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Characterization of clock-related proteins and neuropeptides in *Drosophila littoralis* and their putative role in diapause

Giulia Manoli[1] | Meet Zandawala[1] | Taishi Yoshii[2] | Charlotte Helfrich-Förster[1]

[1]Neurobiology and Genetics, Biocenter, University of Würzburg, Würzburg, Germany

[2]Graduate School of Natural Science and Technology, Okayama University, Okayama, Japan

Correspondence
Charlotte Helfrich-Förster, Neurobiology and Genetics, Biocenter, University of Würzburg, Theodor-Boveri-Weg, 97074 Würzburg, Germany.
Email: charlotte.foerster@biozentrum.uni-wuerzburg.de

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Abstract

Insects from high latitudes spend the winter in a state of overwintering diapause, which is characterized by arrested reproduction, reduced food intake and metabolism, and increased life span. The main trigger to enter diapause is the decreasing day length in summer–autumn. It is thus assumed that the circadian clock acts as an internal sensor for measuring photoperiod and orchestrates appropriate seasonal changes in physiology and metabolism through various neurohormones. However, little is known about the neuronal organization of the circadian clock network and the neurosecretory system that controls diapause in high-latitude insects. We addressed this here by mapping the expression of clock proteins and neuropeptides/neurohormones in the high-latitude fly *Drosophila littoralis*. We found that the principal organization of both systems is similar to that in *Drosophila melanogaster*, but with some striking differences in neuropeptide expression levels and patterns. The small ventrolateral clock neurons that express pigment-dispersing factor (PDF) and short neuropeptide F (sNPF) and are most important for robust circadian rhythmicity in *D. melanogaster* virtually lack PDF and sNPF expression in *D. littoralis*. In contrast, dorsolateral clock neurons that express ion transport peptide in *D. melanogaster* additionally express allatostatin-C and appear suited to transfer day-length information to the neurosecretory system of *D. littoralis*. The lateral neurosecretory cells of *D. littoralis* contain more neuropeptides than *D. melanogaster*. Among them, the cells that coexpress corazonin, PDF, and diuretic hormone 44 appear most suited to control diapause. Our work sets the stage to investigate the roles of these diverse neuropeptides in regulating insect diapause.

KEYWORDS
circadian clock, corazonin, diapause, dormancy, fly, immunohistochemistry, neuroendocrine system, pigment-dispersing factor, seasonal timing

1 | INTRODUCTION

Animals living at high latitudes are exposed to prominent seasonal changes to which they need to adapt to survive. These annual changes are especially harsh in latitudes close to arctic regions, where there are extreme differences in light and temperature conditions throughout the year. Amid the different *Drosophilidae*, members of the *virilis* group (Figure 1a) are some of the best adapted species to such conditions. Some species from this group enter adult diapause that is characterized by reproductive arrest, reduced metabolism, increased

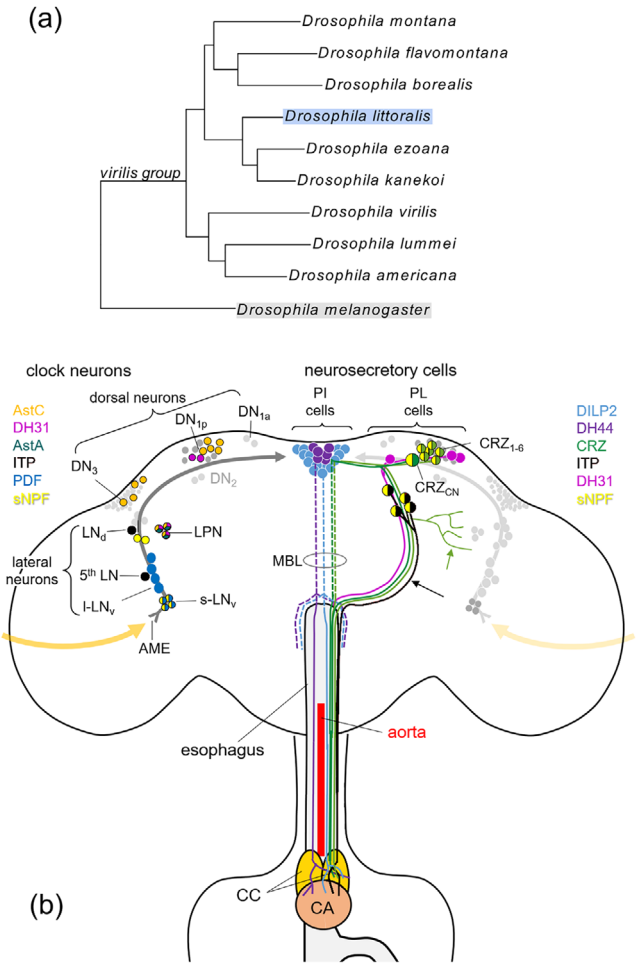

FIGURE 1 Phylogeny of the *virilis* group, and neuropeptide expression in clock- and diapause-related cells in *D. melanogaster* brain. (a) Simplified phylogenetic tree demonstrating the relationship of the *virilis* group to *D. melanogaster* after Yusuf et al. (2022). (b) Expression of selected neuropeptides in the brain of *D. melanogaster*. Neuropeptides of the clock neurons are shown on the left and neuropeptides of the neurohormonal center (PI—pars intercerebralis; PL—pars lateralis) in the right brain hemisphere. As schematically indicated, most clock neurons get light input from the eyes via the accessory medulla (AME, curved yellow arrow) and project to the dorsal protocerebrum, where some are connected to the PL and PI (clock neurons: s-LN_v—small ventrolateral neurons; l-LN_v—large ventrolateral neurons; 5th LN—fifth lateral neuron; LN_d—dorsolateral neurons; LPN—lateral posterior neurons; DN_1, DN_2, DN_3—dorsal neurons). The neurosecretory PI and PL cells project to the corpora cardiaca/allata (CC/CA) complex. The brain is shown from a posterior view and all fibers running in the median bundle (MBL) of the anterior brain are stippled. The *Drosophila* insulin-like peptide 2 (DILP2)- and diuretic hormone 44 (DH44)-expressing cells of the PI run via the MBL to the CC and CA, while the corazonin (CRZ)-expressing cells in the PL innervate only the CC and do so via the MBL and a lateral tract (black arrow). Among the CRZ cells, one large CRZ_{CN} cell and six smaller CRZ_{1-6} cells can be distinguished. The CRZ_{1-6} cells show additional arborizations in the dorsolateral protocerebrum (light green arrow), while the large CRZ_{CN} cell runs only to the CC. The ion transport peptide (ITP) cells in the PL join the lateral tract of the CRZ neurons and like the DILP2 neurons, they innervate the CC and CA. All CRZ and ITP neurosecretory cells additionally express small neuropeptide F (sNPF). The diuretic hormone 31 (DH31) cells are also joining this tract but innervate the CA only.

