Identification of Key Players of the Ecdysteroid Pathway and Detection of Ecdysteroid Synthesizing Tissues in Chelicerates

Insights into the Evolution of Molting in Panarthropoda

Dissertation

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“Normal is an illusion.

What is normal for the spider

is chaos for the fly.”

— Morticia Addams

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Abbreviations

20E	20-hydroxyecdysone
APM	after penultimate molt
β-FTZ-F1	beta-Fushi-tarazu transcription factor 1
BLAST	basic local alignment search tool
°C	degrees Celsius
cDNA	complementary DNA
CNS	central nervous system
CYP	cytochrome P450
CYP18A1	cytochrome 18A1
DAF	Dauer-Abnormal Formation
dib	*disembodied*
D. melanogaster	*Drosophila melanogaster*
dsRNA	double-stranded RNA
EcR	ecdysone receptor
e.g.	exempli gratia/example given
et al.	et alii/and others
etc.	et cetera/and the rest
Fig.	Figure
HR3 & 4	hormone receptor 3 & 4
i.e.	id est/in other words
kbp	kilo-base pair
Mef2	*myocyte enhancer factor 2*
MIH	molt-inhibiting hormone
ml	milliliter
mm	millimeter
mM	millimolar
mRNA	messenger RNA
mya	million years ago
NCBI	National Center for Biotechnology Information
nobo	*noppera-bo*
NPC	Niemann-Pick C
n.s.	not significant
nvd	*neverland*
phm	*phantom*
ponA	ponasterone A
P. pseudoannulata	*Pardosa pseudoannulata*
P. tepidariorum	*Parasteatoda tepidariorum*
PTTH	prothoracicotropic hormone
P. xylostella	*Plutella xylostella*
RNAi	RNA interference
sad	*shadow*
scRNA-seq	single-cell RNA sequencing
shd	*shade*
spo	*spook*
sro	*shroud*
tbx20	*T-box transcription factor 20*
T. urticae	*Tetranychus urticae*
µm	micrometer
UMAP	uniform manifold approximation and projection
UTR	untranslated region

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1. Abstracts

Abstract

Ecdysteroids are crucial hormones that regulate molting and developmental progression in arthropods. While the biosynthesis pathway of these hormones has been extensively studied in insects, functional characterization of the involved components remains limited in chelicerates. This thesis focuses on the early-to-late ecdysteroid pathway genes *neverland* (*Pt-nvd*, Rieske-domain oxygenase), *shroud* (*Pt-sro*, short-chain dehydrogenase/reductase), the cytochrome P450 genes *spook* (*Pt-spo*), *disembodied* (*Pt-dib*), *shadow* (*Pt-sad*) and *shade* (*Pt-shd*) as well as the ecdysteroid-inactivating *CYP18A1* (*Pt-CYP18A1*) in the spider *Parasteatoda tepidariorum*. The aim is to evaluate functional conservation and potential evolutionary diversifications within these molting-related gene families beyond Pancrustacea.

Single-cell RNA sequencing analyses combined with whole-mount *in situ* hybridizations reveal common expression domains in mid-embryonic hemocytes for *Pt-nvd*, *Pt-sro*, *Pt-spo*, *Pt-dib*, *Pt-shd* and *Pt-CYP18A1*, and a later shift to the cardiac vessel or its lumen for *Pt-nvd*, *Pt-sro*, *Pt-dib*, *Pt-sad*, and *Pt-CYP18A1* during late embryogenesis. *Pt-sad* additionally marks neuroectodermal precursors, implicating a function in early neurogenesis. Parental RNA interference against *Pt-sad* disrupts head lobe formation, significantly elevates embryonic lethality and interval until first ecdysis. This indicates essential roles of the gene in both endocrine and morphogenetic processes. Juvenile knockdowns of all studied genes drastically extend molt intervals (up to four-fold) and produce lethal molting defect phenotypes, confirming pathway conservation at the functional level. As in insects, postembryonic expressions of *Pt-nvd*, *Pt-sro*, *Pt-spo*, *Pt-dib* and *Pt-sad* are localized in a common tissue during postembryonic stages. However, instead of a centralized prothoracic gland, spiders appear to utilize hemocytes for ecdysteroidogenesis.

The findings establish hemocytes as previously unrecognized ecdysteroid pathway domains in spiders and reveal spider-specific involvement of *shadow* in neurogenesis. Thereby, this work provides some of the first evidence for ecdysteroid pathway activity in chelicerates, reinforcing functional pathway conservation across panarthropods while exposing evolutionary flexibility of endocrine regulation.

Zusammenfassung

Ecdysteroide sind zentrale Hormone, welche Häutungen und Entwicklungsfortschritte bei Arthropoden steuern. Während der Biosynthese-Pathway bei Insekten umfassend untersucht ist, fehlt es bei Cheliceraten weitgehend an einer funktionellen Charakterisierung beteiligter Komponenten. Diese Arbeit konzentriert sich auf die Gene des frühen bis späten Ecdysteroid-Pathways: *neverland* (*Pt-nvd*, Rieske-Domain Oxygenase), *shroud* (*Pt-sro*, short-chain Dehydrogenase/Reduktase), die Cytochrom P450 Gene *spook* (*Pt-spo*), *disembodied* (*Pt-dib*), *shadow* (*Pt-sad*) und *shade* (*Pt-shd*) sowie das Ecdysteroid-inaktivierende *CYP18A1* (*Pt-CYP18A1*) in der Spinne *Parasteatoda tepidariorum*. Ziel ist es, die funktionelle Konservierung und mögliche evolutionäre Diversifizierungen dieser Genfamilien über die Pancrustacea hinaus zu untersuchen.

Single-cell RNA-Sequenzierungsanalysen in Kombination mit whole-mount *in situ* Hybridisierungen zeigen einheitliche Expressionsdomänen in Hämozyten mittlerer Embryonalstadien für *Pt-nvd*, *Pt-sro*, *Pt-spo*, *Pt-dib*, *Pt-shd* und *Pt-CYP18A1*. Während in der späten Embryogenese die Expression von *Pt-nvd*, *Pt-sro*, *Pt-dib*, *Pt-sad* und *Pt-CYP18A1* im Herzgefäß oder dessen Lumen lokalisiert ist. *Pt-sad* markiert zusätzlich neuroektodermale Vorläufer, was auf eine Funktion in der frühen Neurogenese hindeutet. Parentale RNA-Interferenz gegen *Pt-sad* stört die Kopflappenbildung und erhöht signifikant die embryonale Letalität sowie das Intervall bis zur ersten Häutung. Dies weist auf eine essenzielle Rolle sowohl bei endokrinen als auch morphogenetischen Prozessen hin. Der Knockdown aller untersuchten Gene in Juvenilen verlängert drastisch die Häutungsintervalle (bis zu vierfacher Länge) und führt zu letalen Häutungsdefekten, was die Konservierung des Pathways auf funktioneller Ebene bestätigt. Wie bei Insekten werden *Pt-nvd*, *Pt-sro*, *Pt-spo*, *Pt-dib* und *Pt-sad* postembryonal im selben Gewebe exprimiert. In Spinnen geschieht dies in Hämozyten anstatt einer zentralisierten Prothorakaldrüse. Die Ergebnisse belegen, dass Hämozyten bisher unerkannte Domänen des Ecdysteroid-Pathways bei Spinnen darstellen und deuten außerdem auf eine spinnenspezifische Beteiligung von *shadow* in der Neurogenese hin. Damit liefert diese Arbeit einen der ersten Belege für die Aktivität des Ecdysteroid-Pathways bei Cheliceraten, bestätigt die funktionelle Konservierung des Pathways in Panarthropoden und zeigt gleichzeitig die evolutionäre Flexibilität der endokrinen Regulierung auf.

2. Introduction

Molting as the periodic replacement of the exoskeleton is a defining trait of ecdysozoans that characterizes their growth and metamorphosis (Thummel, 2001; Sullivan & Thummel, 2003). The ecdysis process is regulated by a complex endocrine cascade centered on molting hormones known as ecdysteroids. In insects, the triggering hormone generally is 20-hydroxyecdysone (20E) (Truman, 2019). Synthesized from dietary cholesterol and released in the hemolymph, this hormone initiates the coordinated events of apolysis, interpreted as the separation of old cuticle and ecdysis behavior (White & Ewer, 2014; Daley & Drage, 2016). Studies established that surges of the ecdysteroid hormone at specific developmental times induce waves of gene expression that drive molting and metamorphic transitions (Ou et al., 2016; Pan et al., 2021). For example, a peak of ecdysone at the end of each instar activates the nuclear receptor in *Drosophila melanogaster* larvae initiating the pathway leading to pupation and eclosion (Hill et al., 2013). The importance of ecdysteroids extends beyond molting, influencing reproduction, diapause and other physiological processes in arthropods (Denlinger, 2002; Ameku et al., 2017; Ogihara et al., 2017; Hu et al., 2022).

While the hormonal control of molting has been well characterized in insects (Schumann et al., 2018; Kamiyama & Niwa, 2022), much less is known about this system in other arthropod lineages such as chelicerates like spiders (Qu et al., 2015; Campli et al., 2024). This introduction reviews current knowledge of ecdysteroid biosynthesis with particular focus on key enzyme encoding genes and will elucidate the evolution of the pathway in arthropods. Close attention is given on how conserved components of the ecdysteroidogenic gene network reflect homology within Ecdysozoa, while also illustrating lineage-specific innovations that suggest divergence. After highlighting the knowledge gap regarding molting hormones and ecdysteroidogenesis in spiders, the utilized species *Parasteatoda tepidariorum* is introduced as an emerging model organism. Finally, the objectives and scope of the thesis for studying the roles of the pathway genes in the spider are outlined.

2.1. Molting in Arthropods

The regulation of ecdysis via hormonal signaling represents an apomorphy of the Ecdysozoa, a monophyletic clade comprising arthropods, nematodes, onychophorans, tardigrades, priapulids and other related groups (Fig. 1; Telford et al., 2008). Within Ecdysozoa the molting process is a key innovation enabling the development of a protective exoskeleton, ecological diversification and modular growth strategies (Schmidt-Rhaesa et al., 1998; Peel et al., 2005). In arthropods, molting has facilitated extensive evolutionary radiation, allowing for rapid changes in body size, developmental plasticity and life history adaptation (Giribet & Edgecombe, 2019). These advantages have contributed to the extraordinary morphological and ecological diversity observed across the phylum Arthropoda (Bánki et al., 2023).

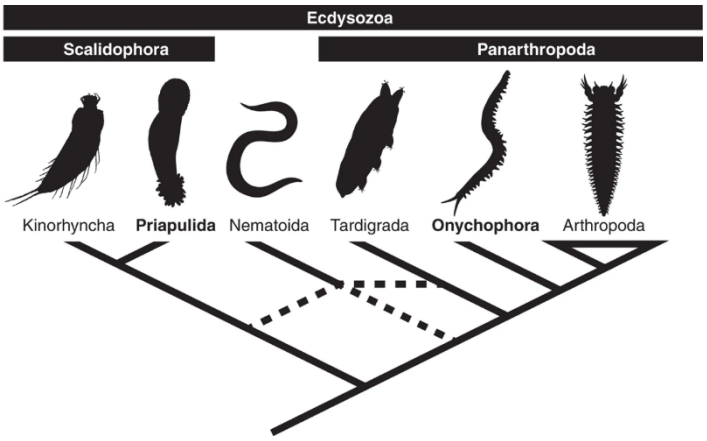

Figure 1 Phylogeny of major ecdysozoan clades (molting animals). Dashed lines indicate unresolved relations (graphic from Sansom, 2016).

All arthropods molt their chitinous exoskeleton periodically in order to grow or to change life stages (Campli et al., 2024). These exuviation events are under endocrine control and involve a well-coordinated order of events at cellular, physiological and behavioral levels (Gilbert et al., 2002; Nijhout et al., 2014; Das, 2015). The molting cycle is classically divided into four stages (Fig. 2; Chiyoda et al., 2023). The intermolt, defined by a fully developed cuticle and low molt-related activity. Secondly the pre-molt or apolysis, when the cuticular chitin and proteins are partially digested and the synthesis of new underlying exocuticle and epicuticle under the stationary exoskeleton begins (Tom et al., 2014; Roer et al., 2015). The shedding of the old exoskeleton is referred to as ecdysis. It is triggered by rupture caused by movement and increased hemolymph pressure in the body (Daley & Drage, 2016). Lastly, post-ecdysis during which the new cuticle is sclerotizing, mineralizing and pigmenting (Andersen, 2010).

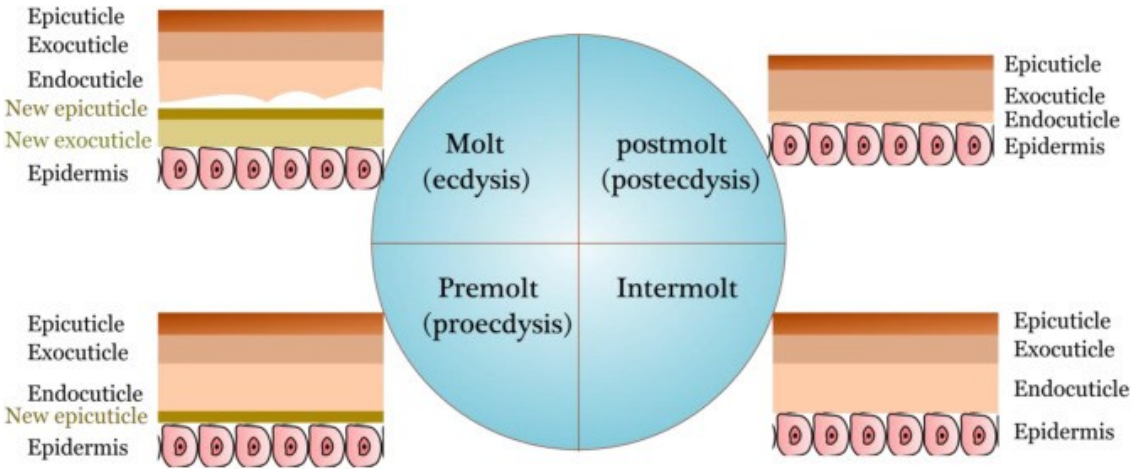

Figure 2 Schematic stages of a complete molting cycle. Cuticle changes over course of intermolt, pre-molt, ecdysis and post-molt on the example of shrimp exoskeleton layers (graphic from Dang et al., 2018).

In insects, endocrine glands synthesize pulses of ecdysteroid hormones that start molt events (Niwa & Niwa, 2016; Ou et al., 2016). The molt cascade in holometabolous insects is initiated by the prothoracic gland which secretes ecdysone, a precursor of the active 20E (Fig. 3; Schumann et al., 2018; Bian et al., 2019), in response to the prothoracicotropic hormone (PTTH) from the brain (Liu et al., 2018). The released ecdysone is converted to 20E in peripheral tissues and afterwards binds to the nuclear ecdysone receptor (EcR) (Kamiyama & Niwa, 2022). This ligand-receptor binding activates transcription of early response genes like *E75*, *hormone receptor 3* (*HR3*) or *beta-Fushi-tarazu transcription factor 1* (*βFtz-f1*) (Bialecki et al., 2002; Parvy et al., 2014), which regulate late genes for molting and metamorphosis (Jindra et al., 2015; Schumann et al., 2018; Thompson, 2021). Through this gene cascade, ecdysteroid pulses regulate the drastic morphological and physiological reorganization that characterizes molts and life stage transitions.

At the end of each molting cycle a decline in ecdysteroid titer, combined with neuropeptides like the ecdysis-triggering hormone and eclosion hormone, induces the irreversible stereotypical ecdysis behavior which frees the animal from its old cuticle (White & Ewer, 2014; De Oliveira et al., 2019; Malhotra & Basu, 2023). This neuroendocrine pathway is conserved across diverse insects and ensures that the timing of ecdysis is tightly linked to hormonal changes (e.g., Roller et al., 2010). Thus, this ancient and intricate network controls molting in arthropods alongside ecdysteroids as the central morphogenetic signal (Niwa & Niwa, 2016).

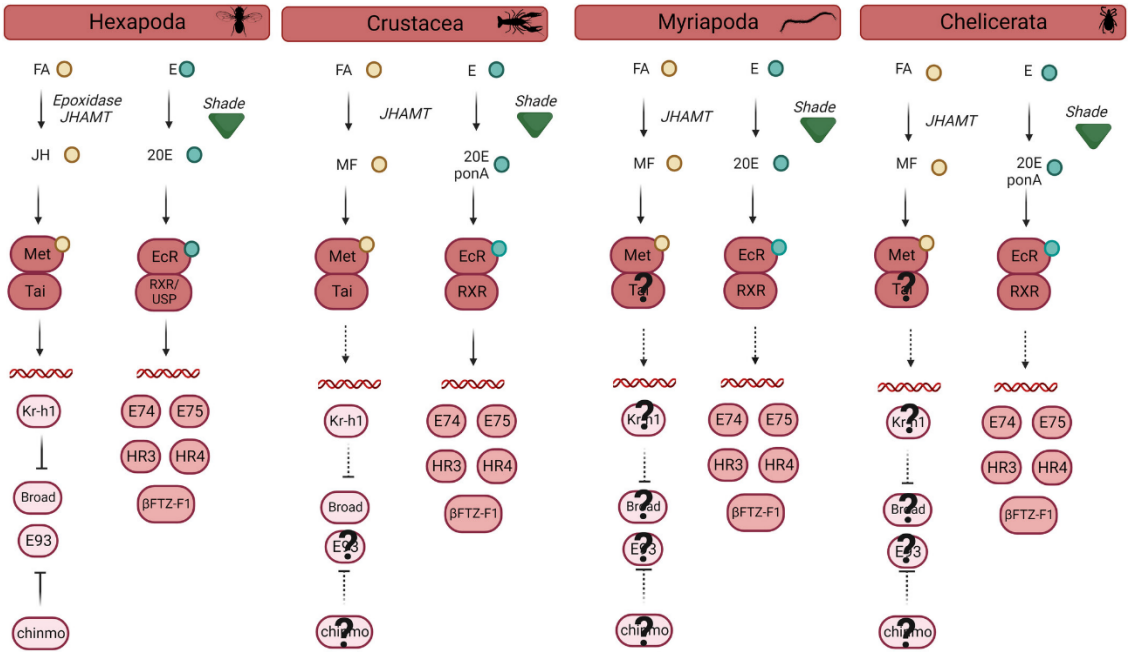

Figure 3 Hormonal signaling cascade triggered by ecdysteroid activation. Farnesoic acid is converted to methyl farnesoate (MF) by juvenile hormone or methyl-transferase in most arthropods, insects require epoxidase to synthesize juvenile hormone (JH). Shade enzymes activate ecdysone into 20-hydroxyecdysone or ponasterone A in some crustaceans and chelicerates. MF/JH binds to the Met receptor. In insects, the Met-Tai complex induces Kr-h1, suppressing Broad and E93 to maintain juvenile status. Insect-specific Chinmo promotes juvenile fate. Knowledge downstream of Met is limited in non-insects. Activated ecdysteroids bind to the EcR/RXR heterodimer to initiate transcription of E74, E75, HR3, HR4 and βFTZ-F1. Question marks indicate unresolved components. 20E, 20-hydroxyecdysone; chinmo, chronologically inappropriate morphogenesis; E, ecdysone; E74/75/93, ecdysone-induced proteins; EcR, ecdysone receptor; FA, farnesoic acid; HR3/4, hormone receptor 3/4; JH, juvenile hormone; JHAMT, juvenile hormone methyl-transferase; Kr-h1, krueppel homolog 1; Met, methoprene-tolerant; MF, methyl farnesoate; ponA, ponasterone A; RXR, retinoid X receptor; ßFTZ-F1, beta fushi-tarazu transcription factor 1; Tai, taiman; USP, ultraspiracle (graphic Campli et al., 2024).

Other arthropod lineages also use ecdysteroids in molt regulation, although the precise endocrine mechanisms vary (Song et al., 2017). In paraphyletic crustaceans, molting is controlled by 20E or an analog, synthesized in the Y-organs located in the cephalothorax (Legrand et al., 2021). Within the X-organ located in the eyestalk, another neuropeptide is secreted (Ventura et al., 2018; Mykles et al., 2021). This molt-inhibiting hormone represses the release of ecdysteroids from the Y-organ during the intermolt phase (Knigge et al., 2021; Mykles, 2021). Myriapods and chelicerates also undergo molting under presumed ecdysteroid regulation, though the identity of the endocrine organs and the associated regulatory circuits remains less characterized (Chipman et al., 2014; Li et al., 2017). This fundamental requirement for ecdysteroid-coordinated molts represents a conserved and defining feature of ecdysozoans and their developmental

biology. Across arthropods, cholesterol or other dietary sterols serve as precursors for the ecdysteroid biosynthesis (Lavrynenko et al., 2015).

2.2. Ecdysteroid Biosynthesis Pathway and Halloween Genes

Arthropods are unable to synthesize cholesterol or related sterols *de novo* and must acquire it through their diet (Clark & Block, 1959; Igarashi et al., 2018). The conversion of dietary sterols into ecdysteroids such as ecdysone and 20E occurs via a multi-step enzymatic pathway of oxidative and reductive transformations (Niwa & Niwa, 2014a, Yamanaka, 2021). Enzymes encoded by the so-called Halloween genes catalyze many of these synthesis steps (Gilbert et al., 2002; Gilbert & Rewitz, 2009; Schumann et al., 2018). First identified in *D. melanogaster* as a set of mutant loci, the Halloween genes caused embryonic lethality and defects in cuticle formation (Jürgens et al., 1984; Wieschaus et al., 1984; Kamiyama & Niwa, 2022). Gene names like *spook*, *phantom*, *disembodied*, *shadow* and *shade* reflect embryonic mutant phenotypes that failed to produce a normal exoskeleton (Chávez et al., 2000; Gilbert, 2004; Van Ekert et al., 2016). The genes encode cytochrome P450 (CYP) monooxygenases that perform sequential hydroxylations in the ecdysteroid biosynthesis pathway and together with additional enzymes convert cholesterol to the bioactive molt hormone (Figure 4; Pan et al., 2021).

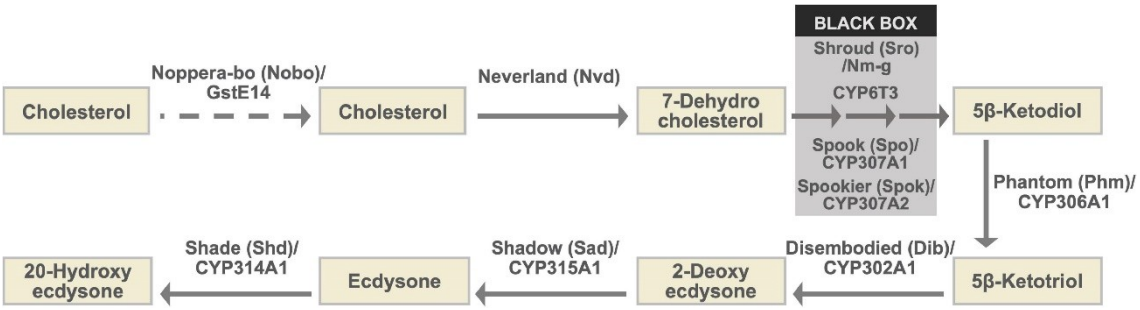

Figure 4 The ecdysteroid biosynthesis pathway in insects. The "Black Box" refers to uncertainties in conversion steps between 7-dehydrocholesterol and 5β-ketodiol. Notably, *spook* and *spookier* are paralogs (e.g., Ono et al., 2006; Komura-Kawa et al., 2015). As endogenous substrates of Nobo are not yet identified its role is indicated by a dashed arrow (graphic from Kamiyama & Niwa, 2022).

The first step of ecdysteroid biosynthesis pathway in insects is conducted by the gene *neverland* (*nvd*), which encodes a Rieske-domain oxygenase (Yoshiyama et al., 2006; Lang et al., 2012). Neverland catalyzes the 7,8-dehydrogenation of cholesterol to produce 7-dehydrocholesterol (Yoshiyama-Yanagawa et al., 2011). The loss of *nvd*

function prevents ecdysone production and arrests development. This phenotype was first demonstrated in *D. melanogaster* and silkworm mutants (Yoshiyama et al., 2006). This *nvd* deficiency phenotype is conserved across insect orders and most pancrustaceans (Qu et al., 2015; Sandlund et al., 2018; Legrand et al., 2021).

Following this initial step, a series of less well-understood transformations occur between 7-dehydrocholesterol and the 5β-ketodiol molecule (Warren et al., 2004). The reactions collectively catalyzing this part of ecdysteroidogenesis are referred to as "Black Box" because intermediates and enzymes involved are not yet completely elucidated (Fig. 4; Gilbert & Warren, 2005; Campli et al., 2024). The involvement of at least two additional genes operating in the Black Box is confirmed, including *shroud* (*sro*) and the Halloween gene *spook* (*spo*) (Niwa et al., 2004; Niwa et al., 2010). Both CYP enzymes Spook (CYP307A1) and its paralog Spookier (CYP307A2, which is restricted to *Drosophilidae*) are likely contributing to one or more of the early conversions after 7-dehydrocholesterol (Namiki et al., 2005; Ono et al., 2006). In *D. melanogaster*, *spook* and *spookier* show partially redundant roles and are expressed in the prothoracic gland during specific developmental timeframes (Rewitz et al., 2007; Sztal et al., 2007). To produce a strong disruption of ecdysone biosynthesis, a loss of function in both genes is required (Namiki et al., 2005; Ono et al., 2006). The exact biochemical reaction catalyzed by Spook/Spookier remains unclear, but some studies indicate participation in converting 7-dehydrocholesterol to a Δ^4-diketone intermediate (Gilbert et al., 2002; Warren et al., 2009). Downstream, the more recently in *D. melanogaster* discovered ecdysteroid pathway gene *sro* (*non-molting glossy* in *Bombyx mori*) encodes a short-chain dehydrogenase/reductase also involved in the Black Box (Niwa et al., 2010). Mutants show an arrest of development due to ecdysteroid deficiency (Niwa &Niwa, 2014a). Shroud is suggested to catalyze a modification on the 3-β-hydroxyl group from a sterol intermediate within the Black Box, potentially the reduction of the 3-oxo-Δ^4-steroid to form 5β-ketodiol (Blais et al., 1996; Niwa et al., 2010). In summary, *nvd*, *spook*/*spookier* and *sro* act successively to synthesize the key intermediate 5β-ketodiol from cholesterol, preparing for the well-studied subsequent steps of ecdysone biosynthesis (Pan et al., 2021; Kamiyama & Niwa, 2022).

The following steps of the pathway are mediated by a group of CYP monooxygenases localized to the endoplasmic reticulum or mitochondria of ecdysteroidogenic glands, like the prothoracic gland in insects or the Y-organ of

crustaceans (Rewitz et al., 2006; Rewitz et al., 2007; Legrand et al., 2021). Phantom (*phm*, CYP306A1) adds a hydroxyl group at C25 of the 5β-ketodiol (Niwa et al., 2004; Warren et al., 2004). Disembodied (*dib*, CYP302A1) then hydroxylates C22, yielding 2-deoxyecdysone (Chávez et al., 2000). Next, Shadow (*sad*, CYP315A1) introduces hydroxyl at C2, converting 2-deoxyecdysone into ecdysone (Warren et al., 2002). Finally, ecdysone is released from the gland into the hemolymph where Shade (*shd*, CYP314A1) hydroxylates the C20 of ecdysone, resulting in the active molting hormone 20E. This gene is predominantly expressed in target tissues such as the fat body (Petryk et al., 2003). The coordinated catalyzation through Phantom, Disembodied, Shadow and Shade completes the biosynthesis of 20E (Liu et al., 2018; Kamiyama & Niwa, 2022). *CYP18A1* encodes a CYP enzyme known to inactivate 20E via 26-hydroxylation, converting the molting hormone into an inactive 20,26-dihydroxyecdysone (Guittard et al., 2011). This inactivation is critical for developmental timing in e.g., *D. melanogaster* as reduction of 20E at the end of larval stages is required for the larva-to-pupa transition (Rewitz et al., 2010). Loss of *CYP18A1* function causes abnormally prolonged molt intervals and pupal lethality, highlighting its role in molting and metamorphosis (Guittard et al., 2011).

Recently, additional factors like a glutathione S-transferase encoded by *noppera-bo* (*nobo*) became evident (Fig. 4; Enya et al., 2014). It is assumed to regulate the intake of dietary cholesterol in the fruit fly (Enya et al., 2015). Mutants of *nobo* phenotypically copy other Halloween gene mutants with lethal molting defects (Enya et al., 2014). Research of *nobo* function outside of a few insect model organisms remains limited, as studies primarily focus on *D. melanogaster* (Koiwai et al., 2020; Ebihara & Niwa, 2023) and some other insect species (Enya et al., 2015; Inaba et al., 2022). There is evidence for additional CYPs, such as CYP6t3 in *D. melanogaster*, possibly exhibiting additional enzymatic functions in the Black Box (Ou et al., 2011; Saito et al., 2016). Nonetheless, enzymes encoded by *nvd*, *spook*/*spookier*, *sro*, *phm*, *dib*, *sad* and *shd* are considered the central ecdysteroid biosynthesis components in insects (Campli et al., 2024). Their expression in the molting gland is tightly coordinated at the transcriptional level to ensure that the ecdysteroid pathway is invoked at specific developmental times (Liu et al., 2018; Bian et al., 2019). This provides the molecular basis for the hormone biosynthesis that regulates arthropod molting.

2.3. Evolution of the Ecdysteroid Pathway in Arthropods

The ecdysteroid hormone biosynthesis pathway for molting is evolutionarily ancient and has undergone significant modifications across ecdysozoans (Qu et al., 2015; Dermauw et al., 2020). While ecdysozoans like nematodes, onychophorans and arthropods molt their exoskeleton, both conservation and divergence are shown in the hormonal and genetic mechanisms (Chipman & Edgecombe, 2019). In arthropods, the central molting hormones are ecdysteroids like ecdysone and 20E, but interestingly other taxa have evolved distinct molting hormones. For example, some nematodes do not utilize ecdysone (Giribet & Edgecombe, 2017; Schumann et al., 2018). Instead, they employ dafachronic acids to regulate their molting events, which are also derived from cholesterol (Aguilaniu et al., 2016; Lažetić & Fay, 2017). Consistent with this research, genomic studies have found no homologs of the Halloween genes involved in their ecdysteroid pathway (Schumann et al., 2018). This suggests that the common ancestor of Ecdysozoa may have lacked the biosynthetic toolkit of arthropods and relied on different enzymes for molt regulation. But a recent study recovered a putative homolog of *sad* in the genome of *Tubiluchus corallicola*, expanding the evidence of biosynthesis components into the early branching priapulid lineage (Lord et al., 2023).

Within Panarthropoda, containing i.e., arthropods, onychophorans and tardigrades, the Halloween genes occur stepwise during evolution (Schumann et al., 2018). Interestingly, a comparative genomic analysis indicates that onychophorans and tardigrades possess only orthologs of the CYP gene *sad* (Schumann et al., 2018). This suggests that intermediate steps in early diverging panarthropods might be carried out by alternative enzymes or a differing molting hormone, as Shadow catalyzes one of the last steps of ecdysteroid synthesis in insects (Warren et al., 2002; Yamakawa & Hejnol, 2024). The complement of CYP monooxygenases is more complete in myriapods, crustaceans and chelicerates, but not always the full network displayed by *D. melanogaster* is present (Qu et al., 2015; Ventura et al., 2018; Dermauw et al., 2020). Certain lineages may lack one or more of these enzymes, potentially using functional analogs or producing different active ecdysteroids (Li et al., 2017; Swall et al., 2021). For example, drosophilids have both *spook* and *spookier*, whereas basal insects and non-insect arthropods possess only *spook* (Ono et al., 2006; Dermauw et al., 2020). Remarkably, a third gene *spookiest* likely originated in the last common ancestor of pancrustaceans, is lost in drosophilids and daphnids (Schumann et al., 2018; Dermauw

et al., 2020). Similarly, the presence of the 20-hydroxylase Shade outside of insects displays variability in genomic studies (Ventrua et al., 2017; Dermauw et al., 2020). Some arthropods might secrete ecdysone derivates as the active molting hormone and other taxa produce 20E (Song et al., 2017; Li et al., 2017). Orthologs of *CYP18A1* are present in diverse major arthropod sub-lineages, indicating an evolutionarily conserved mechanism for ecdysteroid inactivation (Guittard et al., 2011; Schumann et al., 2018; Dermauw et al., 2020). For instance, in the crustacean *Daphnia magna*, it is inversely expressed relative to ecdysteroid peaks (Sumiya et al., 2014). Absence of *CYP18A1* is reported in *Tetranychus urticae* (Grbić et al., 2011) and *Varroa* species suggesting a loss in Acari, coherently with *phm* absence and ponasterone A (ponA) identification.

The early diverging lineage of chelicerates (Dunlop, 2010), composed of i.e., Acariformes, Opiliones, Scorpiones and Araneae, are particularly interesting for conservation studies of the ecdysteroid pathway, as they exhibit distinct life histories and morphologies. Most chelicerates lack the drastic ecological, morphological and physiological alterations of metamorphosis known from holometabolous insects (Bishop et al., 2006; Jindra, 2019). Evidence for answering the question whether chelicerates utilize the same enzymes to produce the molting hormone remains limited and suggests a mixture of conservation and divergence (Schumann et al., 2018; Kamiyama & Niwa, 2022; Campli et al., 2024). Genomic data have hinted at the presence of several Halloween and pathway-related gene homologs in chelicerates (Qu et al., 2015; Li et al., 2017; Schumann et al., 2018; Dermauw et al., 2020).

A recent study on the pond spider *Pardosa pseudoannulata* identified transcripts corresponding to *spo*, *dib*, *sad* and *shd*, providing first insights on the ecdysteroid pathway in araneids (Yang et al., 2021). The relative expression of those genes showed molt interval specific fluctuation, suggesting involvement in the hormone synthesis (Yang et al., 2021). Interestingly *phm* seems absent in the genomes of *P. pseudoannulata* and beyond Mandibulata (Qu et al., 2015; Schumann et al., 2018; Dermauw et al., 2020; Yang et al., 2021). Chelicerates might have an alternative enzyme performing the 25-hydroxylation or rely on a diverging molt triggering hormone. The absence of *phm* combined with biochemical analysis supports the hypothesis that the biologically active molecule in chelicerates is not 20E as in insects, but rather ponA (Grbić et al., 2011; Li et al., 2017 & 2019; Yang et al., 2021). Known from some arthropods and plants, this hormone shares a high configuration similarity to the insect's molting ecdysteroid but

shows a different hydroxylation pattern (Imai et al., 1967; Lachaise & Lafont, 1984; Song et al., 2017). The inconsistent detection of canonical ecdysteroid pathway genes in crustaceans and chelicerates suggests that while the core pathway enzymes of molt hormone production are likely conserved, the molecular components and active hormones may vary. This potentially reflects developmental system drifts with conserved hormonal outcomes despite genetic divergence (True & Haag, 2001).

In summary, an evolutionary perspective indicates that the ecdysteroid biosynthesis pathway has been assembled gradually in the arthropods and might work with lineage-specific modifications. Insects like *D. melanogaster* have served as model organisms for the understanding of this pathway but only represent one branch of Arthropoda while data on chelicerates remains limited. Exploring how this early-branching group achieves similar biochemical function is crucial for the comprehensive understanding of molting endocrinology. This major gap in knowledge provides an opportunity for investigating the ecdysteroid pathway in spiders and evaluating the generality of developmental models, potentially discovering novel regulatory features.

2.4. A Model for Spider Molting and Development

The common house spider *Parasteatoda tepidariorum* (Koch, 1841) has emerged as a powerful model organism for evolutionary developmental biology studies in chelicerates in recent years (Fig. 5E; Hilbrant et al., 2012; Schwager et al., 2017). Offering several practical and scientific advantages, it is an ideal system to bridge the knowledge gap between insect and non-insect models. The cosmopolitan spider is easy to culture in laboratories, has a relatively short generation time of two to three months and prolific reproduction, with each egg sac containing a few hundred embryos (Fig. 5B–D; Oda & Akiyama-Oda, 2020). The genome of *P. tepidariorum* has been sequenced and annotated as part of the i5k arthropod genome project, providing a valuable resource for homolog identification (Schwager et al., 2017; Thomas et al., 2020). Furthermore, a chromosome-level genome was provided by Zhu et al. (2023). In recent years single-cell RNA sequencing data (scRNA-seq data) of multiple embryonic stages became available (Akiyama-Oda et al., 2022; Leite et al., 2024; Medina-Jiménez et al., 2024).

Additionally, a range of crucial molecular and genetic tools are established for *P. tepidariorum*, including gene expression analysis via *in situ* hybridization and functional manipulation utilizing RNA interference (RNAi). Both embryonic RNAi and parental RNAi have proven effective (Akiyama-Oda & Oda, 2006; Kanayama et al., 2010). Through RNAi-based knockdowns, the functions of developmental genes are uncovered, demonstrating the practicability of those studies in spiders (e.g., Akiyama-Oda & Oda, 2006; Kanayama et al., 2011; Pechmann et al., 2017). Studies in this spider have yielded insights into the body axis formation (Schönauer et al., 2016), segmentation mechanisms (Chipman & Edgecombe, 2019) and appendage development (Heingård et al., 2019) by comparing gene networks to insects. The comparative approach has enriched the understanding of conserved versus derived developmental properties in arthropods (Hilbrant et al., 2012). Furthermore, the embryonic development of *P. tepidariorum* is described and staged in detail (Fig. 5A; Mittmann & Wolff, 2012), and therefore accessible for sampling and phenotypic observations on developmental timing and morphological changes resulting from gene knockdowns.

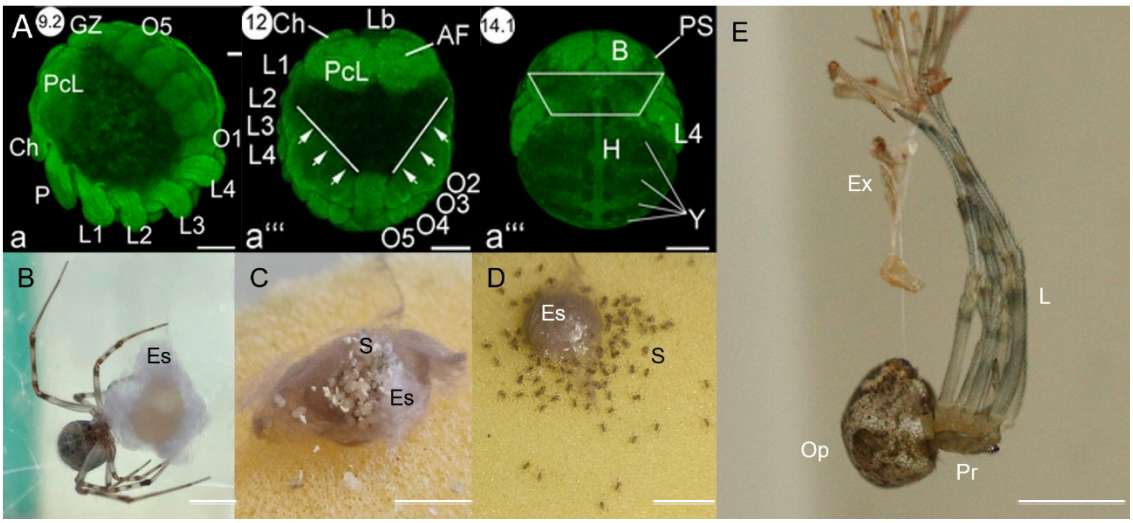

Figure 5 Emerging model organism *Parasteatoda tepidariorum*. (A) Mid-to-late embryogenesis including stages 9.2 when neurogenesis occurs, dorsal closure at stage 12 indicated by arrows and lines representing tissue overgrowing the yolk and finalized cardiogenesis at stage 14.1 with trapeze displaying progress of petiolus formation (combined image of fig. 7a; 11a‴ and 14a‴ from Mittmann & Wolff, 2012). (B) Female producing an egg sac containing hundreds of embryos (provided by M. Strack). (C) Molting spiderlings after hatching (provided by P. Habenicht). (D) Group of spiderlings after emerging from egg sac. (E) Female after ecdysis in post-molt position (provided by L. Elliott). AF; anterior furrow; B, brain; Ch, chelicerates; Es, egg sac; Ex, exuvia; GZ, growth zone; H, heart; L1–4; legs; Lb, labium; O1–5, opisthosomal segments; Op, opisthosoma; P, pedipalp; PcL, precheliceral lobes; Pr, prosoma; S, spiderlings; PS, prosomal shield; Y, yolk compartments. Scale bar: 100 µm for A; 5mm for B–E.

Studying the ecdysteroid biosynthesis pathway of the spider can elucidate if the canonical pathway is conserved or if spiders have evolved divergence in pathway genes, enzyme function or active molting hormone. The spider genome can be mined to identify orthologs of biosynthesis pathway genes and their expression patterns can be studied throughout development. Due to a sufficient background on the spider life cycle, studies on gene function can be performed. Novel phenotypes outside of conserved functions might point to unique roles of ecdysteroids in the spider embryogenesis. In summary, *Parasteatoda tepidariorum* offers a suitable model for this research that provides experimental accessibility with an evolutionary distance to traditional pancrustacean models. This allows to address the question of how conserved the mechanisms of ecdysteroid biosynthesis are across panarthropods.

2.5. Aims, Scope and Overview of the Present Study

Although the hormonal regulation of molting is well-known in insect models, the functional roles of ecdysteroid biosynthesis genes remain largely unexplored in chelicerates (Figure 6; Qu et al., 2015; Pan et al., 2021; Campli et al., 2024). This major and early-diverging arthropod lineage includes e.g. spiders, scorpions and mites (Dunlop, 2010). In particular, the expression dynamics and developmental functions of early-pathway and Black Box genes, comprising *nvd* and *sro*, are poorly studied outside of pancrustaceans. This lack of functional insight has limited the understanding of how conserved these biosynthesis components are across arthropods and whether they play additional roles beyond their established involvement in molting. Addressing this gap is essential to combine the evolutionary history of ecdysteroid biosynthesis pathways and their contribution to developmental processes in non-insects.

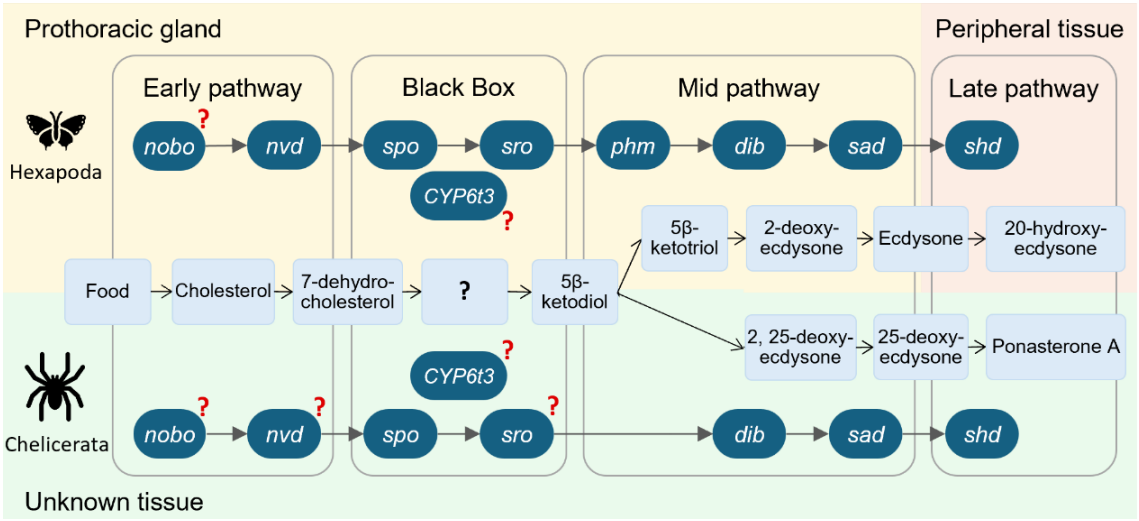

Figure 6 Ecdysteroid biosynthesis pathways of Hexapoda and Chelicerata. In insects the prothoracic gland secrets the specific molt hormone. Further investigation into the secretory tissue in chelicerates is required. *Nobo*, *CYP6t3* and *sro* only show evidence in a few insect models. Ponasterone A might be the bioactive ecdysteroid hormone in Chelicerata as *phm* has not been detected in this lineage. The pathways for Hexapoda and Chelicerata refer to summarized current knowledge. Question marks indicate the need for further clarification. *CYP*, cytochrome P450; *dib*, *disembodied*; *nobo*, *noppera-bo*; *nvd*, *neverland*; *phm*, *phantom*; *sad*, *shadow*; *shd*, *shade*; *spo*, *spook*; *sro*, *shroud*.

This study addresses the characterization of the molecular and functional roles of early-to-late pathway genes involved in ecdysteroid biosynthesis in the common house spider *P. tepidariorum*. Particular emphasis is placed on identifying the tissues in which these genes are expressed during embryogenesis and postembryonic development and to clarify their developmental significance beyond molting. A combination of expression

analysis via *in situ* and scRNA-seq data as well as RNAi is employed to explore when and where these genes are expressed and what phenotypic consequences arise from their disruption.

