system of the photoperiodic timer. The output pathway from the timer modulates the activity of the neurosecretory cells in the brain, which produce hormones and peptides that intervene directly or indirectly in ontogenesis. The cascade of hormonal pathways varies greatly from species to species, as the decision to enter diapause can be made at any developmental stage from periblastula to adult. The details of the modulation between input and output from the photoperiodic clock still linger unknown in the ‘‘black box’’ (Koštál, 2011).

As far as we know, vitamin A-associated opsins and/or the flavoprotein Cryprochrome are the most probable candidates for the photoreceptor pigments in the photoperiodic timer (Goto & Denlinger, 2002; Tamaki et al., 2013). The location of the timer is unknown, but possibly localized near or at the circadian clock center, as the same molecule appears to be the output of both clocks. It is believed that Pigment-Dispersing Factor (PDF), a well-known neurotransmitter of the circadian clock (Renn et al., 1999), may also play a role as a signaling molecule in the output of the photoperiodic timer, which relays information to targeted

neurosecretory cells. However, additional neuromodulators are likely required (Hasebe et al., 2022; Kotwica-Rolinska et al., 2022; Nagy et al., 2019; Shiga & Numata, 2009). The PDF-producing cells are located in the anterior medulla region or, in some hemimetabolous insects, additionally in the lamina in the optic lobes of the brain (Beer et al., 2018; Ikeno et al., 2014; Kotwica-Rolinska et al., 2022; Lee et al., 2009). The PDF-positive neurons have been found to innervate a cluster of neurosecretory cells in the *pars lateralis* (PL) region of the protocerebrum (Hamanaka et al., 2005; Shiga & Numata, 2009). Neurons in the PL and also in the *pars intercerebralis* (PI) play a key role in converting photoperiod information into hormonal signals. These cells, beside paracrine secretion, send neuronal projections to the neurohemal/endocrine organs *corpora cardiaca* (CC) and the endocrine glands *corpora allata* (CA) and control their functions (Hodkova, 1994; Hodkova & Okuda, 2019; Matsumoto et al., 2013; Shiga & Numata, 2001). Current knowledge is graphically summarized in a sketch of the blow fly brain (Fig. 2) (adapted from Meuti & Denlinger, 2013).

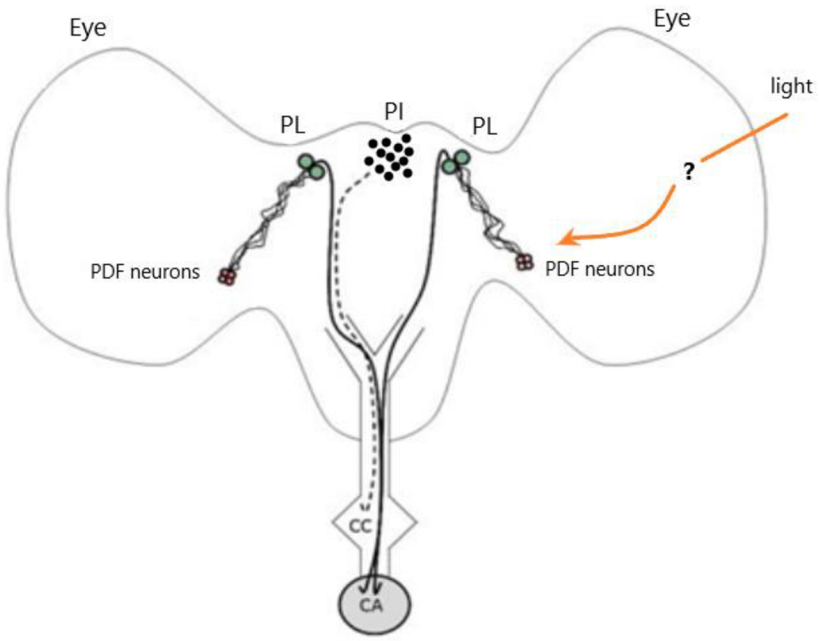

Fig. 2. Schema of the brain of the blow fly, Protophormia terraenovae showing connections among PDF-positive lateral ventral neurons that innervate neurosecretory cells the in pars lateralis (PL). These PL cells innervate the corpora cardiaca (CC) and corpora allata (CA) that produce and/or release key hormones involved in development and diapause. (Modified from Meuti M. (2013)

Adult diapause and the differences between the sexes

Adult or imaginal diapause, commonly called reproductive diapause, is characterized as a cessation of reproduction and massive fat storage. Females terminate oviposition and oocytes development, while in males the lack of mating with receptive females is the most relevant sign. In both sexes, production of the secretions from accessory glands is stopped, and therefore their volume decreases by an order of magnitude. Testes are not a good indicator of ongoing diapause, as are small ovaries. They are sometimes reduced in size, but in some species remain fully developed and contain sperm (Denlinger, 2000; Goto & Matsumoto, 2018; Pener, 1992a). Both sexes do not always undergo diapause. If mating occurs before the onset of diapause, males usually die, and only fertilized females survive until the upcoming season. This strategy is typical for some social insects such as wasps or bees, and the common house mosquito *Culex pipiens* (Denlinger & Armbruster, 2014; Goto & Matsumoto, 2018). When both sexes undergo diapause, mating usually occurs later, after the diapause is terminated (Denlinger, 2000; Denlinger et al., 2012).

The endocrine basis of diapause is considered to be the absence of juvenile hormone (JH), mainly due to studies largely devoted to females. JH is used as the generic name for a group of several sesquiterpenes with pleiotropic effects in addition to reproduction. The chemical structure varies in detail among insect groups. The most common form in insects, in general, is JH-III. JH-0, JH-1, 4-methyl JH-1 and JH-II can be found in Lepidoptera, whereas JHB3 (JH-III bisepoxide) has been discovered in representatives of Diptera (Goodman & Cusson, 2012). Most recently identified JHSB3 (juvenile hormone III skipped bisepoxide) in *Plautia stali* (Kotaki et al., 2012), appears to be the major JH type in Hemiptera. The mode of action of JH is mediated by its nuclear receptor Methoprene-tolerant (Met), which probably also enables its pleiotropic effect in the organisms (Charles et al., 2011; Konopova et al., 2011; Konopova & Jindra, 2007; Miura et al., 2005). Met is a protein from a basic-helix-loop-helix/Per-Arnt-Sim (bHLH-PAS) domain family of transcription factors. To create an active structure of the receptor, the bHLH-PAS domain forms a heterodimer with another partner from this family in response to the presence of JH (Jindra et al., 2013). To date, two functional binding partners of Met are known: Taiman (Tai)

(Charles et al., 2011) and the circadian clock protein Cycle (Bajgar et al., 2013; Shin et al., 2012). Smykal et al., 2014 demonstrated that the Met-Tai complex acts as a receptor in JH signaling during vitellogenesis. RNAi-mediated silencing of Tai effectively disrupted ovarian development and suppresses vitellogenin gene expression in the fat body of *Pyrrhocoris apterus*.

The primary sites of JH synthesis are endocrine glands *corpora allata*, (CA), located in the posterior region of the head near the pharynx. CA usually occurs as a paired organ, but in some insects such as Hemiptera or Dermaptera, it is fused into a single large gland. In the higher Diptera, CA is integrated with CC to form a structure surrounding the aorta, known as the ring gland for its characteristic shape (Goodman & Cusson, 2012). CA is physically connected to CC by two main nerves, and both organs are controlled by neurosecretory cells PI and PL in the brain via neuronal and hormonal pathways (Belles, 2020; Shiga, 2003). How the cells actually regulate CA activity is not entirely clear. Neurons in PI are necessary for vitellogenesis in the blow fly *Protophormia terraenovae*, whereas those in PL suppress vitellogenesis during reproductive diapause (Shiga & Numata, 2001). In contrast, ablation of PI or disruption of innervation of CA *in P. apterus* females stimulates oviposition during diapause, indicating active suppression by PI. Conversely, in reproductive females, PI supports oviposition. (Hodková, 1976). A study on the bean bug *Riptortus pedestris* confirmed the role of PL as a suppressor, but the removal of the PI did not cause any effect on female reproduction (Shimokawa et al., 2008). However, a study by the same author a few years later confirmed the promoting role of PI during oviposition in reproductively active females (Shimokawa et al., 2014). It should be noted that discrepancies between studies may be caused by surgical procedures. The axons of neurosecretory cells are often closely spaced or ramified, so it is easy to disrupt additional, non-targeted ones which could lead to a complex organismal response.

JH level plays a key role in vitellogenesis and ovarian maturation, but its importance in adult males' reproduction is debatable. The males of the blow fly *Protophormia terraenovae* and linden bug *P. apterus* show mating behavior and respond to photoperiod even in absence of JH (Blazkova et al., 2011; Hodkova, 1994; Tanigawa et al., 1999), or its receptor Metoprene-tolerant (Urbanová et al., 2016). The reproductive maturation in leaf beetle *Gastrophysa atrocyanea* is JH independent and an unknown factor is responsible for the maturation of sperm cyst (Ojima et al., 2015).

Ecdysteroid hormones are other discussed regulators with a possible role in reproductive diapause, however, their impact on diapause has been overlooked for a long time. The reason behind this is that the prothoracic gland – the main tissue producing and releasing ecdysteroids, degrades after the adult eclosion. Now, it is known that ecdysteroids are not only produced by the prothoracic gland in larvae, but also by several tissues in adults, where they are primarily involved in the control of reproduction (Dinan, 1997; Lafont et al., 2003; Richard et al., 2001; Soller et al., 1999) Females synthesize ecdysteroids in nurse cells and follicle cells of adult gonads. Ecdysteroids are often present in high concentrations in both vitellogenic oocytes and freshly laid eggs, probably because they are required for the initiation and coordination of vital developmental processes during early embryogenesis before the prothoracic glands begin to differentiate (Lafont et al., 2003) They provide proper follicle cell fate by induction or suppression of apoptosis during eggs maturation in germarium. While a low concentration of ecdysteroids is essential for the normal progression of oogenesis, a high concentration of ecdysteroids caused by nutritional shortage induces apoptosis in the nurse cells. Furthermore, they also control niche formation and cyst cell differentiation at early step of oogenesis (Buszczak et al., 1999; Uryu et al., 2015).

Based on all the current knowledge, it is easy to think about the involvement of ecdysteroids in reproductive diapause, which is suggested in some studies, but the results are sometimes conflicting. In the potato beetle *Leptinotarsa decemlineata*, the titer of ecdysteroids in hemolymph in diapausing females is twice that of reproductively active females (Briers et al., 1982). These results are, however, contradictory to ones shown in the migratory locust *Locusta migratoria*. Diapausing female locusts have a significantly lower titer of ecdysteroids in the hemolymph than those which are reproductively active (Tawfik et al., 2002) and this result is consistent with that obtained in the fruit fly *Drosophila melanogaster* (Denlinger, 2000). Similarly, a recent study on the cabbage beetle *Colaphellus bowringi* points out that a decline in ecdysone levels, caused by long-day diapause-inducing conditions, is necessary for reproductive diapause to occur in adult females (Guo et al., 2021).

In the case of male adults, the sources of ecdysteroids are testes (Gillott & Ismail, 1995; Loeb et al., 1984) and accessory glands (Hentze et al., 2013; Uryu et al., 2015). It is unclear whether ecdysteroids systemically act in the whole body or

only locally at the site of synthesis. Just as in the case of JH, the knowledge of the role of ecdysteroids in adult males is limited.

Since profound metabolic changes and redistribution of energy are an integral part of diapause, insulin/insulin-like signaling (ILS), a major nutrient management system, is also an important part of the diapause phenotype. It is assumed, that JH signaling pathway controlling vitellogenesis could be interconnected with ILS. In red flour beetle *Tribolium castaneum*, reduction in JH synthesis or Met receptor decreased expression of genes encoding insulin-like peptides (ILPs) and resulted in the down-regulation of vitellogenin gene expression. Moreover, JH application to previtellogenic females induced the expression of ILPs, and induced expression of vitellogenin in the fat body (Sheng et al., 2011). ILS is also involved in reproduction regulation in the mosquito *Culex pipiens*. Diapause, which was induced by RNAi-mediated knockdown of insulin receptor or ILPs, was successfully reversed by topical JH or a JH analog application (Sim & Denlinger, 2008).

The linden bug *Pyrrhocoris apterus*

The linden bug, *P. apterus*, belonging to the order Hemiptera is a species widespread in the Palaearctic regions. A rare occurrence has also been described in the USA and India (Kristenová et al., 2011; Socha, 1993). Under the Central European climate, the linden bug belongs to the univoltine insects. The entire development process takes approximately 120 days. A nymph must pass through five larval stages during its life (see Figure 3.), and the adult stage undergoes winter diapause (Honêk & Ŝrámková, 1976) which is characterized by the accumulation of storage proteins (hexamerins) and lipids, cold hardening, and cessation of reproduction in both females and males (Košťál & Šimek, 2000; Socha et al., 1991; Šula et al., 1995). *P. apterus* shows a robust photoperiodic response, even when breeding at constant, relatively high temperatures of 25°C. They are reproductively active under long-day conditions (LD) and diapausing under short-day conditions (SD) (Hodek & Hodkova, 1986; Saunders, 1987). The photoperiodic signal acts via neurosecretory cells mainly in the *pars intercerebralis* in the brain and the signal is further transmitted to the *corpus allatum* (Hodkova, 1976; Hodkova & Okuda, 2019). The photosensitive period during which the linden bug perceives light information extends from the third to the fifth larval instar and

continues until the imago, but in nature, the diapause decision is made mainly during nymphal development at the end of summer (Hodek, 1971; Hodkova, 2015). *P. apterus* is now a well-established model for photoperiodism and diapause. The size of the animal allows for performing surgical procedures that are hardly feasible in *Drosophila melanogaster*. RNA-mediated interference works systemically, and the recent introduction of genetic manipulation by the CRISPR/Cas9 technique makes available even more precise targeting of the studied issue (Kotwica-Rolinska et al., 2019, 2022).

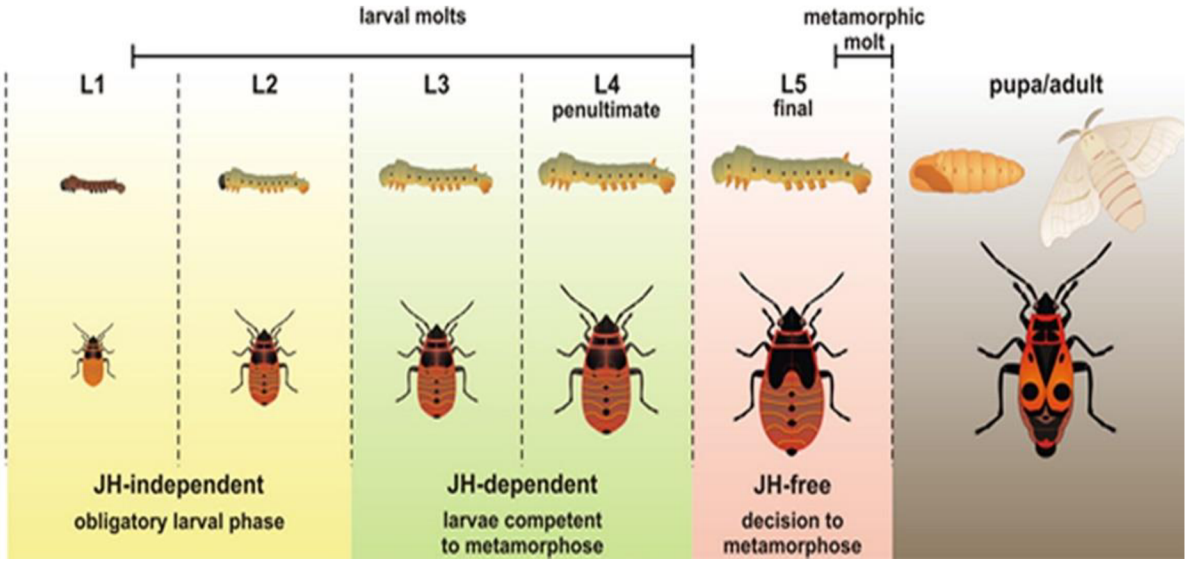

Fig. 3. A comparison of the ontogenesis of a Holometabola representative (*Bombyx mori*) and Hemimetabola *Pyrrhocoris apterus*, and their dependence on the presence of JH in the hemolymph. (Adapted from (Smykal et al., 2014)

Chapter 1

Decrease in *Methoprene tolerant* and *Taiman* expression reduces juvenile hormone effects and enhances the levels of juvenile hormone circulating in males of the linden bug *Pyrrhocoris apterus*

Hejníková M., Paroulek M., Hodková M

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Insect reproductive diapause is associated with a significant decrease in juvenile hormone (JH) production in *corpora allata*. The subsequent series of downstream events lead to the adaptive physiological changes that allow overcoming of harsh environmental conditions, possibly resulting in the death of the organism. Essentially, there is an extensive reduction in metabolism and a cessation of highly energy-consuming processes (Denlinger et al., 2012), such as vitellogenin synthesis in the fat body and consequential ovarian development in females (Raikhel et al., 2005). Vitellogenesis, which is controlled by the juvenile hormone or its receptor Methoprene tolerant (Met), is well studied across the insect species such as *Perillus bioculatus*, *Drosophila virilis*, *Rhodnius prolixus*, *Aedes aegypti*, *P. apterus*, and many more (Adams et al., 2002; Raikhel et al., 2002, 2005; Rauschenbakh et al., 2004; Smykal, Bajgar, et al., 2014; Socha et al., 1991; Wigglesworth, 1936). Met receptor, a member of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) protein family (Ashok et al., 1998), form complex with other bHLH-PAS protein Taiman (Tai) and together act in JH-induced vitellogenesis (Smykal, Bajgar, et al., 2014; Zhang et al., 2011).

Although reproduction and reproductive diapause have been widely studied in females, males have not been in such a center of interest, thus the molecular and neuroendocrine mechanisms of male reproductive dormancy in most insects are still poorly understood. Surprisingly, JH does not appear to be directly involved in spermatogenesis. The studies suggest an unknown factor is responsible for the maturation of sperm cysts (Harano, 2013; Ojima et al., 2015; Wyatt & Davey, 1996). On the other hand, JH is required for the production of male accessory gland proteins (ACPs). These proteins are transferred to the female body during copulation and are capable of altering female mating behavior, feeding patterns, egg production, sperm utilization, adjusting gene expression, and even reducing longevity (Denis et al., 2017; Sirot, 2019).

JH is also involved in the synthesis of storage hexamerin proteins in the fat body. Some of these proteins are synthesized at high levels prior to diapause entry and serve as energy resources during the non-feeding period. The absence of JH significantly enhances their production and release into the hemolymph (Martins et al., 2010; Socha et al., 2004; Socha & Šula, 1992).

In the study "Decrease in Methoprene tolerant and Taiman expression reduces juvenile hormone effects and enhances the levels of juvenile hormone circulating in males of the linden *Pyrrhocoris apterus*" we have demonstrated that knockdown of the genes encoding the juvenile hormone receptor Met, and its binding partner Tai reduces the amount of ACPs in *P. apterus* males. Simultaneously, their knockdown increases the expression and release of hexamerin proteins from the fat body into the hemolymph. These results are comparable to those obtained in allatectomized males. Interestingly, neither of the treatments leading to the disruption of JH signaling had an effect on the fertility or mating behavior in males. We also showed that knock-down of Met extended lifespan as much as allatectomy, whereas knocking down Tai had no such effect. The natural absence of JH induced by short-day conditions (SD) has stronger effects on all these parameters. Loss of Met or Tai impedes JH function but results in the increase of JH level in hemolymph (juvenile hormone III skipped bisepoxide – $JHSB_3$). This study shows that Met and Tai work together in a complex as JH receptor in the regulation of ACPs and hexamerin production in males.

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Decrease in *Methoprene tolerant* and *Taiman* expression reduces juvenile hormone effects and enhances the levels of juvenile hormone circulating in males of the linden bug *Pyrrhocoris apterus*

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ABSTRACT

Juvenile hormone (JH) produced by the corpus allatum (CA) stimulates vitellogenesis and reduces the synthesis of hexamerin proteins in adult females of *Pyrrhocoris apterus*. At present it is unknown whether the signaling pathway involving the JH receptor gene Methoprene tolerant (Met) and its binding partner Taiman (Tai), regulates the synthesis of accessory gland proteins (ACPs) and hexamerin proteins or effects male survival. Knockdown of genes by injecting *Met* dsRNA or *Tai* dsRNA, reduced the amount of ACPs whilst enhancing the amount of *hexamerin* mRNA in the fat body and the release of hexamerin proteins into haemolymph, as occurs after the ablation of CA. Lifespan was enhanced by injecting *Met* but not *Tai* dsRNA. Diapause associated with the natural absence of JH had a stronger effect on all these parameters than the ablation of CA or the knockdown of genes. This indicates there is an additional regulating agent. Both *Met* and *Tai* dsRNA induced a several fold increase in JH (JH III skiped bisepoxide) but a concurrent loss of Met or Tai disabled its function. This supports the view that the Met/Tai complex functions as a JH receptor in the regulation of ACPs and hexamerins.

1. Introduction

Juvenile hormone (JH) produced by the corpus allatum (CA) controls many events in an insect's life, including development and reproduction. JH keeps insects in the juvenile state until the final immature stage when a temporal drop in JH permits metamorphosis to the adult form (Wigglesworth, 1934, 1970; Nijhout, 1994; Gilbert et al., 2000; Jindra et al., 2013). In adult insects, JH has many other functions during reproduction, including the stimulation of oogenesis (Engelmann, 1970, 1990; Raikhel et al., 2005). In diapause the CA is deactivated, resulting in an inhibition in the synthesis and secretion of JH and cessation of reproduction (De Wilde and De Boer, 1969; Darjo, 1976; Hodkova, 1976).

Studies on the molecular basis of JH signaling has revealed that *Methoprene-tolerant* (*Met*), a member of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family of transcription factors (Ashok et al., 1998), is the JH receptor (Jindra et al., 2015b). This was discovered because certain mutants of *Drosophila melanogaster* are resistant to the toxicity of the JH mimic methoprene (Wilson and Fabian, 1986). RNA interference (RNAi)-mediated knockdown of a *Met* ortholog revealed that it is required if JH is to prevent precocious metamorphosis in both holometabolous and the hemimetabolous insects (Konopova and Jindra, 2007; Minakuchi et al., 2008, 2009; Konopova et al., 2011; Lozano and Belles, 2014; Smykal et al., 2014). In adult insects, the molecular basis of JH signaling has been studied mostly in females. The gene *Met* is implicated in oogenesis in different insects (Parthasarathy et al., 2010; Li et al., 2011; Sheng et al., 2011; Zou et al., 2013) including *Pyrrhocoris apterus* (Smykal et al., 2014). In response to JH, Met proteins form complexes with other bHLH-PAS proteins. There is genetic evidence that germ cell-expressed (Gce)/Met acts as a JH receptor during development of *Drosophila melanogaster* (Jindra et al., 2015b). Taiman (Tai) (also called FISC or SRC) is currently the best-known protein partner of Met (Charles et al., 2011; Li et al., 2011; Zhang et al., 2011; Lozano et al., 2014; Jindra et al.,

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2015a). Recently it has been shown that both *Tai* and *Met* are required for JH-induced vitellogenin synthesis in the fat body and ovarian maturation in *P. apterus* (Smykal et al., 2014). On the other hand, JH-induced circadian gene expression in the gut of *P. apterus* females (Bajgar et al., 2013a) requires *Met* and two circadian genes, *cycle* (*cyc*) and *Clock* (*Clk*), but not *Tai* (Bajgar et al., 2013b). Intriguingly, it is possible that Met interacts with many protein partners and so initiates the diverse functions of JH.

The role of JH in male reproduction is less well understood. The clear role of JH in the physiology of the male accessory gland (MAGs) was first suggested by Wigglesworth (1936). Later experiments indicate that JH is involved in the synthesis of male accessory gland proteins (ACPs) in many different insects (Chen, 1984; Gillot, 1988; Yamamoto et al., 1988; Gold and Davey, 1989; Ismail and Gillot, 1994; Herndon et al., 1997). Ablation of CA in males of *P. apterus* results in a decrease in the levels of ACPs, while application of the JH mimic methoprene results in an increase (Socha et al., 2004; Socha, 2006). Recent data indicate that Met and Tai are required for JH to stimulate the growth of MAGs (Urbanova et al., 2016), but their effect on ACPs is unknown. Mating activity in *P. apterus* is not affected by the ablation of CA (Zdarek, 1966, 1968; Blazkova et al., 2011), but diapause males do not mate (Hodkova et al., 1991; Hodkova, 1994). There are no studies on the effect of JH on fertility, but impaired fertility is reported in males of *D. melanogaster* with depleted accessory glands resulting from multiple mating (Lefevre and Johnson, 1962; Heifetz et al., 2001). However, in Met^{27} mutants of *D. melanogaster* the accumulation of protein in accessory glands is less but their fertility is not affected (Wilson et al., 2003). Furthermore, a deficiency in JH enhances the synthesis of hexameric proteins in the fat body and their release into the haemolymph (Bradfield et al., 1990; Jones and Sarkari, 1993). In diapausing males and females of *P. apterus* with a natural deficiency of JH, there is an increase in 70–80 kDa hexameric proteins in the haemolymph (Socha et al., 1991; Sula et al., 1995; Socha and Sula, 1992, 1996). In addition, in females of *P. apterus* from which the CA has been removed, there is an increase in hexameric mRNA in the fat body (Smykal et al., 2014). Finally, that JH affects ageing is indicated by the observation that diapause or the removal of the CA extends lifespan in acridid grasshoppers (Pener, 1972), the monarch butterfly *Danaus plexippus* (Herman and Tatar, 2001; Tatar and Yin, 2001) and *P. apterus* (Hodkova, 2008; Blazkova et al., 2011; Buricova and Hodkova, 2013).

The first aim of this paper is to determine whether RNAi-mediated knockdown of the genes *Met* and *Tai* affect male survival and reproduction (ACPs, hexamerins, fertility) in the same way as the removal of the CA of active males, or the natural absence of JH in diapause males of *P. apterus*.

Although it is known that both *Met* and *Tai* RNAi affect JH function (see above), their effect on the amount of JH is unknown. Therefore, the second aim of this study is to determine the effect of the RNAi-mediated knockdown of the genes *Met* and *Tai* on the level of $JHSB_3$ in the haemolymph of *P. apterus*. The chemical composition of heteropteran JH was only recently identified when JH III skiped bisepoxide ($JHSB_3$), methyl (2R, 3S, 10R)-2,3,10,11 bisepoxyfarnesoate, was identified as a novel JH in the stink bug, *Plautia stali* (Kotaki et al., 2009, 2011; Kaihara et al., 2012).

2. Material and methods

2.1. Experimental animals

Colonies of *P. apterus* (L.) (Heteroptera) were reared at 25 ± 2 °C under either a reproduction-promoting LD photoperiod of 18 h light or a diapause-promoting SD photoperiod of 12 h light/12 h darkness and supplied *ad libitum* with linden seeds and water. All experiments were done using adult males.

Males destined for allatectomy or injection with dsRNA were deprived of food for 24 h after adult ecdysis. Prior to each treatment animals were anesthetized by submerging them in water for 10 min. CA were removed through an incision in the neck membrane or they were injected with 4 μg/2 μl of concentrated dsRNA in Ringer solution. In the case of the control males the neck membrane was cut or they were injected with plain Ringer solution. Males were injected into the abdomen. The adults were provided with food immediately after treatment.

Fourteen day old virgin males were used for the dissection of the accessory glands, fat body and collection of haemolymph. Haemolymph was collected from cut antennae. Dissected tissues and haemolymph were immediately placed in Eppendorf tubes kept in liquid nitrogen, and stored at −85 °C until analyzed.

Males used for determining survival and fertility were kept individually or in pairs in Petri dishes. Each male was provided with a 4 day old virgin female once per week. In the fertility experiments, the previous female was then kept separately and the number of eggs and the percentage that hatched in each batch was recorded. Mortality was recorded three times per week.

2.2. Protein content of accessory glands (ACPs)

Accessory glands were dissected in Ringer solution. In each sample 4 accessory glands were homogenized in 50 μl of 50 mM phosphate buffer (pH 7.0) with 1 mM EDTA. Samples were treated with 5 μl streptomycin sulphate (10%) to remove nucleic acids (Reznick and Packer) and incubated for 15 min at room temperature (RT). The homogenate was then centrifuged at 3000*g* for 10 min at 4 °C. The resulting supernatant was used to determine the protein content using Pierce BCA Protein Assay Kit (ThermoScientific). Bovine serum albumin was used as a standard. A SpectraMax 384 (Molecular Devices) was used to measure absorbance at 562 am. The results are expressed in μg of protein per gland.

2.3. Haemolymph proteins

Polyacrylamide gel electrophoresis with sodium dodecyl sulphate (PAGE SDS) was carried out according to Laemmli (1970). Fresh gels of 10% were prepared by mixing TRIS buffer 8.8 pH with 0.1% SDS and polymerization solution with 30% acrylamide and 0.8% bisacrylamide dissolved in the same TRIS buffer. Gels, 0.7 mm thick, were used in a Bio-Rad electrophoresis apparatus Mini Protean III. Equivalents of 0.2 μl of haemolymph were used for each strip. Electrophoreses were run at 100 V for 2 h. After electrophoresis, the gels were stained with 0.25% Coomassie Brilliant Blue R-250 in ethanol-acetic acid-water for 4 h. Gels were then washed several times with ethanol-acetic acid-water (2.5:1:6.5). Finally, the density of the blue bands corresponding to hexamerin proteins (70–90 kDa) was determined using a densitometer (GS 800, Bio-Rad).

2.4. cDNA cloning

Cloning of *P. apterus* cDNA encoding *Met* (Konopova et al., 2011) and *Tai* (Bajgar et al., 2013a,b) and hexamerin (Smykal et al., 2014) was carried using the methods of the cited authors.

2.5. RNA interference (RNAi)

Double-stranded (dsRNA) was prepared using the The MEGAscript® T7 Kit (Ambion) from plasmids containing the appropriate gene fragments as previously described (Konopova et al., 2011; Bajgar et al., 2013a,b) and 4 μg/2 μl of *Met* or *Tai* dsRNA was

injected into *P. apterus* males. Females were injected with the same amount of dsRNA as males and the effect of the injections evaluated in terms of the inhibition of ovarian maturation. After injection of dsRNA against both *Met* and *Tai*, females did not oviposit for at least 3 weeks and dissection revealed undeveloped ovaries.

2.6. Quantification of mRNA

Fat body was dissected in Ringer solution for the analysis of *hexamerin* mRNA. The whole body was used for the analysis of *Met* and *Tai* mRNA. Total RNA was isolated using Ribozol reagent (Ambresco). After Turbo DNAse (Ambion) treatment, 2 μg of total RNA was used for cDNA synthesis using the SuperScript III reverse transcriptase and oligo (dT) primers (Invitrogen). Relative transcript levels were determined using quantitative PCR and the iQ SYBR Green Supermix kit and a C1000 Thermal Cycler (both Bio-Rad). The transcripts levels were normalized to those of the ribosomal RNA as previously described (Dolezel et al., 2007). The sequences of primers used for quantifying mRNA were those previously described (Konopova et al., 2011; Bajgar et al., 2013a,b; Smykal et al., 2014).

2.7. Identification and quantification of juvenile hormone

In *P. apterus*, $JHSB_3$ levels were analyzed in both *in vitro* incubation of the complex of corpora cardiaca-corpus allatum and in haemolymph. The complex was incubated in Grace's insect tissue culture medium, extracted with hexane and stored at —80 °C prior to analysis. Haemolymph (50 μl) was collected in 500 μl of methanol, homogenized and centrifuged for 5 min at 6000*g*. Extracts were stored at -80 °C prior to analysis. All extracts were then evaporated in a Speed-Vac centrifuge, re-dissolved in 1 ml of 30% methanol and then purified in SPE columns (Oasis HLB, Waters Co., USA). The columns were conditioned with 1 ml of methanol, as recommended by the manufacturer, followed by 1 ml of 30% methanol. After adding the sample, chromatographic columns were washed with 1 ml of 30% methanol and the analytes eluted using 100% methanol. The eluate was dried in a Speed-Vac centrifuge and re-dissolved in the mobile phase used for analysis in the liquid chromatography–mass spectrometry (60% methanol in water buffered with 5 mM ammonium formate). The final volume of all purified samples diluted in the mobile phase was 50 μl.

Chemical analyses were performed using a triple quadrupole mass spectrometer (LTQ, Thermo Electron Corp., USA) in ESI mode. Positive ESI spectra were acquired using the following conditions: spray voltage 2.5 kV, vaporizer temperature 250 °C, capillary temperature 250 °C. Specification of the chromatographic conditions: HPLC column Aquasil C18 RP, 150 × 2.1 mm, 3 μm; flow rate 250 μl/min; gradient 60–100% methanol (0–7 min), followed by isocratic conditioning in 60% methanol with water buffered with 5 mM ammonium formate (7–12 min). The mass spectrometric data were recorded through the following Selected reaction monitoring (SRM) transitions (parent mass *m/z* ? fragment mass *m/z*): 283 ? 233 for $JHSB_3$ (RT 4.06) and 295 ? 263 for JH I (RT 6.15) as internal standard.

For the CC-CA *in vitro* incubation only three calibration levels of the $JHSB_3$ standard were prepared. The main aim of this experiment was to determine whether $JHSB_3$ is present in *P. apterus* and to further develop the analytical method. Thus, only the approximate quantity of $JHSB_3$ synthesized was determined. The calibration curve used for the quantification of $JHSB_3$ in haemolymph (S4) was linear over the range 1–100 pg/μl, the injection volume was 20 μl and sample volume 50 μl. The calibration curve was constructed from the peak area ratio of the $JHSB_3$ standard and JH I as the internal standard at constant level. The internal standard was diluted in the mobile phase that was added to the evaporated extract prior the analysis. The level of JH I in mobile phase was 25 pg/μl (in total l.25 ng per 50 μl sample extract respectively). Correlation = 0.991, used 7 concentration levels, n = 3.

Sample extraction recovery of $JHSB_3$ was evaluated at the level 250 pg of standard added to 50 μl of haemolymph (corresponding to 5 pg per 1 μl on the calibration curve (S4)). The calculated extraction yield was 65%. The levels of $JHSB_3$ presented in this publication were not recalculated to avoid any further interference as we expected that the extraction yield increases with titre of analyte. However, because the differences between samples are quite big, the limitation of accuracy of the JH data is acceptable in this paper. The day to day variation of measurements was within ±1,5%.

2.8. Statistical analysis

Graph Pad Prism 5.0 software was used for all comparisons. For comparing many samples One-Way Anova and Tukey's post hoc tests were used. The difference between two samples was evaluated using Student's *t*-tests. Log-rank test was used to assess the similarity of the longevity of the two groups.

3. Results

3.1. Met and Tai dsRNA inhibit the synthesis of ACPs similar to allatectomy but less than occurs in diapause

The size of accessory glands and the amount of ACPs in adult LD males of *P. apterus* increased with age up to 14 days old (Socha et al., 2004). Therefore, we compared the amount of ACPs in males of this age (Fig. 1A). The amount was greatest in the LD controls, reaching more than 200 μg/gland. Whereas the level in LD males from which the CA was removed (allatectomized) was about one third lower. To determine whether endogenous JH stimulates the synthesis of ACPs via the JH receptor Met and its Tai partner, we examined the accessory glands of males that were injected with double stranded RNA (dsRNA) targeting either protein. Males treated with *Met* or *Tai* dsRNA had an amount of ACP similar to allatectomized LD males (Fig. 1A). This indicates that endogenous JH requires both Met and Tai for stimulating the synthesis of ACPs.

Short day lengths result in inactivation of CA and diapause (Hodkova, 1976). However, the amount of ACP in SD (diapause) controls is almost two thirds lower than that in LD controls and almost one third lower than that in the three experimental groups in LD (allatectomized, treated with *Met* or *Tai* dsRNA). Although ANOVA followed by a Tukey Multiple Comparison Test did not reveal a difference between SD-controls and CAex, Student's *t*-tests revealed very significant differences between SD-controls and the three experimental groups in LD, including CAex (SD vs. CAex, $P = 0.0011$; SD vs. *Met* RNAi, $P < 0.0001$; SD vs. *Tai* RNAi, $P = 0.0069$) (Fig. 1A). This indicates that synthesis of ACP does not depend only on JH, but also on factor(s) responsible for differences between SD diapause and LD allatectomized males or those treated with *Met* or *Tai* dsRNA.

3.2. Met or Tai dsRNA enhance the level of hexamerins similar to allatectomy, but less than occurs in diapause

During SD induced diapause there are high levels of 78 and 82 kDa proteins (hexamerins) in the haemolymph of males of *P. apterus* (Sula et al., 1995). In the present experiments, we determined the effects of diapause, allatectomy and treatment with *Met* or *Tai* dsRNA on the level of hexamerin proteins in the haemolymph and hexamerin mRNA in the fat body of males. The males used were 14 days old as at this age the difference in the level of hexamerin proteins in diapause and non-diapause individuals is greatest (Sula et al., 1995).

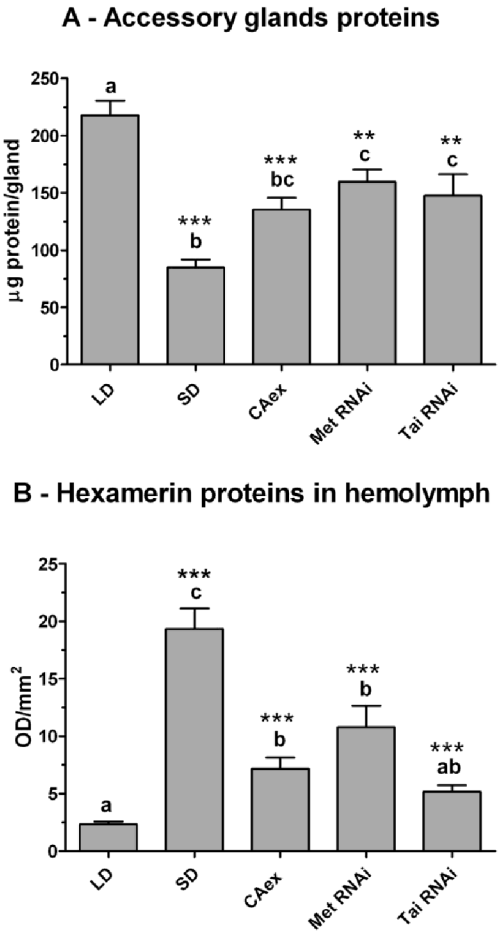

Fig. 1. Changes in the protein content in the accessory gland (A) and hexamerin proteins in the haemolymph of males (B) LD – long day control, SD – short day control, CAex – corpora allata ablated, Met RNAi – Met RNA interference, Tai RNAi – Tai RNA interference. Columns indicate means ± SEM. Differences between means are evaluated using ANOVA followed by Tukey Multiple Comparison Tests. Different letters indicate different means at least at $P < 0.05$ in both A and B. Difference between LD-column and individual means was evaluated using *t*-tests with $P < 0.001$ (*) or $P < 0.001–0.01$ (*) in both (A) and (B). Difference between SD-column and individual means was evaluated using *t*-test with $P = 0.0011$ for SD vs. CAex., $P < 0.0001$ for MetRNAi, $P = 0.0069$ for TaiRNAi in (A). (A) Columns indicate means of µg/gland ± SEM; n = 7–10 for each column. (B) Columns indicate means of optical density (OD)/mm² ± SEM; n = 18–22 for each column.

3.2.1. Level of hexamerin proteins in haemolymph

In LD males, 14 days after allatectomy, the levels of 78 and 82 kDa hexamerin proteins in haemolymph were about three times higher than in LD controls. Injection with *Met* dsRNA resulted in about a four times higher level of hexamerins than in the LD controls (Fig. 1B). By contrast, males injected with *Tai* dsRNA had about a two times higher level of hexamerins than in LD controls. The difference was not significant when evaluated using ANOVA followed by Tukey Multiple Comparison Tests, although Student's *t*-tests revealed highly significant differences (LD vs. Tai RNAi, $P < 0.0001$) (Fig. 1B). However, statistical analysis did not reveal any differences among LD allatectomized, *Met* dsRNA and *Tai* dsRNA males (ANOVA followed by Tukey Multiple Comparison Tests) (Fig. 1B). The results indicate that endogenous JH requires both Met and Tai to suppress hexamerins in haemolymph.