(Continues)

stress resistance, and prolonged life span (Hahn & Denlinger, 2011). In most of the *virilis* group species, day length (also called photoperiod) is the main trigger of diapause, while temperature is less important (Aspi et al., 1993; Lankinen, 1986b; Lumme & Keränen, 1978; Vaze & Helfrich-Förster, 2016; Vesala & Hoikkala, 2011). Therefore, their diapause is also defined as photoperiodic diapause. As such, some species from this group enter adult diapause in late August, when day length is reduced and can maintain this state until May of the following year in natural conditions (Lumme et al., 1974). In comparison, flies living at lower latitudes, such as *Drosophila melanogaster*, enter a state of reproductive dormancy in response to a combination of adverse conditions such as low temperatures combined with food shortage and short photoperiods (Kubrak et al., 2014; Nagy et al., 2018, 2019; Ojima et al., 2018; Saunders et al., 1989). Further, dormancy in these species is not strongly dependent on day length and can be terminated as soon as the environmental conditions improve (Koštál, 2006; Saunders et al., 1989).

The timing of photoperiodic diapause of the *virilis* group requires an internal timing system. As hypothesized by Bünning (1936), the circadian clock is well suited for this role. Indeed, the importance of the circadian clock for photoperiodic responses has been demonstrated for several insects living at high latitudes (reviewed in Denlinger, 2022; Goto, 2022; Saunders, 2020), but such evidence is so far lacking for *Drosophilids*. One reason for this lack of knowledge is the fact that most *virilis* group species possess weak circadian clocks that are unable to maintain rhythmicity under constant darkness (Bahn et al., 2009; Beauchamp et al., 2018; Bertolini et al., 2019; Kauranen et al., 2012; Lankinen, 1986a, 1986b; Menegazzi et al., 2017). In all *Drosophila* species studied so far, the circadian clock in the brain consists of lateral and dorsal clock neurons (LNs and DNs; Figure 1b). In both LNs and DNs, the molecular clock keeps time via at least two feedback loops, which are best described in *D. melanogaster* (reviewed in Helfrich-Förster et al., 2017). In the first loop, the clock proteins PERIOD (PER) and TIMELESS (TIM) block their own transcription by inhibiting their transcriptional activators CLOCK (CLK) and CYCLE (CYC), while in the second loop the clock proteins VRILLE (VRI) and the PAR Domain Protein 1ε (PDP1ε) lead to a rhythmic transcription of the *clock* gene. Another important component of the clock is the light-sensitive CRYPTOCHROME (CRY), which is expressed in about half of the LNs and DNs of all *Drosophila* species investigated so far (Hermann et al., 2013). CRY has an important role in transmitting light information directly to the molecular clock network, and in addition to that, most of the clock neurons get light input from the eyes via the accessory medulla (Figure 1b; Helfrich-Förster, 2020; Li et al., 2018). The clock neurons predominantly use neuropeptides for neural communication (Table 1) and once more, their expression is best described in *D. melanogaster* (Figure 1b; Ma et al., 2021; Reinhard, Bertolini, et al., 2022; Reinhard, Schubert, et al., 2022). Among the neuropeptides expressed

TABLE 1 Selected neuropeptides in circadian clock neurons.

Neuropeptide	Expression in clock neurons	Function of neuropeptide/clock neuron	References
Pigment-dispersing factor (PDF)	4 s-LN_vs 4 l-LN_vs	Rhythmic control of morning activity; activation of insulin-like-peptide-positive cells in the pars intercerebralis (PI) and inhibition of dormancy	Helfrich-Förster et al., 2000; Nagy et al., 2019; Renn et al., 1999
Short neuropeptide F (sNPF)	4 s-LN_vs	Rhythmic control of eclosion; sleep promotion; activation of insulin-like-peptide-positive cells in the PI and inhibition of dormancy	Nagy et al., 2019; Selcho et al., 2017; Shang et al., 2013
Ion transport peptide (ITP)	1 LN_d 5th LN	Rhythmic control of evening activity; sleep promotion	Hermann-Luibl et al., 2014
Allatostatin C (AstC)	~15 DN_3s ~4 DN_{1p}s 3 LPNs	Adaptation of evening activity to short and long photoperiods; rhythmic control and stimulation of oogenesis; sleep control; temperature-sensing; inhibition of dormancy at high environmental temperature	Diaz et al., 2019; Meiselman et al., 2022; Reinhard, Bertolini, et al., 2022; Reinhard, Schubert, et al., 2022; Zhang et al., 2021
Diuretic hormone 31 (DH31)	~7 DN_{1p}s 3 LPNs	Temperature sensing and preference; sleep control; involved in the free running rhythm	Goda et al., 2016, 2019; Reinhard, Bertolini, et al., 2022; Reinhard, Schubert, et al., 2022

Abbreviations: 5th LN, fifth lateral neuron; DN_{1p}s, posterior dorsal neurons; DN_3s, dorsal neurons; l-LN_vs, large ventrolateral neurons; LN_d, dorsolateral neurons; LPNs, lateral posterior neurons; s-LN_vs, small ventrolateral neurons.

in the clock network, the pigment-dispersing factor (PDF) from the small ventrolateral neurons (s-LN_vs), a group of lateral clock neurons, plays an important role in maintaining robust rhythmicity under constant conditions in *D. melanogaster* (reviewed in Helfrich-Förster, 2017; Shafer & Yao, 2014; Top & Young, 2018). Interestingly, PDF appears to be absent from the s-LN_vs of the *virilis* group species, which could explain their weak rhythmicity under constant conditions (Bahn et al., 2009; Beauchamp et al., 2018; Helfrich-Förster et al., 2020; Kauranen et al., 2012; Menegazzi et al., 2017). In contrast to *D. melanogaster*, the expression of other neuropeptides in the clock neurons, such as ion transport peptide (ITP), short neuropeptide F (sNPF), allatostatin-A, allatostatin-C (AstC), and diuretic hormone 31 (DH31) (Figure 1b), has not yet been investigated in any species of the *virilis* group.