The investigation is structured in four parts. The first focuses on the early-pathway and Black Box genes *neverland* (*Pt-nvd*) and *shroud* (*Pt-sro*) respectively, which act upstream of the CYP pathway genes. In *P. tepidariorum*, orthologs for both genes are recovered from the genome and expression in "extraembryonic tissue" and heart-related structures is observed. Their knockdown resulted in molting impairments in juveniles. These findings indicate a conserved function in pathway initiation, as well as a potentially and previously unrecognized role in hematopoiesis.

The second part investigates a CYP enzyme encoded by the gene *shadow* (*Pt-sad*). Expression analyses utilizing whole-mount *in situ* hybridization reveal dynamic and stage-specific messenger-RNA (mRNA) activity in the central nervous system and cardiogenic mesoderm. Knockdowns via parental RNAi demonstrate involvement in neurogenesis and heart development, leading to embryonic lethality and developmental delays. Postembryonic knockdown results in severe molting defects, prolonged molt intervals and reduced growth, further confirming *sad*'s requirement for molting, while highlighting its role in coordinating endocrine and morphogenetic processes.

The third part presents a comparative analysis of the CYP enzymes Spook, Disembodied and Shade, key components of the ecdysteroid biosynthesis pathway across arthropods. Integrated embryonic and postembryonic expression studies identify stage- and tissue-specific activation, including peripheral domains such as hemocytes and reproductive tissues. Functional analysis via RNAi highlights their crucial roles in molting. These findings indicate both the evolutionary conservation of the canonical endocrine ecdysteroid pathway and its potential divergent plasticity.

The last part focuses on *CYP18A1*, a gene known to mediate ecdysteroid inactivation via 26-hydroxylation. In *P. tepidariorum*, expression analyses reveal a dynamic pattern, with mRNA first localized in embryonic hemocytes and in postembryonic midgut epithelium. RNAi causes delayed or incomplete lethal molts, suggesting that *Pt-CYP18A1* is required for proper ecdysis. These results support a conserved role of *CYP18A1* in molting hormone inactivation across arthropods and suggest an additional function in hemocyte formation.

Together, these analyses offer the first comprehensive characterization of genes involved in the ecdysteroid pathway in a chelicerate and provide insights into the potential sites of ecdysone synthesis in *P. tepidariorum*. The results support that these genes are not only conserved in their ecdysteroidogenesis function within Ecdysozoa but have additional roles in the regulation of embryonic and postembryonic development. This highlights the importance of broad comparative studies beyond insect model organisms to recognize the evolutionary variability of hormonal synthesis systems across Ecdysozoa.

3. Results

Every chapter within the results begins with a short description of:

- The aim of the respective study in relation to the thesis
- The authors and their contributions to the manuscripts
- The status of the study in publishing

The structure of this result section follows the ecdysteroid pathway. First, the early molt-related non-Halloween genes involved in the pathway are discussed. Then the mid pathway gene *sad* is examined in more detail, as it is considered the most ancient gene in ecdysteroid biosynthesis. In the third chapter, the remaining Halloween genes involved in the spider ecdysteroid pathway are compared. The last chapter focuses on another cytochrome which is responsible for the degradation of the active hormone and thus initializes the onset of molting.

3.1. Involvement of *neverland* and *shroud* in Hematopoiesis and Ecdysteroid Biosynthesis in the Spider *Parasteatoda tepidariorum*

This chapter elucidates the roles of the early ecdysteroid biosynthesis genes *neverland* and *shroud* in the spider *Parasteatoda tepidariorum*. The embryonic expression patterns are analyzed via single-cell RNA sequencing data. Expression validation and analyses on embryos and juveniles were performed using *in situ* hybridization, and RNA interference-mediated knockdowns were conducted on juveniles. Results indicate that *neverland* and *shroud* contribute to the molting process and suggest involvement beyond ecdysis, expanding the knowledge on hemocyte formation in spider embryogenesis.

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Author contributions to this research:

Denise Klinkenbuß: Conceptualization, Data curation, Formal analysis, Validation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Chetan Munegowda: Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Sandra Treffkorn: Methodology, Writing – review & editing.

Georg Mayer: Conceptualization, Funding acquisition, Project administration, Writing – review & editing.

Nikola-Michael Prpic-Schäper: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Status: **Finalized Manuscript** for Submission in Developmental Biology

Involvement of *neverland* and *shroud* in Hematopoiesis and Ecdysteroid Biosynthesis in the Spider *Parasteatoda tepidariorum*

Highlights

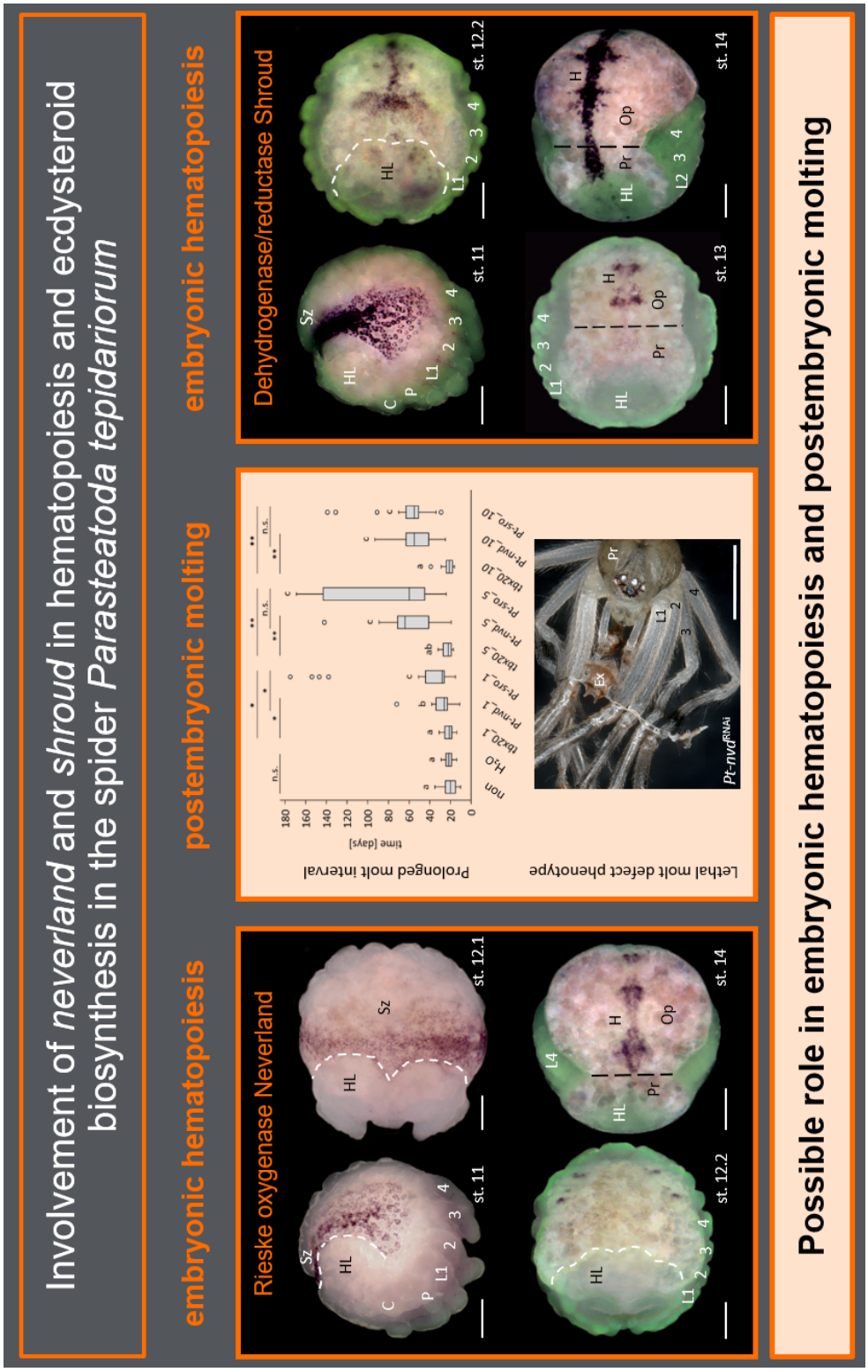
- *noppera-bo* and *CYP6t3* are not detectable in the spider genome.
- Possible function of *Pt-nvd* and *Pt-sro* in embryonic hemocyte formation.
- Postembryonic expression suggests roles in hematopoietic tissue and molting.
- RNAi with prolonged and defective molts, support a role in molting.

Abstract

Ecdysteroid hormones regulate critical developmental processes in arthropods, including diapause, metamorphosis and molting. The biosynthesis of the molting hormone from dietary cholesterol is a tightly regulated pathway involving the Rieske-domain oxygenase Neverland, the short-chain dehydrogenase/reductase Shroud and cytochrome P450 monooxygenases. Understanding the molecular roles of the early pathway genes *neverland* and *shroud* will provide crucial insights into the evolutionary conservation in arthropods. Therefore, this study aims to elucidate the roles and localization of *neverland* (*Pt-nvd*) and *shroud* (*Pt-sro*) expression in embryonic and postembryonic stages of the spider *Parasteatoda tepidariorum* and to assess their contribution in ecdysis. Phylogenetic analysis recovered both genes in the spider genome, while *noppera-bo* and *CYP6t3*, other early pathway genes, were undetectable. Employed *in situ* hybridization revealed similar spatiotemporal activity, including possible hematopoietic tissue of embryos and hemocytes in hemolymph carrying vessels in juveniles, as well as patterns in the prosomal neuroectoderm. RNA interference was conducted to explore the effects on molts, resulting in disrupted molt cycles and molting defects in juveniles, supporting a role in ecdysteroidogenesis. These findings highlight the impact of *neverland* and *shroud* in the spider. While the involvement of these genes in the ecdysteroid pathway is presumably conserved in arthropods, the expression in hemocytes may indicate diversification in endocrine tissues. These outcomes provide a first step for future research on ecdysteroidogenesis and hematopoiesis in arachnids.

Keywords: embryogenesis, postembryonic, Black Box, molting, arachnid

Graphical Abstract



3.1.1. Introduction

The molting hormones of Ecdysozoa regulate critical life events such as diapause, metamorphosis and molting (Thummel, 2001; Denlinger, 2002). In arthropods, ecdysone as the pivotal ecdysteroid hormone is synthesized from dietary cholesterol via a complex biosynthetic pathway (Niwa & Niwa, 2016; Yamanaka, 2021). Disruptions of ecdysteroidogenesis result in impaired cuticle formation, molt defects and broader developmental abnormalities, underscoring the critical role of precise pathway regulation (Jürgens et al., 1984; Wieschaus et al., 1984).

Recently, the glutathione S-transferase encoded by *noppera-bo* (*nobo*) was found to be involved in the intake of cholesterol in the fruit fly *Drosophila melanogaster* (Enya et al., 2014). However, knowledge of *nobo*'s function in other species remains limited, as research primarily focuses on a few insects (Enya et al., 2015; Koiwai et al., 2020; Inaba et al., 2022; Ebihara & Niwa, 2023). Similarly, the cytochrome P450 (CYP) gene *CYP6t3* was recovered in the fruit fly, with unresolved involvement in the biosynthesis Black Box and an uncertain presence beyond the genus (Saito et al., 2016; Shimell & O'Connor, 2022). The initial ecdysteroid biosynthesis step is catalyzed by the Rieske-domain oxygenase Neverland, converting dietary cholesterol into 7-dehydrocholesterol (Yoshiyama-Yanagawa et al., 2011; Kamiyama & Niwa, 2022). Following this, within the "Black Box" of biosynthesis, the short-chain dehydrogenase/reductase Shroud and cytochrome P450 (CYP) monooxygenase Spook transform the intermediate into 5ß-ketodiol (Namiki et al., 2005; Niwa et al., 2010; Knigge et al., 2021; Pan et al., 2021; Kamiyama & Niwa, 2022), although the precise mechanisms remain unresolved (Campli et al., 2024). The subsequent conversion steps are catalyzed by the CYP monooxygenases Phantom, Disembodied and Shadow (Gilbert et al., 2002; Gilbert, 2004; Gilbert & Rewitz, 2009; Schumann et al., 2018). Afterwards, ecdysone is released into the hemolymph and converted by Shade into the active molt hormone 20-hydroxyecdysone (Petryk et al., 2003; Liu et al., 2018). With the exception of *shade* being expressed in midgut, Malpighian tubes and fat body (Gilbert, 2004), synthesis-associated genes, including *neverland* (*nvd*) and *shroud* (*sro*), are predominantly expressed in the endocrine prothoracic gland of insects and Y-organ of crustaceans (Yoshiyama et al., 2006; Mykles, 2011; Legrand et al., 2021).

The ecdysteroid pathway and its localization remains poorly understood in non-insect arthropods like chelicerates (Niwa & Niwa, 2014a; Schumann et al., 2018; Pan et al., 2021; Campli et al., 2024). Within this early-diverging arthropod group (Dunlop, 2010), the presence of multiple CYP genes (*spook*, *disembodied*, *shadow* and *shade*) is reported (Qu et al., 2015; Dermauw et al., 2020). But the secreting tissues of e.g. spiders remain unknown as no homologous structure to the prothoracic gland is yet discovered (Sawadro et al., 2017). Notably, Yang et al. (2021) uncovered a steady expression increase in CYP monooxygenase coding genes during the intermolt of juvenile pond spiders (*Pardosa pseudoannulata*) with significantly higher levels in the abdomen of spiderlings. A recent study of *shadow* in embryonic and postembryonic development of *Parasteatoda tepidariorum* showed possible multifunctionality of ecdysteroid pathway genes, including neurogenesis and cardiogenesis, in addition to molt-related functions (Klinkenbuß et al., 2025). Despite the presence of a *nvd* sequence in *P. tepidariorum* recovered by phylogenetic analyses of Schumann et al. (2018), its tissue-specific expression, functional role and involvement in ecdysteroid biosynthesis remains unknown. Similarly, *sro* has not yet been studied in spiders. The common house spider, *P. tepidariorum*, has become an important model organism for investigating developmental processes (McGregor et al., 2008; Turetzek et al., 2024). Investigating the function of *Pt-nvd* and *Pt-sro* presents an opportunity to extend the understanding of the pathway's functional diversity across arthropods and its evolution in non-model organisms.

In this study, the following key questions are addressed: (1) which tissues exhibit mRNA expressions of genes at the junction between early ecdysteroid pathway and Black Box in embryogenesis and postembryonic stages of *P. tepidariorum*? (2) What are the potential associated developmental roles of *Pt-nvd* and *Pt-sro*? (3) Are the genes involved in spider molting? Exploring these questions will provide insights into the evolutionary conservation of early ecdysteroid pathway genes in arthropods.

3.1.2. Results

3.1.2.1. Identification and verification of early ecdysteroid pathway genes

Full-length orthologs of the ecdysteroid pathway genes *Pt-nvd* (NCBI accession: XM_043045290.1) and *Pt-sro* (NCBI accession: XM_043042224.1) were identified and verified via cloning and sequencing. The genes contain an open reading frame of 1,680 and 1,005 base pairs, encoding proteins of 559 and 334 amino acids, respectively. Gene organization analysis predicts that *Pt-nvd* consists of nine exons, while *Pt-sro* occurs in two isoforms, the longer one comprising five and the short isoform three exons (Fig. 7). Both isoforms encode identical proteins but differ in their 5´UTR.

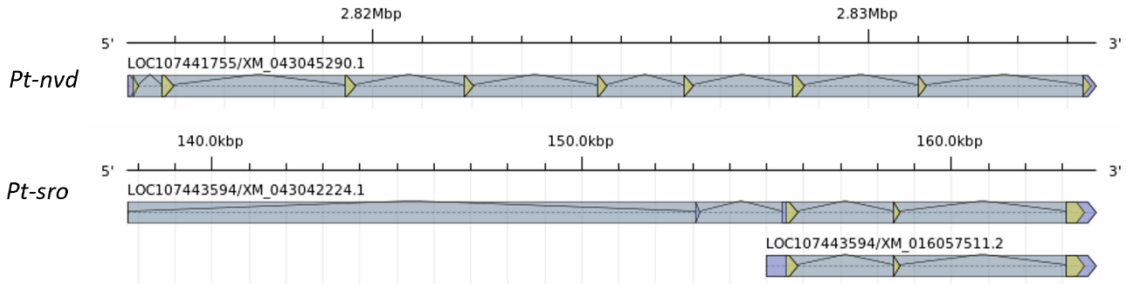


Figure 7 Genomic organization of *Pt-nvd* and *Pt-sro*. Gene location and exon-intron structure spanning ~20 kbp in *Pt-nvd*, and 26 and 9 kbp in *Pt-sro*, respectively. Exonic regions (E1–E9) shown in yellow, introns in grey and untranslated regions in blue.

No orthologs were recovered for the glutathione S-transferase gene *nobo* or CYP gene *CYP6t3* from the *P. tepidariorum* genome. The presumed *nvd* and *sro* orthologs cluster together in their respective trees and form monophyletic groups against the outgroups (Fig. 8). Within the designated *nvd* cluster, *Pt-nvd* forms a monophyletic group with the chelicerates and the myriapod, showing higher similarity to those of Arachnopulmonata (Fig. 8A). In the resulting tree of *sro* orthologs, Pt-*sro* is grouped within the chelicerate cluster (except *Trichonephila clavipes*; Fig. 8B).

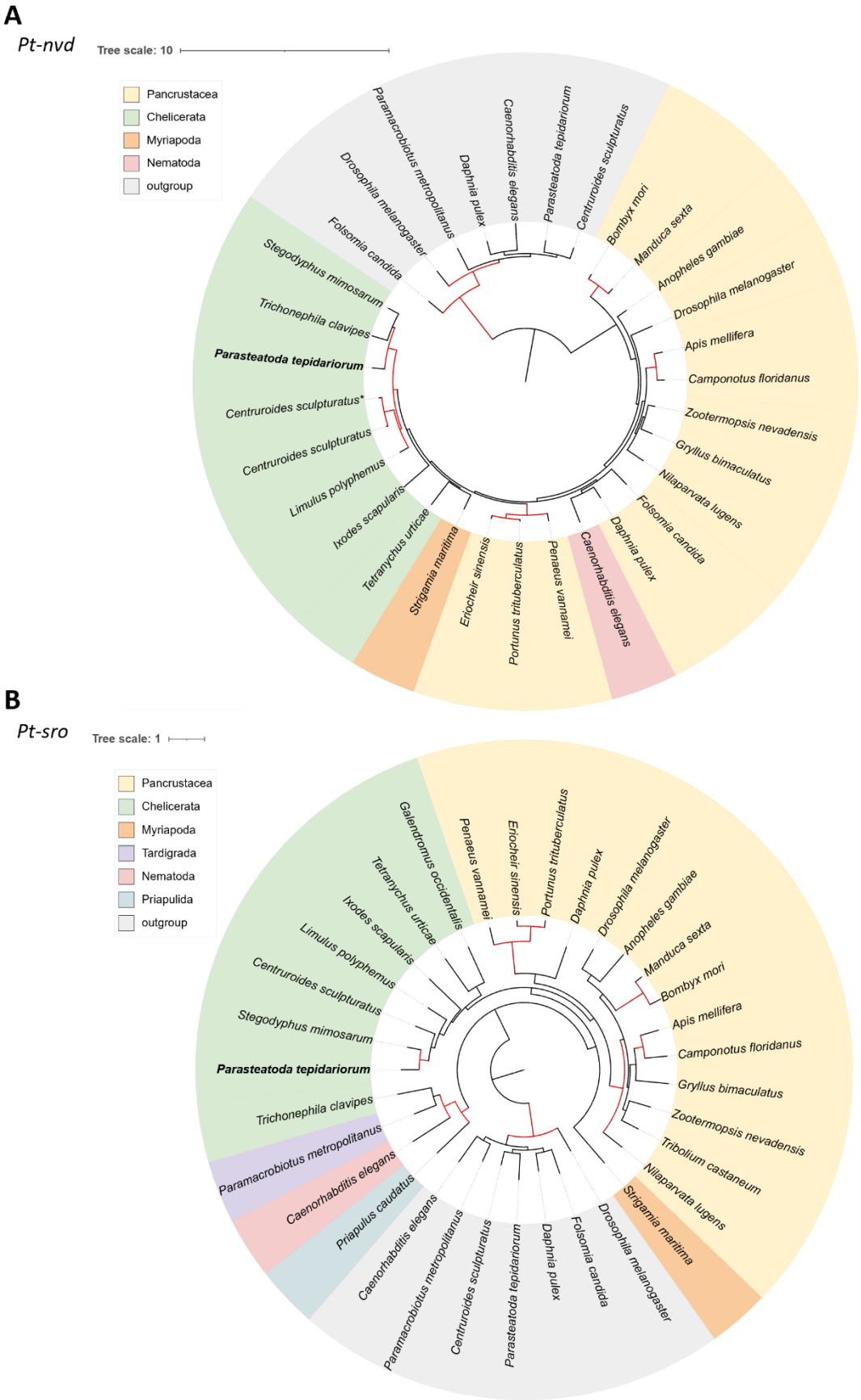


Figure 8 Phylogenetic conservation of *Pt-nvd* and *Pt-sro* in ecdysozoans. (A) Protein-based gene tree of *nvd* orthologs and (C) *sro* orthologs from various ecdysozoans (Supplementary Tab. 1, 2). Red branches indicate bootstrap support values greater than 80 obtained from 10,000 pseudoreplicates. Asterix indicates gene duplication.

3.1.2.2. Expression analyses of Pt-nvd and Pt-sro during embryogenesis

Uniform manifold approximation and projection plot (UMAP) of *Pt-nvd* and *Pt-sro* expression from single-cell RNA sequencing (scRNA-seq) data containing embryonic stages 7 to 9 (Leite et al., 2024) displays a small number of expressing cells (Fig. 9A–A’’). Expression in these stages was detected in cluster 5 (Fig. 9C), the cells of which are defined as “dorsal” tissue. Number of *Pt-sro* mRNA expressing cells is approximately 2.5 times higher than *Pt-nvd*. Additionally, the expression of *Pt-sro* is evident in cluster 20, associated with extra-embryonic cells contributing to the endoderm.

UMAP projection of *Pt-nvd* and *Pt-sro* from scRNA-seq data containing pooled embryonic stages 10 to 12 (Medina-Jiménez et al., 2024a) exhibit a wide range of expressing cells (Fig. 9B–B’’) and assigned clusters (Fig. 9D). For both genes, mRNA was found in cluster V, exhibiting the developing central nervous system with suggested epithelial-to-mesenchymal transitions-like processes in neuronal precursor determination. *Pt-nvd* is also identified in cluster I assigned to dorsal tissue and ectoderm patterning of developing appendages and cluster XXII representing the stomodaeum and ventral midline. *Pt-sro* is additionally expressed in a multitude of clusters, including neuronal precursors (cluster XI), early differentiating neurons (clusters VI, VIII, X and XVII), differentiating and differentiated neurons (clusters II and XII) and sensory nervous system of the head (cluster XIX). Ectoderm patterning in the developing appendages is another cluster assigned function of *Pt-sro* (clusters IV and XV). Furthermore, the gene is detected in cells of the ventral sulcus (cluster XIV) and interestingly in cluster XXI corresponding to functions in midgut development, yolk metabolism, immune response and hematopoiesis.

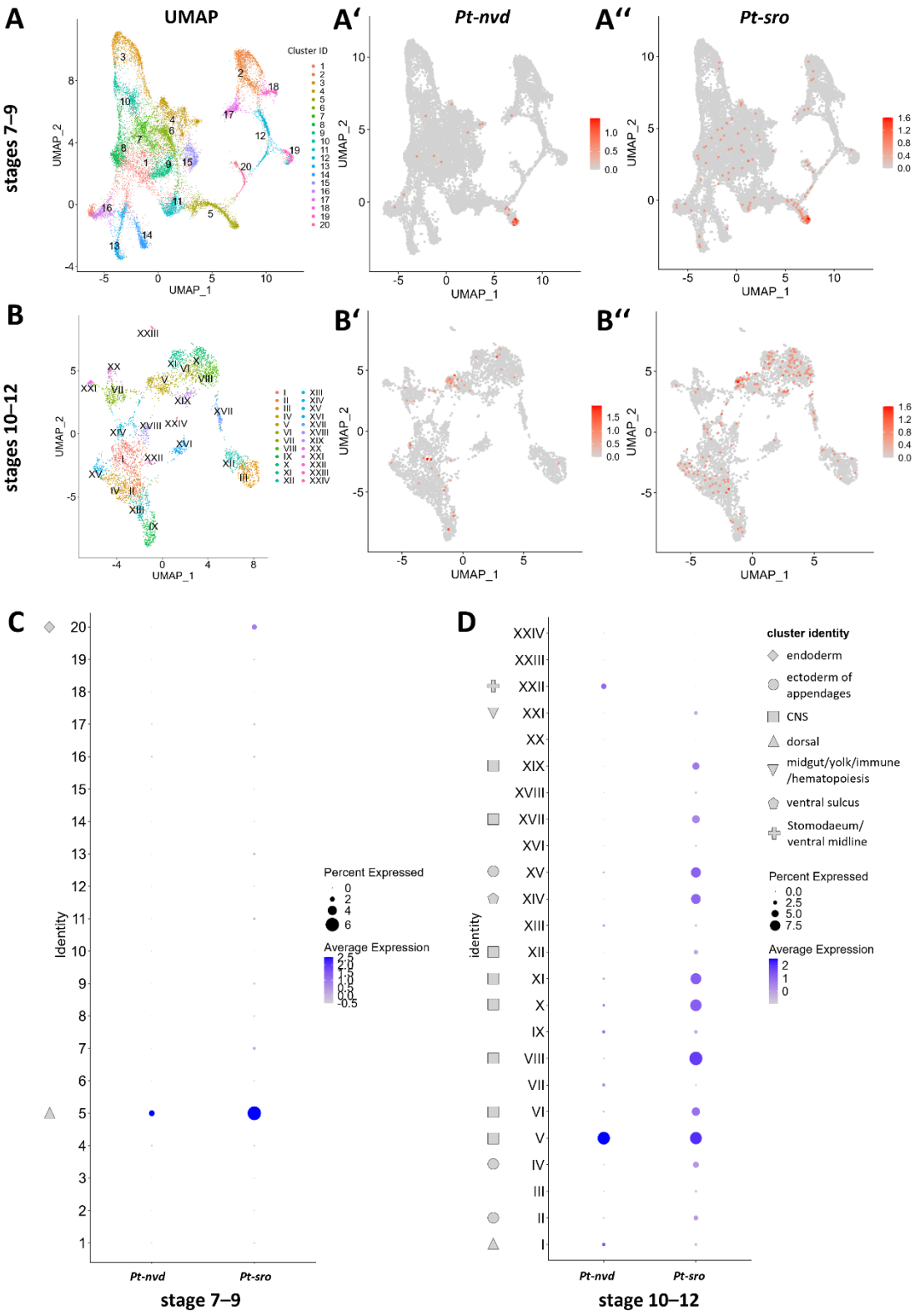


Figure 9 *Pt-nvd* and *Pt-sro* expression in embryogenesis from scRNA-seq data. UMAP for rPCA integrated embryonic stages 7 to 9 (Leite et al., 2024) shows (A) the cell clusters, (A') cells expressing *Pt-nvd* and (A'') cells expressing *Pt-sro*. UMAP for embryonic stages 10 to 12 (Medina-Jiménez et al., 2024a) shows the (B) cell clusters, (B') cells expressing *Pt-nvd* and (B'') cells expressing *Pt-sro*. Red gradient indicates expression level. (C) Dot plots show expression of *Pt-nvd* and *Pt-sro* in clusters for rPCA integrated embryonic stages 7 to 9, and (D) stages 10 to 12. Percentage of cells per cluster expressing the gene corresponds to the dot size, and average expression level to the blue gradient. Symbols next to axis indicate summarized similar cluster identity.

mRNA localization of *Pt-nvd* and *Pt-sro* using whole-mount *in situ* hybridization during embryogenesis reveals dynamic expression loci (Fig. 10, 11). Both genes displayed expression in the thin yolk covering tissue from embryonic stages 8 to 11 (Fig. 10A–D; 11A–D). The expression progresses similarly, but more pronounced in *Pt-sro*. At stage 8, staining begins in several large cells in the yolk covering tissue (Fig. 10A, 11A) and increases in number and staining intensity over stages 9 and 10 (Fig 10B, C; 11B, C). Expression localizes dorsally towards the gap between the posterior head lobe margin and segment addition zone at stage 11 (Fig. 10D, 11D). With retraction of the segment addition zone at dorsal closure initiation (stage 12), stained cells accumulate in the cleft formed by tergites anterodorsally overgrowing the yolk (Fig. 10E, 11E). Subsequently, *Pt-nvd* and *Pt-sro* expressions form dorsal bilateral symmetric clusters in the margin of yolk overgrowing tissue (Fig. 10F, 11F).

At stage 13, after constriction of pro- and opisthosoma, the expression extends anteriorly into a tube-like pattern, reaching from the posterior opisthosomal region to the head lobes (Fig. 10G, 11G). Along the tubular pattern, recurring triangular thickenings occur (Fig. 10H, I; 11H, I). The size of expressing cells decreased progressively with embryonic development, transforming from large cells in the yolk covering tissue (Fig. 10B', E'; 11A', D', H') to minute cells in the heart-related structure (Fig. 10H', 11H').

During late embryonic stages *Pt-nvd* and *Pt-sro* display differing bilateral symmetric expression patterns in the forming brain (Fig. 12). *Pt-nvd* shows two small cell clusters at the posterior periphery of the developing brain lobes (Fig. 12A–C) at stages 13 and 14, whereas *Pt-sro* demonstrates a more dynamic expression development (Fig. 12D–F). At stage 13, expression arises in four cell clusters (Fig. 12D) that increase in size (Fig. 12E) and ultimately form ring-shaped patterns covering almost the entire lobes at stage 14 (Fig. 12F).

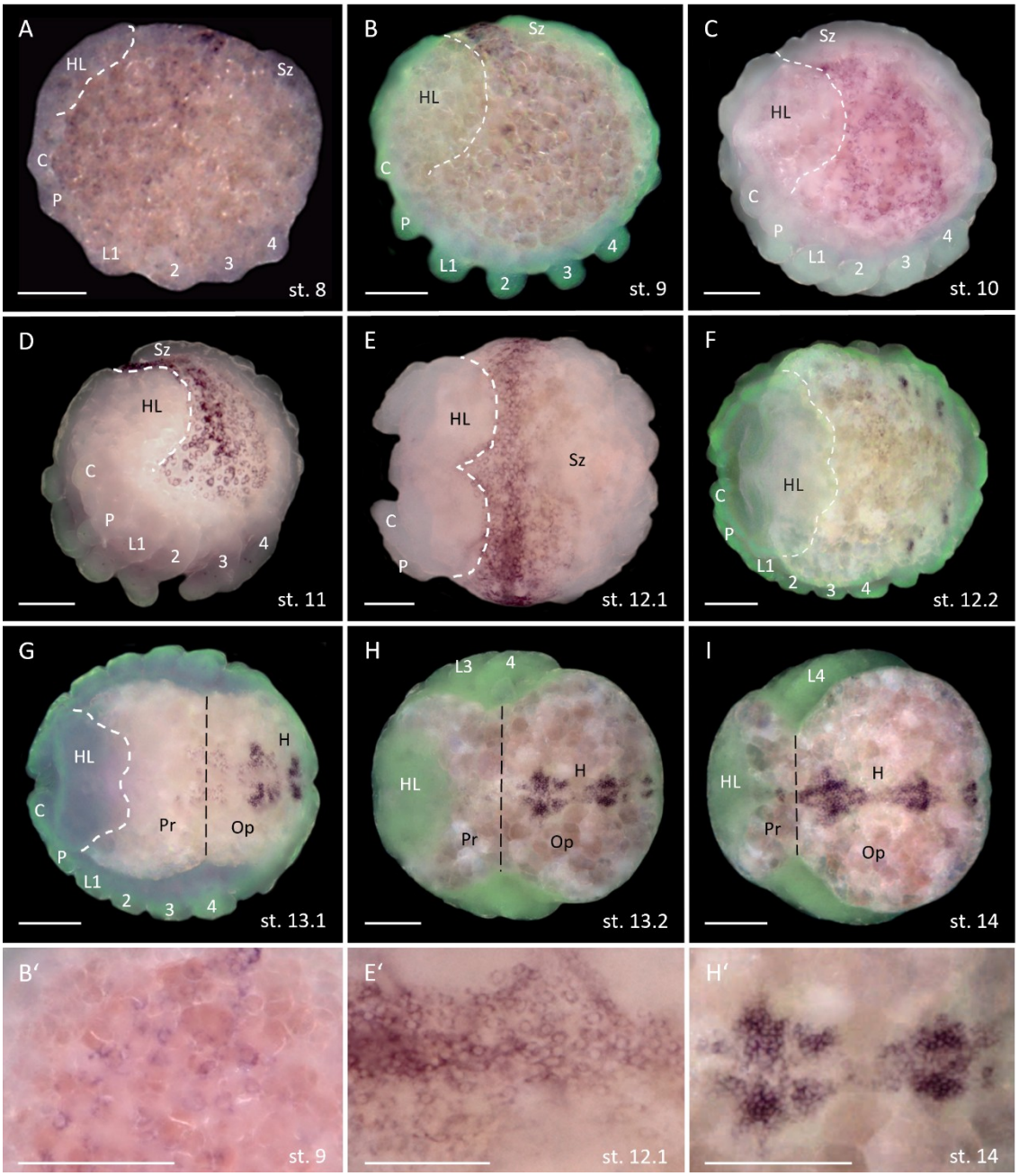


Figure 10 Embryonic expression of the ecdysteroid pathway gene *Pt-nvd*. Lateral view with dorsal facing up (A–D); dorsal view with anterior to the left (E–I). Nuclear marker SYTOX in green (B, C, F–I). (A–D) Increasing expression in single cells covering the yolk from stages 8 to 11. (E) Expression in yolk covering cells intensifying at stage 12.1. (F) Clustered expression on the margin of yolk overgrowing dorsal tissue at stage 12.2. (G–I) Expression located in the posterior to anterior developing cardiogenic tissue in stages 13.1 to 14. (B', E', H') Zoomed in images of stained cells at stages 9, 12.1 and 14. C, chelicera; HL, head lobes; H, heart; L1–L4, legs 1 to 4; Op, opisthosoma; P, pedipalp; Pr, prosoma; Sz, segment addition zone. Scale bar: 100 µm.

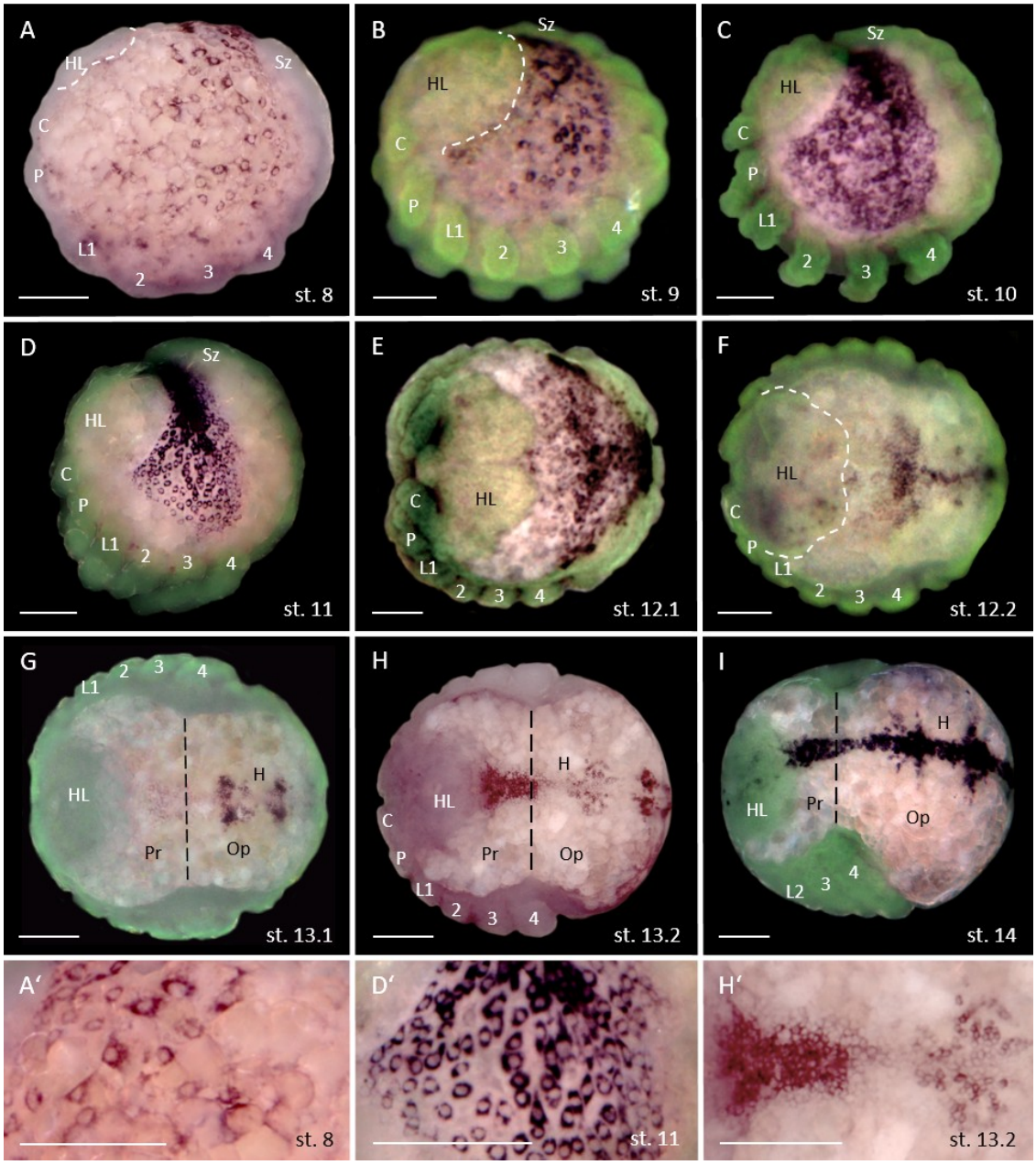


Figure 11 Embryonic expression of the ecdysteroid pathway gene *Pt-sro*. Lateral view with dorsal facing up (A–D); dorsal view with anterior to the left (E–I). Nuclear marker SYTOX in green (B–G; I). (A–E) Strong mRNA expression in single cells covering the yolk from stages 8 to 12.1. (F) Dynamic expression is displayed at stage 12.2. (G–I) Expression of *Pt-sro* located in the cardiogenic tissue of stages 13.1 to 14. (A', D', H') Zoomed in images of stained cells at stages 8, 11, and 13.2. C, chelicera; HL, head lobes; H, heart; L1–L4, legs 1 to 4; Mt, Malpighian tubes; Op, opisthosoma; P, pedipalp; Pr, prosoma; Sz, segment addition zone. Scale bar: 100 µm.

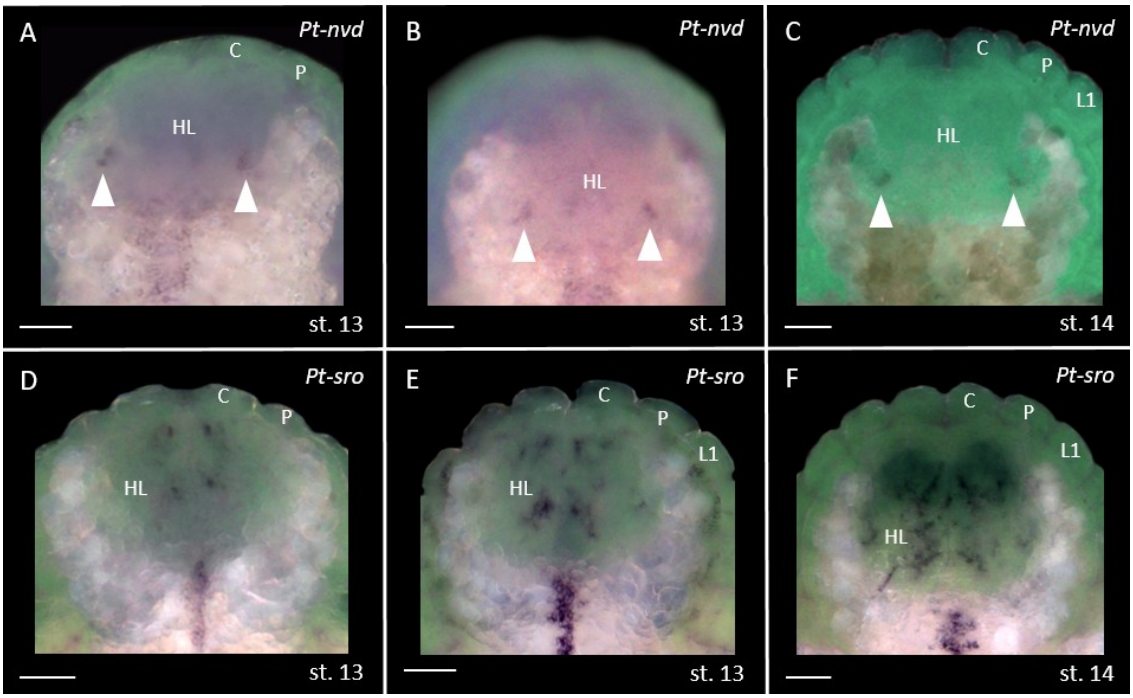


Figure 12 Expression of pathway genes *Pt-nvd* and *Pt-sro* in embryonic brain development. Dorsal view with anterior facing up. Nuclear marker SYTOX in green. (A–C) Expression of *Pt-nvd* (in purple) as two small cell clusters in the posterior periphery of the brain lobes (arrowheads) at stage 13 and 14. (D–F) Dynamic expression of *Pt-sro* in the brain lobes at stages 13 and 14. C, chelicera; HL, head lobes; L1, first leg pair; P, pedipalp. Scale bar: 50 µm.

3.1.2.3. Expression of Pt-nvd and Pt-sro during postembryonic development

For this study, time between molts in juveniles is divided into four stages, the post-, inter- and pre-molt as well as ecdysis (Fig. 2). In the intermediate stage, between the fourth and fifth molt, no distinct *Pt-nvd* expression is visible throughout the supra- and subesophageal ganglion of the prosoma (Fig. 13A, C). During the pre-molt interval, expression is detected in the distal periphery of the mushroom body within the supraesophageal ganglion and the dorsal visceral cortex resembling structure (Fig 13B). In the opisthosoma, singular cells expressing mRNA are visible in the midgut tissue and heart at the end of posterior extension (Fig. 13D, arrow). In cross-sections, these cells are more apparent in the heart and efferent vessels extending towards the location of the Malpighian tubes (Fig. 13E). Prosoma staining of *Pt-sro* is located predominantly in the subesophageal ganglion of the brain (Fig. 13F–H). In cross-sections distinct bilateral-symmetric cell clusters (salt-and-pepper pattern) become evident (Fig. 13H). mRNA is detected in the distal periphery of the mushroom body and the visceral cortex resembling structure in some sections (Fig. 13G, H). Posterior prosoma expression is likely caused by staining of hemolymph leaking from the anteriorly extending aorta.