The level of hexamerin proteins in SD controls of the same age (14 days) were about 7 times higher than in LD controls and 2–4 times higher than in LD males, either allatectomized or treated with *Met* or *Tai* dsRNA (Fig. 1B). This indicates that the level of hexamerins in the haemolymph of SD diapause males, with naturally inactive CA, are much greater than in LD allatectomized males or those treated with *Met* or *Tai* dsRNA. Again, there is a great difference between the LD experimental groups and SD diapause controls.

3.2.2. Level of hexamerin mRNA in fat body

Hexamerins released into the haemolymph are synthesized in fat body. Therefore, levels of *hexamerin* mRNA in the fat body of males were determined (Fig. 2A). *Hexamerin* mRNA levels are lowest in LD controls, highest in SD controls and intermediate in LD males with ablated CA or treated with *Met* or *Tai* dsRNA. Thus changes in the level of *hexamerin* mRNA in the fat body show similar trends to those in the haemolymph, although not statistical significantly so. There are significant differences between LD and other groups (Student's *t*-test), but differences between SD and LD experimental groups were not statistically significant.

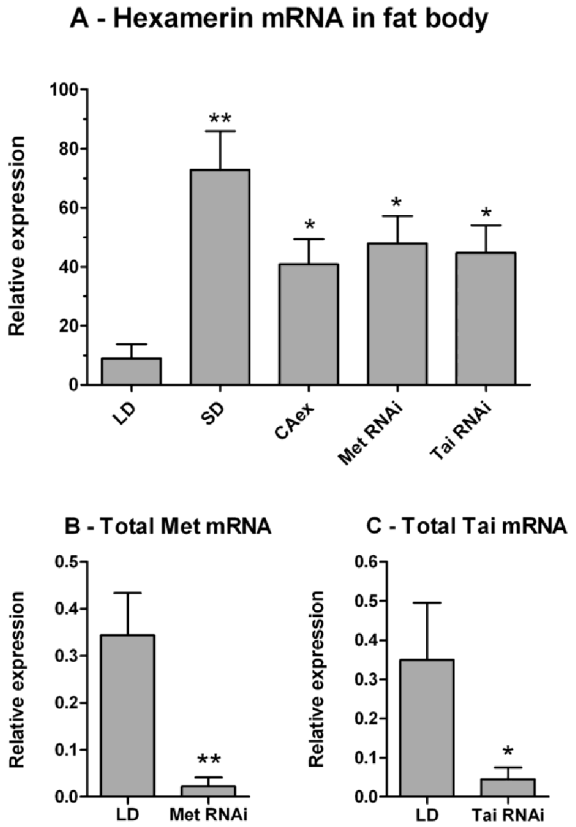

Fig. 2. Changes in the expression of *Hexamerin* mRNA in the fat body (A), total *Met* mRNA (B) and total *Tai* mRNA LD – long day control, SD – short day control, CAex – corpora allata ablated, Met RNAi – Met RNA interference, Tai RNAi – Tai RNA interference. Columns indicate means of relative expression ± SEM. A – *Hexamerin* mRNA was isolated from the fat body of males aged 14 days. Males were allatectomized or injected with dsRNA at age 24 h. (B, C) Total mRNA was isolated from the whole body of males aged 14 days. Difference between LD-column and individual means was evaluated using *t*-tests with $P < 0.001–0.01$ (*) or $P < 0.01–0.05$ (*). Columns indicate means of relative expression ± SEM. Relative expression is normalized to *rp49*. (A) n = 3–4, (B) n = 5–6, (C) n = 5–6.

3.2.3. Level of Met and Tai mRNA

Injection of *Met* dsRNA or *Tai* dsRNA depleted *Met* mRNA ca. 94% (15.7x, Fig. 2B) and *Tai* mRNA ca. 87% (7.7x, Fig. 2C).

3.6. Allatectomy and Met dsRNA increase lifespan less than diapause, and Tai dsRNA has no effect

Allatectomy of paired LD-males considerably prolonged their lifespan. Virgin LD-controls lived as long as allatectomized paired LD-males. In contrast, allatectomy of LD-virgins did not affect their lifespan with the exception of about one third of the individuals that lived slightly longer than the virgin LD-controls (Fig. 3A). Thus, CA affected the lifespan of LD-males mainly through sexual interactions. On the other hand, injection with *Met* dsRNA prolonged their lifespan in both virgin and paired males (Fig. 3C). This indicates that the *Met* dsRNA prolongs lifespan independently of sexual interactions. Finally, injection with *Tai* dsRNA did not affect both virgin and paired males (Fig. 3D). This indicates that *Tai* dsRNA does not prolong lifespan either through sexual interactions or other means.

Diapause SD-males do not mate. Lifespan of virgin males was not significantly different from that of males paired with diapause females (Fig. 3B). The CA is inactive and its removal had only a slight or no effect on lifespan of either virgin or paired males.

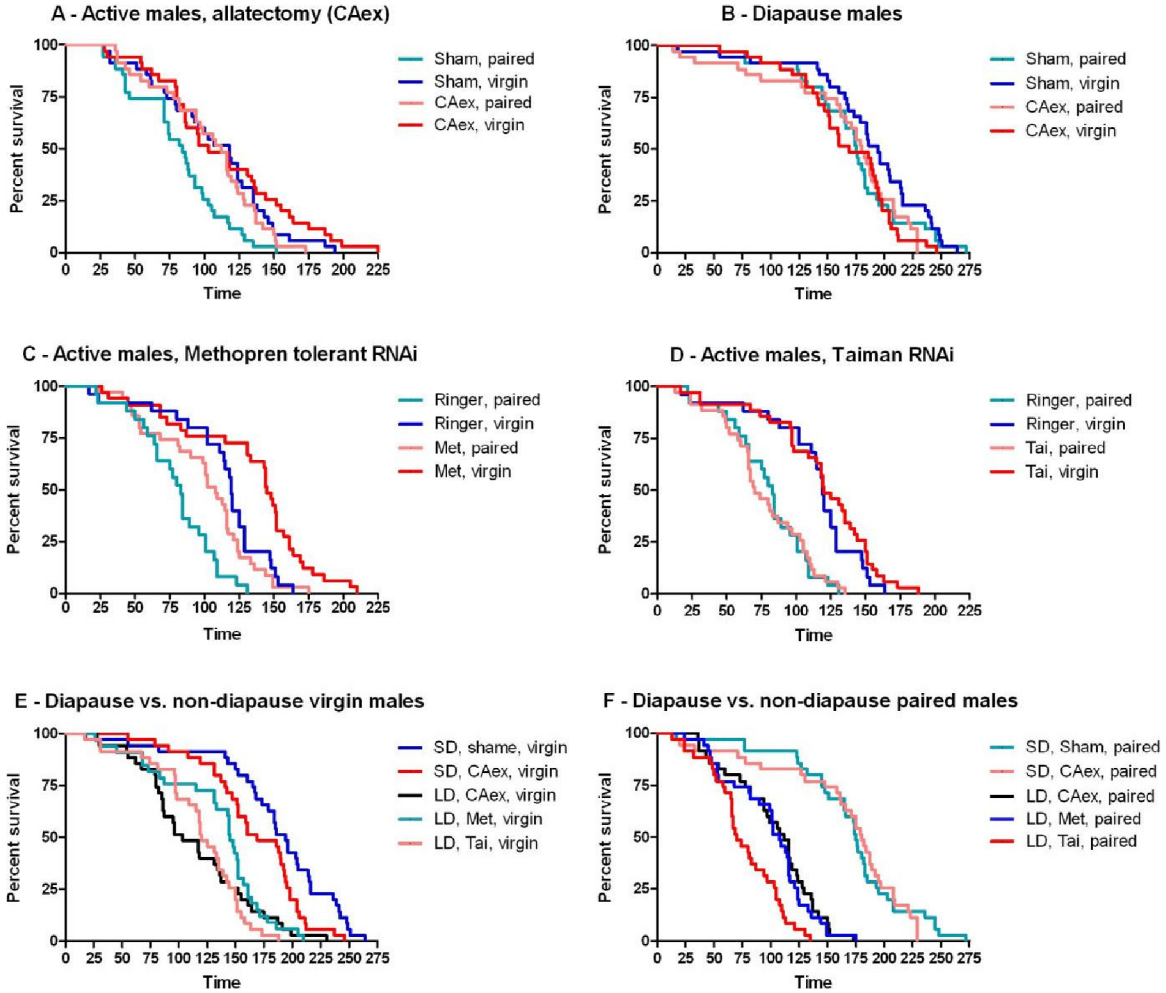

Fig. 3. Survival of active, LD-males after CA ablation (A), diapause, SD-males after CA ablation (B) active, LD males with *Met* RNAi (C), Active, LD-males with *Tai* RNAi (D), and comparison of SD-males with LD-CA ablation, LD-Met RNAi or LD-Tai RNAi males (E, F) (A) LD-sham-operated, paired, n = 35, median = 84; LD-sham-operated, virgin, n = 35, median = 118; LD-CAex, paired, n = 35, median = 112; LD-CAex, virgin, n = 35, median = 103; curve comparison: operated paired, vs. CA paired, P = 0.0062; operated paired vs. CA virgin, P = 0.0005; operated paired vs. operated virgin, P = 0.0019. (B) SD-sham-operated, paired, n = 35, median = 175; SD-sham-operated, virgin, n = 35, median = 194; SD-CAex, paired, n = 35, median = 180; SD-CAex, virgin, n = 35, median = 169; curve comparison: CA paired vs operated virgin, P = 0.0343; CA virgin vs. operated virgin,P = 0.0100. (C) LD-Ringer, paired, n = 25, median = 83; LD-Ringer, virgin, n = 25, median = 119; LD-Met, paired, n = 35, median = 108; LD-Met, virgin, n = 35, median = 145; curve comparison: Ringer paired vs. Met paired, P = 0.0026; Ringer paired vs. Met virgin, P < 0.0001; Ringer paired vs. Ringer virgin, P < 0.0001; Met paired vs. Met virgin, P < 0.0001; Met virgin vs. Ringer virgin, P = 0.0013. (D) LD-Ringer, paired, n = 25, median = 83; LD-Ringer, virgin, n = 25, median = 119; LD-Tai, paired, n = 35, median = 70; LD-Tai, virgin, n = 35, median = 120; curve comparison: Ringer paired vs. Tai virgin, P < 0.0001; Ringer paired vs. Ringer virgin, P < 0.0001; Tai paired vs. Tai virgin, P < 0.0001; Tai paired vs. Ringer virgin, P < 0.0001. (E) SD-CAex. virgin vs. LD-CAex virgin, P = 0.0001, vs. LD-Met virgin, 0.0017, vs. LD-Tai virgin P < 0.0001; SD-virgin vs. LD-CAex virgin,vs. LD-Met virgin, vs. LD-Tai virgin, in all cases P < 0.0001. (F) SD-CAex paired vs. LD-CAex paired, vs. LD-Met paired, vs. LD-Tai paired, in all cases P < 0.0001; SD-paired LD-CAex paired, vs. LD-Met paired, vs. LD-Tai paired, in all cases P < 0.0001. Numbers of replicates and medians in (E), (F) are given in (A), (B), (C), (D). Comparison of survival curves was made using the Log-rank (Mantel-Cox) Test. Only significant differences are shown.

Diapause SD-males lived significantly longer than LD-males, even if the lifespan of LD-virgins was greatly prolonged by allatectomy or treatment with *Met* dsRNA (Fig. 3E, F). A comparison of the survival curves using Log-rank (Mantel-Cox) is presented in Fig. 3.

3.7. Allatectomy, Met and Tai dsRNA do not significantly affect fertility

Allatectomized LD males mate as frequently as LD-controls (Blazkova et al., 2011) and diapause SD-males do not mate (Hodkova, 1994). To determine whether a low level of protein in the accessory glands is associated with the low fertility of LD-males, they were provided with a new 4 day old female each week. These females were subsequently kept separately and the number of eggs laid and the number that hatched were recorded for every egg batch. There was no significant difference in either the fecundity or fertility. Here we present only the fecundity in terms of the first egg batch of the first three females (Fig. 4A) and the percentage that hatched (Fig. 4B). The percentage of eggs

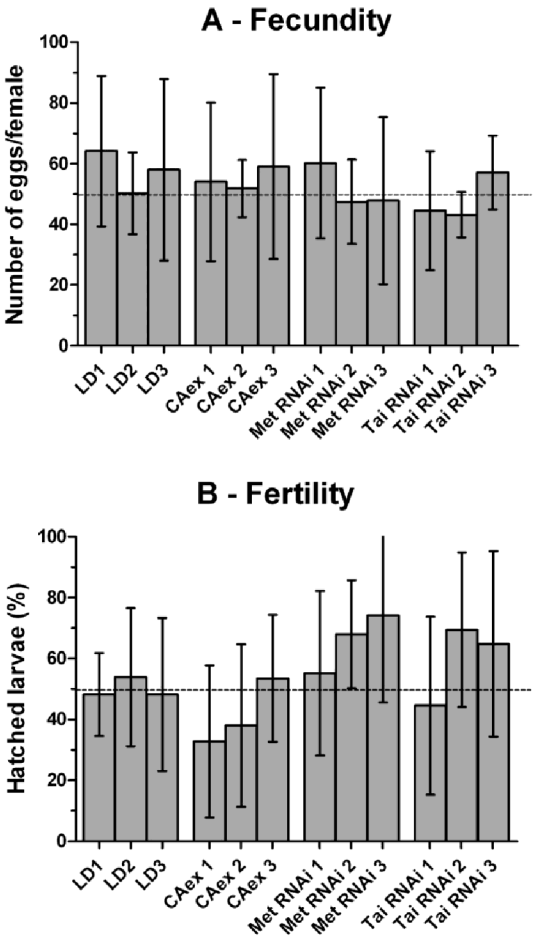

Fig. 4. Fecundity and fertility of females paired with treated males. LD – long day control, CAex – corpora allata ablated, Met RNAi – Met RNA interference, Tai RNAi – Tai RNA interference. (A) Fecundity of the first 3 females (each female spent 1 week with the male) in the first batch. Columns indicate means of number of eggs laid by 1 female ± SEM, n = 12–15. (B) Percentage of eggs in the first batch of the first 3 females that hatched. Columns indicate means of the percentage of eggs that hatched in the first batch of 1 female ± SEM, n = 12–15. ANOVA followed by Tukey Multiple Comparison Tests revealed no significant differences.

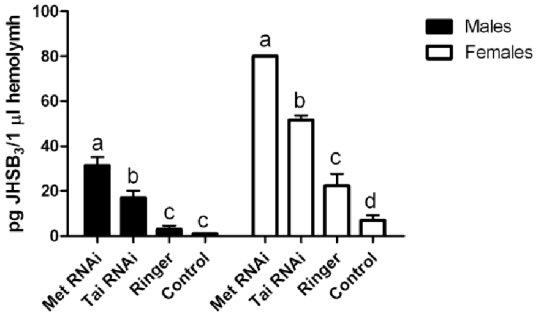

Fig. 5. Changes in $JHSB_3$ in haemolymph after injection of *Met* dsRNA, *Tai* dsRNA or Ringer. Columns indicate means ± SEM. Differences between means were evaluated using ANOVA followed by Tukey Multiple Comparison Tests, n = 3. Different letters indicate statistically different values at least at $P < 0.05$.

of the controls that hatched was slightly higher than that of pairs with CA ablated males and lower than for pairs with males treated with *Met* or *Tai* dsRNA. However, the difference is not significant because the results were very variable.

3.8. Met and Tai dsRNA greatly enhance the level of JH in the haemolymph

Triple quadrupole mass spectrometer analysis revealed that it is highly likely that $JHSB_3$ is present both in the secretion of the CA *in vitro* and the haemolymph of *P. apterus*. In total we incubated 500 CA for 2 2 h, to receive about 50 ng of experimental sample. Mass spectra (S1, S2, Teal and Gomez-Simuta (2002)) and retention time (S3) of the incubated sample as well as the haemolymph were identical with that of the $JHSB_3$ standard (a gift by Toyomi Kotaki). This result suggests that the JH of *P. apterus* is identical to $JHSB_3$ previously found in *P. stali* (Kotaki et al., 2009, 2011; Kaihara et al., 2012), although the chirality of $JHSB_3$ in *P. apterus* remains to be determined.

Next we determined the effect of *Met* and *Tai* dsRNA on the quantity of $JHSB_3$ in the haemolymph, using a calibration curve (S4). S5 presents chromatograms of the most characteristic samples for each category. The 14-day old males injected with *Met* dsRNA had 10 times more and those injected with *Tai* dsRNA had a 6 times more $JHSB_3$ in their haemolymph than the controls injected with Ringer solution (Fig. 5, n = 3). For 14-day old females, the level of $JHSB_3$ in their haemolymph increased almost 4 times after injection with *Met* dsRNA and almost 3 times after injection with *Tai* dsRNA compared to those injected with Ringer solution (Fig. 5, n = 3).

4. Discussion

4.1. Regulation of ACPs and hexamerins in LD-males

Allectomy in *P. apterus* results in a lower quantity of total soluble protein in the accessory glands (ACPs) of males (Socha et al., 2004; Socha, 2006). Furthermore, the separation of the ACPs on SDS-PAGE reveal that all protein fractions were lower following allatectomy (Socha et al., 2004). This indicates that JH has a wide-ranging effect on protein synthesis in the accessory glands of males. Recent findings demonstrate that JH-induced growth of MAGs in *P. apterus* is inhibited by *Met* or *Tai* dsRNA (Urbanova et al., 2016). The data presented indicate that injection of *Met* or *Tai* dsRNA reduced the quantity of ACPs in the same way as ablation of the CA (Fig. 1A). This indicates that Met and Tai transduce

the JH signal that enhances the expression of ACP. In contrast, allatectomy enhances the quantity of hexamerin protein in the haemolymph of *P. apterus* (Socha et al., 1991). The data presented demonstrate that injection of *Met* or *Tai* dsRNA enhanced the amount of hexamerin protein in the haemolymph (1B) and *hexamerin* mRNA (2A) in the fat body, similar to that which resulted from the ablation of CA. This indicates that Met and Tai transduce the JH signal that inhibits hexamerin expression. While Tai itself does not bind with JH, the hormonal receptor Met interacts with Tai in the presence of JH (Charles et al., 2011). Although JH greatly increased after injection with *Met* or *Tai* dsRNA ($JHSB_3$, Fig. 5) the function of JH was blocked. This is not surprising as these two major findings indicate that Met and Tai are needed to transduce the JH signal in *P. apterus*. Previous reports indicate that Met and Tai promote JH-induced synthesis of *vitellogenin* mRNA and ovarian maturation, and reduce the synthesis of *hexamerin* mRNA in the fat body of females of *P. apterus* (Smykal et al., 2014). Similarly, in the gut of adult females of *Aedes aegypti*, both Met and Tai are required for normal expression of JH-induced trypsin gene (Li et al., 2011).

4.2. Regulation of survival in LD-males

Consistent with earlier results (Blazkova et al., 2011) ablating the CA greatly prolonged the lifespan of paired males (Fig. 3A). The production of ACPs is not important in determining longevity as allatectomy had only a slight effect on survival of virgins (Fig. 3A) but a large effect on the level of ACPs (Fig. 1A). Therefore, it is likely that the CA shortened the lifespan of paired males mainly through sexual interactions. However, mating itself did not mediate the negative effect of CA, because the absence of CA had no significant effect on the mating activity of *P. apterus* (Zdarek, 1966, 1968; Blazkova et al., 2011). The mechanism by which sexual interactions affect lifespan is not known. It is possible that courtship behaviour is influenced by the CA. Notably, the main cost of sexual interactions arises from courtship e.g. in males of *D. melanogaster* (Cordts and Partridge, 1996) or *Ceratitis capitata* (Papandopoulos et al., 2010).

Injection with *Met* dsRNA prolonged the median lifespan of virgin and paired males by a similar value (Fig. 3C). This indicates that Met shortens lifespan independently of sexual interactions. In females of *Tribolium castaneum*, Met stimulates vitellogenin gene expression through components of the insulin-like peptide signaling pathway (Sheng et al., 2011). Hypothetically, Met may shorten life through the CA-independent insulin signaling pathway in LD-males of *P. apterus*. Indeed, in LD-females of *P. apterus*, a CA-independent effect of insulin producing cells in the pars intercerebralis shortens their life (Hodkova, 2008).

Injection of *Tai* dsRNA did not affect male lifespan (Fig. 1D). In contrast to its regulation of ACPs and hexamerins, Tai was not a partner of Met for regulation of longevity by JH or through other pathways. Earlier reports indicate that although Met and Tai are required for the synthesis of *vitellogenin* mRNA in *P. apterus* (Smykal et al., 2014) or in repression of metamorphosis in *Blattella germanica* (Lozano et al., 2014), Tai is not necessary for JH-dependent synthesis of mRNA of some other genes. For example, the expression of the circadian gene *Pdp1* by JH in the gut requires *clock*, *cycle* and *Met*, while *Tai* appears to have no role (Bajgar et al., 2013b). The anti-metamorphic effect of JH is inhibited by injection of *Met* dsRNA, but the partner of Met is unknown in *P. apterus* (Konopova et al., 2011; Smykal et al., 2014).

4.3. Regulation of fertility

Earlier experiments indicate that allatectomy does not reduce mating (Zdarek, 1966, 1968; Blazkova et al., 2011). Fertility of allatectomized males was not reduced. Although mating behaviour of males treated with *Met* and *Tai* dsRNA was not analyzed in the present study, their fertility was slightly, although not significantly, higher than that of the control males. It is likely that a low amount of protein in accessory fluid does not result in a decrease in the percentage of fertilized eggs. Perhaps, under natural conditions, males fertilize more females. It is also possible that the development of larvae or the next generation may be affected. Data in the literature indicate that a deficiency in JH either decreases fertility of males (e.g. *T. castaneum* treated with *Met* dsRNA or JHAMT dsRNA) or has no effect (e.g. Met^{27} null mutants of *D. melanogaster*), although ACPs are reduced in both cases (Wilson et al., 2003; Parthasarathy et al., 2009).

4.4. Regulation of JH

$JHSB_3$ was synthesized by the CA *in vitro* (S1–S3) and was present also in the haemolymph (S5, Fig. 5) of *P. apterus*. A higher amount of $JHSB_3$ in females compared to males might be related to the difference in the volume of their CAs, which are nearly twice as large in females as in males (Hodkova and Socha, 2006; Socha and Hodkova, 2006).

In order to function JH requires Met and Tai proteins, receptors for JH (Jindra et al., 2013, 2015a). Met binds with JH and forms a complex with Tai (FISC, SRC) and together they regulate JH-response genes by binding with JH-response elements in DNA (Kayukawa et al., 2012; Li et al., 2014; Ojani et al., 2016). Previous studies demonstrate that allatectomy (absence of JH) (Konopova et al., 2011), diapause with natural absence of JH (Bajgar et al., 2013b) or depletion of *Met* or *Tai* mRNA (genes for JH receptor) (Smykal et al., 2014) result in a marked reduction in the *transcription factor Krüppel-homolog 1 (Kr-h1)* mRNA in adults of *P. apterus*, assuming that JH is required for stimulating *Kr-h1* mRNA. On the other hand, the level of *Met* mRNA is significantly higher in diapause compared with reproductive adults of *P. apterus* (Bajgar et al., 2013b), Presently we show how depletion of *Met* or *Tai* mRNAs influences the JH titre. An increase in $JHSB_3$ caused by injection of Ringer solution without dsRNA (3 times) was most probably an injury effect. However, injection of Ringer solution with *Met* or *Tai* dsRNA resulted in an even greater increase in $JHSB_3$ in both males (10 and 6 times, respectively) and females (3.6 and 2.3 times, respectively) of 14-day old *P. apterus*, (Fig. 5). This is consistent with the hypothesis of Grutenko et al. (2000) that impaired JH reception in *Drosophila* mutants Met^{27}, carrying a null allele of the *Met* gene, prevented JH titre-mediated regulation of the JH degradation system, which may result in an elevated JH level. JH was reported to have a negative effect on the synthesis of JH in the CA of *D melanogaster* (Richard and Gilbert, 1991). Met/Tai complex possibly acts as a JH sensor in the CA and might negatively regulate JH synthesis. It is important that even a greatly enhanced level of $JHSB_3$ does not influence the amount of ACPs and hexamerins when Met or Tai proteins are substantially reduced. This result is consistent with the fact that the Met/Tai complex functions as a receptor of JH needed to transduce the JH signal (Konopova and Jindra, 2007; Charles et al., 2011; Li et al., 2011; Konopova et al., 2011; Zhang et al., 2011; Jindra et al., 2013, 2015a; Smykal et al., 2014).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2016.08.009.

References

Ashok, M., Turner, C., Wilson, T.G., 1998. Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. Proc. Natl. Acad. Sci. U.S.A. 95, 2761–2766.

Bajgar, A., Dolezel, D., Hodkova, M., 2013a. Endocrine regulation of non-circadian behavior of circadian genes in insect gut. J. Insect Physiol. 59, 881–886.

Bajgar, A., Jindra, M., Dolezel, D., 2013b. Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. Proc. Natl. Acad. Sci. U.S.A. 110, 4416–4421.

Blazkova, H., Provaznik, J., Hodkova, M., 2011. Novel roles for the corpus allatum hormone in the cost of sexual interactions in the linden bug *Pyrrhocoris apterus*. J. Insect Physiol. 57, 529–535.

Bradfield, J.Y., Berlin, R.L., Keeley, L.L., 1990. Contrasting modulations of gene expression by a juvenile hormone analog. Insect Biochem. 20, 105–111.

Buricova, M., Hodkova, M., 2013. Relationship between locomotor activity, oxidative damage and life span in males and females of the linden bug, *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). Eur. J. Entomol. 110, 443–449.

Charles, J.-P., Iwema, T., Epa, V.C., Takaki, K., Rynes, J., Jindra, M., 2011. Ligand binding properties of a juvenile hormone receptor, Methoprene-tolerant. Proc. Natl. Acad. Sci. U.S.A. 108, 21128–21133.

Chen, P.S., 1984. The functional morphology and biochemistry of insect male accessory glands and their secretions. Annu. Rev. Entomol. 29, 233–255.

Cordts, R., Partridge, L., 1996. Courtship reduces longevity of male *Drosophila melanogaster*. Anim. Behav. 52, 269–278.

Darjo, A., 1976. Activité des corpora allata et controle photoperiodique de la maturation ovarienne chez *Locusta migratoria*. J. Insect Physiol. 22, 347–355.

Dolezel, D., Sauman, I., Kostal, V., Hodkova, M., 2007. Photoperiodic and food signals control expression pattern of the clock gene, period, in the linden bug, *Pyrrhocoris apterus*. J. Biol. Rhythms 22, 335–342.

Engelmann, F., 1970. The Physiology of Insect Reproduction. Pergamon Press, Oxford, New York.

Engelmann, F., 1990. Hormonal control of arthropod reproduction. In: Epple, A., Scanes, C.G., Stetson, M.H. (Eds.), Comparative Endocrinology. Wiley-Liss, New York, pp. 357–364.

Gilbert, L.I., Granger, L.A., Roe, R.M., 2000. The juvenile hormones: historical facts and speculations on future research directions. Insect Biochem. Mol. Biol. 30, 617–644.

Gillot, C., 1988. Arthropoda-Insecta. In: Adiyodi, H.G., Adiyodi, R.G. (Eds.), Reproductive Biology of Invertebrates, III. Wiley III, Chichester, pp. 319–471 (Accessory Sex Glands).

Gold, S.M.W., Davey, K.G., 1989. The effect of juvenile hormone on protein synthesis in the transparent accessory gland of male *Rhodnius prolixus*. Insect Biochem. 19, 139–143.

Grutenko, N.E., Wilson, T.G., Monastirioti, M., Rauschenbach, I.Y., 2000. Stress-reactivity and juvenile hormone degradation in *Drosophila melanogaster* strains having stress-related mutation. Insect Biochem. Mol. Biol. 30, 775–783.

Heifetz, Y., Tram, U., Wolfner, M.F., 2001. Male contributions to egg production: the role of accessory gland products and sperm in *Drosophila melanogaster*. Proc. R. Soc. Lond. B: Biol. Sci. 268, 175–180.

Herman, W.S., Tatar, M., 2001. Juvenile hormone regulation of longevity in the migratory monarch butterfly. Proc. R. Soc. Lond. B: Biol. Sci. 268, 2509–2514.

Herndon, L.A., Chapman, T., Kalb, J.M., Levin, S., Partridge, L., Wolfner, M.F., 1997. Mating and hormonal triggers regulate accessory gland gene expression in male *Drosophila*. J. Insect Physiol. 43, 1117–1123.

Hodkova, M., 1976. Nervous inhibition of corpora allata by photoperiod in *Pyrrhocoris apterus*. Nature 263, 521–523.

Hodkova, M., 1994. Photoperiodic regulation of mating behaviour in the linden bug, *Pyrrhocoris apterus*, is mediated by a brain inhibitory factor. Experientia 50,742–744.

Hodkova, M., 2008. Tissue signaling pathways in the regulation of life-span and reproduction in females of the linden bug, *Pyrrhocoris apterus*. J. Insect Physiol. 54, 508–517.

Hodkova, M., Zieglerova, J., Hodek, I., 1991. Diapause in males of *Pyrrhocoris apterus* (L.) (Heteroptera) and its dependence on photoperiod and the activity of females. Zool. Jb. Syst. 118, 279–285.

Hodkova, M., Socha, R., 2006. Endocrine regulation of the reproductive arrest in the long-winged females of the linden bug, *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). Eur. J. Entomol. 103, 523–529.

Ismail, S.M., Gillot, C., 1994. A high affinity, juvenile hormone-binding protein in the long hyaline tubules of male *Melanoplus sanguinipes*. Insect Biochem. Mol. Biol. 24, 739–745.

Jindra, M., Belles, X., Shinoda, T., 2015a. Molecular basis of juvenile hormone signaling. Curr. Opin. Insect Sci. 11, 39–45.

Jindra, M., Palli, S.R., Riddiford, L.M., 2013. The juvenile hormone signaling pathway in insect development. Annu. Rev. Entomol. 58, 181–204.

Jindra, M., Uhlirova, M., Charles, J.P., Smykal, V., Hill, R.J., 2015b. Genetic evidence for function of the bHLH-PAS protein Gce/Met as a juvenile hormone receptor. PLoS Genet. 11, e1005394.

Jones, G., Sarkari, N., 1993. Basic isoforms of hemolymph storage proteins expressed during larval metamorphosis. Arch. Insect Biochem. Physiol. 24, 55–64.

Kaihara, K., Kotaki, T., Numata, H., Ohfune, Y., Shinada, T., 2012. Structure-activity relationship of novel juvenile hormone, $JHSB_3$, isolated from the stink bug, *Plautia stali*. Tetrahedron 68, 106–113.

Kayukawa, T., Minakuchi, C., Namiki, T., Togawa, T., Yoshiyama, M., Kamimura, M., Mita, K., Imanishi, S., Kiuchi, M., Ishikawa, Y., Shinoda, T., 2012. Transcriptional regulation of juvenile hormone-mediated induction of Krüppel homolog 1, a repressor of insect metamorphosis. Proc. Natl. Acad. Sci. U.S.A. 29, 11729–11734.

Konopova, B., Jindra, M., 2007. Juvenile hormone resistance gene methoprene-tolerant controls entry into metamorphosis in the beetle *Tribolium castaneum*. Proc. Natl. Acad. Sci. U.S.A. 104, 10488–10493.

Konopova, B., Smykal, V., Jindra, M., 2011. Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. PLoS One 6, e28728.

Kotaki, T., Shinada, T., Kaihara, K., Ohfune, Y., Numata, H., 2009. Structure determination of a new juvenile hormone from a heteropteran insect. Org. Lett. 11, 5234–5237.

Kotaki, T., Shinada, T., Kaihara, K., Ohfune, Y., Numata, H., 2011. Biological activities of juvenile hormone III skipped bisepoxide in last instar nymphs and adults of a stink bug, *Plautia stali*. J. Insect Physiol. 57, 147–152.

Lefevre, G., Johnson, V.B., 1962. Sperm transfer. Storage, displacement and utilization in *Drosophila melanogaster*. Genetics 47, 1719–1736.

Li, M., Mead, E.A., Zhu, J., 2011. Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. Proc. Natl. Acad. Sci. U.S.A. 108, 638–643.

Li, M., Liu, P., Willey, J.D., Ojani, R., Bevan, D.R., Li, J., Zhu, J., 2014. A steroid receptor coactivator acts ad the DNA-biding partner of the methoprene-tolerant protein in regulating juvenile hormone response genes. Mol. Cell. Endocrinol. 394, 47–58.

Lozano, J., Belles, X., 2014. Role of Methoprene-tolerant (Met) in adult morphogenesis and in adult ecdysis of *Blattella germanica*. PLoS One 9, e103614.

Lozano, J., Kayukava, T., Shinoda, T., Belles, X., 2014. A role for Taiman in insect metamorphosis. PLoS Genet. 10, e1004769.

Minakuchi, C., Namiki, T., Shinoda, T., 2009. Krüppel homolog 1, an early juvenile response gene downstream of Methoprene-tolerant, mediates its anti-metamorphic action in the red flour beetle *Tribolium castaneum*. Dev. Biol. 325, 341–350.

Minakuchi, C., Zhou, X., Riddiford, L.M., 2008. Krüppel homolog 1 (Kr-h1) mediates juvenile hormone action during metamorphosis of Drosophila melanogaster. Mech. Dev. 125, 91–105.

Nijhout, H., 1994. Insect Hormones. Princeton University Press, Princeton.

Ojani, R., Liu, P., Fu, X., Zhu, J., 2016. Protein kinase C modulates transcriptional activation by the juvenile hormone receptor methoprene-tolerant. Insect Biochem. Mol. Biol. 70, 44–52.

Papandopoulos, N.T., Liedo, P., Mller, H.-G., Wang, J.-L., Molleman, F., Carey, J.R., 2010. Cost of reproduction in male medflies: the primacy of the sexual courting in extreme longevity reduction. J. Insect Physiol. 56, 283–287.

Parthasarathy, R., Tan, A., Sun, Z., Chen, J., Rainkin, M., Palli, S.R., 2009. Juvenile hormone regulation of male accessory gland activity in the red flour beetle, *Tribolium castaneum*. Mech. Dev. 126, 563–579.

Parthasarathy, R., Sun, Z., Bai, H., Palli, S.R., 2010. Juvenile hormone regulation of vitellogenin synthesis in the red flour beetle, *Tribolium castaneum*. Insect Biochem. Mol. Biol. 40, 405–414.

Pener, M.P., 1972. The corpus allatum in adult acridids: the interrelation of its functions and possible correlations with the life cycle. In: Hemming, C.F., Taylor, T.H.C. (Eds.), Proceedings of the International Study Conference on the Current and Future Problems of Acridology. Centre for Overseas Pest Research, London, pp. 135–147.

Raikhel, A.S., Bown, M.R., Bellés, X., 2005. Hormonal control of reproductive processes. In: Gilbert, L.I., Iatrou, K., Gill, S.S. (Eds.), Comprehensive Insect Science. Elsevier/Pergamon, Amsterdam, pp. 433–491.

Richard, D.S., Gilbert, L.I., 1991. Reversible juvenile-hormone inhibition of ecdysteroids and juvenile-hormone synthesis by the ring gland of *Drosophila melanogaster*. Experientia 47, 1063–1066.

Sheng, Z., Xu, J., Bai, H., Zhu, F., Palli, S.R., 2011. Juvenile hormone regulates vitellogenin gene expression through insulin-like peptide signaling pathway in the red flour beetle, *Tribolium castaneum*. J. Biol. Chem. 286, 41924–41936.

Smykal, V., Bajgar, A., Provaznik, J., Fexova, S., Buricova, M., Takaki, K., Hodkova, M., Jindra, M., Dolezel, D., 2014. Juvenile hormone signaling during reproduction and development of the linden bug, *Pyrrhocoris apterus*. Insect Biochem. Mol. Biol. 45, 69–76.

Socha, R., 2006. Endocrine control of wing morph-related differences in mating success and accessory gland size in male firebugs. Anim. Behav. 71, 1273–1281.

Socha, R., Hodkova, M., 2006. Corpus allatum volume-dependent differences in accessory gland maturation in long- and short-winged males of *Pyrrhocoris apterus* (Hemiptera: Pyrrhocoridae). Eur. J. Entomol. 103, 27–32.

Socha, R., Sula, J., 1992. Voltinism and seasonal changes in haemolymph protein pattern of *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae) in relation to diapause. Physiol. Entomol. 17, 370–376.

Socha, R., Sula, J., 1996. Differences in haemolymph proteins in relation to diapause and wing dimorphism in *Pyrrhocoris apterus* (L.) (Heteroptera: Pyrrhocoridae). J. Comp. Physiol. B. 166, 382–387.

Socha, R., Sula, J., Kodrik, D., 2004. Wing morph-related differences in developmental pattern of accessory gland proteins in adult males of *Pyrrhocoris apterus* (L.) and their endocrine control. J. Insect Physiol. 50, 893–901.

Socha, R., Sula, J., Kodrik, D., Gelbic, I., 1991. Hormonal control of vitellogenin synthesis in *Pyrrhocoris apterus* (L.) (Heteroptera). J. Insect Physiol. 37, 805–816.

Sula, J., Kodrik, D., Socha, R., 1995. Hexameric haemolymph protein related to adult diapause in the red firebug, *Pyrrhocoris apterus* (L.) (Heteroptera). J. Insect Physiol. 41, 793–800.

Tatar, M., Yin, C.-M., 2001. Slow aging during insect reproductive diapause: why butterflies, grasshoppers, and flies are like worms. Exp. Gerontol. 36, 733–738.

Teal, P.E.A., Gomez-Simuta, Y., 2002. Juvenile hormone: action in regulation of sexual maturity in Caribean fruit flies and potential use in improving efficacy of sterile insect control technique for tephritid fruit flies. IOBC/vprs Bull. 25, 167–180.

Urbanova, V., Bazalova, O., Vaneckova, H., Dolezel, D., 2016. Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, *Pyrrhocoris apterus*. Insect Biochem. Mol. Biol. 2016. http://dx.doi.org/10.1016/j. ibmb..01.003.

Wigglesworth, V.B., 1934. The physiology of ecdysis in *Rhodnius prolixus* (Hemiptera). II. Factors controlling moulting and metamorphosis. Q. J. Microsc. Sci. 77, 191–222.

Wigglesworth, V.B., 1936. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). Q. J. Microsc. Sci. 79, 91–121.

Wigglesworth, V.B., 1970. Insect Hormones. Oliver Boyd, Edinburgh.

De Wilde, J., De Boer, S.A., 1969. Humoral and nervous pathways in photoperiodic induction of diapause in *Leptinotarsa decemlineata*. J. Insect Physiol. 15, 661–675.

Wilson, T.G., DeMoor, S., Lei, J., 2003. Juvenile hormone involvement in *Drosophila melanogaster* male reproduction as suggested by the Methoprene-tolerant^{27} mutant phenotype. Insect Biochem. Mol. Biol. 33, 1167–1175.

Wilson, T.G., Fabian, J., 1986. A *Drosophila melanogaster* mutant resistant to a chemical analog of juvenile hormone. Dev. Biol. 118, 190–201.

Yamamoto, K., Chadarevian, A., Pellegrini, M., 1988. Juvenile hormone action mediated in male accessory glands of *Drosophila* by calcium and kinase C. Science 239, 916–919.

Zdarek, J., 1966. Hormonal control of mating behaviour in Pyrrhocoris *apterus*. In: Proceedings of the International Symposium in Insect Endocrines, Brno, pp. 51–61.

Zdarek, J., 1968. Le comportement d'accouplement a la fin de la diapause imaginale et son controle hormonal dans le cas de la punaise *Pyrrhocoris apterus* L. (Pyrrhocoridae, Heteroptera). Ann. Endocr. Paris 29, 703–707.

Zhang, Z., Xu, J., Sheng, Z., Sui, Y., Palli, S.R., 2011. Steroid receptor co-activator is required for juvenile hormone signal transduction through a bHLH-PAS transcription factor, methoprene tolerant. J. Biol. Chem. 286, 8437–8447.