In all *Drosophila* species studied, the CRY-expressing circadian clock neurons project to the dorsal protocerebrum (Hermann et al., 2013), where they may signal to the neuroendocrine system in the pars intercerebralis (PI) and pars lateralis (PL) (indicated as a single curved gray arrow in Figure 1b). The PI and PL of *D. melanogaster* comprise several neurosecretory cells, which produce multiple neuropeptides (Figure 1c; Table 2) and regulate various aspects of dormancy/diapause (Nässel & Zandawala, 2020). The PL consists of three sets of neurosecretory cells that produce (1) DH31, (2) ITP and sNPF, or (3) corazonin (CRZ) and sNPF. The PI, on the other hand, contains several sets of neurosecretory cells, including those producing *Drosophila* insulin-like peptides (DILPs) or diuretic hormone 44 (DH44) (Figure 1b). These neurosecretory cells project to the corpora cardiaca (CC), corpora allata (CA), or the anterior gut and release neuropeptides into the hemolymph (Figure 1b; Table 2). The secreted neuropeptides regulate different aspects of diapause/dormancy such as prolongation of life span, feeding, fecundity, egg production/laying, metabolism, osmotic homeostasis, and stress resistance (Table 2). Recent work has also identified neural pathways linking the circadian clock (Figure 1b) to the neuroendocrine system during dormancy. For instance, PDF and sNPF from the s-LN_vs signal to the DILP-positive neurons and inhibit the dormancy response to short days (Nagy et al., 2019). Further, AstC from some dorsal neurons (DN_3s) (Figure 1b) inhibits the dormancy response to low temperatures via still unknown cholinergic neurons (Meiselman et al., 2022). Lastly, multiple clock neurons provide inputs to DH31-positive neurosecretory cells, which in turn regulate reproductive dormancy by modulating juvenile hormone (JH) biosynthesis in the CA (Kurogi et al., 2023). Hence, most of our current knowledge on the link between the circadian clock and photoperiodic responses stems from work done in *D. melanogaster* despite them exhibiting a shallow diapause that is highly variable and often does not involve the entire population (Saunders et al., 1989). Therefore, it is imperative to study this phenomenon in species, such as those from the *virilis* group, that exhibit more pronounced overwintering responses.

To close this gap, here we characterized the circadian clock network and part of the neurohormonal system that might control diapause in the *virilis* group species *Drosophila littoralis*. *Drosophila littoralis* is a well-investigated model for diapause timing and photoperiodic responses (Lankinen, 1986a, 1986b; Lankinen & Forsman, 2006; Lumme & Oikarinen, 1977; Lumme et al., 1974; Salminen at al. et al., 2015). We mapped the distribution of various neuropeptides in *D. littoralis* that were shown to be expressed in either the clock neurons or neurosecretory cells of *D. melanogaster*. Among these peptides, we laid a special focus on CRZ, because it belongs to the superfamily of gonadotropin-releasing hormones that control reproductive maturation in vertebrates (Andreatta et al., 2020; Zandawala et al., 2018) and because CRZ was shown to be involved in diapause and reproduction control of different insects (Gospocic et al., 2017; Shiga et al., 2003; Tsuchiya et al., 2021). Our results reveal that the overall anatomy of the circadian clock network and the neurohormonal center in the PI/PL is well conserved between *D. melanogaster* and *D. littoralis*.

TABLE 2 Selected neuropeptides in neurosecretory cells of the pars intercerebralis (PI) and pars lateralis (PL).

Neuropeptide	Expression in PI/PL (per hemisphere)	Function of neuropeptide/ neurosecretory cell	References
Drosophila insulin-like peptide 2 (DILP2), *Drosophila* Insulin-like peptide 3 (DILP3)	7 PI cells	Control of growth, metabolism, reproduction, stress responses, and life span; prevention of dormancy/diapause	Nässel & Vanden Broeck, 2016; Nässel & Zandawala, 2019; Ohhara et al., 2018;
Diuretic Hormone 44 (DH44)	3 PI cells	Nutrient sensing; stimulating feeding and food storage in the crop, involved in circadian rhythmicity	Cavanaugh et al., 2014; Dus et al., 2015; Ohhara et al., 2018
Corazonin (CRZ)	7 PL cells (one large CRZ_{CN} neuron and six smaller neurons; all of them coexpress sNPF)	Controls reproductive maturation in invertebrates; is involved in diapause control of different insects; suppresses egg-laying in the ant species *Harpegnathos saltator* and *D. melanogaster*; is involved in energy and osmotic homeostasis, life span, stress resistance, and coordinates aspects of fecundity in *D. melanogaster*	Bergland et al., 2012; Choi et al., 2005; Gospocic et al., 2017; Kapan et al., 2012; Kubrak et al., 2016; Nässel et al., 2013; Shiga et al., 2003; Tsuchiya et al., 2021; Zandawala et al., 2021
Ion transport peptide (ITP)	4 PL cells	Prevents the fly from water loss by promoting thirst and repressing excretion; suppresses feeding and regulates water and energy balance, which is most important during desiccating winter conditions during which the flies are in dormancy	Dircksen, 2009; Gáliková et al., 2018
Diuretic hormone 31 (DH31)	3 PL cells	Works antagonistically to DILP2 by repressing juvenile hormone biosynthesis; inhibits vitellogenesis leading to reproductive dormancy	Kurogi et al., 2022

However, there are clear differences in neuropeptide expression between corresponding neurons in these two species, which could explain the differences in their dormancy response.

2 | MATERIALS AND METHODS

2.1 | Fly strains, husbandry, and entrainment

The *D. littoralis* stock was collected in Kilpisjärvi, Finland (69°N) (Lankinen, 1986a). The flies were fed on cornmeal/agar medium supplemented with yeast. For keeping the flies in the reproductive state, *D. littoralis* flies were reared at 23°C under light–dark (LD) cycles of 20-h light and 4-h darkness (LD 20:04). Wild-type *D. melanogaster* flies (Canton-S) were reared at 25°C under LD 12:12. Most staining experiments were carried out on reproductive flies. The flies were usually collected 1 h after lights on (ZT1). In the case of CRY immunostaining, the flies were kept in constant darkness (DD) after an initial entrainment to LD cycles for at least 3 days and collected at circadian time 1 (CT1) on the third day of DD.

2.2 | Diapause induction

To induce diapause in *D. littoralis*, the flies were transferred to 10°C and LD 08:16. For comparing PDF and CRZ staining intensity in reproductive and diapausing flies, a group of 60 female virgin flies was collected and entrained either to diapause-inducing conditions (LD 08:16, 10°C) or reproductive conditions (LD 20:04, 23°C). To assess only the effect of the photoperiod, we exposed another group of flies to diapausing photoperiods (LD 08:16) or reproductive photoperiods (LD 20:04) at the same temperature of 16°C. After 3 weeks of entrainment, flies were collected at ZT1 to evaluate the diapausing state. Each fly was singly dissected in phosphate-buffered saline (PBS) to verify the status of the ovaries and fat bodies. The flies were considered as diapausing when no oocyte at stage 14 was present (Kurogi et al., 2023). This state can also be defined as stage II ovaries following the description of Lankinen et al. (2022). The brains of the reproductive and diapausing flies were used for fluorescent immunocytochemistry and quantitative polymerase chain reaction (qPCR) analysis as described in Sections 2.5 and 2.7, respectively.