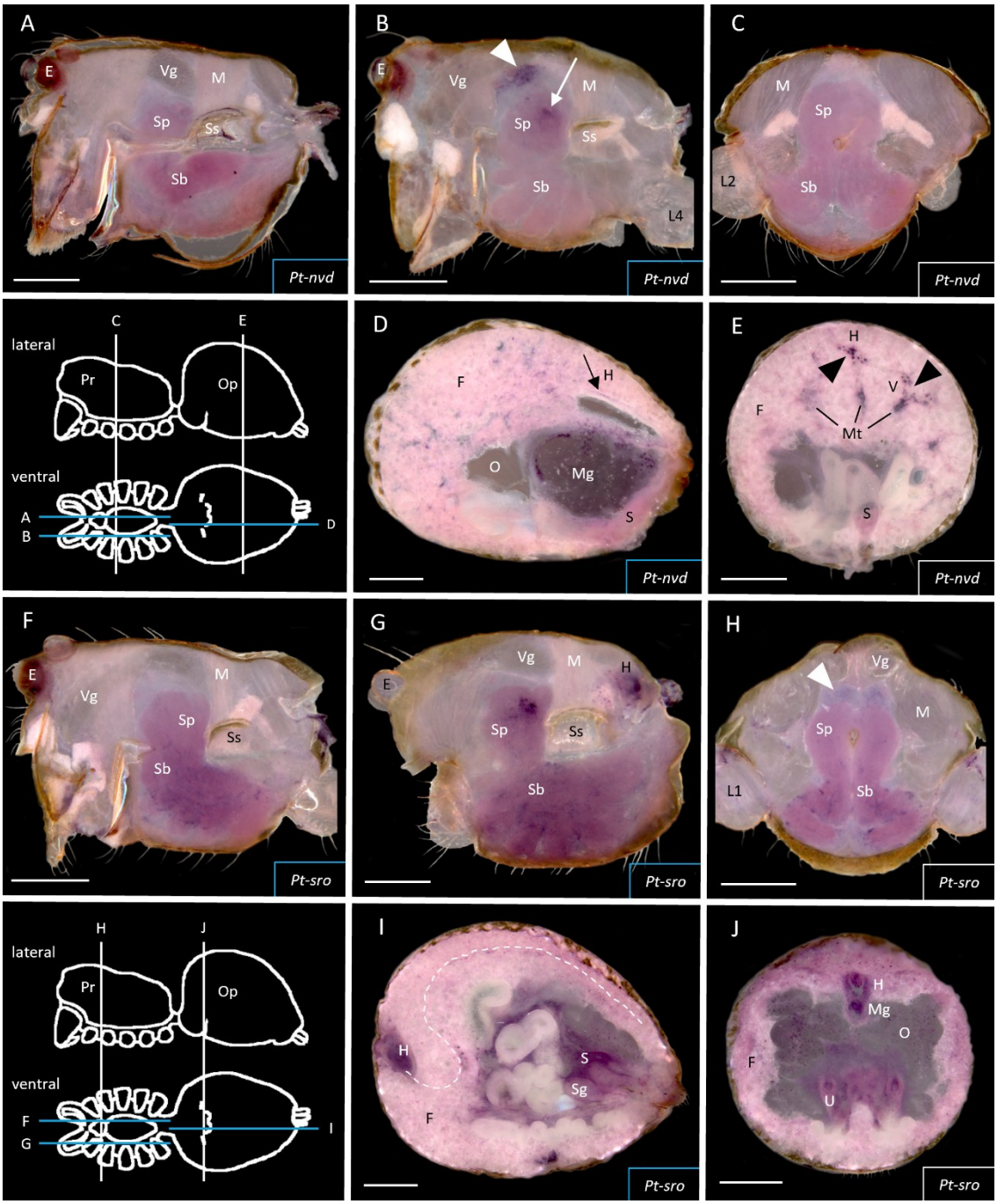


Figure 13 Postembryonic expression of *Pt-nvd* and *Pt-sro*. Expression of *Pt-nvd* (A–E) and *Pt-sro* (F–J) in purple, shown in prosomata (A–C, F–H) and opisthosomata (D, E, I, J). The morphological schemes [redrawn after Foelix (1996)] indicate section planes. Anterior is left in the scheme; dorsal is top in all images. (A) No distinct *Pt-nvd* expression. (B) Expression in distal section of visceral cortex (white arrowhead) and supraesophageal ganglion (arrow). (C) No distinct expression posterior to first leg pair. (D) Expression in heart (arrow) and midgut in opisthosoma. (E) Expression in heart and efferent vessels (black arrowheads). (F) Expression of *Pt-sro* in subesophageal ganglion. (G) Expression in distal part of supraesophageal ganglion. Posterior expression likely corresponds by aorta extending in prosoma. (H) Expression in cell clusters of subesophageal ganglion and visceral cortex (white arrowhead). (I) Expression in heart (dashed line). Staining between organs is likely artificial due to adhesive property of leaked silk. (J) Expression in the heart and ovary, artificial staining in midgut caused by gut contents. B, book lungs; E, eye; F, fat body; H, heart; L1–L4, legs 1 to 4; M, muscles; Mg, midgut; Mt; Malpighian tubes; Op, opisthosoma; O, ovary; Pr, prosoma; S, silk; Sb, subesophageal ganglion; Sg, silk gland; Sp, supraesophageal ganglion; Ss, sucking stomach; U, uterus; V, vessel; Vg, venom gland. Scale bar 200 µm.

In sagittal opisthosoma sections, an expression is visible in the heart tissue extending dorsally from the petiolus to the spinnerets (Fig. 13I, dashed line). The intense staining outside of distinct tissues is presumably artificial and caused by the adhesive properties of leaked silk components, also detected in some controls (Supplementary Fig. 3). The heart-related *Pt-sro* expression is more apparent in the opisthosomal cross-section (Fig. 13J). Expression is also accumulated in scattered dots covering the ovary.

3.1.2.4. Effect of Pt-nvd and Pt-sro RNA interference on molting

RNA interference (RNAi) of *Pt-nvd* and *Pt-sro* was conducted on subadult males, defined as the interval between penultimate and final molt, to evaluate the effects on the molting process (Fig. 14). For the molt duration, control groups (no injection, H_2O and *tbx-20*) with average molting times of 21.5 to 22.8 days were compared to double-stranded RNA (dsRNA) treatments of both ecdysteroid pathway genes. Significantly prolonged molt intervals were observed in *Pt-nvd* and *Pt-sro* groups (Fig. 14A), being most distinct at later injection times (5 and 10 days). Additionally, molt-related mortality was only present in specimens treated with dsRNA of the ecdysteroid pathway genes, being most severe for treatments 5 days after penultimate molt. The *Pt-nvd_5* group also resulted in the most severe molt delay, with a median of 64 days. Groups treated with dsRNA of *Pt-sro* displayed multiple outliers surviving up to 175 days in subadult stage before initiation of ultimate molt. However, no significant deviation was detectable between *Pt-nvd* and *Pt-sro* treatments, except for dsRNA injections one day after penultimate molt. Dead spiders either displayed absent molt initiation, resulting in death long after controls completed the molting interval or defects during the molt process. These lethal molting defects prevented the separation between old and new cuticles of intricate structures, including the legs, bulbs (Fig. 14C) and book lungs (Fig. 14D). Variation of post-molt survival time between controls and dsRNA treatment with *Pt-nvd* and *Pt-sro* was highly significant (Fig. 14B). Spiders of control groups survived on average 74.2 to 77.9 days, while most effected pathway gene injection group (5 days) survived only 36.2 days. The *Pt-nvd_10* and *Pt-sro_10* groups exhibit survival times closest to the controls.

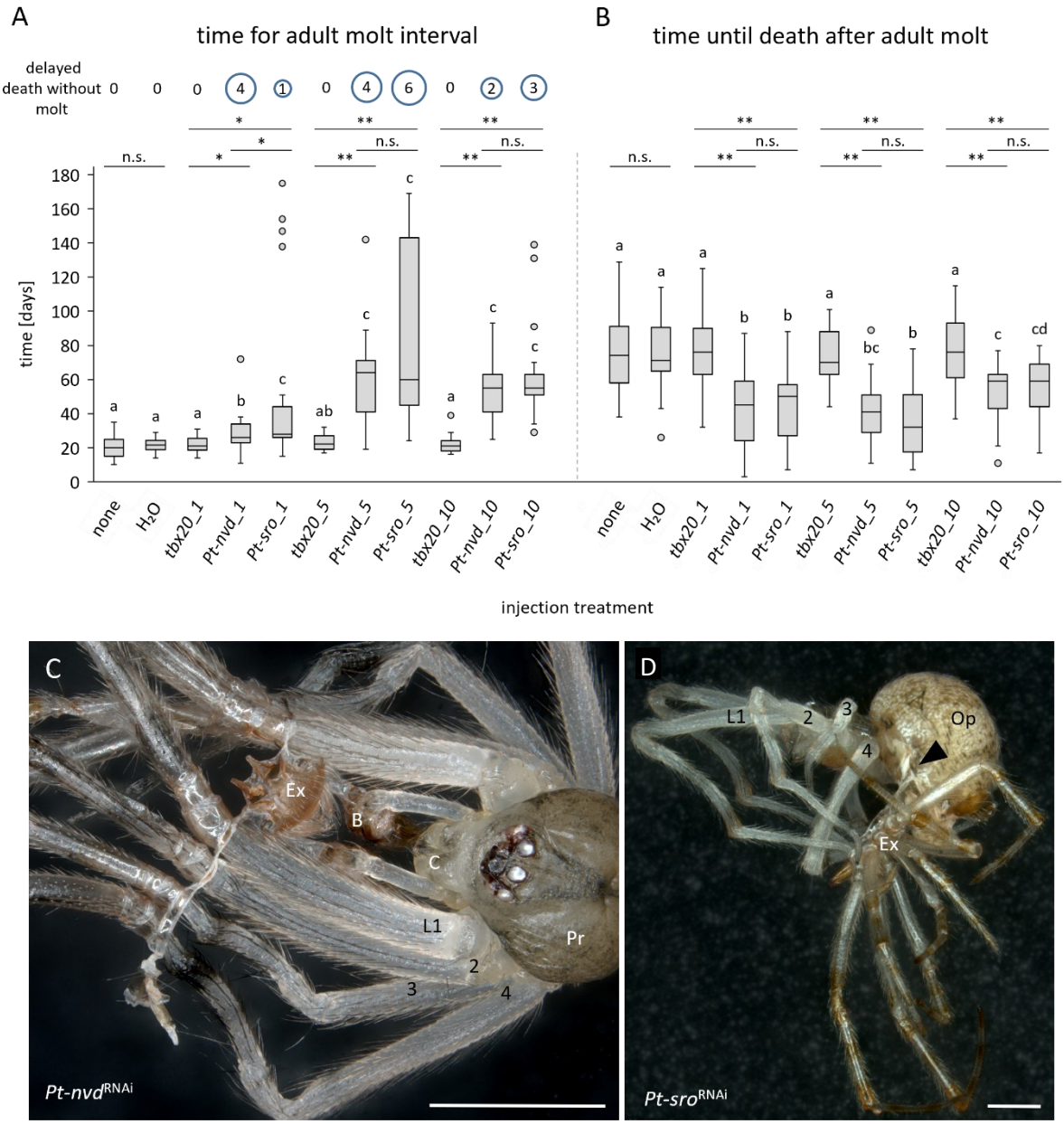


Figure 14 Effect of *Pt-nvd* and *Pt-sro* RNAi on subadult males after penultimate molt (APM). (A) Time for molting from subadult to adult in subadults subjected to different treatments: none (no injection), H_2O (control, one day APM), dsRNA of *tbx20* (gene control), *Pt-nvd* and *Pt-sro* at multiple time points (1, 5, 10 days APM). N > 20 for all treatment groups. (B) Lifespan after the last molt in spiders subjected to different treatments. Asterisks indicate significant differences at P>0.05, n.s. (not significant); *, P<0.05; **, P<0.001. Letters above bars (a, b, c, d) indicate significant differences between groups. (C) Molting defect after RNAi with *Pt-nvd* dsRNA. Fine bulb structure unable to separate. (D) Molting defects after *Pt-sro* dsRNA treatment. Book lung tissue inseparable (arrowhead). B, bulb; C, chelicera; Ex, exuvia; L1–4, legs 1 to 4; Op, opisthosoma; Pr, prosoma. Scale bar: 2 mm.

3.1.3. Discussion

3.1.3.1. Identification and phylogeny of early pathway genes Pt-nvd and Pt-sro

In *D. melanogaster*, the glutathione S-transferase encoding *noppera-bo* (*nobo*) plays a direct role in cholesterol uptake for ecdysteroid hormone synthesis (Enya et al., 2014). However, *nobo* was not recovered from the spider genome. As arthropods diversified (Giribet & Edgecombe, 2019), the ecdysteroid pathway appears to have undergone evolutionary modifications. With reports only from Diptera and Lepidoptera (Ebihara & Niwa, 2023), *nobo* possibly has evolved first within insects, suggesting the presence of alternative cholesterol uptake pathways in ecdysteroidogenesis for spiders. On the same note, *CYP6t3* was previously only studied in the fruit fly with conflicting results regarding its involvement in ecdysteroid biosynthesis (Saito et al., 2016; Shimell & O'Connor, 2022). *CYP6t3* was also absent from the spider genome.

The identification and phylogenetic analysis of full-length *Pt-nvd* and *Pt-sro* orthologs provides valuable insights into the conservation and evolution of early ecdysteroid pathway genes in arthropods, marking the first report of *sro* and expression profiling of *nvd* and *sro* in spiders. The gene organization of *Pt-nvd* exhibits nine exonic regions. *Pt-sro* displays two isoforms of five and three exons, respectively. Both isoforms are predicted to encode for the same protein. These findings contribute to the limited knowledge of intron-exon structures in spider pathway genes, previously only studied in cytochrome P450 genes (Yang et al., 2021). For comparison, *nvd* in *D. melanogaster* has six exons, while *sro* contains two (NCBI Genome Data Viewer, version 3.49). In the hemimetabolous insect *Acyrthosiphon pisum*, *nvd* seems absent, while *sro* is retained with two isoforms, each comprising five exons. The observed gene structure variations across arthropods indicate that, while the ecdysteroid biosynthesis pathway is functionally conserved, modifications occur.

Protein-based gene tree analysis of *nvd* orthologs formed a monophyletic group including sequences from chelicerates and the myriapod *Strigamia maritima*. Within this cluster *Pt-nvd* showed higher similarity to sequences from Arachnopulmonata, suggesting its conservation across chelicerates and underscoring the retention of key ecdysteroid pathway genes in arachnids. This is consistent with previous studies, where *nvd* plays a central role in the biosynthesis (Yoshiyama-Yanagawa et al., 2011; Kamiyama & Niwa, 2022). Similarly, all *sro* orthologs of included chelicerates, except

for *Trichonephila clavipes*, form a cluster indicating evolutionary consistency. The gene was initially discovered in *D. melanogaster* as a critical "Black Box" enzyme (Niwa et al., 2010), and identification in the spider confirms it is not an insect-specific innovation. Similar to insects, chelicerate orthologs of *sro* likely participate in converting cholesterol-derived intermediates into ecdysone (Niwa et al., 2010; Pan et al., 2021). These findings align with studies showing highly conserved ecdysteroid pathway genes in Pancrustacea, with modifications in gene presence or structure occurring across distant clades (Lafont et al., 2012; Wan et al., 2014; Schumann et al., 2018; Yang et al., 2021).

3.1.3.2. Involvement in hematopoietic cell formation during embryogenesis

Expression analyses from scRNA-seq data of rPCA integrated embryonic stages 7 to 9 (Leite et al., 2024) display *Pt-nvd* restricted to cluster 5, and *Pt-sro* in clusters 5 and 20. Cluster 20 corresponds to mRNA expression in extra-embryonic cells contributing to the endoderm, defined by marker genes like *fuchi, GPCPD*, *HSP* and *g12621* (Iwasaki-Yokozawa et al., 2022). Cluster 5 markers are mostly dorsal determinants like *BMPR*, *noggin-D* and *GATA1*, they represent cells in the extra-embryonic region considered to originate from the mesenchymal cumulus underneath epithelial cells (Akiyama-Oda & Oda, 2003 & 2006). *Pt-nvd* and *Pt-sro* patterns obtained via whole-mount *in situ* hybridization were consistent with the expected cell clusters generated from scRNA-seq. Both genes exhibit expression in cells corresponding to this cluster in embryonic stages 8 and 9 with stronger detection in *Pt-sro*. In scRNA-seq data obtained from early embryogenesis stage 5 the markers also corresponded to a cluster defined as cumulus-related endodermal and mesodermal tissue (Supplementary Figure 1; Akiyama-Oda & Oda, 2010; Akiyama-Oda et al., 2022). Corresponding cells from data of stages 7 to 9 further show *hepatocyte-nuclear factor-4* and *serpent*/*GATA4* expression (Feitosa et al., 2017; Iwasaki-Yokozawa et al., 2022). serpent/GATA4 acts as a canonical hematopoietic transcription factor in *D. melanogaster* and other arthropods. It is necessary for hemocyte lineage specification and differentiation (Lebestky et al., 2000; Waltzer et al., 2002). Cells expressing this gene are strongly linked to hemocyte progenitors or early hematopoietic activity (Sorrentino et al., 2005; Muratoglu et al., 2006). Combined with the observation that both clusters 5 and 20 exhibit enhanced G1 phase cells, indicating proliferation and differentiation of progenitor cells in the study of Leite et al. (2024), the involvement of *Pt-nvd* and *Pt-sro* in hemocyte formation is likely.

The cells on the spider extra-embryonic region expressing *Pt-nvd* and *Pt-sro* might correspond to pre-hemocytes with potential conservation between insects and chelicerates. These cells around the dorsal head region may give rise to mesodermal hemocyte progenitors. Likewise, in *D. melanogaster* one origin of embryonic hemocytes is restricted to a region within the head mesoderm (Holz et al., 2003; De Velasco et al., 2006; Spahn et al., 2014). Similar to *neverland* and *shroud*, the heart marker *Mef2.1* (cluster 19, Leite et al., 2024) displays expression in dorsal cells located around the forming head lobes which migrate over the extra-embryonic region during mid-embryonic stages. *Mef2.1* participates in mesenchymal heart tube formation in late spider embryogenesis after dorsal closure (Janssen & Damen, 2008; Mittmann & Wolff, 2012). Compared to its tubular pattern, *Pt-nvd* and *Pt-sro* display a domain within the dorsal heart tube, further supporting a possible role in hemolymph or hemocyte formation. This finding points toward a connection of hematopoiesis and endocrine function in the spider. Therefore, the linkage of the ecdysone secreting prothoracic gland and the hemocyte progenitors both originating from embryonic head mesoderm in *D. melanogaster* is intriguing (Tepass et al., 1994; Holz et al., 2003; De Velasco et al., 2006). The spider's molting hormone might be synthesized within the hemocytes or a specialized subset of hemocyte-forming mesoderm (without necessarily implying a direct role in hematopoiesis). This would allow ecdysteroids to act locally on developing hemocytes or to be efficiently released into the hemolymph (Tingvall et al., 2001; Jacobs et al., 2014). This is supported by observations in insects wherein ecdysone signaling can influence immune response and hemocytes during development (Regan et al., 2013; Rus et al., 2013; Vieira et al., 2024). In either case, this study suggests a previously overlooked topological and ontogenetical connection between the endocrine molting gland-equivalent in spiders and hematopoietic or circulatory systems in embryos. This finding enables investigation into whether ecdysteroids have functional roles in spider hemocyte development or immune system function.

Expression analysis of pooled scRNA-seq data of embryonic stages 10 to 12 (Medina-Jiménez et al., 2024a) indicates *Pt-nvd* expression in dorsal tissue, stomodaeum/ventral midline and a neuroectoderm cluster, while *Pt-sro* is expected in midgut/yolk/immune/hematopoiesis, ventral sulcus and a variety of ectodermal clusters. When validating the predicted gene expressions via whole-mount *in situ* hybridization, expressions only occurred in the cells covering the yolk, later displaying mRNA in heart-

related tissues. The inconsistencies between the expected expression from scRNA-seq and observed expression might be attributed to the pooled sequencing of stages 10 to 12 in combination with low sequencing depth. First, cells covering the yolk could be underrepresented in the pooled sample due to dissociation sensitivity or rare cell types resulting in reduced capture during library preparation (Zheng et al., 2017; Young & Behjati, 2020). According to Medina-Jiménez et al. (2024a) these cells may be disproportionately removed during sample preparation in their study. Secondly, clustering algorithms tend to prioritize dominant transcriptional signatures, such as abundant ectodermal cell populations in stage 10 to 12, while masking subtle differences from rare cell types leading to incorrect clustering or omission of rare cell populations (Haque et al., 2017). Lastly, low sequencing saturation (~11%; Medina-Jiménez et al., 2024a) may limit detection of low-abundance transcripts, diminishing the interpretability of cell clustering resolution. This could lead to unresolved heterogeneity causing artificial mega-clusters as seen in Medina-Jiménez et al. (2024a). Taken together, these factors likely contribute to the discrepancies observed between the scRNA-seq and *in situ* results. Despite improvements in the reanalyzed scRNA-seq dataset (Supplementary Figure 2; Medina-Jiménez et al., 2024b), obtained *in situ* hybridization data does not align with predicted expression domains, highlighting the need for experimental mRNA expression validation in complex embryonic tissues.

In stages 13 and 14, RNA was detectable in some parts of the nervous system, showing varying domains in the developing brain. *Pt-sro* displays a more complex expression, forming ring-like patterns in late embryogenesis, while *Pt-nvd* presents two small dots in the posterior periphery of the brain. The role in the brain might be to establish sensory structures or pathways as it occurs during late embryogenesis (Mittmann & Wolf, 2012; Schomburg et al., 2015; Baudouin Gonzalez et al., 2025).

3.1.3.3 Involvement of ecdysteroid pathway genes during postembryonic development

A conserved feature of *nvd* and *sro* function appears in ovaries of (sub-)adult females in spiders and insects (Niwa et al., 2010). Expression analyses via whole-mount *in situ* hybridization on postembryonic stages indicate expression of *Pt-sro* in developing ovarian tissue in the opisthosoma. This points towards a role in the reproductive metabolism, like support of oocyte maturation or vitellogenesis in maturing females. Consistent with ovarian follicle cells producing ecdysteroids for egg development in

insects (Gilbert et al., 2002; Uryu et al., 2015) a preliminary study on the spider *P. pseudoannulata* also revealed pathway gene transcripts in spiderling abdominal tissues and adult ovaries (Yang et al., 2021).

Additionally, the gene transcripts enriched in the dorsal heart tube and associated pericardial tissue of the opisthosoma indicate involvement in the spider's circulatory system. The spatial restriction of *Pt-nvd* and *Pt-sro* within the hemolymph caring vessel implies that this structure actively synthesizes ecdysteroid precursors, while directly releasing them into the hemolymph. This contrasts with the known synthesis function of the prothoracic gland in insects which is composed of the *corpora cardiac* and *corpora allata*. No analogous organs are discovered in spiders up to this date (Sawadro et al., 2017). Furthermore, spatial expression levels of the pathway genes *spook*, *disembodied*, *shadow* and *shade* were significantly higher in the opisthosoma than in the prosoma in *P. pseudoannulata* spiderlings (Yang et al., 2021). This pronounced abdominal localization weakens the hypothesis of a functional prothoracic gland equivalent in the prosoma. Having ecdysteroid-producing cells associated with the circulatory system could ensure a quick endocrine response as the heart's activity would rapidly distribute ecdysteroids to all tissues and trigger molting. This localization of *Pt-nvd* and *Pt-sro* contrasts with holometabolous insects where orthologous ecdysteroid-producing cells are sequestered in specialized endocrine glands rather than the heart (Yoshiyama et al., 2006; Niwa et al., 2010). Insect mutants demonstrate that *nvd* and *sro* are indispensable for raising ecdysteroid titers and enabling successful molting (Saito et al., 2016). Notably, the functional role of these genes to synthesize crucial ecdysteroids for molting is likely conserved, whether in a gland or in hematopoietic tissue.

Prior to wild-type molting *P. tepidariorum* assumes an inverted molting position secured by silk threads. The molt is initiated by the rupture of the old prosomal shield along the edges (Collatz & Mommsen, 1974). Limb extraction occurs sequentially through rhythmic contractions. After freeing of the limbs, the spider carefully withdraws the opisthosoma (Stewart & Martin, 1982). Subsequently, the spider remains immobile, actively pumping hemolymph to expand the new cuticle. The soft exoskeleton gradually sclerotizes over subsequent hours (Politi et al., 2021). RNA interference with *nvd* and *sro* demonstrates functional evidence to essential roles in molting. Knockdowns lead to ecdysteroid-deficient phenotypes that display significant delays in molt intervals. This developmental delay when molt is due might be caused by disruption in ecdysteroid

signaling. Defects resemble the known molting hormone deficiencies studied in insects. In lepidopterans, RNAi significantly reduces 20-hydroxyecdysone titers and causes prolonged development durations and failure to properly pupate (Peng et al., 2019). The lethal phenotypes shown in this study, resulting from cuticle separation issues, are potentially provoked by defective cuticle formation on molecular and cellular levels. These findings extend to insects, with lethal knockdowns reinforcing that once the ecdysone titer falls below a critical threshold, the molting process cannot proceed (Kamimura et al., 2012). The significantly reduced post-molt survival time thereby implicates how disrupted ecdysteroid biosynthesis potentially influences the health and longevity. Comparing RNAi treatments targeting *Pt-nvd* and *Pt-sro*, no statistically significant differences in molting delay or lethality were observed. This outcome is not unexpected as both genes function within ecdysteroidogenesis and act sequentially in the early steps. Therefore, disruption of the genes is likely to impair the pathway at similar levels, leading to comparable phenotypic effects. This functional redundancy in phenotypic outcome underscores the interdependent nature of early ecdysteroid pathway components.

3.1.3.4. Broader implications for ecdysteroid pathway evolution

The presence of *Pt-nvd* and *Pt-sro* in *P. tepidariorum* confirms that the components of the biosynthesis pathway are ancient and likely present in the common ancestor of chelicerates and mandibulates. The conservation of *nvd* in all analyzed arthropods is well documented (Yoshiyama-Yanagawa et al., 2011; Schumann et al., 2018) and this research extends this conservation to the functional level in spiders. Comparative analyses even proposed that a Neverland-like Rieske-domain oxygenase already existed in the last common bilaterian ancestor, originally serving in the general sterol metabolism (Schumann et al., 2018). The reutilization of the original enzyme for synthesis of the molting hormone might represent a case of evolutionary co-option of an ancient metabolic function into a new developmental role in arthropods.

Discovered in 2010, the gene *shroud* demonstrates one of the intermediate steps of Black Box ecdysteroid biosynthesis (Niwa et al., 2010), and the verification of an ortholog in the spider suggests deep evolutionary roots in arthropods rather than uniqueness to insects. Hence, it is conceivable that a big part of the hormonal pathway was established by the time of arthropod diversification, which underscores the

conservation of physiological mechanisms included in ecdysteroid pathway output across Arthropoda (Ogihara et al., 2017; Knigge et al., 2021; Kamiyama & Niwa, 2022). Interestingly evolutionary flexibility is observed in this pathway regarding enzymatic gene differences and variability in anatomical context of ecdysteroidogenesis. Provided data suggest that spiders like *P. tepidariorum* localize *Pt-nvd* and *Pt-sro* expression in discrete cells rather than a single centralized gland like pancrustaceans. This diversity likely reflects adaptation to different body plans and life histories and contributes to a more detailed understanding of ecdysteroid endocrinology in a broader evolutionary context. Future research should aim to elucidate the regulatory interactions between *Pt-nvd*, *Pt-sro* and other ecdysteroid biosynthesis components to establish a comprehensive gene network, while also validating their functions across arachnid species. These comparative analyses will give insights into hormonal regulation across ecdysozoan lineages and contribute to broadening the understanding of developmental and endocrine evolution in invertebrates.

3.1.4. Conclusion

Here, the first comprehensive characterization of the early ecdysteroid biosynthesis genes *neverland* (*Pt-nvd*) and Black Box gene *shroud* (*Pt-sro*) in the chelicerate model *Parasteatoda tepidariorum* is provided. These genes are evolutionarily conserved, demonstrate expression in spatiotemporally restricted domains during embryonic as well as postembryonic development and are functionally crucial for successful molting. The expression patterns suggest endocrine activity within a subset of mesodermal cells, which likely resemble embryonic precursors of hemocytes and heart-associated tissue. Therefore, indicating a developmental link between hormonal regulation and hematopoietic processes in spiders. RNAi-mediated knockdown of the genes disrupted the molting process and thereby reinforces the role in ecdysteroidogenesis known from insects by suggesting that spiders rely on a precisely timed internal ecdysteroid pulse for developmental transitions. The absence of *nobo*, differences in *sro* isoforms and distributed expression domains in *P. tepidariorum* highlight clade-specific evolutionary adaptations within a deeply conserved molt-related endocrine pathway. Future studies including biochemical validation of enzyme function and investigation into ecdysteroid regulation of hemocyte formation are necessary to build a comparative work across chelicerates, myriapods and crustaceans that illuminates how hormonal systems developed across the arthropod tree.

3.1.5. Materials and Methods

3.1.5.1. Animal husbandry and tissue fixation

Specimens of *Parasteatoda tepidariorum* (Koch, 1841) were kept at 25°C in *D. melanogaster* breeding tubes covered with Ceaprene plugs and filled one-third with coconut fiber. Spiders were fed with *D. melanogaster* and supplied with water weekly. Developmental stages of synchronously developing embryos in cocoons were determined according to Mittmann & Wolff (2012). For embryonic fixation, eggs were dechorionized with hypochloride solution (DanKlorix) for 2 to 3 minutes. After washing several times with distilled water, embryos were fixed in 8 ml fixing solution containing 4 ml heptane, 0.5 ml 37 % formaldehyde and 3.5 ml PEMS (100 mM PIPES, 1 mM EDTA, 1 mM $MgSO_4$). After fixation for 6 to 15 hours, embryos were washed with PEMT (PEMS + 0.01 % TWEEN-20), stepwise transferred to 100 % methanol and stored at -20°C. For fixation of postembryonic tissues, all appendages were removed and tissues fixed for 24 hours in 4 % formaldehyde in phosphate-buffered saline with 0.01 % TWEEN-20. Subsequently sections were made at 80–120 µm thickness in 8 % low melt agarose gel (Roth, Germany) using a Leica VT1000 S vibratome. Fixation was repeated for 30 min post-sectioning and agarose remnants were removed before *in situ* hybridization.

3.1.5.2. Genomic and gene tree analysis

Putative homologs of the ecdysteroid pathway genes *nvd* and *sro* in *P. tepidariorum* were identified using BLASTp (NCBI) (Altschul et al., 1997) with the annotated sequences from *D. melanogaster* (*nvd* FBgn0287185; *sro* FBgn0262112) as queries. The identity of *Pt-nvd* and *Pt-sro* sequences was confirmed by reciprocal BLASTp search against the *D. melanogaster* genome (GCF_000001215.4). From the *P. tepidariorum* genome assembly (NCBI accession: GCF_000365465.3) and its corresponding annotation, the gene structures of the ecdysteroid pathway genes *Pt-nvd* and *Pt-sro* were visualized using GenomeTools (version 1.6.2; Gremme et al., 2013). To generate trees of protein sequences from putative homologs of Nvd, Sro and corresponding outgroups (*apoptosis-inducing factor 3* and *3-dehydrosphinganine reductase*), sequences of 27 species were retrieved from NCBI and Ensembl Metazoa release 60 (myriapod, Yates et al., 2022) (accession in Supplementary Table 1, 2). This

data includes eleven insects, four crustaceans, a myriapods, eight chelicerates, a tardigrade, priapulid and nematode. The sequences were aligned with Clustal Omega (version 1.2.4; Sievers et al., 2011) and trimmed using ClipKIT (version 2.3.0; Steenwyk et al., 2020) in kpic-smart-gap mode. The best-fit amino acid substitution models were identified using ModelTest-NG (version 0.1.7; Darriba et al., 2020), with "LG+I+G4" and "LG+I+G4+F" as best-fit models for Nvd and Sro, respectively. Maximum-likelihood trees were generated using RAxML-NG (version 1.2.2; Kozlov et al., 2019) and the best-fit model specified by *--model* along with additional parameters: *--all --bs-trees 10000 --seed 1*. The trees were visualized with iTol (version 6.8.1; Letunic and Bork, 2021).

3.1.5.3. Gene expression analysis in single-cell data sets

To identify expressions of *Pt-nvd* and *Pt-sro* in embryonic developmental stages 7 to 12 and the corresponding cell types, the scRNA-seq data for stages 7 to 9 (Leite et al., 2024) and stages 10 to 12 (Medina-Jiménez et al., 2024a) were used. The genes were identified by reciprocal BLASTp search (version 2.11.0+) in the gene annotation generated by Leite et al. (2024) for rPCA integrated stages 7 to 9 and by NCBI Gene ID in stages 10 to 12 scRNA-seq data. Seurat (version 5.1.0; Hao et al., 2024) package in R was used to visualize scRNA-seq data by generating UMAPs and dot plots.

3.1.5.4. Molecular cloning and in situ hybridization

Primers for *Pt-nvd* and *Pt-sro* (Table 1) were ordered from Eurofins Genomics. Specificity and potential for dimer formation were checked using the online tools Multiple Primer Analyzer (ThermoFisher) and Primer-BLAST (NCBI). The primers for *Pt-sro* were designed to target the common region for both isoforms. The specificity of the designed primers was confirmed via Primer-BLAST (Ye et al., 2012). Total RNA from embryonic stages 6 to 14 was isolated with TRIzol® Reagent (ambion®, California) and cDNA was synthesized using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Germany) according to the kit manual. PCR amplicons were cloned into pCRII vector using the Dual Promoter TA Cloning® (Invitrogen, Massachusetts). Vectors were used for transformation of NEB® 5-alpha competent *Escherichia coli* (NEB, Germany). Plasmid DNA was purified using the

Plasmid NucleoSpin® Kit for DNA, RNA and protein purification (MACHEREY-NAGEL, Germany). The inserts were sequenced at LGC Genomics.

Table 1 Primer sequences of the ecdysteroid biosynthesis pathway genes *neverland* and *shroud* from *P. tepidariorum* used for cloning and dsRNA synthesis.

Gene	Name	Forward Primer	Reverse Primer
neverland (*nvd*)	*Pt-nvd*	CTTGGATCATCACTGTCTTCGGC	GCTCCTCATTATCGGCATGGTAC
shroud (*sro*)	*Pt-sro*	GATCGGTGGCTTAACATTGCTG	GGCAGCCTTCGAAAGAAATCG
T-box20	*tbx20*	CCTTATTCTGCGGCCATGTC	CCCGTGAATGGGGAATCTGG
dsRNA	ds_T7	TAATACGACTCACTATAGGG	-
dsRNA	ds_T7M13	-	TAATACGACTCACTATAGGGCAG GAAACAGCTATGAC

Probes for *in situ* hybridization were prepared using T7- or SP6-RNA-polymerases, RNAse inhibitor and DIG RNA Labeling Mix (Roche Diagnostics, Germany). The whole-mount *in situ* hybridization protocol was executed for embryos as well as pro- and opisthosomata of juveniles according to the methodology outlined by Prpic et al. (2008) for fixed tissues. Post-fixation and proteinase K treatment on all samples were excluded from the procedure, as these steps did not yield discernible enhancements. Embryos were counter-stained with nucleic marker SYTOX-Green (Invitrogen).

3.1.5.5. dsRNA synthesis and RNA interference

DNA templates for double-stranded RNA (dsRNA) synthesis were generated via PCR on the pCRII vector using a T7 primer and a M13 primer supplemented with the T7 promotor sequence (Table 1). dsRNA for *Pt-nvd* (dsNvd) and *Pt-sro* (dsSro) was synthesized using the MEGAscriptTM T7 High Yield Transcription Kit (Invitrogen), following the manufacturer's recommended protocol. To investigate the RNAi effect on individual animals, dsRNA injections were performed on subadult males. Subadults were treated once either 1, 5, 10 or 15 days (N > 20 each) after the penultimate molt (APM). Specimens received 1 µl of dsNvd, dsSro or control dsRNA (*tbx20*). Additionally, 25 subadult males injected with H_2O one day APM served as injection control. Time until next molt was compared to observed intervals of wild-type males (N = 30).

3.1.5.6. Microscopy and imaging

Images were captured with a ZEISS AxioZoom.V16 Microscope including a PlanNeoFluar z1x/0.25F objective with the Axiocam 305 Color Camera in bright field and GFP filter setting. The images generated in Z-stack mode of the ZEN microscopy software (blue edition; Carl Zeiss Microscopy GmbH) were combined in the Helicon Focus program for focus stacking (version 7.7.5).

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3.1.6. References

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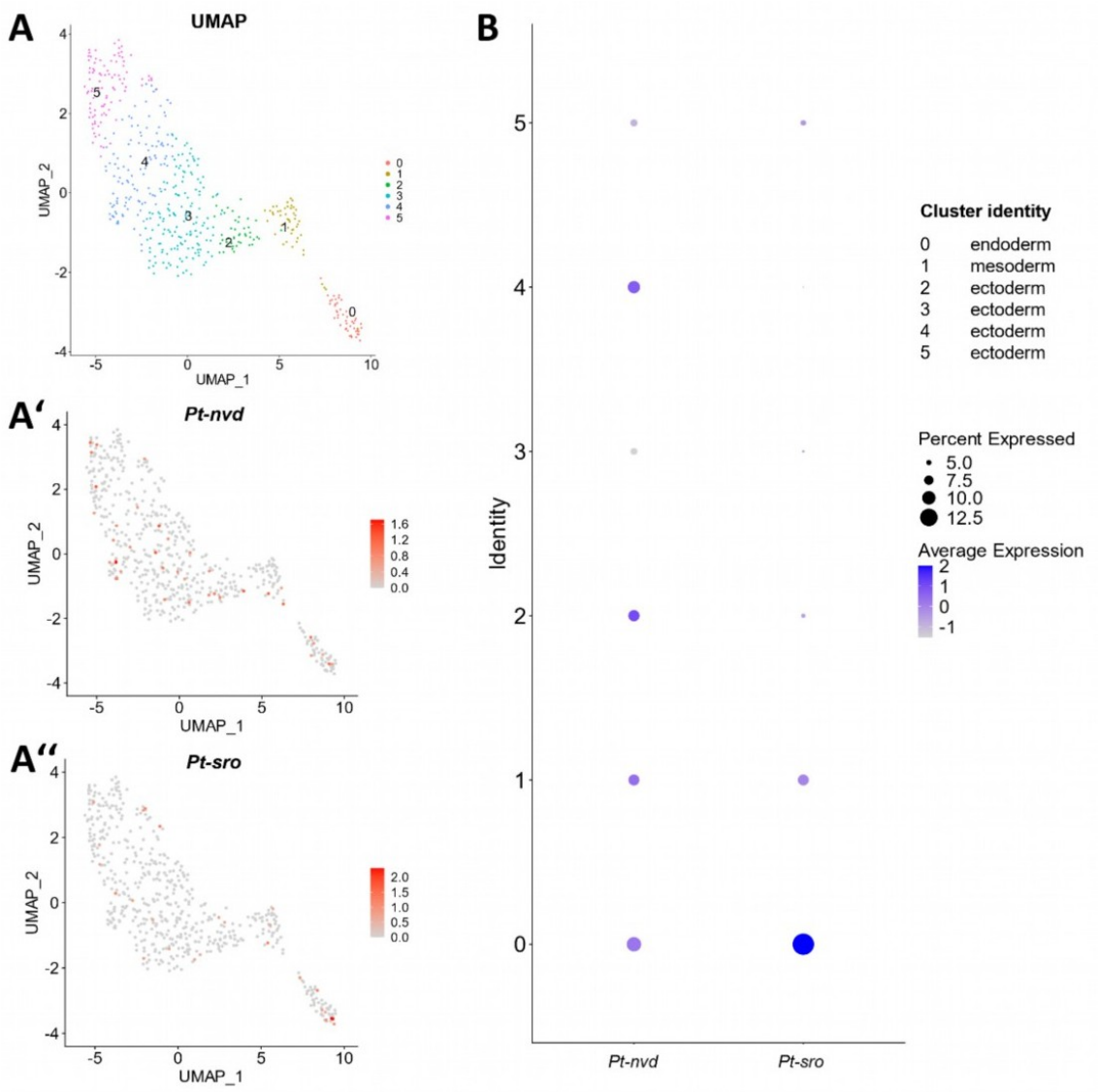
3.1.7. Supplementary Information

Supplementary Table 1 Protein accession of *nvd* orthologs identified in the genomes of various ecdysozoans for protein-based gene tree. Asterisk indicates gene duplication.

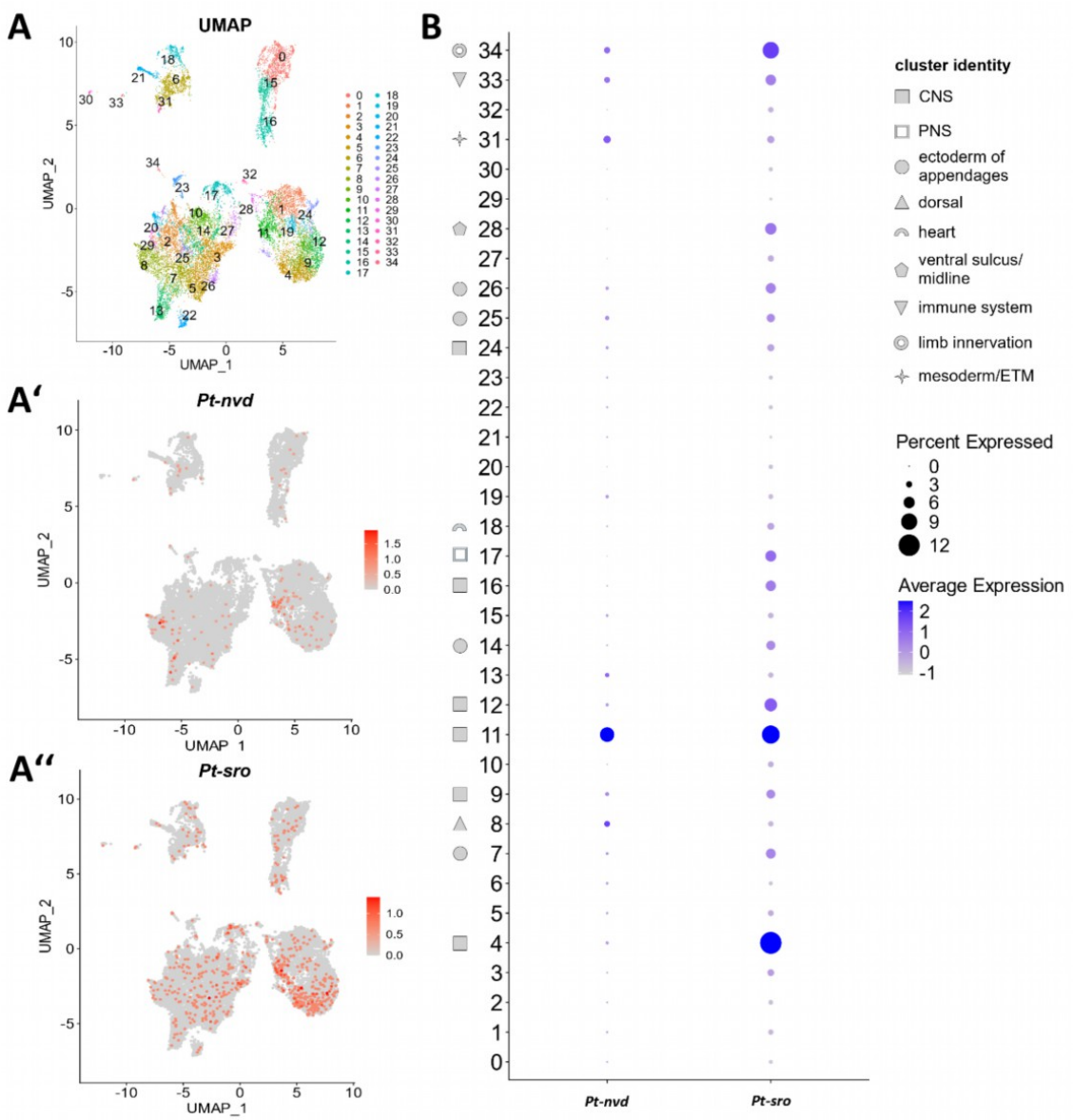
Group	Species	Accession	Gene name
Insecta	*Drosophila melanogaster*	NP_651725.1	cholesterol 7-desaturase
	Anopheles gambiae	XP_061499038.1	cholesterol 7-desaturase nvd
	Bombyx mori	XP_012547344.1	cholesterol 7-desaturase nvd isoform X1
	Manduca sexta	XP_030035493.1	cholesterol 7-desaturase
	Tribolium castaneum	-	-
	Apis mellifera	XP_026296485.1	cholesterol 7-desaturase
	Gryllus bimaculatus	GLH13445.1	3-chlorobenzoate-3,4-dioxygenase oxygenase subunit
	Zootermopsis nevadensis	XP_021913807.1	cholesterol 7-desaturase
	Camponotus floridanus	XP_011256681.1	cholesterol 7-desaturase
	Nilaparvata lugens	XP_022199322.2	cholesterol 7-desaturase nvd
	Folsomia candida	XP_021949071.1	cholesterol 7-desaturase isoform X1
"Crustacea"	*Penaeus vannamei*	XP_069980673.1	cholesterol 7-desaturase nvd
	Daphnia pulex	XP_046441659.1	cholesterol 7-desaturase nvd-like
	Portunus trituberculatus	XP_045129360.1	cholesterol 7-desaturase nvd-like
	Eriocheir sinensis	XP_050690570.1	cholesterol 7-desaturase nvd-like
Myriapoda	*Strigamia maritima*	SMAR010370-PA	unnamed
Chelicerata	*Limulus polyphemus*	XP_022241673.1	cholesterol 7-desaturase-like isoform X1
	Ixodes scapularis	XP_029846293.2	cholesterol 7-desaturase nvd
	Tetranychus urticae	XP_015795304.1	cholesterol 7-desaturase
	Parasteatoda tepidariorum	XP_042901224.1	cholesterol 7-desaturase nvd
	Centruroides sculpturatus	XP_023243683.1	cholesterol 7-desaturase-like
	*Centruroides sculpturatus**	XP_023211401.1	cholesterol 7-desaturase-like
	Stegodyphus mimosarum	KFM69336.1	3-ketosteroid-9-alpha-hydroxylase oxygenase subunit, partial
	Galendromus occidentalis	-	-
	Trichonephila clavipes	GFX74105.1	cholesterol 7-desaturase
Nematoda	*Caenorhabditis elegans*	-	-
Priapulida	*Priapulus caudatus*	-	-
Tardigrada	*Paramacrobiotus metropolitanus*	-	-
outgroup	*Drosophila melanogaster*	CAA21128.1	EG:22E5.5
	Folsomia candida	XP_021952244.1	apoptosis-inducing factor 3
	Daphnia pulex	XP_046442713.1	apoptosis-inducing factor 3-like isoform
	Parasteatoda tepidariorum	XP_015926857.1	apoptosis-inducing factor 3
	Centruroides sculpturatus	XP_023213679.1	apoptosis-inducing factor 3-like
	Paramacrobiotus metropolitanus	XP_055331403.1	apoptosis-inducing factor 3-like isoform
	Caenorhabditis elegans	NP_505112.1	Rieske domain-containing protein

Supplementary Table 2 Protein accession of *sro* orthologs identified in the genomes of various ecdysozoans for protein-based gene tree.