Zou, Z., Saha, T.T., Roy, S., Shin, S.W., Backman, T.W.H., Girke, T., White, K.P., Raikhel, A.S., 2013. Juvenile hormone and its receptor, methoprene-tolerant, control the dynamics of mosquito gene expression. Proc. Natl. Acad. Sci. U.S.A. 110, E2173–E2181.

Supplementary online material:

title:

Decrease in *Methoprene tolerant* and *Taiman* expression reduces juvenile hormone effects and enhances the levels of juvenile hormone circulating in males of the linden bug *Pyrrhocoris apterus*

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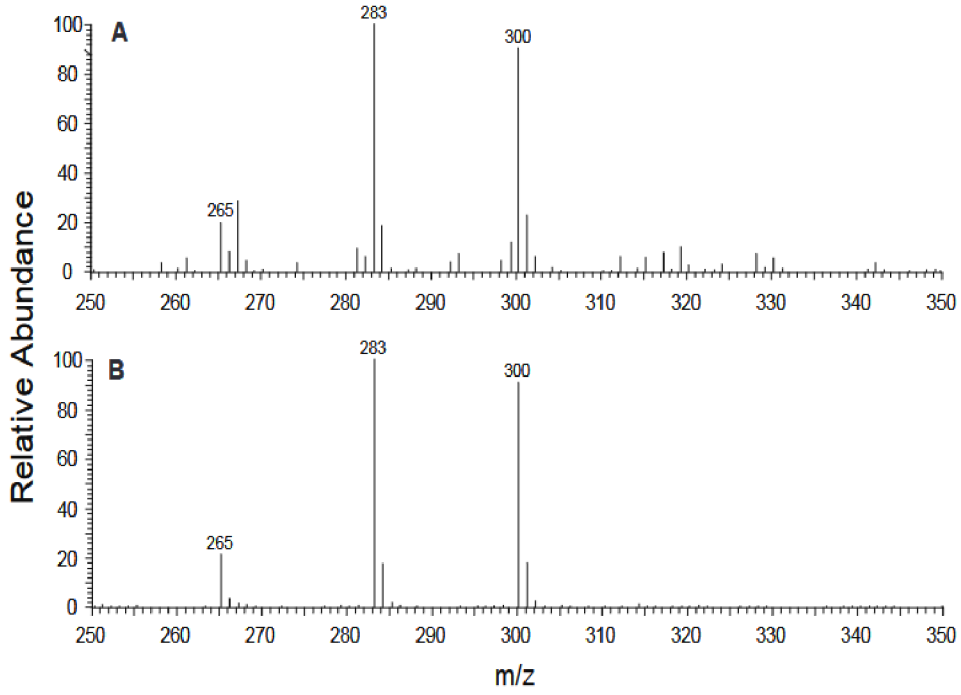
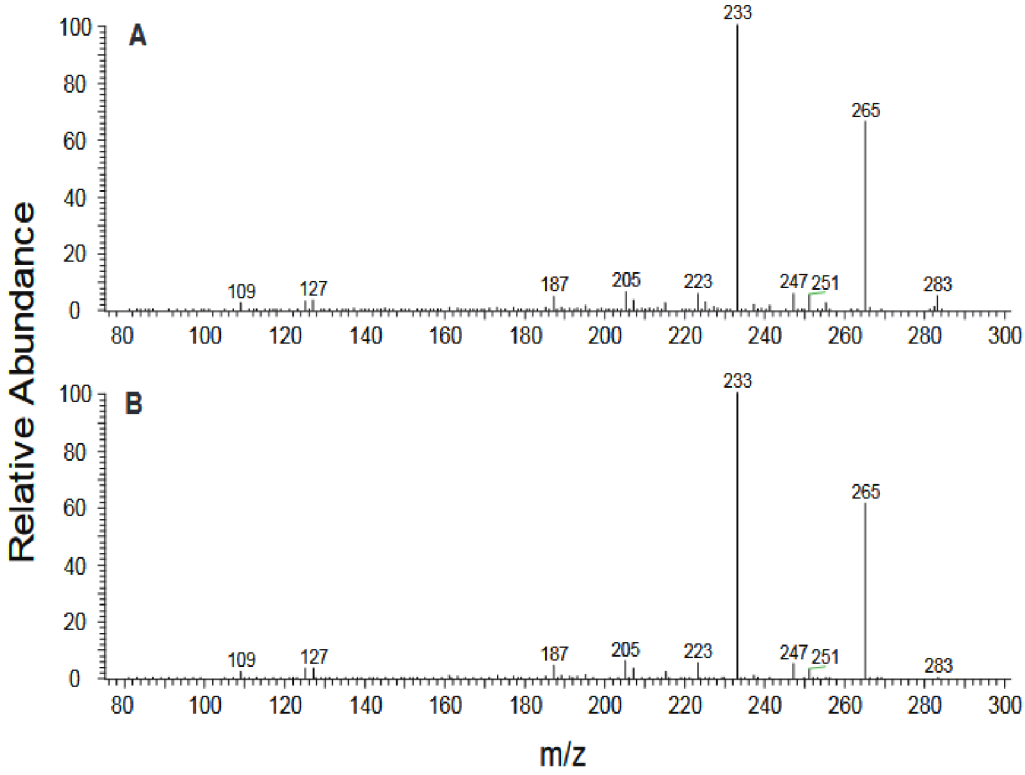
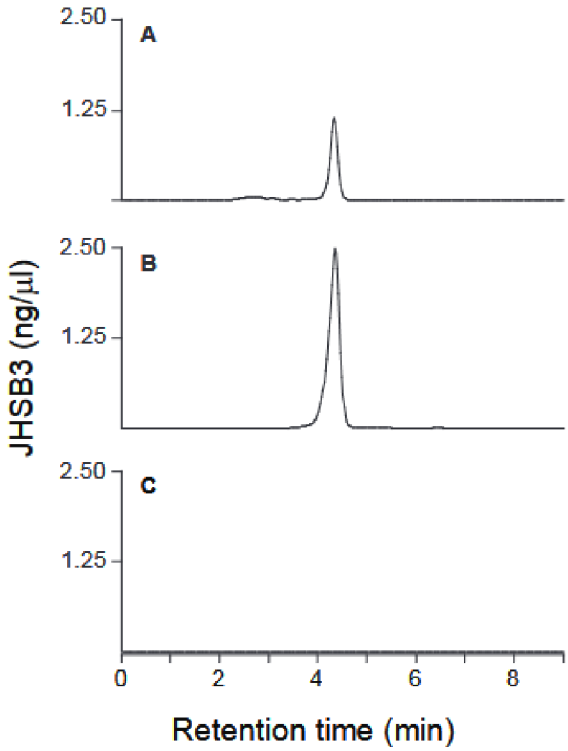


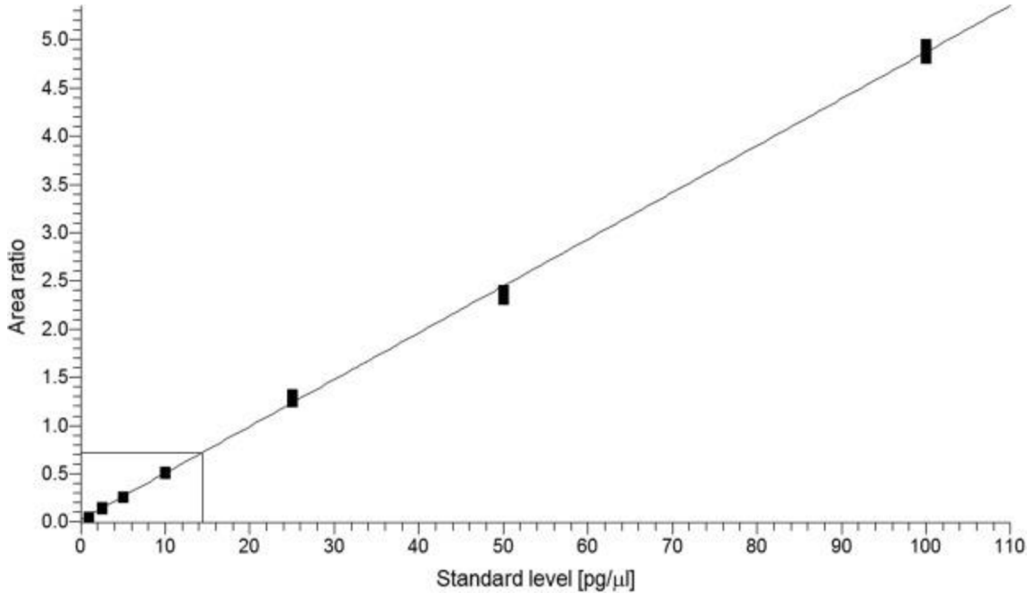
Supplementary Fig. S1. Positive ESI full scan mass spectra of naturally produced JHSB3 and its corresponding standard in mass range from 250 to 350 (m/z). Both mass spectra were obtained by coeluting peaks in retention time 4.35 min. (A) extracted sample from CC-CA in vitro incubation and (B) JHSB3 standard. The most abundant ions were the molecular ion (M+H+) m/z = 283, the ammonium adduct (M+H+NH4+) m/z = 300 and the fragments resulting from ring cleavage of epoxide (M+H+−HOH) m/z = 265. Despite the preceding SPE purification there is considerable background noise in the spectrum of the concentrated sample.



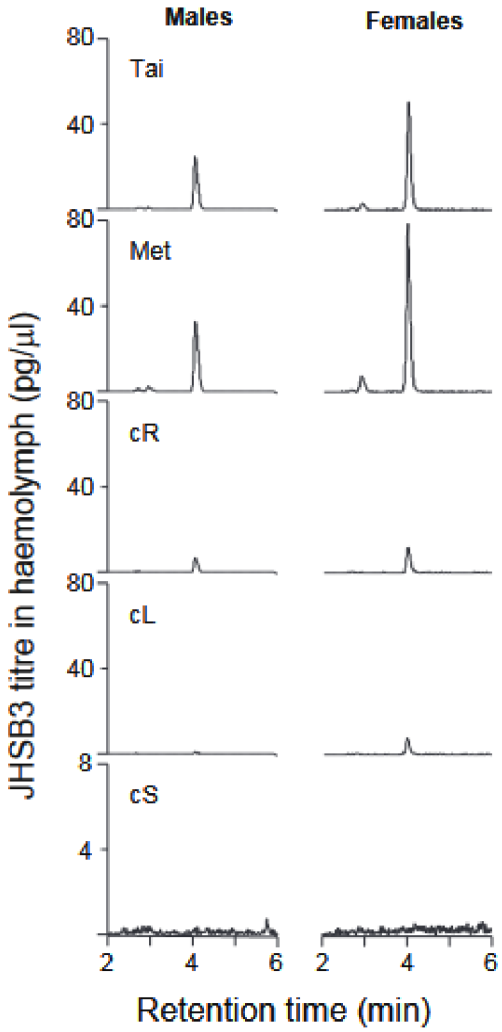
Supplementary Fig. S2. Collision-induced dissociation tandem mass spectrometry of the product from the CC-CA in vitro incubation in mass range from 80 to 300 (m/z). Comparison of the MS/MS spectra of (A) incubation extract and (B) JHSB3 standard. Molecular ion m/z = 283 was selected as a precursor ion. Diagnostic ions used for identification were used according to interpretation of Teal and Gomez-Simuta (2002). The most abundant ions were similar as those obtained from GC/MS with chemical ionization and included m/z = 265 (M+H+−HOH), 251 (M+H+−CH3OH), 233 (M+H+−CH3OH−HOH), 205 (M+H+−CH3OH−HOH−CO) and 187 (M+H+-CH3OH−HOH−CO−HOH).



Supplementary Fig. S3. Chromatograms showing production of JHSB3 (retention time 4.35 min) by the corpora cardiaca-corpus allatum complex of P. apterus in vitro. (A) CC-CA in vitro incubation extract, (B) JHSB3 standard, 2.5 ng/µl and (C) blank. The chromatogram was constructed as an amount of fragment ion 233 resulting from parent ion 283.



Supplementary Fig. S4. The calibration curve was linear over the range 1–100 pg/µl, injected volume was 20 µl and the sample volume 50 µl. The calibration curve was constructed from the peak area ratio of JHSB3 standard and JH I as the internal standard at constant level 25 pg/µl. Correlation = 0.991, n = 3. The mass spectrometric data were recorded through the following Selected reaction monitoring (SRM) transition (parent mass m/z → fragment mass m/z): 283 → 233 for JHSB3 and 295 → 263 for JH I as internal standard.



Supplementary Fig. S5. Chromatograms showing comparison of the levels of JHSB3 (retention time 4.05 min) in haemolymph of males and females of P. apterus. (Tai) Taiman RNAi, (Met) Methoprene tolerant RNAi, (cR) control Ringer, (cL) control LD and (cS) control SD.

Chapter 2

Sexual dimorphism of diapause regulation in the hemipteran bug *Pyrrhocoris apterus*

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As mentioned earlier, insects can alter their physiology in an attempt to survive hostile environmental conditions. One of the main strategies is adult reproductive diapause. The decision on when to initiate preparation for the upcoming seasonal changes, associated with low temperatures and a lack of food resources, is crucial especially for animals living in temperate zones. The shortening of daylight is the most reliable cue to predict upcoming unfavored conditions. Therefore, insects had to evolve a mechanism to measure the ratio of daylight to darkness (photoperiod). The point at which a maximum of 50 % of the population initiate diapause is known as critical photoperiod (CPP). Although photoperiod is the main factor that triggers diapause, the temperature is an additional element modulating the response to photoperiod and can significantly affect CPP (Bell & Bowley, 1980; Gomi, 1997; Hong & Platt, 1975; Saunders, 2014; Saunders et al., 1989; Tauber & Tauber, 1970). Nearly all current knowledge about reproductive diapause is based on studies of females. Thus, reproductive diapause is best defined by the inactivity of the *corpora allata* (CA) and subsequent absence or low titer of the juvenile hormone (JH), whereas the activity of CA and high JH levels lead to diapause termination (Kotaki & Yagi, 1989; Pener, 1992b). The clear neuroendocrine mechanism of reproductive diapause has not been shown in males yet. Moreover, in some insect species males do not undergo diapause at all (Denlinger & Armbruster, 2014; Pener, 1992b). Therefore, the most prominent feature of male diapause is their failure to mate with receptive females (Denlinger et al., 2012).

In the study "Sexual dimorphisms of diapause regulation in the hemipteran bug *Pyrrhocoris apterus*", we focused particularly on the reproductive diapause of males and described the differences between the sexes. Surprisingly, males with no JH reception or gland *corpus allatum* (CA), i.e., without a JH source, are capable of reproduction triggered by a long photoperiod. This situation is not observed in females. Under short photoperiod (SD), no JH was detected in diapausing adults of either sex. However, reproduction can be orchestrated through the JH receptor complex Methoprene-tolerant and Taiman by application of the JH analog – methoprene. Both males and females become reproductive after treatment. During the reproductive cycle of females, the titer of JH in the hemolymph varies over time and significantly differs from that of males. Unexpectedly, we also detected JH and key enzymes of the JH synthetic pathway in the accessory glands (MAGs), even in males without CA. There is a possibility

that the MAGs could be another site for JH synthesis in *P. apterus* as was previously shown in long-horned beetle (Tian et al., 2010) or mosquitoes (Borovsky et al., 1994), or simply it serves as a JH storage site (Clifton et al., 2014; Shirk et al., 1976). In either case, further detailed research is needed. Interestingly, we also observed a sex-dependent response to low temperature-dependent termination of diapause under SD conditions. After only one week at 0°C followed by a transfer to a moderate temperature of 25 °C, males resumed reproduction within a few days, while females remained in diapause. The different time frame for the termination of the reproductive diapause caused by low temperatures seems to be the result of the above-mentioned dimorphism.

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Sexual dimorphism of diapause regulation in the hemipteran bug *Pyrrhocoris apterus*

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ABSTRACT

Diapause is one of the major strategies for insects to prepare for and survive harsh seasons. In females, the absence of juvenile hormone (JH) is a hallmark of adult reproductive diapause, a developmental arrest, which is much less characterized in males. Here we show that juvenile hormone III skipped bisepoxide ($JHSB_3$) titers in hemolymph remarkably differ between reproductive males and females of the linden bug *Pyrrhocoris apterus*, whereas no JH was detected in diapausing adults of both sexes. Like in females, ectopic application of JH mimic effectively terminated male diapause through the canonical JH receptor components, Methoprene-tolerant and Taiman. In contrast to females, long photoperiod induced reproduction even in males with silenced JH reception or in males with removed *corpus allatum* (CA), the JH-producing gland. $JHSB_3$ was detected in the accessory glands (MAG) of reproductive males, unexpectedly, even in males without CA. If there is a source of $JHSB_3$ outside CA or a long-term storage of $JHSB_3$ in MAGs remains to be elucidated. These sex-related idiosyncrasies are further manifested in different dynamics of diapause termination in *P. apterus* by low temperature. We would like to propose that this sexual dimorphism of diapause regulation might be explained by the different reproductive costs for each sex.

1. Introduction

Insects living in temperate regions survive seasonal adversities either by slowing or completely halting development. One of the most widespread adaptations is diapause, a programmed developmental arrest occurring at a species-specific developmental stage. Diapause is either obligatory, entered regardless of prevailing environmental cues, or facultative, which is triggered by signals that are not themselves adverse (Denlinger et al., 2017; Hodek 2002; Kostal 2006). The most reliable seasonal cue is the day-to-night ratio, known as the photoperiod. It is perceived by photoreceptors, from which the information is further relayed to the photoperiodic timer (also known as the photoperiodic clock) where the day/night length is measured and the decision to enter diapause is made (Denlinger et al., 2017; Goto 2013; Kostal 2011; Saunders 2020; Dolezel, 2015). The output from the timer involves various hormonal pathways (Denlinger et al., 2012; Schiesari and O'Connor 2013) which, depending on the developmental stage, might involve ecdysteroid signaling (Guo et al., 2021; Poupardin et al., 2015), insulin-like peptides (Sim and Denlinger 2008, 2013), juvenile hormone (JH) (Chippendale 1977), and additional neuroendocrine pathways that may be integrated.

JH, a key hormone needed for insect reproduction, was first identified, as its name suggests, for its role in metamorphosis (Wigglesworth 1934). However, data from ametabolous insects indicate JH served primarily as a reproductive signal, which was only secondarily recruited to control insect metamorphosis (Charles et al., 2011). Consistently with the role of JH in reproduction, its absence or noticeably low JH levels is a hallmark of the adult reproductive diapause (Holters 1981). However, JH regulates multiple aspects of insect life, including color polyphenism (Suzuki and Nijhout, 2006), caste differentiation (De Wilde and Beetsma, 1982), and aggression (Pearce et al., 2001).

Canonical JH reception relies on the Methoprene-tolerant (MET)

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protein (Charles et al., 2011; Jindra et al., 2015; Miura et al., 2005), and its depletion prevents vitellogenesis and female reproduction in various insects (Guo et al., 2014; Li et al., 2011; Marchal et al., 2014; Smykal et al., 2014). MET is a transcription factor of the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) family (Ashok et al., 1998) interacting with another bHLH-PAS protein TAIMAN (TAI; also known as SRC or FISC) in a JH-dependent manner (Li et al., 2011). Yet another bHLH-PAS protein, circadian clock transcription factor CYCLE (CYC), was identified to interact with MET and induce rhythmic gene expression in response to JH (Shin et al., 2012). Genetic interactions between CYC, MET, and the third circadian bHLH-PAS protein, CLOCK (CLK), were proposed as a gut autonomous JH-dependent response in the linden bug *Pyrrhocoris apterus* (Bajgar et al., 2013a; Bajgar et al., 2013b). The connection of the circadian clock and JH signaling was suggested by JH-dependent expression of circadian clock genes (Dolezel et al., 2008). In male *P. apterus*, MET and TAI are essential for the JH-dependent growth of male accessory glands (MAG), with the photoperiodic signal perception requiring CLK and CYC (Urbanova et al., 2016). Surprisingly, *Met* knockdown did not prevent *P. apterus* males from mating. This contrasts with a recent study on the desert locust *Schistocerca gregaria*, where either knockdown of *Met* or *tai* completely abolished males' copulation (Holtof et al., 2021). Similar importance of JH was described in the grasshopper *Anacridium aegyptium*, in which male reproduction required active *corpus allatum* (CA), the gland synthesizing JH (Greenfield and Pener 1992).

The decision between diapause or non-diapause trajectories remarkably influences fitness. Therefore, the accurate assessment of seasonal cues is important. Indeed, a geographical cline in the critical day length has been observed in several species (Danilevskii 1965; Lankinen 1986; Paolucci et al., 2016). Critical day length is defined as the inflection point in a photoperiodic response curve and usually corresponds to the value when 50% of maximum diapause incidence is induced. In some insects, a shift corresponding to recent climate changes was described (Bradshaw and Holzapfel 2006; Honek et al., 2020; Matsuda et al., 2018). Furthermore, the energy investment into reproduction differs between males and females. In some vespids, coccinellids and mosquitoes, pre-diapause mating occurs followed by diapause of fertilized females, whereas males do not survive the winter (Hodek and Landa, 1971; Sulaiman and Service, 1983; Danilevskii, 1965; Tauber et al., 1986). In post-diapause mating strategies, both sexes initiate diapause together, but the diapause termination in males is often less stringent (Pener 1983), or perhaps males do not diapause (Connin 1971; Wellso 1981).

Given the contradicting data on the roles of JH signaling in male reproduction (Holtof et al., 2021; Urbanova et al., 2016), we sought to explore commonalities and differences of male and female reproductive diapause regulation in the linden bug *P. apterus*. When we generated laboratory conditions corresponding to those of natural diapause termination occurring during winter, we identified marked differences between male and female diapause. Furthermore, males' diapause termination seemed to be affected by *Met* silencing. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS) we detected Juvenile hormone III skipped bisepoxide ($JHSB_3$) as the only JH in *P. apterus* and quantified its titers in reproductive and diapause males and females. The combination of microsurgical interventions, RNA interference (RNAi), and JH mimic administration confirmed a JH-independent regulation of male reproductive diapause.

2. Methods and materials

2.1. Experimental animals

Pyrrhocoris apterus of Oldrichovec strain (Pivarciova et al., 2016) were reared at constant temperature 25 ± 0.5 °C either under long day (LD) photoperiod of 18 h light/6 h darkness (reproductive conditions) or short day (SD) photoperiod of 12 h light/12 h darkness (reproductive diapause-inducing conditions). Insects were supplied with water and linden seeds *ad libitum*. All experiments were done using adult males. Females served as positive or negative controls in RNA interference (RNAi) and microsurgical experiments.

2.2. Diapause termination by low temperature

Groups of *P. apterus* males and females were reared under diapause-inducing conditions (SD photoperiod 12 h light and 12 h darkness at 25 ± 0.5 °C) since the early larval stage. One month after adult ecdysis (AAE), gradual acclimation was used (Ditrich et al., 2018; Kostal et al., 2008): bugs were kept one week in SD photoperiod with temperature cycles of 20 °C during the light phase/10 °C during the night phase, followed by one week in SD with temperature cycling of 15 °C during the light phase/5 °C during the night phase. On the third week of the experiment, insects were kept in SD conditions at a constant temperature of 0 °C. The groups of experimental animals were transferred to 25 °C: after 6 days at 15/5 °C followed by 1 week at 0 °C, they spend 1, 3, 4, 6, 9, or 13 weeks at 0 °C. The photoperiodic conditions remained the same (SD). In each subgroup males were either injected with a) dsRNA Met, b) Ringer's solution or c) left intact. Males were then analyzed in regard to their ability to mate.

2.3. Corpus allatum extirpation (CAx)

Two- and ten-day after adult ecdysis males reared under LD or SD condition were used for the surgical removal of CA, the allatectomy. Males were anesthetized by immersion in water for approximately 10 min. A small incision was cut in the neck membrane on the dorsal part of the body, and CA were removed using fine forceps. In the case of the control Sham-operated animals, only the incision was made. Intact control animals were anesthetized and left without any intervention. After surgery, males were kept individually in Petri dishes supplied with linden seeds and water, and then tested for their mating ability.

2.4. Corpus allatum measurement and photography

Corpora allata (CA) were dissected in Ringer's solution, transferred into a drop of Ringer's solution on a slide, and covered by a coverslip. Every CA was photographed under a 100× magnification (Olympus SZX16). The images were analyzed by ImageJ software, and the area of the CA was measured in pixels. To verify the successful removal of the CA, brains + CA from CAx animals and controls were dissected in Ringer's solution, stained with safranin O and photographed under an 85× magnification (Olympus SZX16).

2.5. Mating experiment and photoperiod transfers

SD→LD transfer: The groups of experimental males (CAx, sham-operated, intact) from SD conditions were transferred to the LD conditions one day after surgical treatment. As a control, one set of CAx, sham-operated, and intact males was left in SD. One reproductive virgin female from LD (4 days AAE) was added to each male. The male's mating success was evaluated during the next 12 h. Females were replaced every day by new ones, until all individual males started mating. *LD→SD transfer*: three-day AAE reproductive males (CAx, sham-operated, and intact) reared under LD conditions were transferred into SD conditions. Each male was provided with one AAE reproductive female every second day. Mating was evaluated, and percentages of mating males were recorded.

2.6. RNAi and JH mimic treatments

An RNAi approach was used to downregulate the expression of the JH receptors *Met* and *tai* (Bajgar et al., 2013b). *Met* and *tai* fragments were cloned in the pGem T-Easy vector (Promega, Table S1). The

inserted sequences were modified by PCR to incorporate the T7 promoter sequence (Smykal et al., 2014a). Double-stranded RNAs (dsRNAs) were prepared using the T7 MEGAscript kit (Ambion), according to the manufacturer's instructions. Synthesized dsRNAs were diluted with Ringer's solution to a final concentration of 3 μg/μl. Two-day AAE diapause males were injected with 1 μl of dsRNA into the abdomen and kept separately afterward. One μl injections of Ringer's saline solution were used as negative controls. Injected males remained under SD conditions during the entire experiment. Three days after the injection, males were anesthetized by submersion in water and methoprene (JH mimic) was applied on the thorax at doses of 2 μg or 4 μg. Two μl of pure acetone served as a negative control. Four-day AAE reproductive females were individually added to each male two days after methoprene or acetone application, male's mating success was evaluated during the next 12 h. Females were replaced every day by new ones. The knockdown efficiency was tested in a subset of males using quantitative reverse transcription polymerase chain reaction (qRT PCR) using cDNA synthesized from head extracts of experimental and control males.

2.7. $JHSB_3$ measurement

Five μl of hemolymph were collected from the clipped antennae of *P. apterus* adult males and females using silanized glass capillary. Hemolymph was transferred immediately to ice-cold silanized glass vials (ThermoFisher). Ten μl of a JH III d3 internal standard (6.25 pg/μl in acetonitrile) and 600 μl of hexane were added to each sample. Samples were vortexed for 1 min at 25 °C and centrifuged for 5 min 2000 g at 4 °C. The upper hexane phase was transferred to a new silanized vial and stored at −20 °C until further use. The $JHSB_3$ amounts present in the hemolymph were quantified by liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) (Ramirez et al., 2020).

2.8. RNA isolation and qRT PCR

Accessory glands were dissected in Ringer's solution for the analysis of JHAMT expression. Total RNA was isolated using TRIzol reagent (Invitrogen) and treated by HL-dsDNase (ArcticZymes). One μg of the treated total RNA was used for a reverse transcription reaction using the One-Step Fast GrandMaster® Kit (TATAA Biocentre). Relative transcript levels were determined using quantitative PCR with TATAA SYBR® GrandMaster® Mix (TATAA Biocentre) and a C1000 Thermal Cycler (Bio-Rad). Transcripts levels were normalized using the ribosomal protein 49 mRNA (*rp49*) as previously described (Dolezel et al., 2007). Primer sequences are listed in Table S2.

3. Results

3.1. Low-temperature exposure terminates diapause differently in males and females

Diapause males and females were gradually cooled to 0 °C. After exposure to 0 °C for 1, 3, 4, 6, 9, and 13 weeks, linden bugs were transferred to ambient temperature (25 °C). After transferred, all females exposed to 0 °C for one week remained in diapause. Longer exposures to 0 °C terminated diapause, and, depending on the duration of cold exposure, the percentage of egg-laying females gradually increased (Fig. 1A). Nine- and thirteen-week exposures induced reproduction in more than 90% of females. Males also gradually increased reproduction with prolonged 0 °C exposure; however, diapause was remarkably weaker. Even after 1 week at 0 °C, 50% of males mated (Fig. 1B).

We also tested whether JH signaling is necessary for mating of males in which diapause was terminated by low temperature. Injection of *dsMet* RNA slightly prolonged the onset of mating in males with diapause terminated after 1 week at 0 °C (Fig. 1C). However, longer

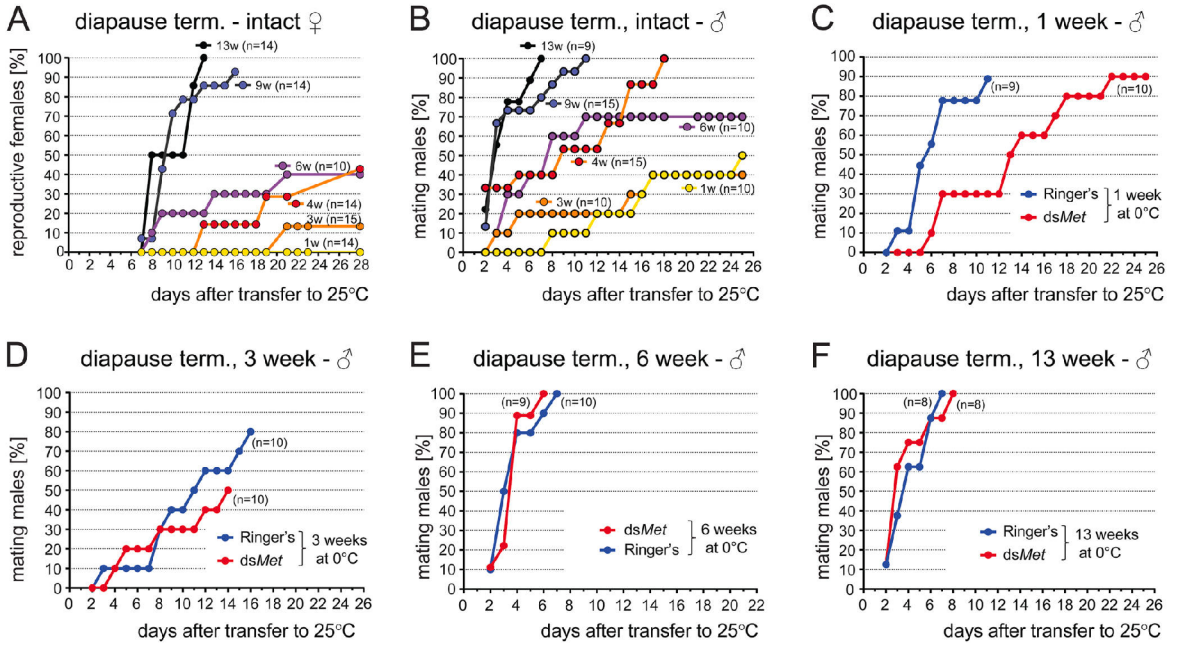

Fig. 1. Diapause termination by low temperature differs between *P. apterus* males and females. Diapause insects were gradually acclimated to 0 °C, where they spend 1, 3, 4, 6, 9, or 13 weeks at 0 °C under short photoperiod (SD). Then, bugs were transferred to 25 °C and SD, and egg laying (females) or mating capacity (males) were tested. Double strand *Met* RNA (ds*Met*) or Ringer's solution were injected immediately after transfer to 25 °C. (*A*) All females exposed to 0 °C for one week remained in diapause, whereas longer exposures gradually increased the percentage of reproductive females, reaching >90% and 100% after 9 and 13 weeks at 0 °C, respectively. (*B*) In males, even one-week exposure to 0 °C terminated diapause in 50% of intact males. (*C-F*) *Met* silencing did not reduce the percentage of mating males.

exposures to 0 °C resulted in accelerated mating, and the differences between silenced a control bugs diminished (Fig. 1D–F).

3.2. JH mimic stimulates mating in diapause P. apterus males through canonical JH reception

Given the observed impact of *dsMet* RNA on diapause termination in males, we decided to test whether application of JH mimic can influence male reproductive behavior. Indeed, the application of the JH mimic methoprene to diapause males elicited mating, whereas all control males exposed only to a vehicle (acetone) remained in diapause (Fig. 2A). Successful mating seemed to be dose-dependent, with the strongest responses observed with 2 μg and 4 μg of methoprene, whereas 0.5 μg and 1 μg induced mating only in 40% and 50%, respectively. Our next experiment addressed whether the components of the canonical JH receptor, MET and TAI, are involved in this JH-induced mating. Although all control males injected with Ringer's solution responded to JH-mimic by mating, 100% of *Met* knock-down males (Fig. 2B) and females (Fig. S1A) remained in diapause. Similarly, 100% and 80% of control males responded to JH-mimic, whereas only 30% and 25% of *tai* knock-down males mated upon JH mimic administration, and 10% or 15% of controls mated even after exposure to the vehicle (Fig. 2C). Comparable trends were observed in females (Fig. S1B). Thus, JH-induced mating requires the canonical JH reception relying on MET and TAI.

3.3. Photoperiod changes trigger mating independently of JH signaling

Since MET and TAI are essential for the JH mimic-induced switch from diapause to mating under short photoperiod, we asked whether JH signaling is also needed during male reproduction under long photoperiod. First, diapause males raised under short photoperiod were injected with *Met* or *tai* dsRNAs, one day later transferred to long photoperiod and their mating tested on a daily basis. The expression levels of *Met* and *tai*, measured in entire head extracts by qRT PCR, indicated that the mRNA titers dropped after dsRNA treatment to approximately 17% (*tai*) and 29% (*Met*) of the control levels (Fig. S2). Surprisingly, all *tai* and 95% of *Met* knockdown males successfully mated, albeit with a slight delay when compared to intact and Ringer's solution-injected controls (Fig. 2D). Notably, parallel RNAi experiments on female siblings completely prevented their vitellogenesis (1-day AAE females from LD, 10 females per *Met* and *tai* dsRNA). We further addressed the role of JH signaling by microsurgical removal of *corpus allatum* (CAx); an operation that reliably prevents reproduction in *P. apterus* females (Smykal et al., 2014a). CA was removed from diapause males either 2 or 10 days after adult ecdysis (AAE). Males were then transferred to long photoperiod conditions, and their mating performance tested. Not only did the vast majority of CAx males mated, but the mating of the 10 d AAE CAx group took place earlier after the transfer than the mating of sham-operated and intact sibling controls (Fig. 2E and F). In parallel, 10 females from LD where allatectomized and 10 were sham operated. Two CAx females died, whereas the remaining eight CAx females did not develop eggs. In contrast, nine sham-operated females laid eggs and one died. When reproductive males were transferred from LD to SD, their mating continued in SD for the rest of their lives. Not only were the intact and sham-operated males mating, but also the CAx males were able to reproduce successfully, even 74 days after transfer (Figs. S3A and B). Taken together, our experiments suggest that the CA is not essential for the photoperiod-induced mating in *P. apterus* males.

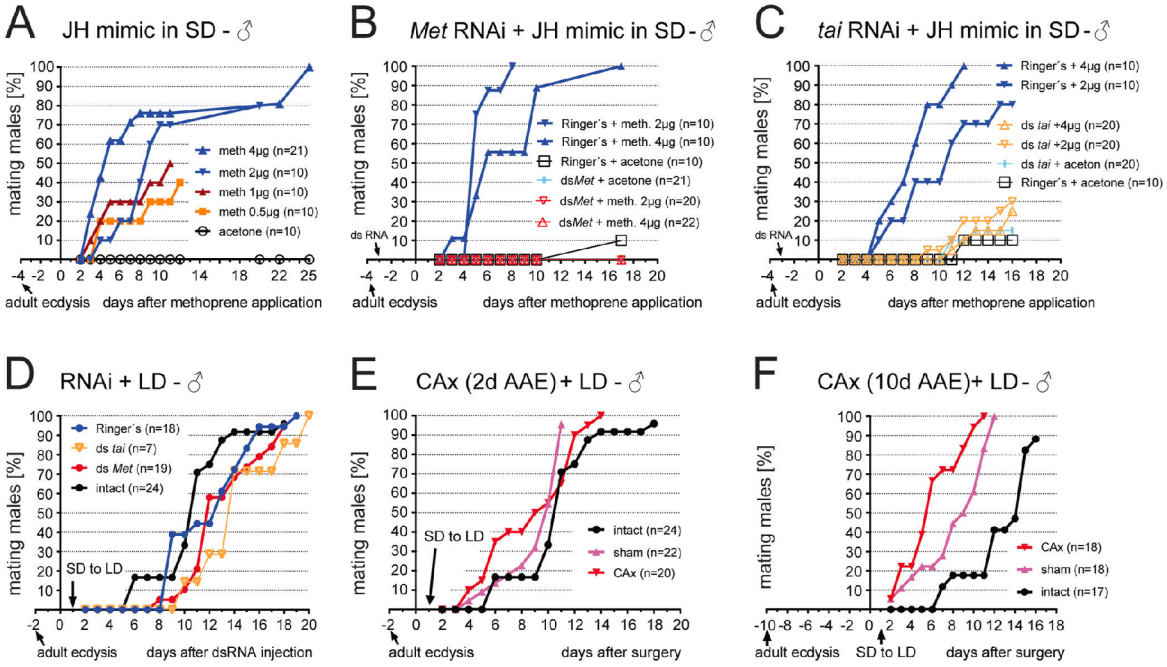

Fig. 2. Either the JH mimic methoprene or long photoperiod effectively terminates reproductive diapause in *P. apterus* males. Diapausing males from short photoperiod conditions (SD) were exposed either to JH mimic or to long photoperiods (LD), and these treatments were further combined with JH receptor's silencing (*Met* RNAi, *tai* RNAi) or with allatectomy (CAx). (*A*) Ectopic application of JH mimic ended reproductive diapause of males kept in SD; whereas the vehicle, acetone, had no impact on diapause. (*B*) *Methoprene tolerant* (*Met*), and (*C*) *taiman* (*tai*), are essential components of JH mimic-induced reproduction; whereas the control males injected with Ringer's solution fully responded to JH mimic. (*D*) Photoperiod changes terminated male diapause independently of JH signaling, as well as (*E*, *F*) in males without *corpus allatum* (CAx), the canonical source of JH. Stress, including sham operation, influenced diapause termination.

3.4. Photoperiod-dependent $JHSB_3$ titers differ between male and female *P. apterus*

We employed a well-established liquid chromatography-mass spectrometry (LC-MS/MS) approach (Villalobos-Sambucaro et al., 2020) to measure JH in male and female adult *P. apterus*. Our analysis identified JH III skipped bisepoxide, $JHSB_3$ as the only detectable JH homolog. The results are in full agreement with the previous, yet technically distinct detection approach used in *P. apterus* (Hejnikova et al., 2016), as well as other Hemiptera species (Ando et al., 2020; Kotaki et al., 2009; Matsumoto et al., 2021). In the hemolymph of reproductive linden bug females, $JHSB_3$ titers peaked five days AAE, and then dropped on day 6 (Fig. 3A, Table S3). In contrast to females, $JHSB_3$ titers in males were approximately 10 times lower, and without any obvious peak. Instead, the $JHSB_3$ titers gradually increased with time, reaching maximal levels on days 9–10 (Fig. 3B, Table S4). No $JHSB_3$ was detectable in either females or males undergoing diapause. The limit of detection for $JHSB_3$ is 1.1 fmol (7.8 pg/ml) in the injected sample (Ramirez et al., 2020).