2.3 | In silico identification of neuropeptide and clock genes

We identified the neuropeptide and clock genes of interest in the *D. littoralis* genome (Kim et al., 2021) using the following procedure. First, we obtained the amino acid sequences of these proteins in *D. melanogaster* using FlyBase (v FB2022_05; Gramates et al., 2022) and used them to perform protein BLAST (Altschul et al., 1990) against the *D. virilis* reference sequence protein database. This step was necessary since *D. littoralis* protein sequences have not yet been predicted. Next, we used *D. virilis* protein sequences as a query to mine the *D. littoralis* whole- genome shotgun database via tBLASTn (Kim et al., 2021). This search was more accurate than using *D. melanogaster* sequences as a query since *D. littoralis* is more closely related to *D. virilis* than *D. melanogaster*. To determine the intron–exon boundaries in the genomic hits, we used the Splice Site Prediction tool (from Berkeley *Drosophila* Genome Project [https://www.fruitfly.org/seq_tools/splice.html],

NNSPLICE v0.9). The exons were assembled in silico to generate putative coding sequences, which were then translated to proteins using the Translate tool (from Expasy [https://web.expasy.org/translate/]). Finally, *D. littoralis*-predicted protein sequences were aligned to *D. virilis* and *D. melanogaster* sequences using the multiple sequence alignment tool Clustalomega (from EMBL-EBI [https://www.ebi.ac.uk/Tools/msa/clustalo/]) and the identity percentage was assessed using the Snapgene software (v5.2.2 [www.snapgene.com]).

NCBI Reference Sequences used in this study are as follows:

PER: *D. melanogaster* (NP_525056.2), *D. virilis* (XP_002056806.1), *D. littoralis* contig (JAEIGF010000591.1);
CRY: *D. melanogaster* (AAF55649.1), *D. virilis* (XP_002053627.1), *D. littoralis* contig (JAEIGF010000592.1);
AstC: *D. melanogaster* (NP_523542.1), *D. virilis* (XP_002051772.1), *D. littoralis* contig (JAEIGF010000612.1);
CRZ: *D. melanogaster* (NP_524350.1), *D. virilis* (XP_002058438.1), *D. littoralis* contig (JAEIGF010000418.1);
DH31: D. melanogaster (NP_523514.1), D. virilis (XP_002057568.2), D. littoralis contig (JAEIGF010000612.1);
DH44: *D. melanogaster* (NP_001097725.2), *D. virilis* (XP_002056345.1), *D. littoralis* contig (JAEIGF010000592.1);
DILP2: *D. melanogaster* (NP_524012.1), *D. virilis* (XP_002047065.1), *D. littoralis* contig (JAEIGF010000408.1);
DILP3: *D. melanogaster* (NP_648360.2), *D. virilis* (XP_015031225.1), *D. littoralis* contig (JAEIGF010000408.1);
KR-H1: *D. melanogaster* (NP_477467), *D. virilis* (XP_002051467.1), *D. littoralis* contig (JAEIGF010000057.1);
ITP: *D. melanogaster* (NP_001163293.1), *D. virilis* (XP_015029224.1), *D. littoralis* contig (JAEIGF010000297.1);
PDF: *D. melanogaster* (NP_524517.1), *D. virilis* (XP_015027941.2), *D. littoralis* contig (JAEIGF010000593.1);
sNPF: *D. melanogaster* (NP_724239.1), *D. virilis* (XP_002057815.1), *D. littoralis* contig (JAEIGF010000059.1);
αTUB84B: *D. melanogaster* (NP_476772.1), *D. virilis* (XP_002054644.1), *D. littoralis* contig (JAEIGF010000418.1).

2.4 | Primary antibodies

All primary antibodies, their source, and the used dilutions are listed in Table 3. Except for the newly generated ITP-antibody (see Section 2.5), all primary antibodies are well characterized and have been extensively used in different insects including *D. melanogaster* and other fly species. The amino acid sequences they are directed against are indicated in Table 3.

To investigate the degree to which the amino acid sequences of the neuropeptides AstC, CRZ, DH31, DH44, PDF, ITP, sNPF, and DILPs and the clock proteins PER and CRY1 are conserved in *D. littoralis*, we identified them in silico as described in Section 2.2 and compared the sequences with those of *D. melanogaster* and *D. virilis*. As shown in Figure 3, the sequences of most neuropeptides turned out to be highly conserved if not identical.

To assess the specificity of the antibodies that we used, we searched the genome of *D. littoralis* using tblastn to find sequences other than the addressed neuropeptides that could be recognized by the antibodies used. We blasted both the antigen and whole-neuropeptide sequences using algorithm parameters of low stringency: a word size of two and an expected threshold of one. Even with these parameters, we were not able to find any further hits in the genome. Only the neuropeptide sequences against which the antibodies had been generated were identified. This suggests that our antibodies are specific.

Regarding the specificity of the anti-PDH antibody, a recent study by Veenstra (2021) showed that this antibody cross-reacts with calcitonin-A in *Locusta migratoria*. To rule out this possibility in *D. littoralis*, we searched the *D. littoralis* genome for calcitonin-A- and calcitonin-B-like sequences. Our genome searches failed to identify calcitonin-like sequences in *D. littoralis*. This suggests that our anti-PDF immunostaining represents authentic PDF expression.

2.5 | Generation and characterization of an antibody against ITP

The antibody against ITP was generated in guinea pig by Scrum Inc. The part of the C-terminal of ITP, EMDKYNEWRDTL, was chemically synthesized and the N-terminal was coupled to keyhole limpet hemocyanin. Three guinea pigs were immunized using a conventional method. The specificity of the three different sera was tested on brains of *D. melanogaster* by immunocytochemistry as described in Section 2.6. All immunostaining with the three different sera showed exactly the same staining pattern as reported in previous studies (Hermann-Luibl et al., 2014). Finally, we selected the serum with the lowest background signals for further analysis. We tested the specificity of the antibody by costaining the brain with anti-ITP_{rabbit} (Table 3), both in *D. melanogaster* and in *D. littoralis*. The newly generated antibody reliably stained the ITP-positive neurosecretory cells and the dorsolateral neuron (LN_d) and fifth lateral neuron (5th LN) in both species (Figure 2a,b).

2.6 | Fluorescent immunocytochemistry

Immunocytochemistry was performed on young female and male flies according to the protocol of Schubert et al. (2018). All images are based on brains from female flies, although we found no differences between the two sexes. If not otherwise stated, flies were collected 3–10 days posteclosion, fixed for 3.5 h (3 h for *D. melanogaster*) in 4% paraformaldehyde dissolved in PBS containing 0.5% Triton ×100 (PBST), and dissected after rinsing three times for 10 min each in PBS. Brains were blocked in 5% normal goat serum in PBST (normal donkey serum in case of anti-PER-goat) overnight at 4°C. The brains were then incubated in the primary antibody mix for 2 days at 4°C and an additional day at 20°C. After washing them in PBST, the brains were incubated in the secondary antibody mix (Table 4) overnight at 4°C and subsequently washed in PBST. Finally, brains were embedded in Vectashield (Vector Laboratories) on glass slides with spacers. Samples

TABLE 3 Primary antibodies.