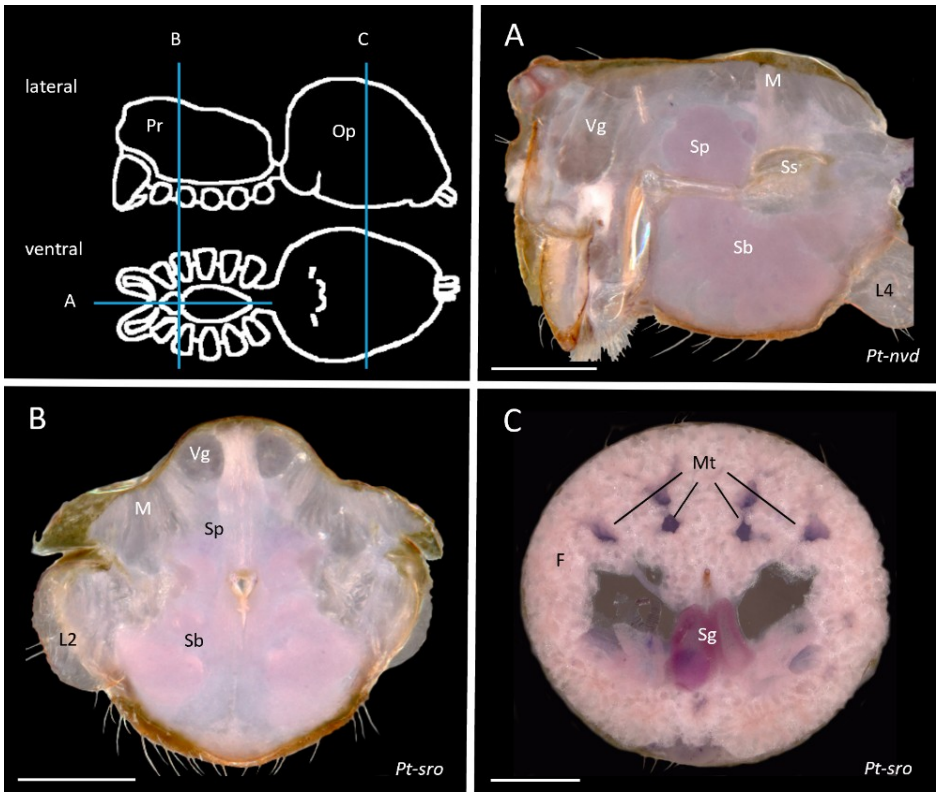
Group	Species	Accession	Gene name
Insecta	*Drosophila melanogaster*	NP_651725.1	short-chain dehydrogenase/reductase
	Anopheles gambiae	XP_316858.5	retinol dehydrogenase 7
	Bombyx mori	NP_001171333.1	SDR family oxidoreductase shroud
	Manduca sexta	XP_037293906.1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial
	Tribolium castaneum	XP_973118.1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial
	Apis mellifera	XP_016767782.1	estradiol 17-beta-dehydrogenase 2
	Gryllus bimaculatus	GLH13347.1	Dehydrogenase/reductase SDR family protein 7-like
	Zootermopsis nevadensis	XP_021913325.1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial
	Camponotus floridanus	XP_025262119.1	retinol dehydrogenase 7
	Nilaparvata lugens	XP_022200477.2	D-beta-hydroxybutyrate dehydrogenase, mitochondrial
	Folsomia candida	-	-
"Crustacea"	*Penaeus vannamei*	XP_027222633.1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial
	Daphnia pulex	XP_046463855.1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial-like
	Portunus trituberculatus	XP_045111858.1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial-like
	Eriocheir sinensis	XP_050712783.1	short-chain dehydrogenase/reductase
Myriapoda	*Strigamia maritima*	SMAR011122-PA	unnamed
Chelicerata	*Limulus polyphemus*	XP_013791754.1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial-like
	Ixodes scapularis	XP_040063156.1	retinol dehydrogenase 7
	Tetranychus urticae	XP_015790279.1	retinol dehydrogenase 16
	Parasteatoda tepidariorum	XP_015912997.1	short-chain dehydrogenase/reductase family 9C member 7
	Centruroides sculpturatus	XP_023220594.1	D-beta-hydroxybutyrate dehydrogenase mitochondrial-like
	Stegodyphus mimosarum	KFM74352.1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial, partial
	Galendromus occidentalis	XP_003743507.1	retinol dehydrogenase 5-like
	Trichonephila clavipes	GFW34123.1	17-beta-hydroxysteroid dehydrogenase type 6
Nematoda	*Caenorhabditis elegans*	NP_505941.3	Retinol dehydrogenase 7
Priapulida	*Priapulus caudatus*	XP_014680661.1	D-beta-hydroxybutyrate dehydrogenase, mitochondrial-like
Tardigrada	*Paramacrobiotus metropolitanus*	XP_055346861.1	retinol dehydrogenase 16-like
outgroup	*Drosophila melanogaster*	NP_651363.1	3-dehydrosphinganine reductase
	Folsomia candida	XP_021954517.1	3-ketodihydrosphingosine reductase isoform X1
	Daphnia pulex	XP_046449073.1	3-ketodihydrosphingosine reductase-like
	Parasteatoda tepidariorum	XP_015908034.1	3-ketodihydrosphingosine reductase
	Centruroides sculpturatus	XP_023225867.1	3-ketodihydrosphingosine reductase-like
	Paramacrobiotus metropolitanus	XP_055335232.1	3-ketodihydrosphingosine reductase-like
	Caenorhabditis elegans	NP_001294377.1	3-dehydrosphinganine reductase



Supplementary Figure 1 *Pt-nvd* and *Pt-sro* expression at embryonic stage 5 from scRNA-seq data. UMAP for embryonic stage 5 (Akiyama-Oda et al., 2022) shows (A) the clusters from presumptive endodermal, mesodermal and ectodermal cells, (A') cells expressing *Pt-nvd* and (A'') cells expressing *Pt-sro*. Red gradient indicates expression level. (B) Dot plot shows expression of *Pt-nvd* and *Pt-sro* in all clusters. Percentage of cells per cluster expressing the gene corresponds to the dot size, and their average expression level to the blue gradient.



Supplementary Figure 2 *Pt-nvd* and *Pt-sro* expression at embryonic stages 10 to 12 from reanalyzed scRNA-seq data. UMAP for pooled embryonic stages 10 to 12 (Medina-Jiménez et al., 2024b) shows (A) the cell clusters, (A') cells expressing *Pt-nvd* and (A'') cells expressing *Pt-sro*. Red gradient indicates expression level. (B) Dot plot shows expression of *Pt-nvd* and *Pt-sro* in all clusters. Percentage of cells per cluster expressing the gene corresponds to the dot size, and average expression level to the blue gradient.



Supplementary Figure 3 Sense control of *Pt-nvd* and *Pt-sro* on postembryonic sections in the spider *Parasteatoda tepidariorum*. Dorsal is up in all images. The morphological scheme [redrawn after Foelix (1996)] indicates section planes. (A) Centered longitudinal section using *Pt-nvd* and (B) cross section using *Pt-sro* sense probe in prosoma. (C) Cross section at the level of silk glands in the opisthosoma using *Pt-sro* sense probe. Note that staining (magenta) in silk glands is a presumed artefact due to the adhesiveness of silk residues. F, fat body; L1–4, leg pairs 1 to 4; M, muscles; Mt, Malpighian tubes; Sb, subesophageal ganglion; Sg, silk gland; Sp, supraesophageal ganglion; Ss, sucking stomach; Vg, venom gland. Scale bars: 200 µm.

3.2. The Halloween Gene *shadow* is Involved in Embryonic Development and Postembryonic Molting in the Spider *Parasteatoda tepidariorum*

Here the functional involvement of the mid-ecdysteroid pathway gene *shadow* in the spider *Parasteatoda tepidariorum* is invested. Utilization of expression analyses on embryonic and postembryonic stages via whole-mount *in situ* hybridization, in combination with functional analyses through parental and juvenile RNA interference-mediated knockdown suggests a multifunctional role beyond molt involvement. Results indicate the relevance of *shadow* is neurogenesis and cardiogenesis during spider embryogenesis as well as in successful molting.

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Author contributions to this research:

Denise Klinkenbuß: Data curation, Formal analysis, Validation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Sandra Treffkorn: Investigation, Methodology, Writing – review & editing.

Georg Mayer: Conceptualization, Formal analysis, Funding acquisition, Project administration, Writing – review & editing.

Nikola-Michael Prpic-Schäper: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Status: **Under Review** in Developmental Biology

The Halloween Gene *shadow* is Involved in Embryonic Development and Postembryonic Molting in the Spider *Parasteatoda tepidariorum*

Highlights

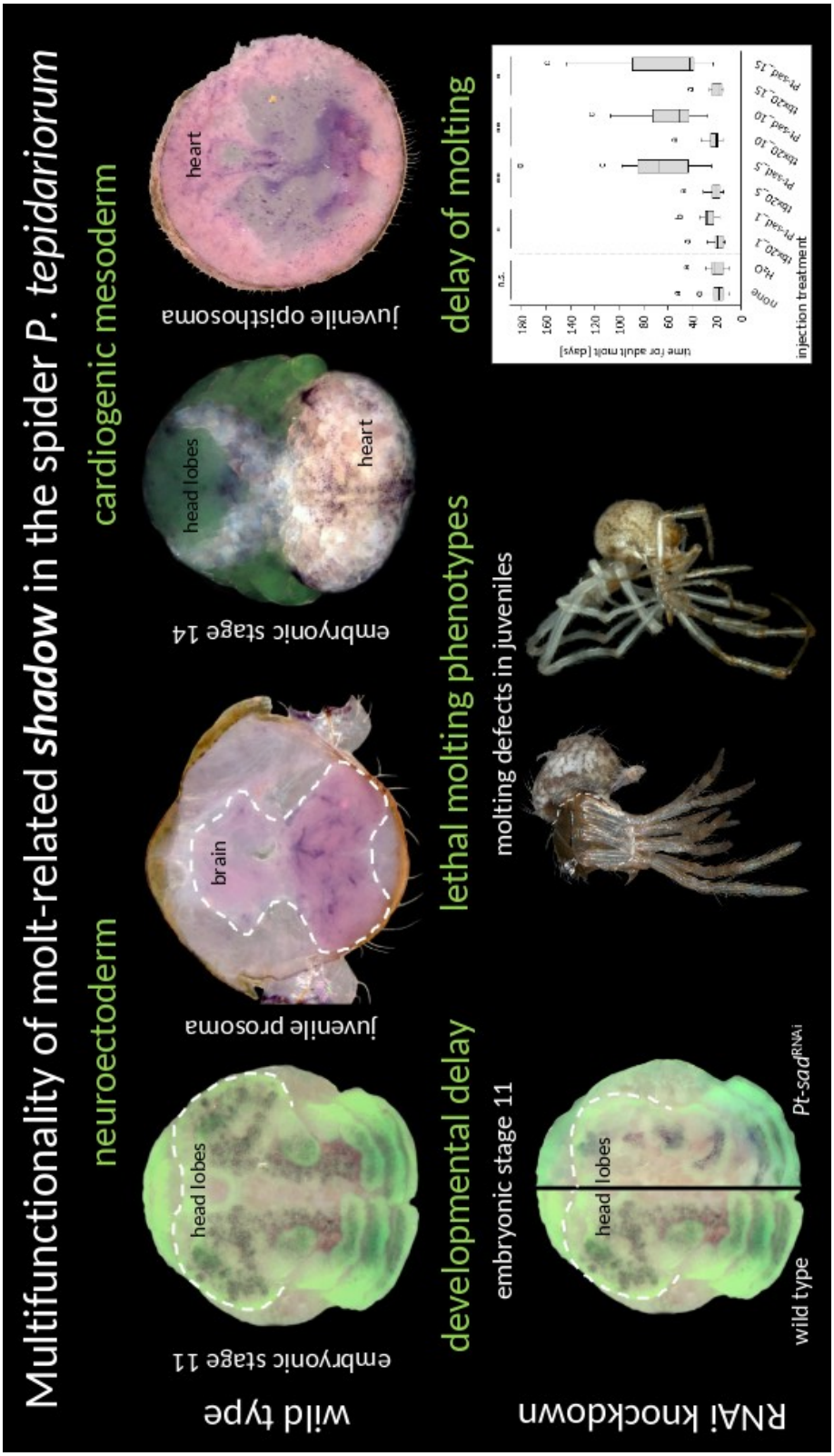
- *Pt-sad* is dynamically expressed in neuroectoderm and cardiogenic mesoderm.
- Expression indicates multifunctionality in embryonic and postembryonic stages.
- Parental RNAi results in increased embryonic mortality and developmental delays.
- Postembryonic knockdowns result in molt defects, suggesting a role in ecdysis.

Abstract

Ecdysteroids are key hormones that regulate molting and developmental processes in arthropods, including insect metamorphosis. Understanding the molecular roles of the genes involved in ecdysteroid biosynthesis could provide crucial insights into the evolutionary conservation of regulation and regulatory modifications in arthropods. While extensively studied in insects, the functions of the so-called Halloween genes, which encode enzymes responsible for the conversion of cholesterol to ecdysone and 20-hydroxyecdysone, remain unclear in arachnids. Therefore, this study aims to elucidate the functions of the Halloween gene *shadow* (*Pt-sad*) in the spider *Parasteatoda tepidariorum* in both embryonic and postembryonic stages and assess its potential role in molting. Employed *in situ* hybridization to characterize the expression patterns of *Pt-sad* and RNA interference-mediated knockdown were conducted to explore its effects on development and molting. Knockdown of *Pt-sad* resulted in increased embryonic mortality and developmental delays, while it disrupted molt cycles in the postembryonic stages, supporting a role in ecdysteroidogenesis. Expression analysis revealed *Pt-sad* activity in specific regions of embryos and juveniles, including the central nervous system, appendages and heart. These findings highlight the multifunctionality of *shadow* in the spider. While the involvement of this gene in the ecdysteroid pathway is most likely conserved in arthropods, this role may have diversified in spiders to include additional, unique functions that differ from their insect counterparts. These results pave the way for future research on ecdysteroid biosynthesis and molting in arachnids.

Keywords: ecdysis, ecdysteroid pathway, embryogenesis, RNAi, arachnid, araneid

Graphical Abstract



3.2.1. Introduction

Ecdysteroids are key hormones that regulate molting, metamorphosis and various other developmental processes within the arthropod lineage (Sullivan & Thummel, 2003; Ishimoto et al., 2009; Niwa & Niwa, 2014a). In insects, particularly the fruit fly *Drosophila melanogaster*, these hormones are mostly synthesized by cytochrome P450 (CYP) enzymes (Gilbert et al., 2002; Niwa & Niwa, 2014b). The enzymes, encoded by the Halloween genes, play a crucial role in the biosynthesis pathway of the molting hormone. In this pathway, cholesterol is stepwise converted to ecdysone and its active derivative 20-hydroxyecdysone (20E) via the Rieske-domain oxygenase Neverland, the short-chain dehydrogenase/reductase Shroud and the CYP monooxygenases Spook, Phantom, Disembodied, Shadow and Shade (Gilbert et al., 2002; Rewitz et al., 2006a; Niwa et al., 2010). Studies on *D. melanogaster* and other insect models have shown that disruption of the expression of these genes leads to defects in molting, cuticle formation and development, highlighting their critical role in the ecdysteroid biosynthesis pathway (Jürgens et al., 1984; Wieschaus et al., 1984; Van Ekert et al., 2016). One of these Halloween genes, *shadow* (*sad*, *CYP315A1*), is synthesized in the prothoracic gland of *D. melanogaster* larvae and converts 2-deoxyecdysone to ecdysone. The intermediate is then released into the hemolymph, where it is activated by *shade* to form the molting hormone 20E (Gilbert et al., 2002; Petryk et al., 2003).

While the ecdysteroid pathway has been extensively studied in insects (Campli et al. 2024), its functions in non-model and non-insect arthropods such as chelicerates, a group that diverged early in arthropod evolution (Dunlop, 2010), remain poorly understood. Genomic studies of Halloween genes belonging to the CYP family have revealed the presence of *spook*, *disembodied*, *shadow* and *shade* in chelicerates, while *phantom* appears to be absent (Schumann et al., 2018; Yang et al., 2021). This absence suggests a divergence in the chelicerate ecdysteroid pathway and is consistent with the observation that spiders and mites may utilize ponasterone A instead of 20E as the active hormone (Li et al., 2019; Yang et al., 2021). Furthermore, there is little to no information on the localization and function of *shadow*, therefore it is unclear whether this gene plays a role during embryogenesis in arachnids. Investigating the molecular mechanisms of arachnid development may improve the understanding of evolutionary conservation and modifications of fundamental pathways in arthropods.

The common house spider, *Parasteatoda tepidariorum*, has emerged as a promising model system for studying the developmental biology of arachnids (McGregor et al., 2008; Turetzek et al., 2024). Males of *P. tepidariorum* typically undergo five and females six molts after emerging from the cocoon to reach adulthood via a final molt. Including the single molt that occurs within the cocoon, the total number of molts is six for males and seven for females (Miyashita, 1987). Understanding the organization and mechanisms of the ecdysteroid system in the spider, together with an in-depth characterisation of the Halloween gene *shadow* (*Pt-sad*), may provide broader insights into the evolution of hormonal control in arthropods. Functional analysis of *Pt-sad* might help to elucidate how disruptions in ecdysteroid synthesis affect molting. On the other hand, localization of *Pt-sad* in embryonic and postembryonic stages may reveal cells and tissues that produce the Shadow enzyme and are responsible for ecdysteroid biosynthesis.

In this study, the following questions are address: (1) which tissues exhibit mRNA expression of the Halloween gene *Pt-sad* in embryonic and postembryonic stages of *P. tepidariorum*? (2) What are potential roles of *Pt-sad* during embryogenesis and in juvenile stages? (3) Does *Pt-sad* influence the molting process in the spider? Addressing these questions will provide insights into the evolutionary conservation and multifunctionality of molt-related genes in arthropods.

3.2.2. Results

3.2.2.1. Identification and verification of Pt-sad ortholog

The full-length ortholog of the Halloween gene *Pt-sad*, identified in previous genomic studies (Schumann et al., 2018; Dermauw et al., 2020; NCBI accession: XM_016054492.2), was used and verified by cloning. The gene contains an open reading frame of 1,557 base pairs and encodes a protein of 518 amino acids. Gene structure analysis indicates that *Pt-sad* consists of eight exons (Fig. 15A). The deduced protein sequences of 24 putative *sad* orthologs (Supplementary Table 3) from other members of Chelicerata (including spiders, mites, scorpions and horseshoe crabs) and Pancrustacea were aligned for phylogenetic analysis. In the resulting phylogeny, *Pt-sad* groups with *sad* orthologs from other spider species within the chelicerate clade (Fig. 15B). Multiple alignment revealed conserved structural features of *Pt-sad*, including an N-terminal proline/glycine (P/G)-rich domain and five characteristic CYP motifs: Helix-C, Helix-I, Helix-K, PERF motif and heme-binding domain (Fig. 15B).

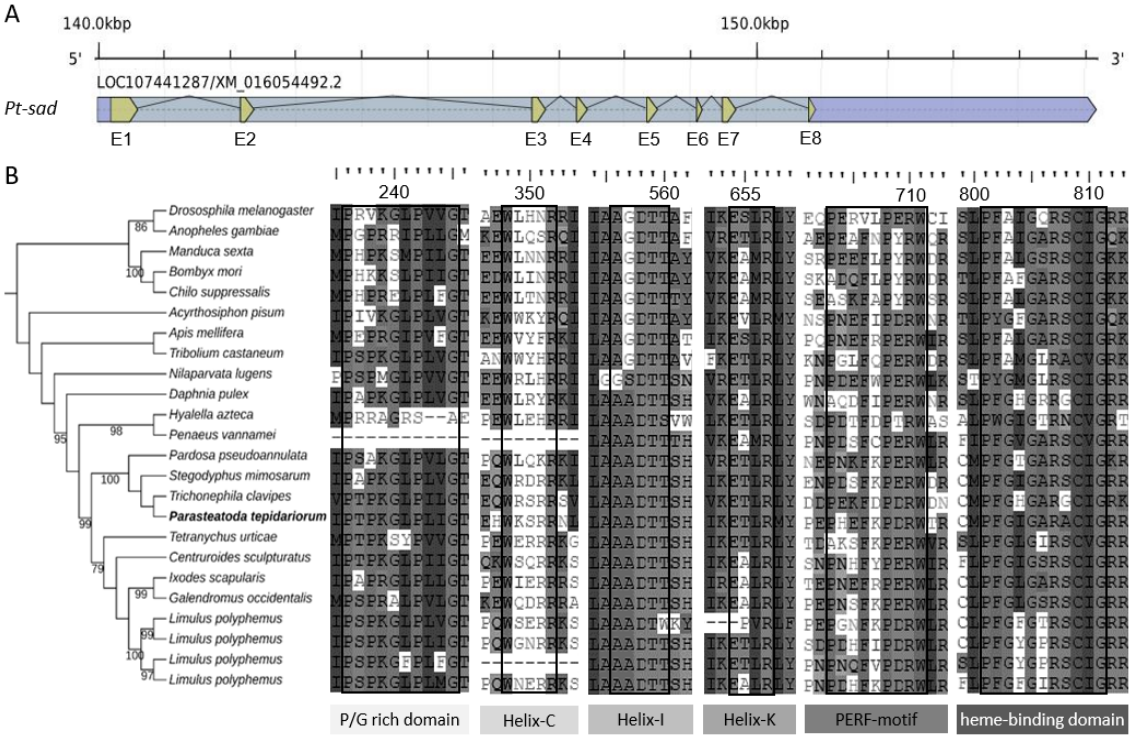


Figure 15 Genomic organization and phylogenetic conservation of the *Pt-sad* gene in arthropods. (A) Genomic location and exon-intron structure spanning ~15 kbp. Exons (E1–E8) are shown as yellow triangles, introns as lines and untranslated regions in blue. (B) Phylogenetic tree and multiple sequence alignment of *Pt-sad* orthologs from different arthropods. Numbers at nodes indicate bootstrap support values greater than 70 obtained from 1,000 pseudoreplicates. The sequence alignment highlights conserved domains across species, including the P/G-rich domain, Helix-C, Helix-I, Helix-K, PERF motif and the heme-binding motif within boxes.

3.2.2.2. Expression of Pt-sad during embryogenesis

The expression pattern of *Pt-sad* during embryogenesis is dynamic and comprises several expression loci. Expression begins in the middle and distal parts of the appendages at embryonic stage 8 (Supplementary Figure 4), persists until stage 11 (Fig. 16A–F), and is absent by stage 12 (Fig. 16G–I). The expression in the appendages is most likely in mesodermal tissue.

Strong expression is also observed in the developing nervous system, including numerous invagination sites in the head lobes and the ventral neuroectoderm, giving rise to neurons and sensory structures (Fig. 16A–F). In the ventral neuroectoderm expression begins as multiple dots per hemisegment at stage 9 (Fig. 16D, arrows exemplary show the visible dots in L2 hemisegment). The segmental pattern increases in complexity as more invagination sites form at stage 10 (Fig. 16E), giving rise to the paired segmental invaginations at stage 11, which are the ganglion *anlagen* of the ventral nerve cord (Fig. 16F). A similar profile is observed in the head lobes, where expression begins in a subset of invagination sites at stage 9. This is followed by upregulation throughout much of the neuroectoderm during development and forming of a ring-like pattern at stage 11 (Fig. 16A–C). Additionally, a bilaterally symmetric pair of dots appears in the non-neurogenic ectoderm (Fig. 16C, arrowhead). Interestingly, both expressions disappear in the head lobes by embryonic stage 12 (Fig. 16G) and are replaced by a single, distinct expression domain at the anterior margin of the forming brain in stages 12 to 14 (Fig. 16G–I, arrowheads).

A strong dorsal expression of *Pt-sad* appears at stage 12, following completion of dorsal closure and heart formation. The pattern consists of repeated clusters in the arched margin of the tissue overgrowing the yolk (Fig. 16G, dashed circle). At stage 13, when the constriction between pro- and opisthosoma occurs after dorsal closure, *Pt-sad* mRNA is detectable at the posterior end of the opisthosoma, forming a dorsal tube-like pattern that gradually expands anteriorly (Fig. 16H). During the last embryonic stage (stage 14), this expression develops into repeated blotches branching from the central tube-like pattern (Fig. 16I).

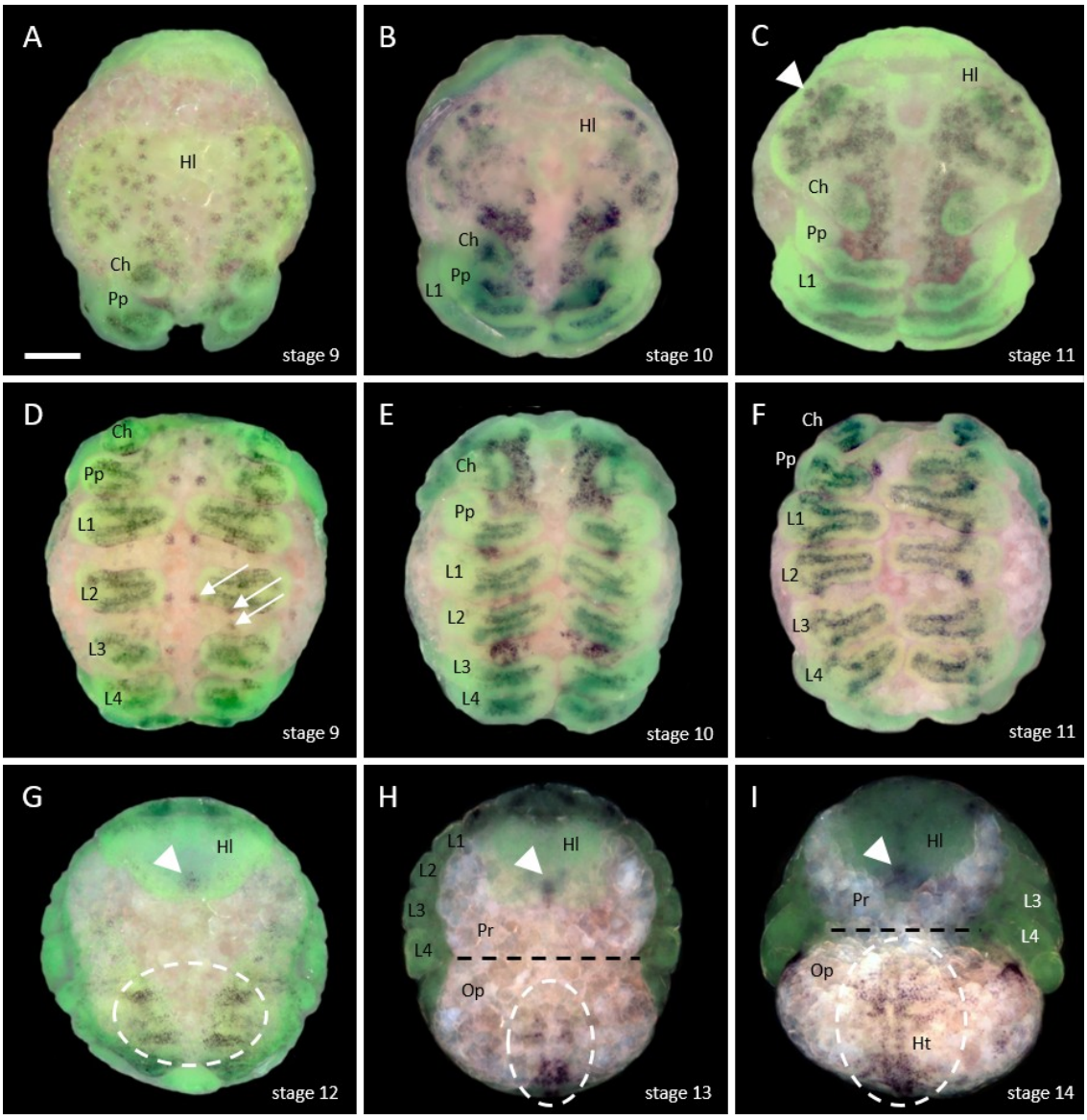


Figure 16 Embryonic expression of the Halloween gene *Pt-sad*. Anterior view (A–C) with dorsal facing up; ventral (D–C) and dorsal (G–I) views with anterior facing up. Nuclear marker SYTOX in green. (A–C) Expression of *Pt-sad* in the developing head lobes starting as a dotted pattern and increasing into a ring-like shape from stages 9 to 11. (D–F) Expression in the developing ventral neuroectoderm transforms from dot-like invagination sites (arrows) at stage 9 to lines of invaginated ganglion anlagen of the ventral nerve cord at stage 11. (G–I) Expression of *Pt-sad* located in the cardiogenic mesoderm of stages 12 to 14 (dashed circles). Ch, chelicera; Hl, head lobes; Ht, heart tube; L1–L4, legs 1 to 4; Op, opisthosoma; Pp, pedipalp; Pr, prosoma. Scale bar: 100 µm (in A for A–I).

3.2.2.3. Effect of Pt-sad RNA interference on embryonic development

To evaluate the role of *Pt-sad* in embryogenesis of the spider *P. tepidariorum*, RNAi (RNA interference) knockdown experiments were performed. Embryos from parental RNAi (dsSad) were examined for alterations in the expression pattern of *Pt-sad* and for visible morphological defects compared to embryos from H_2O-treated females.

In the control group, 92.1 % of embryos exhibited a wild-type phenotype (Fig. 17A), while the remaining 7.9 % did not reach the hatching event, consistent with a previous study (Turetzek et al., 2015). Of these 7.9 %, 3.2 % were unfertilized eggs, 1.8 % delayed and 2.9 % dead embryos (Fig. 17A). In contrast, embryos from parental dsSad treatment showed an increase in unfertilized eggs (5.0 %) and embryonic mortality (19.7 %), along with a higher proportion (25.4 %) of delayed embryos (Fig. 17A). Embryos were classified as delayed if their developmental stage at the time of fixation was at least three stages behind the cocoon average.

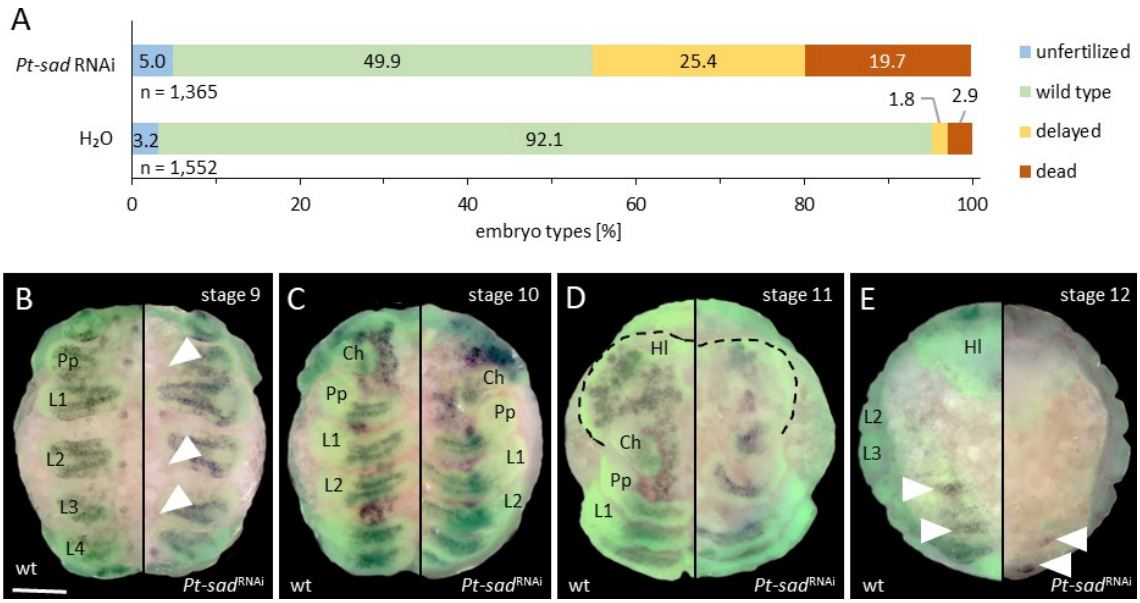


Figure 17 Effect of *Pt-sad* RNAi on embryogenesis. (A) Distribution of embryo types after *Pt-sad* RNAi treatment and control. Embryos that are at least three developmental stages behind the cocoon average are considered delayed. (B–E) Expression of *Pt-sad* (dark purple stain) in RNAi-treated (right) and wild-type embryos (left). Nuclear marker SYTOX in green. Ventral (B, C) and dorsal (E) views with anterior facing up, and (D) anterior view with dorsal facing up. (B) Wild-type embryo (left) showing expression in the invagination sites in the ventral neuroectoderm (round dots near the midline). Note that expression is partially absent in the parental RNAi-treated embryo (arrowheads on the right). (C) *Pt-sad* expression is strongly affected in the invagination sites of the cheliceral segment compared to a higher number in wild type. (D) Note the lack of expression in most of the neuroectoderm and reduced size of head lobe in the parental RNAi-treated embryo (right side). Expression is clearly seen in invagination sites of the wild-type embryo. (E) Expression in the developing heart region (arrowheads) is delayed in the parental RNAi-treated embryo (right). Ch, chelicera; Hl, head lobes; Ht, heart tube; L1–L4, legs 1 to 4; Pp, pedipalp; *Pt-sad*^{RNAi}, parental RNAi-treated embryos; wt, wild type. Scale bar: 100 µm (in B for B–E).

In situ hybridization on parental RNAi-treated embryos revealed considerable downregulation of *Pt-sad* (Fig. 17B–E). The invagination sites were affected, with some hemi-segmental dots missing in stage 9 embryos (Fig. 17B). Furthermore, the upregulation throughout the ventral neuroectoderm that coincides with the formation of

further invagination sites, as it occurs in wild-type embryos at stage 10, was substantially impaired in the parental dsSad-treated embryos (Fig. 17C). Except at stage 12, *Pt-sad* expression appears to be less affected in the developing limbs than in the neuroectoderm (Fig. 17B–E). At stage 11, wild-type embryos exhibit proper head lobe formation with mRNA staining covering almost the entire lobe. In contrast, knockdown embryos show disturbed expression, often characterized by asymmetric and locally restricted patches. Consistent with downregulation, head lobe development is visibly reduced (Fig. 17D). In addition, reduced staining is observed in the dorsal region of stage 12 knockdown embryos, corresponding to the cardiogenic tissue in wild type (Fig. 17E).

3.2.2.4. Expression of Pt-sad in postembryonic stages

In prosoma sections of juveniles in the last third of the interval between the fourth and fifth molt, *Pt-sad* expression occurs predominantly in the subesophageal ganglion and near the commissural midline. Here, distinct bilateral symmetric clusters of expression form a salt-and-pepper pattern (Fig. 18A, B) with thin lines of expression extending to the commissural midline (Supplementary Fig. 5). In the supraesophageal ganglion, expression is found in the distal region of the mushroom body (Fig. 18B, arrowhead). In the central nervous system of the posterior prosoma part, expression is restricted to two small cell clusters in the subesophageal ganglion (Fig. 18C, arrowheads).

Pt-sad expression in the opisthosoma of spiderlings is clearly visible as scattered dots covering the entire sectioned ovary. *Pt-sad* mRNA has also accumulated in cells of the midgut epithelium. In more posterior sections of the opisthosoma, the same dotted expression can be detected in the ovary (Fig. 18D). A light dotted expression was also visible in the whole fat body of the opisthosoma (Fig. 18D, E). In addition, a thin layer of expression frames the heart tube (Fig. 18E). The intense purple staining in the silk glands is most likely an artifact caused by the adhesive properties of the silk components, as it is also present in sense controls (Supplementary Fig. 6).

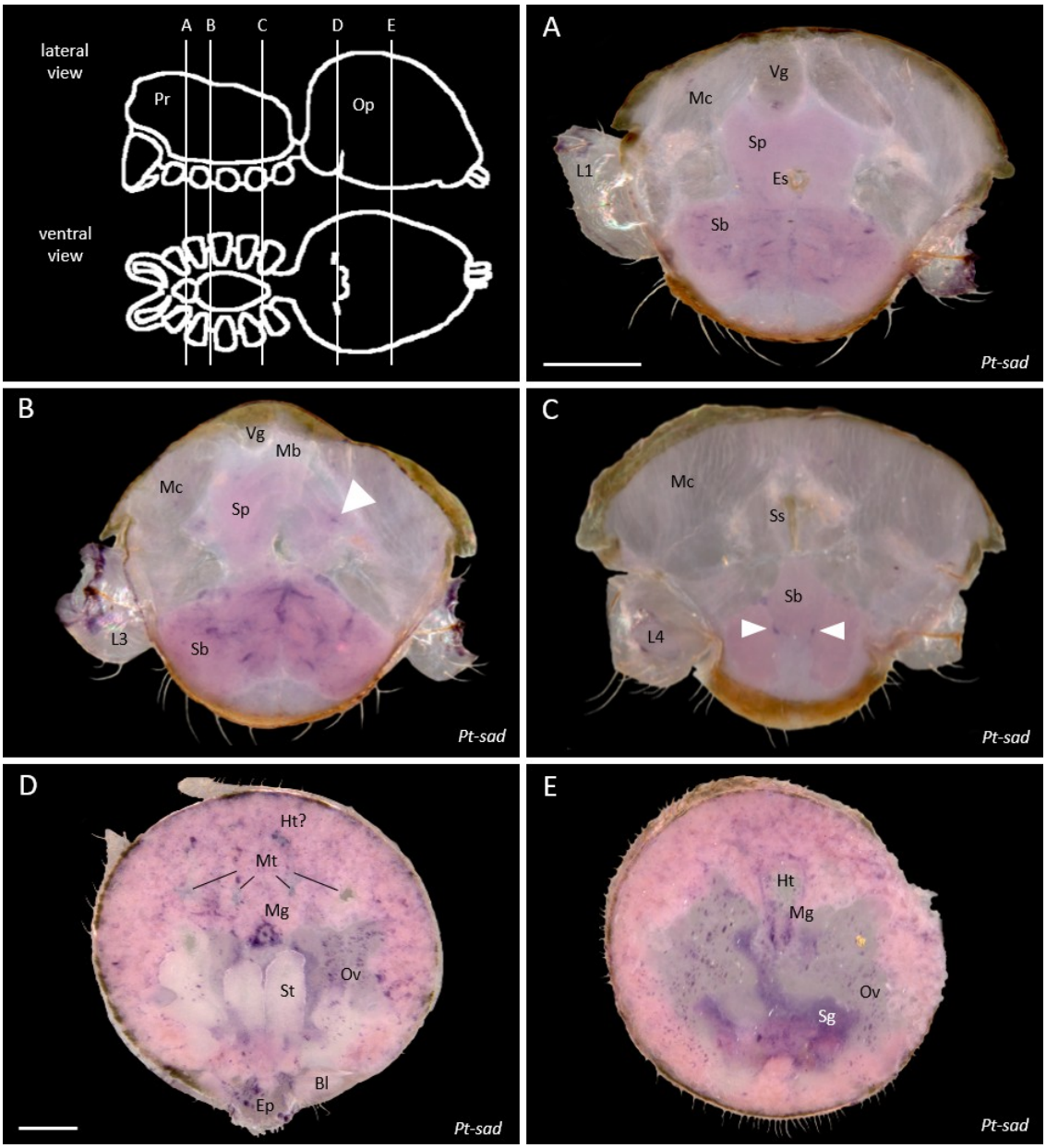


Figure 18 Postembryonic expression of the Halloween gene *Pt-sad*. The section planes are indicated in the morphological scheme [redrawn after Foelix (1996)]. Anterior is left in the diagram; dorsal is top in all images. (A–E) Expression of *Pt-sad* (dark purple stain) in prosoma (A–C) and opisthosoma (D, E) of juveniles. (A) Expression in subesophageal ganglion in section posterior to pedipalps. (B) Expression in subesophageal ganglion and distal part of supraesophageal ganglion (arrowhead) between first and second pair of legs. (C) Expression in cells of the subesophageal ganglion in section between third and fourth pair of legs. (D) Section at the level of book lungs. (E) Section at the level of silk glands. Bl, book lungs; Es, esophagus; Ep, epigyne; Ht, heart tube; L1–L4, legs 1 to 4; Mb, mushroom body; Mc, muscles; Mg, midgut; Mt, Malpighian tubes; Op, opisthosoma; Ov, ovary; Pr, prosoma; Sb, subesophageal ganglion; Sg, silk gland; Sp, supraesophageal ganglion; Ss, sucking stomach; St, spermatheca; Vg, venom gland. Scale bar 200 µm (in A for A–C, in D for D, E).

3.2.2.5. Effect of Pt-sad RNA interference on postembryonic development and molting

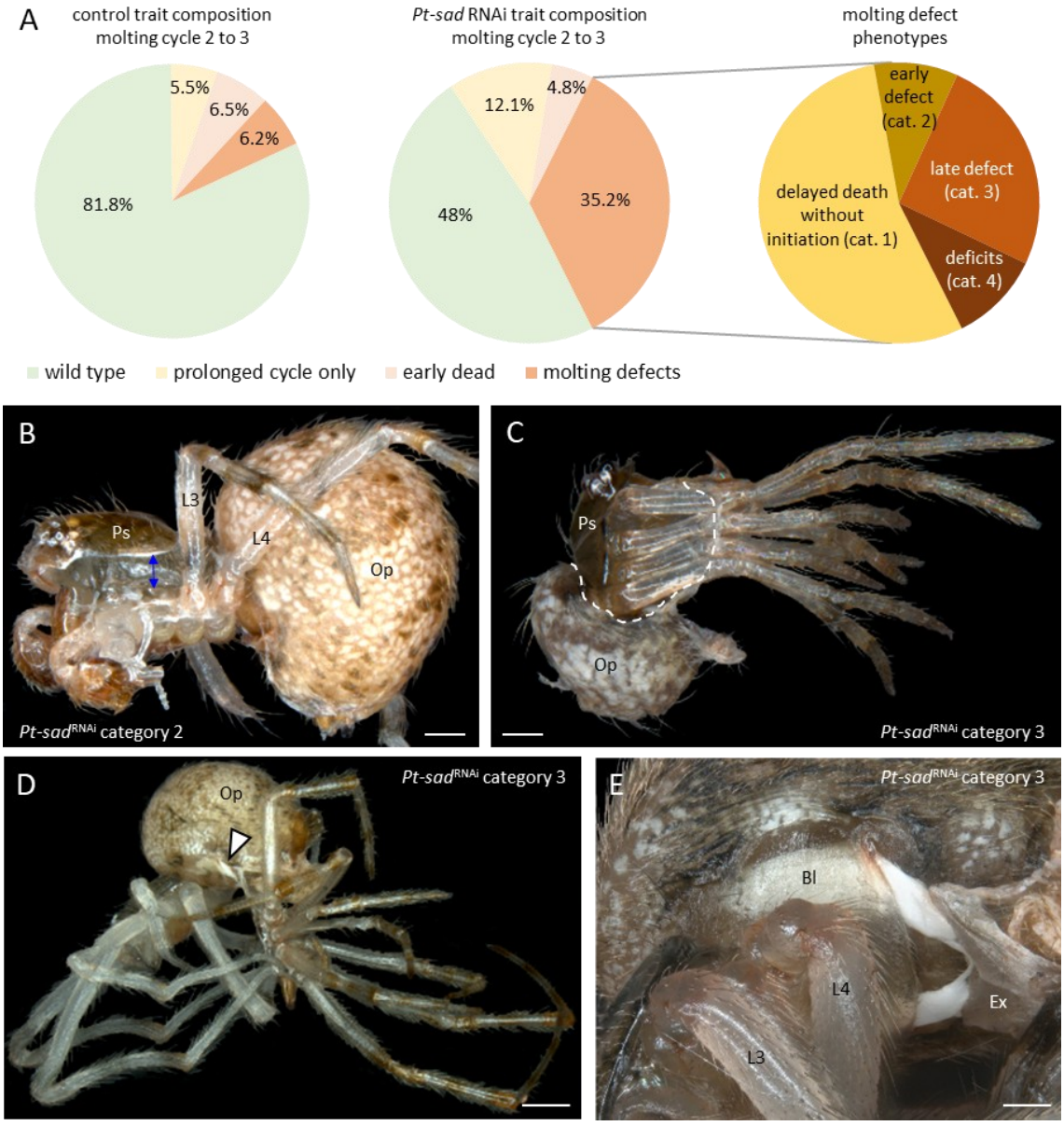


Figure 19 Effects of *Pt-sad* parental RNAi on postembryonic development and molting. (A) Summary of RNAi effect in control and *Pt-sad* RNAi-treated spiders exemplary for molting cycles two to three. Detailed chart shows percentages of molting defect phenotypes in RNAi group. (B–E) *Pt-sad* RNAi-treated spiders displaying molting defect phenotypes. Lateral view, anterior to the left (B, D, E) or up (C). (B) Early molting defect, prosomal shield not detachable (blue arrow), first and second legs dissected for clarity. (C, D) Late molting defect, legs (dashed line) and book lungs (arrowhead) not separable from exuvial cuticle. (E) Detail of stuck book lung cuticles. Bl, book lung; Ex, exuvia; L1–4, walking legs 1 to 4; Op, opisthosoma; Ps, prosomal shield. Scale bars: 200 µm (B), 500 µm (C, D) and 100 µm (E).