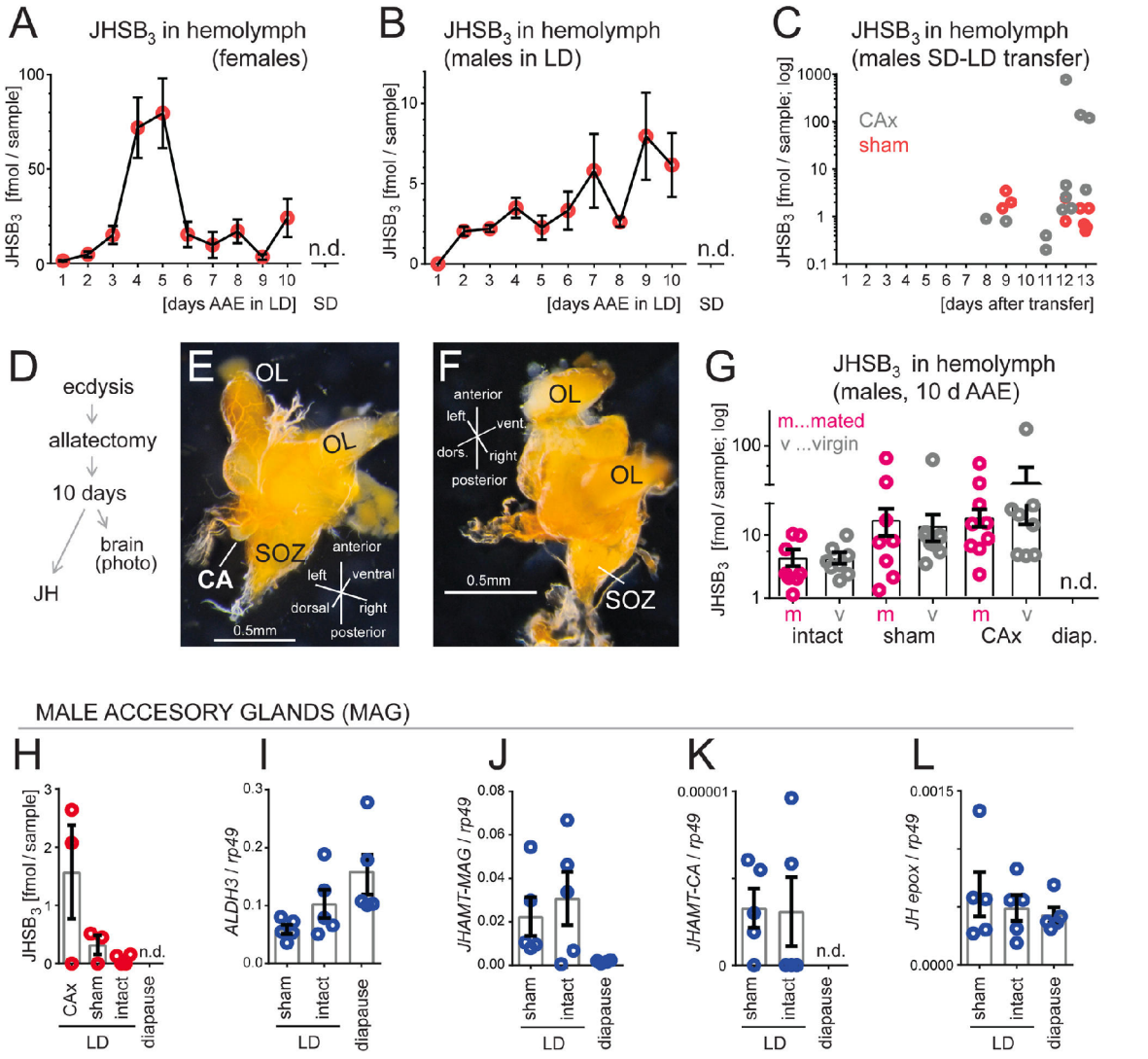

Fig. 3. Titers of $JHSB_3$ in adult males and females *P. apterus* (*A*) A clear peak, matching the peak of vitellogenic development, was detected in reproductive females kept under long photoperiod (LD) for 10 days after adult ecdysis (AAE). Each value represents the mean ± SEM of 4–6 biological samples (for individual titers see Table S3). (*B*) $JHSB_3$ levels gradually raised in reproductive males, albeit not to the higher titers detected in females (n = 3–6, titers in Table S4). (*C*) $JHSB_3$ is detected in the hemolymph of sham-operated and allatectomized (CAx) males after transferring them from short (SD) to long photoperiod (LD) (n = 6 CAx and 6 sham for each day after transfer, titers in Tables S5 and S6). (*E*) Typical images of brains of control males with *corpus allatum* (CA) and (*F*) allatectomized males without CA, (OL-optic lobe, SOZ-suboesophageal zone). (*G*) Comparison of $JHSB_3$ titers in the hemolymph of males with and without CA 10 days AAE (n = 8–9, titers in Table S7). $JHSB_3$ was not detected in diapausing males (diap., n = 6). (*H*) $JHSB_3$ was present in dissected male accessory glands (MAG) of reproductive males, showing high titers in CAx males and non-detectable values in MAG of diapause males (n = 3–6, titers in Table S8). (*I-J*) Quantitative real time PCR expression levels of (*I*) farnesal dehydrogenase (ALDH3), (*J*) MAG-type of *juvenile hormone acid methyltransferase* (JHAMT), (*K*) CA-type JHAMT, and (*L*) methyl farnesoate epoxidase (n = 3 × 5 for panels I–J). The house keeping gene *rp49* served as a control.

3.5. $JHSB_3$ Is detectable in males transferred to LD

Having identified a clear difference in $JHSB_3$ levels in the hemolymph of reproductive and diapause males from LD and SD conditions, we studied $JHSB_3$ titers in males transferred from short to long photoperiods. Although no juvenoids were detectable in the majority of samples, some males sacrificed at the end of the transfer (days 9, 12, and 13) contained $JHSB_3$ (Fig. 3C, Fig. S4, and Table S5). Surprisingly, $JHSB_3$ titer was identified even in males without CA, the gland established as a source of JH (Fig. 3C, Fig. S4, and Table S6). Next, we designed a specific experiment aiming to understand the roles of the CA and mating in males' JH synthesis (Fig. 3D). Intact, CAx, and sham-operated males of mated and virgin groups were sacrificed 10 days after the operation. The successful extirpation of CA was confirmed in all CAx males, whereas intact glands were clearly present in all sham-operated and control males (Fig. 3E and F). Neither $JHSB_3$ nor any other juvenoid was detected in diapause males, whereas $JHSB_3$ was present in intact, sham-operated, and even CAx males from LD conditions (Fig. 3G, Table S7). These results suggest that a source of $JHSB_3$ might exist outside the CA in *P. apterus* males. Since male accessory glands (MAG) of moth contain JH (Shirk et al., 1976) and MAGs of mosquitoes contain (Clifton et al., 2014) and even might synthesize this hormone (Borovsky, 1994), we explored whether we could detect JH in the MAG of *P. apterus*. Indeed, extracts from MAG of reproductive males contained $JHSB_3$, whereas no JH was detected in MAGs of diapause males (Fig. 3H). Interestingly, qRT PCR analysis revealed mRNA

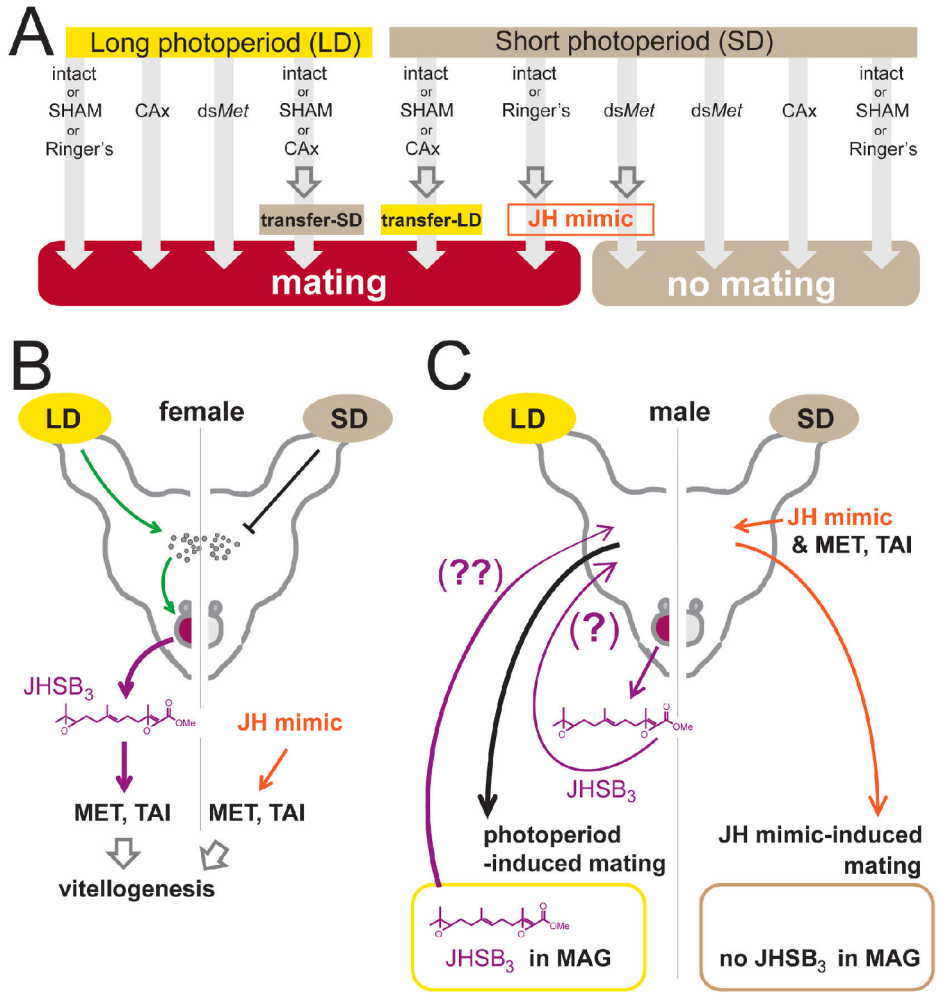

Fig. 4. Either long photoperiod or juvenile hormone (JH) is sufficient to induce mating in *P. apterus* males. (*A*) Summary of all treatments used and their impact on male's mating capacity. Males were either transferred from reproduction-promoting LD (long-day) or from diapause-inducing and -maintaining SD (short-day) photoperiods. Experimental interventions included microsurgical removal of *corpus allatum* (CAx), sham-operation serving as a control (SHAM), injection of *Met* dsRNA (ds*Met*), injection of Ringer's solution, and no treatment at all (intact). Some of these treatments were followed by a change in photoperiod (transfer-SD/LD) or ectopic application of methoprene (JH mimic). (*B*) Model of the regulation of diapause in females. The long photoperiod (LD) is perceived by the compound eye, from which the signal is relayed (green arrow) to neurosecretory cells located in the *pars intercerebralis* (grey dots), which further activate *corpus allatum* (in purple) that synthesizes $JHSB_3$. The humoral systemic signal induces vitellogenesis via activation of the JH receptor components, MET and TAI. In females, the short photoperiod (SD) inhibits the activity of the CA, although the diapause state can be overridden by JH mimic treatment. (*B*) In males, the LD signal is sufficient to induce mating, even without CA or active JH signaling. Thus, $JHSB_3$ detected in males under LD conditions might be either functionally redundant, or this JH further feeds back to the brain and modulates its activity. The latter hypothesis is supported by the JH mimic-induced mating observed in males from SD conditions, which are JH free. This hypothetical brain-localized induction requires the JH receptor components, MET and TAI.

expression of several JH biosynthetic enzymes in the MAG (Fig. 3I-L), in particular two *JHAMTs* were significantly expressed in MAGs of reproductive males but showed none or extremely low gene expression in MAGs of diapause males (Fig. 3J and K). JH epoxidase, an enzyme critical for biosynthesis of the fully active JH (Nouzova et al., 2021), was expressed at comparable levels in diapause and reproductive MAGs (Fig. 3L).

4. Discussion

This study addresses the regulation of reproductive diapause in *P. apterus* males using a combination of reverse genetic, allatectomy, and JH mimic application (summarized in Fig. 4A). As in females, ectopic application of a JH mimic induces male reproduction via the canonical JH receptor consisting of MET and TAI. Long photoperiod induces reproduction in both sexes; however, detailed analyses point to important sex-specific differences (Fig. 4B and C). In females, both the JH-producing gland CA and the JH receptor are absolutely essential for reproduction (Smykal et al., 2014a), whereas the situation is more complex in males. Methoprene, a potent JH mimic applied to diapausing males induces their successful mating. The JH receptor components MET and TAI are essential for this induction, as is clearly indicated by their knockdown when no Met^{RNAi} male mated even 19 days after dsRNA administration. However, *Met* silencing in diapause males prior to their transfer to long photoperiod did not prevent their mating, which reached more than 50% in less than 14 days and 100% in 20 days. A similar situation was observed after *tai* silencing, although 25–30% of tai^{RNAi} males mated when exposed to JH mimic. These data are in line with previously reported CA-independent (Hejnikova et al., 2016) and MET-independent reproduction (Urbanova et al., 2016) in *P. apterus* males, but contrast with a recent study on the desert locust *S. gregaria*, where either knockdown of *Met* or *tai* completely abolished males' copulation behavior (Holtof et al., 2021).

RNAi treatment often results in a partial gene knockdown. Nevertheless, we observed levels of gene silencing similar to those previously reported in experiments on *Met* and *tai* knockdowns in *P. apterus* that led to clear reproductive and developmental phenotypes (Smykal et al., 2014a,b). In addition, the successful silencing of JH signaling was confirmed in experiments where methoprene did not induce mating even 17 days after its administration. Furthermore, we performed parallel control experiments on female siblings that completely obliterated oogenesis. A second possible concern might be the allatectomy. Indeed, the size of the CA makes its reliable elimination challenging in some insect species (see Nouzova et al., 2012, Fig. 6, for an example of mosquito CA). However, the size and anatomy of *P. apterus* with one visibly separated CA allow its reliable and complete removal from the *corpora cardiaca* and the brain.

Similar to *S. gregaria*, JH induces growth of *P. apterus* MAG (Urbanova et al., 2016) and influences protein content in this gland (Hejnikova et al., 2016). The importance of MAG's size and its content are unclear, as *P. apterus* males start mating *circa* one week after their transfer to LD or, in the case of CAx males, even slightly earlier (Fig. 2D–F), a the time when the MAGs of transferred males are comparable in size to glands of diapausing males (Urbanova et al., 2016). Whether MAG content including $JHSB_3$ might be transferred to *P. apterus* females is unknown, however, a similar mechanism was reported for proteins (Dottorini et al., 2007), ecdysteroids (Pondeville et al., 2008), and JH (Clifton et al., 2014; Park et al., 1998) in other insect. Notably, the protein content differs between MAG of reproductive and diapause *P. apterus* (Hejníková et al., 2016), and similarly differs between short- and long-wing morphs (Socha et al., 2004). This wing polyphenism is most abundant during hot summer days, results in delayed reproduction and dispersal behavior of long-wing morph (Honek 1995) and is regulated by the insulin signaling cascade (Smykal et al., 2020).

The JHAMT-like 'MAG' is related to *R. prolixus* RPRC004476-RA, a protein that was originally annotated as JHAMT (Mesquita et al., 2015), although it later turned out not to be expressed in the CA (Villalobos-Sambucaro et al., 2020). In the light of our findings, it will be interesting to see whether this gene is instead expressed in some other *R. prolixus* tissues, especially in MAG. The possibility that $JHSB_3$ is synthesized in the MAG remains unproved. An alternative explanation is that $JHSB_3$ is only stored in MAGs, which, in case of allatectomized diapausing males, would require long-storage of $JHSB_3$ synthesized before metamorphosis. On the other hand, the role of $JHSB_3$ in *P. apterus* males is also undefined and requires further studies.

In *Drosophila* males, JH is essential for courtship memory retention during an early-adult-specific period (Lee et al., 2017). However, *P. apterus* diapausing males transferred to LD initiated mating earlier than $JHSB_3$ synthesis increases. Thus, $JHSB_3$ might rather be important later by affecting subsequent mating attempts, or may have additional reproduction-independent role(s). In the honeybee workers, fat body energy metabolism is influenced by JH and vitellogenin (Wang et al., 2012). In *P. apterus* females, the diapause-to-reproduction transition is connected to a dramatic energy expenditure signified by suppressed hexamerine expression and strong expression of vitellogenins (Smykal et al., 2014a). Coincidentally, in *P. apterus* females $JHSB_3$ titers peak and drop consistently with the timing of the vitellogenic cycle (Fig. 3A; Ždárek, 1970).

The exact mechanism behind reproductive diapause regulation is not fully understood, although growing evidence supports involvement of the circadian clock genes during the photoperiodic induction (Ikeno et al., 2011a, b; Ikeno et al., 2010; Kotwica-Rolinska et al., 2017), and photoperiodic termination (Urbanová et al., 2016). Both, the photoperiodic response curves for the induction and termination are steep, and the critical day length slightly longer for the termination (Saunders, 1983). However, the photoperiodic termination of diapause in *P. apterus* has been studied mainly under a laboratory setup. In the field conditions, diapause is primarily terminated during the winter by exposure to low temperature, after which the linden bugs remain in a quiescence state and are ready to reproduce whenever temperature rises (Hodek, 1971). These sex-specific differences in *P. apterus* diapause termination by low temperature are consistent with a different reproductive cost, with each sex investing differentially into reproduction. Whereas ovarian growth is energetically demanding, mating of males requires much less energy investment. Thus, males that mate first will have the best opportunity to transfer their genes to the next generation. This selection pressure on trying to mate as early as possible seems to be a plausible explanation for the origin of JH-independent signaling triggering male mating in *P. apterus*. Notably, a fraction of females in this species overwinter fertilized and successfully reproduce afterward (Socha 2010). Therefore, despite the highly conserved role of JH in insect reproduction, some species-specific idiosyncrasies evolved to optimize species- and sex-specific performance under given environmental conditions.

Author contribution

M.H. perform all *P. apterus* experiments; D.D. and M.H. designed the study and interpreted results; M.N., F.N, F.F.L. and C.R. performed and analyzed JH measurements. D.D. wrote the manuscript with input from all co-authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2022.103721.

References

Ando, Y., Matsumoto, K., Misaki, K., Mano, G., Shiga, S., Numata, H., Kotaki, T., Shinada, T., Goto, S.G., 2020. Juvenile hormone III skipped bisepoxide, not its stereoisomers, as a juvenile hormone of the bean bug *Riptortus pedestris*. Gen. Comp. Endocrinol. 289, 113394. https://doi.org/10.1016/j.ygcen.2020.113394.

Ashok, M., Turner, C., Wilson, T.G., 1998. Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. Proc. Natl. Acad. Sci. U.S.A. 95, 2761–2766. https://doi.org/10.1073/pnas.95.6.2761.

Bajgar, A., Dolezel, D., Hodkova, M., 2013a. Endocrine regulation of non-circadian behavior of circadian genes in insect gut. J. Insect Physiol. 59, 881–886. https://doi.org/10.1016/j.jinsphys.2013.06.004.

Bajgar, A., Jindra, M., Dolezel, D., 2013b. Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. Proc. Natl. Acad. Sci. U.S.A. 110, 4416–4421. https://doi.org/10.1073/pnas.1217060110.

Borovsky, D., Carlson, D.A., Hancock, R.G., Rembold, H., van Handel, E., 1994. De novo biosynthesis of juvenile hormone III and I by the accessory glands of the male mosquito. Insect Biochem. Mol. Biol. 24, 437–444.

Bradshaw, W.E., Holzapfel, C.M., 2006. Climate change. Evolutionary response to rapid climate change. Science 312, 1477–1478. https://doi.org/10.1126/science.1127000.

Charles, J.P., Iwema, T., Epa, V.C., Takaki, K., Rynes, J., Jindra, M., 2011. Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. Proc. Natl. Acad. Sci. U.S.A. 108, 21128–21133. https://doi.org/10.1073/pnas.1116123109.

Chippendale, G.M., 1977. Hormonal regulation of larval diapause. Annu. Rev. Entomol. 22, 121–138.

Clifton, M.E., Correa, S., Rivera-Perez, C., Nouzova, M., Noriega, F.G., 2014. Male *Aedes aegypti* mosquitoes use JH III transferred during copulation to influence previtellogenic ovary physiology and affect the reproductive output of female mosquitoes. J. Insect Physiol. 64, 40–47. https://doi.org/10.1016/j.jinsphys.2014.03.006.

Connin, R.V., Hoopingarner, R.A., 1971. Sexual behavior and diapause of the cereal leaf beetle, *Oulema melanopus*. Ann. Entomol. Soc. Am. 64, 655–660.

Danilevskii, A.S., 1965. Photoperiodism and Seasonal Development of Insects, First English edn. Oliver & Boyd, Edinburgh.

Denlinger, D.L., Hahn, D.A., Merlin, C., Holzapfel, C.M., Bradshaw, W.E., 2017. Keeping time without a spine: what can the insect clock teach us about seasonal adaptation? Philos. Trans. R. Soc. Lond. B Biol. Sci. 372 https://doi.org/10.1098/rstb.2016.0257.

Denlinger, D.L., Yocum, G.D., Rinehart, J.P., 2012. Hormonal control of diapause. Insect Endocrinol. 430–463.

De Wilde, J., Beetsma, J., 1982. The physiology of caste development in social insects. Adv. Insect Physiol 16, 167–246.

Ditrich, T., Janda, V., Vaneckova, H., Dolezel, D., 2018. Climatic variation of supercooling point in the linden bug *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). Insects 9. https://doi.org/10.3390/insects9040144.

Dolezel, D., Sauman, I., Kost'ál, V., Hodková, M., 2007. Photoperiodic and food signals control expression pattern of the clock gene, period, in the linden bug, *Pyrrhocoris apterus*. J. Biol. Rhythm. 22, 335–342. https://doi.org/10.1177/0748730407303624.

Dolezel, D., Zdechovanova, L., Sauman, I., Hodkova, M., 2008. Endocrine-dependent expression of circadian clock genes in insects. Cell. Mol. Life Sci. 65, 964–969. https://doi.org/10.1007/s00018-008-7506-7.

Dolezel, D., 2015. Photoperiodic time measurement in insects. Curr. Opin. Insect Sci. 7, 98–103.

Dottorini, T., Nicolaides, L., Ranson, H., Rogers, D.W., Crisanti, A., Catteruccia, F., 2007. A genome-wide analysis in *Anopheles gambiae* mosquitoes reveals 46 male accessory gland genes, possible modulators of female behavior. Proc. Natl. Acad. Sci. U.S.A. 104, 16215–16220. https://doi.org/10.1073/pnas.0703904104.

Goto, S.G., 2013. Roles of circadian clock genes in insect photoperiodism. Entomol. Sci. 16, 1–16. https://doi.org/10.1111/Ens.12000.

Greenfield, M.D., Pener, M.P., 1992. Alternative schedules of male reproductive diapause in the grasshopper *Anacridium aegyptium* (L.): effects of the corpora allata on sexual behavior (Orthoptera: Acrididae) J. Insect Behav. 5, 245–261.

Guo, S., Tian, Z., Wu, Q.W., King-Jones, K., Liu, W., Zhu, F., Wang, X.P., 2021. Steroid hormone ecdysone deficiency stimulates preparation for photoperiodic reproductive diapause. PLoS Genet. 17, e1009352 https://doi.org/10.1371/journal.pgen.1009352.

Guo, W., Wu, Z., Song, J., Jiang, F., Wang, Z., Deng, S., Walker, V.K., Zhou, S., 2014. Juvenile hormone-receptor complex acts on Mcm4 and Mcm7 to promote polyploidy and vitellogenesis in the migratory locust. PLoS Genet. 10, e1004702 https://doi.org/10.1371/journal.pgen.1004702.

Hejnikova, M., Paroulek, M., Hodkova, M., 2016. Decrease in Methoprene tolerant and Taiman expression reduces juvenile hormone effects and enhances the levels of juvenile hormone circulating in males of the linden bug *Pyrrhocoris apterus*. J. Insect Physiol. 93–94, 72–80. https://doi.org/10.1016/j.jinsphys.2016.08.009.

Hodek, I., 2002. Controversial aspects of diapause development. Eur. J. Entomol. 99, 163–173.

Hodek, I., 1971. Termination of adult diapause in *Pyrrhocoris apterus* (Heteroptera-Pyrrhocoridae) in field. Entomol. Exp. Appl. 14, 212.

Hodek, I., Landa, V., 1971. Anatomical and histological changes during dormancy in two Coccinelidae. Entomophaga 16, 239–251.

Holters, W., 1981. Untersuchungen zur Steuerung des Kurztagund Langtag-Prozesses der Gonadenreifung bei *Pterostichus angustatus* (Col: Carabidae). Zool. Jb. Physiol. 85, 426–461.

Holtof, M., Van Lommel, J., Gijbels, M., Dekempeneer, E., Nicolai, B., Vanden Broeck, J., Marchal, E., 2021. Crucial role of juvenile hormone receptor components methoprene-tolerant and taiman in sexual maturation of adult male desert locusts. Biomolecules 11. https://doi.org/10.3390/biom11020244.

Honěk, A., 1995. Factors and consequences of a nonfunctional alary polymorphism in *Pyrrhocoris apterus* (Heteroptera, Pyrrhocoridae). Res. Popul. Ecol 37 (1), 111–118.

Honěk, A., Martínková, Z., Pekar, S., 2020. How climate change affects the occurrence of a second generation in the univoltine *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). Ecol. Entomol. 45, 1172–1179. https://doi.org/10.1111/een.12903.

Ikeno, T., Numata, H., Goto, S.G., 2011a. Circadian clock genes period and cycle regulate photoperiodic diapause in the bean bug *Riptortus pedestris* males. J. Insect Physiol. 57, 935–938. https://doi.org/10.1016/j.jinsphys.2011.04.006.

Ikeno, T., Numata, H., Goto, S.G., 2011b. Photoperiodic response requires mammalian-type cryptochrome in the bean bug *Riptortus pedestris*. Biochem. Biophys. Res. Commun. 410, 394–397. https://doi.org/10.1016/j.bbrc.2011.05.142.

Ikeno, T., Tanaka, S.I., Numata, H., Goto, S.G., 2010. Photoperiodic diapause under the control of circadian clock genes in an insect. BMC Biol. 8, 116. https://doi.org/10.1186/1741-7007-8-116.

Jindra, M., Uhlirova, M., Charles, J.P., Smykal, V., Hill, R.J., 2015. Genetic evidence for function of the bHLH-PAS protein Gce/Met as a juvenile hormone receptor. PLoS Genet. 11, e1005394 https://doi.org/10.1371/journal.pgen.1005394.

Kostal, V., 2006. Eco-physiological phases of insect diapause. J. Insect Physiol. 52, 113–127. https://doi.org/10.1016/j.jinsphys.2005.09.008.

Kostal, V., 2011. Insect photoperiodic calendar and circadian clock: independence, cooperation, or unity? J. Insect Physiol. 57, 538–556. https://doi.org/10.1016/j.jinsphys.2010.10.006.

Kostal, V., Tollarova, M., Dolezel, D., 2008. Dynamism in physiology and gene transcription during reproductive diapause in a heteropteran bug, *Pyrrhocoris apterus*. J. Insect Physiol. 54, 77–88. https://doi.org/10.1016/j.jinsphys.2007.08.004.

Kotaki, T., Shinada, T., Kaihara, K., Ohfune, Y., Numata, H., 2009. Structure determination of a new juvenile hormone from a heteropteran insect. Org. Lett. 11, 5234–5237. https://doi.org/10.1021/ol902161x.

Kotwica-Rolinska, J., Pivarciova, L., Vaneckova, H., Dolezel, D., 2017. The role of circadian clock genes in the photoperiodic timer of the linden bug *Pyrrhocoris apterus* during the nymphal stage. Physiol. Entomol. 42, 266–273. https://doi.org/10.1111/phen.12197.

Lankinen, P., 1986. Geographical variation in circadian eclosion rhythm and photoperiodic adult diapause in *Drosophila littoralis*. J. Comp. Physiol. A Sens. Neural Behav. Physiol. 159, 123–142. https://doi.org/10.1007/Bf00612503.

Lee, S.S., Ding, Y., Karapetians, N., Rivera-Perez, C., Noriega, F.G., Adams, M.E., 2017. Hormonal signaling cascade during an early-adult critical period required for courtship memory retention in drosophila. Curr. Biol. 27, 2798–2809 e2793. https://doi.org/10.1016/j.cub.2017.08.017.

Li, M., Mead, E.A., Zhu, J.S., 2011. Heterodimer of two bHLH-PAS proteins mediates juvenile hormone-induced gene expression. Proc. Natl. Acad. Sci. U.S.A. 108, 638–643. https://doi.org/10.1073/pnas.1013914108.

Marchal, E., Hult, E.F., Huang, J., Pang, Z., Stay, B., Tobe, S.S., 2014. Methoprene-tolerant (Met) knockdown in the adult female cockroach, *Diploptera punctata* completely inhibits ovarian development. PLoS One 9, e106737. https://doi.org/10.1371/journal.pone.0106737.

Matsuda, N., Tanaka, K., Watari, Y., Shintani, Y., Goto, S.G., Nisimura, T., Izumi, Y., Numata, H., 2018. Northward expansion of the bivoltine life cycle of the cricket over the last four decades. Global Change Biol. 24, 5622–5628. https://doi.org/10.1111/gcb.14436.

Matsumoto, K., Kotaki, T., Numata, H., Shinada, T., Goto, S.G., 2021. Juvenile hormone III skipped bisepoxide is widespread in true bugs (Hemiptera: Heteroptera). R. Soc. Open Sci. 8, 202242 https://doi.org/10.1098/rsos.202242.

Mesquita, R.D., Vionette-Amaral, R.J., Lowenberger, C., Rivera-Pomar, R., Monteiro, F.A., Minx, P., Spieth, J., Carvalho, A.B., Panzera, F., Lawson, D., Torres, A.Q., Ribeiro, J.M., Sorgine, M.H., Waterhouse, R.M., Montague, M.J., Abad-Franch, F., Alves-Bezerra, M., Amaral, L.R., Araujo, H.M., Araujo, R.N., Aravind, L., Atella, G.C., Azambuja, P., Berni, M., Bittencourt-Cunha, P.R., Braz, G.R., Calderon-Fernandez, G., Carareto, C.M., Christensen, M.B., Costa, I.R., Costa, S.G., Dansa, M., Daumas-Filho, C.R., De-Paula, I.F., Dias, F.A., Dimopoulos, G., Emrich, S.J., Esponda-Behrens, N., Fampa, P., Fernandez-Medina, R.D., da Fonseca, R.N., Fontenele, M., Fronick, C., Fulton, L.A., Gandara, A.C., Garcia, E.S., Genta, F.A., Giraldo-Calderon, G.I., Gomes, B., Gondim, K.C., Granzotto, A., Guarneri, A.A., Guigo, R., Harry, M., Hughes, D.S., Jablonka, W., Jacquin-Joly, E., Juarez, M.P., Koerich, L.B., Latorre-Estivalis, J.M., Lavore, A., Lawrence, G.G., Lazoski, C., Lazzari, C.R., Lopes, R.R., Lorenzo, M.G., Lugon, M.D., Majerowicz, D., Marcet, P.L., Mariotti, M., Masuda, H., Megy, K., Melo, A.C., Missirlis, F., Mota, T., Noriega, F.G., Nouzova, M., Nunes, R.D., Oliveira, R.L., Oliveira-Silveira, G., Ons, S., Pagola, L., Paiva-Silva, G.O., Pascual, A., Pavan, M.G., Pedrini, N., Peixoto, A.A., Pereira, M.H., Pike, A., Polycarpo, C., Prosdocimi, F., Ribeiro-Rodrigues, R., Robertson, H.M., Salerno, A.P., Salmon, D., Santesmasses, D., Schama, R., Seabra-Junior, E.S., Silva-Cardoso, L., Silva-Neto, M.A., Souza-Gomes, M., Sterkel, M., Taracena, M.L., Tojo, M., Tu, Z.J., Tubio, J.M., Ursic-Bedoya, R., Venancio, T.M., Walter-Nuno, A.B., Wilson, D., Warren, W.C., Wilson, R.K., Huebner, E., Dotson, E.M., Oliveira, P.L.,

2015. Genome of *Rhodnius prolixus*, an insect vector of Chagas disease, reveals unique adaptations to hematophagy and parasite infection. Proc. Natl. Acad. Sci. U. S.A. 112, 14936–14941.

Miura, K., Oda, M., Makita, S., Chinzei, Y., 2005. Characterization of the *Drosophila* Methoprene-tolerant gene product - juvenile hormone binding and ligand-dependent gene regulation. FEBS J. 272, 1169–1178. https://doi.org/10.1111/j.1742-4658.2005.04552.x.

Nouzova, M., Brockhoff, A., Mayoral, J.G., Goodwin, M., Meyerhof, W., Noriega, F.G., 2012. Functional characterization of an allatotropin receptor expressed in the corpora allata of mosquitoes. Peptides 34 (1), 201–208. https://doi.org/10.1016/j.peptides.2011.07.025.

Nouzova, M., Edwards, M.J., Michalkova, V., Ramirez, C.E., Ruiz, M., Areiza, M., DeGennaro, M., Fernandez-Lima, F., Feyereisen, R., Jindra, M., Noriega, F.G., 2021. Epoxidation of juvenile hormone was a key innovation improving insect reproductive fitness. Proc. Natl. Acad. Sci. U.S.A. 118, e2109381118 https://doi.org/10.1073/pnas.2109381118.

Paolucci, S., Salis, L., Vermeulen, C.J., Beukeboom, L.W., van de Zande, L., 2016. QTL analysis of the photoperiodic response and clinal distribution of period alleles in *Nasonia vitripennis*. Mol. Ecol. https://doi.org/10.1111/mec.13802.

Park, Y.I., Shu, S., Ramaswamy, S.B., Srinivasan, A., 1998. Mating in *Heliothis virescens*: transfer of juvenile hormone during copulation by male to female and stimulation of biosynthesis of endogenous juvenile hormone. Arch. Insect Biochem. Physiol. 38, 100–107. https://doi.org/10.1002/(SICI)1520-6327(1998)38:2<100::AID-ARCH6>3.0.CO;2-X.

Pearce, A.N., Huang, Z.Y., Breed, M.D., 2001. Juvenile hormone and aggression in honey bees. J. Insect Physiol. 47, 1243–1247. https://doi.org/10.1016/s0022-1910(01)00109-3.

Pener, M.P., Orshan, L., 1983. The reversibility and flexibility of the reproductive diapause in males of a "short day" grasshopper, Oedipoda miniata. In: Brown, V.K., Hodek, I. (Eds.), Diapause and Life Cycle Strategies in Insects. Dr. W. Junk, The Hague, The Netherlands, 67-85.: 67-85.

Pivarciova, L., Vaneckova, H., Provaznik, J., Wu, B.C., Pivarci, M., Peckova, O., Bazalova, O., Cada, S., Kment, P., Kotwica-Rolinska, J., Dolezel, D., 2016. Unexpected geographic variability of the free running period in the linden bug *Pyrrhocoris apterus*. J. Biol. Rhythm. https://doi.org/10.1177/0748730416671213.

Pondeville, E., Maria, A., Jacques, J.C., Bourgouin, C., Dauphin-Villemant, C., 2008. *Anopheles gambiae* males produce and transfer the vitellogenic steroid hormone 20-hydroxyecdysone to females during mating. Proc. Natl. Acad. Sci. U.S.A. 105, 19631–19636. https://doi.org/10.1073/pnas.0809264105.

Poupardin, R., Schottner, K., Korbelova, J., Provaznik, J., Dolezel, D., Pavlinic, D., Benes, V., Kostal, V., 2015. Early transcriptional events linked to induction of diapause revealed by RNAseq in larvae of drosophilid fly, *Chymomyza costata*. BMC Genom. 16, 720. https://doi.org/10.1186/s12864-015-1907-4.

Ramirez, C.E., Nouzova, M., Michalkova, V., Fernandez-Lima, F., Noriega, F.G., 2020. Common structural features facilitate the simultaneous identification and quantification of the five most common juvenile hormones by liquid chromatography-tandem mass spectrometry. Insect Biochem. Mol. Biol. 116, 103287.

Saunders, D.S., 1983. A diapause induction termination asymmetry in the photoperiodic responses of the linden bug, *Pyrrhocoris apterus* and an effect of near-critical photoperiods on development. J. Insect Physiol. 29, 399–405.

Saunders, D.S., 2020. Dormancy, Diapause, and the role of the circadian system in insect photoperiodism. Annu. Rev. Entomol. 65, 373–389. https://doi.org/10.1146/annurev-ento-011019-025116.

Schiesari, L., O'Connor, M.B., 2013. Diapause: delaying the developmental clock in response to a changing environment. Curr. Top. Dev. Biol. 105, 213–246. https://doi.org/10.1016/B978-0-12-396968-2.00008-7.

Shin, S.W., Zou, Z., Saha, T.T., Raikhel, A.S., 2012. bHLH-PAS heterodimer of methoprene-tolerant and Cycle mediates circadian expression of juvenile hormone-induced mosquito genes. Proc. Natl. Acad. Sci. U.S.A. 109, 16576–16581. https://doi.org/10.1073/pnas.1214209109.

Shirk, P.D., Dahm, K.H., Roller, H., 1976. The accessory sex glands as the repository for juvenile hormone in male cecropia moths. Zeitschrift Naturforschung. Sect. C, Biosci. 31, 199–200.

Sim, C., Denlinger, D.L., 2008. Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*. Proc. Natl. Acad. Sci. U.S.A. 105, 6777–6781. https://doi.org/10.1073/pnas.0802067105.

Sim, C., Denlinger, D.L., 2013. Insulin signaling and the regulation of insect diapause. Front. Physiol. 4, 189. https://doi.org/10.3389/fphys.2013.00189.

Smykal, V., Bajgar, A., Provaznik, J., Fexova, S., Buricova, M., Takaki, K., Hodkova, M., Jindra, M., Dolezel, D., 2014a. Juvenile hormone signaling during reproduction and development of the linden bug, *Pyrrhocoris apterus*. Insect Biochem. Mol. Biol. 45, 69–76. https://doi.org/10.1016/j.ibmb.2013.12.003.

Smykal, V., Daimon, T., Kayukawa, T., Takaki, K., Shinoda, T., Jindra, M., 2014b. Importance of juvenile hormone signaling arises with competence of insect larvae to metamorphose. Dev. Biol. 390 (2), 221–230. https://doi.org/10.1016/j.ydbio.2014.03.006.

Smykal, V., Pivarci, M., Provaznik, J., Bazalova, O., Jedlicka, P., Luksan, O., Horak, A., Vaneckova, H., Benes, V., Fiala, I., Hanus, R., Dolezel, D., 2020. Complex evolution of insect insulin receptors and homologous decoy receptors, and functional significance of their multiplicity. Mol. Biol. Evol. 37, 1775–1789.

Socha, R., Sula, J., Kodrik, D., 2004. Wing morph-related differences in developmental pattern of accessory gland proteins in adult males of *Pyrrhocoris apterus* (L.) and their endocrine control. J. Insect Physiol. 50, 893–901.

Socha, R., 2010. Pre-diapause mating and overwintering of fertilized adult females: new aspects of the life cycle of the wing-polymorphic bug *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). Eur. J. Entomol. 107, 521–525.

Sulaiman, S., Service, M.W., 1983. Studies on hibernating populations of the mosquito *Culex pipiens* L. in southern and northern England. J. Nat. Hist. 17, 849–857.

Suzuki, Y., Nijhout, H.F., 2006. Evolution of a polyphenism by genetic accommodation. Science 311, 650–652.

Tauber, M.J., Tauber, C.A., Masaki, S., 1986. Seasonal Adaptation in Insects. Oxford University Press, New York, 1986.

Urbanova, V., Bazalova, O., Vaneckova, H., Dolezel, D., 2016. Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, *Pyrrhocoris apterus*. Insect Biochem. Mol. Biol. 70, 184–190. https://doi.org/10.1016/j.ibmb.2016.01.003.

Villalobos-Sambucaro, M.J., Nouzova, M., Ramirez, C.E., Eugenia Alzugaray, M., Fernandez-Lima, F., Ronderos, J.R., Noriega, F.G., 2020. The juvenile hormone described in *Rhodnius prolixus* by Wigglesworth is juvenile hormone III skipped bisepoxide. Sci. Rep. 10, 3091. https://doi.org/10.1038/s41598-020-59495-1.

Wang, Y., Brent, C.S., Fennern, E., Amdam, G.V., 2012. Gustatory perception and fat body energy metabolism are jointly affected by vitellogenin and juvenile hormone in honey bees. PLoS Genet. 8, e1002779 https://doi.org/10.1371/journal.pgen.1002779.