Antibody	Antigen	Source	Dilution	Host species	Reference
Anti-PER (dN-19)	*Drosophila melanogaster* N-terminus of PER	Santa Cruz Biotechnology	1:200	Goat (polyclonal)	Cat# sc-15720; Shiga & Numata, 2009; RRID: AB_654018
Anti-CRY	*Drosophila melanogaster* His-tagged form of full-length CRY	Todo T. Osaka University	1:1000	Rabbit (polyclonal)	Yoshii et al., 2008; RRID: AB_2314242
Anti-PDP1	*Drosophila melanogaster* PDP1-α	Blau J. New York University	1:1000	Rabbit (polyclonal)	Reddy et al., 2000; RRID: AB_2569283
Anti-AstC	*Manduca sexta* AstC (VRFRQCYFNPISCF-O)	Veenstra J. Université de Bordeaux	1:250	Rabbit (polyclonal)	Veenstra et al., 2008; RRID: AB_2753141
Anti-CRZ	*Periplaneta americana* full-length CRZ (pQTFQYSRGWT-NH_2)	Veenstra J. Université de Bordeaux	1:1000	Rabbit (polyclonal)	Veenstra & Davis, 1993; RRID: AB_2532101
Anti-DH31	*Drosophila melanogaster* full-length DH31	Veenstra J. Université de Bordeaux	1:500	Rabbit (polyclonal)	Park et al., 2008; RRID: AB_2569126
Anti-DH44	*Drosophila melanogaster* full-length DH44	Veenstra J. Université de Bordeaux	1:1000	Rabbit (polyclonal)	Cabrero et al., 2002
Anti-ITP	*Drosophila melanogaster* C-terminus of ITP (EMDKYNEWRDTL-NH_2)	Yoshii T. Okayama University	1:1000	Guinea pig (polyclonal)	This study
Anti-ITP	*Drosophila melanogaster* C-terminus of ITP (CEMDKYNEWRDTL-NH_2)	Dircksen H. Stockholm University	1:5000	Rabbit (polyclonal)	Dircksen et al., 2008; RRID: AB_2567966
Anti-PDF C7	*Drosophila melanogaster* full-length PDF (NSELINSLLSLPKNMNDA-NH_2)	DSHB	1:1000	Mouse (monoclonal)	Cyran et al., 2005; RRID: AB_760350
Anti-PDF cricket	*Gryllus bimaculatus* full-length PDF (NSEIINSLLGLPKVLNDA-NH_2)	Tomioka K. Okayama University	1:2000	Rabbit (polyclonal)	Abdelsalam et al., 2008; RRID: AB_2916037
Anti-βPDH	*Uca pugilator* full-length βPDH (NSELINSILGLPKVMNDA-NH_2)	Dircksen H. Stockholm University	1:1000	Rabbit (polyclonal)	Dircksen et al., 1987; RRID: AB_2315091
Anti-sNPF	*Drosophila melanogaster* sNPF Precursor peptide (DPSLPQMRRTAYDDLLEREL)	Veenstra J. Université de Bordeaux	1:1000	Rabbit (polyclonal)	Johard et al., 2008; RRID: AB_2315341

TABLE 4 Secondary polyclonal antibodies (Thermo Fisher Scientific).

Antibody	Concentration	Host species	Reference
Alexa Fluor 488 (anti-goat)	1:400	Donkey	Cat# A-11055, RRID:AB_2534102
Alexa Fluor 555 (anti-goat)	1:400	Donkey	Cat# A-21432, RRID:AB_2535853
Alexa Fluor 555 (anti-mouse)	1:400	Donkey	Cat# A-31570, RRID:AB_2536180
Alexa Fluor 647 (anti-mouse)	1:400	Donkey	Cat# A-31571, RRID:AB_162542
Alexa Fluor 488 (anti-rabbit)	1:400	Donkey	Cat# A-21206, RRID:AB_2535792
Alexa Fluor 647 (anti-rabbit)	1:400	Donkey	Cat# A-31573, RRID:AB_2536183
Alexa Fluor 488 (anti-guinea pig)	1:400	Goat	Cat# A-11073, RRID:AB_2534117
Alexa Fluor 488 (anti-rabbit)	1:400	Goat)	Cat# A-11070, RRID:AB_2534114
Alexa Fluor 635 (anti-rabbit)	1:400	Goat	Cat# A-31577, RRID:AB_2536187
Alexa Fluor 635 (anti-mouse)	1:400	Goat	Cat# A-31575, RRID:AB_2536185