Compared to wild-type molts (Supplementary Video 1), RNAi-treated animals exhibit postembryonic developmental defects and molting abnormalities, collectively referred to as molting defect phenotypes (Fig. 19). These lethal phenotypes are classified into four categories based on observations between the second and third molt. In category 1, molt initiation was completely absent, resulting in death long after the control animals had completed the cycle. Category 2 included an early molt defect characterized by failure to shed the prosomal shield (Fig. 19B). Category 3 defects occurred later in the process and prevented the separation of old and new cuticles of complex structures such as legs and book lungs (Fig. 19C–E). The category 4 phenotype showed an apparently normal molt followed by severe locomotory impairments (Supplementary Video S2). The most common defect phenotype in the interval between the second and third molt is category 1 (19.2 %), followed by category 3 (8.9 %). Early molting defects (category 3) and post-molt deficits (category 4) occurred in 3.4 % and 3.7 % of cases (Fig. 19A).

In addition, due to the defects, mortality in the first and second molt cycles significantly increased in parental RNAi juveniles (Fig. 20A). Furthermore, the time between the first and second molt was significantly prolonged. The intervals between the third and fourth molt, and the fourth and fifth molt were also prolonged (Fig. 20B). A reduction in body mass is indicated by a significant decrease of ~10 % in body weight compared to control subadult males and females (Fig. 20C).

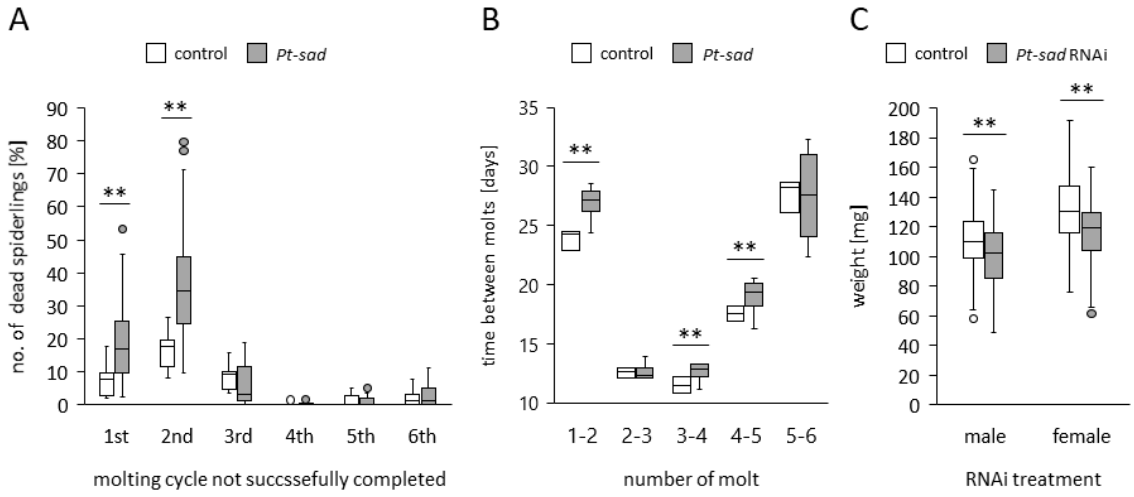


Figure 20 Effects of *Pt-sad* RNAi on postembryonic molting success, molting intervals and weight. (A) Percentage of spiderlings that failed to successfully complete the first through sixth molt. (B) Time intervals between the first to sixth molts. (C) Weight comparison of subadult male and female spiders in the sixth molt interval, 100 days after hatching. Asterisks indicate significant differences at **, P<0.001.

Finally, RNAi was performed on subadult males, the stage between the penultimate and final molt, to evaluate the effect of dsSad on molt duration and post-molt survival in individual animals. Significantly prolonged time to adult molt was observed in the dsSad-treated groups, especially at later injection times after the penultimate molt (days 5, 10 and 15), compared to controls (non-injected, H_2O, *tbx-20*). The *Pt-sad*_5 treatment resulted in the greatest delay, while early treatment (*Pt-sad*_1) resulted in a moderate but still significantly prolonged molt cycle (Fig. 21A). Post-molt survival times also varied between treatment groups. In particular, *Pt-sad*_1 resulted in significantly shorter survival compared to the control and late injections (days 10 and 15). *Pt-sad*_15 group showed prolonged survival similar to controls (Fig. 21B).

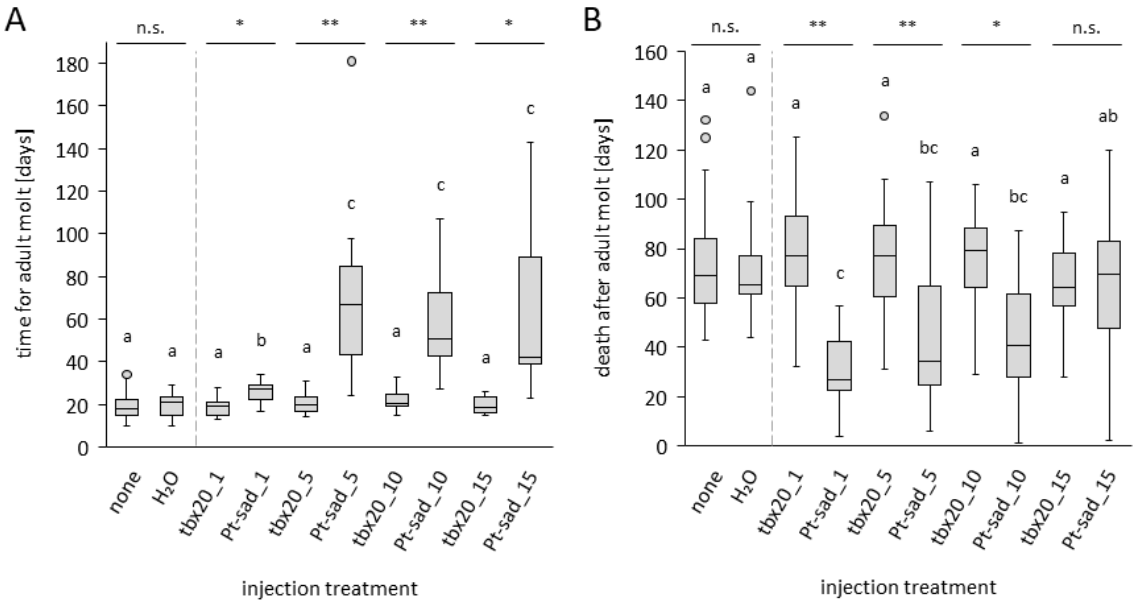


Figure 21 Effect of *Pt-sad* RNAi on subadult males after penultimate molt. (A) Time for molting from subadult to adult in spiderlings subjected to different treatments: none (no injection), H_2O (control, one day after penultimate molt), dsRNA of *tbx-20* (gene control) and *Pt-sad* at multiple time points (1, 5, 10, 15 days after penultimate molt). (B) Lifespan after the last molt in spiders subjected to different treatments. Asterisks indicate significant differences at P>0.05., n.s. (not significant); *, P<0.05; **, P<0.001. Letters above bars (a, b, c) indicate significant differences between groups.

3.2.3. Discussion

3.2.3.1. Pt-sad and phylogeny

The verification of a single full-length *Pt-sad* ortholog provides insight into the conservation and evolution of molting-related gene pathways in arthropods. The gene structure observed in *P. tepidariorum* comprising eight exonic regions is consistent with the *sad* of the pond spider *Pardosa pseudoannulata* (Yang et al., 2021). Phylogenetic analysis grouped *Pt-sad* with other chelicerate orthologs, with the highest similarity observed within araneid species. The characteristic CYP motifs, including the proline/glycine-rich domain, the Helix-C, Helix-I, Helix-K, PERF motif and the heme-binding domain, were identified in *Pt-sad*, indicating functional conservation of the enzyme (Lewis et al., 1998; Rewitz et al., 2006a; Rewitz et al., 2006b; Wan et al., 2014). These conserved features play pivotal roles in ecdysteroidogenesis and molting processes (Schumann et al., 2018) and other aspects of developmental regulation (Rewitz et al., 2006b; Lamb & Waterman, 2013).

3.2.3.2. Dynamic expression and RNA interference suggest a role of Pt-sad in different developmental processes

Pt-sad expression in the medial and distal leg areas at stages 8 to 11, followed by its absence later, suggests a role in the specification of leg muscle precursors. Such spatial and temporal expression dynamics are consistent with the patterns of other early appendage genes observed by Medina-Jimenez et al. (2024). In their study, marker genes such as *laminin*, *papilin* and *integrin alpha-PS2* of cluster VII showed similar expression to *Pt-sad*, suggesting a possible role in epithelial-to-mesenchymal transition (EMT) and development into visceral mesoderm cells.

The stereotypic pattern of *Pt-sad* in the invagination sites, emerging at embryonic stage 9, indicates its involvement in neurogenesis (Stollewerk et al., 2001). Parental RNAi-treated embryos (dsSad) showed variation in the presence and absence of these stereotypic cell clusters in the ventral neuroectoderm compared to wild-type expression. A reduction of invagination sites was also reported in RNAi experiments with the proneural gene *ASH1* in the spider *Cupiennius salei* (Stollewerk et al., 2001). With greater complexity in the subsequent stage in the wild type, corresponding to the increasing number of invagination sites, this pattern is also known from neurogenesis-

related genes such as *Krüppel*, *delta-2*/*notch*, or members of the Sox family (Haenlin et al., 1994; Stollewerk, 2002; Oda et al., 2007; Akiyama-Oda & Oda, 2016; Bonatto Paese et al., 2018; Schomburg et al., 2020). The impaired upregulation throughout the ventral neuroectoderm of dsSad embryos seen during this stage contrasts with the uniform expression in wild types. Especially the expression in the cheliceral segment is affected in knockdowns. This disruption is an indicator that *Pt-sad* not only affects molting and hormonal regulation, as it is commonly assumed for Halloween genes (Campli et al., 2024) but also plays a role in establishing neural segmentation (Clark et al., 2019). At stage 11, the numerous dots transform to a more lateral stripe of invaginating epithelial vesicles forming the neuromeres of the developing ventral nerve cord, suggesting a role in the specification of neurons (Stollewerk et al., 2001; Stollewerk, 2004).

Moreover, the expression in the head lobes at stage 9, followed by upregulation throughout the neuroectoderm in stages 10 and 11**,** supports the notion that *Pt-sad* plays an important role in the formation and differentiation of the central nervous system (Stollewerk et al., 2001; Stollewerk et al., 2003). dsSad embryos display substantially reduced and asymmetric expression in the head lobes, correlating with morphological defects, such as decreased head lobe size. These results are consistent with those of Chávez et al. (2000) in *D. melanogaster*, where the Halloween genes showed a similar phenotype in mutants. This observation suggests that *Pt-sad* may play a role in ensuring proper cell differentiation and patterning during neurogenesis. Additionally, a bilaterally symmetric pair of expression dots was identified in the non-neurogenic ectoderm (arrowhead in Fig. 16C) of wild types. This may correspond to the primary eye primordia that form during brain differentiation (Schomburg et al., 2015; Baudouin-Gonzalez et al., 2022; Janeschik et al., 2022). The absence of head lobe expression after stage 11 implies a role of *Pt-sad* primarily in the establishment and specification of neurons (Stollewerk et al., 2001; Stollewerk et al., 2003) and sensory structures (Schomburg et al., 2015; Baudouin-Gonzalez, 2022). Interestingly, a new cluster arises at the anterior rim of the developing brain in later embryonic stages, suggesting an additional role in late brain differentiation, although the function remains unclear.

A prominent expression of *Pt-sad* emerges dorsally in the opisthosoma at stage 12, correlating with the movement of the tergites leading to dorsal closure (Mittmann & Wolff, 2012). Repeated expression in clusters that overgrow the yolk and fuse into a longitudinal expression domain suggests a role in cardiogenesis (Janssen & Damen,

2008; Mittmann & Wolff, 2012). *Pt-sad* knockdown resulted in reduced dorsal expression in stage 12 embryos. This suggests a previously unreported role for *Pt-sad* in cardiogenic mesoderm specification. However, the molecular interactions linking *Pt-sad* to mesodermal differentiation, as well as the evolutionary pressures driving its functional diversification in arthropods as a whole, require further investigation. By stage 13, *Pt-sad* expression is seen in a tube-like structure that extends anteriorly in bilateral segmentally repeated triangular blotches. Their shape and position resemble the alary muscles in insects (Wasserthal & Wasserthal, 1977; Glenn et al., 2010; Bataillé et al., 2024). This similarity suggests a role of *Pt-sad* analogous to known cardiogenic marker genes such as *myocyte enhancer factor 2* (*Mef2*), *ryanodine receptor* (*ryr*) and *lethal 2*, represented in developing visceral mesoderm tissue (Leite et al., 2024; Medina-Jimenez et at., 2024). This pattern may show functional parallels to pathways involving *tinman/Nkx2-5*, which have been associated with cardiogenesis in other arthropods and bilaterians (Janssen & Damen, 2008; Zaffran et al., 2006; Qian et al., 2011).

In summary, the expression pattern of *Pt-sad* reveals a highly dynamic and complex patterning with potential roles in diverse developmental processes, such as muscle precursor specification, formation of neural and sensory structures and cardiogenesis. The transition from early expressions in neurogenic regions to later expressions in cardiogenic mesodermal areas suggests multifunctionality during embryogenesis. The significant increase in embryonic mortality and developmental delay observed in *Pt-sad* parental RNAi-treated embryos highlights its essential role in embryogenesis. This is consistent with previous studies on Halloween genes in insects, where knockdowns or knockouts impaired the viability by interfering with embryonic morphogenesis, ecdysteroid synthesis and molt interval duration (Wan et al., 2015; Yamanaka et al., 2013; Chávez et al., 2000). Furthermore, in contrast to studies on *D. melanogaster*, where ecdysteroid deficiency mainly affects cuticle formation (Jürgens et al., 1984; Wieschaus et al., 1984), this study suggests that *Pt-sad* has a broader role in head lobe development and cardiogenesis in spiders.

3.2.3.3. The roles of Pt-sad during postembryonic development and molting

Expression of *Pt-sad* in juveniles suggests developmental roles beyond its involvement in embryogenesis. The spatial distribution of *Pt-sad* expression in the prosoma and opisthosoma indicates its involvement in neural and other tissues, such as the midgut and ovary. In the juvenile prosoma, *Pt-sad* is prominently expressed in a reticular pattern in the subesophageal ganglion, which converges towards the commissural midline. This expression suggests a role in neuronal differentiation or neural integration. From the neurogenesis in the spider *C. salei* it is known, that during embryogenesis secondary invaginating cells, not showing any features of differentiation, are arrested in an immature state by formation of epithelial vesicles (Stollewerk, 2004). This process is crucial for facilitating the production of postembryonic neurons. Interestingly, one of the products of ecdysteroid synthesis, namely ecdysone, also regulates transcription factors that generate a diversity of neural precursors in *D. melanogaster* embryos and larvae (Syed et al., 2017). Given the crucial role of ecdysone in regulating the timing of temporal transitions of postembryonic neuroblasts in the developing central nervous system (Truman & Riddiford, 2023), it is possible that *Pt-sad* also serves as a regulatory node between ecdysteroid production and downstream effects in the postembryonic differentiation of neurons in the spider. The additional expression detected in the mushroom body suggests an extended role in the regulation of higher order neural functions, possibly related to sensory processing (Babu & Barth, 1984; Steinhoff et al., 2024).

In the opisthosoma, *Pt-sad* was detected as scattered dots in the ovary and midgut, similar to results from the bug *Cyrtorhinus lividipennis*, suggesting a possible role in midgut specification and aspects of reproductive organ formation and function (Hu et al., 2022). This observation is consistent with the emerging role of ecdysteroid pathway-related genes in the regulation of reproductive organ development in arthropods (Ameku et al., 2017; Peng et al., 2019; Zhou et al., 2020). A thin domain of *Pt-sad* expression framing the heart suggests a potential role in the further heart development in postembryonic stages, possibly as a continuation of its role during embryogenesis. Future research should explore the molecular interactions of *Pt-sad* within these tissues to better understand its role in the broader developmental context of arachnids and to elucidate its evolutionary conservation and diversification.

Parental *Pt-sad* RNAi treatment (dsSad) showed effects on postembryonic development, as evidenced by an increase in mortality rates and molting defect phenotypes. The molting defects observed fell into four categories. First, failure of molt initiation was the most common defect, affecting 19.2 % of parental RNAi-treated juveniles, highlighting the essential role of *Pt-sad* in enabling molt initiation and successful ecdysis. Failure to detach the prosomal shield, incomplete separation of complex cuticular structures and locomotor impairment after an apparently normal molt were less severe defect phenotypes, in which at least some aspects of ecdysis were initiated. Prolonged average molting time and arrest of molting followed by death without ecdysis (11.1 %) were also observed in the grasshopper *Locusta migratoria* after silencing the *sad* homolog (Zhang et al., 2021).

Mortality was significantly increased during the first and second molting cycles, which coincided with longer molting intervals. Wan et al. (2015) showed similar results when knocking down *sad* in the planthopper *Laodelphax striatellus*. This suggests that *Pt-sad* knockdown affects the timing or titter of ecdysteroid production, which is essential for the induction of molting. Previous knockdown of *chitin synthase* in the ladybird *Henosepilachna vigintioctopunctata*, which is critical for chitin synthesis, also resulted in an increase in molt defect phenotypes leading to lethality (Jiang et al., 2021). Given a similar effect in obtained RNAi experiments, these results indicate the involvement and function of *Pt-sad* in the spider ecdysteroid pathway. In addition, a significant reduction in body size of approximately 10 % in parental RNAi-treated offspring was observed, indicating an effect on overall growth and likely resource allocation due to problems in hormonal signaling.

The duration of ecdysis after the penultimate molt was significantly prolonged in all dsSad-treated juveniles, especially at later injection times (days 5, 10 and 15), with the *Pt-sad*_5 group showing the greatest delay. These results indicate that *Pt-sad* activity, which is essential for the accumulation of the molt-inducing ecdysteroid hormone titer, is most likely not initiated immediately after the previous molt. Post-molt survival times varied between the dsSad groups, with *Pt-sad*_1 treatment resulting in the shortest survival times, while later treatment (day 15) showed survival times comparable to the control groups. Overall, the two values, the time to reach the adult molt after penultimate molt and the survival time after the adult molt, roughly added up to an approximate life span of about 100 days. This means that the RNAi-affected individuals, while technically

remaining subadults, actually continue to age normally. This strongly suggests that *Pt-sad* RNAi specifically interferes with molting, but not with other processes related to aging. Thus, *Pt-sad*-controlled molting appears to be relatively low in the hierarchy of processes that control aging in spiders and progression through the postembryonic stages.

In summary, the expression of *Pt-sad* in juvenile spiders highlights its roles in neural development, reproductive organ formation and cardiogenesis, while RNAi-mediated knockdown experiments demonstrated that *Pt-sad* is critical for successful molting, with knockdown resulting in molting defect phenotypes. Future research should explore how *Pt-sad* regulates developmental processes such as molting and neurogenesis, while investigation of other ecdysteroid pathway-related genes and comparative studies in other ecdysozoans may reveal its evolutionary diversification.

3.2.4. Conclusion

This study provides insights into the multifunctional role of *Pt-sad*, a homolog of the molt-related Halloween gene *sad* in the spider *P. tepidariorum*. The observed expression patterns suggest a contribution to neural development and cardiogenesis in embryogenesis and postembryonic stages, with the latter showing additional expression in the ovary. In addition, the results indicate evolutionary changes in Halloween gene functions in spiders, including roles different from those observed in insects. RNAi experiments revealed spider-specific functions of *sad*, while also highlighting its conserved role in molting and development across arthropods. *Pt-sad* is essential for molting and participates in ecdysteroid hormone synthesis, also demonstrated in other arthropods like insects and crustaceans. Future research should focus on the molecular mechanisms associated with the ecdysteroid biosynthesis pathway and the molting process to elucidate the complexity of arachnid development.

3.2.5. Materials and Methods

3.2.5.1. Animal husbandry and embryo fixation

Specimens of *Parasteatoda tepidariorum* (Koch, 1841) were kept at 25°C ± 1°C in *Drosophila melanogaster* polystyrene breeding tubes covered with Ceaprene plugs and filled to one-third with coconut fiber. Spiders were fed *D. melanogaster* and supplied with water once a week. Cocoons containing up to 200 synchronously developing embryos were opened with forceps and developmental stages were determined according to Mittmann & Wolff (2012). For fixation, eggs were dechorionated with hypochlorite solution (DanKlorix) for 2–3 minutes. After several washes with distilled water (dH_2O) the embryos were fixed in 8 mL fixative (4 mL heptane, 0.5 mL 37 % formaldehyde, 3.5 mL PEMS [100 mM Pipes, 1 mM EDTA, 1 mM $MgSO_4$]) for 5 to 12 hours depending on the developmental stage. Embryos were washed with PEMT (PEMS containing 0.01 % Tween-20), transferred stepwise to 100 % methanol and stored at −20°C.

3.2.5.2. Phylogenetic analysis

A putative homolog of the *P. tepidariorum* Halloween gene *sad* (*Pt-sad*) was identified using BLASTp (NCBI) (Altschul et al., 1997) with the published *D. melanogaster sad* sequence (Rewitz et al., 2007; NP_650123.1) as query. The identity of the *Pt-sad* sequence was tested by a reciprocal BLASTp search against the *D. melanogaster* genome and verified by cloning. From the *P. tepidariorum* genome assembly (NCBI accession: GCF_000365465.3) and its annotation, the gene structure of *Pt-sad* (XM_016054492.2) was visualized using GenomeTools (version 1.6.2). A protein dataset of *sad* homologs from 21 species obtained from NCBI, including eight insects, three crustaceans, four spiders, three mites, one scorpion and one horseshoe crab, was used for the phylogenetic tree (Supplementary Table 3). A maximum likelihood tree was generated with RAxML-NG v. 1.2.0 (with settings --model LG+G8+F --tree pars{10} --bs-trees 1000) based on the multiple sequence alignment generated with Muscle v5.1 (Edgar, 2021). Trees were visualized using iTol v. 6.8.1 (Letunic and Bork, 2021).

3.2.5.3. Molecular cloning

Primers for a fragment of *Pt-sad* and the RNAi control gene *T-box transcription factor 20* (*tbx-20*) (Supplementary Table 4) were ordered from Eurofins Genomics and checked for dimers and target specificity using the online tools Tm Calculator (NEB), Multiple Primer Analyzer (ThermoFisher) and Primer-BLAST (NCBI). *tbx-20* is known for involvement in *D. melanogaster* leg development and embryonic heart formation in spiders (Svendsen et al., 2009; Janssen & Damen, 2008). Total RNA from embryonic stages 8 to 14 was isolated using the TRIzol® Reagent (ambion®, California). cDNA was synthesized using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics GmbH, Germany) according to the kit instructions. PCR-amplified DNA fragments were cloned into the pCRII vector using the Dual Promoter TA Cloning® (Invitrogen, California). The vectors were used for transformation of NEB® 5-alpha competent *Escherichia coli* (NEB, Germany). Plasmid DNA was purified using the Plasmid NucleoSpin® Kit for DNA, RNA and protein purification (MACHEREY-NAGEL, Germany). The nucleotide sequence of the insert was determined by commercial services (LGC sequencing).

3.2.5.4. Whole-mount in situ hybridization

Probes for *in situ* hybridization were prepared using T7 or Sp6 RNA polymerases, RNAse inhibitor and DIG RNA Labeling Mix (Roche Diagnostics, Germany). The whole-mount *in situ* hybridization (WISH) protocol was executed for fixed embryos as described by Prpic et al. (2008), with the following minor modifications. Post-fixation and proteinase K treatment were excluded from the procedure, as these steps did not result in detectable enhancements. Embryos were counterstained with the DNA marker SYTOX Green (Invitrogen, California). WISH on pro- and opisthosomata of postembryonic specimens in the last third of the molt interval between the fourth and fifth molt was performed after removal of all appendages prior to fixation. Tissues were fixed in 4 % formaldehyde in PBS-T for 24 hours and then sectioned at 80–120 µm thickness using a vibratome (Leica VT1000 S). Repeat fixation after sectioning and WISH on sectioned tissues was performed according to Prpic et al. (2008) with minor adjustments (see above).

3.2.5.5. RNA interference

The DNA template for RNA synthesis was generated via PCR using a T7 primer and an M13 primer supplemented with the T7 promotor sequence. Double-stranded RNA (dsRNA) probes targeting *Pt-sad* and *tbx-20* were synthesized using the MEGAscriptTM T7 High Yield Transcription Kit (Invitrogen, California) according to the manufacturer's protocol. Parental RNAi (Akiyama-Oda & Oda, 2006) was performed by injecting mature virgin females with a total of 2 µL of *Pt-sad* dsRNA (dsSad) at a concentration of >2.5 mg/µL in dH_2O. A total of 16 females received four injections at two-day intervals. Six females injected with dH_2O served as controls. The day after the first injection, females were mated with virgin males, with successful mating confirmed when bulb-to-epigyne contact was observed. The consecutive cocoons two to four from each female were used for further analysis. Cocoons from eight dsSad and three H_2O treated females were incubated, fixed as described above and used for WISH. The offspring of the other eight dsSad and three H_2O-injected females were incubated until hatching and immediately separated afterwards. Animals were checked daily for molting events and mortality for 100 days. All specimens that successfully completed the sixth molt were then starved for one week and weighed for size comparison.

To investigate the effect of RNAi on individual animals, additional dsRNA injections were performed on subadult males, defined as the stage between the penultimate and final molt (Quade et al., 2019). Subadults were treated once either 1, 5, 10 or 15 days (N > 20 each) after the penultimate molt by receiving 1 µL of dsSad or control dsRNA (*tbx-20*). These cohorts are referred to in this paper as *Pt-sad*_1, *tbx20*_1, *Pt-sad*_5, *tbx20*_5, *Pt-sad*_10, *tbx20*_10, *Pt-sad*_15 and *tbx20*_15, respectively. Additionally, 12 subadult males injected with H_2O after one day served as injection controls. Time to next molt was compared to the interval observed in wild-type males (N = 40). Descriptive statistics, including means, standard deviations and variance, were calculated using Microsoft® Excel® (version 2410). In addition, t-tests were performed using built-in functions.

3.2.5.6. Microscopy and imaging

Images were captured with a ZEISS AxioZoom.V16 Microscope and PlanNeoFluar z1x/0.25F objective in bright field and under the GFP filter setting with the Axiocam 305 Color Camera. The Z-stack mode of the ZEN microscopy software (blue edition; Carl Zeiss Microscopy GmbH) and the Helicon Focus program for focus stacking (version 7.7.5) were used to combine the images.

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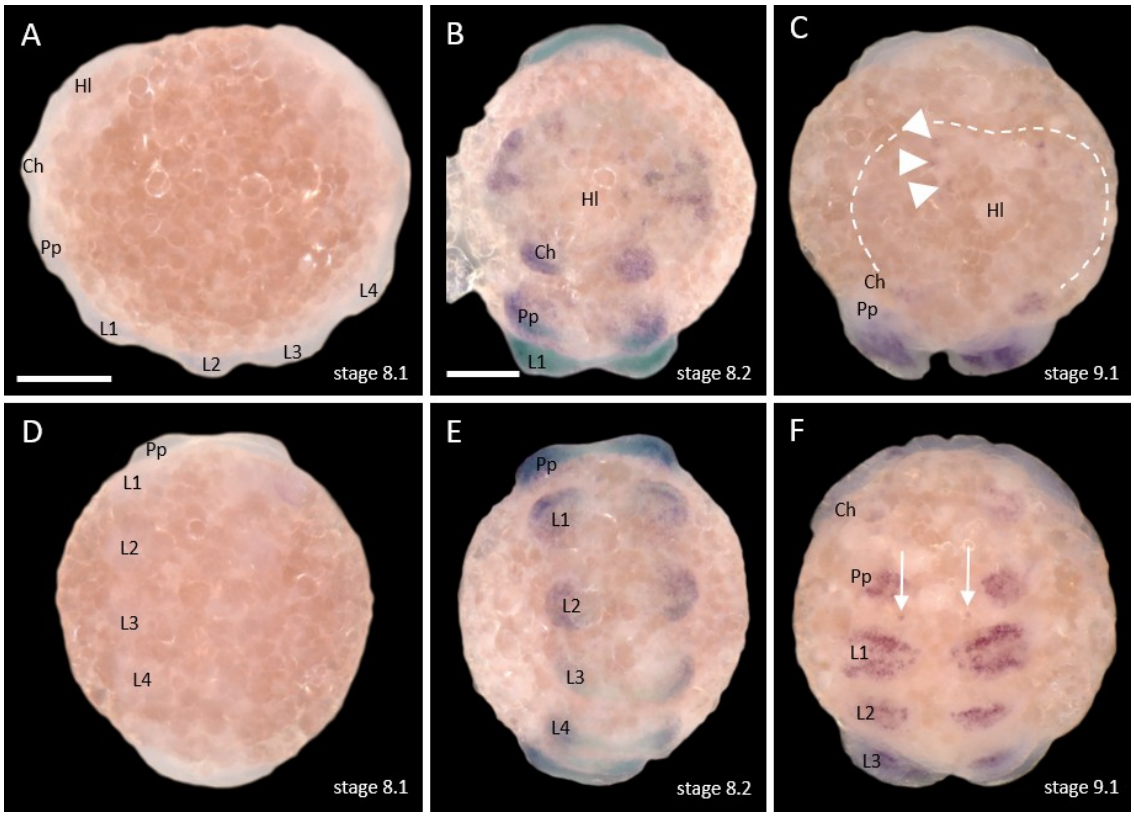
3.2.7. Supplementary Information

Supplementary Table 3 Protein accessions of homologs of the Halloween gene *shadow* identified in the genomes of various arthropods.

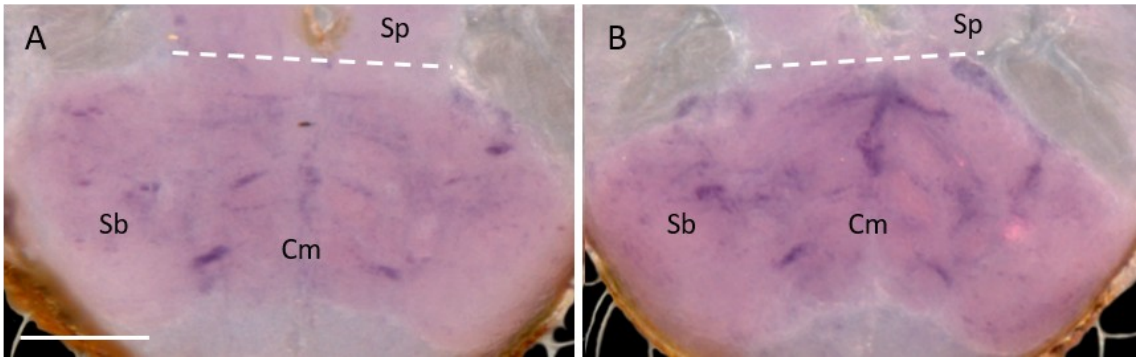
Taxa	Species	Accession
Insecta	*Drosophila melanogaster*	FBpp0081949
	Anopheles gambiae	XP_310837.6
	Manduca sexta	XP_030027247.1
	Bombyx mori	XP_021207530.1
	Chilo suppressalis	UUA80965.1
	Acyrthosiphon pisum	XP_001944183.2
	Apis mellifera	XP_395360.3
	Tribolium castaneum	XP_008193656.1
	Nilaparvata lugens	XP_022207767.2
"Crustacea"	*Daphnia pulex*	EFX70970.1
	Hyalella azteca	XP_018019751.1
	Penaeus vannamei	XP_027238669.1
Arachnida	***Parasteatoda tepidariorum***	**XP_015909978.1**
	Pardosa pseudoannulata	QIS60146.1
	Stegodyphus mimosarum	KFM70608.1
	Trichonephila clavipes	PRD32337.1
Acari	*Tetranychus urticates*	XP_015783494.1
	Ixodes scapularis	XP_002411841.2
Scorpiones	*Galendromus occidentalis*	XP_003743235.1
	Centruroides sculpturatus	XP_023243581.1
Xiphosura	*Limulus polyphemus*	XP_022243138.1
		XP_013794786.1
		XP_013779180.1
		XP_013775772.1

Supplementary Table 4 Primer sequences of the ecdysteroid biosynthesis pathway gene *shadow* from *P. tepidariorum* used for cloning and dsRNA synthesis.

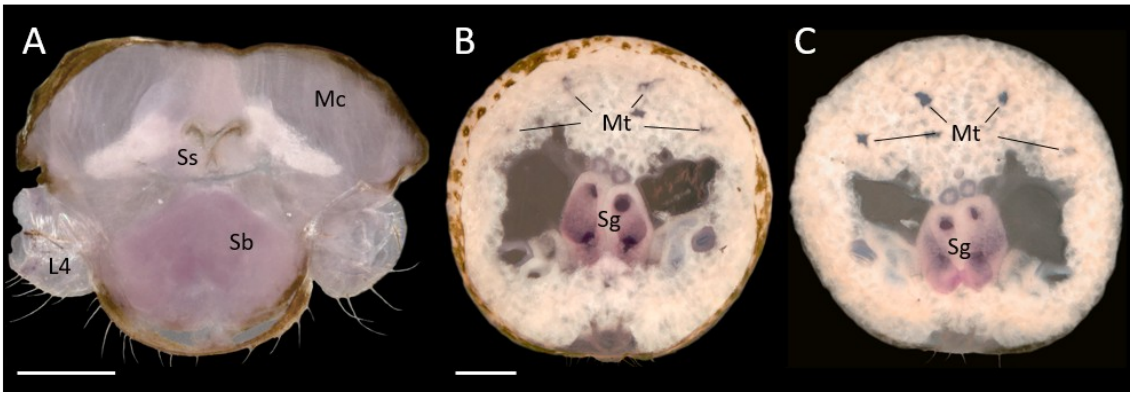
Gene	Name	Forward Primer	Reverse Primer
shadow (*sad*)	*Pt-sad*	GCATGGTCCAGATTTCAACGGTG	GGCTTGAACTCATGTGGTTCAGG
T-box20	*tbx20*	CCTTATTCTGCGGCCATGTC	CCCGTGAATGGGGAATCTGG
dsRNA	ds_T7	TAATACGACTCACTATAGGG	-
dsRNA	ds_T7M13	-	TAATACGACTCACTATAGGGCAG GAAACAGCTATGAC



Supplementary Figure 4 Embryonic expression of the Halloween gene *Pt-sad*. Lateral (A) and anterior (B, C) views with dorsal facing up, and ventral view (D–F) with anterior facing up. Nuclear marker SYTOX in green (B, E). (A, D) No expression in germ band detectable at stage 8. (B, C) Emerging expression in head lobes at stages 8.2 and 9.1 (arrowhead) in the developing invagination sites. (E) Expression in the forming appendage buds. (F) *Pt-sad* in appendages and first expression in ventral invagination site in posterior part of pedipalp hemisegment (arrows). Ch, chelicera; Hl, head lobes; L1–L4, legs 1 to 4; Pp, pedipalp. Scale bar: 100 µm (in A, in B for B–F).



Supplementary Figure 5 Detail of postembryonic expression of the Halloween gene *Pt-sad* in subesophageal ganglion sections. Dorsal is facing up. Dashed lines indicate boundary between supra- and subesophageal ganglion. (A) Bilaterally symmetric expression clusters (dark purple stain) and thin lines of expression expanding towards the commissural midline in subesophageal ganglion in section posterior to pedipalps and (B) between first and second pair of legs. Cm, commissural midline; Sb, subesophageal ganglion; Sp, supraesophageal ganglion. Scale bar: 100 µm (in A for A, B).



Supplementary Figure 6 *Pt-sad* sense control on postembryonic sections in the spider *Parasteatoda tepidariorum*. Dorsal is up in all images. (A) Section between third and fourth leg pair in the prosoma. (B, C) Sections at the level of silk glands in the opisthosoma, staining (magenta) in silk glands presumed artifact due to the adhesiveness of silk residues. L4, leg pair 4; Mc, muscles; Mt, Malpighian tubes; Sb, subesophageal ganglion; Sg, silk gland; Ss, sucking stomach. Scale bars: 200 µm (in A, in B for B, C).

Supplementary Video S1: Molting in wild-type specimen of the spider *Parasteatoda tepidariorum*. Video sped up six times.

Supplementary Video S2: Male RNAi-mediated knockdown spider one day after adult molt showing a molting defect phenotype of category 4 displayed by erratic movements. Video slowed down six times.

Videos are available on the provided flash drive.

3.3. Cytochrome P450s *spook*, *disembodied* and *shade* in Embryonic Development and Molting in the Spider *Parasteatoda tepidariorum*

In this part, functional involvement of the early ecdysteroid pathway gene *spook*, mid pathway gene *disembodied* and late pathway gene *shade* in the spider *Parasteatoda tepidariorum* are investigated. Utilization of expression analyses via scRNA-seq and whole-mount *in situ* hybridization, in combination with functional analyses through juvenile RNA interference-mediated knockdown suggests multifunctional role beyond molt involvement. Results indicate that all three genes are relevant for successful molting, while also indicate involvement in hemocyte formation during embryogenesis.

Denise Klinkenbuß, Nikola-Michael Prpic-Schäper

Author contributions to this research:

Denise Klinkenbuß: Data curation, Formal analysis, Validation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Nikola-Michael Prpic-Schäper: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Status: **First Manuscript** for Submission

Cytochrome P450s *spook*, *disembodied* and *shade* in Embryonic Development and Molting in the Spider *Parasteatoda tepidariorum*

Highlights

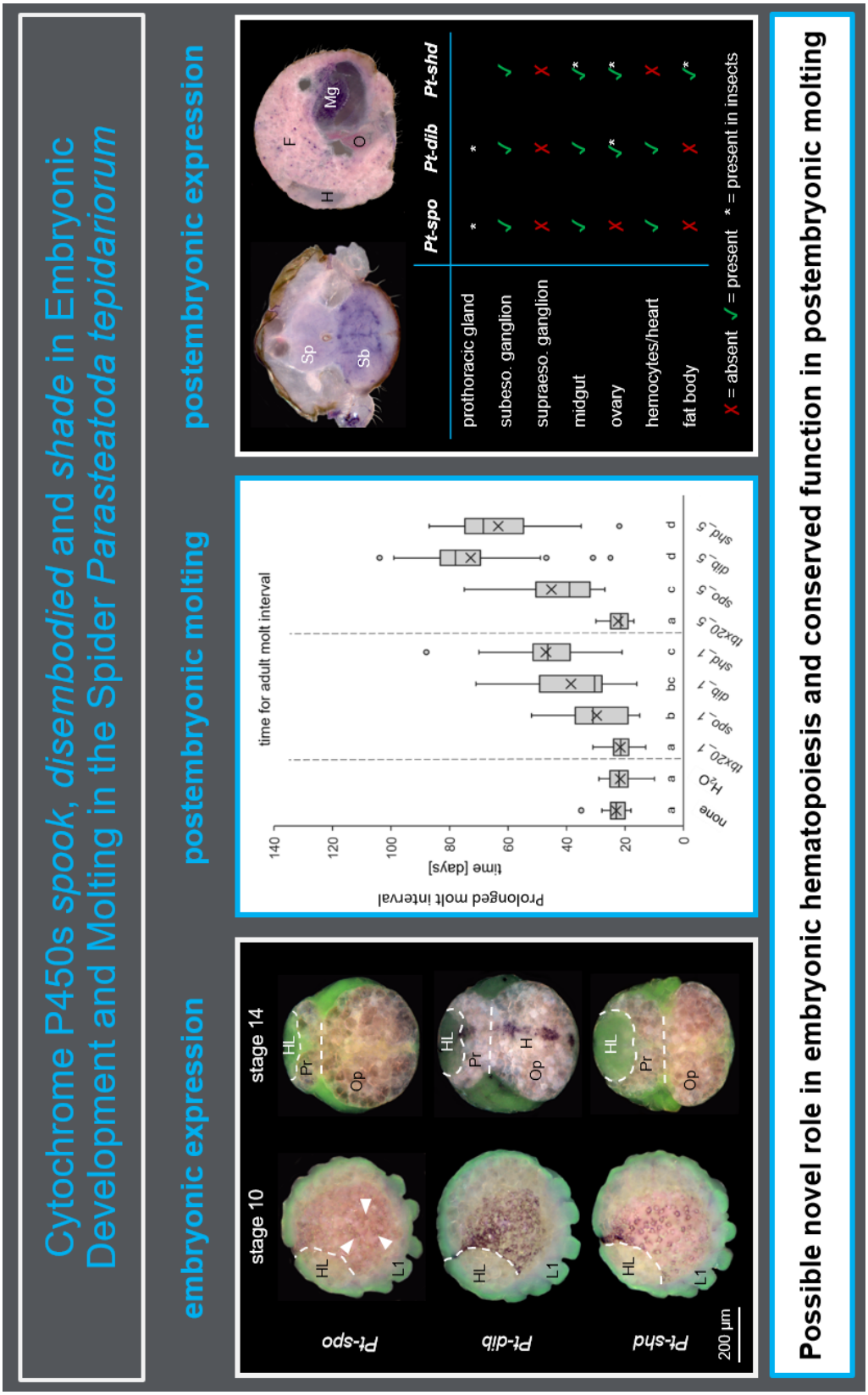
- Ortholog of *phantom* CYP P450 is absent from the spider genome.
- Embryonic expression of studied genes suggests involvement in hematopoiesis.
- Conserved juvenile expression domains of *Pt-shd* in ovary and fat body.
- RNAi of *Pt-spo*, *Pt-dib* and *Pt-shd* result in prolonged molt intervals.

Abstract

Ecdysteroid hormones are key drivers for developmental transitions in arthropods. Their biosynthesis is well studied in insects and depends on a stepwise pathway including the Rieske-domain oxygenase Neverland, the short-chain dehydrogenase/reductase Shroud and multiple cytochrome P450 enzymes known as Halloween genes. To elucidate the conservation of the ecdysteroid pathway in postembryonic stages and involvement in embryogenesis in chelicerates, a comparative analysis of the three cytochrome genes *spook*, *disembodied* and *shade* is employed in the spider *Parasteatoda tepidariorum*. The spatiotemporal expression of all genes is detected in mid-embryonic stages in cells covering the yolk, possibly related to hemocyte formation. *Pt-spo* and *Pt-shd* transcripts diminish in late-embryogenesis. Further non-traditional expression domains of *Pt-spo* and *Pt*-dib in juveniles include hemocytes, suggesting potentially deviated roles in hormone production or signaling, whereas the enrichment of *Pt-shd* in ovary and fat body highlights a conserved function. RNA interference underscores the essential nature of these genes by causing molting delay explainable through slowed ecdysteroid biosynthesis. These findings support an evolutionary model in which Spook, Disembodied and Shade ensure proper ecdysteroid biosynthesis for chelicerate development, while also hinting at diversified, tissue-specific roles. The results lay a foundation for deeper investigations into ecdysteroid biosynthesis and molting processes in arachnids.

Keywords: ecdysteroid, Halloween gene, molting, RNAi, arachnid

Graphical abstract



3.3.1. Introduction

Ecdysteroid molting hormones regulate growth and developmental transitions for embryogenesis, molting and metamorphosis in arthropods (Thummel, 2001; Ishimoto et al., 2009). The most studied bioactive ecdysteroid is 20-hydroxyecdysone (20E), which is produced through multi-step enzymatic conversions (Niwa & Niwa, 2016). These steps are catalyzed by the so-called Halloween genes (Gilbert et al., 2002; Rewitz et al., 2006b). The genes were originally identified in *Drosophila melanogaster* mutants and display a lack of cuticle deposition leading to lethal phenotypes in embryogenesis (Jürgens et al., 1984; Wieschaus et al., 1984). Each Halloween gene corresponds to a specific cytochrome P450 monooxygenase (CYP) in the ecdysone biosynthetic pathway that converts dietary cholesterol into 20E via a chain of hydroxylations and oxidations (Namiki et al., 2005; Warren et al., 2004; Chávez et al., 2000; Warren et al., 2002; Petryk et al., 2003).