Wellso, S.G., Hoxie, R.P., 1981. Diapause and non-diapause behavior of the cereal leaf beetle. Entomol. Exp. Appl. 30, 19–25.

Wigglesworth, V.B., 1934. Memoirs: the Physiology of Ecdysis in *Rhodnius prolixus* (Hemiptera). II. factors controlling moulting and 'metamorphosis. J. Cell Sci. s2–77, 191–222. https://doi.org/10.1242/jcs.s2-77.306.191.

Zdarek, J., 1970. Mating behaviour in the bug, *Pyrrhocoris apterus* L. (Heteroptera): Ontogeny and its environmental control. Behaviour 37, 253–268.

Supplementary online material:

title:

Sexual dimorphism of diapause regulation in the Hemipteran bug *Pyrrhocoris apterus*

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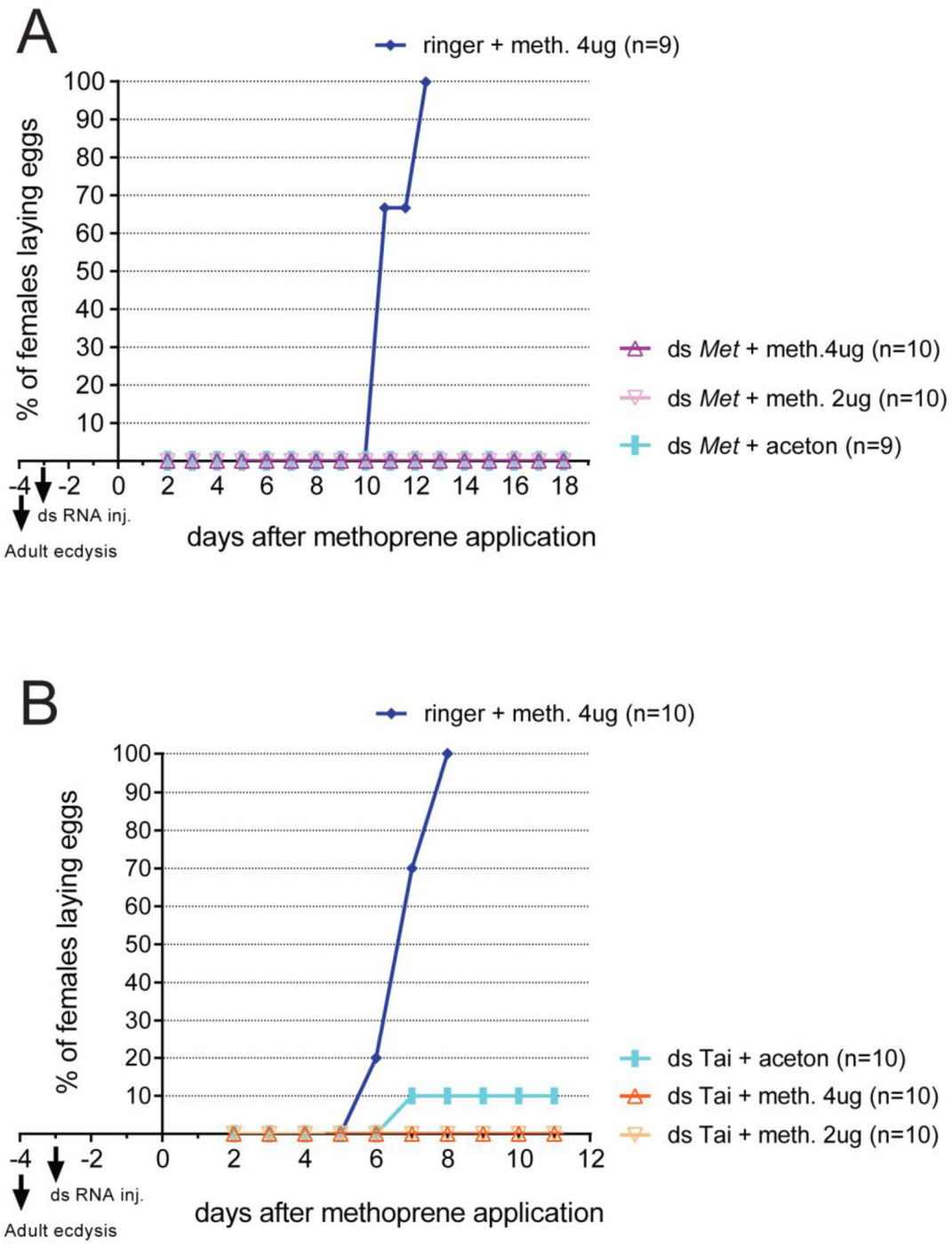

Fig. S1. JH controls *P. apterus* female reproduction. While topical application of methoprene stimulated egg laying in diapause females injected with ringer buffer, silencing JH signaling by injection of double stranded RNA for *Methoprene tolerant* (*Met*) (A) or *taiman* (*tai*) (B) abolished egg laying and could not be "overridden" by methoprene treatment.

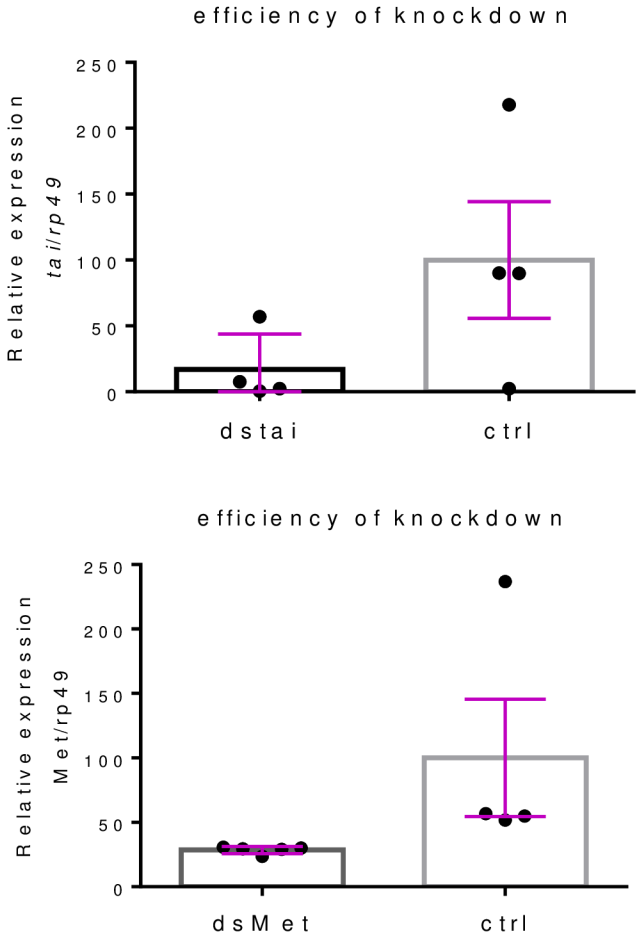

Fig. S2 Knockdown efficiency measured by RT qPCR in entire heads after RNAi of 10d AAE *P. apterus* males from SD conditions. Four to five biological replicates were measured. Each dot corresponds to the levels measured in one head when *rp49* served as a housekeeping gene. The grey columns correspond to means and magenta bars indicate SEMs. The mean calculated for the control (ctrl) group corresponds to 100% on y-axis. In case of *tai*, the mean in silenced group is 16.91 % of the controls. In *Met,* the mean of silenced group is 28.59 % of the controls.

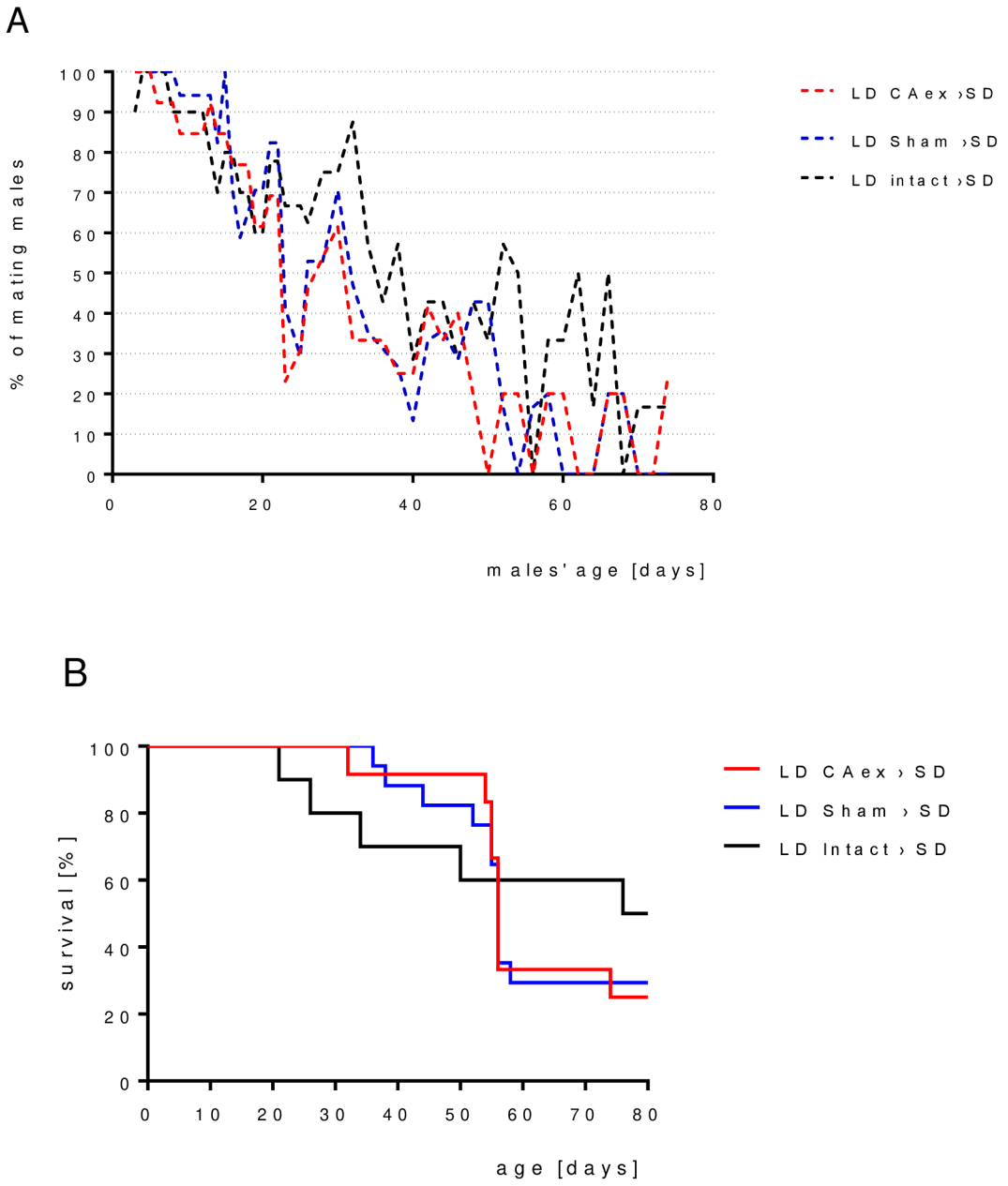

Fig. S3 *P. apterus* males transferred from long (LD) to short day (SD) conditions continue reproducing for the rest of their life. (*A*) The percentage of mating males gradually decreases over the time in both experimental allatectomized males (LD CAx) and control groups of intact and sham operated males (LD intact, LD Sham). (*B*) Survival rates observed in males for the duration of the experiment. Initial n = 10-17.

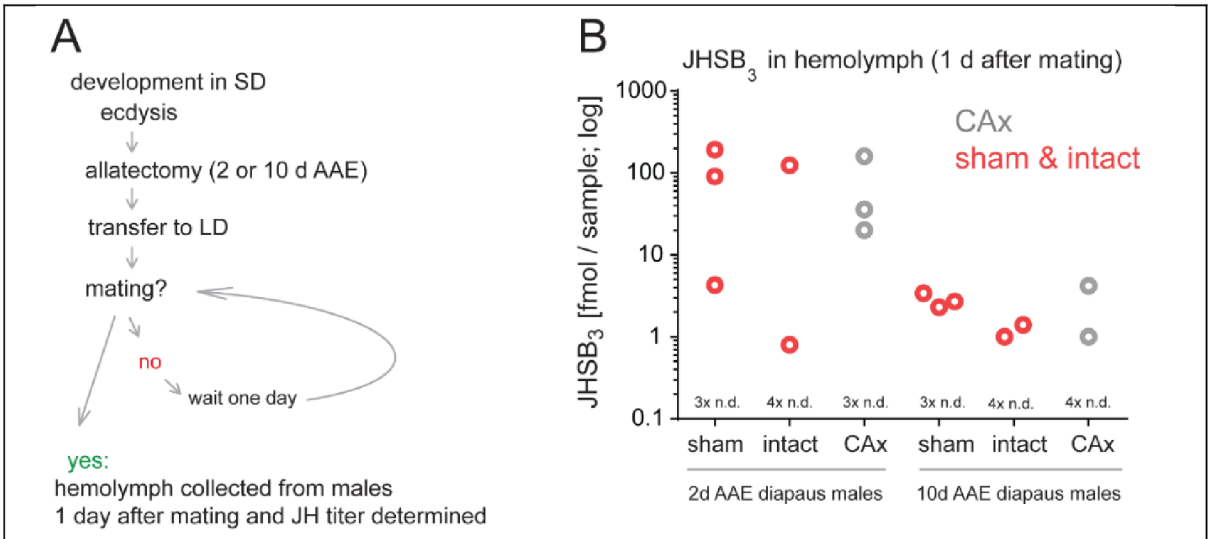

Fig. S4 Some but not all males that mated after been transferred from SD to LD produced $JHSB_3$. (*A*) Scheme of the experiment. Diapausing males from SD conditions were allatectomized (CAx) either 2 or 10 days after adult ecdysis (AAE), and transferred to LD. Then, their mating ability was tested on a daily basis. Mating males were sacrificed, hemolymph isolated, and $JHSB_3$ titers determined. (*B*) $JHSB_3$ titers detected in the hemolymph of sham-operated, intact, and allatectomized (CAx) males one day after mating (therefore, as their initiated mating on different day after the transfer, their age differs). Number of males that did not contain measurable amount of any JH is specified for each group as "n.d.". Mating of these males is depicted in Fig. 2E and

Supplementary Information Tables

Table S1. Primers used to amplify and clone template to produce dsRNA fragments for RNAi.

gene	forward	reverse
Met	ATGGTATCCTCATCTCCTAAG	GTGTGTTGATGCAGATGAATG
tai	GCACATAAAGGAGCAAGAAGCA	CAGGAGCCAAGAGGAAAACC

Table S2. Primers used for qRT PCR.

target	forward	reverse
rp49	CCGATATGTAAAACTGAGGAGAAAC	GGAGCATGTGCCTGGTCTTTT
JHAMT-'CA' (GDFI01024537)	GTGGAGGACAAAAGGCAAATAG	TCGAAACCTGCATTCTGAAATAAC
JHAMT-'MAG' (GDFI01048469)	ATCTTCCGGCCGAGTCTAAA	AAGAGAACTGATCGTTGCCG
ALDH3 (GFOX01019093)	CTTCCCTTTGGCGGAGTC	TGGGTATCTCGCACTTGCAA
EPOX (GFOX01104698)	AAACTGGTGAAGTGGTGCAG	ACGGGCTTTAGGATCTTCGA

Table S3. JHSB3 titers identified in hemolymph of females kept under long photoperiod.

	after adult ecdysis (AAE) [days], females in LD									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
JHSB3 [fmol / sample]	1.7	9.8	22	110	130	9.7	44	11.6	2.2	4.1
	0.9	5.8	5.7	18.1	120	44	2.3	21	5.7	69.1
	1.2	4.3	33	44	12.7	6.1	1.1	9.4	1.3	35
	1.8	2.1	18	120	49	26	4.1	46.2	9.5	19.2
		1.9	1.9	58	100	1.8	2.4	11.2	0.9	4.2
			10	81	65	4.4	4.5	2.5	1.1	13.1

Table S4. JHSB3 titers identified in hemolymph of males from long photoperiod

	after adult ecdysis (AAE) [days]; males in LD									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
JHSB3 [fmol / sample]	0	2.1	2.6	3.3	3.5	2.7	1	2.5	3.3	0
	0	1.5	2.3	4.1	2.4	1.5	2.4	2.7	6.4	7.5
	0	2.5	1.6	5.4	0.9	6.8	12.6	2.3	6.3	4.8
	0		2.3	3.4		2.3	13.4	3.7	15.8	6.2
	0			0.8			2.6	1.9		12.3
	0			4			2.8			

Table S5. JHSB3 titers identified in hemolymph of sham operated males after transfer from short to long photoperiod.

	Time since transfer from SD to LD [days]; sham operated males												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
JHSB3 [fmol / sample]	0	0	0	0	0	0	0	0	2	0	0	2.5	1.5
	0	0	0	0	0	0	0	0	0	0	0	0	0.5
	0	0	0	0	0	0	0	0	3.5	0	0	0	0.6
	0	0	0	0	0	0	0	0	1.5	0	0	0.8	0.7
	0	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0	1.5

Table S6. JHSB3 titers identified in hemolymph of CAx operated males after transfer from short to long photoperiod.

	Time since transfer from SD to LD [days]; CAx males												
	1	2	3	4	5	6	7	8	9	10	11	12	13
JHSB3 [fmol / sample]	0	0	0	0	0	0	0	0	0	0	0	1.1	0.1
	0	0	0	0	0	0	0	0	0.8	0	0	2.6	0
	0	0	0	0	0	0	0	0	0.1	0	0	768	0
	0	0	0	0	0	0	0	0	0.1	0	0.2	4.6	3.7
	0	0	0	0	0	0	0	0	0	0	0.4	1.5	139
	0	0	0	0	0	0	0	0.9	0	0	0	0	28.4

Table S7. JHSB3 titers in males 10 d after adult ecdysis (AAE) which were intact, sham operated (sham), or with removed CA (CAx). As a reference, titers from intact diapause males kept in SD are shown (SD intact).

	long photoperiod (LD)			SD
	intact	**Sham**	**CAx**	**intact**
JHSB3 [fmol /sample]	3.38	10	25.62	0
	10	6.8	14.42	0
	3.84	7.4	20.89	0
	6.8	11	29.66	0
	3.7	3.5	163.91	0
	1.88	10	4.84	0
	4.73	66	4.7	
	2.4	9.2	4.83	
		5.4		

Table S8. JHSB3 titers in male accessory glands (MAGs) isolated from males 10 d after adult ecdysis (AAE) were intact, sham operated (sham), or with removed CA (CAx). As a reference, titers from MAG of intact diapause males kept in SD are shown (SD intact).

	long photoperiod (LD)			SD
	intact	**sham**	**CAx**	**intact**
JHSB3 [fmol /sample]	0	0	0	0
	0.16	0.45	2.64	0
	0	0.52	2.07	0
	0.13			0
				0
				0

Chapter 3

Functional analysis and localization of a thyrotropin-releasing hormone-type neuropeptide (ELFa) in hemipteran insects

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2020

In an effort to shed more light on male reproductive diapause, or diapause in general, and to add another piece of the puzzle to the endocrine control mechanism, we aimed to investigate the newly identified putative neuropeptide in the transcriptome of *P. apterus* - TVGTEFLamide (EFLa). EFLamide was first predicted in silico from the spider mite genome (Veenstra et al., 2012) and its existence and localization were later confirmed by descriptive characterization in *Locusta migratoria* (Veenstra & Šimo, 2020). One interesting thing about EFLa is that, together with its receptor, it has been identified as an ortholog of vertebrate thyrotropin-releasing hormone (TRH) (Bauknecht & Jékely, 2015; Veenstra & Šimo, 2020). TRH is a highly conserved neuropeptide in vertebrates. It is a major regulator of the synthesis, secretion, and biological activity of thyroid-stimulating hormone (TSH), also called thyrotropin, from the anterior pituitary gland. TSH then stimulates the thyroid gland to produce the thyroid hormones thyroxine (T4) and triiodothyronine (T3), which are essential for the proper function of most tissues, including the liver, bones, cardiovascular system, and brain development. In addition, in mammals, TRH also has a neuromodulatory function in the control of food intake, sleep, locomotor activity, immunity, and thermogenesis. Subsequent studies in other vertebrate models have shown that TRH evinces diverse species-specific action. For example, in adult frogs, TRH is a minor stimulator of TSH release but the major for growth hormone and prolactin. In fish, TRH controls growth hormone and prolactin levels but does not affect TSH secretion (Ben-Shlomo & Melmed, 2011; Fekete & Lechan, 2014; Galas et al., 2009). The biological importance of TRH-like neuropeptides in invertebrates has only recently been demonstrated. The nematode *Caenorhabditis elegans* deficient in TRH-like peptides or receptor show a decrease in growth and have a reduced number of offspring (van Sinay et al., 2017).

In the study "Functional analysis and localization of a thyrotropin-releasing hormone-type neuropeptide (ELFa) in hemipteran insects", we examined whether EFLamide has any biological role in a representative of the hemipteran insects *P. apterus*. For this purpose, complete EFLa null mutants were engineered by CRISPR/Cas9 technology. Immunohistochemistry revealed that 2-3 EFLa cells were localized in close proximity to the surface of the lateral protocerebrum. Axons from the cell bodies run to the medial protocerebrum where they intensively ramify. However, EFLa gene knockout has been shown to have no impact on development. Embryos, nymphs, and adults are fully viable with no visible

anatomical discrepancy. Adult reproduction and the number of offspring are not affected, nor are diapause initiation or termination, circadian rhythms, locomotor activity, or lifespan. In addition, our phylogenetic analysis discovered that EFLa encoding transcripts are produced by alternative splicing of a gene that also produces Prohormone-4. A detailed comparison of EFLa-encoding genes in arthropods shows an interesting link between EFLa and Prohormone-4 in several insect species (*Lygus*, *Bemisia*, and *Locusta*). Despite the recent discovery of the EFLa family in insects and null mutants of EFLa in *Pyrrhocoris apterus,* the only mutants of this gene in arthropods, we have not been able to clarify the role of the peptide or even link it to diapause.

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Functional analysis and localisation of a thyrotropin-releasing hormone-type neuropeptide (EFLa) in hemipteran insects

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ABSTRACT

EFLamide (EFLa) is a neuropeptide known for a long time from crustaceans, chelicerates and myriapods. Recently, EFLa-encoding genes were identified in the genomes of apterygote hexapods including basal insect species. In pterygote insects, however, evidence of EFLa was limited to partial sequences in the bed bug (*Cimex*), migratory locust and a few phasmid species. Here we present identification of a full length EFLa-encoding transcript in the linden bug, *Pyrrhocoris apterus* (Heteroptera). We created complete null mutants allowing unambiguous anatomical location of this peptide in the central nervous system. Only 2–3 EFLa-expressing cells are located very close to each other near to the surface of the lateral protocerebrum with dense neuronal arborization. Homozygous null EFLa mutants are fully viable and do not have any visible defect in development, reproduction, lifespan, diapause induction or circadian rhythmicity. Phylogenetic analysis revealed that EFLa-encoding transcripts are produced by alternative splicing of a gene that also produces Prohormone-4. However, this Proh-4/EFLa connection is found only in Hemiptera and *Locusta*, whereas EFLa-encoding transcripts in apterygote hexapods, chelicerates and crustaceans are clearly distinct from Proh-4 genes. The exact mechanism leading to the fused *Proh-4/EFLa* transcript is not yet determined, and might be a result of canonical cis-splicing, cis-splicing of adjacent genes (cis-SAG), or trans-splicing.

1. Introduction

Neuropeptides are the most diverse and largest class of neuronally-secreted signaling molecules. These peptides affect a plethora of biological processes, ranging from development to physiology and behavior, acting as neurotransmitters, neuromodulators or neurohormones.

Despite remarkable sequence diversity, all neuropeptides share the following properties: (*i*) they are derived from larger preprohormone proteins that contain an N-terminally positioned signal peptide that targets the precursor protein for secretion (Douglass et al., 1984). (*ii*) They are derived from preprohormones following cleavage at dibasic sites recognized by convertases (Veenstra, 2000), and (*iii*) they often are subject to post-translational modifications, with the most common modification being conversion of a C-terminal glycine to an amide group (Eipper et al., 1992).

The neuropeptide signaling is known in Bilateralia and occurs even in cnidarians (Jekely, 2013; Elphick et al., 2018). Many neuropeptide signaling pathways are conserved across taxa, however, the minimal sequence similarity preserved in already short peptide sequences frequently prevents unambiguous detection of any relationship. Often, the evolution of neuropeptide receptors helps to reveal relationships between the peptide ligands (Mirabeau and Joly, 2013).

The neuropeptide toolkit of arthropods, one of the richest and most diverse groups of organisms, is remarkably diverse. However, functional research is strongly biased towards holometabola and particularly to *Drosophila melanogaster*, an amazing model organism with unprecedented experimental tools. Importantly, holometabola have lost a significant number of neuropeptide signaling pathways (Hansen et al., 2010; Veenstra, 2014). Thus, comparative research on basal insect groups became rewarding for uncovering the ancestral neuropeptide toolkit and it is also the crucial first step for planning functional experiments. In recent years, remarkable progress of next generation sequencing paved the way for *in silico* discoveries of neuropeptides from genome drafts and transcriptomes of insects (Veenstra, 2019; Tanaka

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et al., 2014; Predel et al., 2018), non-pterygote hexapods (Derst et al., 2016), chelicerates (Veenstra et al., 2012) and crustaceans (Veenstra, 2016). In some cases, even several new putative insect neuropeptides were discovered in one species (Liessem et al., 2018).

Remarkable progress of receptor deorphanization in recent years further shed light on evolution of neuropeptide signaling. Identification of a receptor often revealed relatedness that cannot be deduced from the sequence of ligands. One such example includes EFLamides (EFLa), neuropeptides originally predicted *in silico* from the genome of the mite *Tetranychus urticae* (Veenstra et al., 2012), which later were found to be orthologs of thyrotropin-releasing hormone (TRH) (Bauknecht and Jékely, 2015; Van Sinay et al., 2017). A recent study further confirmed that the EFLa receptor (EFLaR) from a polyneopteran insect species, *Locusta migratoria*, is activated by a physiologically relevant concentration of *Locusta* EFLa (Veenstra and Šimo, 2020).

Our study was performed on the linden bug, *Pyrrhocoris apterus*, a heteropteran species that has been used in research for more than 5 decades (reviewed by Socha, 1993). Early experiments provided the remarkable discovery of the paper factor, a Juvenile hormone (JH)-mimicking compound produced in North American trees (Slama and Williams, 1966). The role of JH was further revealed in the context of development (Konopova et al., 2011), reproduction (Smykal et al., 2014) and circadian clock gene expression (Dolezel et al., 2008; Bajgar et al., 2013a,b). Although some neuropeptides were described and characterized in *P. apterus*, including two adipokinetic hormones (Kodrik et al., 2000, 2002), adipokinetic hormone receptor (Ibrahim et al., 2017), and three insulin-like peptides (Smykal et al., 2020), a detailed neuropeptide inventory is not yet available for this species. During our analysis of a *P. apterus* transcriptome a new neuropeptide candidate, TVGTEFLamide (EFLa), was identified. Our goal was to test if this new candidate fulfils criteria to be considered as a putative neuropeptide, to pinpoint where it is expressed, and (ideally) identify its role in *P. apterus* biology. Therefore, we have created complete *EFLa* null mutants in *P. apterus*, the first EFLa/TRH-like mutants in arthropods, and analyzed their development, lifespan, reproduction and circadian phenotypes. In addition, we performed detailed phylogenetic comparison of EFLa-coding genes in arthropods, mapped the presence of EFLa and its receptor on insect phylogeny, and discovered an interesting connection between EFLa and Prohormone-4 (Proh-4) in several insect species.

2. Materials and methods

2.1. Insect rearing

Laboratory strain Oldrichovec (Pivarciova et al., 2016), which has been kept in the laboratory for more than 55 generations since 2010 and phenotypically corresponds to wild type bugs, was used in all experiments, including gene editing and subsequent backcrosses. For simplicity, it is abbreviated as wild type (*wt*) throughout this study. *P. apterus* were maintained in the laboratory at 25 °C under a diapause preventing long day photoperiod consisting of 18 h light and 6 h dark phase (LD 18:6). If the ability to diapause was tested, bugs were reared from early developmental stages at 25 °C under short day photoperiod (12 h light and 12 h dark phase, briefly SD 12:12).

2.2. Gene editing –EFLa null mutants

EFLa null mutants were engineered by CRISPR/Cas9 approach, where non-homologous-end-joining repair (NHEJ) mechanism resulted in a deletion removing sequence coding for the putatively active peptide. The detailed protocol including gRNA sequence, embryo injection and mutant detection is published elsewhere (Kotwica-Rolinska et al., 2019). Founder mutants were backcrossed to *wt* strain (identical to the strain where the mutations were induced), heterozygous offspring were identified by PCR and used again in subsequent backcross to *wt* to remove any off-target mutations. Heterozygotes resulting from the 6th backcross were mated together and resulting homozygotes were used to establish a clean mutant line. Heterozygous and homozygous bugs were identified by PCR. Seven mutant lines were originally established and up to three of them were further phenotypically characterized.

2.3. Duration of development

Homozygous mutants of $EFla^{08}$, $EFla^{011}$ and $EFLa^{016}$ (numbers reflect order during the screening process) were single self-crossed to obtain homozygous eggs or back-crossed to *wt*, to obtain heterozygous eggs. Single crosses of *wt* bugs were used for controls. All developmental events were recorded daily. When a clutch of eggs was laid, parents were transferred to a new Petri dish. For egg development, duration is determined for the entire clutch, not for individual eggs. Afterwards, exuviae were counted and removed daily. For presentation, the number of all individuals of particular developmental stage of the same genotype was set as 100% and the daily percentage of newly emerged bugs was plotted.

2.4. Duration of oviposition cycles

The mutants were prepared identically to experiment 2.3. Adult virgin females of *wt* (controls), heterozygotes and homozygotes of $EFLa^{011}$ and $EFLa^{016}$ lines were put separately into Petri dishes within 24 h after adult ecdysis and then egg-laying was recorded daily. When a clutch of eggs was laid, the date was recorded and eggs were removed. Recording was carried out until the fifth consecutive oviposition cycle.

2.5. Lifespan

The mutants were prepared identically to experiment 2.3. Female virgin bugs of *wt* (controls), heterozygotes and homozygotes of $EFla^{011}$ and $EFLa^{016}$ lines were collected at the day of adult ecdysis and kept individually in Petri dishes (diameter 70 mm). Petri dishes were kept at 25 °C under LD 18:6 on the same shelf in the same incubator to ensure as identical conditions as possible and all mutants and controls were reared and analyzed in parallel (± one week). The number of dead females was controlled daily.

2.6. Diapause phenotype

Homozygous $EFla^{011}$mutants were back-crossed to wild type. Heterozygotes resulting from this backcross were self-mated and their offspring was reared from the second instar in diapause-promoting conditions (SD 12:12 and 25 °C). Adult females were kept individually to detect any egg laying. Females were dissected two weeks after adult ecdysis and the ovarian morphology was determined. Females with small ovaries without eggs and vitellogenic follicles were scored as diapausing, whereas females laying eggs or having large ovaries were considered as reproductive (see Smykal et al., 2014 for ovarian morphology images). The genotype of females was determined by PCR afterwards.

2.7. Locomotor activity and circadian rhythmicity

Homozygous $EFla^{011}$mutants were back-crossed to wild type. Heterozygotes resulting from this backcross were self-mated and resulting male offspring at 3–5 days after adult ecdysis were individually transferred to test tubes (diameter 25 mm, length 150 mm), equipped with a water reservoir on one side and a peeled linden seed (*Tilia cordata*) wrapped in textile mesh attached to the other side. Tubes were placed in LAM25 monitors (Trikinetics, Waltham, MA, USA) horizontally with infrared beams crossing the tube in the middle. Locomotor activity was recorded in 5 min bins. Bugs were entrained for five days to the photo-regime LD 18:6 and then released to constant dark at 25 °C

for 12 days. The genotype of males was determined by PCR after the experiment.

The daily profile of locomotor activity was analyzed as previously (Kotwica-Rolinska et al., 2017) in ActogramJ software (Schmid et al., 2011). Briefly, activity of all individuals of a particular genotype was averaged, smoothed (Gaussian smooth 3) and displayed using Graphpad7 (Prism) software.

Lomb-Scargle periodogram in ActogramJ was used to determine the rhythmicity of bugs and the length of the free running period (τ) in constant conditions and double-plotted actograms were further controlled by eye. Three categories were defined: (1) *rhythmic* males: periodogram peak crossed the significance threshold and PN value calculated by ActogramJ software was > 35. (2) *complex* males: periodogram peak crossed the significance line and PN values were > 35 but more than one periodicity value was found. (3) *arrhythmic* males: periodogram peak did not cross the significance line or periodogram peak crossed the significance line but PN value was < 35 (as described in Pivarciova et al., 2016; Kaniewska et al., 2020).

2.8. Statistical analysis

Statistical analysis was performed in the GraphPad software (Prism). One-way ANOVA was used to analyze differences in the development, locomotor activity and τ. Two-way ANOVA was used to analyze the effect of the EFLa mutation on the duration of oviposition cycles. Survival was analyzed by the Mantel–Cox log-rank test.

2.9. In silico data mining

EFLa preprohormone mRNA was identified manually in the *P. apterus* transcriptome. First, a library of all putative proteins was built from the transcriptome (brain, fat body, gut), including interior open reading frames. Then, signal P was used to determine proteins containing signal peptide. This dataset of putatively exported proteins was prospected for candidate biologically active peptides containing EFLG motif followed by a cleavage site (KR, RR). The search for EFLa prohormone orthologs was done in GenBank using BLAST-P and T-BLAST-N algorithms in non-redundant protein sequences, reference RNA sequences, and in Transcriptome Shotgun Assemblies (TSA) with various taxonomic limits to optimize the search. Similarly, *Proh-4* encoding transcripts were identified using various query sequences. Additional EFLa preprohormone sequences were retrieved from supplementary material of Ders at al. (2016). The nucleotide and protein sequences were aligned in Geneious 11 (Biometters).

Signal-P 5.0 (http://www.cbs.dtu.dk/services/SignalP/) (Almagro Armenteros et al., 2019) and NeuroPred (http://stagbeetle.animal.uiuc.edu/cgi-bin/neuropred.py) (Southey et al., 2006) were used to predict preprohormone processing and identify expected peptides. These peptides were aligned either in Geneious or manually and the alignment was used to plot the consensus as a Logo (http://weblogo.berkeley.edu/logo.cgi).

To retrieve receptors of EFLa and ETH, respectively, TSAs, non-redundant protein sequences, whole genome shotgun sequences (wgs) and genomes available in GenBank were analyzed using BLAST-P and T-BLAST-N algorithms. Retrieved sequences were aligned as proteins to known receptors (MAFFT, Geneious 11) and their relationship to a particular G protein-coupled receptor (GPCR) group was further determined using phylogenetic analysis (Fast tree, Geneious 11).

2.10. cDNA cloning and RNA interference (RNAi)

EFLa/Proh-4 transcript fragment was amplified with gene-specific primers (Fw: 5′-CCCGCCGGACACCAGAGA-3′ and Rev: 5′-AGTCCTCG TCGTAGCCGTCAAGAC-3′), 329 bp product was cloned into pGEM-T-easy plasmid (Promega) and verified by sequencing. PCR was used to amplify the insert and replace SP6 with T7 promoter. Double-stranded RNA (dsRNA) was prepared from PCR template using the T7 RNA polymerase with the MEGAscript kit (Thermo Fisher Scientific) and injected into *P. apterus* adults as described previously (Bajgar et al., 2013a). Adults received 3 µl of dsRNA at a concentration of 2–4 µg/µl in Ringer's solution; control animals were injected with heterologous dsRNA derived from bacterial β-galactosidase (*lacZ*) gene or with the Ringer's solution alone.

2.11. mRNA quantification

Analyzed tissues (brain, gut, fat body) were dissected in RNAse-free Ringer's solution and total RNA was isolated with Trizol reagent, residual genomic DNA removed with Turbo DNAse and 1 µg of total RNA was used for cDNA synthesis using SuperScript III reverse transcriptase (all from Thermo Fisher Scientific). Relative transcript levels were measured by reverse transcription quantitative PCR (RT qPCR) using qPCR 2_ SYBR Master Mix (Top Bio) and a C1000 Thermal Cycler (Bio-Rad). *EFLa* and *Proh-4* common forward primer (5′-GACGGTGCCTGC ATCTCCAT-3′) was combined either with *EFLa*-specific reverse (5′-AGTTCCGACGGTCCTCTTCAAA-3′), or with *Proh-4*-specific reverse (5′-CCAGCGGGGCAAGAGCATC-3′) primer. Both measured transcripts were normalized to relative levels of *ribosomal protein 49* (*rp49*) mRNA (Dolezel et al., 2007). Primer efficiency was evaluated by an RT qPCR method on a six-point standard curve prepared separately from purified PCR products for every gene tested.

2.12. EFLa antibody & whole mount immunohistochemistry

Polyclonal antibody against putative *P. apterus* EFLa was produced in rat (Moravian Biotech, Czech Republic). Immunization was done with TVGTEFLa peptide, amino-terminally coupled to keyhole limpet haemocyanine (KLH) via glutaraldehyde. Brains were fixed overnight in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. Afterwards, brains were subjected to several washes in 0.3% TX-100 in PBS (PBST) and incubated for 1 h in blocking solution consisting of 5% normal goat serum (NGS) in PBST. Next, brains were incubated overnight at 4 °C with primary rat anti-EFLa antiserum diluted 1:1000 in 5% NGS in PBST. Brains were washed several times with PBST and incubated overnight at 4 °C with fluorescent secondary goat anti-rat AlexaFluor 488 antibody (ThermoFisher Scientific) diluted 1:1000 in the blocking solution. Brains were then washed several times in PBST and mounted in Vectashield mounting medium (Vector Laboratories). The samples were imaged under Laser Scanning Confocal Microscope FluoView FV1000 (Olympus) using objective UPLSAPO 10× or UPLSAPO 20xO, correction of brightness in depth. All confocal images were processed and analyzed by ImageJ software (NIH). 3D models were reconstructed by stitching of the particular frames in the software XuvStitch (XuvTools). The compound image was then analyzed in the software Imaris (Bitplane) by using modules: Easy 3D, Surpass - Surfaces, Surpass - Ortho Slicer and Animation and scanned under Series (Olympus). The specificity of the antibody was tested on *P. apterus* EFLa null mutants generated by CRISPR/Cas9.