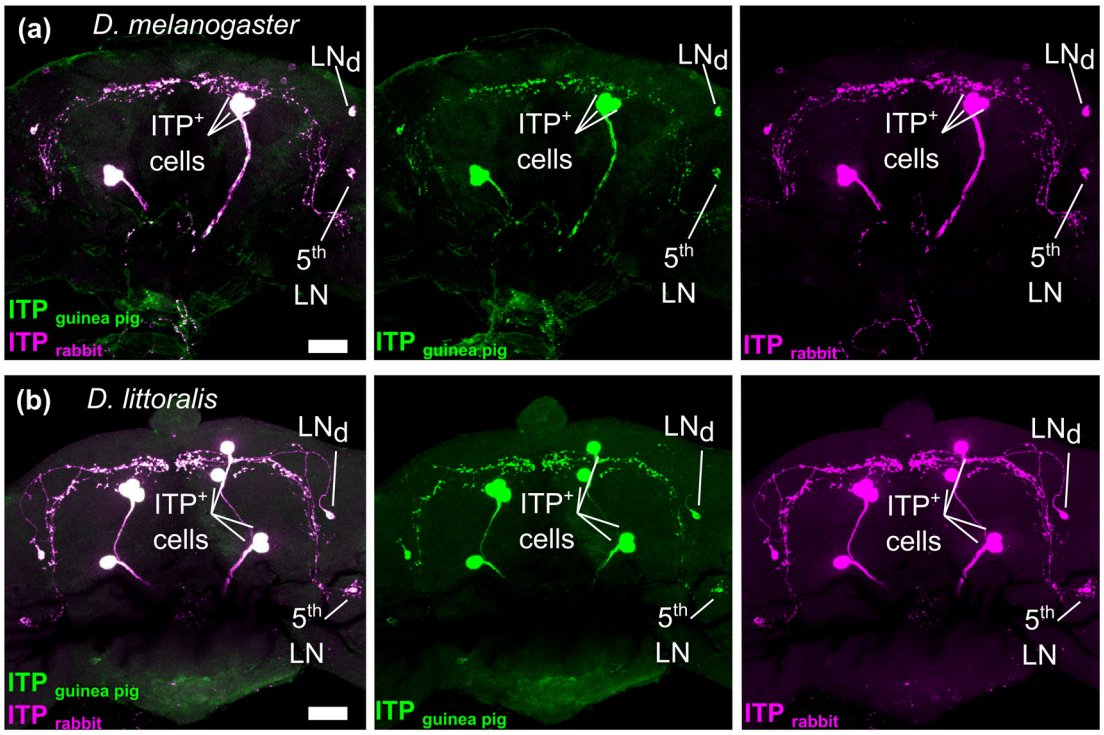

FIGURE 2 Validation of the newly generated anti-$ITP_{guinea\ pig}$ by colocalization with anti-ITP_{rabbit}. (a) *Z* stack of 45 confocal planes showing the colocalization of the two antibodies in the central brain of *D. melanogaster*. (b) *Z* stack of 57 confocal planes, showing the colocalization of the two antibodies in the central brain of *Drosophila littoralis*. The anti-$ITP_{guinea\ pig}$ generated in this study and the anti-ITP_{rabbit} label the two pairs of ITP-positive clock neurons (the dorsolateral neuron [LN_d] and fifth lateral neuron [5th LN]) and the four pairs of ITP-positive neurosecretory cells in the pars lateralis in both species. Scale bars: 50 μm in panels (a) and (b). ITP, ion transport peptide.

were scanned with a Leica SPE confocal microscope (Leica Microsystems) with a photomultiplier tube and solid-state lasers (488, 532, and 635 nm) for excitation. A 20-fold glycerol immersion objective (HC PL APO; Leica Microsystems) was used for the scans. The confocal stacks had a 2-μm *z*-step size and 1024 × 1024 pixels with a pixel size of 537 × 537 nm and a voxel size of 0.537 × 0.537 × 2 μm.

The images were processed using Fiji (v1.53c; Schindelin et al., 2012). Only changes in brightness and contrast were performed on the images. At least 10 brains were scanned for each staining performed.

2.7 | Quantification of immunostaining intensity

For CRZ and PDF immunostaining in the PL, staining intensity and size of the neurosecretory cell bodies were quantitatively compared between sister groups of *D. littoralis* female flies entrained to reproductive or diapausing conditions (as described in Section 2.2). The entrainment and immunostaining of the two groups were conducted simultaneously and confocal pictures were acquired maintaining the same laser intensity and general settings. The raw images were quantified in Fiji as follows: For each cell body, the focal plane showing the largest diameter was determined, and the cell body outlines were manually defined as region of interest (ROI) using the polygon selection tool. The cell area and its mean pixel intensity were measured. The mean pixel intensities were corrected by the mean background intensity that was measured in the closest nonstained area to the neurons using an ROI with a comparable size to the cell bodies.

2.8 | quantitative PCR

The relative transcript level of *Crz*, *Pdf*, *Kr-h1* (*Kruppel homolog 1*), and *Dilp2* in reproductive and diapausing *D. littoralis* females was assessed by qPCR. Each gene sequence was manually retrieved as described in Section 2.3. The used primers are listed in Table 5 and were designed with GENtle (v1.9.4 [http://gentle.magnusmanske.de/]). Primers were ordered from Merck (Merck KGaA). The total RNA of five fly heads for each biological replicate was extracted with the Quick-RNA MicroPrep Kit (Zymo Research). The heads were first smashed in the lysis buffer at ZT1 and subsequently frozen at −20°C until extraction. The RNA was then extracted following the manufacturer's instructions. After the extraction, the mRNA was immediately reverse-transcribed into cDNA using the QuantiTect Reverse Transcription Kit (Qiagen). The qPCR reactions were performed in the Rotor-Gene Q machine (Qiagen) using the SensiFAST SYBR No-Rox Kit (Bioline). Three biological replicates for each reproductive condition and two technical replicates for each sample were performed. The relative transcript level was calculated with the ΔΔCT equation using *tubulin 84 B* as the housekeeping gene.

FIGURE 3 Alignments and antibody recognition sequences of the predicted mature neuropeptides allatostatin-C (AstC), corazonin (CRZ), diuretic hormone 31 (DH31), diuretic hormone 44 (DH44), ion transport peptide (ITP), and pigment-dispersing factor (PDF) and the partial precursors of short neuropeptide F (sNPF), *Drosophila* insulin-like peptide 2 (DILP2), and DILP3 in *Drosophila melanogaster* (*mel*), *Drosophila virilis* (*vir*), and *Drosophila littoralis* (*lit*). The sequence conservation is shown as bar on top (black: completely conserved; shaded gray: well-conserved to

(Continues)

TABLE 5 Primers used in this study.

Gene	Sequence 5t'to 3t'	Primer Melting Temperature (Tm)
aTub84B F	CAACCAGATGGTCAAGTGCGATCC	72°C
aTub84 R	CACAACAGTGGGTGGCTGGTAGTT	70°C
Crz F	TGCTGCCCCTGTTCCTCTTCA	71°C
Crz R	CACAAAGTGCTGCAGTTGCAAC	68°C
Dilp2 F	GCGCGCTCTGTGACAAAGGATTCAATCGT	78°C
Dilp2 R	GGTGGCGGTCAGCGAGTTGAGGACATT	78°C
Kr-h1 F	TTTGGCTACAATCATGTGCTCAAGC	70°C
Kr-h1 R	GGATGAGTTGGAAACGCTGCTG	70°C
Pdf F	GCTACGTGGAAAAGGAGTATAATCGG	67°C
Pdf R	CGTGTCATGTTTTTGGGTAAGCTGAGC	70°C

FIGURE 3 (Continued)
little-conserved [the darker, the more conserved]; white: not conserved), and the consensus sequences are shown below in bold letters. Regions that correspond to the antigen sequences used to generate the antibodies are circled in green in the *D. melanogaster* sequences. Amino acids that do not match the *D. melanogaster* reference are highlighted in pink.

2.9 | Statistical analysis and figures

The statistical analysis and plot designing were performed with R (v4.0.3), using RStudio (v1.3.1093). The data were first tested for normal distribution with the Shapiro–Wilk test, and based on the result, a Wilcoxon rank-sum test was performed for the signal intensity (Figure 10b–f) and transcript analyses (Figure 10g). Plots were generated using the ggplot2 package (Wickham, 2016). The alignment pictures (Figure 3) was created using Snapgene software (v5.2.2 [www.snapgene.com]).

3 | RESULTS AND DISCUSSION

3.1 | Sequence similarities of clock proteins and neuropeptides between *D. melanogaster* and *D. littoralis*

To assess the sequence similarity between the *D. littoralis* and *D. melanogaster* clock proteins and neuropeptides, we performed an in silico analysis using the NCBI Reference Sequences indicated in Section 2. Subsequently, we compared the predicted *D. littoralis* neuropeptide and clock protein sequences with those of *D. virilis* and *D. melanogaster*. As expected from the phylogeny (Figure 1), the identity percentage was always highest between *D. littoralis* and *D. virilis* (Table 6; Figure 3).