The CYP genes *spook* (*spo*), *phantom* (*phm*), *disembodied* (*dib*), *shadow* (*sad*) and *shade* (*shd*) are conserved across most arthropods (Pan et al., 2021; Campli et al., 2024). Orthologs have been identified in numerous insect orders and even in crustaceans and chelicerates, indicating that the ecdysteroid pathway is an ancient developmental mechanism (Schumann et al., 2018). In holometabolous insects the Halloween genes are expressed at multiple life stages to support each molting cycle. For instance, *D. melanogaster* embryos and early larvae rely on the enzyme Spook for ecdysone biosynthesis, but intriguingly, *spo* expression is greatly reduced after embryogenesis (Ono et al., 2006). Instead, the fruit fly utilizes a paralogous enzyme called Spookier during larval stages. This demonstrates an evolutionary innovation/duplication unique to higher dipterans that partitions ecdysone production between embryonic and postembryonic development (Namiki et al., 2005; Ono et al., 2006; Van Ekert et al., 2016). By contrast, *dib* and *shd* are single-copy in *D. melanogaster* and are presumed to function through all life stages in insects (Petryk et al., 2003, Gilbert & Rewitz, 2009). Indeed, expression profiling in the moth *Plutella xylostella* showed continuous and adult-stage upregulation of *dib* and *shd* in ovaries, underscoring their role at multiple transitions including reproduction (Ameku et al., 2017; Peng et al., 2019).

In insects, the spatial expression pattern of most Halloween genes in postembryonic stages is localized to the prothoracic glands, an endocrine organ synthesizing and secreting ecdysone (Bian et al., 2019). The inactive ecdysone is converted to active 20E via expression of *shd* detected in peripheral tissues such as the epidermis, midgut, Malpighian tubules and fat body (Petryk et al., 2003). This spatial division of synthesis appears to be common in holometabolous insects. In hemimetabolous insects and non-insect arthropods the ecdysteroidogenic tissues are less well characterized. Recent genomic surveys suggest that chelicerates lack a *phantom* ortholog, implying a modified pathway (Kamiyama & Niwa, 2022). Nonetheless, the genes *spo*, *dib* and *shd* were recovered from spider genomes and relative expression analyses were investigated (Schumann et al., 2018; Yang et al., 2021). As maternal contributions of the ecdysteroid hormone are insufficient for completion of embryogenesis (Sonobe & Yamada, 2004), arthropod embryos must produce a hormone pulse during late development to initiate cuticle secretion and hatching (Bordes-Alléaume & Sami, 1987). Therefore, zygotic expressions of *spo*, *dib* and *shd* should be required. However, understanding of embryonic expression domains of these genes is spare (Chávez et al., 2000; Petryk et al., 2003; Namiki et al., 2005). The question arises whether chelicerates like spiders express these genes during embryogenesis in analogous patterns and tissues.

In this research the Halloween genes *spook*, *disembodied* and *shade* are studied during embryogenesis, with an emphasis on the mid-to-late embryonic stages, when organogenesis and cuticle formation are initiated, in the spider *Parasteatoda tepidariorum* (Mittmann & Wolff, 2012). To map the spatial expressions, single-cell RNA sequencing profiles were combined with *in situ* hybridization data. Additionally, embryonic results are merged with functional studies utilizing RNA interference knockdowns on juveniles to include the developmental roles of *spo*, *dib* and *shd* in postembryonic stages. Collectively, these integrative data are expected to clarify when and where each gene operates during spider development and to establish a foundation for future comparative work on chelicerate ecdysteroidogenesis.

3.3.2. Results

3.3.2.1. Identification and verification of mid-to-late ecdysteroid pathway genes

Full-length orthologs of the mid-to-late ecdysteroid pathway genes *Pt-spo* (NCBI accession: XM_016071382.2), *Pt-dib* (XM_043057079.1) and *Pt-shd* (XM_043047939.1) were identified and verified via cloning and sequencing. The genes contain an open reading frame of 1,560, 1,335 and 1,572 base pairs, encoding proteins of 519, 444 and 523 amino acids, respectively. The genes consist of 8 or 9 exons for the isoforms of *Pt-spo*, 5 exonic regions for both *Pt-dib* isoforms and 9 exons for *Pt-shd* according to gene organization analysis (Fig. 22). The two isoforms for *Pt-spo* are predicted to encode two different proteins, however the primers used in this study were designed to match the sequence portion common to both. The two isoforms of *Pt-dib* encode for identical proteins but differ in their 3´ untranslated region (UTR).

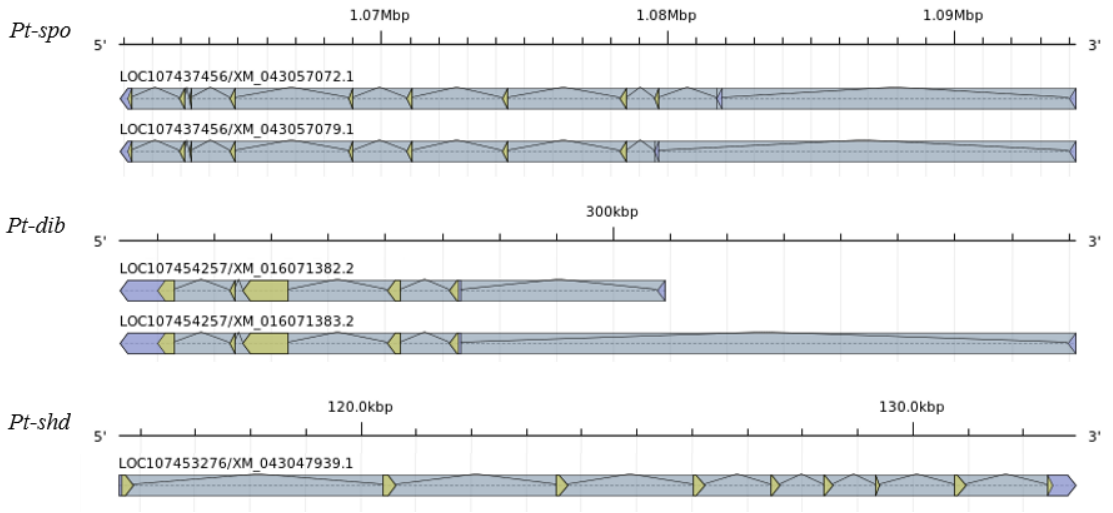


Figure 22 Genomic organization of the Halloween genes *Pt-spo*, *Pt-dib* and *Pt-shd*. Exon-intron structures consist of 33 kbp in *Pt-spo*, 16.5 and 9.5 kbp in *Pt-dib*, and 17.5 kbp in *Pt-shd,* respectively. Exonic regions displayed in yellow, introns in grey and UTR is indicated in blue.

The presumed Halloween gene sequences of *P. tepidariorum* cluster together with their respective orthologs from other panarthropods (Fig. 23; Supplementary Table 5). The characteristic distinction of CYPs in (1) mitochondrial, including *disembodied*, *shadow* and *shade*, and (2) CYP2-related clan, consisting of *spook*, *phantom* and *CYP18A1*, was recovered from the protein-based gene tree. The *spookier* and *spookiest* sequences are nested within the *spook* cluster, while *dib* and *shd* are branching from the same ancestral node (Fig. 23). No *phantom* sequence was detected in the spider genome.

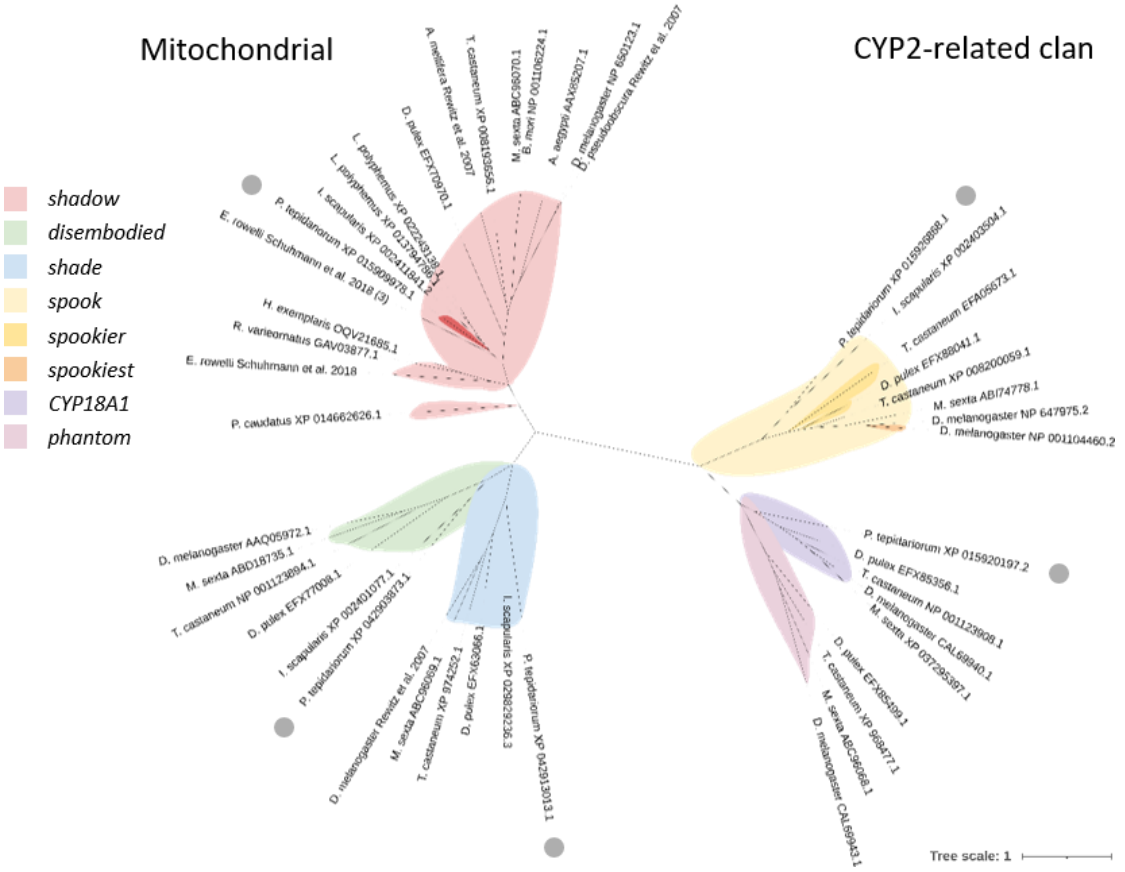


Figure 23 Protein-based gene tree of molt-related cytochrome P450s in Panarthropoda. Halloween gene orthologs of *P. tepidariorum* group with designated gene clusters of other ecdysozoans (Supplementary Table 5). Distinction of mitochondrial and CYP2-related clan was recovered. Grey dots indicate localization of spider sequence within clusters.

3.3.2.2. Expression analysis of molt-related cytochromes during embryogenesis

Uniform manifold approximation and projection plot (UMAP) from single-cell RNA sequencing (scRNA-seq) data of rPCA integrated embryonic stages 7 to 9 (Leite et al., 2024) displays distinct expression of *Pt-spo*, *Pt-dib* and *Pt-shd* in cluster 5 defined as “dorsal” tissue (Fig. 24A). *Pt-spo* and *Pt-shd* indicate a similar number of transcribing cells, while *Pt-dib* expression levels are doubled. Additionally, minor expression of *Pt-dib* is evident in the endoderm cluster 20 and *Pt-sad* expressing cells appear in the mesodermal cluster 17.

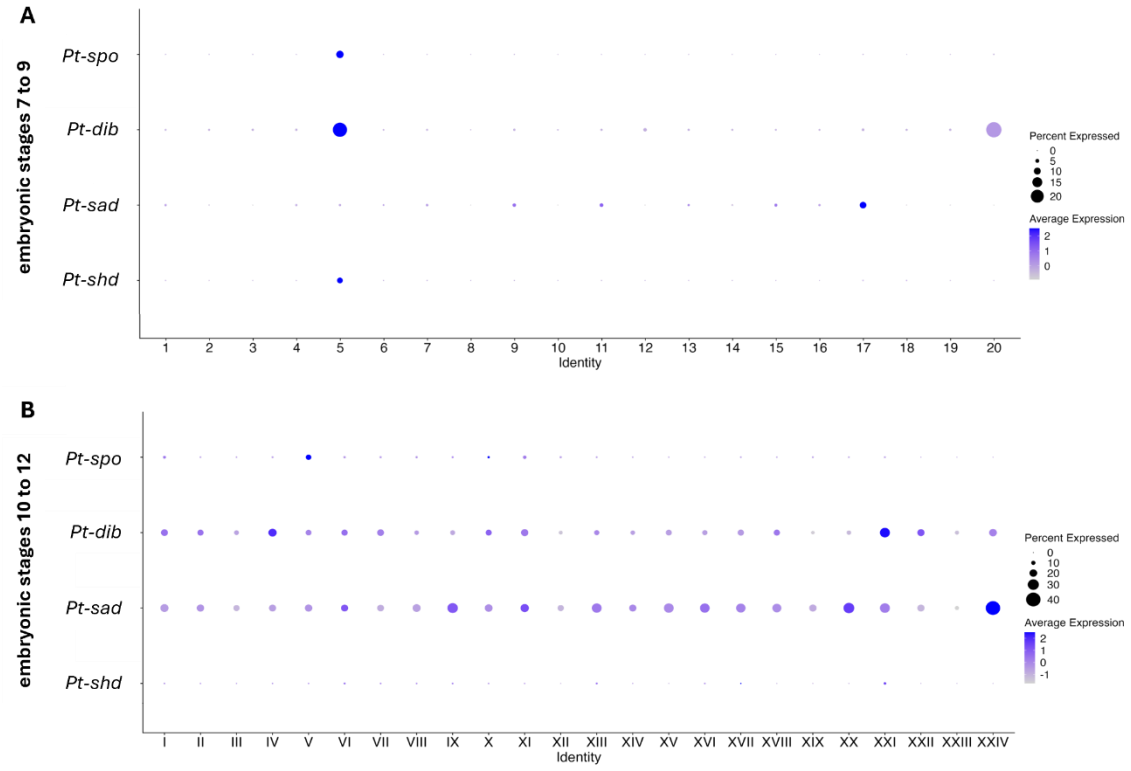


Figure 24 scRNA-seq based expression analysis of molt-related cytochromes in embryogenesis. Dot plots show (A) expression of genes in clusters for rPCA integrated embryonic stages 7 to 9 (Leite et al., 2024) and (B) pooled embryonic stages 10 to 12 (Medina-Jiménez et al., 2024). Percentage of expressing cells per cluster corresponds to dot size and average expression levels are display by blue gradient. For UMAP of rPCA integrated stages 7 to 9 see Supplementary Figure 7.

The pooled embryonic scRNA-seq data of stages 10 to 12 (Medina-Jiménez et al., 2024) revealed *Pt-spo* expression in cluster V corresponding to cells of the central nervous system (CNS). *Pt-dib* and *Pt-sad* share similar expression evidence in multiple clusters (Fig. 24B). Both exhibit expressing cells in cluster I, identified as "dorsal" tissue, mesodermal cluster VII, midgut/yolk/immune cluster XXI, stomodaeum/ventral midline cluster XXII and possible leg innervation cluster XXIV. Additionally, both genes display expression in mega-clusters defined by Medina-Jiménez et al. (2024), including ectoderm appendages (*Pt-dib*: II, IV, XVIII and *Pt-sad*: II, IV, XIII, XV, XVIII) and CNS (*Pt-dib*: V, VI, X, XI, XVII and *Pt-sad*: V, VI, VIII, X, XI, XVII, XIX). *Pt-sad* is furthermore present in cells of cluster IX belonging to opisthosomal appendages, cluster XIV representing the ventral sulcus and cluster XX defined by heart tissue. Cells expressing *Pt-shd* were not recovered from mid-to-late embryonic stages.

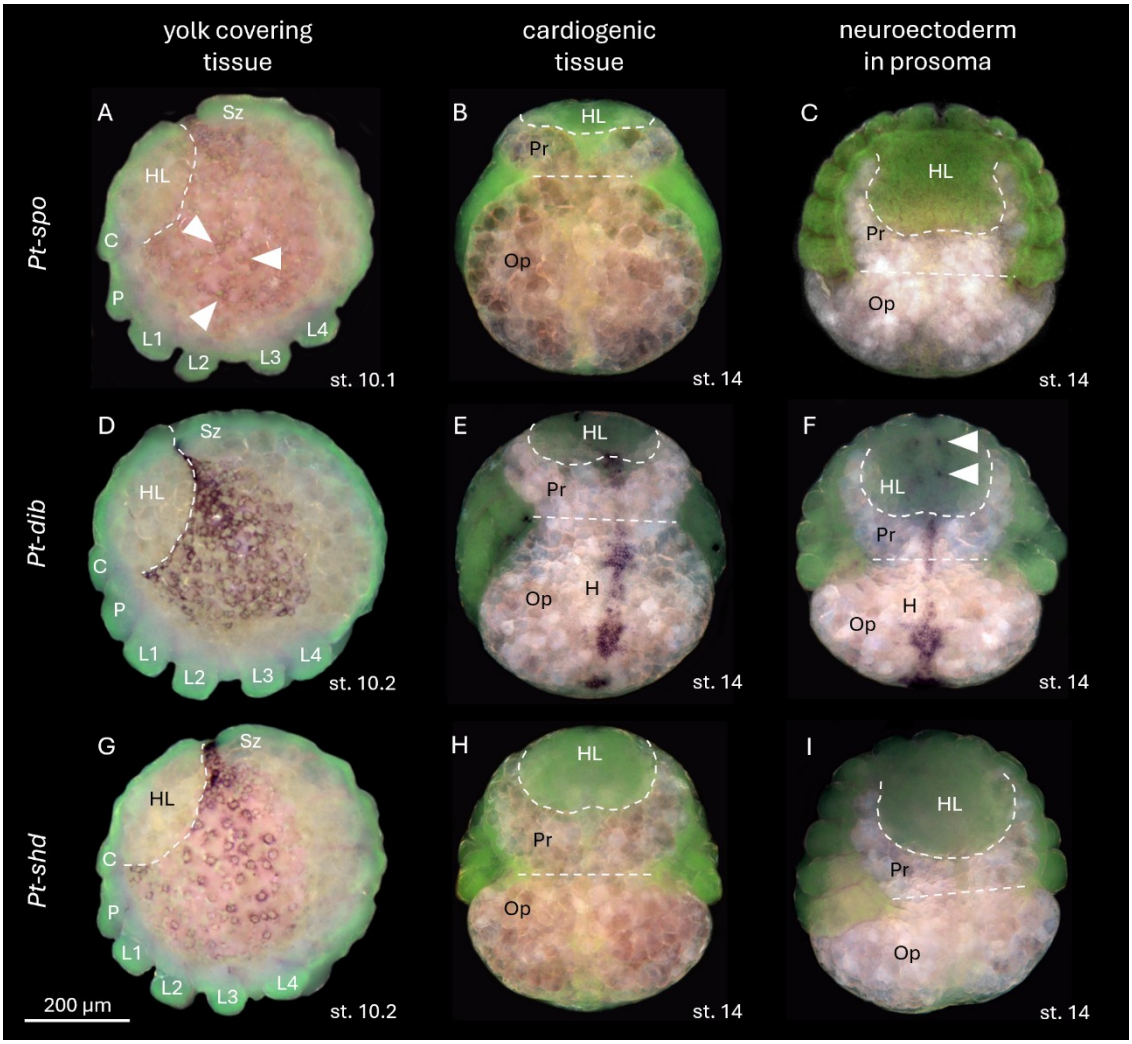


Figure 25 Embryonic expression of ecdysteroid pathway-related Halloween genes. Lateral view with dorsal facing up (A, D, G); dorsal view with anterior up (B & C, E & F, H & I). Nuclear marker SYTOX in green. (A) *Pt-spo* expression indicated in cells covering the yolk at stage 10 (arrowheads). (B, C) Note the absence during later embryogenesis. (D) Expression of *Pt-dib* located in yolk covering cells at stage 10, (E) in centered dorsal cardiogenic tissue and (F) in bilateral symmetric pattern in head lobes at stage 14 (arrowheads). (G) *Pt-shd* expression in single cells covering the yolk. (H, I) Note absence in following embryonic development. Dashed line indicates restriction between pro- and opisthosoma, and dashed form marks rim of forming head lobes. C, chelicera; HL, head lobes; H, heart; L1–L4, legs 1 to 4; Op, opisthosoma; P, pedipalp; Pr, prosoma; Sz, segment addition zone.

Patterns of the ecdysteroid pathway genes observed in embryonic stages of *P. tepidariorum* via whole-mount *in situ* hybridization reveal dynamic shifts throughout development. Staining in the "extraembryonic tissue" is detected for the pathway genes *Pt-dib*, *Pt-shd* and faintly for *Pt-spo* (Fig. 25A, D, G). *Pt-dib* and *Pt-shd* display a leopard-like pattern in large cells of the yolk covering tissue (Fig. 25D, G). The staining is most intense for *Pt-dib*, followed by *Pt-shd* and *Pt-spo*. Expression of *Pt-spo* and *Pt-shd* diminishes over the course of embryonic development and is absent during late embryonic stages (Fig. 25B, C; H, I).

After dorsal closure at stages 13 and 14, when the constriction between pro- and opisthosoma occurs, *Pt-dib* mRNA is detectable on the opisthosoma and forms a dorsal tube-like pattern. This vessel gradually expands anterior until it reaches into the prosoma at stage 14 when pre-cuticle secretion takes place. In this final stage of embryogenesis, repeated blotches branching from the central vessel appear (Fig. 25E). *Pt-dib* additionally presents a bilateral symmetric expression domain consisting of four dots arranged in a square-like configuration in the brain lobes (Fig. 25F, arrowheads).

3.3.2.3. Expression of Pt-spo, Pt-dib and Pt-shd during postembryonic development

The spatial distribution of *Pt-spo*, *Pt-dib* and *Pt-shd* in juvenile specimens was assessed to identify potential domains of ecdysteroidogenesis (Fig. 26). All three molt-related CYP genes exhibited constant, molt interval-independent mRNA expression within the subesophageal ganglion of the prosoma (Fig. 26A, C, E). The detected signals were organized in bilaterally symmetric clusters and presented a “salt-and-pepper” distribution. In contrast to embryonic expressions, *Pt-spo* displayed the most prominent patterning (Fig. 26A).

Multiple expression domains were also evident in the opisthosomata. *Pt-spo* displayed noticeable patterns within the hemocytes of heart efferent vessels (arrowheads, Fig. 26B) and in cells covering the midgut. The posterior staining is most likely artificial and caused by leaked silk components from the silk glands. Expression in hemocytes within the heart tube is also observed for *Pt-dib*, in addition to faint ubiquitous expression in the developing ovary (Fig. 26D). No mRNA was detected in the midgut. Observed signals of *Pt-shd* included large singular cells embedded in the fat body (see exemplary arrowheads Fig. 26F), ubiquitous staining of the ovary and particularly intense staining in epithelium cells covering the midgut (Fig. 26F). In contrast, no *Pt-shd* expression was detected in hemocytes.

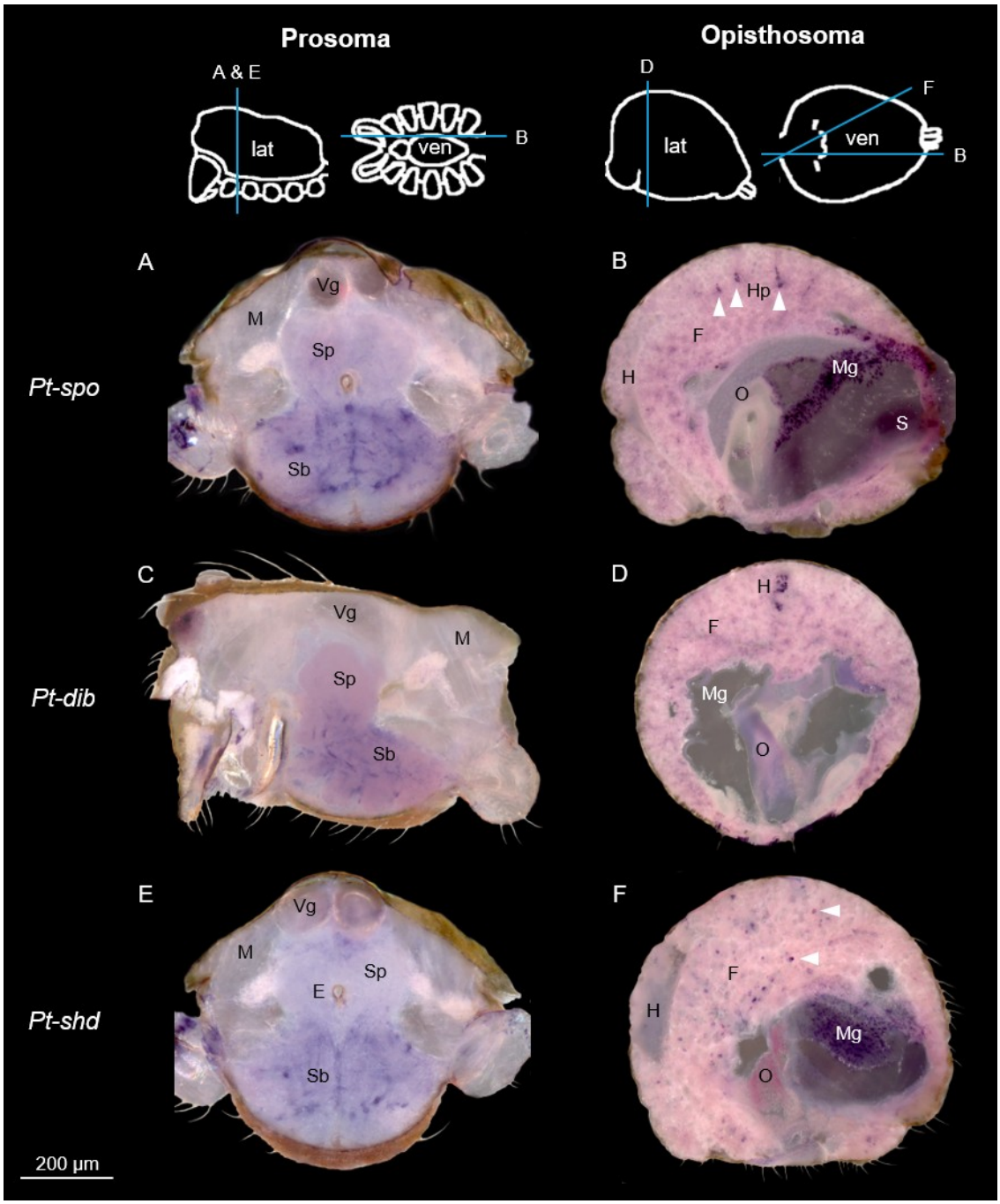
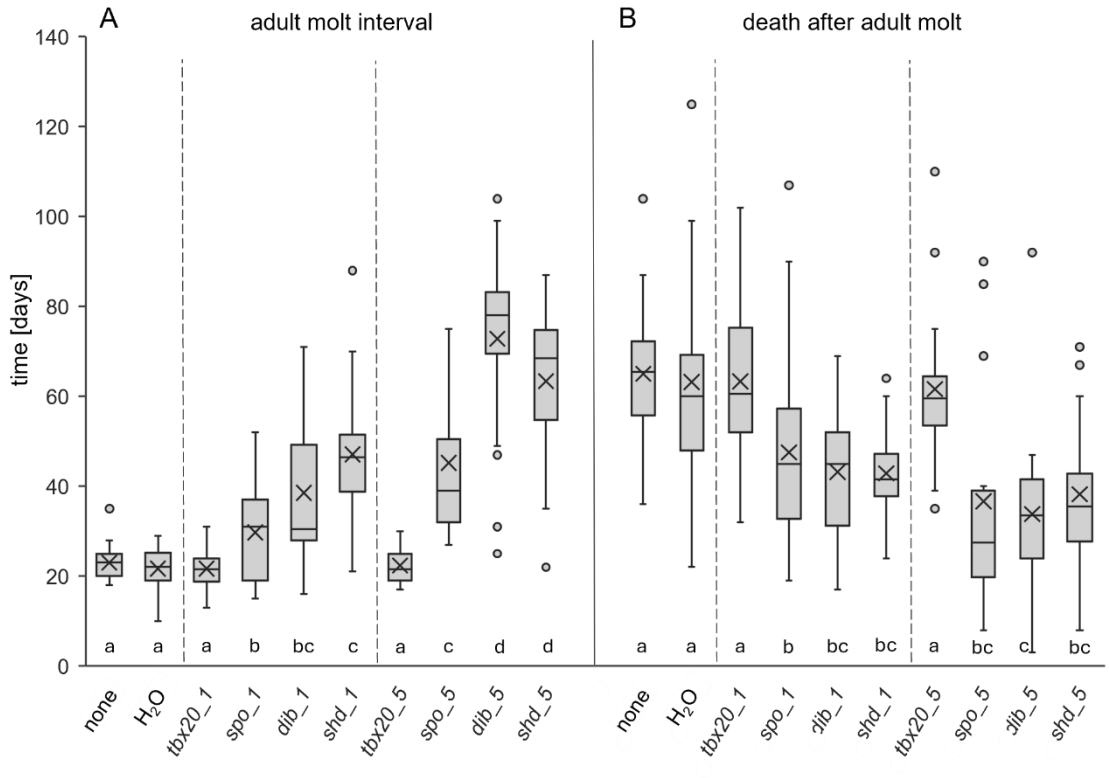


Figure 26 Postembryonic expression of molt-related cytochromes *Pt-spo*, *Pt-dib* and *Pt-shd*. Morphological schemes redrawn after Foelix (1996). Anterior is left in the diagrams; dorsal is top in all images. (A–B) Expression (dark purple stain) of *Pt-spo* with (A) staining in the subesophageal ganglion of the prosoma and (B) midgut and hemocytes (arrowheads) in the opisthosoma. Note artificial posterior staining caused by leaked silk. (C–D) Expression of *Pt-dib* with (C) staining in the subesophageal ganglion of the prosoma and (D) hemocytes within the heart tube in addition to ovary in the opisthosoma. (E–F) Expression of *Pt-shd* with (E) staining in the subesophageal ganglion of the prosoma and (F) in cells of the fat body, ubiquitously in the ovary and in cells covering the midgut of the opisthosoma. E, esophagus; F, fat body; H, heart; Hp, heart pockets; lat, lateral view; M, muscle; Mg, midgut; O, ovary; S, silk; Sb, subesophageal ganglion; Sp, supraesophageal ganglion; ven, ventral view; Vg, venom gland.

3.3.2.5. Molt-related cytochrome RNA interference effects ecdysis and lifespan



C

	none	H_2O	*tbx20*_1	*spo*_1	*dib*_1	*shd*_1	*tbx20*_5	*spo*_5	*dib*_5	*shd*_5
none		n.s.	n.s.	*	***	***	n.s.	***	***	***
H_2O	n.s.		n.s.	**	***	***	n.s.	***	***	***
*tbx20*_1	n.s.	n.s.		**	***	***	n.s.	***	***	***
*spo*_1	**	*	*		n.s.	***	*	**	***	***
*dib*_1	***	**	**	n.s.		n.s.	***	n.s.	***	***
*shd*_1	***	**	***	n.s.	n.s.		***	n.s.	***	**
*tbx20*_5	n.s.	n.s.	n.s.	*	**	***		***	***	***
*spo*_5	***	**	**	n.s.	n.s.	n.s.	**		***	**
*dib*_5	***	***	***	*	n.s.	n.s.	***	n.s.		n.s.
*shd*_5	***	***	***	n.s.	n.s.	n.s.	***	n.s.	n.s.	

Figure 27 Effects of *Pt-spo*, *Pt-dib* and *Pt-shd* RNAi on subadult males after penultimate molt (APM). (A) Time for molting from subadult to adult in subadults subjected to different treatments: none (no injection), H_2O (control, 1 day APM), dsRNA of *tbx20* (gene control), dsRNA of *Pt-spo*, *Pt-dib* and *Pt-shd* at multiple time points (1 & 5 days APM). N = 20 for all treatment groups. (B) Lifespan after the last molt in spiders subjected to different treatments. Letters under bars (a, b, c, d) indicate significant differences between groups. (C) Pair-wise significance matrix for the injection treatments. White background for 'adult molt interval' and grey background for 'death after adult molt'. Asterisks indicate significant differences at P>0.05, n.s. (not significant); P<0.05, *; P<0.01, **; P<0.001, ***.

To assess the functional significance of *Pt-spo*, *Pt-dib* and *Pt-shd* during ecdysteroid biosynthesis and molting, knockdown studies via RNA interference were conducted. The molting interval in wild type, H_2O and *Pt-tbx20* controls varied insignificantly between 21.7 and 23 days. Knockdowns with gene-specific dsRNA injection one day after the penultimate molt (APM) resulted in 29.35 % (*Pt-spo*) to 104.78 % (*Pt-shd*) prolongation in molting intervals. Meanwhile injections 5 days APM increased molt intervals by 96.62 % (*Pt-spo*) to 216.52 % (*Pt-dib*) compared to wild types. The latest observed non-lethal molting occurred 104 days APM in a *Pt-dib*_5 treated individual. In general, only two lethal molting defects were detected, both appearing in *Pt-spo*_5. Most CYP gene treatments show no significant difference to each other. Average adult life span after ultimate molt averaged between 61.6 and 65 days in controls. Individuals injected one day APM displayed a decrease in survival time of 26.85 % in *Pt-spo* to 34 % in *Pt-shd*. Injections 5 days APM yielded reductions compared to control average of 41.15 % in *Pt-shd* and up to 48 % in *Pt-dib*. No age-dependent differences considering added molt and survival time were detected between control and treatment groups ($p = 0.41$).

3.3.3. Discussion

3.3.3.1. Evolutionary conserved ecdysteroid pathway components

In the phylogenetic analysis gene sequences of *Pt-spo*, *Pt-dib* and *Pt-shd* clustered with their respective orthologs from *D. melanogaster* and other arthropods. This confirms the previous study of Yang et al. (2021), which detected conserved domains of ecdysteroid pathway genes in spiders. The same study also identified numbers of exonic regions identical to *Pt-spo*, *Pt-shd* and the long isoform of *Pt-dib* in the pond spider *Pardosa pseudoannulata*. Interestingly some holometabolous insects utilize a *spo* duplicate (*spookier*) for larval biosynthesis (Namiki et al., 2005; Ono et al., 2006; Van Ekert et al., 2016). This stage-specific specialization seems reserved to higher dipterans, as only a single copy is observed in *P. tepidariorum* and in most insects, crustaceans and other chelicerates (Namiki et al., 2005; Marchal et al., 2011; Yuan et al., 2021). As expected, no ortholog of *phantom* was observed in the spider. This confirms that the gene originated first in Mandibulata and supports the usage of Ponasterone A as the active molting hormone in chelicerates (Niwa et al., 2004; Iga & Kataoka, 2012; Campli et al., 2024). After previous studies reported their absence, here the presence of *dib* and *shd* was indicated in *Ixodes scapularis* (Qu et al., 2015; Schumann et al., 2018; Rotenberg et al., 2020; Qi et al., 2023).

3.3.3.2. Canonical and novel transcription domains of pathway cytochromes

Expected patterns from scRNA-seq for embryonic stages 7 to 9, indicating expression in the dorsal tissue covering the yolk (Leite et al., 2024), are detected in later stages via *in situ* hybridization. Expression of *Pt-dib* in embryonic stage 10 matches with predicted scRNA-seq data and represents cells covering the yolk (Medina-Jiménez et al., 2024). The absence of *Pt-shd* in the scRNA-seq data also consists with *in situ* hybridization results. The remaining expression predictions from scRNA-seq data of stages 10 to 12, especially in central nervous system and ectoderm appendage clusters, do not resemble observed patterns. This could be caused by low sequencing depth (~11 %) or disproportionate removal of yolk-covering tissue (Medina-Jiménez et al., 2024). While the exact tissue identity needs confirmation, the spatiotemporal expression strongly suggests that spider embryos transcriptionally activate the genes *Pt-spo*, *Pt-dib* and *Pt-shd* in mid-development to ensure hemocyte formation. This observation is based

on the later location of those expressing cells within the boundaries of the heart tube (Janssen & Damen, 2008, Mittmann & Wolff, 2012) and raises the possibility that hemocytes themselves contribute to embryonic ecdysteroid synthesis or metabolism. Such findings would expand the classical view of a centralized ecdysteroid source and suggest the presence of a more distributed system in spiders. During late embryonic development *Pt-spo* and *Pt-shd* expressions cease, matching the reported lack of mRNA expression in *D. melanogaster* in the prothoracic gland (Berkeley Drosophila Genome Project; Tomancak et al., 2002; Tomancak et al., 2007; Hammonds et al., 2013). Studies on the moth *Plutella xylostella* also display some degree of embryonic gene expression. Expression appears to be stronger for *shd* than *spo* and *dib*, while mRNA levels further increase during larval and adult stages (Peng et al., 2019).

In larval *D. melanogaster*, *dib* and the *spo* duplicate *spookier* are strongly expressed in the prothoracic gland, which acts as a dedicated endocrine tissue (Warren et al., 2002). No prothoracic gland analog is known from spiders (Sawadro et al., 2017). Therefore, Yang et al. (2021) speculated that a specific tissue in the abdomen is responsible for ecdysteroid biosynthesis, functionally similar to the classical prothoracic glands in insects. The *Pt-spo* and *Pt-dib* positive cells observed within the heart tube and efferent vessels in juvenile opisthosomata of *P. tepidariorum* are reminiscent of hemocytes (Sherman et al., 1973; Fukuzawa et al., 2008; Soares et al., 2015) and might represent the predicted endocrine tissue. Expression in hemocytes circulating throughout the body of juveniles raises the intriguing possibility that these cells could contribute to ecdysteroid biosynthesis or metabolism as mobile sites of ecdysteroid conversion. The flexibility of retaining the catalytic core yet reallocating steps among tissues shows how the pathway could have been remodeled during arthropod evolution to fit lineage-specific physiology and life history.

Interestingly, in fruit fly larvae *shd* is expressed in peripheral sites for E20 activation, such as epidermis, midgut, Malpighian tubules and fat body, rather than in the prothoracic gland (Petryk et al., 2003; Rewitz et al., 2006b; Rewitz et al., 2007; Niwa & Niwa, 2016). The peripheral expression of *shd* is considered conserved in insects. For instance, in *Manduca sexta* larvae *shd* is also strongly expressed in the midgut, Malpighian tubules, epidermis and fat body, with negligible expression in the prothoracic gland (Rewitz et al., 2006a; Ou et al., 2016). The spider data fit the same pattern. If hemocytes act as the chelicerate endocrine tissue, the absence of *Pt-shd* in these cells,

combined with its presence in ovary, fat body and midgut, points to a conserved division of steps in ecdysteroidogenesis. Thus, the spatial separation between *spo* and *dib* in the endocrine tissue, and *shd* in widespread tissue appears to be a general feature (Petryk et al., 2003; Niwa et al., 2005). Testing of this model will require hemocyte-specific knockdowns of pathway genes and *in vitro* assays confirming ecdysteroidogenic activity. Nonetheless, the data presented provides an entry point for studying how developmental and hematopoietic mechanisms are hormonally integrated across Arthropoda.

When targeting these genes in arthropods, researchers have uncovered roles in reproduction in addition to development. Expression and function of one or multiple Halloween genes is reported in ovaries of adult females in the fruit fly (Buszczak et al., 1999), diamondback moth *P. xylostella* (Peng et al., 2019), the desert locust *Schistocerca gregaria* (Marchal et al., 2011), the tick *Ornithodoros moubata* (Ogihara et al., 2015), the spider mite *Tetranychus urticae* (Wang et al., 2023) and the spider *P. pseudoannulata* (Yang et al., 2021), which correlates with the known role of ecdysteroids in stimulating vitellogenin production and uptake into oocytes. Moreover, the expression of *Pt-shd* in the fat body is comparable to results from the spider *Tegenaria atrica*, which demonstrated ecdysteroid hormone roles in stimulating *vitellogenin* production in the fat body and in facilitating yolk protein accumulation in oocytes (Pourié & Trabalon, 2003). The pronounced expression in the ovary demonstrated in this study, supports the notion that the ecdysteroid hormone plays important gonadotropic roles in the reproductive physiology of female spiders. Collectively, this suggests that the ancestral molting-hormone pathway has been co-opted for reproduction, allowing the same CYP enzymes to regulate diverse life-history transitions that require synchronized cell proliferation, differentiation and gene-regulatory shifts (Sieglaff et al., 2005; Ameku & Niwa, 2016).

3.3.3.3. Functional involvement of Halloween genes in spider molting hormone pathway

In the rice planthoppers *Sogatella furcifera* and *Laodelphax*, oral delivery of *dib* double-stranded RNA results in impaired nymphal development, displayed by molting failure and high nymph mortality (Wan et al., 2014). This indicates that lowering transcript levels can critically reduce 20E titers and thereby disrupt the molting process. These phenotypes mirror what has been documented in other insects, where *shadow* knockdown in *P. xylostella* prolonged the larval period and reduced pupation rate (Peng et al., 2019), and *spo* knockdown in *Tribolium castaneum* leads to nymphal delays in

development (Takaki et al., 2020). The results of RNAi-mediated knockdown in *P. tepidariorum* consistently demonstrate that each of the studied CYP genes is indispensable for wild-type molting intervals, confirming their roles in ecdysteroid biosynthesis even 500 million years after the chelicerate and pancrustacean split (Dunlop, 2010). Therefore, the results highlight both the deep conservation and the developmental plasticity of arthropod molting endocrinology (Rewitz et al., 2007; Niwa & Niwa, 2016). The observation of delayed development in many RNAi experiments, rather than absolute arrest, suggests that partial knockdown yields suboptimal ecdysteroid levels that postpone the molts without completely preventing them. This could imply a threshold effect in hormonal control where development can proceed at a slower speed if hormone titers are low but will fail entirely below a critical threshold.

Future work should extend single-cell transcriptomics, RNAi and comparative endocrinology across myriapods, crustaceans and additional chelicerates to characterize the full set of tissues in which these enzymes operate. Beyond standard EvoDevo insights, detailed knowledge of *spo*, *dib* and *shd* regulation may guide precision pest-management strategies, as RNAi of these bottleneck enzymes already yields increased lethality and reproductive failure in multiple target species (Marchal et al., 2012; Kong et al., 2014; Yu et al., 2014; Sugahara et al., 2017; Paredes-Montero et al., 2022).

3.3.4. Conclusion

Spook, Disembodied and Shade are fundamental players in the ecdysteroid biosynthesis pathway that underlies arthropod development. Their sequences and ecdysteroid-producing functions are strongly conserved in Panarthropoda. Functional knockdown of each gene leads to severely prolonged molting intervals. This consistent phenotype across diverse arthropods implies the conserved mechanism of ecdysteroid-regulated development. This study also reveals a non-traditional expression domain of ecdysteroidogenic CYP genes in hemocytes. This pattern indicates the possibility of decentralized hormone production. The findings warrant further investigation, as they could redefine the understanding of endocrine regulation in arthropods. Therefore, this work provides a foundation for future research into the spatial regulation of molting hormone production and its link with other physiological systems.

3.3.5. Materials and Methods

3.3.5.1. Animal care and sample preparation

Parasteatoda tepidariorum was maintained at 25°C in polystyrene *Drosophila* tubes (with Ceaprene plugs) containing coconut fiber substrate. Individuals were fed *D. melanogaster* and were watered weekly. Synchronized embryonic stages were identified via Mittmann & Wolff (2012). For fixation, eggs were dechorionated in sodium hypochlorite (DanKlorix) for 2–3 minutes, rinsed thoroughly in distilled water and immersed in 8 ml fixative [4 ml heptane, 0.5 ml 37 % formaldehyde, 3.5 ml PEMS (100 mM PIPES, 1 mM EDTA, 1 mM $MgSO_4$)]. Fixation was carried out for 6–15 hours, followed by several washes with PEMT (PEMS + 0.01 % TWEEN-20), dehydration in methanol and storage at -20°C. After appendages removal, postembryonic samples were fixed in 4 % formaldehyde in PBS with 0.01 % TWEEN-20 for 24 hours and sectioned (80–120 µm) in 8 % low-melt agarose using a Leica VT1000 S vibratome. Afterwards, a second fixation step (30 min) was performed, and residual agarose was removed before *in situ* hybridization.

3.3.5.2. Identification and phylogenetic analysis of candidate genes

Candidate homologs of *spo*, *dib* and *shd* were identified via BLASTp (Altschul et al., 1997) using *Drosophila melanogaster* protein sequences (FBgn0003486, FBgn0000449 and FBgn0003388, respectively). Orthology was confirmed by reciprocal BLASTp against the *D. melanogaster* genome (GCF_000001215.4). Gene models were visualized using GenomeTools v1.6.2 (Gremme et al., 2013) on the *P. tepidariorum* genome (GCF_000365465.3). Protein sequences from arthropod species, including insects, crustaceans, myriapods, chelicerates and basal ecdysozoans were retrieved from Rewitz et al. (2007) and NCBI (Supplementary Table 5). Alignments were performed with Clustal Omega (v1.2.4; Sievers et al., 2011) and model selection was done with ModelTest-NG (v0.1.7, Darriba et al., 2020). Maximum-likelihood trees were built with RAxML-NG (v1.2.2, Kozlov et al., 2019) using the best-fit substitution models and 1,000 bootstrap replicates. Tree visualization was created in iTOL v6.8.1 (Letunic & Bork, 2021).