3. Results

3.1. EFLa gene and transcripts in P. apterus

During exploration of *P. apterus* transcriptome, a putative new neuropeptide precursor was identified. The following sequence criteria indicated that the peptide might be biologically relevant: (*i*) the preprohormone starts with a clearly predicted signal peptide, (*ii*) the putative neuropeptide, TVGTEFLG, is surrounded by convertase cleavage sites, and (*iii*) the last amino acid of the peptide, glycine, could serve as amidation signal, a hallmark of many neuropeptides (Fig. 1A). Therefore, the peptide was named EFLa to indicate its connection with other EFLa-type neuropeptides. Closer *in silico* analysis discovered transcripts

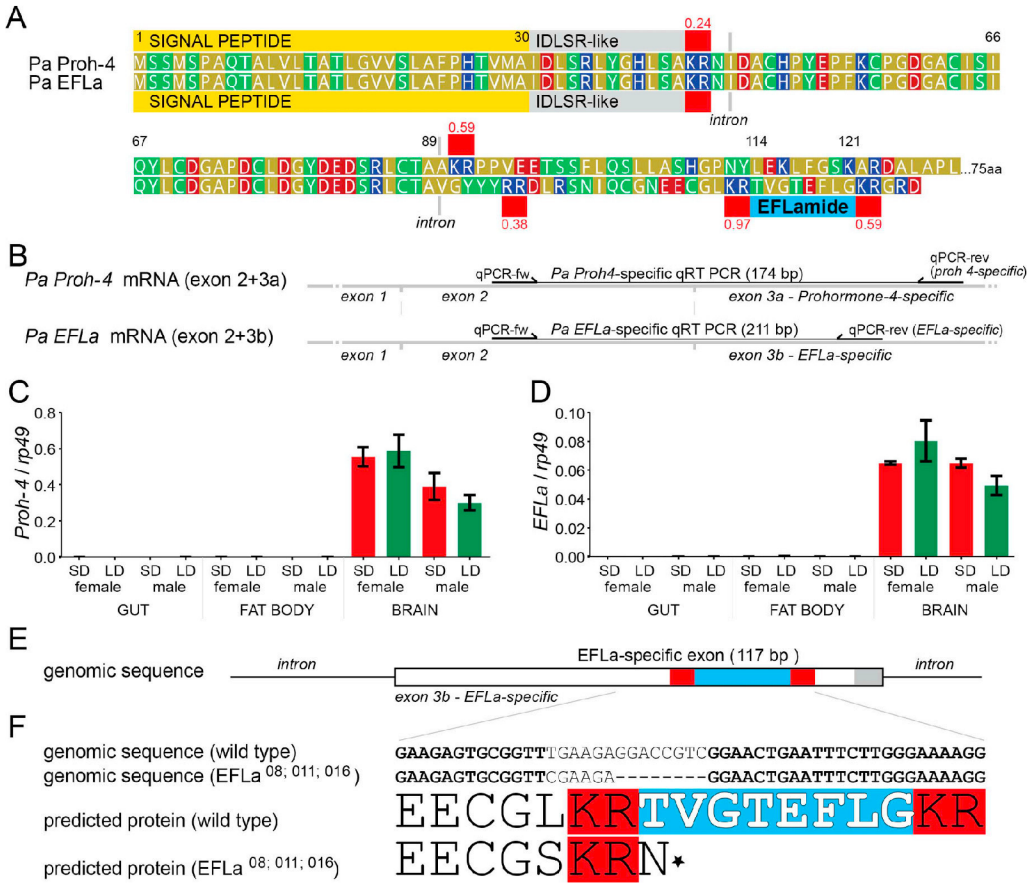

Fig. 1. EFLa is a neuropeptide which results from alternative splicing of the *Prohormone-4* gene in *P. apterus*. (A) Alignment of Proh-4 and EFLa preprohormone protein sequences. Signal peptide is shown in yellow (SignalP 5.0 prediction), convertase cleavage sites are highlighted in red with values indicating NeuroPred cleavage prediction scores. Both preprohormones contain a 12 aa peptide (IDLSRLYGHLSA) located between the signal peptide and first cleavage site. The EFLa preprohormone contains an 8 aa peptide (TVGTEFLG) motif surrounded by two cleavage sites. (B) Detailed scheme of mRNA indicates the position of intron-exon boundaries and transcript-specific reverse primers used (C,D) to confirm brain-specific expression of *Proh-4* and *EFLa* (graphs show mean ± SEM). LD - long day, SD - short day. In both transcripts, the expression was significantly different in brains when compared to other tissues (One-way ANOVA, $p < 0.001$), but the differences between sexes or photoperiods were not significant ($p > 0.05$). (E) 117 bp long exon 3 codes for *EFLa* peptide, its non-coding untranslated terminal region (UTR) is shown in gray. (E,F) Detail of the genomic sequence in wild type and EFLa null mutants, where 8 bp deletion results in a stop codon (shown as asterisk). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

identical with the EFLa mRNA in the first nucleotides coding for the signal peptide and the predicted IDLSRF-like neuropeptide, but distinct at the 3′ end, where EFLa is encoded. IDLSRF-like peptide is highly conserved amino acid motif characteristic for Prohormone-4 (Proh-4) found first in the honey bee (Hummon et al., 2006). BLAST search in GenBank and detailed protein alignments revealed high similarity of *EFLa* transcripts with *Proh-4* in *P. apterus*, suggesting that *EFLa*-coding exon is fused downstream of the first two *Proh-4* exons. Indeed, a physical presence of both transcripts in brain tissue was confirmed by PCR on brain cDNA followed by Sanger sequencing and independently with different pair of primers by RT qPCR (Fig. 1B, C,D).

Since typical neurohormones and neuropeptides are preferentially expressed in the Central Nervous System (CNS), we quantified *EFLa* and *Proh*-4 by RT qPCR with universal forward and transcript-specific reverse primers. The minimal expression levels in gut and fat body contrasted with the clear expression in brains of both sexes. The amount of *Proh-4* was approximately 10-times the level of *EFLa* (Fig. 1C and D).

To further shed some light on the role of this putative neuropeptide, null mutants were engineered in *P. apterus* (for technical details see Kotwica-Rolinska et al., 2019). Briefly, Cas9 was guided to *EFLa*-specific exon 3b (Fig. 1E) to cleave right upstream of the peptide-coding sequence. Deletion of eight nucleotides resulted in a frameshift and premature stop codon that removed the entire amino acid motif of EFLa (Fig. 1F). Three mutants with identical deletions were retrieved and separately backcrossed to wild type strain for up to eight generations. Two or three of the mutant strains were used in further experiments to clarify the effect of the mutation and distinguish it from the genetic background.

3.2. EFLa is expressed in 2–3 cells of each brain hemisphere

Polyclonal antibodies raised against the TVGTEFLa peptide labeled approximately twenty neuronal cell bodies in the wild type adult brain. Majority of these cells were labeled also in the EFL^{011} and EFL^{016} mutants (all EFLa mutants are homozygous viable), indicating that the antibodies cross-react with other antigens. However, intensive staining detected in a bilateral cluster of two to three neurons located in the lateral protocerebrum were not observed in the mutants indicating their

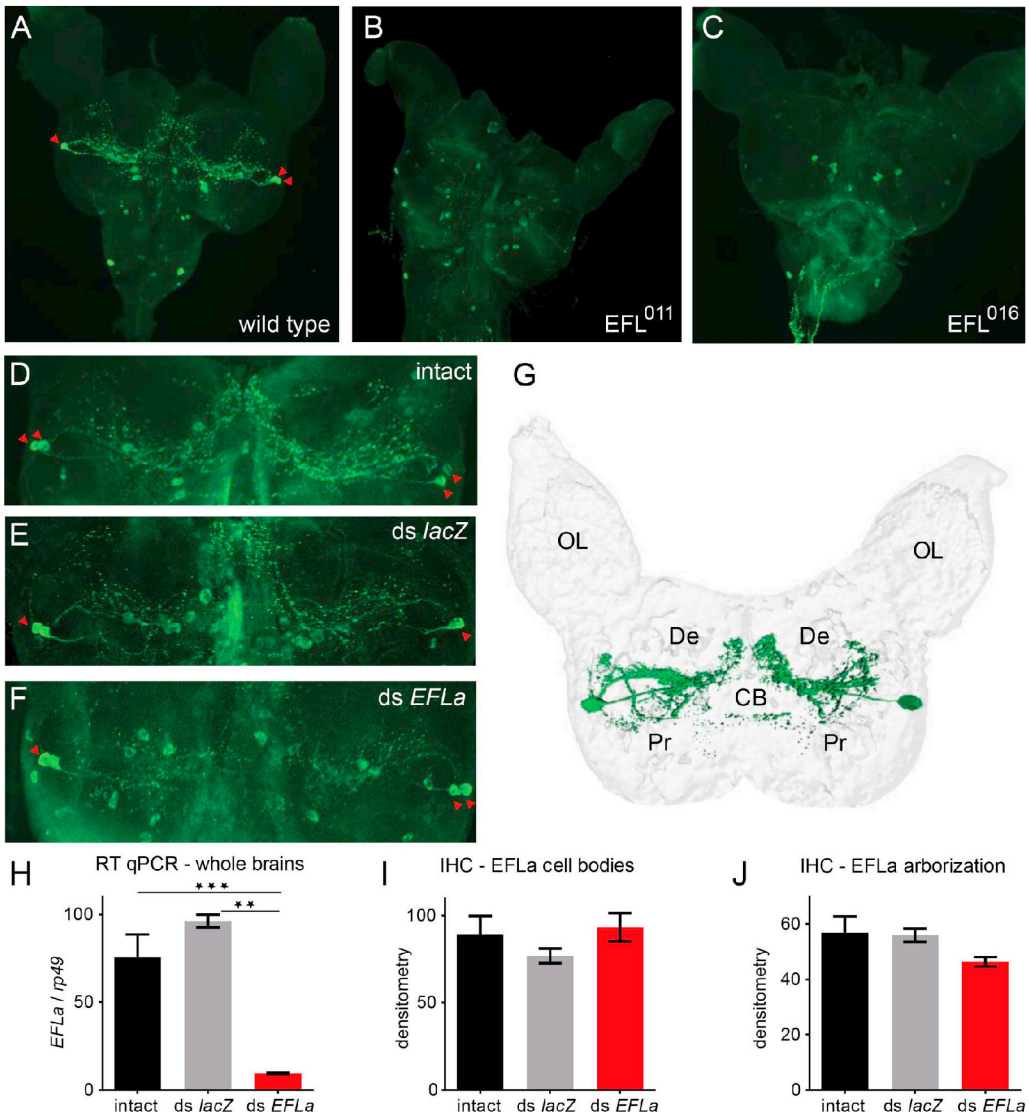

Fig. 2. Localisation of immunoreactivity with anti EFLa antibody in the brain of *P. apterus*, with red arrowheads labelling EFLa-expressing cell bodies (A–C) Dorsal view of the whole brain of wild type, *EFLa*^{011}, and *EFLa*^{016} mutants, respectively. (D) Detailed image of EFLa positive neurons and their neuronal projections. (E, F) Comparable EFLa immunoreactivity was observed in control bugs injected with double strand RNA (ds) for *lacZ* (E), and with ds*EFLa* (F). Red arrows indicate the EFLa-specific signal in a bilateral cluster of neurons in the lateral protocerebrum. (G) Extracted EFLa-specific immunoreactivity in a 3D reconstruction of the brain. (H) The efficiency of gene silencing by dsRNA was confirmed by RT qPCR, yet the IHC signal was strong in EFLa-specific cell bodies of RNAi animals (I), and only marginally reduced in their arborizations (J) (One-way ANOVA $p > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

EFLa-specificity (Fig. 2A–C). The EFLa-specific cell bodies were located very close to each other near to the surface of the lateral protocerebrum. Neuronal projections rising from those cells run to the medial protocerebrum where they intensively ramify. This ramification gives off a dense neuronal arborization, which projects in several directions (Fig. 2G): (1) anteriorly to the midline of the ipsilateral proto- and deutocerebrum, (2) posteriorly around the central body to the contralateral hemispheres, (3) laterally to the dorsal protocerebrum and to the frontoventral deutocerebrum. These lateral branches turn to the posterior brain passing ventrally of the EFLa specific neurons and merging with a neuronal network in the posterior brain.

In parallel, RNA interference was used to silence expression of EFLa. Although the knockdown was reasonably efficient, resulting in ~90% reduction in mRNA level that persisted low for 3, 7 and 10 days (Fig. 2H), EFLa immunoreactivity in cell bodies of *bona-fide* EFLa neurons was not affected even 10 days after *EFLa* dsRNA injection (Fig. 2F and I), and the immunoreactivity was only marginally reduced in arborizations (Fig. 2F and J). Cumulatively this suggests that EFLa might be a stable peptide with a long half-live.

3.3. *EFLa null-mutants are fully viable with no obvious developmental defects*

We have used all assays available in our lab for *P. apterus* to

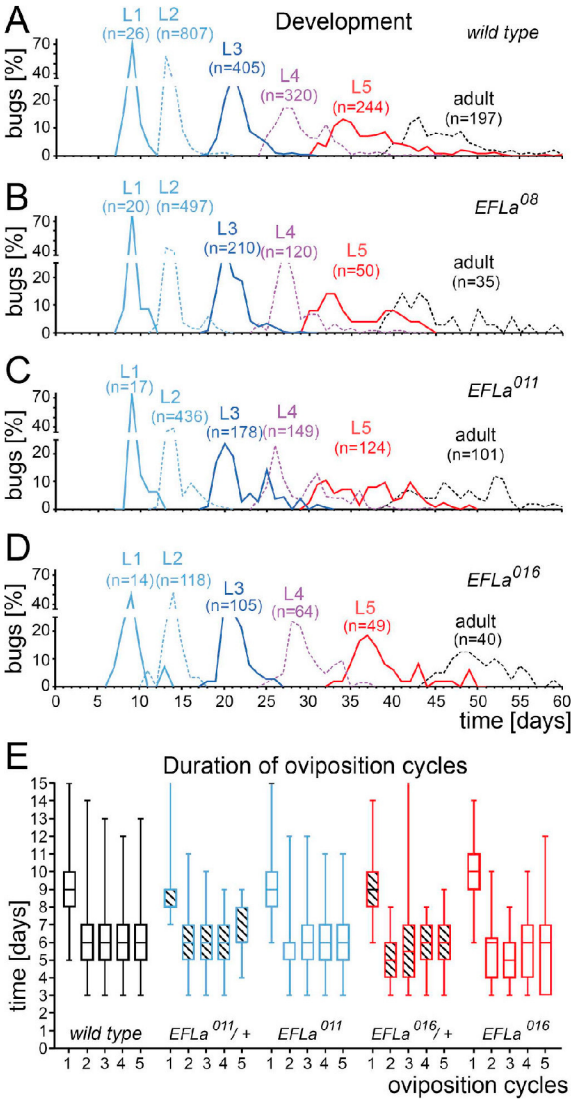

Fig. 3. Comparison of development in wild-type and EFLa-mutant *P. apterus*. (A–D) Timing of transition to a new instar is shown on x-axis. Time '0' corresponds to egg laying. Transition to L1 indicating egg development is determined for the entire clutch, not for individual eggs. Sum of all individuals of particular developmental stage of the same genotype was set as 100% and the daily percentage of newly emerged bugs is plotted. (E) Duration of first five oviposition cycles shown as mean, 25–75 percentile (box), and min-max (whiskers) is not significantly different between tested genotypes (Two-way ANOVA $p > 0.05$).

investigate the physiological roles of EFLa. The development from the egg to the adult takes 40–50 days at 25 °C in wild type animals. Comparable duration of all instars and the entire development was observed in all three mutants (Fig. 3A–D) and also in heterozygotes (data not shown).

P. apterus wild type females lay the first batch of eggs approximately on the ninth day after adult eclosion and afterwards are able to lay eggs in several oviposition cycles separated by six days when kept at 25 °C with a diapause-preventing long photoperiod. Comparable duration was determined for the $EFLa^{011}$ and $EFLa^{016}$ homozygotes and heterozygotes (Fig. 3E). To further characterize possible roles of *EFLa*, lifespan was compared between wild types and $EFLa^{011}$ and $EFLa^{016}$ homozygotes and heterozygotes. The survival of virgin females at reproduction-promoting conditions of LD and 25 °C is not affected by loss of EFLa (Fig. 4A and B).

3.4. Circadian clock and photoperiodic time measurement are not influenced by EFLa

Adult *P. apterus* bugs are active mostly during the long day (LD) with a relatively broad activity peak. Both homozygous mutants show comparable timing of activity rise and duration through the photophase of the long photoperiod (Fig. 5A). The average locomotor activity differs between $EFLa^{011}$ and $EFLa^{016}$, but is not different from the wild type (Fig. 5B). Therefore, these differences seem to be resulting from the genetic background and are not caused by the lack of EFLa.

Then we tested the ability of *P. apterus* to detect short days (SD) and to communicate this information further downstream. Under short photoperiod, wild type bugs undergo reproductive arrest, diapause, which is characterized by small ovaries without eggs. Indeed, ~96% of *wt* females are diapausing in SD conditions and a comparable amount of $EFLa^{011}$ homozygotes (94.4%) and heterozygotes (96%) enter diapause too (Fig. 5C).

The free running period of locomotor activity recorded under constant dark conditions (DD) differs between $EFLa^{011}$ and $EFLa^{016}$, but this difference can be clearly attributed to genetic background, because one mutant ($EFLa^{011}$) produces a faster circadian clock than the wild type line, whereas the trend is completely the opposite in the second mutant (Fig. 5D). Neither the percent rhythmicity, nor the average activity in DD is influenced by loss of EFLa (Fig. 5E and F).

3.5. Evolution of EFLa in arthropods

The link between *Proh-4* and *EFLa* observed in *P. apterus* prompted us to explore the evolution of EFLa-coding genes. Orthologous full length *EFLa* transcripts were identified in *Lygus* (Heteroptera), in *Bemisia* (Sternorrhyncha) and in *Locusta* (Polyneoptera). In all cases, the EFLa preprohormone also contained the highly conserved IDLSR-like peptide characteristic for Proh-4 (Fig. 6A). Indeed, *Lygus*, *Bemisia* and *Locusta* Proh-4 are identical to EFLa preprohormone in their first 85, 82 and 90 amino acids, respectively (Fig. 6B). Proh-4 sequences are highly conserved, which allowed us to unambiguously explore its possible co-evolution with EFLa. Clearly, EFLa prohormones of basal hexapods, Crustacea and Chelicerata are neither identical, nor even similar to Proh-4. Thus, the connection of EFLa with Proh-4 is an evolutionary novelty found only in Hemiptera and *Locusta* (Fig. 6A). Whether EFLa exists in holometabolan insects is unclear. However, our focused attempts to identify EFLa or even alternative splicing of *Proh-4* were repeatedly unsuccessful in Holometabola, Psocodea and Thysanoptera.

There is one EFLa peptide encoded in *Bemisia* (SIGTEFLG) and *Pyrrhocoris* (TVGTEFLG) preprohormones that differ in two initial amino acids. In *Lygus*, two identical TVGTEFLG sequences are found and the same arrangement exists in the partial sequence from *Lopidea* (both species belong to heteropteran family *Miridae*) indicating that tandem EFLa organization is not an artifact of the transcriptome assembly. The organization of EFLa preprohormones in basal hexapods is more diverse. The number of EFLa motifs is varies and reaches up to 18 paracopies in several species (Fig. 6A, Derst et al., 2016). The lowest copy number might be as low as 4 in *Tetranychus* (Veenstra et al., 2012).

3.6. Presence of EFLa-receptor (EFLaR) and ligand in insects

Our inability to repeatedly find EFLa transcripts and genes in holometabolan insects, aphids, and some polyneopteran orders, prompted us to explore the distribution of EFLaR in major insect lineages. Although absence of a particular gene may reflect just an imperfect

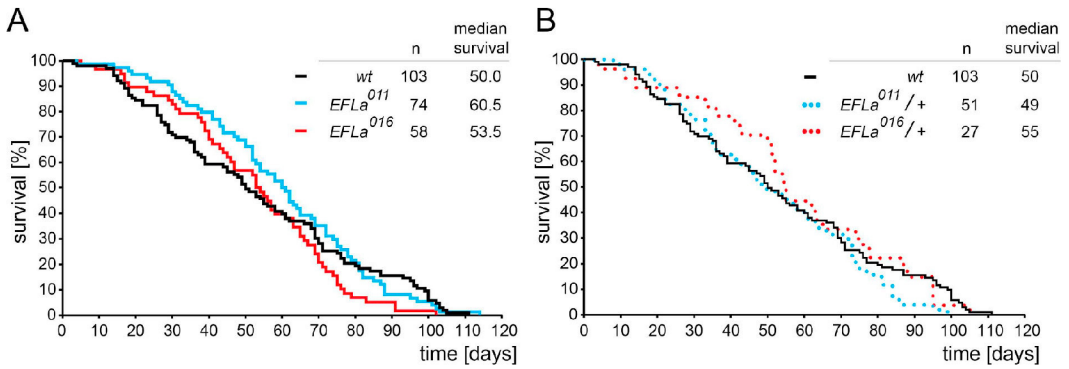

Fig. 4. Comparison of lifespan of adult reproductive virgin wild-type and EFLa-mutant *P. apterus* females, determined in LD regime and 25 °C for homozygotes (A) and heterozygotes (B). y-axis indicates percent survival, the actual n is shown above the graph for all genotypes. Survival is not significantly affected by mutation of *EFLa* gene (Mantel–Cox log-rank test).

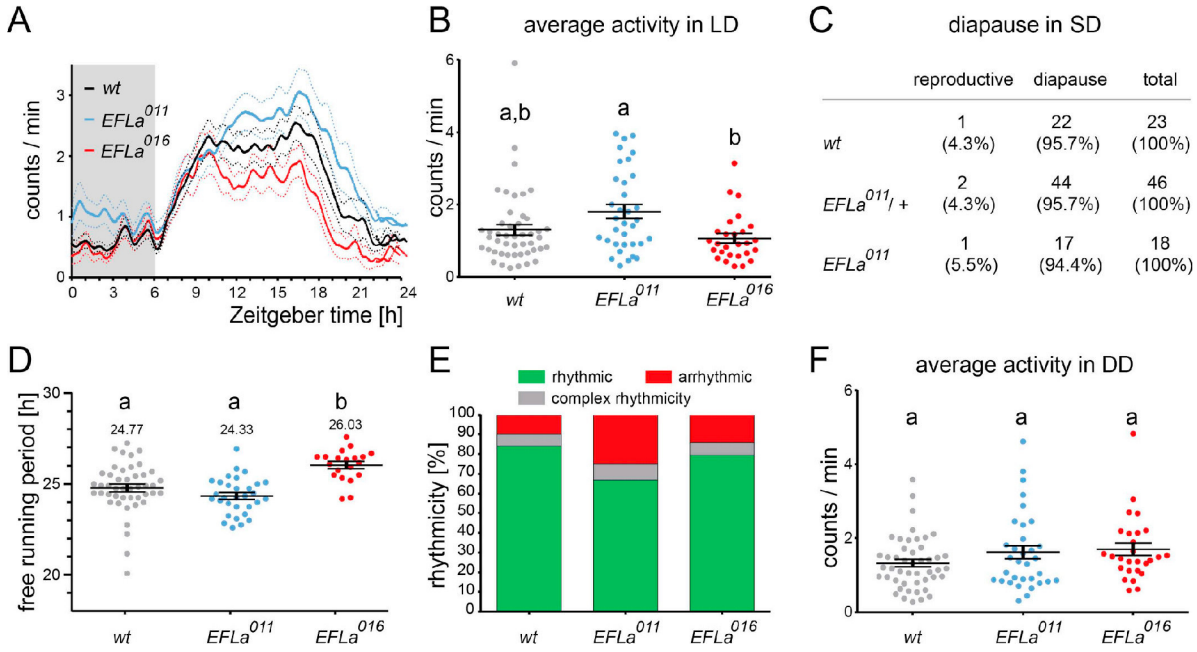

Fig. 5. Locomotor activity, circadian clock and diapause in wild-type and EFLa-mutants. (A) Locomotor activity in long day (LD) regime (gray background corresponds to dark) shown as mean ± SEM. (B) The average activity in LD is not different between wt and mutants, although mutants differ between each other (One-way ANOVA). (C) Photoperiodic induction of diapause is identical in wt and *EFLa*^{011} homozygotes and heterozygotes, respectively. (D) The free running period and (E) percent rhythmicity determined from 10 days in constant dark (DD). (F) The average activity in DD is not different between tested genotypes. Different small letter above categories indicate statistical difference $p < 0.05$ (One-way ANOVA).

genome assembly, or, in the case of the transcriptomic analysis result from low expression of the gene in the sequenced tissue, repeated failure to identify EFLaR in multiple species belonging to one taxonomic group becomes informative. We also plotted the presence of the receptor for ecdysis triggering hormone (ETH), the closest relative of EFLaR.

Despite our systematic search in genomes, transcriptomes and proteomes of several holometabolan insects, we were not able to find EFLaR and EFLa (representative species are shown in Fig. 7; the only EFLaR sequence retrieved from *Ragoletis* clusters clearly with mite sequences, and is, therefore interpreted as a result of interspecific contamination of the sequenced material). Similarly to Holometabola, no EFLaR and EFLa was identified in Psocodea and Thysanoptera.

A more complicated scenario was observed in Hemiptera, a group consisting of three orders: Sternorrhyncha, Auchenorrhyncha and Heteroptera (Johnson et al., 2018). In basal Sternorrhyncha, such as *Bemisia* and *Diaphorina*, both EFLaR and EFLa are found (Fig. 7). However, in the apical Sternorrhyncha, Aphids, we repeatedly failed to identify either the receptor or the ligand, although aphids belong to one of the most sequenced insect groups. A comparable situation were observed in Auchenorrhyncha, where ligand and receptor was identified in Cicadomorpha, but in a sister group containing planthoppers (Fulgoromorpha), only the receptor was identified.

In Polyneoptera, a monophyletic assembly represented here by Orthoptera, Blattodea and Phasmatodea, a picture analogous to situation observed previously in Hemiptera was found. Both receptor and

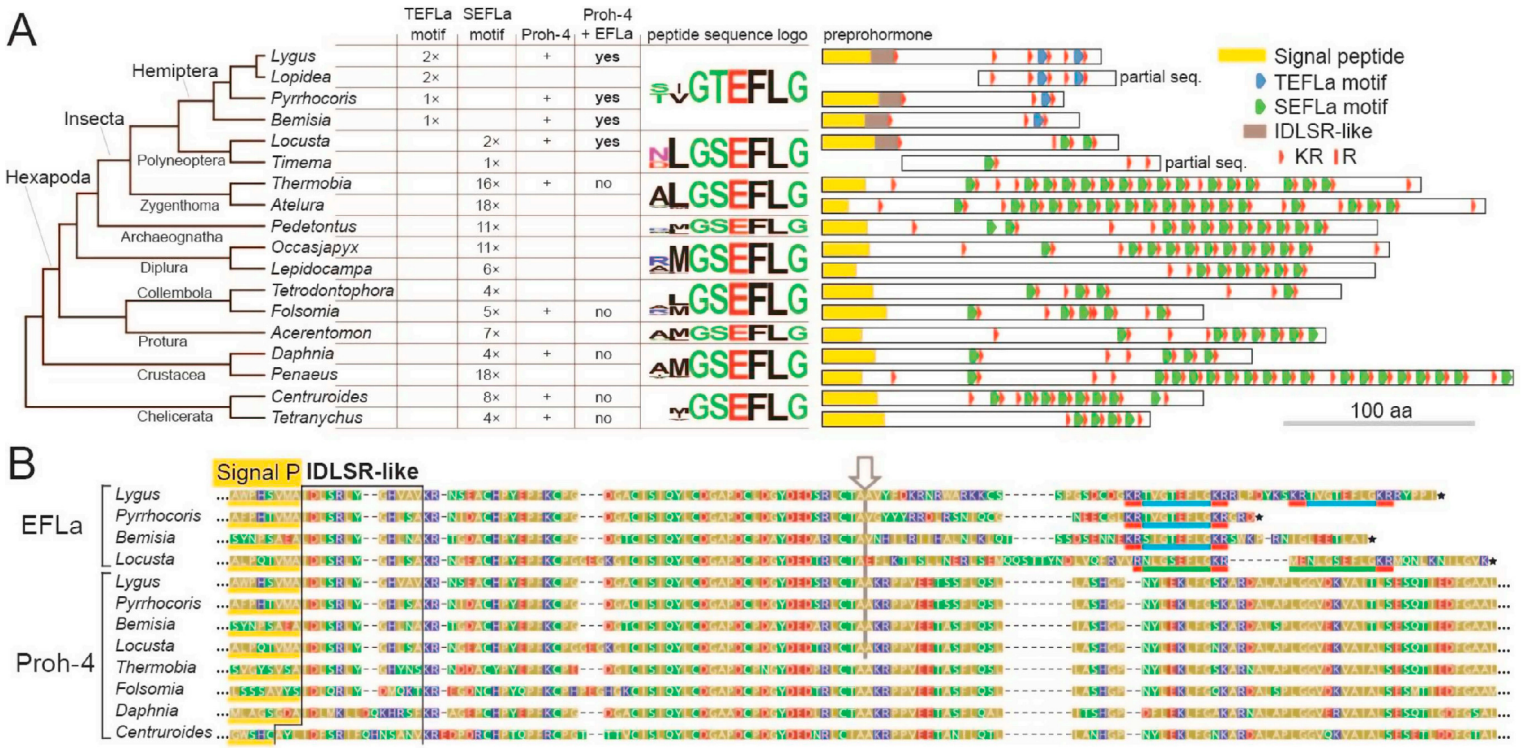

Fig. 6. Co-evolution of EFLa with Proh-4 is found in *Locusta* and Hemiptera but not in basal hexapods. (A) Simplified tree illustrates the phylogenetic relationship between analyzed species. The table shows number of TEFLa or SEFLa motifs predicted within each preprohormone. Proh-4 was identified in representative species (+) and its alternative fusion to EFLa transcript (Proh-4 + EFLa) is either identified (yes) or excluded (no). The peptide sequence logo shows frequency of amino acids in predicted EFLa peptides from particular species and groups of species. Schematic depiction of EFLa preprohormones from each species indicates the presence and position of important sequence features. In Hemiptera and Locusta, 1 or 2 EFLa motifs are found in the preprohormone, which also encodes for IDLSR-like peptide. Multiple SEFLa repeats found in preprohormones in Chelicerata, Crustacea and wingless hexapods (Protura, Collembola, Diplura, Archaeognatha, and Zygenthoma) are never combined with IDLSR-like peptide. (B) Detail of protein alignment indicates shared prohormone region between EFLa and Proh-4 in Hemiptera (from the N-terminus up to the arrow). Asterisk indicates the C-terminus of the protein. See supplement (Fig. S1) for alignment of all EFLa sequences with Proh-4.

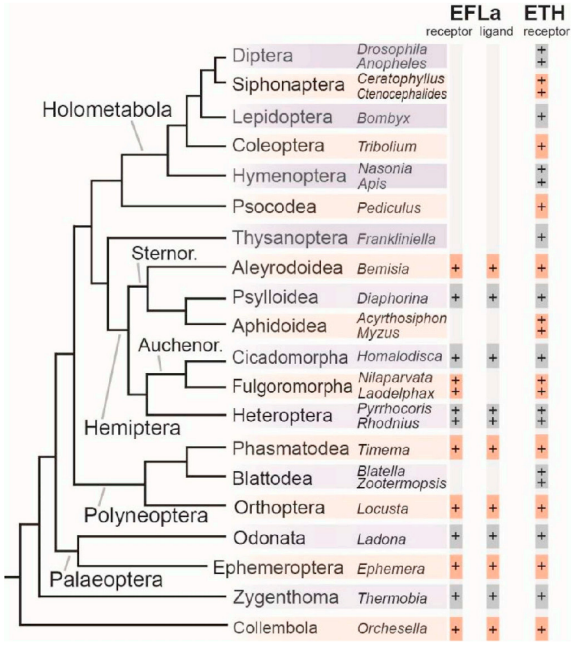

Fig. 7. Phylogenetic distribution of EFLa-like receptor and its ligand in insects. Simplified insect phylogeny according to recent molecular data (Misof et al., 2014; Johnson et al., 2018; Wipfler et al., 2019) indicates insect groups with particular species where EFLa receptor, ligand and related ETH receptor were (+) or were not (empty box) identified. Presence indicates confidence that identified sequence is reliably assigned as a particular receptor homolog, however, even partial sequences were included, if the analysis was reliable. Thus, this scheme should be interpreted as an indication of where the EFLa receptor gene is present, but it does imply that the gene is also functional. See supplement for actual sequences. Sternor. – Sternorrhyncha. Auchenor. - Auchenorrhyncha.

ligand were identified in *Locusta* (Orthoptera) and *Timema* (Phasmatodea), but, despite our repeated effort, neither ligand nor receptor was retrieved from any Blattodea, including species with sequenced genomes and reasonably covered brain transcriptomes.

Altogether, available data suggest five possible independent losses of EFLa signaling in insects (Fig. 7): (*i*) Holometabola together with order Psocodea; (*ii*) order Thysanoptera, thrips; (*iii*) superfamily Aphidoidea, aphids; (*iv*) infraorder Fulgoromorpha, planthoppers; and (*v*) order Blattodea with cockroaches and termites.

4. Discussion

This study and concurrent research on locusts (Veenstra and Šimo, 2020) provide the first analysis of EFLa-type neuropeptide expression in insects, although genes encoding EFLa-type neuropeptides were previously identified in basal hexapods including insects such as the firebrat *Thermobia domestica* and *Atelura formicaria* (Derst et al., 2016), the bed bug *Cimex lectularius*, locust, and phasmids (Predel et al., 2018; Veenstra, 2019). Various EFLa-encoding genes and related FFamide, FLamide and FVamide genes have been identified in crustaceans, mollusks and annelids (Conzelmann et al., 2013; Veenstra, 2010, 2011, 2012).

To elucidate the function of EFLa, complete null mutants were engineered in *P. apterus* by CRISPR/Cas9 technology. One key aspect of any reverse-genetic experiment is to ensure that the desired mutation, such as the EFLa removal, is not accompanied by any unintentional changes in the genome, the off target mutations. This is usually solved by outcrossing the mutant line to *wt* strain. While this backcrossing can be efficiently done in *D. melanogaster*, the generation time of many emerging model organisms is significantly longer and thus protracts the CRISPR/Cas9 experiment, which might be a serious practical limitation for the research. In our case, 6 rounds of backcrosses were used. With subsequent amplification of the "cleaned" lines, the procedure took ~15 months to obtain a sufficient number of individuals for phenotypic characterization.

Up to three independently backcrossed EFLa mutant lines were used in experiments and compared to *wt*. Our data indicate that EFLa removal does not affect development duration and adult bugs are phenotypically normal, including no anatomical defect observed, their locomotion is comparable to *wt*, and their lifespan is not affected. EFLa mutants are able to discriminate between long and short photoperiods to enter reproductive diapause and their circadian clock is functional. Since the expression pattern is reasonably similar in *P. apterus* and *Locusta*, it is plausible to suggest that comparable modulatory roles of EFLa are shared between species, although, of course, details might differ. Interestingly, EFLa expression pattern in *Locusta* prompted Veenstra and Šimo (2020) to suggest "… that the ELFamide neurons exert a modulatory input onto the navigation system of the locust by simultaneous targeting several stages of the sky compass system in the locust brain." Whether EFLa is involved in some sort of neuromodulation in *P. apterus* is unknown. Since *P. apterus* is not a migratory species, neither its navigation nor its sky compass has been studied, to our knowledge.

Obviously, a neuropeptide or neuromodulator might affect a plethora of biological phenomena, thus negative results observed in a limited number of assays do not indicate that EFLa has no function in *P. apterus*. However, even this eventuality is plausible, because EFLa and its receptor were identified only in some insects, whereas they are absent and thus most likely lost in others (see caveats below). Since unused ligand or receptors can slowly accumulate deleterious mutations, early steps in this process cannot be excluded in *P. apterus*. When we map the presence of EFLa ligands and its receptors on insect phylogeny, up to five possible loses of either both or only the ligand are suggested (Fig. 7). Nevertheless, it is problematic to prove loss of a gene in general and particularly if it codes for a short ligand, therefore, any negative data need to be interpreted carefully. Yet, if a gene is repeatedly missing in all representatives of a particular monophyletic lineage, its absence becomes meaningful. In the case of planthoppers, EFLaR is still present (Tanaka et al., 2014) and only the ligand is missing, which invites speculation that the early steps of signaling loss are captured. The second hemipteran group where EFLa signaling was independently lost, aphids, lacks both the receptor and ligand. Given the agricultural importance of planthoppers and aphids, reasonable number of transcriptomes and genomes from additional species is expected in future and will facilitate further confirmations. Moreover, the short ligand-coding sequences can be retrieved even from raw Illumina reads, thus the challenging process of genome assembly will not prevent either EFLa identification, or will further support its likely absence in planthoppers.

A similarly interesting situation is seen in Polyneoptera. This assemblage of insect orders includes Orthoptera, where activation of EFLa receptor by EFLa was functionally confirmed for *Locusta* (Veenstra and Šimo, 2020) and phasmids with only partially identified EFLa genes (Veenstra, 2019). However, in cockroaches and termites, representatives of the most apical polyneopteran order, Blattodea (Wipfler et al., 2019), neither ligand nor receptor is identified (Fig. 7). It is worth noting that polyneopteran genomes are one of the largest in animals, which does not prevent or even limit their sequencing, yet the assembly is often complicated. However, with remarkable interest in social insects, termite genomic and transcriptomic data are quickly growing and will clarify our hypothesis.

Our study points to another interesting and perhaps even provocative finding, the fusion of *EFLa* mRNA downstream of sequence

originating from *Proh-4*. In fact, the connection of Proh-4 and EFLa was independently identified by Veenstra and Šimo (2020) in *Locusta*, however, interpreted as an artifact generated during the transcript assembly. At that time, their cautious approach was completely correct and even now we cannot entirely exclude the possibility that the fused *Proh-4/EFLa* encoding transcripts are artifacts. However, in the light of new data, the connection of *EFLa* and *Proh-4* seems to be reasonably supported. Firstly, the physical presence of existing mRNA was confirmed in *P. apterus* by two independent PCR experiments, the first aiming at confirmation of transcript sequences, and the second addressing the expression level of both transcripts. Secondly, the fused *Proh-4/EFLa* encoding transcripts were found in four insect species (*P. apterus*, *Lygus*, *Bemisia*, *Locusta*) each of them coded by slightly different RNA sequence due to different codon usage, yet, the *Proh-4* and *EFLa* fusion occurs in an identical position (coding for amino acids RLCTA, Fig. 6) in all four species. Nevertheless, the exact process of how *EFLa*-specific exon is connected to the *Proh-4* sequence is unclear. The possible mechanism might involve canonical cis-splicing, cis-splicing of adjacent genes (cis-SAG), or even trans-splicing, a molecular mechanism relatively rare in insects (Kong et al., 2015), although even examples of trans-spliced neuropeptides were reported in mosquitoes (Robertson et al., 2007). The quality of available genome assemblies for *P. apterus*, *Lygus*, *Bemisia*, and *Locusta* does not allow us to clarify this issue at this point, but, with emerging sequencing technologies one can expect that this task will be unambiguously solved in near future.

Another standing question is dating when the connection of *Proh-4* and *EFLa* originated during the insect evolution. Solving this issue also strongly depends on high quality transcriptomes and genomes. In chelicerates, crustaceans, basal hexapods and basal insects including early winged Ephemeroptera, well conserved *Proh-4* do no overlap with *EFLa*-coding transcripts and these transcripts seem to code all essential prohormone properties, including the signal peptide, thus they are very likely full transcripts (Supplementary Fig. S1). Therefore, the most plausible still provisional estimation dates the connection of *EFLa* with *Proh-4* to the common ancestor of Polyneoptera and Hemiptera, which is approximately 380 Mya according to the most recent phylogenies (Misof et al., 2014; Johnson et al., 2018).

Both our and the concurrent study by Veenstra and Šimo (2020) confirmed that the genes with two or even only one EFLa motif copy are expressed in insects. It will be very interesting to see the expression pattern and level of EFLa in basal hexapods, where genes with as many as 18 paracopies are frequently found. Despite the confirmation and identification of EFLa-positive neurons, the role of the peptide in insects is elusive. Although it might be possible that EFLa has lost entirely its function, it is equally conceivable that the neuropeptide has some specific role that is not obvious under normal rearing conditions.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2020.103376.

References

Almagro Armenteros, J.J., Tsirigos, K.D., Sonderby, C.K., Petersen, T.N., Winther, O., Brunak, S., von Heijne, G., Nielsen, H., 2019. Signalp 5.0 improves signal peptide predictions using deep neural networks. Nat. Biotechnol. 37, 420–423. https://doi.org/10.1038/s41587-019-0036-z.

Bajgar, A., Jindra, M., Dolezel, D., 2013a. Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. Proc. Natl. Acad. Sci. U.S.A. 110, 4416–4421. https://doi.org/10.1073/pnas.1217060110.

Bajgar, A., Dolezel, D., Hodkova, M., 2013b. Endocrine regulation of non-circadian behavior of circadian genes in insect gut. J. Insect Physiol. 59, 881–886. https://doi.org/10.1016/j.jinsphys.2013.06.004.

Bauknecht, P., Jékely, G., 2015. Large-scale combinatorial deorphanization of *Platynereis* neuropeptide GPCRs. Cell Rep. 12, 684–693. https://doi.org/10.1016/j.celrep.2015.06.052.

Conzelmann, M., Williams, E.A., Krug, K., Franz-Wachtel, M., Macek, B., Jékely, G., 2013. The neuropeptide complement of the marine annelid *Platynereis dumerilii*. BMC Genom. 14, 906.

Derst, C., Dircksen, H., Meusemann, K., Zhou, X., Liu, S., Predel, R., 2016. Evolution of neuropeptides in non-pterygote hexapods. BMC Evol. Biol. 16, 51. https://doi.org/10.1186/s12862-016-0621-4.