Although PER is a key component of the molecular clock, it was found to be not well conserved between *D. melanogaster* and the *virilis* group. However, its N-terminal domain was highly conserved across species. Since this is the sequence against which the *D. melanogaster* PER antibody was generated, it allowed us to use this antibody in *D. littoralis*. In contrast, CRY was well-conserved across all species. This was also true for the majority of neuropeptides except for DILP2 and DILP3 (Table 6; Figure 3). Consequently, except for insulin-like peptides, all antibodies raised against *D. melanogaster* neuropeptides successfully labeled the relevant neuropeptidergic neurons in *D. littoralis* (see Figures 4–8).

TABLE 6 Percent identity of different proteins and mature neuropeptides.

Identity %		
Protein/mature neuropeptide	*Drosophila littoralis–Drosophila melanogaster*	*Drosophila littoralis–Drosophila virilis*
PER	**54%**	84%
CRY	82%	96%
AstC	100%	100%
CRZ	100%	100%
DH31	96%	93%
DH44	98%	98%
DILP2	**35%**	97%
DILP3	**48%**	96%
ITP	97%	100%
PDF	100%	100%
sNPF	100%	100%

Note: Identities lower than 70% are bold.
Abbreviations: AstC, allatostatin-C; CRZ, corazonin; DH31, diuretic hormone 31; DH44, diuretic hormone 44; DILP2, *Drosophila* insulin-like peptide 2; DILP3, *Drosophila* insulin-like peptide 3; ITP, ion transport peptide; PDF, pigment-dispersing factor; sNPF, short neuropeptide F.

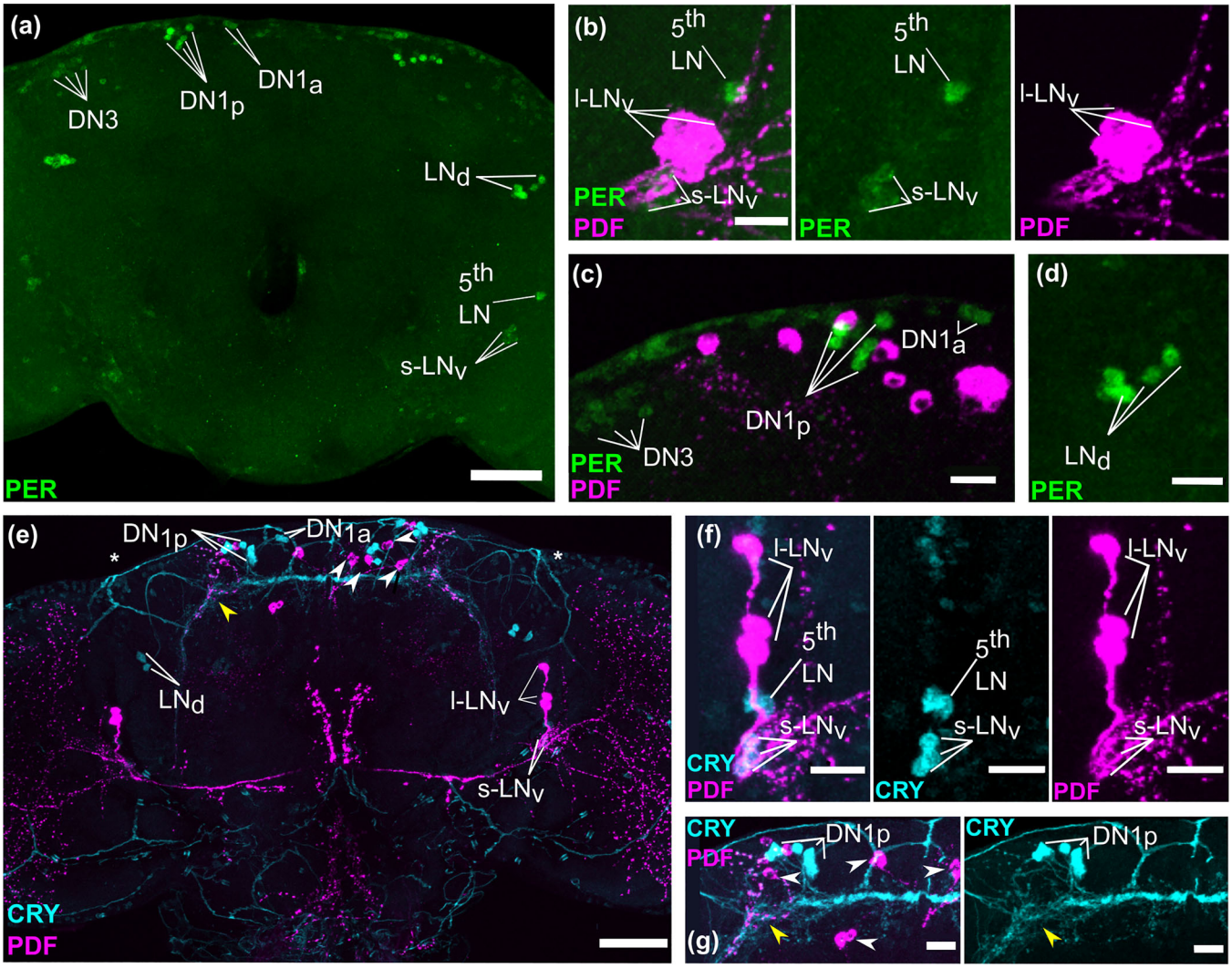

FIGURE 4 Clock neurons in the brain of *Drosophila littoralis* immunostained by anti-PER (green), anti-CRY (cyan), and anti-PDF C7 (magenta). (a) *Z* stack of 46 confocal planes. PER-like immunoreactive clock neurons in the central brain of a reproductive fly at ZT1 (1 h after lights on) under light–dark cycles of 20-h light and 4-h darkness. PER is present in every cluster except the DN_2 and the large ventrolateral neurons (l-LN_v). (b–d) Magnifications of panel (a). (b) *Z* stack of 12 confocal planes. PER and PDF staining of the fifth lateral neuron (5th LN), small ventrolateral neurons (s-LN_v), and l-LN_v (right brain hemisphere). PER is present in the 5th LN and the s-LN_v; however, it is not colocalizing with PDF in the l-LN_v. (c) *Z* stack of 14 confocal planes. PER immunostaining in the left dorsal brain is showing the DN_3, posterior dorsal neurons (DN_{1p}), and anterior dorsal neurons (DN_{1a}). PDF signal is present in the dorsal part of the brain but it is not colocalizing with PER. (d) *Z* stack of 13 confocal planes. PER immunostaining in the dorsolateral neuron (LN_d) (right brain hemisphere). (e) *Z* stack of 120 confocal planes. CRY and PDF immunostaining in the central brain. CRY is present in DN_1, LN_d, and s-LN_v, but not colocalizing with PDF in the dorsal brain (white arrowheads). A yellow arrowhead marks the s-LN_v projections to the dorsal brain. Additional signal outside the clock network (asterisks) is visible in this brain; due to the morphology of this structure, it is most likely trachea. (f, g) Magnifications of panel (e). (f) *Z* stack of 31 confocal planes. CRY is expressed in the 5th LN and s-LN_vs, but not in the l-LN_vs. (g) *Z* stack of 40 confocal planes. CRY is present in the DN_1s and the PDF fibers from s-LN_vs (yellow arrowhead) but not in the PDF-positive dorsal cells (white arrowhead). Scale bars: 50 μm in panels (a) and (e); 15 μm in panels (b–d), (f), and (g). PER, PERIOD; CRY, CRYPTOCHROME; PDF, pigment-dispersing factor; DN_1, DN_2, DN_3, dorsal neurons.