3.3.5.3. Single-cell RNA-seq analysis

To assess gene expression in developing embryos, scRNA-seq datasets of rPCA integrated stages 7 to 9 (Leite et al., 2024) and pooled stages 10 to 12 (Medina-Jiménez et al., 2024) were analyzed. Genes were identified by reciprocal BLASTp (v2.11.0+) or Gene ID matching. Data visualization (UMAPs, dot plots) was performed using the Seurat package (v5.1.0; Hao et al., 2024) in R.

3.3.5.4. Cloning and in situ hybridization

Primer design for *Pt-spo*, *Pt-dib* and *Pt-shd* (Table 2) was conducted using Primer-BLAST and Multiple Primer Analyzer (ThermoFisher), with *Pt-spo* and *Pt-dib* primers targeting a region common to both isoforms. Total RNA from stages 6 to 14 was extracted using TRIzol® (Ambion®) and cDNA was synthesized with the Transcriptor High Fidelity cDNA Kit (Roche). PCR products were cloned into the pCRII vector using the Dual Promoter TA Cloning® Kit (Invitrogen) and transformed into NEB® 5-alpha competent *Escherichia coli*. After plasmid purification with the NucleoSpin® Kit for DNA, RNA and protein purification (MACHEREY-NAGEL), sequencing (LGC Genomics) confirmed insert identity. *In situ* probes were synthesized using T7 or SP6 RNA polymerases and DIG RNA Labeling Mix (Roche). Whole-mount *in situ* hybridization followed the protocol of Prpic et al. (2008), excluding post-fixation and proteinase K treatment. Embryos were counterstained with SYTOX Green (Invitrogen).

Table 2 Primer sequences of the cytochrome P450 genes *spook*, *disembodied* and *shade* from *P. tepidariorum* used for cloning and dsRNA synthesis.

Gene	Name	Forward Primer	Reverse Primer
spook (*spo*)	*Pt-spo*	CAACGCTTGATGGAAACCGC	CGGTGTCTGTTGTTGCCAC
disembodied (*dib*)	*Pt-dib*	GACTTATGTCGGGCATGCATTC	GGCATTCCCTTTCAAAGACAAGC
shade (*shd*)	*Pt-shd*	CCTGGACCAAGACCTTCAATACC	GCCGTAAAATAGCTCCTGAAATGC
T-box20	*tbx20*	CCTTATTCTGCGGCCATGTC	CCCGTGAATGGGGAATCTGG
dsRNA	ds_T7	TAATACGACTCACTATAGGG	-
dsRNA	ds_T7M13	-	TAATACGACTCACTATAGGGCAG GAAACAGCTATGAC

3.3.5.5. RNA interference

RNA templates were amplified from pCRII vectors using T7 and modified T7-M13 primers (Table 2). Double-stranded transcripts were synthesized with the MEGAscript™ T7 Kit (Invitrogen). Subadult males received 1 µl of dsRNA (targeting *Pt-spo*, *Pt-dib*, *Pt-shd* or control gene *tbx20*) at 1 or 5 days post-penultimate molt (N = 20 per group). H_2O-injected controls (N = 20, 1 day after penultimate molt) and untreated wild-type males (N = 20) were used for reference. Molt timing and survival rate were monitored post-treatment.

3.3.5.6. Microscopy and imaging

Specimens were imaged with a ZEISS AxioZoom.V16 microscope using a PlanNeoFluar z1x/0.25F objective and an Axiocam 305 Color Camera. Brightfield and GFP channel images were acquired via Z-stack mode in ZEN Blue software and merge function in Helicon Focus (v7.7.5) for focus stacking.

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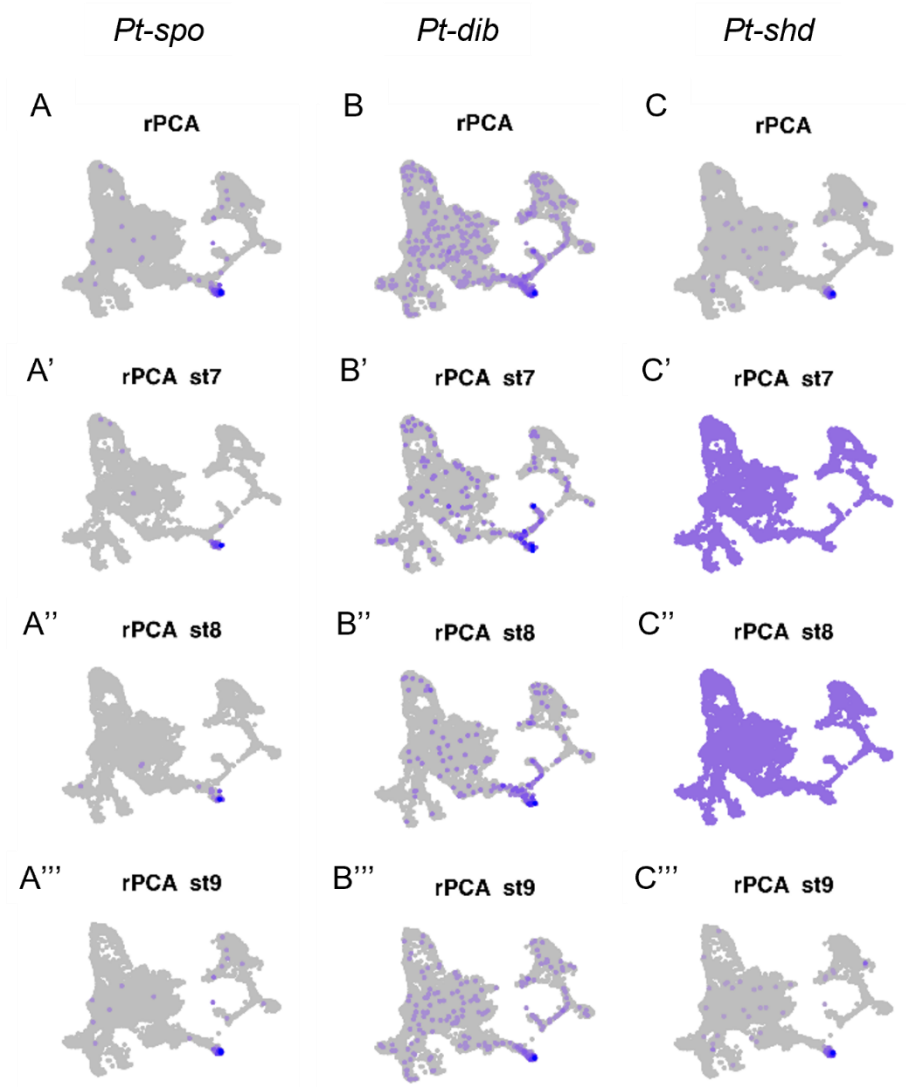
3.3.7. Supplementary Information

Supplementary Table 5 Protein accession of cytochrome P450 orthologs identified in the genomes of various ecdysozoans for protein-based gene tree.

Gene	Species	Accession	Gene name
CYP18A1	*Drosophila melanogaster*	CAL69940.1	P450 Cyp18a1
	Manduca sexta	XP_037295397.1	P450 Cyp18a1
	Tribolium castaneum	NP_001123908.1	P450 Cyp18a1
	Daphnia pulex	EFX85356.1	18a1/hypothetical protein DAPPUDRAFT
	Parasteatoda tepidariorum	XP_015920197.2	P450 Cyp18a1
disembodied	*Drosophila melanogaster*	Rewitz et al., 2007	CYP302A1
	Manduca sexta	ABC96069.1	CYP302A1
	Tribolium castaneum	XP_974252.1	Predicted CYP302A1
	Ixodes scapularis	XP_029829236.3	CYP302A1
	Daphnia pulex	EFX63066.1	CYP302A1
	Parasteatoda tepidariorum	XP_042913013.1	CYP302A1
phantom	*Drosophila melanogaster*	CAL69943.1	CYP306A1
	Manduca sexta	ABC96068.1	CYP306A1
	Tribolium castaneum	XP_968477.1	Predicted CYP 306A1
	Daphnia pulex	EFX85499.1	CYP306A1
shade	*Drosophila melanogaster*	AAQ05972.1	CYP314A1
	Manduca sexta	ABD18735.1	CYP314A1
	Tribolium castaneum	NP_001123894.1	CYP314A1
	Ixodes scapularis	XP_002401077.1	Putative CYP314A1
	Daphnia pulex	EFX77008.1	CYP314A1
	Parasteatoda tepidariorum	XP_042903873.1	CYP314A1
shadow	*Drosophila melanogaster*	NP_650123.1	CYP315A1
	Drosophila pseudoobscura	Rewitz et al., 2007	CYP315A1
	Aedes aegypti	AAX85207.1	CYP315A1
	Apis mellifera	Rewitz et al., 2007	CYP315A1
	Manduca sexta	ABC96070.1	CYP315A1
	Bombyx mori	NP_001106224.1	CYP315A1
	Tribolium castaneum	XP_008193656.1	Predicted CYP315A1
	Daphnia pulex	EFX70970.1	Putative CYP315A1
	Ixodes scapularis	XP_002411841.2	CYP315A1
	Limulus polyphemus	XP_022243138.1	CYP315A1-like
	Limulus polyphemus	XP_013794786.1	CYP315A1-like
	Parasteatoda tepidariorum	XP_015909978.1	CYP315A1
	Euperipatoides rowelli	Schumann et al., 2018	scaffold29685
	Euperipatoides rowelli	Schumann et al., 2018	scaffold175979
	Hypsibius exemplaris	OQV21685.1	Sterol 26-hydroxylase
	Ramazzottius varieornatus	GAV03877.1	Hypothetical protein RvY 315
	Priapulus caudatus	XP_014662626.1	CYP315A1
spook	*Drosophila melanogaster*	NP_647975.2	CYP307A1
	Manduca sexta	ABI74778.1	CYP307A1
	Tribolium castaneum	XP_008200059.1	CYP307A1
	Ixodes scapularis	XP_002403504.1	Putative CYP307A1
	Parasteatoda tepidariorum	XP_015926868.1	CYP307A1
spookier	*Tribolium castaneum*	EFA05673.1	CYP307B1
	Daphnia pulex	EFX88041.1	CYP307B1
spookiest	*Drosophila melanogaster*	NP_001104460.2	CYP307A2



Supplementary Figure 7 UMAP for rPCA integrated scRNA-seq data of embryonic stages 7 to 9 (Leite et al., 2024). (A) UMAP for *Pt-spo* of stages 7 to 9 with individual (A') stage 7, (A'') stage 8 and (A''') stage 9. (B) UMAP for *Pt-dib* of stages 7 to 9 with individual (B') stage 7, (B'') stage 8 and (B''') stage 9. (C) UMAP for *Pt-shd* of stages 7 to 9 with individual (C') stage 7, (C'') stage 8 and (C''') stage 9.

3.4. Functional Conservation of *CYP18A1* in Ecdysteroid Degradation and Molt Completion in the Spider *Parasteatoda tepidariorum*

This chapter aims to elucidate the functional involvement of the pre-molt cytochrome P450 monooxygenases CYP18A1 in the spider *Parasteatoda tepidariorum*. Utilization of expression analyses on embryonic and postembryonic stages via *in situ* hybridization, in combination with functional analyses through juvenile RNA interference-mediated knockdown suggests conserved function in ecdysteroid degradation. Results indicate that *Pt-CYP18A1* is relevant for successful molt completion and support a role in hemocyte formation during embryogenesis.

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Author contributions to this research:

Denise Klinkenbuß: Data curation, Formal analysis, Validation, Investigation, Methodology, Visualization, Writing – original draft, Writing – review & editing.

Nikola-Michael Prpic-Schäper: Conceptualization, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Status: **First Draft**

Functional Conservation of *CYP18A1* in Ecdysteroid Degradation and Molt Completion in the Spider *Parasteatoda tepidariorum*

Highlights

- Embryonic expression of *Pt-CYP18A1* suggests involvement in hematopoiesis.
- Postembryonic expression implies evolutionarily conserved function.
- RNA interference results in prolonged molt intervals and defect phenotypes.

Abstract

Cytochrome P450 CYP18A1 is a known pre-molt ecdysteroid-inactivating enzyme in insects, but its role in arachnids remains unexplored. Here *CYP18A1* is investigated in the common house spider *Parasteatoda tepidariorum* during embryonic and postembryonic development. While using whole-mount *in situ* hybridization on mid-to-late embryonic stages, dynamically expressed *Pt-CYP18A1* is found in presumptive hematopoietic cells. This spatiotemporal expression combined with patterns in the postembryonic midgut epithelium suggests that *Pt-CYP18A1* might modulate molt hormone levels in developing hemocytes and juvenile tissues like fat body and midgut epithelium. RNA interference knockdown of *Pt-CYP18A1* resulted in spiderlings with delayed or disrupted molts, further implicating involvement in ecdysteroid regulation. These findings provide the first insight into the function of *CYP18A1* in a spider and support the evolutionary conservation of ecdysteroid inactivation mechanisms across arthropods.

Keywords: hormone, inactivation, embryo, RNAi, *in situ*

3.4.1. Introduction

Molting in arthropods is controlled by ecdysteroid hormones, principally ecdysone and its active derivative 20-hydroxyecdysone (20E) (Gilbert et al., 2002; Gilbert et al., 2004). These hormones regulate developmental transitions such as molting and metamorphosis (Sullivan & Thummel, 2003; Truman, 2019). In insects, high titers of 20E trigger each molt, while titer declines allow ecdysis (Jindra et al., 2013). Embryos of diverse arthropods are equipped with maternal ecdysteroids in the yolk to guide early development (Brown et al., 2009). However, much less is known about how ecdysteroid levels are regulated in non-insect arthropods such as arachnids (Campli et al., 2024). Spiders and their chelicerate relatives appear to use a form of ecdysteroid called ponasterone A (ponA) as the predominant molting hormone rather than the typical insect 20E (Grbić et al., 2011). Despite this difference, chelicerates possess the canonical ecdysone receptor (EcR) and retinoid X receptor (RXR) hormone heterodimer (Thomson et al., 2009; Chipman et al., 2014; Qu et al., 2015). Furthermore, recent genomic analyses indicate they retain homologs of many ecdysteroid pathway genes (Schumann et al., 2018). This suggests that fundamental mechanisms of ecdysteroid signaling may be conserved across Arthropoda.

One key aspect of ecdysteroid regulation is hormone inactivation (Fig. 28). In insects, ecdysteroid titers are reduced partly by metabolic inactivation pathways including C26 hydroxylation, which converts active ecdysteroids into 26-hydroxyecdysteroids and further to 26-oic acids (Davies et al., 2006; Rewitz & Gilbert, 2008; Guittard et al., 2011). A cytochrome P450 enzyme (CYP18A1) has been identified as the mediating 26-hydroxylase and therefore ecdysteroid-degrading enzyme in insects (Guittard et al., 2011; Li et al., 2014). For example, in *Drosophila melanogaster* loss-of-function or overexpression of *CYP18A1* is lethal at the larval-to-pupal transition, due to failure of reducing hormone levels and consequently improper metamorphic timing (Rewitz et al., 2010; Guittard et al., 2011). Notably, *Bombyx mori* has a second paralog (*CYP18B1*), but *CYP18A1* remains the conserved part in 20E inactivation (Li et al., 2014). Importantly, genome-wide studies have now identified *CYP18A1* homologs in all studied panarthropods, including chelicerates (Qu et al., 2015; Schumann et al., 2018). This implies that a common ancestral mechanism for hormonal inactivation existed in

early arthropod evolution. Whether spider CYP18A1 functions analogously to insects in controlling ecdysteroid levels is an open question addressed by this study.

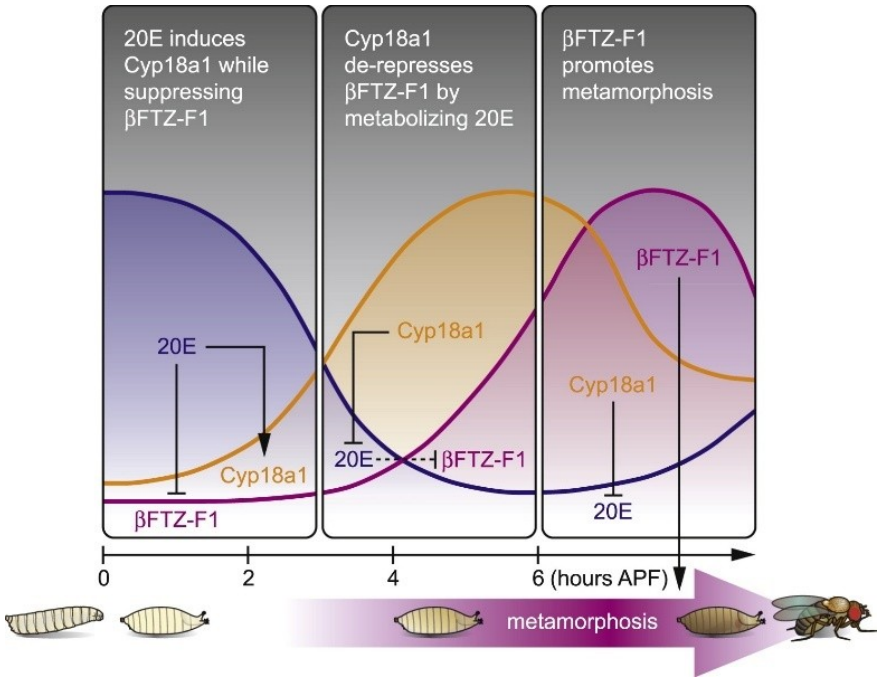

Figure 28 CYP18A1-mediated inactivation of 20-hydroxyecdysone (20E) sets the timing for βFTZ-F1 (beta-Fushi-tarazu transcription factor 1) upregulation during the first six hours after pupariation in *D. melanogaster*. Lines show relative levels of 20E (blue), CYP18A1 (orange) and the orphan nuclear receptor βFTZ-F1 (magenta). Graphic obtained from Rewitz et al. (2010).

Parasteatoda tepidariorum provides an excellent model to explore this question in a spider. Its embryogenesis has been staged and characterized, with 14 defined stages from the fertilized egg to pre-hatching embryo (Hilbrant et al., 2012; Mittmann & Wolff, 2012). Here, the expression pattern of *Pt-CYP18A1* during embryonic and postembryonic stages is examined to answer the question of gene involvement and role in chelicerates. By comparing the findings to known arthropod model organisms, the evolutionary conservation of ecdysteroid hormone functions is elucidated.

3.4.2. Results

3.4.2.1. CYP18A1 expression in embryonic hemocytes and postembryonic periphery

Pt-CYP18A1 is expressed in multiple domains over the course of development (Fig. 29). First whole-mount *in situ* hybridization revealed a population of singular cells covering the yolk during mid-embryonic stages 9 to 11. At late stage 9, *Pt-CYP18A1* transcripts were faintly detectable in cells spread across the yolk in the germband proximity (Fig. 29A, arrowheads). By stage 10, the expression expanded and dispersed, with many individual *Pt-CYP18A1*-positive cells now visible throughout the "extraembryonic" tissue (Fig. 29B). At stage 11, the pattern shifts and expressing cells are notably accumulated at the dorsal margin between head lobes and segment addition zone (Fig. 29C). In late-embryonic development (stage 12 to hatching) mRNA is localized in cells within the dorsal heart tube. During dorsal closure at stage 12, a stripe of *Pt-CYP18A1* transcribing cells seems condensed along the forming dorsal midline (Fig. 29D). In the next developmental stage, this dorsal expression morphed into a segment-like pattern reminiscent of the forming heart tube lumen (Fig. 29E). No significant expression was observed in other tissues like developing limbs or neuroectoderm throughout stages 9 to 12. At stage 14 (pre-hatching), strong expression is observed dorsally in an anterior-to-posterior running cell cluster, coincident with the fully formed heart (Fig. 29F). These cells appear to reside adjacent to or within the cardiac tissue. Interestingly, additional expression loci are present in the head lobes at stage 13 (Fig. 29G) and in visual organ domains at pre-hatching stage (arrowheads, Fig. 29H) (Schomburg et al., 2015).

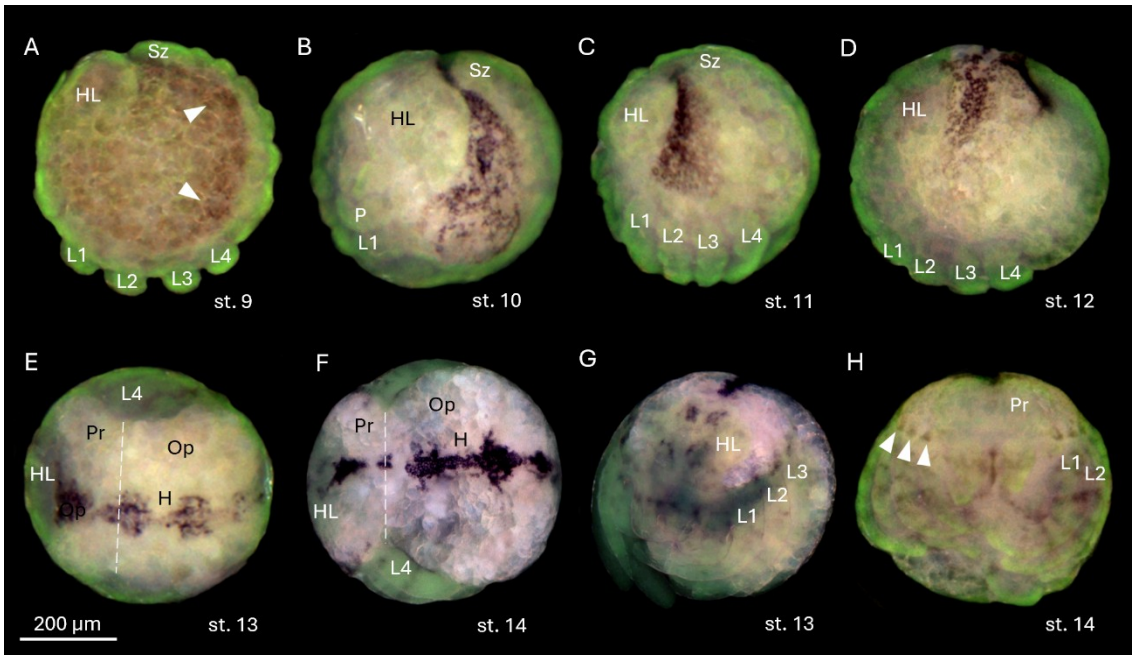

Figure 29 Spatiotemporal expression of *CYP18A1* during mid-to-late embryogenesis in *P. tepidariorum*. mRNA staining (purple) with nuclear counterstain (green) from stages 9 to 14. Anterior is left in images A to F. Lateral view with dorsal facing up (A–D); dorsal view (E, F) and anterior view with dorsal facing up (G, H). (A–C) Increasing and dispersing expression in single cells covering the yolk from stages 9 (arrowhead) to 11. (D) Expression condensed during dorsal closure at stage 12. (E–F) Expression in the lumen of the posterior-to-anterior developing cardiogenic tissue at stages 13 to 14. (G) Four expression domains in the head lobes. (H) mRNA localization in visual organ domains. HL, head lobes; H, heart; L1–L4, legs 1 to 4; Op, opisthosoma; P, pedipalp; Pr, prosoma; Sz, segment addition zone.

Molt interval-independent expression is detected covering the subesophageal ganglion in the typical "salt-and-pepper" pattern in the juvenile prosoma (Fig. 30A). No mRNA was detected in the supraesophageal ganglion. In the opisthosoma, singular cells expressing *Pt-CYP18A1* are visible in the epithelium covering the midgut tissue (Fig. 30B). Additional staining is observed in the gut tissue (arrowhead). Faint staining is visible throughout the fat body dorsally surrounding the digestive system. The intense ventral staining outside of distinct inner organs is presumably artificial, caused by probe-absorbing properties of the book lung cuticles.

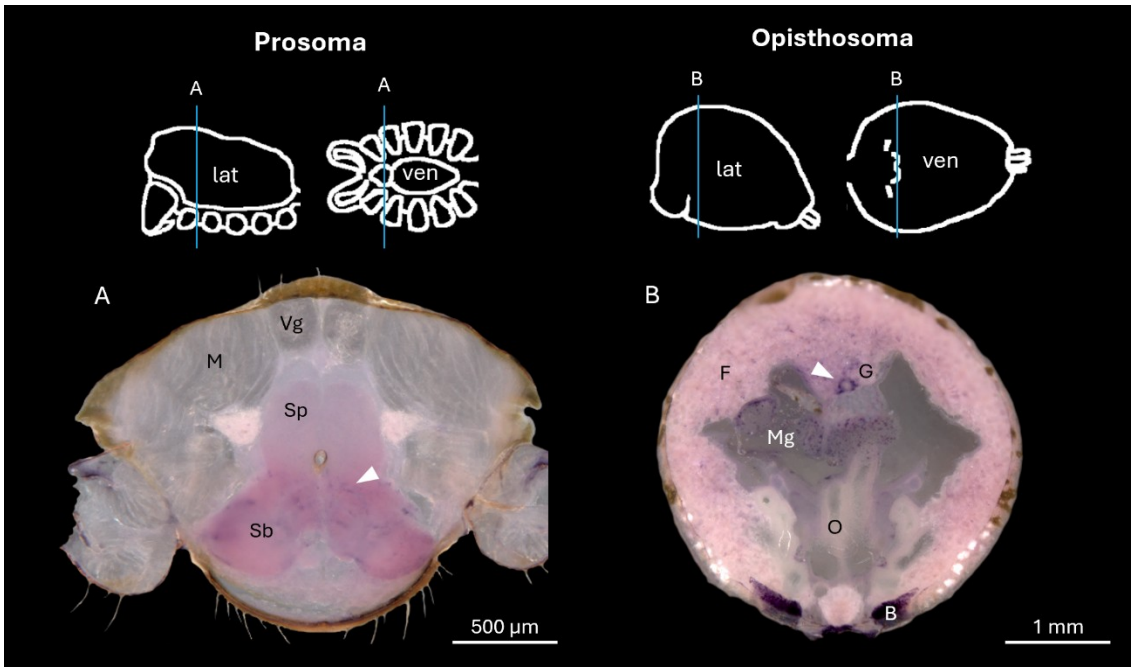

Figure 30 Postembryonic expression of *Pt-CYP18A1*. Expression shown in purple. The morphological schemes [redrawn after Foelix (1996)] indicate section planes. Anterior is left in the schemes; dorsal is top in all images. (A) Expression in subesophageal ganglion of the prosoma. (B) Expression in fat body and midgut epithelium in the opisthosoma. Ventral staining in book lungs is likely artificial due to probe absorbing property of cuticula. B, book lungs; F, fat body; G, gut; M, muscles; Mg, midgut; O, ovary; Sb, subesophageal ganglion; Sp, supraesophageal ganglion; Vg, venom gland.

3.4.2.2. CYP18A1 knockdown in postembryonic stages suggests role in molting

To investigate the functional significance of *Pt-CYP18A1*, RNA interference (RNAi) knockdown experiments were performed. Double-stranded RNA targeting *Pt-CYP18A1* was injected into subadult males 5 days after the penultimate molt. When comparing RNAi-treated subadults to wild type or *tbx20*-injected controls, effects on molting became apparent (Fig. 31). *Pt-CYP18A1*-RNAi spiderlings showed significant molting delay compared to controls ($p < 0.001$). No significant differences were observed comparing wild type to control treatment ($p = 0.38$). Several knockdown spiderlings experienced lethal molting defect phenotypes with the exuvia not separatable from delicate structures. In contrast, control spiderlings molted normally and without delay. Beyond molting, no obvious defects in other development aspects were observed in the *Pt-CYP18A1*-RNAi survivors.

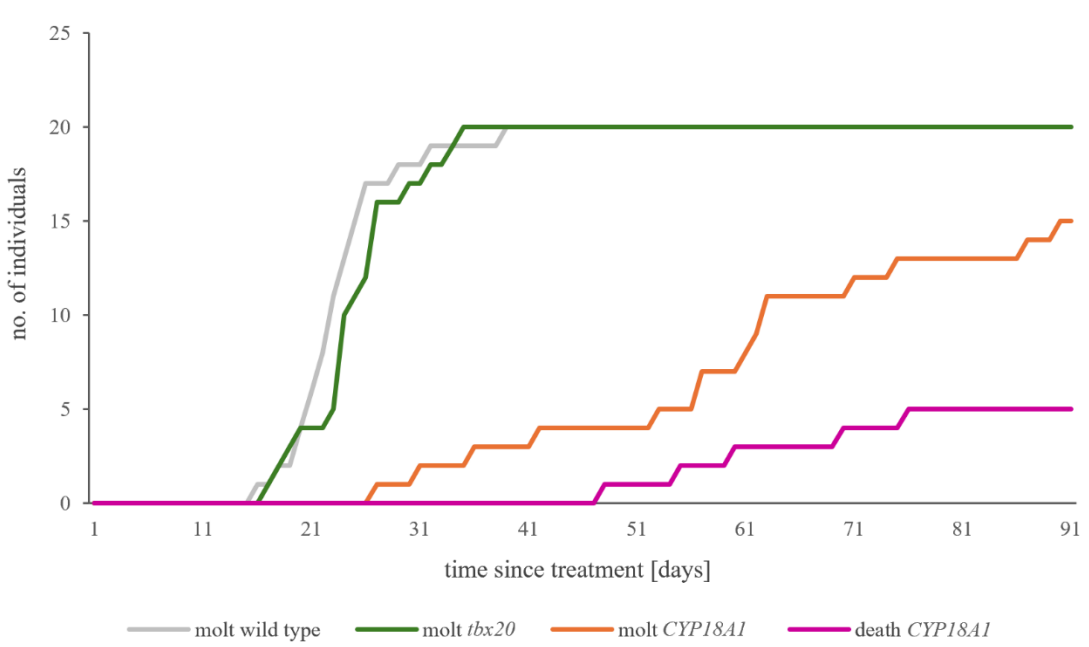

Figure 31 Impact of *Pt-CYP18A1* RNAi knockdown on subadult male molting. Time for molting from subadult to adult in subadults subjected to different treatments: wild type (no injection), dsRNA *tbx20* (control gene; injected 5 days after penultimate molt (APM)) and dsRNA of *Pt-CYP18A1* (5 days APM). N = 20 for all treatment groups. Magenta line indicates lethal molting events in *Pt*-CYP18A1 treatment group.

3.4.3. Discussion

In insect models, CYP18A1 is a pivotal enzyme that deactivates 20E by 26-hydroxylation (Chen et al., 1994; Rewitz et al., 2010; Li et al., 2014). This activity is critical at the end of molting cycles to ensure proper termination of ecdysteroid signals and allow molting and metamorphosis to proceed (Hurban & Thummel, 1993; Davies et al., 2006). RNAi knockdowns in *P. tepidariorum* are consistent with these findings, as spiderlings with reduced *CYP18A1* function exhibit delayed or impaired molting, strongly implying that ecdysteroid inactivation was insufficient. These results support the hypothesis that *Pt-CYP18A1* metabolic function is conserved in spiders and therefore required to reduce active molting hormone levels to permit molting.

Main expression domains included hematopoietic cells during mid-to-late embryogenesis and cells covering the midgut epithelium in postembryonic stages. Juvenile mRNA detection might correspond to observation in holometabolous insect larvae, where ecdysteroid inactivation is suspected in fat body, midgut and Malpighian tubules (Berkeley Drosophila Genome Project; Tomancak et al., 2002; Tomancak et al., 2007; Hammonds et al., 2013). The restricted expression of *Pt-CYP18A1* in embryonic hemocytes is consistent with a role in modulating molt hormone levels. In the pre-hatching spider embryo, terminating ecdysteroid signals by heart-associated *CYP18A1*-positive cells may be necessary to exit embryogenesis. This would be analogous to an immune cell role, where residual developmental signals are removed once they have fulfilled their function. These results add to a growing recognition that the endocrine and immune systems might be interlinked in arthropod development (Karpac et al., 2011; Nunes et al., 2021). The expression in embryonic hemocytes also suggests a role beyond immunity, perhaps in developmental timing. For example, the hematopoietic niche of *D. melanogaster* uses ecdysone signaling to control the balance between maintaining hemocyte progenitors and triggering their differentiation (Ramesh et al., 2021). If this mechanism is conserved, spider hemocyte progenitors might also respond to ecdysteroid levels during embryogenesis. It may be an early evolved case of co-opting immune cells to help coordinate developmental transitions, ensuring that tissue remodeling is synchronized with the hormonal cycle.

The identification of the expressing cells as hemocytes is based on morphology, position and timing. Future work could combine *Pt-CYP18A1 in situ* hybridization with immunostaining or markers for hemocytes to confirm their identity. Similarly, co-localization with heart muscle markers like *Mef2* (Janssen & Damen, 2008; Leite et al., 2024) would clarify whether *Pt-CYP18A1* is in the embryonic hemolymph or in adjacent pericardial cells.

3.4.4. Conclusion

The ecdysteroid-inactivating enzyme CYP18A1 is developmentally expressed in the spider *Parasteatoda tepidariorum* in patterns that suggest a conserved function in controlling molt hormone levels. *Pt-CYP18A1* is active in embryonic hemocytes and later in midgut epithelium cells, pointing to these sites as key for hormonal down-regulation before hatching and in molt hormone degradation. RNA-interference knockdown indicates that reduction of CYP18A1 disturbs normal molting, mirroring the enzyme's essential role during insect metamorphosis. Together, the results support the idea that ecdysteroid catabolism is an ancient, conserved component of arthropod development, ensuring transition timing by removing hormonal signals. This study lays the groundwork for further comparative studies into how arthropods from different lineages achieve the balance of hormone signaling required for successful development.

3.4.5. Material and Methods

3.4.5.1. Animal care and tissue preparation

Parasteatoda tepidariorum was bred at 25 °C in fruit fly culture tubes containing one-third coconut fiber. Spiders received *Drosophila melanogaster* and water once a week. Embryonic staging followed the series of Mittmann and Wolff (2012). Eggs were dechorionated for 2 to 3 minutes in commercial hypochlorite, rinsed in distilled water, and fixed in 8 ml fixing solution containing 4 ml heptane, 0.5 ml 37 % formaldehyde and 3.5 ml PEMS (100 mM PIPES, 1 mM EDTA, 1 mM $MgSO_4$) for up to fifteen hours. Post-fixation washes with PEMS including 0.01 % TWEEN-20, stepwise dehydration in methanol and storage at −20 °C were performed. Juvenile pro- and opisthosomata were fixed 24 hours in 4 % formaldehyde in PBS, embedded in 8 % low-melt agarose, vibratome sectioned at 80–120 µm (Leica VT1000 S), re-fixed for 30 minutes and removed from agarose residuals before *in situ* hybridization.

3.4.5.2. Homolog search, cloning and in situ probes

The *CYP18A1* candidate was retrieved from the *P. tepidariorum* genome (NCBI accession: GCF_000365465.3) by BLASTp (NCBI; Altschul et al., 1997) using the *D. melanogaster* sequence (FBgn0010383) as query. Reciprocal BLASTp against the fly genome (GCF_000001215.4) confirmed identity. Total RNA from embryonic stages 6 to14 was extracted with TRIzol® Reagent. cDNA was synthesized with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Target fragment was amplified with primers (Table 3) tested for specificity (Multiple Primer Analyzer, ThermoFisher) and cloned into pCRII vector using the Dual Promoter TA Cloning® Kit (Invitrogen). Afterwards, the vector was transformed into NEB® 5-alpha competent *Escherichia coli*. Plasmid was purified with the Plasmid NucleoSpin® Kit for DNA, RNA and protein purification (MACHEREY-NAGEL) and sequence-verified (LGC Genomics). DIG-labelled RNA probes were generated with T7 or SP6 polymerase (Roche). Whole-mount *in situ* hybridization followed the protocol of Prpic et al. (2008), omitting post-fixation and proteinase K steps. Embryos were counterstained with SYTOX Green (Invitrogen).

Table 3 Primer sequence of the ecdysteroid degradation gene *CYP18A1* in *P. tepidariorum* used for cloning and dsRNA synthesis.

Gene	Name	Forward Primer	Reverse Primer
CYP18A1	*Pt-CYP18A1*	CAAGAGGCGTGAGTAATGTGG	CCTTCGACCGACAGAAAATGG
T-box20	*tbx20*	CCTTATTCTGCGGCCATGTC	CCCGTGAATGGGGAATCTGG
dsRNA	ds_T7	TAATACGACTCACTATAGGG	-
dsRNA	ds_T7M13	-	TAATACGACTCACTATAGGGCAG GAAACAGCTATGAC

3.4.5.4. RNA interference

Template DNA for dsRNA production was amplified from pCRII vector with T7 and T7-tagged M13 primers (Table 3). dsRNA was synthesized with the MEGAscriptTM T7 High Yield Transcription Kit (Invitrogen). Subadult males received a single 1 µl injection with either dsRNA of *CYP18A1* or *tbx20* (control gene) 5 days after the penultimate molt (N = 20). Time to ultimate molt was compared to untreated subadult males (N = 20).

3.4.5.5. Microscopy

Bright-field and GFP images were acquired with a Zeiss AxioZoom.V16 Microscope and Axiocam 305 Color Camera. Z-stacks collected in ZEN microscopy software (blue edition; Carl Zeiss Microscopy GmbH) were merged with the Helicon Focus program (version 7.7.5).

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3.4.6. References

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4. Discussion

In this thesis, several molt-related ecdysteroid biosynthesis pathway genes in the common house spider *Parasteatoda tepidariorum* were investigated. The early-pathway genes *neverland* (*Pt-nvd*) and *shroud* (*Pt-sro*), the mid-pathway cytochrome P450 (CYP) gene *shadow* (*Pt-sad*), the mid-to-late pathway CYP genes *spook* (*Pt-spo*), *disembodied* (*Pt-dib*) and *shade* (*Pt-shd*), as well as the hormone-degradation gene *CYP18A1* (*Pt-CYP18A1*) were identified and characterized. The results confirm their presence in the spider genome and reveal spatiotemporal expression patterns and functional roles in development. In summary, (1) *Pt-nvd* and *Pt-sro* are expressed in cells of the heart lumen and brain lobes during embryogenesis. In postembryonic stages staining occurred in putative hematopoietic cells, suggesting a role in hematopoiesis. RNA interference (RNAi) led to prolonged or defective molts, implementing a molting function for these genes. (2) *Pt-sad* is expressed in embryonic cardiogenic mesoderm with additional staining in neuroectodermal precursors and limb buds, as well as hemocytes of postembryonic stages. Parental RNAi caused increased embryonic lethality and developmental delays. Surviving juveniles showed disrupted molt cycles, indicating an essential role for ecdysteroid production. (3) mRNA for the CYP genes *Pt-spo*, *Pt-dib* and *Pt-shd* was detected in presumptive hemocyte precursors. In juveniles *Pt-spo* and *Pt-dib* were prominently expressed in hemocytes, while *Pt-shd* was enriched in peripheral tissues like the midgut epithelium, ovary and fat body. RNAi led to severe molting delay. (4) *CYP18A1* also displayed expressions in embryonic hemocyte precursors and postembryonic midgut epithelium. RNAi-mediated knockdown resulted in molting defects and molt interval prolongation. Collectively, these findings demonstrate that the spider ecdysteroid pathway is functionally conserved but possesses alterations that indicate endocrine tissue decentralization and additional roles beyond molting.

Building on these results, the following insights will be discussed: **(a)** the conservation and divergence of ecdysteroid pathway genes across Panarthropoda, including the absence or modification in non-insect lineages; **(b)** broader evolutionary context, comparing spiders to distant ecdysozoans like tardigrades; **(c)** the discovery of novel expression domains of these genes in spiders (digestive, nervous and circulatory systems); **(d)** evidence that the hematopoietic cells in spiders may serve as ecdysteroid-producing centers; and lastly **(e)** hypotheses for the regulatory networks governing molt hormone synthesis in arachnids.

4.1. Conservation and Divergence of the Ecdysteroid Pathway in Panarthropoda

The findings reinforce that the core enzymes for ecdysteroid biosynthesis are ancient and broadly conserved to enable molting across arthropods. Nearly all major pathway genes known from insects were identified in the spider *P. tepidariorum*. This suggests that the capacity to synthesize a form of ecdysone from dietary sterols was already present in the arachnid lineage and likely the common ancestor of Arthropoda. Indeed, genomic surveys have found orthologs of these genes across many arthropod lineages, from insects to crustaceans and chelicerates (Qu et al., 2015; Schumann et al., 2018; Yamanaka, 2021; Campli et al., 2024). The essential nature of this pathway has placed strong evolutionary constraints on its components, evidenced by lethal molting defects in diverse species when disrupting the ecdysteroid pathway genes (Chávez et al., 2000; Niwa et al., 2004; Ameku et al., 2017; Peng et al., 2019; Yang et al., 2021). Functional RNAi-mediated knockdowns to assess the effects of ecdysteroid pathway genes and ecdysteroid-inactivating gene in *P. tepidariorum* also resulted in prolonged molting intervals (Fig. 32). This phenotype is often paired with high lethality during molts and is consistent with reported insect ecdysteroid deprivation (Kamimura et al., 2012; Peng et al., 2019; Zhang et al., 2021). The results on *shroud* represent the first functional confirmation of the gene role outside of insects.

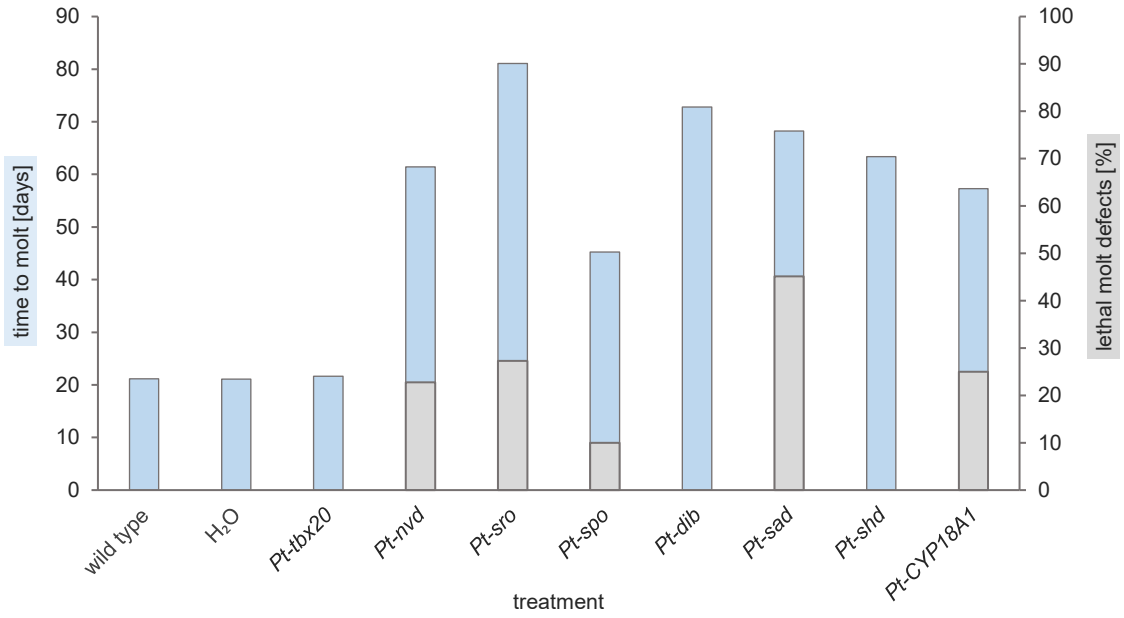


Figure 32 Summarized molt interval prolongation and occurrence of lethal molting defect phenotypes resulting from RNA interference-mediated knockdown treatment with dsRNA of ecdysteroid pathway genes 5 days after penultimate molt in subadult males of *P. tepidariorum*.

Despite this overall conservation, significant divergences and lineage-specific absences of certain pathway components were observed (Fig. 33). One of the more obvious differences is the absence of the *phantom* gene in chelicerates (Qu et al., 2015; Dermauw et al., 2020; Yang et al., 2021), confirmed in this study for the spider *P. tepidariorum*. Genomic and phylogenetic analyses indicate that *phm* likely evolved later in the pancrustacean lineage, perhaps first appearing in insects (Schumann et al., 2018). It encodes a critical step in the canonical insect pathway (Niwa et al., 2004; Warren et al., 2004). Thereby, the absence implies a modified ecdysteroid pathway where spiders may not add the C25 hydroxyl group that defines ecdysone and instead synthesize an analogous molt hormone. A potential candidate is ponasterone A (ponA). This ecdysteroid lacks the C25 hydroxyl and was identified in various chelicerates and some crustaceans (Ueno et al., 1992; Qu et al., 2015; Li et al., 2017; Yang et al., 2021).