Dolezel, D., Sauman, I., Kostal, V., Hodkova, M., 2007. Photoperiodic and food signals control expression pattern of the clock gene, period, in the linden bug, *Pyrrhocoris apterus*. J. Biol. Rhythm. 22, 335–342. https://doi.org/10.1177/0748730407303624.

Dolezel, D., Zdechovanova, L., Sauman, I., Hodkova, M., 2008. Endocrine-dependent expression of circadian clock genes in insects. Cell. Mol. Life Sci. 65, 964–969. https://doi.org/10.1007/s00018-008-7506-7.

Douglass, J., Civelli, O., Herbert, E., 1984. Polyprotein gene expression: generation of diversity of neuroendocrine peptides. Annu. Rev. Biochem. 53, 665–715.

Eipper, B.A., Stoffers, D.A., Mains, R.E., 1992. The biosynthesis of neuropeptides: peptide alpha-amidation. Annu. Rev. Neurosci. 15, 57–85.

Elphick, M.R., Mirabeau, O., Larhammar, D., 2018. Evolution of neuropeptide signalling systems. J. Exp. Biol. 221https://doi.org/10.1242/jeb.151092. jeb151092.

Hansen, K.K., Stafflinger, E., Schneider, M., Hauser, F., Cazzamali, G., Williamson, M., Kollmann, M., Schachtner, J., Grimmelikhuijzen, C.J., 2010. Discovery of a novel insect neuropeptide signaling system closely related to the insect adipokinetic hormone and corazonin hormonal systems. J. Biol. Chem. 285, 10736–10747.

Hummon, A.B., Richmond, T.A., Verleyen, P., Baggerman, G., Huybrechts, J., Ewing, M.A., Vierstraete, E., Rodriguez-Zas, S.L., Schoofs, L., Robinson, G.E., Sweedler, J.V., 2006. From the genome to the proteome: uncovering peptides in the *Apis* brain. Science 314, 647–649. https://doi.org/10.1126/science.1124128.

Ibrahim, E.A.S., Hejníková, M., Shaik, H.A., Doležel, D., Kodrík, D., 2017. Adipokinetic hormone activities in insect body infected by entomopathogenic nematode. J. Insect Physiol. 98, 347–355. https://doi.org/10.1016/j.jinsphys.2017.02.009.

Jekely, G., 2013. Global view of the evolution and diversity of metazoan neuropeptide signaling. Proc. Natl. Acad. Sci. U. S. A. 110, 8702–8707. https://doi.org/10.1073/pnas.1221833110.

Johnson, K.P., Dietrich, C.H., Friedrich, F., Beutel, R.G., Wipfler, B., Peters, R.S., Allen, J.M., Petersen, M., Donath, A., Walden, K.K.O., et al., 2018. Phylogenomics and the evolution of hemipteroid insects. Proc. Natl. Acad. Sci. U. S. A. 115, 12775–12780.

Kaniewska, M.M., Vaneckova, H., Dolezel, D., Kotwica-Rolinska, J., 2020. Light and temperature synchronizes locomotor activity in the linden bug, *Pyrrhocoris apterus*. Front. Physiol. https://doi.org/10.3389/fphys.2020.00242. (in press).

Kodrik, D., Socha, R., Simek, P., Zemek, R., Goldsworthy, G.J., 2000. A new member of the AKH/RPCH family that stimulates locomotory activity in the firebug, *Pyrrhocoris apterus* (Heteroptera). Insect Biochem. Mol. Biol. 30, 489–498. https://doi.org/10.1016/S0965-1748(00)00025-4.

Kodrik, D., Simek, P., Lepsa, L., Socha, R., 2002. Identification of the cockroach neuropeptide Pea-CAH-II as a second adipokinetic hormone in the firebug *Pyrrhocoris apterus*. Peptides 23, 585–587. https://doi.org/10.1016/S0196-9781(01)00627-1.

Kong, Y., Zhou, H., Yu, Y., Chen, L., Hao, P., Li, X., 2015. The evolutionary landscape of intergenic trans-splicing events in insects. Nat. Commun. 6, 8734. https://doi.org/10.1038/ncomms9734.

Konopova, B., Smykal, V., Jindra, M., 2011. Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. PloS One 6 (12). https://doi.org/10.1371/journal.pone.0028728.

Kotwica-Rolinska, J., Pivarciova, L., Vaneckova, H., Dolezel, D., 2017. The role of circadian clock genes in the photoperiodic timer of the linden bug, *Pyrrhocoris apterus*, during the nymphal stage. Physiol. Entomol. 42, 266–273. https://doi.org/10.1111/phen.12197.

Kotwica-Rolinska, J., Chodakova, L., Chvalova, D., Kristofova, L., Fenclova, I., Provaznik, J., Bertolutti, M., Wu, B.C., Dolezel, D., 2019. CRISPR/Cas9 Genome editing introduction and optimization in the non-model insect *Pyrrhocoris apterus*. Front. Physiol. 10, 891. https://doi.org/10.3389/fphys.2019.00891.

Liessem, S., Ragionieri, L., Neupert, S., Buschges, A., Predel, R., 2018. Transcriptomic and neuropeptidomic analysis of the stick insect, *Carausius morosus*. J. Proteome Res. 6, 2192–2204. https://doi.org/10.1021/acs.jproteome.8b00155.

Mirabeau, O., Joly, J.S., 2013. Molecular evolution of peptidergic signaling systems in 418 bilaterians. Proc. Natl. Acad. Sci. U.S.A. 110, E2028–E2037. https://doi.org/10.1073/pnas.1219956110.

Misof, B., et al., 2014. Phylogenomics resolves the timing and pattern of insect evolution. Science 346, 763–767. https://doi.org/10.1126/science.1257570.

Pivarciova, L., Vaneckova, H., Provaznik, J., Wu, B.C., Pivarci, M., Peckova, O., Bazalova, O., Cada, S., Kment, P., Kotwica-Rolinska, J., Dolezel, D., 2016. Unexpected geographic variability of the free running period in the linden bug, *Pyrrhocoris apterus*. J.

Biol. Rhythm. 31, 568–576. https://doi.org/10.1177/0748730416671213.
Predel, R., Neupert, S., Derst, C., Reinhardt, K., Wegener, C., 2018. Neuropeptidomics of the bed bug *Cimex lectularius*. J. Proteome Res. 17, 440–454. https://doi.org/10.1021/acs.jproteome.7b00630.
Robertson, H.M., Navik, J.A., Walden, K.K., Honegger, H.W., 2007. The bursicon gene in mosquitoes: an unusual example of mRNA trans-splicing. Genetics 176, 1351–1353. https://doi.org/10.1534/genetics.107.070938.
Schmid, B., Helfrich-Forster, C., Yoshii, T., 2011. A new ImageJ plug-in "ActogramJ" for chronobiological analyses. J. Biol. Rhythm. 26, 464–467. https://doi.org/10.1177/0748730411414264.
Slama, K., Williams, C.M., 1966. Paper factor as an inhibitor of embryonic development of european bug Pyrrhocoris *apterus*. Nature 210, 329–330. https://doi.org/10.1038/210329a0.
Smykal, V., Bajgar, A., Provaznik, J., Fexova, S., Buricova, M., Takaki, K., Hodkova, M., Jindra, M., Dolezel, D., 2014. Juvenile hormone signaling during reproduction and development of the linden bug, *Pyrrhocoris apterus*. Insect Biochem. Mol. Biol. 45https://doi.org/10.1016/j.ibmb.2013.12.003. 69e76.
Smýkal, V., Pivarči, M., Provazník, J., Bazalová, O., Jedlička, P., Lukšan, O., Horák, A., Vaněčková, H., Beneš, V., Fiala, I., Hanus, R., Doležel, D., 2020. Complex evolution of insect insulin receptors and homologous decoy receptors, and functional significance of their multiplicity. Mol. Biol. Evol. https://doi.org/10.1093/molbev/msaa048. (in press).
Socha, R., 1993. Pyrrhocoris apterus (Heteroptera) - an experimental model species - a review. Eur. J. Entomol. 90, 241–286.
Southey, B.R., Amare, A., Zimmerman, T.A., Rodriguez-Zas, S.L., Sweedler, J.V., 2006. Neuropred: a tool to predict cleavage sites in neuropeptide precursors and provide the masses of the resulting peptides. Nucleic Acids Res. 34, W267–W272. https://doi.org/10.1093/nar/gkl161.
Tanaka, Y., Suetsugu, Y., Yamamoto, K., Noda, H., Shinoda, T., 2014. Transcriptome analysis of 460 neuropeptides and G-protein coupled receptors (GPCRs) for neuropeptides in the brown planthopper *Nilaparvata lugens*. Peptides 53, 125–133. https://doi.org/10.1016/j.peptides.2013.07.027.
Van Sinay, E., Mirabeau, O., Depuydt, G., Van Hiel, M.B., Peymen, K., Watteyne, J., Zels, S., Schoofs, L., Beets, I., 2017. Evolutionarily conserved TRH neuropeptide pathway regulates growth in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. U.S.A. 114, E4065–E4074. https://doi.org/10.1073/pnas.1617392114.
Veenstra, J.A., 2000. Mono- and dibasic proteolytic cleavage sites in insect neuroendocrine peptide precursors. Arch. Insect Biochem. Physiol. 43, 49–63. https://doi.org/10.1002/(SICI)1520-6327(200002)43:2<49::AID-ARCH1>3.0.CO;2-M.
Veenstra, J.A., 2010. Neurohormones and neuropeptides encoded by the genome of *Lottia gigantea*, with reference to other mollusks and insects. Gen. Comp. Endocrinol. 167, 86–103.
Veenstra, J.A., 2011. Neuropeptide evolution: neurohormones and neuropeptides predicted from the genomes of *Capitella teleta* and *Helobdella robusta*. Gen. Comp. Endocrinol. 171, 160–175.
Veenstra, J.A., 2014. The contribution of the genomes of a termite and a locust to our understanding of insect neuropeptides and neurohormones. Front. Physiol. 5, 454.
Veenstra, J.A., 2016. Similarities between decapod and insect neuropeptidomes. PeerJ 4, e2043. https://doi.org/10.7717/peerj.2043.
Veenstra, J.A., 2019. Two Lys-vasopressin-like peptides, EFLamide, and other phasmid neuropeptides. Gen. Comp. Endocrinol. 278, 3–11. https://doi.org/10.1016/j.ygcen.2018.04.027.
Veenstra, J.A., Šimo, L., 2020. The TRH-ortholog EFLamide in the migratory locust. Insect Biochem. Mol. Biol. 116. https://doi.org/10.1016/j.ibmb.2019.103281.
Veenstra, J.A., Rombauts, S., Grbić, M., 2012. In silico cloning of genes encoding neuropeptides, neurohormones and their putative G-protein coupled receptors in a spider mite. Insect Biochem. Mol. Biol. 42, 277–295. https://doi.org/10.1016/j.ibmb.2011.12.009.
Wipfler, B., Letsch, H., Frandsen, P.B., Kapli, P., Mayer, C., Bartel, D., Buckley, T.R., Donath, A., Edgerly-Rooks, J.S., Fujita, M., et al., 2019. Evolutionary history of Polyneoptera and its implications for our understanding of early winged insects. Proc. Natl. Acad. Sci. U. S. A. 116, 3024–3029. https://doi.org/10.1073/pnas.1817794116.

Title: **Functional analysis and localization of a thyrotropin-releasing hormone-type neuropeptide (EFLa) in hemipteran insects**

Short title: EFLa in Hemiptera

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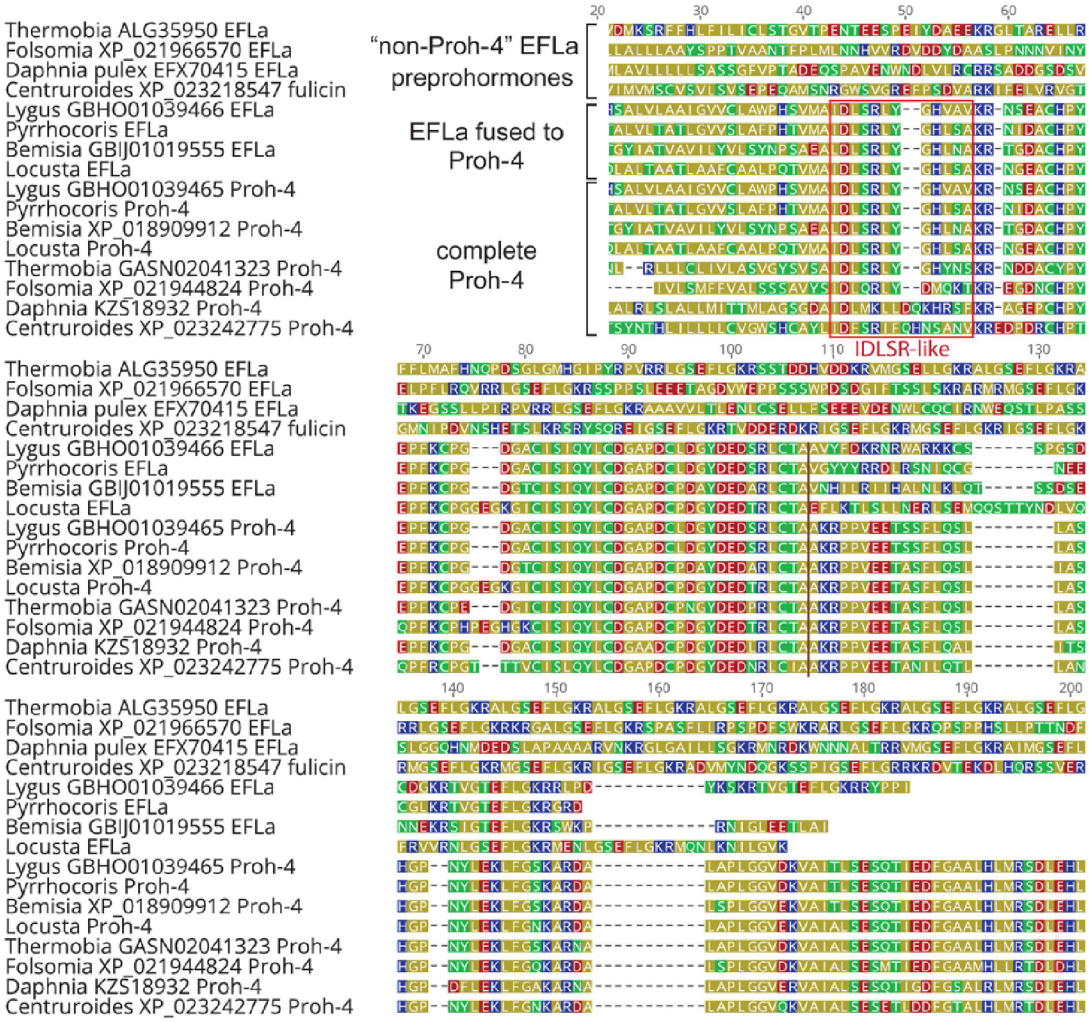

Fig. S1. Protein alignment of Proh-4 preprohormones, EFLa fused to Proh-4 preprohormones and EFLa preprohormones that do not show similarity to Proh-4. The IDLSRF-like motif is highlighted by red box and the brown line indicates the end of identities between Proh-4 and EFLa fused to Proh-4 sequences.

EFLa and Prohormone-4 protein sequences:

>Lygus GBHO01039466 EFLa
MSCSHSALVLAAIGVVCLAWPHSVMAIDLSRLYGHVAVKRNSEACHPYEPFKCPGDGACISIQYLCDGAPD
CLDGYDEDSRLCTAAVYFDKRNRWARKKCSSPGSDCDGKRTVGTEFLGKRRLPDYKSKRTVGTEFLGKR
RYPPI

>Pyrrhocoris EFLa
MSSMSPAQTALVLTATLGVVSLAFPHTVMAIDLSRLYGHLSAKRNIDACHPYEPFKCPGDGACISIQYLCD
GAPDCLDGYDEDSRLCTAVGYYYRRDLRSNIQCGNEECGLKRTVGTEFLGKRGRD

>Bemisia GBIJ01019555 EFLa
MTGYIATVAVILYVLSYNPSAEALDLSRLYGHLNAKRTGDACHPYEPFKCPGDGTCISIQYLCDGAPDCPD
AYDEDARLCTAVNHILRIIHALNLKLQTSSDSENNEKRSIGTEFLGKRSWKPRNIGLEETLAI

>Locusta EFLa
MVRACAQLALTAATLAAFCAALPQTVMAIDLSRLYGHLSAKRNGEACHPYEPFKCPGGEGKGICISIQYLC
DGAPDCPDGYDEDTRLCTAEFLKTLSLLNERLSEMQQSTTYNDLVQFRVVRNLGSEFLGKRMENLGSEFL
GKRMQNLKNILGVK

>Thermobia ALG35950 EFLamide
MVDMKSRFFHLFILICLSTGVTPENTEESPEIYDAEEKRGLTARELLRFFLMAFHNQPDSGLGMHGIPYRPV
RRLGSEFLGKRSSTDDHVDDKRVMGSELLGKRALGSEFLGKRALGSEFLGKRALGSEFLGKRALGSEFLGK
RALGSEFLGKRALGSEFLGKRALGSEFLGKRALGSEFLGKRSLGSEFLGKRSDEFDKRVMGSEFLGKRALG
SEFLGKRALGSEFLGKRDSESDYLEKKALGSEFLGKRALGSEFLGKRALGSEFLGKRTPEQEENLENHKEV
VAEPWTFEGSPEHNSTQILLLERKKRFA

>Folsomia XP_021966570 EFLa
MSEPRQAQHLTSSLIASALLALLLAAYSPPTVAANTFPLMLNNHVVRDVDDYDAASLPNNNVINYELPFLR
QVRRLGSEFLGKRSSPPSLEEETAGDVWEPPSSSWPDSDGIFTSSLSKRARMRMGSEFLGKRRLGSEFLGKR
KRGALGSEFLGKRSPASFLLRPSPDFSWKRARLGSEFLGKRQPSPPHSLLPTTNDF

>Daphnia pulex EFX70415 EFLa
MRMEILQHHSACQRMLAVLLLLLSASSGFVPTADEQSPAVENWNDLVLRCRRSADDGSDSVTKEGSSLLPI
RPVRRLGSEFLGKRAAAVVLTLENLCSELLFSEEEVDENWLCQCIRNWEQSTLPASSSLGGQHNMDEDSLA
PAAAARVNKRGLGAILLSGKRMNRDKWNNNALTRRVMGSEFLGKRAIMGSEFLGKRAIMGSEFLGKRGY
NGRSNGLSGPVKI

>Centruroides XP_023218547 fulicin
MSKMLEVTYSRQIVLMAVIMVMSCVSVLSVSEPEQAMSNRGWSVGREFPSDVARKIFELVRVGTGMNIPD
VNSHETSLKRSRYSQREIGSEFLGKRTVDDERDKRIGSEFLGKRMGSEFLGKRIGSEFLGKRMGSEFLGKRM
GSEFLGKRIGSEFLGKRADVMYNDQGKSSPIGSEFLGRRKRDVTEKDLHQRSSVERV

>Lygus GBHO01039465 Proh-4
MSCSHSALVLAAIGVVCLAWPHSVMAIDLSRLYGHVAVKRNSEACHPYEPFKCPGDGACISIQYLCDGAPD
CLDGYDEDSRLCTAAKRPPVEETSSFLQSLLASHGPNYLEKLFGSKARDALAPLGGVDKVAITLSESQTIED
FGAALHLMRSDLEHLRSVFMAVENGDLGMLKSLGIKDSELGDVKFFLEKLVNTGFLD

>Pyrrhocoris apterus Proh-4
MSSMSPAQTALVLTATLGVVSLAFPHTVMAIDLSRLYGHLSAKRNIDACHPYEPFKCPGDGACISIQYLCD
GAPDCLDGYDEDSRLCTAAKRPPVEETSSFLQSLLASHGPNYLEKLFGSKARDALAPLGGVDKVAITLSES
QTIEDFGAALHLMRSDLEHLRSVFMAVENGDLGMLKSLGIKDSELGDVKFFLEKLVNTGFLD

>Bemisia tabaci XP_018909912 Proh-4
MTGYIATVAVILYVLSYNPSAEALDLSRLYGHLNAKRTGDACHPYEPFKCPGDGTCISIQYLCDGAPDCPD
AYDEDARLCTAAKRPPVEETASFLQSLIASHGPNYLEKLFGSKARDALSPLGGVEKVAITLSESQTIEDFGA
ALHLMRSDLEHLRSVFIAVENGDLGMLKSLGIKDSELGDVKFFLEKLVNTGFLD
>Locusta migratoria Proh4
MVRACAQLALTAATLAAFCAALPQTVMAIDLSRLYGHLSAKRNGEACHPYEPFKCPGGEGKGICISIQYLC
DGAPDCPDGYDEDTRLCTAAKRPPVEETASFLQSLLASHGPNYLEKLFGNKARDALAPLGGVEKVAIALSE
SQTIEDFGAALHLMRSDLEHLRSVFMAVENGDLGMLKSLGIKDSELGDVKFFLEKLVNTGFLD

>Thermobia domestica GASN02041323 Proh-4
MAPRSNLRLLLCLIVLASVGYSVSAIDLSRLYGHYNSKRNDDACYPYEPFKCPEDGICISIQYLCDGAPDCP
NGYDEDPRLCTAAKRPPVEETASFLQSLLASHGPNYLEKLFGSKARNALAPLGGVDKVAIALSESQTIEDFG
AALHLMRSDLEHLRSVFMAVENGDLGMLKSLGIKDSELADVKFFLEKLVNTGFLD

>Folsomia candida XP_021944824 Proh-4
MISKMVYCGIVLSMFFVALSSSAVYSIDLQRLYDMQKTKREGDNCHPYQPFKCPHPEGHGKCISIQYLCDG
APDCPDGYDEDTRLCTAAKRPPVEETASFLQSLLASHGPNYLEKLFGQKARDALSPLGGVDKVAIALSESM
TIEDFGAAMHLLRTDLDHLKSVFVAVENGDLGRLKSLGIKDSEIGDVRFFLERLISTGFID

>Daphnia magna KZS18932 Proh-4
MIAVKLGLIDSTSSSSRTHLALRLSLALLMITTMLAGSGDAIDLMKLLDQKHRSFKRAGEPCHPYEPFKCPG
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GVERVAIALSESQTIGDFGSALRLMRSDLEHLRSIFAGIQSGDMTALASLGIKDAELGDVKFFLEKLIKTGFL
D

>Centruroides sculpturatus XP_023242775 Proh-4
MAKSTSYNTHLILLLLCVGWSHCAYLIDFSRIFQHNSANVKREDPDRCHPTQPFRCPGTTTVCISLQYLCDG
APDCPDGYDEDNRLCIAAKRPPVEETANILQTLLANHGPNYLEKLFGNKARDALAPLGGVQKVAIALSESE
TLDDFGTALHLMRTDLEHLRSVLTAVESGDLSLLKALGIRDSELADVKVFLDKLVSTGFMD

>Lopidea amorphae GCXF01014748 EFLa_partial
IAALFYEKRNGRWSRQKCNSPGSDCDGKRTVGTEFLGKRGMPDPKSKRTVGTEFLGKRRLPESFPQSLKSS

>Timema genevievae GFPR01023314 EFLa-pertial
FLQKEDEQLQDEFSHLIYASNDKQTGSTGLRFKWIQLRSTRDLGSEFLGKRSTKDFTRTLRPSNVFGKKHFA
KALSLYRNISKSNIMFSKILYDLCGLNNFSKSENKLKNIFKTLEVTEKRSLGLEFLGKRKPFK

>Occasjapyx japonicus EFLa [GenBank:GAXJ01010969.1]
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>Pedetontus okajimae EFLa
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RRMGSEFLGKRRMGSEFLGKRMMGSEFLGKRMMGSEFLGKRGLGSEFLGKRALGSIDANHSEPKQNEKYI
KQL

>Penaeus vannamei XP_027222476 EFLa
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RAMGSEFLGKRAMGSEFLGKRAMGSEFLGKRAMGSEFLGKRAMGSEFLGKRAMGSEFLGKRFVPGSLDS
ASVDTKRAVGSEFLG

>Tetrodontophora bielanensis GAXI01153395.1 EFLa
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>Tetranychus urticae XP_015790582 EFLa
MIQSVAKHNSVSPSSSVLLNALIFFMSVSMVHSNSPYKSDVTADEMRLTNSNLITDLVQYLKDASTFNERFR
HSQCCPELGYYLNGQESRSASSLPSSSSPSLSALLFSGSSLMPTSSSSSASQPLGLDKRMGSEFLGKRVGSEFL
GKRVGSEFLGKRMGSEFLGRRKRSV

>Ephemera danica AYNC02025439 EFLa
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GKRRMGSEFLGKRRLSSEFLGKRRMGSEFLGKRSMGSEFLGKRDLENNAYENENTNADILEHIFNQSFNDQ
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ATKDSSHRQNNLVRLSGMKPTSSAVHRSKNM

>Orchesella cincta (Collembola) EFLa (from Derst at al., 2016)
…SEFLGKRGMGSEFLGKRGMGSEFLGKRGMGSEFLGKRRMGSEFLGKRRMGSEFLGKRDGDLDSFYPES
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DNDMDLLTSHSGVGKVRDT…

> Homalodisca vitripennis JJNS02005461 isolate HVIT.00 contig_5461
SCGSGDRYGDKRTVGTEFLGKR

>Ladona fulva APVN02023242.1 contig_23242, whole genome shotgun
QQREKGLVKWGKDASTAMQPQTAVLYQKKYLGSEFLGKRNLGSEFLGKR

EFLa-Receptor protein sequences

>Ladona fulva (Veenstra and Simo 2020) deduced from (genome KZ308327.1 join (3063995..3064244,
3078489..3078674,3096652..3096904,3101217..3101370,3206328..3206488,3208169..3208364)
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LYSCPWLGLTETKPLKYRGHPEVRFCDFKRPRQEYLAYFFADLVVFYIIPLLLSCVLYILIARVLFARRVVK
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TLMCGKKSELWGLAYGRSQPPHSSCTLTRQSTQVQRNAPLKTECK

>Bemisia tabaci XP_018902557 TRHR
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VLMGRTLLNTHRPSNSEHRKSSSSSRAQVVKMLAVIVALFATLWLPYRGMLVYNSVASLLNKQKFMDLW
FLLFAKTCVFINSAINPILYNVMSSKFRAAFREFLICSTRSSRNSGQGGSSSTFNLSTIRSFRSSQNSTAIKRTD
DPQIKDLGF

>Diaphorina citri AWT50634 TRHR
MITLNNNVSINDTFDFLYFDHDLNLTDCMANFTDLIDLQELQCNQTIVKEMFSLEYRIIGTIFQSIIFIVGLLG
NIMVVSVVYKIRSMRTPTNCYLVSLSIADLMVLIAAVPNELVSYYQEKYTWLWGNYGCKMSVFLQYLGIN

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VVIVFATLWLPYRLMLVYNSFAAMLSQPKFMDLWFIMFAKTCVFINSAINPILYNAMSTKFRRAFSRFLVC
RSPDYNTSTGRLSTVTSMTSLSGRRKVNINGVLVRRNTEYPIRRNTEF

>Ephemera danica (Veenstra and Simo 2020) KZ497744.1 TRHR
MNTTEVCLDCPSPYNLSNSTNSSEIEDYDLPQYYSYRYRIIGTFFQGIVFLVGVLGNVMVVIVVKRTQSMHS
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HPMKAHALCTVNRAKRITLAVWMFAVLYCSPWLGLTITKPLIYRGEPPARCCDFKLPREEYLAYFFADLIV
FYLIPLLLSCVLYSLIARVLFVVKMLAVVVAVFATLWLPYRGMLVYNSFAVVPEDKFMDLWFLMFAKTCV
FINSAINPILYNALFKFRRAFQRTLTCKRRGRNECAMSTGMHNTTRRTGNIGLSTYSTTHNAGATTLAGNSA
SGF

>Homalodisca vitripennis JJNS02005999.1 EFLaR
LSQGIVFLVGILGNLMVMVVVKRTRSMHSPTNCYLVSLAMADMVVLVASVPNEILSYYLVGNHWLWGDL
GCSLFVFFQNLGINSSSLGLIAFTFERYMAICHPMKAHKVCTQRRAKKIIFSVWVLAVLYCSPWLFLTTTKP
LNYRGFPQARFCDFKRPRDEYLPYYFTDLVVFYLVPLFVSCVLYGLIARTLLNRRVIRAAGKASSTVVLTEA
ASSSRSQVV

>Laodelphax striatellus QKKF02020908.1 EFLaR
MDTSNESTDIYDVDYDYNRTFQTNCSYPLPYYYSFRYRLIGTVFQTIIFTSGCLGNIMVITIVSRCRTMRTPT
NCYLTSLAVADIMVLVASVPLNIASYYVVSEQWLLPDITCSLVIYLQYLGINCSTLSLVAFTVERYIAICHPM
KAHKMCTLHRAKRIILVTWLIAIVYCTPWFTTVGMRSINYRHFSIKKCDFLYNRDVYFRFYFTDLVVFYIFP
LLLTCVLYGLIMRTLMRRSGGIGRCVRKNSVKAEIQSRMQVVKMLALVVALFAILWLPYRGLVVYNSLAS
MFGGALYMQPWFLMFAKTSISAINPILYNAMSTKFRNEFRKLLVCGGSEDTARHNLGSSRCHTASNISRTH
VKVTDLEL

>Locusta migratoria QGT41395 EFLaR
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VCGARSMRSPTNCYLVSLAVADCLVLVASVPNEIASYYLVGNQWLWGDAGCAAFVFSQNLGINASALSL
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AAFAALWLPYRGLLVYNSFATLLSGDKYMDLWFLLFAKTCVFVNSAINPILYNAMSAKFRRAFRRALLRC
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>Nilaparvata lugens_A45 EFLaR
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RYIAICHPMKAHKMCTLHRAKRIILFTWVIAIFYCTPWFTTVGTRSIDYRHFTIKKCDFLYNRDVYFRFYFTD
LIVFYIFPLLLTCVLYGLIMRTLMRRSGGIGRSATVRKNSVKAEIQSRMQVVKMLALVVALFAILWLPYRGL
LVYNSLASMFGGALYMQPWFLMFAKTCIFINSAINPILYNAMSTKFRNEFRKLLVCGGSEDTRHNLGGSSR
YQTASNLSRIHTKVTDLEL

>Orchesella cincta ODM93884 EFLaR
MAKPLETVLYEDPIYFSESYRLIGTTIQGLIFLVGVLGNLLVVFVVARSRSMWCPTNCYLVSLAVADCIVLV
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NQPFNGSPPIIIIGNRRCVELQPNFRTSSRSQVVKMLGFVSALFAVLWLPYRGLLVYNSVAQEKYMNLWYL
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>Rhodnius prolixus GECK01070320 EFLaR
MKAHKICTIGRAKKITVGMWVFAIFYCSPWLFLTETVPVNYRGYPEIHQCIFKRPREEYLVGFFLDLVLFYV
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FMTLFGKSPFMDLWYLMFAKTCVYINSAINPFLYNAMSTKFRRAFHRMLVCKRATRPPVITISTVAGPSLSS
ASSSHRETTTVSVV

>Tetranychus urticae_tetur21g02030 EFLaR
MFMLIFLVTTIWIILSICCCCVQLTKITKESTKMDPFFNTFDNLPLFSNDSLLESLNTTDLANSLLAGCDYVTD
TANRLTDNSSTLLTSSSSSLVSLFSSSLALNSPSSPYFQSSPLSSSSSSSPYPSPLSLSSSSILSSASSSSVLSLSPSS
SISSSSLNSANSYDPATGTASPAVVAAAAGFSPETYCNNLNTTFCKDPSYYAISYRIIGTIFQGFILIIGVLGNI
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SITAFTVERYIAICHPMKAQKVCTVHRAKRIILNVWIFACLYNSPWFFLTKTEPICYRSLEDSNLETCTFAWS
RKYYLGYFFSDLVLFYIFPLLLSCVLYGLMARVLFNNPLSKTMGASTSSSSSTSTIITGITAKSNQANSSSTSN
HSHTNNTHHHHQSSHSTSQTLAQSTHSKKISSQSNDSSRVQVVKMLVVIVAVFATLWLPYRALLVYNSMT
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>Thermobia domestica (Veenstra and Simo 2020) EFLaR
MNKTSNILWNCSEYLTSTEDEFSATNTNLNCTNISVDSVTNNTASLTNQTELPDPEYFSYTYRAIGTFFQGIV
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ETH-Receptor protein sequences

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>Tribolium castaneum NP_001076792 ETHR
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MCLGVIGNVMVPIVIFKTKDMRNSTNIFLVNLSVADLMVLLVCTPTVLVEVNSRPETWVLGREMCKAVPF
VELTVAHASVLTILAISFERYYAICKPLKAGYICTKTRASLICLLAWFIAALFTSPMLVIAEYKQLDYFDGSK

VPACHTLANTFWSALYFLTIIFLFFIIPLIILLILYCIIAKNLMSNAATLVLNKHIDNISLRARKQVVLMLGTVV
LSFFLCLIPFRVFILWIILVPEEQVYHLEIEKYYNILYFCRIMVYLNSAINPILYNLMSSKFRTGFIICSESNRKF
YFKRARNGTFSTTANSCRSSTFRNNRDEYNVCFRPRNNSILIKNFNDSPESNRSNEIDGPEK

>Zootermopsis nevadensis XP_021916331 ETHR
KWIIARNCDRGWRYVFVSEASTRATTVTIATAYAGGLEAAGNSTITAAYLYPNFSGTEGVGNETTGTEIPVI
PAYIRITSMIFCFVILGIGVAGNIMVPIVILKTKDMRNSTNIFLMNLSVADLMVLLVCTPTVLVEVNTKPETW
VLGEEMCKIVPFVELTVAHASVLTILAISFERYYAICEPLKASYVCTKARALIICLLAWLFAALFTSPILLIAQ
YDPFREYLDGSLVPACLTEVNTFWSTFFFIMIISLFFLVPLLVLIVLYAAMARHLMADPVTSAVKDLESTNTR
ARKQVVLMLGTVVLSFFICLIPFRVFTVWFIFVSAEQVHSLGVEGYYNILYFCRTMLYLNSAVNPILYNLMS
SKFRNGFMRVCGLRRRDAVLLRRGTTSTSAYSSTRRCNSFGMTHRSSTEFSWRSASRYSVDSTQSSSPRSER
RLRSTSFRRSVMIRTSILKRNGSCIQDDVMNCIEMRPCPESFILGHLRRYYPILGVADYHHELDLDGSTVAVC
FTQADTFWTAFFFILSIVIFFVVPFVILLVLYTIIARHLMTHTGIIAPPCRYGSYTSGTQHTALRYRKQVVVML
GTVVLSFFLCLMPFRAFTLWIIVAPEEAVFSLDIEEYYNLLYFCRVMTYINSAINPILYNVMSSKFRDGFTRL
CGLTSRNRAEVYLGRKGTLNTVSTTTTTNCSSGLQDSIWRRSVKMTLPESRTFKRTQSTHCGDVKGINNAT
CILLRSKSHLVCSTRNNVVRTGDEDGRRGHEESYV

>Pyrrhocoris apterus ETHR
MIEAITNDTSAAAEAGGGGGNSSYSGGLLFPPYVRTVYILLCVLILGIGIVGNVMVPLVIVKTKDMRNSTNIF
LMNLSIADLMVLLVCTPTVLVEVNSAPLIWVLGEEMCKAVPFVELTVAHASVLTILAISFERYYAICEPLRA
GYVCTKTRAMLICLLAWAFAALFTSPVLIVADYREEEYIDGTNVTTCLMDANDFWPSFYFTASITVFFCLPL
AILVGVYFVIARHLMSNPGLVAPNSHSSALRYRRQVVLMLGTVVLSFFICLLPFKAFTLYVIYASPEFIVSMG
LQTFYNILYICRIMLYLNSAVNPILYNLMSSKFRDGFRKLLGIGNHDKNLKRKGTVTTTTLTSSTRTNSSEGG
LLRKSVVRVISLEDSGKFDNSLTTTIKILNNSKSDESYV

>Pyrrhocoris apterus ETHRb
MIEAITNDTSAAAEAGGGGGNSSYSGGLLFPPYVRTVYILLCVLILGIGIVGNVMVPLVIVKTKDMRNSTNIF
LMNLSIADLMVLLVCTPTVLVEVNSAPLIWVLGEEMCKAVPFVELTVAHASVLTILAISFERYYAICEPLRA
GYVCTKTRAMLICLLAWAFAALFTSPITVIAEYSMASNNGTLVPACFTNSDTTWRKVFFYSIITIFFILPLSILI
VLYTVIALHLIRDPGTVNSGDGVNLRARRQVVMMLATVVLSFFLCLLPFRVFMVWALIAPNDLVALGEQP
YLLLLYFCRLLHYLNSAINPILYNLMSSKFRQGFGRLVGFRRKRHLLLLRHRATFSSTFSQSSTRFQRNSPDL
SWRGASFDSRHRNGSIRRSVILKSSVLQSKKSEPITQPESNV

Discussion and summary

This thesis is devoted to the regulation of reproductive diapause, with the main focus on males of the linden bug, *P. apterus*. Perhaps because of a clearly recognizable phenotype – cessation of oviposition, which is considered a fundamental sign of reproductive diapause – attention is given primarily to females. In contrast, little is known about the reproductive diapause of males. In fact, it can be inferred that the mechanism of diapause may differ to some extent between the sexes. This could be due to the enormous energy requirements that females must expend for egg production.

The first chapter is therefore dedicated to the basic parameters of diapause, i.e., the production of energy reserves in the form of hexameric proteins and the cessation of JH synthesis, and the associated shutdown of reproduction. The second chapter deals in more detail with the effect of the JH in connection with photoperiod and low temperature as a modulator of diapause. Finally, the third chapter provides the first analysis of a very promising neuropeptide – EFLa – with potential regulatory function in reproductive diapause. Unfortunately, any biological effects of EFLa have not been discovered yet, despite the complete null mutants we have created.

Allatectomy, surgical removal of CA, and RNAi down-regulation of *Met* or *Tai* reduce the amount of total male's ACPs. This confirms the findings of another study in which male accessory gland growth is inhibited by knocking down the JH receptor (Urbanová et al., 2016). These results suggest that JH acts through the Met-Tai complex in male accessory glands. Similarly, in the linden bugs females, it was shown that both Met and Tai are crucial for ovarian development (Smykal, Bajgar, et al., 2014). Three decades earlier, Socha demonstrated increased levels of hexamerin storage proteins in the hemolymph of allatectomized females (Socha et al., 1991). Here, in males, the level of hexamerins, as well as their expression in the fat body increases significantly after both allatectomy and silencing of *Met* or *Tai*. Allatectomy suppresses mating behavior in the blow fly *Phormia regina* by more than twofold in both sexes and reduces the fertility of males (Yin et al., 1999). After topical application of JH or its analog methoprene, males of the Caribbean fruit fly, *Anastrepha suspensa*, start mating significantly earlier than controls (Teal et al., 2000). Moreover, in males of the desert locust, *Schistocerca gregaria*, lack of JH or Met or Tai completely disrupt mating and reduces relative testes weight (Holtof et al., 2021). We did not observe any effects of the JH signaling pathway on the mating activity of reproductively active *P.*

apterus males under long-day conditions, but topical application of a JH analog to diapausing males under short-day conditions helps them to restore their mating activity. We speculated that females may disadvantage allatectomized males in mate choice. However, females select their mating partner more or less randomly, and males's allatectomy does not affect this choice (Hejnikova, unpublished). It is likely that spermatogenesis in adult males is JH-independent and low ACPs levels do not affect fertilization. Males transferred from short-day to long-day conditions are able to initiate reproduction within a week, or even slightly earlier in allatectomized males, which is earlier than JH production increase in CA. It remains unclear what role JH and accessory glands play in adult male reproduction. In some insect species, the contents of accessory glands, including JH, have been shown to be transported into the female during mating (Herndon et al., 1997; Shirk et al., 1980; South & Lewis, 2011) and modulate behavioral and physiological changes in the female. These changes include loss of mating receptivity, stimulation of oogenesis and oviposition, increased feeding and sleeping activity, induction of immune responses, and decrease lifespan, as was well documented in the fruit fly, *Drosophila melanogaster* or mosquito *Anopheles gambiae* (Baldini et al., 2012; Chapman & Davies, 2004; Ram & Wolfner, 2007). It is also worth considering that possibly larval development or the next generation of adults may be affected by a lack of ACPs or JH.

Although *P. apterus* shows a robust response to photoperiod (Hodek, 1971; Hodek & Hodkova, 1986; Hodkova, 1994; Numata et al., 1993; Saunders, 1987; Syrová et al., 2003; Urbanová et al., 2016), temperature appears to be a fundamental modulator of diapause intensity. The importance of the effect of temperature on diapause was noted earlier in females, in which the intensity of diapause was higher after thermoperiod treatment than at constant temperature (Kalushkov et al., 2001). In general, the longer the animals were exposed to low temperature, the faster the diapause termination was observed. However, a sexual dimorphism was evident in this case. After only one week at the low temperature, all females remained in diapause, whereas males started mating seven days after the temperature rise, at the same time, they do not need a presence of JH or its receptor, although some impact of Met silencing on the speed of diapause termination was observed. Surprisingly, reproductive males transferred from long-day to short-day photoperiod at a constant temperature, do not stop reproducing but, on the contrary, are willing to mate basically for the

rest of their lives. This can be explained by the existing selection pressure. The males' sole task is to fertilize the females and pass on their genes to the next generation. So, keeping the reproductive status after transfer to short day conditions as adults, they possibly maximize the chances of fertilizing the females. Once the female is receptive, males must be ready to copulate, otherwise, they risk losing the competition among other males. Perhaps, the mere presence of a receptive female may be the decisive stimulus to not undergo diapause. This theory is supported by a study in which in the constant presence of active females, males transferred to short-day remained active longer than males kept with diapausing females (Hodkova et al., 1991).

P. apterus males appear to have JH-independent diapause induction and maintenance. However, it would be interesting to prove unequivocally whether JH is synthesized in accessory glands. Indeed, we detected JH in the hemolymph and accessory glands, and all the necessary enzymes of the JH synthetic pathway in the accessory glands of allatectomized males. Synthesis of JH by accessory glands has already been demonstrated *de novo* in the mosquito *Aedes aegypti* (Borovsky et al., 1994). However, long-term storage of JH cannot be excluded. A direct influence on the male diapause by some circadian clock genes (especially *Clock*, *cycle,* and *pigment-dispersing factor*, *pdf*) is also possible. It has been already shown that *Clock* genes silenced by RNAi prevent diapausing males from switching to reproductive mode after transfer from short-day to long-day photoperiod (Urbanová et al., 2016). The RNA interference-mediated knockdown of the *Clock* and *cycle* during the nymphal stage led to reproductive diapause in adult females (Kotwica-Rolinska et al., 2017), further adult *pdf* null mutant females are reproductive under diapause triggering conditions (Kotwica-Rolinska et al., 2022). Whether males would show the same phenotype remains a question. However, the newly established CRISPR/Cas9 tool for our non-model insect *P.apterus* (Kotwica-Rolinska et al., 2019) will hopefully help us uncover more details about male diapause in the future.

Bibliography

Adams, T. S., Filipi, P. A., & Yi, S. X. (2002). Effect of age, diet, diapause and juvenile hormone on oogenesis and the amount of vitellogenin and vitellin in the twospotted stink bug, Perillus bioculatus (Heteroptera: pentatomidae). *Journal of Insect Physiology*, *48*(4). https://doi.org/10.1016/S0022-1910(02)00069-0

Ashok, M., Turner, C., & Wilson, T. G. (1998). Insect juvenile hormone resistance gene homology with the bHLH-PAS family of transcriptional regulators. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(6). https://doi.org/10.1073/pnas.95.6.2761

Bajgar, A., Jindra, M., & Dolezel, D. (2013). Autonomous regulation of the insect gut by circadian genes acting downstream of juvenile hormone signaling. *Proceedings of the National Academy of Sciences of the United States of America*, *110*(11), 4416–4421. https://doi.org/10.1073/pnas.1217060110

Baldini, F., Gabrieli, P., Rogers, D. W., & Catteruccia, F. (2012). Function and composition of male accessory gland secretions in Anopheles gambiae: A comparison with other insect vectors of infectious diseases. In *Pathogens and Global Health* (Vol. 106, Issue 2, pp. 82–93). https://doi.org/10.1179/2047773212Y.0000000016

Bauknecht, P., & Jékely, G. (2015). Large-scale combinatorial deorphanization of platynereis neuropeptide GPCRs. *Cell Reports*, *12*(4). https://doi.org/10.1016/j.celrep.2015.06.052

Beer, K., Kolbe, E., Kahana, N. B., Yayon, N., Weiss, R., Menegazzi, P., Bloch, G., & Helfrich-Förster, C. (2018). Pigment-Dispersing Factor-expressing neurons convey circadian information in the honey bee brain. *Open Biology*, *8*(1). https://doi.org/10.1098/rsob.170224

Bell, C. H., & Bowley, C. R. (1980). Effect of photoperiod and temperature on diapause in a Florida strain of the tropical warehouse moth Ephestia cautella. *Journal of Insect Physiology*, *26*(8). https://doi.org/10.1016/0022-1910(80)90128-6

Bell, R. A., Kelly, T. J., Masler, E. P., Thyagaraja, B. S., DeMilo, A. B., & Borkovec, A. B. (1990). Endocrinology of Embryogenesis and Late Embryonic Diapause in the Gypsy Moth, Lymantria Dispar. In *Insect Neurochemistry and Neurophysiology · 1989 ·*. https://doi.org/10.1007/978-1-4612-4512-4_39

Belles, X. (2020). Hormones involved in the regulation of metamorphosis. In *Insect Metamorphosis*. https://doi.org/10.1016/b978-0-12-813020-9.00006-5

Ben-Shlomo, A., & Melmed, S. (2011). Hypothalamic Regulation of Anterior Pituitary Function. In *The Pituitary* (pp. 21–45). Elsevier Inc. https://doi.org/10.1016/B978-0-12-380926-1.10002-1

Blazkova, H., Provaznik, J., & Hodkova, M. (2011). Novel roles for the corpus allatum hormone in the cost of sexual interactions in the linden bug Pyrrhocoris apterus. *Journal of Insect Physiology*, *57*(4), 529–535. https://doi.org/10.1016/j.jinsphys.2011.02.007

Borovsky, D., Carlson, D. A., Hancock, R. G., Rembold, H., & van Handel, E. (1994). De novo biosynthesis of juvenile hormone III and I by the accessory glands of the male mosquito. *Insect Biochemistry and Molecular Biology*, *24*(5). https://doi.org/10.1016/0965-1748(94)90038-8

BRIERS, T., PEFEROEN, M., & LOOF, A. de. (1982). Ecdysteroids and adult diapause in the Colorado potato beetle, Leptinotarsa decemlineata. *Physiological Entomology*, *7*(4). https://doi.org/10.1111/j.1365-3032.1982.tb00313.x

Buszczak, M., Freeman, M. R., Carlson, J. R., Bender, M., Cooley, L., & Segraves, W. A. (1999). Ecdysone response genes govern egg chamber development during mid-oogenesis in Drosophila. *Development*, *126*(20). https://doi.org/10.1242/dev.126.20.4581

Chapman, T., & Davies, S. J. (2004). Functions and analysis of the seminal fluid proteins of male Drosophila melanogaster fruit flies. In *Peptides* (Vol. 25, Issue 9). https://doi.org/10.1016/j.peptides.2003.10.023

Charles, J. P., Iwema, T., Epa, V. C., Takaki, K., Rynes, J., & Jindra, M. (2011). Ligand-binding properties of a juvenile hormone receptor, Methoprene-tolerant. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(52). https://doi.org/10.1073/pnas.1116123109

Clifton, M. E., Correa, S., Rivera-Perez, C., Nouzova, M., & Noriega, F. G. (2014). Male Aedes aegypti mosquitoes use JH III transferred during copulation to influence previtellogenic ovary physiology and affect the reproductive output of female mosquitoes. *Journal of Insect Physiology*, *64*(1), 40–47. https://doi.org/10.1016/j.jinsphys.2014.03.006

Danks, H. v. (2002). The range of insect dormancy responses. *European Journal of Entomology*, *99*(2). https://doi.org/10.14411/eje.2002.021

Denis, B., Claisse, G., le Rouzic, A., Wicker-Thomas, C., Lepennetier, G., & Joly, D. (2017). Male accessory gland proteins affect differentially female sexual

receptivity and remating in closely related Drosophila species. *Journal of Insect Physiology*, *99*. https://doi.org/10.1016/j.jinsphys.2017.03.008

Denlinger, D. L. (1986). Dormancy in Tropical Insects. *Annual Review of Entomology*, *31*(1). https://doi.org/10.1146/annurev.en.31.010186.001323

Denlinger, D. L. (2000). Molecular regulation of insect diapause. In *Cell and Molecular Response to Stress* (Vol. 1, Issue C, pp. 259–275). Elsevier. https://doi.org/10.1016/S1568-1254(00)80020-0

Denlinger, D. L., & Armbruster, P. A. (2014). Mosquito diapause. *Annual Review of Entomology*, *59*, 73–93. https://doi.org/10.1146/annurev-ento-011613-162023

Denlinger, D. L., Yocum, G. D., & Rinehart, J. P. (2012). Hormonal Control of Diapause. In *Insect Endocrinology* (pp. 430–463). Elsevier. https://doi.org/10.1016/B978-0-12-384749-2.10010-X

Dinan, L. (1997). Ecdysteroids in Adults and Eggs of the House Cricket, Acheta domesticus (Orthoptera: Gryllidae). In *Biochem. Physiol* (Vol. 116, Issue 2).

Dolezel, D. (2015). Photoperiodic time measurement in insects. In *Current Opinion in Insect Science* (Vol. 7, pp. 98–103). Elsevier Inc. https://doi.org/10.1016/j.cois.2014.12.002

Emerson, K. J., Dake, S. J., Bradshaw, W. E., & Holzapfel, C. M. (2009). Evolution of photoperiodic time measurement is independent of the circadian clock in the pitcher-plant mosquito, Wyeomyia smithii. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology*, *195*(4), 385–391. https://doi.org/10.1007/s00359-009-0416-9

Fekete, C., & Lechan, R. M. (2014). Central regulation of hypothalamic-pituitary-thyroid axis under physiological and pathophysiological conditions. In *Endocrine Reviews* (Vol. 35, Issue 2, pp. 159–194). Endocrine Society. https://doi.org/10.1210/er.2013-1087

Fielding, D. (2006). Optimal diapause strategies of a grasshopper, Melanoplus sanguinipes. *Journal of Insect Science*, *6*(2), 1. http://creativecommons.org/licenses/by/2.5

Galas, L., Raoult, E., Tonon, M. C., Okada, R., Jenks, B. G., Castaño, J. P., Kikuyama, S., Malagon, M., Roubos, E. W., & Vaudry, H. (2009). TRH acts as a multifunctional hypophysiotropic factor in vertebrates. In *General and*

Comparative Endocrinology (Vol. 164, Issue 1, pp. 40–50). Academic Press Inc. https://doi.org/10.1016/j.ygcen.2009.05.003

Gillott, C., & Ismail, P. M. (1995). In vitro synthesis of ecdysteroid by the male accessory reproductive glands, testis and abdominal integument of the adult migratory grasshopper, melanoplus sanguinipes. *Invertebrate Reproduction and Development*, *27*(1). https://doi.org/10.1080/07924259.1995.9672435

Gomi, T. (1997). Geographic variation in critical photoperiod for diapause induction and its temperature dependence in Hyphantria cunea Drury (Lepidoptera: Arctiidae). *Oecologia*, *111*(2). https://doi.org/10.1007/s004420050220

Goodman, W. G., & Cusson, M. (2012). The Juvenile Hormones. In *Insect Endocrinology* (pp. 310–365). Elsevier. https://doi.org/10.1016/B978-0-12-384749-2.10008-1

Goto, S. G. (2013). Roles of circadian clock genes in insect photoperiodism. In *Entomological Science* (Vol. 16, Issue 1, pp. 1–16). https://doi.org/10.1111/ens.12000

Goto, S. G. (2022). Photoperiodic time measurement, photoreception, and circadian clocks in insect photoperiodism. *Applied Entomology and Zoology*. https://doi.org/10.1007/s13355-022-00785-7

Goto, S. G., & Denlinger, D. L. (2002). Short-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: period, timeless, cycle and cryptochrome. *Journal of Insect Physiology*, *48*(8). https://doi.org/10.1016/S0022-1910(02)00108-7

Goto, S. G., & Matsumoto, K. (2018). Photoperiodism, insects. In *Encyclopedia of Reproduction* (pp. 420–425). Elsevier. https://doi.org/10.1016/B978-0-12-809633-8.20587-6

Guo, S., Tian, Z., Wu, Q. W., King-Jones, K., Liu, W., Zhu, F., & Wang, X. P. (2021). Steroid hormone ecdysone deficiency stimulates preparation for photoperiodic reproductive diapause. *PLoS Genetics*, *17*(2). https://doi.org/10.1371/JOURNAL.PGEN.1009352

Hamanaka, Y., Yasuyama, K., Numata, H., & Shiga, S. (2005). Synaptic connections between pigment-dispersing factor-immunoreactive neurons and neurons in the pars lateralis of the blow fly Protophormia terraenovae. *Journal of*

Comparative Neurology, *491*(4), 390–399. https://doi.org/10.1002/cne.20712

Harano, K.-I. (2013). Effects of juvenile hormone analog on physiological and behavioral maturation in honeybee drones. *Apidologie*, *44*(5). https://doi.org/10.1007/s13592-013-0208-7ï

Hasebe, M., Kotaki, T., & Shiga, S. (2022). Pigment-dispersing factor is involved in photoperiodic control of reproduction in the brown-winged green bug, Plautia stali. *Journal of Insect Physiology*, *137*. https://doi.org/10.1016/j.jinsphys.2022.104359

Hentze, J. L., Moeller, M. E., Jørgensen, A. F., Bengtsson, M. S., Bordoy, A. M., Warren, J. T., Gilbert, L. I., Andersen, O., & Rewitz, K. F. (2013). Accessory Gland as a Site for Prothoracicotropic Hormone Controlled Ecdysone Synthesis in Adult Male Insects. *PLoS ONE*, *8*(2). https://doi.org/10.1371/journal.pone.0055131

Herndon, L. A., Chapman, T., Kalb, J. M., Lewin, S., Partridge, L., & Wolfner, M. F. (1997). Mating and hormonal triggers regulate accessory gland gene expression in male Drosophila. *Journal of Insect Physiology*, *43*(12). https://doi.org/10.1016/S0022-1910(97)00062-0

Hodek, I. (1971). Sensitivity of larvae to photoperiods controlling the adult diapause of two insects. *Journal of Insect Physiology*, *17*(2). https://doi.org/10.1016/0022-1910(71)90205-8

Hodek, I., & Hodkova, M. (1986). Diapause development and photoperiodic activation in starving females of Pyrrhocoris apterus (Heteroptera). *Journal of Insect Physiology*, *32*(7). https://doi.org/10.1016/0022-1910(86)90090-9

Hodková, M. (1976). Nervous inhibition of corpora allata by photoperiod in Pyrrhocoris apterus. *Nature*, *263*(5577). https://doi.org/10.1038/263521a0

Hodkova, M. (1994). Photoperiodic regulation of mating behaviour in the linden bug, Pyrrhocoris apterus, is mediated by a brain inhibitory factor. *Experientia*, *50*(8). https://doi.org/10.1007/BF01919375

Hodkova, M. (2015). Why is the number of days required for induction of adult diapause in the linden bug Pyrrhocoris apterus fewer in the larval than in the adult stage? *Journal of Insect Physiology*, *77*, 39–44. https://doi.org/10.1016/j.jinsphys.2015.04.001

Hodkova, M., & Okuda, T. (2019). Three kinds of regulatory signals for production of juvenile hormone in females of the linden bug, Pyrrhocoris apterus. *Journal of Insect Physiology*, *113*, 17–23. https://doi.org/10.1016/j.jinsphys.2019.01.002

Hodkova, M., Zieglerova, J., & Hodek, I. (1991). Diapause in males of Pyrrhocoris apterus (L.) (Heteroptera) and its dependence on photoperiod and the activity of females. *Zoologische Jahrbucher: Abteilung Fur Systematik, Okologie Und Geographie Der Tiere*, *118*(3–4).

Holtof, M., van Lommel, J., Gijbels, M., Dekempeneer, E., Nicolai, B., Broeck, J. vanden, & Marchal, E. (2021). Crucial role of juvenile hormone receptor components methoprene-tolerant and taiman in sexual maturation of adult male desert locusts. *Biomolecules*, *11*(2), 1–21. https://doi.org/10.3390/biom11020244

Honêk, A., & Ŝrámková, K. (1976). Behavioral regulation of developmental cycle in Pyrrhocoris apterus L. (Heteroptera: Pyrrhocoridae). *Oecologia*, *24*(3), 277–281. https://doi.org/10.1007/BF00345479

Hong, J. W., & Platt, A. P. (1975). Critical photoperiod and daylength threshold differences between northern and southern populations of the butterfly Limenitis archippus. *Journal of Insect Physiology*, *21*(5). https://doi.org/10.1016/0022-1910(75)90129-8

Ikeno, T., Numata, H., Goto, S. G., & Shiga, S. (2014). Involvement of the brain region containing pigment-dispersing factor-immunoreactive neurons in the photoperiodic response of the bean bug, Riptortus pedestris. *Journal of Experimental Biology*, *217*(3), 453–462. https://doi.org/10.1242/jeb.091801

Jindra, M., Palli, S. R., & Riddiford, L. M. (2013). The juvenile hormone signaling pathway in insect development. In *Annual Review of Entomology* (Vol. 58, pp. 181–204). https://doi.org/10.1146/annurev-ento-120811-153700

Joplin, K. H., Yocum, G. D., & Denlinger, D. L. (1990). Diapause specific proteins expressed by the brain during the pupal diapause of the flesh fly, Sarcophaga crassipalpis. *Journal of Insect Physiology*, *36*(10). https://doi.org/10.1016/0022-1910(90)90051-G

Kalushkov, P., Hodková, M., Nedvěd, O., & Hodek, I. (2001). Effect of thermoperiod on diapause intensity in Pyrrhocoris apterus (Heteroptera Pyrrhocoridae). *Journal of Insect Physiology*, *47*(1). https://doi.org/10.1016/S0022-1910(00)00091-3

Konopova, B., & Jindra, M. (2007). *Juvenile hormone resistance gene Methoprene-tolerant controls entry into metamorphosis in the beetle Tribolium castaneum*. www.pnas.org/cgi/content/full/

Konopova, B., Smykal, V., & Jindra, M. (2011). Common and distinct roles of juvenile hormone signaling genes in metamorphosis of holometabolous and hemimetabolous insects. *PLoS ONE*, *6*(12). https://doi.org/10.1371/journal.pone.0028728

Koštál, V. (2006). Eco-physiological phases of insect diapause. In *Journal of Insect Physiology* (Vol. 52, Issue 2, pp. 113–127). https://doi.org/10.1016/j.jinsphys.2005.09.008

Koštál, V. (2011). Insect photoperiodic calendar and circadian clock: Independence, cooperation, or unity? In *Journal of Insect Physiology* (Vol. 57, Issue 5, pp. 538–556). https://doi.org/10.1016/j.jinsphys.2010.10.006

Košťál, V., & Šimek, P. (2000). Overwintering strategy in Pyrrhocoris apterus (Heteroptera): The relations between life-cycle, chill tolerance and physiological adjustments. *Journal of Insect Physiology*, *46*(9). https://doi.org/10.1016/S0022-1910(00)00056-1

Koštál, V., Stetina, T., Poupardin, R., Korbelová, J., & Bruce, A. W. (2017). Conceptual framework of the eco-physiological phases of insect diapause development justified by transcriptomic profiling. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(32), 8532–8537. https://doi.org/10.1073/pnas.1707281114

Kotaki, T., Shinada, T., & Numata, H. (2012). Structure determination of a natural juvenile hormone isolated from a heteropteran insect. In *Psyche (London)*. Hindawi Limited. https://doi.org/10.1155/2012/924256

Kotaki, T., & Yagi, S. (1989). Hormonal Control of Adult Diapause in the Brown-Winged Green Bug, Plautia stali SCOTT (Heteroptera: Pentatomidae). *Applied Entomology and Zoology*, *24*(1). https://doi.org/10.1303/aez.24.42

Kotwica-Rolinska, J., Chodakova, L., Chvalova, D., Kristofova, L., Fenclova, I., Provaznik, J., Bertolutti, M., Wu, B. C. H., & Dolezel, D. (2019). CRISPR/Cas9 genome editing introduction and optimization in the non-model insect Pyrrhocoris apterus. *Frontiers in Physiology*, *10*(JUL). https://doi.org/10.3389/fphys.2019.00891

Kotwica-Rolinska, J., Damulewicz, M., Chodakova, L., Kristofova, L., & Dolezel, D. (2022). Pigment Dispersing Factor Is a Circadian Clock Output and Regulates Photoperiodic Response in the Linden Bug, Pyrrhocoris apterus. *Frontiers in Physiology*, *13*. https://doi.org/10.3389/fphys.2022.884909

Kotwica-Rolinska, J., Pivarciova, L., Vaneckova, H., & Dolezel, D. (2017). The role of circadian clock genes in the photoperiodic timer of the linden bug Pyrrhocoris apterus during the nymphal stage. *Physiological Entomology*, *42*(3), 266–273. https://doi.org/10.1111/phen.12197

Kristenová, M., Exnerová, A., & Štys, P. (2011). Seed preferences of Pyrrhocoris apterus (Heteroptera: Pyrrhocoridae): Are there specialized trophic populations? *European Journal of Entomology*, *108*(4). https://doi.org/10.14411/eje.2011.075

Lafont, R., Dauphin-Villemant, C., & Blais, C. (2003). Ecdysteroids, Overview. In *Encyclopedia of Hormones*. https://doi.org/10.1016/b0-12-341103-3/00046-2

Lee, C. M., Su, M. T., & Lee, H. J. (2009). Pigment dispersing factor: An output regulator of the circadian clock in the German cockroach. *Journal of Biological Rhythms*, *24*(1), 35–43. https://doi.org/10.1177/0748730408327909

Lees, A. D. (1956). The Physiology and Biochemistry of Diapause. *Annual Review of Entomology*, *1*(1). https://doi.org/10.1146/annurev.en.01.010156.000245

Loeb, M. J., Brandt, E. P., & Birnbaum, M. J. (1984). Ecdysteroid production by testes of the tobacco budworm, Heliothis virescens, from last larval instar to adult. *Journal of Insect Physiology*, *30*(5). https://doi.org/10.1016/0022-1910(84)90094-5

MacRae, T. H. (2016). Stress tolerance during diapause and quiescence of the brine shrimp, Artemia. In *Cell Stress and Chaperones* (Vol. 21, Issue 1). https://doi.org/10.1007/s12192-015-0635-7

Martins, J. R., Nunes, F. M. F., Cristino, A. S., Simões, Z. L. P., & Bitondi, M. M. G. (2010). The four hexamerin genes in the honey bee: Structure, molecular evolution and function deduced from expression patterns in queens, workers and drones. *BMC Molecular Biology*, *11*. https://doi.org/10.1186/1471-2199-11-23

Matsumoto, K., Numata, H., & Shiga, S. (2013). Role of the brain in photoperiodic regulation of juvenile hormone biosynthesis in the brown-winged green bug Plautia stali. *Journal of Insect Physiology*, *59*(4), 387–393. https://doi.org/10.1016/j.jinsphys.2013.01.007

Meuti, M. E., & Denlinger, D. L. (2013). Evolutionary links between circadian clocks and photoperiodic diapause in insects. *Integrative and Comparative Biology*, *53*(1), 131–143. https://doi.org/10.1093/icb/ict023

Miura, K., Oda, M., Makita, S., & Chinzei, Y. (2005). Characterization of the Drosophila Methoprene -tolerant gene product. *FEBS Journal*, *272*(5). https://doi.org/10.1111/j.1742-4658.2005.04552.x

Moraiti, C. A., & Papadopoulos, N. T. (2017). Obligate annual and successive facultative diapause establish a bet-hedging strategy of Rhagoletis cerasi (Diptera: Tephritidae) in seasonally unpredictable environments. In *Physiological Entomology* (Vol. 42, Issue 3). https://doi.org/10.1111/phen.12206

Nagy, D., Cusumano, P., Andreatta, G., Anduaga, A. M., Hermann-Luibl, C., Reinhard, N., Gesto, J., Wegener, C., Mazzotta, G., Rosato, E., Kyriacou, C. P., Helfrich-Förster, C., & Costa, R. (2019). Peptidergic signaling from clock neurons regulates reproductive dormancy in drosophila melanogaster. *PLoS Genetics*, *15*(6). https://doi.org/10.1371/journal.pgen.1008158

Nelson, R. J., Denlinger, D. L., & Somers, D. E. (2010). Photoperiodism: The Biological Calendar. In *Photoperiodism: The Biological Calendar*. Oxford University Press. https://doi.org/10.1093/acprof:oso/9780195335903.001.0001

Numata, H., Saulich, A. H., & Volkovich, T. A. (1993). Photoperiodic Responses Of The Linden Bug, Pyrrhocoris-Apterus, Under Conditions Of Constant-Temperature And Under Thermoperiodic Conditions. *Zoological Science*, *10*(3).

Ojima, N., Ishiguro, S., An, Y., Kadosawa, T., & Suzuki, K. (2015). Male reproductive maturity and diapause termination in the leaf beetle Gastrophysa atrocyanea. *Physiological Entomology*, *40*(4), 277–283. https://doi.org/10.1111/phen.12112

Pener, M. P. (1992a). Environmental cues, endocrine factors, and reproductive diapause in Male insects. *Chronobiology International*, *9*(2). https://doi.org/10.3109/07420529209064521

Pener, M. P. (1992b). Environmental Cues, Endocrine Factors, and Reproductive Diapause in Male Insects. In *Chronobiology International* (Vol. 9, Issue 2).

Raikhel, A. S., Brown, M. R., & Belles, X. (2005). Hormonal Control of Reproductive Processes. In *Comprehensive Molecular Insect Science* (Vols. 3–6). https://doi.org/10.1016/B0-44-451924-6/00040-5

Raikhel, A. S., Kokoza, V. A., Zhu, J., Martin, D., Wang, S. F., Li, C., Sun, G., Ahmed, A., Dittmer, N., & Attardo, G. (2002). Molecular biology of mosquito vitellogenesis: From basic studies to genetic engineering of antipathogen immunity. *Insect Biochemistry and Molecular Biology*, *32*(10). https://doi.org/10.1016/S0965-1748(02)00090-5

Ram, K. R., & Wolfner, M. F. (2007). Seminal influences: Drosophila Acps and the molecular interplay between males and females during reproduction. *Integrative and Comparative Biology*, *47*(3), 427–445. https://doi.org/10.1093/icb/icm046

Rauschenbakh, I. Y., Adon'eva, N. v, Gruntenko, N. E., Karpova, E. K., & Faddeeva, N. v. (2004). PHYSIOLOGY OF DEVELOPMENT Juvenile Hormone Controls Oviposition and Fertility in Drosophila virilis during Starvation. In *Russian Journal of Developmental Biology* (Vol. 35, Issue 6).

Renn, S. C. P., Park, J. H., Rosbash, M., Hall, J. C., & Taghert, P. H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in Drosophila. *Cell*, *99*(7). https://doi.org/10.1016/S0092-8674(00)81676-1

Richard, D. S., Jones, J. M., Barbarito, M. R., Cerula, S., Detweiler, J. P., Fisher, S. J., Brannigan, D. M., & Scheswohl, D. M. (2001). Vitellogenesis in diapausing and mutant Drosophila melanogaster: Further evidence for the relative roles of ecdysteroids and juvenile hormones. *Journal of Insect Physiology*, *47*(8). https://doi.org/10.1016/S0022-1910(01)00063-4

Ruberson, J. R., Shen, Y. J., & Kring, T. J. (2000). Photoperiodic Sensitivity and Diapause in the Predator Orius insidiosus (Heteroptera: Anthocoridae). In *Ann. Entomol. Soc. Am* (Vol. 93, Issue 5). https://academic.oup.com/aesa/article/93/5/1123/9136

Saunders, D. S. (1965). Larval Diapause of Maternal Origin: Induction of Diapause in Nasonia Vitripennis (Walk.) (Hymenoptera: Pteromalidae). *Journal of Experimental Biology*, *42*(3). https://doi.org/10.1242/jeb.42.3.495

Saunders, D. S. (1987). Insect photoperiodism: the linden bug, Pyrrhocoris apterus, a species that measures daylength rather than nightlength. *Experientia*, *43*(8). https://doi.org/10.1007/BF01951677

Saunders, D. S. (2014). Insect photoperiodism: Effects of temperature on the induction of insect diapause and diverse roles for the circadian system in the photoperiodic response. In *Entomological Science* (Vol. 17, Issue 1, pp. 25–40). https://doi.org/10.1111/ens.12059

Saunders, D. S., Henrich, V. C., & Gilbert, L. I. (1989). Induction of diapause in Drosophila melanogaster: photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(10). https://doi.org/10.1073/pnas.86.10.3748

Schebeck, M., Dobart, N., Ragland, G. J., Schopf, A., & Stauffer, C. (2022). Facultative and obligate diapause phenotypes in populations of the European spruce bark beetle Ips typographus. *Journal of Pest Science*, *95*(2). https://doi.org/10.1007/s10340-021-01416-w

Sheng, Z., Xu, J., Bai, H., Zhu, F., & Palli, S. R. (2011). Juvenile hormone regulates vitellogenin gene expression through insulin-like peptide signaling pathway in the red flour beetle, Tribolium castaneum. *Journal of Biological Chemistry*, *286*(49), 41924–41936. https://doi.org/10.1074/jbc.M111.269845

Shiga, S. (2003). Anatomy and functions of brain neurosecretory cells in diptera. In *Microscopy Research and Technique* (Vol. 62, Issue 2, pp. 114–131). https://doi.org/10.1002/jemt.10366

Shiga, S., & Numata, H. (2001). Anatomy and functions of the brain neurosecretory neurons with regard to reproductive diapause in the blow fly Protophormia terraenovae. In *Insect Timing: Circadian Rhythmicity to Seasonality*. https://doi.org/10.1016/b978-044450608-5/50038-6

Shiga, S., & Numata, H. (2009). Roles of PER immunoreactive neurons in circadian rhythms and photoperiodism in the blow fly, Protophormia terraenovae. *Journal of Experimental Biology*, *212*(6), 867–877. https://doi.org/10.1242/jeb.027003

Shimokawa, K., Numata, H., & Shiga, S. (2008). Neurons important for the photoperiodic control of diapause in the bean bug, Riptortus pedestris. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and*

Behavioral Physiology, *194*(8), 751–762. https://doi.org/10.1007/s00359-008-0346-y

Shimokawa, K., Numata, H., & Shiga, S. (2014). Pars intercerebralis promotes oviposition in the bean bug, Riptortus pedestris (Heteroptera: Alydidae). *Applied Entomology and Zoology*, *49*(4), 525–528. https://doi.org/10.1007/s13355-014-0281-z

Shin, S. W., Zou, Z., Saha, T. T., & Raikhel, A. S. (2012). bHLH-PAS heterodimer of methoprene-tolerant and Cycle mediates circadian expression of juvenile hormone-induced mosquito genes. *Proceedings of the National Academy of Sciences*, *109*(41), 16576–16581. https://doi.org/10.1073/pnas.1214209109

Shirk, P. D., Bhaskaran, G., & Röller, H. (1980). The transfer of juvenile hormone from male to female during mating in the Cecropia silkmoth. *Experientia*, *36*(6). https://doi.org/10.1007/BF01970138

Shirk, P. D., Dahm, K. H., & Röller, H. (1976). The Accessory Sex Glands as the Repository for Juvenile Hormone in Male Cecropia Moths. In *Zeitschrift fur Naturforschung - Section C Journal of Biosciences* (Vol. 31, Issues 3–4). https://doi.org/10.1515/znc-1976-3-421

Sim, C., & Denlinger, D. L. (2008). Insulin signaling and FOXO regulate the overwintering diapause of the mosquito Culex pipiens. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(18). https://doi.org/10.1073/pnas.0802067105

Sirot, L. K. (2019). On the evolutionary origins of insect seminal fluid proteins. In *General and Comparative Endocrinology* (Vol. 278, pp. 104–111). Academic Press Inc. https://doi.org/10.1016/j.ygcen.2019.01.011

Ślusarczyk, M. (2004). Environmental plasticity of fish avoidance diapause response in Daphnia magna. In *J. Limnol* (Vol. 63).

Smykal, V., Bajgar, A., Provaznik, J., Fexova, S., Buricova, M., Takaki, K., Hodkova, M., Jindra, M., & Dolezel, D. (2014). Juvenile hormone signaling during reproduction and development ofthe linden bug, pyrrhocoris apterus. *Insect Biochemistry and Molecular Biology*, *45*(1), 69–76. https://doi.org/10.1016/j.ibmb.2013.12.003

Smykal, V., Daimon, T., Kayukawa, T., Takaki, K., Shinoda, T., & Jindra, M. (2014). Importance of juvenile hormone signaling arises with competence of insect

larvae to metamorphose. *Developmental Biology*, *390*(2). https://doi.org/10.1016/j.ydbio.2014.03.006

Socha, R. (1993). Pyrrhocoris apterus (Heteroptera) - an experimental model species: a review. *European Journal of Entomology*, *90*(3).

Socha, R., & Šula, J. (1992). Voltinism and seasonal changes in haemolymph protein pattern of Pyrrhocoris apterus (Heteroptera: Pyrrhocoridae) in relation to diapause. *Physiological Entomology*, *17*(4), 370–376. https://doi.org/https://doi.org/10.1111/j.1365-3032.1992.tb01034.x

Socha, R., Šula, J., & Kodrík, D. (2004). Wing morph-related differences in developmental pattern of accessory gland proteins in adult males of Pyrrhocoris apterus (L.) and their endocrine control. *Journal of Insect Physiology*, *50*(10). https://doi.org/10.1016/j.jinsphys.2004.07.001

Socha, R., Šula, J., Kodrík, D., & Gelbič, I. (1991). Hormonal control of vitellogenin synthesis in Pyrrhocoris apterus (L.) (Heteroptera). *Journal of Insect Physiology*, *37*(11), 805–816. https://doi.org/10.1016/0022-1910(91)90077-D

Soller, M., Bownes, M., & Kubli, E. (1999). *Control of Oocyte Maturation in Sexually Mature Drosophila Females*. http://www.idealibrary.com

Sonobe, H., Maotani, K., & Nakajima, H. (1986). Studies on embryonic diapause in the pnd mutant of the silkworm, Bombyx mori: Genetic control of embryogenesis. *Journal of Insect Physiology*, *32*(3). https://doi.org/10.1016/0022-1910(86)90061-2

South, A., & Lewis, S. M. (2011). The influence of male ejaculate quantity on female fitness: A meta-analysis. *Biological Reviews*, *86*(2). https://doi.org/10.1111/j.1469-185X.2010.00145.x

Šula, J., Kodrík, D., & Socha, R. (1995). Hexameric haemolymph protein related to adult diapause in the red firebug, Pyrrhocoris apterus (L.) (Heteroptera). *Journal of Insect Physiology*, *41*(9). https://doi.org/10.1016/0022-1910(95)00025-P

Syrová, Z., Doležel, D., Šauman, I., & Hodková, M. (2003). Photoperiodic regulation of diapause in linden bugs: Are period and Clock genes involved? *Cellular and Molecular Life Sciences*, *60*(11), 2510–2515. https://doi.org/10.1007/s00018-003-3227-0

Tamaki, S., Takemoto, S., Uryu, O., Kamae, Y., & Tomioka, K. (2013). Opsins are involved in nymphal photoperiodic responses in the cricket Modicogryllus siamensis. *Physiological Entomology*, *38*(2), 163–172. https://doi.org/10.1111/phen.12015

Tanigawa, N. A., Shiga, S., & Numata, H. (1999). Role of the corpus allatum in the control of reproductive diapause in the male blow fly, Protophormia terraenovae. *Zoological Science*, *16*(4), 639–644. https://doi.org/10.2108/zsj.16.639

Tauber, M. J., & Tauber, C. A. (1970). Photoperiodic induction and termination of diapause in an insect: Response to changing day lengths. *Science*, *167*(3915). https://doi.org/10.1126/science.167.3915.170

Tawfik, A. I., Tanaka, Y., & Tanaka, S. (2002). Possible involvement of ecdysteroids in photoperiodically induced suppresion of ovarian development in a Japanese strain of the migratory locust, Locusta migratoria. In *Journal of Insect Physiology* (Vol. 48). www.elsevier.com/locate/jinsphys

Taylor, L. R., Tauber, M. J., Tauber, C. A., & Masaki, S. (1987). Seasonal Adaptations of Insects. *The Journal of Animal Ecology*, *56*(1). https://doi.org/10.2307/4831

Teal, P. E. A., Gomez-Simuta, Y., & Proveaux, A. T. (2000). *Mating experience and juvenile hormone enhance sexual signaling and mating in male Caribbean fruit flies* (Vol. 97, Issue 7). PNAS. www.pnas.orgcgidoi10.1073pnas.060034397

Tian, L., Ji, B. zhong, Liu, S. wen, Jin, F., Gao, J., & Li, S. (2010). Juvenile hormone III produced in male accessory glands of the longhorned beetle, Apriona Germari, is transferred to female ovaries during copulation. *Archives of Insect Biochemistry and Physiology*, *75*(1). https://doi.org/10.1002/arch.20385

Urbanová, V., Bazalová, O., Vaněčková, H., & Dolezel, D. (2016). Photoperiod regulates growth of male accessory glands through juvenile hormone signaling in the linden bug, Pyrrhocoris apterus. *Insect Biochemistry and Molecular Biology*, *70*, 184–190. https://doi.org/10.1016/j.ibmb.2016.01.003

Uryu, O., Ameku, T., & Niwa, R. (2015). Recent progress in understanding the role of ecdysteroids in adult insects: Germline development and circadian clock in the fruit fly Drosophila melanogaster. *Zoological Letters*, *1*(1). https://doi.org/10.1186/s40851-015-0031-2

van Sinay, E., Mirabeau, O., Depuydt, G., van Hiel, M. B., Peymen, K., Watteyne, J., Zels, S., Schoofs, L., & Beets, I. (2017). Evolutionarily conserved TRH neuropeptide pathway regulates growth in Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(20), E4065–E4074. https://doi.org/10.1073/pnas.1617392114

van Vliet, S. (2015). Bacterial Dormancy: How to Decide When to Wake Up. In *Current Biology* (Vol. 25, Issue 17). https://doi.org/10.1016/j.cub.2015.07.039

Veenstra, J. A., & Šimo, L. (2020). The TRH-ortholog EFLamide in the migratory locust. *Insect Biochemistry and Molecular Biology*, *116*. https://doi.org/10.1016/j.ibmb.2019.103281

Wigglesworth, V. B. (1936). The function of the Corpus Allatum in the Growth and Reproduction of Rhodnius prolixus (Hemiptera). *J. Cell Sci*, *79*(313), 91–121. https://doi.org/https://doi.org/10.1242/jcs.s2-79.313.91

Wyatt, G. R., & Davey, K. G. (1996). Cellular and Molecular Actions of Juvenile Hormone. II. Roles of Juvenile Hormone in Adult Insects. *Advances in Insect Physiology*, *26*(C). https://doi.org/10.1016/S0065-2806(08)60030-2

Yin, C. M., Qin, W. H., & Stoffolano, J. G. (1999). Regulation of mating behavior by nutrition and the corpus allatum in both male and female Phormia regina (Meigen). *Journal of Insect Physiology*, *45*(9). https://doi.org/10.1016/S0022-1910(99)00047-5

Zhang, Z., Xu, J., Sheng, Z., Sui, Y., & Palli, S. R. (2011). Steroid receptor co-activator is required for juvenile hormone signal transduction through a bHLH-PAS transcription factor, methoprene tolerant. *Journal of Biological Chemistry*, *286*(10), 8437–8447. https://doi.org/10.1074/jbc.M110.191684

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