Another notable difference is the absence of the *noppera-bo* (*nobo*) gene in the spider. Recently discovered in *D. melanogaster*, it encodes a glutathione S-transferase to facilitate cholesterol trafficking into steroidogenic tissues (Enya et al., 2014). However, *nobo* has been functionally characterized in the fruit fly and a few holometabolous insects (Enya et al., 2015; Koiwai et al., 2020; Inaba et al., 2022; Ebihara & Niwa, 2023) but is not detected outside this lineage. Its absence suggests reliance on different and possibly more generalized mechanisms for sterol uptake and transport. For example, spiders might utilize conserved lipid transporters, such as lipoproteins or Niemann-Pick type C (NPC) proteins, to deliver extremely hydrophobic cholesterol to ecdysteroidogenic cells (Jouni et al., 2002; Bialistoky et al., 2019). The independent origin of *nobo* in insects underscores that especially peripheral or regulatory factors of the ecdysteroid pathway have been gained or lost in different ecdysozoan lineages.

Likewise, insects have evolved multiple *spook* paralogs (Spook, Spookier and Spookiest) that partition in ecdysone production between life stages (Ono et al., 2006). Higher dipteran flies express *spook* in embryos but switch to the duplicate *spookier* for larval ecdysone synthesis (Namiki et al., 2005; Van Ekert et al., 2016; Dermauw et al., 2020). Spiders and other chelicerates appear to retain a single *spo* ortholog functioning across stages, reflecting how gene duplication events in specific insect lineages led to novel regulatory strategies, presumably subfunctionalization (Schumann et al., 2018; Dermauw et al., 2020). Beyond arthropods, gene presence/absence patterns become even more divergent. For instance, some parasitic mites are reported to have lost the *neverland*

gene (Cabrera et al., 2015; Techer et al., 2019) and onychophorans and tardigrades only possess a subset of the pathway genes (Yamakawa & Hejnol, 2024).

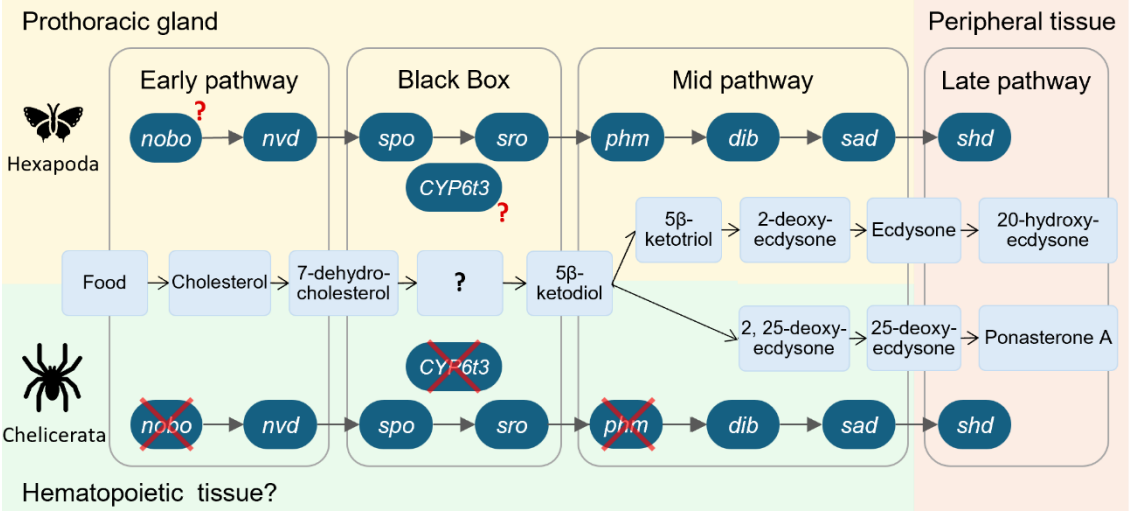


Figure 33 Updated ecdysteroid biosynthesis pathway of insects and chelicerates. In chelicerates hematopoietic tissue is suggested as secretory origin of the molt hormone. Evidence for *nvd* and *sro* was recovered while *nobo* and *CYP6t3* seem absent in spiders. *phm* has not been detected in Chelicerata. Differentially modified ecdysteroid Ponasterone A might be the active chelicerate hormone. The two rows refer to summarized knowledge in Hexapoda and Chelicerata. Question marks indicate the need for further clarification; crosses highlight reported absence. *CYP*, *cytochrome P450*; *dib*, *disembodied*; *nobo*, *noppera-bo*; *nvd*, *neverland*; *phm*, *phantom*; *sad*, *shadow*; *shd*, *shade*; *spo*, *spook*; *sro*, *shroud*.

4.2. Evolutionary Implications: Panarthropods, Nematodes and Priapulida

How is molting hormonally controlled across Ecdysozoa? The conservation of ecdysteroid pathway genes in spiders aligns with emerging evidence that panarthropods, including arthropods, tardigrades and onychophorans, might share a common ancestral ecdysteroid hormone for molting regulation. In particular, tardigrades and earlier branching ecdysozoans like nematodes and priapulids provide a fascinating case.

Genomic studies reveal that tardigrades are missing homologs of most of the canonical CYP genes like *spo*, *phantom*, *dib* and *shd* (Schumann et al., 2018), suggesting that the animals might lack the classic ecdysone-producing pathway entirely. However, a recent study by Yamakawa & Hejnol (2024) demonstrated that molting is regulated by ecdysteroids in tardigrades. In *Hypsibius exemplaris*, endogenous ecdysteroid levels rise and fall with the molting cycle. The study confirms that tardigrades utilize the ecdysteroid degradation gene *CYP18A1* and an ecdysone/ecdysone receptor (EcR) system for molt regulation. This fact supports the presence of ecdysteroid-dependent molting in the last common ancestor of Panarthropoda during the Ediacaran Period (635

to 541 mya) (Howard et al., 2022). This evolutionary paradox raises the question of how they produce the molting hormone. As the pathway gene *sad* was detected in tardigrades in recent studies (Schumann et al., 2018; Yamakawa & Hejnol, 2024), one theory is the presence of highly divergent enzyme analogs that perform the necessary conversions but are not recognizable as CYP homologs. Alternatively, in combination with the short-chain dehydrogenase/reductase Sro detected in the tardigrade *Paramacrobiotus metropolitanus* in this study, an ecdysteroid precursor might be subsequently modified non-enzymatically. The presence of *sro* and *sad* supports the ancient ecdysteroid pathway hypothesis, pushing its origin back to at least the Precambrian (~540 to 560 mya, before the Cambrian explosion), when the panarthropod lineage formed (Howard et al., 2022).

In contrast to panarthropods, free-living nematodes like *Caenorhabditis elegans* represent a lineage of ecdysozoans that largely abandoned ecdysone for molting (Malakhov, 1986; Giribet & Edgecombe, 2017). The *EcR* gene is absent (Taubenheim et al., 2021; Yamakawa et al., 2025) and none of the canonical ecdysteroid pathway CYPs are represented in its genome (Schumann et al., 2018). Genomic analyses indicate multiple independent losses of the ecdysone/EcR system in nematode evolution (Yamakawa et al., 2025). Instead, they have developed a different hormonal system for molt control. It utilizes sterols called dafachronic acids with signaling through the distinct Dauer-Abnormal Formation gene 12 nuclear receptor (DAF-12; Hochbaum et al., 2011; Aguilaniu et al., 2016; Lažetić & Fay, 2017). The biosynthesis of dafachronic acid co-opts some ancestral ecdysteroid pathway enzymes like the cholesterol metabolism-initiating Rieske-domain oxygenase DAF-36/*neverland* (Yoshiyama-Yanagawa et al., 2011). Downstream, a CYP called DAF-9 converts precursors to dafachronic acid, fulfilling a hormonal role somewhat analogous to the canonical pathway CYPs in insects (Gerisch & Antebi, 2004). The contrast in pathway conservation is potentially dictated by the environmental niche and life history which favors endocrine strategies. Nematodes evolved small body sizes and often short, continuous growth without dramatic metamorphosis. This perhaps reduced reliance on pulses of molting hormone, whereas panarthropods maintained their episodic growth and more complex life cycles.

The recent genome assembly of the priapulan *Tubiluchus corallicola* (Lord et al., 2023) extends comparative coverage to one of the earliest-branching ecdysozoan phyla. A single-copy ortholog of the CYP *shadow* was recovered. This implies that the mitochondrial hydroxylase was already present in the priapulan stem lineage, while much of the upstream CYP toolkit evolved later in Panarthropoda. The present study also recovered a *shroud* sequence for *Priapulus caudatus*. These findings suggest that the Black Box and mid-to-late step of ecdysteroidogenesis are the most ancient and refractory to loss.

In summary, the evolutionary overview suggests that ecdysteroid signaling originated early in the panarthropod lineage and was likely present in the ecdysozoan ancestor to some extent. It has been subject to gene losses, replacements and innovations in various clades. Spiders retain the ancestral core ecdysteroid pathway genes. Studying (1) how tardigrades and priapulids manage molting with only one CYP enzyme or (2) how nematodes replaced the EcR system, can provide insights into the minimum essential components for molting and how endocrine control of development evolved.

4.3. Tissue-Specific Expression and Potential Non-Molting Roles

A novelty of this research is the broader temporospatial expression of ecdysteroid pathway genes detected in spider embryogenesis and juvenile stages compared to insects (Fig. 34). In holometabolous insects, the enzymes Neverland, Phantom, Disembodied and Shadow are restricted to the prothoracic gland during embryonic and pre-molting stages (Chavez et al., 2000; Warren et al., 2002; Warren et al., 2004; Yoshiyama-Yanagawa et al., 2011). The Spook enzyme is limited to the amnioserosa in mid embryogenesis and prothoracic gland in pre-molt larval stages of *D. melanogaster* (Ono et al., 2006). Shade is only expressed in postembryonic peripheral tissues like epidermis, midgut, Malpighian tubules and fat body (Petryk et al., 2003). This spatial division of central production versus peripheral activation is an identification feature of insect molting physiology. By contrast, *P. tepidariorum* displayed expression in multiple tissues, hinting at a decentralized strategy for ecdysteroidogenesis in spiders. Notably mRNA was detected in digestive and reproductive organs, the nervous system and circulatory system in embryonic and postembryonic development. These patterns suggest that the enzymes may occupy additional, non-molting roles. Below, the gene expressions in each major tissue and potential functions therein are discussed.

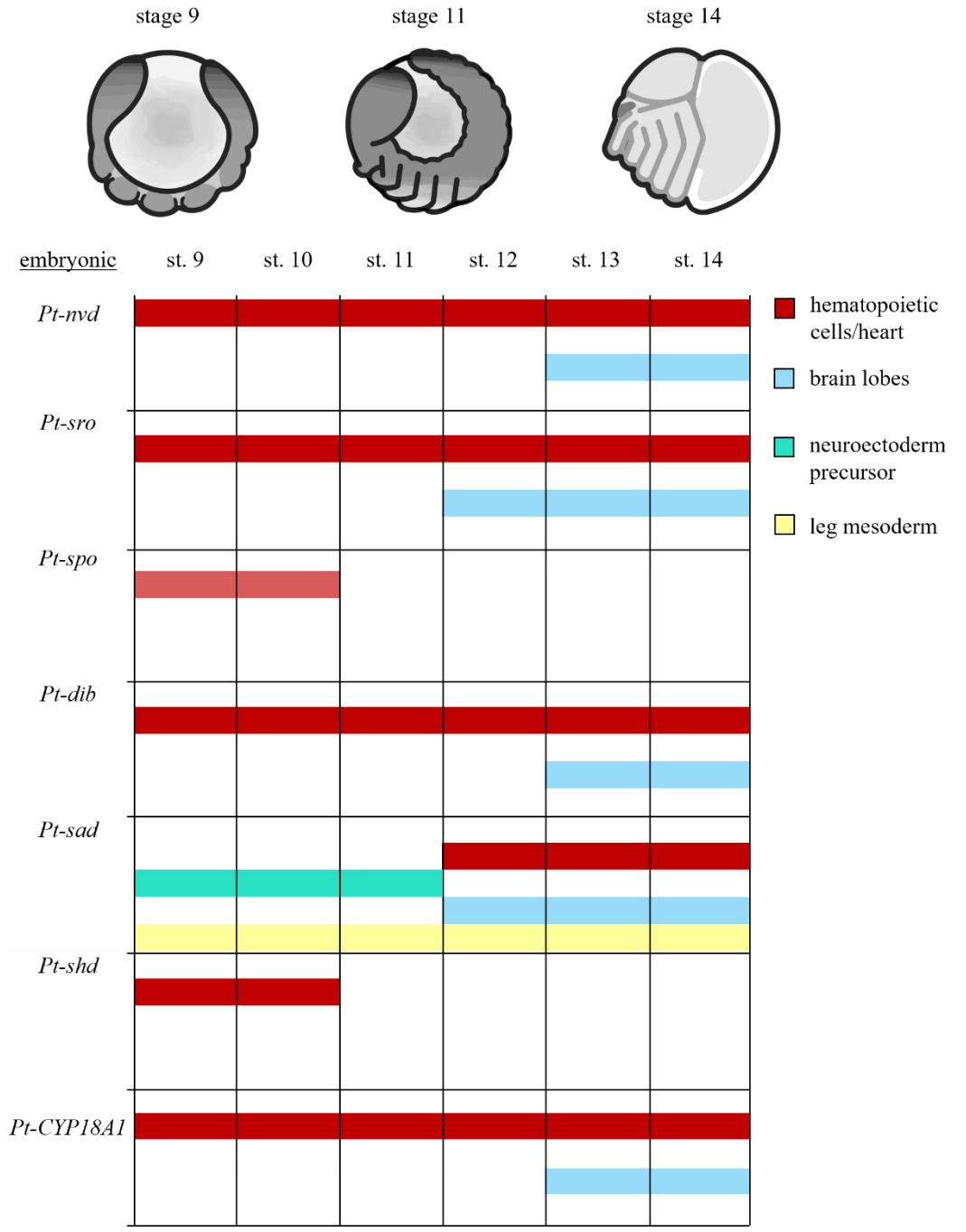
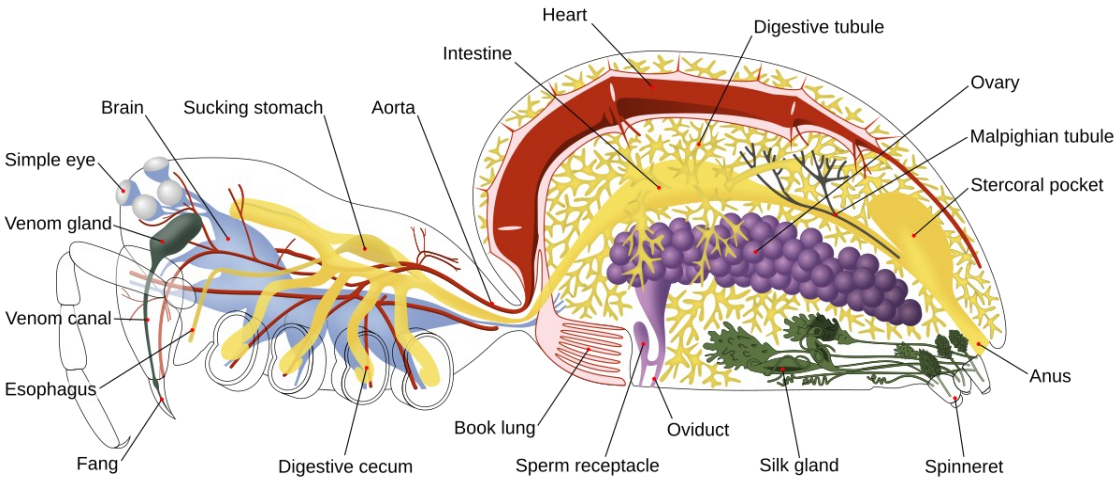


Figure 34 Summarized findings of ecdysteroid pathway gene expression via *in situ* hybridization during mid-to-late embryogenesis of *P. tepidariorum* (embryonic schemes: Wikimedia Commons, see online reference section).

4.3.1. Digestive and Reproductive Tissues

Expression of ecdysteroid pathway genes in juvenile spider digestive and reproductive organs suggest additional roles related to metabolism and reproduction (Fig. 35). The genes *Pt-spo*, *Pt-sad*, *Pt-shd* and *Pt-CYP18A1* are strongly expressed in the midgut epithelium cells. These midgut-associated cells likely correspond to digestive midgut diverticula and serve as hepatopancreas-like organs (Laino et al., 2009). The

presence of Shade in this tissue aligns with its known role in peripheral ecdysone activation (Petryk et al., 2003; Rewitz et al., 2006; Niwa & Niwa, 2016). Expression in the spider midgut suggests a similar activation domain for the molt hormone. Additionally, activation in the midgut may regulate pre-molt behavior like decreasing food intake before molting. Performed experiments also showed *Pt-shd* transcripts in cells distributed in the opisthosoma, representing fat body cells and again reflecting insect data (Smith & Mitchell, 1986; Petryk et al., 2003). Furthermore, expression domains in the spider midgut epithelium and fat body for *CYP18A1* align with previously observed patterns in *D. melanogaster*, suggesting peripheral molt hormone inactivation (Guittard et al., 2011).



POSTEMBRYONIC	*Pt-nvd*	*Pt-sro*	*Pt-spo*	*Pt-dib*	*Pt-sad*	*Pt-shd*	*Pt-CYP18A1*
prothoracic gland (in insects)	*	*	*	*	*		
hemocytes/heart	✓	✓	✓	✓	✓	✗	✗
fat body	✗	✗	✗	✗	✗	✓*	✓*
midgut	✗	✗	✓	✗	✓	✓*	✓*
ovary	✗*	✓*	✗	✓*	✓*	✓*	✗
subesophageal ganglion	✗	✓	✓	✓	✓	✓	✓
supraesophageal ganglion	✗	✓	✗	✗	✗	✗	✗
mushroom body	✓	✓	✗	✗	✓	✗	✗
visceral cortex resembling	✓	✓	✗	✗	✗	✗	✗

✗ = absent in spiders ✓ = present in spiders * = present in insects

Figure 35 Summarized mRNA localization of ecdysteroid pathway genes in postembryonic development of the spider *Parasteatoda tepidariorum*. Spider scheme displaying internal anatomy is redesigned from Comstock (1920) by Ryan Wilson, who included additional anatomical information from Foelix (1996), and was taken from Wikimedia Commons (see online references).

In reproductive tissues of juvenile female spiders, expression of *Pt-sro*, *Pt-dib*, *Pt-sad* and *Pt-shd* was detected in the developing ovaries. Previous studies also indicated important roles of ecdysteroids in female reproduction (Ono et al., 2006; Niwa et al., 2010; Sieber & Spradling, 2015), like ovarian tissue maturation, preparation for yolk uptake and oocyte growth (Garcia et al., 2025). In moths, grasshoppers and spider mites, pathway genes are required for vitellogenesis and oocyte maturation (Peng et al., 2019; Schellens et al., 2022; Wang et al., 2023). Ovarian follicle cells in *D. melanogaster* also produce ecdysone to coordinate egg development with physiology (Berg et al., 2024). The activity in ovarian tissues of juvenile spiders suggests autonomous production of ecdysteroids to regulate ovary functions, like yolk deposition after reaching maturity. This would represent a conserved reproductive role for ecdysteroids, independent of molting. Taken together, the expression in digestive and reproductive organs underscores that ecdysteroid pathway genes in spiders have expanded roles beyond molting. They likely contribute to the regulation of metabolism, digestion and reproduction.

4.3.2. Nervous System Expression

Surprisingly, multiple ecdysteroid pathway genes were found in neural tissues of the spider, suggesting that they partake in the development or function of the nervous system. In embryos, *Pt-nvd*, *Pt-sro*, *Pt-dib*, *Pt-sad* and *Pt-CYP18A1* are expressed in the region of forming head lobes (Fig. 34). *Pt-sad* plays a particular role in embryonic neurogenesis as it shows strong expression in neuronal invagination sites (Stollewerk et al., 2001; Stollewerk et al., 2003). This is noteworthy because in insects, while the prothoracic gland is partly of ectodermal origin and associated with the head, the ecdysteroid pathway enzymes themselves are not typically expressed in neural tissue (Sánchez-Higueras et al., 2014; Campli et al., 2024). From a developmental point of view, the early embryonic expression of *Pt-sad* in the neuroectoderm could be tied to patterning or morphogenesis of the arachnid nervous system (Stollewerk et al., 2003; Stollewerk, 2016). Molting hormones are required in late insect embryogenesis to trigger head involution, cuticle formation and hatching behaviors (Chávez et al., 2000; Petryk et al., 2003; Scanlan et al., 2023). If spider embryos similarly require an ecdysteroid pulse to complete development, the central nervous system (CNS) expression might coordinate this event through neurosecretory cells. In insects, a set of neurons in the brain produces the prothoracicotropic hormone (PTTH) to stimulate ecdysone synthesis (McBrayer et al., 2007; Rewitz et al., 2009). In spiders, if hemocytes are the primary ecdysteroid

producers, the brain might directly or indirectly modulate them. It is intriguing to speculate that *Pt-sad* expression in the spider brain could mark neurons that themselves release hormones or an activating cofactor into circulation. Although speculative, this could represent an alternate evolutionary solution where neurons both initiate and partially execute hormone production.

The postembryonic spider data indicates a more complex pattern with the nervous system probably being both a source and a target of ecdysteroids. One possibility is that the expression in neural tissue relates to developmental patterning. For instance, ecdysone is known to coordinate aspects of neurodevelopment in insects, like neuronal pruning and reorganization during metamorphosis (Kirilly et al., 2009; Truman & Riddiford, 2023). Spiders do not undergo complete metamorphosis, but ecdysteroid surges might influence neural maturation at each molt (Napiórkowska & Kobak, 2017). A well-documented role of ecdysteroids in postembryonic insects is the modulation of neural functions like behavior, learning and memory. Matsumura et al. (2023) summarized that genes for ecdysteroid signaling are expressed in a regulated manner in the mushroom body of honeybee brains. Additionally, ecdysteroid receptors are expressed in the insect brain and studies have shown that 20-hydroxyecdysone (20E) signaling in adult *D. melanogaster* brains is required for processes such as long-term memory formation (Ishimoto et al., 2009; Ishimoto et al., 2013). These findings illustrate that ecdysteroids can function as neuromodulators outside of developmental molting contexts. By analogy, the presence of ecdysteroid pathway enzymes in the spider nervous system could imply local synthesis or turnover of hormones within the brain, potentially affecting neural physiology or behavior. These locally limited expression sites could control developmental transitions in behavior or regulate feeding around molting.

In summary, the expressions of ecdysteroid pathway genes in the spider CNS point to multifunctionality. This is supported by RNAi-mediated *Pt-sad* knockdowns resulting in broader developmental effects beyond just molting defects. The use of these genes in the spider nervous system may display how an ancestral hormonal pathway can be repurposed or integrated into new developmental programs. Both the transcription profiles reported by Yang et al. (2021) and *in situ* hybridization results of this study demonstrate that pathway gene expression is less pronounced in the prosoma compared to the opisthosoma. This implies that molt-related ecdysteroidogenesis occurs primarily in non-CNS tissues.

4.3.3. Circulatory System: Hemocytes and Cardiac Organogenesis

Large cells in the “extraembryonic tissue” covering the yolk in *P. tepidariorum* during mid-embryogenesis exhibit transcription of all ecdysteroid pathway genes, except for *Pt-sad* (Fig. 34). The temporal and spatial localization of the expressing pathway genes correlates with mRNA transcripts observed for presumptive circulatory system precursors of the spider embryo. Single-cell RNA-seq (Leite et al., 2024) assigns these yolk covering cells to clusters enriched with hematopoietic regulators like serpent/GATA4 (Muratoglu et al., 2007; Spahn et al., 2014). Those clusters are characterized by high proportions of G1 phase cells, indicating potential proliferative hemocyte progenitors (Leite et al., 2024). The profile of *Pt-nvd*, *Pt-sro*, *Pt-spo*, *Pt-dib*, *Pt-shd* and *Pt-CYP18A1* expressing cells occupy a similar dorsal head-mesoderm position as insect hemoblasts and potentially share expression of the GATA hematopoietic program (Holz et al., 2003; Sorrentino et al., 2005), indicating a conserved early wave of hematopoiesis.

As dorsal closure completes during late-embryogenesis, the gene expression of *Pt-nvd*, *Pt-sro*, *Pt-dib* and *Pt-CYP18A1* becomes restricted in a tube with bilaterally symmetric and segmentally repeated widenings. Comparison to cardiogenic markers such as *myocyte enhancer factor 2* (*Mef2*) (Janssen & Damen, 2008; Leite et al., 2024) situates these cells within the tinman/Nkx2-5-positive cardiogenic mesoderm common to arthropods and vertebrates (Zaffran et al., 2006; Janssen & Damen, 2008; Qian et al., 2011; Mittmann & Wolff, 2012). Ultrastructural studies in spiders have described this endocardial epithelium as mitotically active and as blebbing pro-hemocytes into the hemocoel (Seitz, 1972; Sherman, 1973). These cells might present an embryonic hematopoietic niche, comparable to the insect lymph gland and larval “hematopoietic pockets”. In *D. melanogaster* larvae, hematopoiesis is initiated by embryonically differentiated hemocytes that migrate into segmentally repeated epidermal-mesodermal pockets, where they proliferate (Makhijani, et al., 2011). A role in cardiogenic mesoderm specification is suggested for *Pt-sad* when compared to the previously described cardiogenic markers like *Mef2* and *ryanodine receptor* (*ryr*) (Leite et al., 2024). The shape and position of its expression domain resembles the alary muscles in insects (Wasserthal & Wasserthal, 1977; Glenn et al., 2010; Bataillé et al., 2024).

In juveniles the expression for *Pt-sad* in the heart-associated mesoderm seems consistent compared to embryonic development. Expression of the other ecdysteroid

biosynthesis pathway genes (*nvd*, *sro*, *spo* and *dib*) consists in small cells inside the heart lumen and efferent vessels, confirming continuity between hemocyte progenitors and mature hemocytes. Spiders appear to rely solely on this vessel-based system for lifelong hemocyte production.

Placing the spider strategy within a wider arthropod framework, exhibits heart-associated hematopoiesis as characteristic for crustaceans and early insect embryos, while Holometabola also utilize a separate lymph gland (Grigorian & Hartenstein, 2013). The spider therefore represents the likely primitive state, where hemoblasts migrate dorsally, integrate into the forming heart and continuously supply the circulation. Lineage-tracing of cells covering the yolk into the heart epithelium and knockdown-based disruption of *serpent* or other early factors will evaluate this model directly and clarify the conservation of hemocyte formation across Arthropoda. The consistent presence of multiple pathway enzymes in hemocytes and heart-related tissue strongly suggests that hemocytes themselves could be ecdysteroid-producing cells in spiders.

4.4. Hemocytes as Endocrine Producers: A New Model for Arachnid Molting

Given the evidence above, here a model is proposed, in which spider hemocytes function as the principal ecdysteroid-producing "organ," similar to the prothoracic gland of insects. The idea mirrors the observed expression data and aligns with functional observations. RNAi of each ecdysteroid pathway gene produces delayed and/or lethal molts accompanied by pre-molt lethargy. This is reminiscent of ecdysteroid deficiency phenotypes in insects (Jürgens et al., 1984; Wieschaus et al., 1984; Kamiyama & Niwa, 2022). One can hypothesize that reducing expression in hemocytes decreases their ability to produce sufficient molting hormone to initiate ecdysis.

A hemocyte-centered endocrine system would represent a remarkable analog to crustaceans and insects, where the paired Y-organs or prothoracic gland remain the primary source of *de novo* ecdysone synthesis (Yoshiyama et al., 2006; Chang & Mykles, 2011; Mykles, 2011; Legrand et al., 2021). What might be the evolutionary advantage of hemocyte-based ecdysteroidogenesis? Firstly, hormone production would become spatially ubiquitous, ensuring synchronous exposure to all tissues. Secondly, hemocytes could be directly modulated by nutritional and immune signals. Thereby infection or starvation could down-regulate ecdysteroid output and consequently postpone molting until conditions improve. Comparable immune/endocrine cross-talk is documented in

insects, where septic injuries or infections elevate cytokines that suppress PTTH release and delay or abort molting (Regan et al., 2013; Rus et al., 2013; Ghosh et al., 2015; Nunes et al., 2021). This integration could be a benefit, ensuring the spider does not attempt to molt at a vulnerable time.

From an evolutionary perspective, hemocyte-based ecdysteroidogenesis may represent either a retained ancestral state or a chelicerate innovation. It is tempting to consider that early ecdysozoans use circulating cells to manage hormonal molts. The discovery of a molting "neuro-center" rather than a discrete gland in tardigrades (Yamakawa & Hejnol, 2024) implies that early ecdysozoans relied on simple distribution systems. Independently, the insect lineage later consolidated ecdysteroid synthesis into a specialized gland for greater efficiency during complete metamorphosis (Niwa & Niwa, 2016; Bian et al., 2019). Arachnids may represent an intermediate endocrine state with a centralized neural timer for signals combined to a decentralized, hemocyte-executed biosynthesis. This is speculative, but it underscores how studying spiders can fill gaps in our understanding of endocrine organ evolution.

The absence of *Pt-shd* transcripts in hemocytes but their presence in ovary, fat body and midgut, suggests a two-step system analogous to insects (Petryk et al., 2003; Rewitz et al., 2006; Yang et al., 2021). If spider hemocytes secrete hormone precursors, these might be hydroxylated to the bioactive molting hormone in peripheral tissues with detected juvenile *Pt-shd* expression. Such division of synthesizing hemocytes and activating peripheral tissues would be functionally akin to the insect system. Intriguingly, a transcriptomic survey on the tick *Ixodes scapularis* now reports ecdysteroid pathway gene expression in hemocyte clusters (Rolandelli et al., 2024). Together, the evidence suggests that hemocytes may constitute a conserved ecdysteroidogenic trait across Chelicerata. The here presented work therefore provides a foundation for rethinking the molting endocrine system in chelicerates. This gives rise to new research questions, including the search for the regulating signals of hemocyte ecdysteroidogenesis.

4.5. Hypotheses and Evolutionary Considerations

The absence of canonical enzymes like Phantom and Nobo in spiders and the distributed nature of ecdysteroidogenic tissues suggest that the molt regulatory network in spiders differs significantly from the insect pathway. There, the molting hormone pathway is tightly controlled by neuroendocrine signals. The brain produces PTTH,

which binds to receptors on prothoracic gland cells and triggers a cAMP (cyclic adenosine monophosphate) signaling cascade, upregulating molt-related CYP transcription and ecdysteroidogenesis (Song et al., 2017). Upregulation results in an ecdysone pulse which, after peripheral conversion to 20E, activates the ecdysone receptor complex (EcR/USP) which coordinates a defined gene cascade resulting in ecdysis (Thummel, 1996). The prothoracic gland is also exposed to inhibitory factors, like dopamine or neuropeptides that are able to suppress ecdysone synthesis and thereby cause molt delay (Riddiford et al., 2003; Niwa & Niwa, 2014b). Do spiders have analogous regulatory mechanisms? We can only hypothesize, but several points are worth considering.

4.5.1. Neuroendocrine Control and PTTH

Neuropeptide screens of non-insect genomes (including the crustacean *Parhyale hawaiensis* and the spider *P. tepidariorum*) failed to detect a PTTH-like precursor (De Oliveira et al., 2019). However, spiders do possess a complex brain with numerous neuropeptides identified, some of which could perform a PTTH-like function. The tardigrade study by Yamakawa & Hejnol (2024) provides an intriguing clue. A region in the protocerebrum that appears homologous to the insect PTTH-producing center was detected, suggesting an ancestral origin of the molt control center in the brain. If spiders share this ancestral feature, they might possess neurons in the synganglion (fusion of supra- and subesophagial ganglion) that respond to developmental cues and signal to peripheral tissues for ecdysteroid synthesis initiation. Studies on crustaceans established that the molt-inhibiting hormone (MIH) released from the X-organ of the eyestalk suppresses ecdysteroid synthesis in the Y-organ, which resembles the functional analog of the insect prothoracic gland (Chang & Mykles, 2011). Spiders lack eyestalks but could have MIH-like factors from other centers (perhaps the subesophageal ganglion) to prevent premature hormone release. The observation that *Pt-sad* and other genes are expressed in the brain raises the possibility that this structure itself contributes some of the ecdysteroid pathway steps or at least monitors it.

4.5.2. Transcriptional Regulation of Halloween Genes

In insect prothoracic glands, early cascade genes like ecdysone-inducible gene 74 (E74) and 75 (E75) are first targets of the EcR-RXR complex (EcR/retinoid X receptor) (Hill et al., 2013; Song et al., 2017). Afterwards, several conserved transcription factors including the β-Fushi-tarazu transcription factor 1 (βFTZ-F1) and hormone receptor 3 & 4 (HR3 & 4) as well as signaling pathways like Ras/ERK (rat sarcoma/extracellular-signal-regulated kinase), coordinate the up- and downregulation of ecdysteroid pathway genes during molting cycles (Truman & Riddiford, 2002; Rewitz et al., 2009; Niwa & Niwa, 2016; Truman, 2019; Pan et al., 2021). Do spider hemocytes receive similar transcriptional instructions? No direct experimental data on the spider molting hormone regulatory network is available yet. Homologous regulators might be expected in the spider genome. For instance, orphan nuclear receptor FTZ-F1 and the EcR-RXR complex are present in many arthropods (Yamada et al., 2000; Nakawaga et al., 2007; Zhang et al., 2015; Schumann et al., 2018). Identified EcR in the spider *P. pseudoannulata* likely targets the presumably conserved downstream genes like *E74* and *E75* when induced by the molt hormone (Yang et al., 2023). The presence of these early-response genes suggests that the downstream regulatory cascade is intact. Another question is how the upstream ecdysteroid pathway genes are regulated to generate the hormone pulse. One hypothesis is that some ecdysteroid pathway genes of the spider might be rate-limiting steps that get acutely upregulated to produce a triggering pulse.

4.5.3. Alternative Pathways and Precursors

The lack of *phantom* means the molting hormone pathway of spiders and other chelicerates might produce an analogous precursor which is subsequently converted into ponA and has a molt-inducing effect (Grbić et al., 2011; Li et al., 2017; 2019; Yang et al., 2021). On the other hand, *phm* detection in the copepod *Lepeophtheirus salmonis* and the mite *Varroa destructor* are uncertain, but the active molting hormone 20E and not ponA were extracted from the latter (Sandlund et al., 2018; Humble et al., 2019; Techer et al., 2019). The variable observation of *phm* and ponA in crustaceans and chelicerates (Legrand et al., 2021) suggests alternative pathways either through a plurality of unidentified enzymes or flexibility in biochemical reactions. While the overall process remains conserved, the developmental gene repertoire for ecdysteroid biosynthesis can change over the course of evolution, hinting to the possibility of developmental system

shifts (True & Haag, 2001). To determine if chelicerates predominantly utilize ponA or 20E, investigations of the EcR-RXR complex are necessary. Spiders possibly evolved an alternative route, involving enzymes that are not part of the canonical pathway. From a regulatory point, this means spiders would not need to regulate the *phm* gene but might have to regulate the supply of 7-dehydrocholesterol and other intermediates differently. It also means any transcriptional regulators that specifically target *phm* in insects, for example the homeodomain transcription factors Antennapedia and POU-M2 in the silkworm *Bombyx mori* (Meng et al., 2015), would be absent or repurposed in spiders.

4.5.4. Cholesterol Uptake and Storage

Without *nobo*, spiders must manage cholesterol uptake via other mechanisms. In insects, *nobo* is transcriptionally upregulated to increase sterol import for molting (Enya et al., 2015). Spiders might regulate cholesterol availability through other routes, like the *NPC1* transporter or lipoproteins (Cunningham et al., 2000; Laino et al., 2011; Zheng et al., 2018). Regulation of uptake may also be mediated through behavior. It is known that spiders stop feeding before molts (Moon & Tillinghast, 2020), which likely prevents new cholesterol intake and could force the use of stored sterols from the midgut/fat body (Molina et al., preprint). Thus, an interesting speculation is that in the absence of *nobo* spiders rely on timed feeding versus fasting as a coarse control on internal cholesterol levels for ecdysteroid production. The exact regulation of this is unknown, but potentially hormonally controlled, e.g., insulin signaling might link nutrient status to molt hormone synthesis similar to insects (Colombani et al., 2005; Gu et al., 2009; Pan et al., 2021).

4.6. Evolutionary Perspective: Molting and its Hormonal Control in Ecdysozoa

Integrating all available data reveals a molt hormone pathway that is anciently conserved across arthropods yet remains evolutionarily flexible. The identification of key biosynthesis genes in chelicerates fills an important gap between well-studied insect models and more basal ecdysozoans like tardigrades and nematodes. This work supports the idea that the last common ancestor of Panarthropoda already evolved an enzymatic pathway to produce molting hormones from cholesterol. This ancestor likely possessed at least one CYP enzyme and the short-chain dehydrogenase/reductase Shroud. Interestingly, partial remnants of the canonical insect pathway in nematodes and

priapulids (e.g., DAF-36/*nvd*; Yoshiyama-Yanagawa et al., 2011) also support a common origin of sterol-based molting in the earliest ecdysozoans.

The evolutionary success of the species-rich superphylum (Ecdysozoa) results from the fact that periodic molting is a remarkably effective strategy with versatile hormone regulation solutions. Tardigrades, as early-branching panarthropod lineages, appear to have limited enzymes but manage hormonal function (Yamakawa & Hejnol, 2024), while in pancrustaceans the full complement of ecdysteroid pathway genes is established (Campli et al., 2024). The complexity seen in insects, including feedback loops, precise timing and stage-specific roles of duplicated genes, might be a later elaboration for holometabolous development. This study broadens the perspective to chelicerates, which represent an interesting mix of primitive and derived traits. They display a possibly ancestral distribution of hormone-producing cells (hemocytes) rather than a discrete gland. This stands in contrast to pancrustaceans, which have centralized steroid production (Niwa & Niwa, 2016; Ou et al., 2016). From an EvoDevo standpoint, comparing molting hormones of spiders, insects and tardigrades provides a case study of deep homology that has been modified in certain descendent lineages. Specifically, the potential role of hemocytes in hormone production and involvement of ecdysteroids in processes like immunity blurs the line between the endocrine and immune response. Perhaps early in ecdysozoan evolution, the molting hormone had dual roles, coordinating molts and acting as a stress hormone (like cortisol in vertebrates; Adamo, 2012).

Further comparative studies are necessary. Do scorpions also lack *phantom* and rely on hemocytes for ecdysone? Do multiple independent evolutionary paths occur within Chelicerata? Broadening to Myriapoda, the question arises whether the insect-like pattern or something closer to spiders is present. Spiders emerge as a model where one can see the evolutionary versatility of a hormonal system: (1) they use a different ecdysteroid (ponA), (2) lack certain enzymes (Phantom, Nobo) and (3) possibly distribute hormone production among hemocytes and other tissues while still successfully regulate molting. These insights deepen our understanding of how the fundamental process of ecdysozoan molting has been shaped over hundreds of millions of years. They provide a platform for future studies to explore the evolution of endocrine regulation in this diverse animal clade and to explore the power of natural selection in maintaining essential developmental processes while allowing genetic innovation.

5. Conclusion

This thesis identifies and functionally characterizes six ecdysteroid biosynthesis genes and one ecdysteroid-inactivating gene in the common house spider *Parasteatoda tepidariorum*. RNAi-mediated knockdowns of *Pt-nvd*, *Pt-sro*, *Pt-spo*, *Pt-dib*, *Pt-sad*, *Pt-shd* and *Pt-CYP18A1* prolong or disrupt molts. This demonstrates that, despite 550 million years of divergence to insects, the core ecdysteroid pathway remains predominantly retained in spiders. Yet the pathway also shows lineage-specific innovations. Two canonical insect components, *phantom* and *noppera-bo*, are absent which indicates ponA as the active molting hormone. Rather than being confined to a discrete prothoracic gland, spider ecdysteroid pathway genes show a broad spatiotemporal expression. mRNA is detected in multiple organ systems, including the midgut diverticula, fat body, ovary, nervous system and most prominently the circulating hemocytes and heart tube. These findings support a decentralized endocrine anatomy in which mobile hemocytes synthesize the inactive molting hormone, subsequently activated by *shade* in peripheral tissues. The proposed model may represent a retained ancestral state or a chelicerate innovation. Functionally, it would allow systemic hormone distribution while linking molt timing to nutritional status.

Finally, comparative genomics place the spider data in a wider EvoDevo context. All ecdysozoans included in this study displayed at least one CYP (*shadow*) and the short-chain dehydrogenases/reductases Shroud, implying that ecdysteroid controlled molting arose deep in the Precambrian period. The independent losses of enzymes show that endocrine modules can be rewired. Nevertheless, the molting behavior itself is maintained, even in nematodes where the ecdysone/EcR is replaced with dafachronic-acid/DAF-12 signaling. Thus, spiders exemplify an evolutionary strategy in which the ancestral enzymatic process is retained, while ecdysteroid products have been remodeled. Therefore, several hypotheses emerge: (1) Spider hemocytes autonomously convert cholesterol into ecdysteroids; (2) neuroendocrine cues may stimulate hemocyte ecdysteroidogenesis, analogous to insect PTTH or nutrient-sensitive insulin signals; (3) the loss of *phantom* suggests alternative enzymology.

6. Outlook

Elucidating ecdysteroidogenesis in *Parasteatoda tepidariorum* has opened multiple experimental and comparative routes, extending well beyond this thesis and inviting future interdisciplinary exploration.

1. Validating hemocytes as a distributed endocrine organ

   The expression data and RNAi phenotypes collectively suggest that circulating hemocytes synthesize and secrete ecdysteroids. A priority for future work is to combine cell-specific transcriptomics (e.g., single-cell RNA sequencing of isolated hemocytes) with *ex vivo* ecdysteroid measurements (LC–MS/MS) to quantify the production of 20E or ponA. Hemocyte-targeted knockdowns would provide direct comparison between hemocyte activity and hormone titers. Such experiments will define whether spiders rely exclusively on hemocytes as mobile cell populations or potentially utilize sessile pericardial cells for ecdysteroid output.

2. Dissect the mechanisms of upstream neuroendocrine control

   The absence of a canonical prothoracic gland implies that the spider brain or subesophageal ganglion must modulate hemocyte ecdysteroidogenesis through a yet unknown signal. Analyzing the synganglion transcriptome for secreted peptides that peak prior to an ecdysteroid pulse could reveal a PTTH or MIH analogue in chelicerates. Parallel proteomic profiling of hemocytes during artificial peptide stimulation will identify second-messenger pathways involved in the ecdysteroid pathway gene transcription.

3. Biochemical reconstruction of the "*phantom*-less" pathway

   Chelicerates evidently bypass the Phantom step and appear to terminate the hormone synthesis with ponA. Reconstituting each reaction in cell cultures, then feeding isotopically labelled cholesterol to engineered cell lines that express spider *nvd* → *sro* → *spo* → *dib* → *sad* → *shd* will determine intermediates and might allow activity tests on the spider EcR. This biochemical map will help clarify how modifications were gained or lost during arthropod evolution.

4. Comparative EvoDevo across chelicerates and basal ecdysozoans

   With robust gene models established for *P. tepidariorum*, orthology can be compared to scorpions, mites, horseshoe crabs etc. to observe the completeness of ecdysteroid pathway genes, study their developmental dynamics and identify associated regulatory motifs. Combining fossil-integrated phylogeny with gene shifts could elucidate the emergence or loss of *phantom*, *nobo* and *spook* paralogs against ecological shifts such as predatory lifestyles and terrestrialization.

5. Ecdysteroid RNAi approaches for pest control

   Understanding non-insect ecdysteroid pathways also has practical implications for pest control and aquaculture, where precise molting manipulation is desirable. For mites, first studies on effectiveness and feasibility of molt interfering pest control via knockdowns suggest gene and species depending success (Niu et al., 2018; Wang et al., 2023; Li et al., 2025). Releasing dsRNA for RNAi-knockdowns that target these highly conserved genes could unintentionally harm non-target beneficial invertebrates, including pollinators.

Advancing this research will move the field beyond descriptive gene datasets and toward an integrative understanding of how endocrine, immune and metabolic networks co-evolved to coordinate molting across diverse ecdysozoans.

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Figure 34:

Embryonic scheme stage 9: File:202211 Common house spider prosomal limb buds embryo. https://commons.wikimedia.org/wiki/File:202211_Common_house_ spider_ prosomal_limb_buds_embryo.svg. Last: 06.06.2025 at 02:12 pm.

Embryonic scheme stage11: File:202211 Common house spider brain defferrentiation embryo. https://commons.wikimedia.org/wiki/File:202211_Common_house_spider_ brain_defferrentiation_embryo.svg. Last: 06.06.2025 at 02:10 pm.

Embryonic scheme stage 14: File:202211 Common house spider ventral closure embryo. https://commons.wikimedia.org/wiki/File:202211_Common_house_spider_ventral_clo sure_embryo.svg#filee. Last: 06.06.2025 at 02:08 pm.

Figure 35:

Spider scheme internal anatomy: original diagram from *The Spider Book* (1912, 1920) by John Henry Comstock with additional anatomical information from *Biology of Spiders* (1996) by Rainer F. Foelix. License: https://creativecommons.org/licenses /by/3.0/. Source: https://upload.wikimedia.org/wikipedia/commons/2/22/Spider_ internal_anatomy-en.svg. Last visited: 11.06.2025 at 01:32 pm.