3.2 | PER and CRY expression in the clock neurons of *D. littoralis* show a rather similar pattern to *D. melanogaster*, but PDF expression differs

To date, the clock neurons of the *virilis* group have been identified with antibodies against PDP1 and CRY (Hermann et al., 2013) as the commonly used *Drosophila* PER antibody (Stanewsky et al., 1997) did not recognize the *virilis* group PER (Hermann et al., 2013). Here, we used a commercially available PER antibody directed against the well-conserved N-terminus of *D. melanogaster* PER (Table 3). This antibody labeled putative PER-expressing cells in *D. littoralis*, allowing us to detect the different clock neuron clusters (Figure 4). As previously reported by Hermann et al. (2013), the *virilis* group possesses most, if not all, of the clock neurons described in *D. melanogaster* (Figure 4a–d).

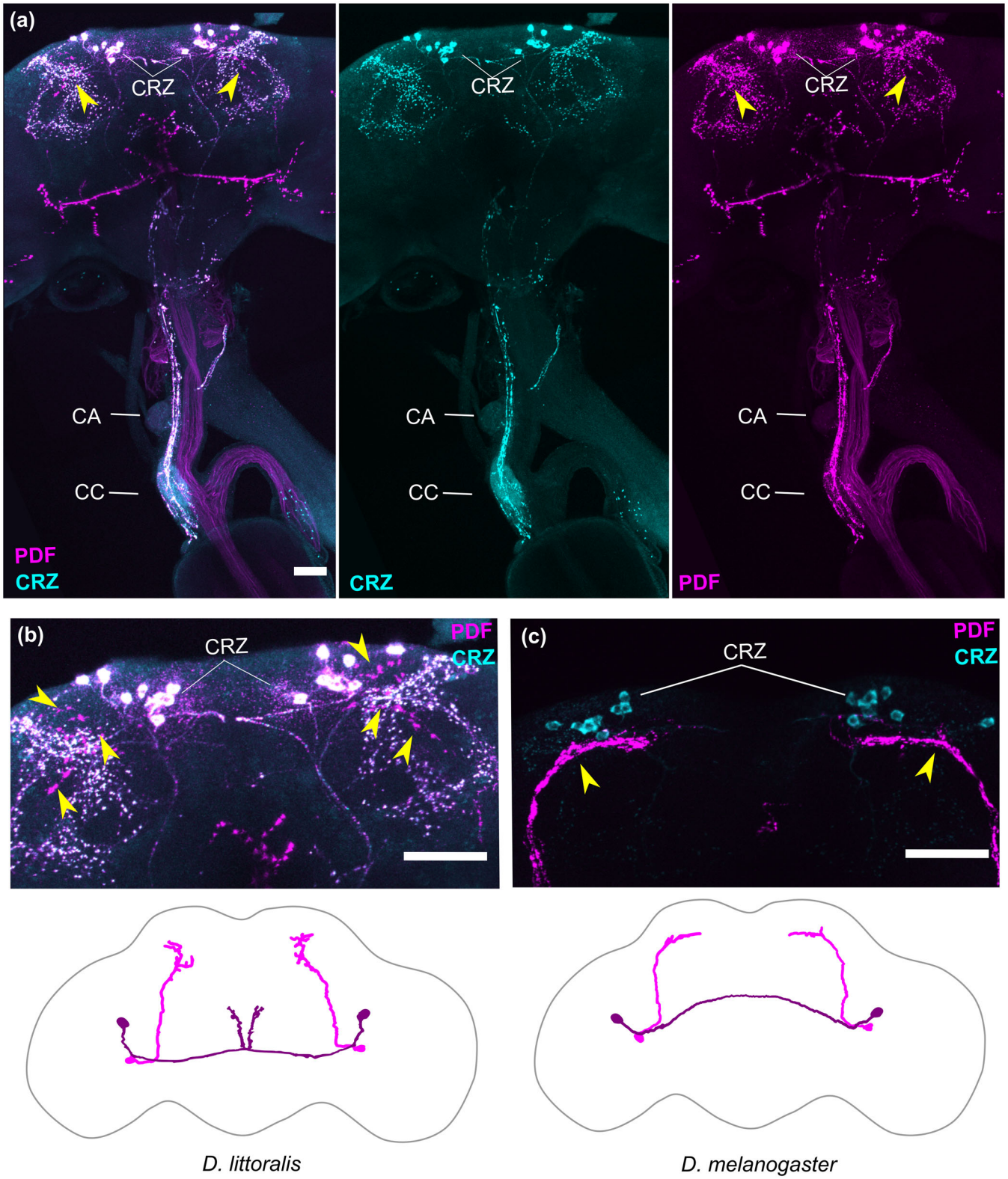

FIGURE 5 Pigment-dispersing factor (PDF) and corazonin (CRZ) staining in *Drosophila littoralis* and *Drosophila melanogaster*. (a) *Z* stack of 33 confocal planes. PDF is expressed in CRZ neurosecretory cells of *D. littoralis*. PDF and CRZ fibers are in the proximity of the corpus allatum (CA) and are innervating the corpora cardiacum (CC). Yellow arrowheads point to the small ventrolateral neuron (s-LN_v) terminals in the dorsal brain. (b) Upper panel: Magnification of panel (a); CRZ neurons are double-labeled by CRZ and PDF antibodies and therefore appear in white. The terminals from the s-LN_vs are only labeled by PDF and faintly stained in magenta (yellow arrowheads). Lower panel: reconstruction of PDF-positive fibers in *D. littoralis*. The s-LN_v projections are shown in light magenta, and those coming from the large ventrolateral neurons (l-LN_vs) in dark magenta. (c) Upper panel: *Z* stack of 30 confocal planes; dorsal brain of *D. melanogaster* double-labeled with CRZ and PDF

(Continues)

FIGURE 5 (Continued)
antibodies. The PDF-positive s-LN_v terminals in the dorsal brain are strongly stained (yellow arrowheads). Lower panel: reconstruction of PDF-positive fibers in *D. melanogaster*. Labeling as in panel (b). Scale bars: 150 μm in panel (a); 50 μm in panels (b) and (c).

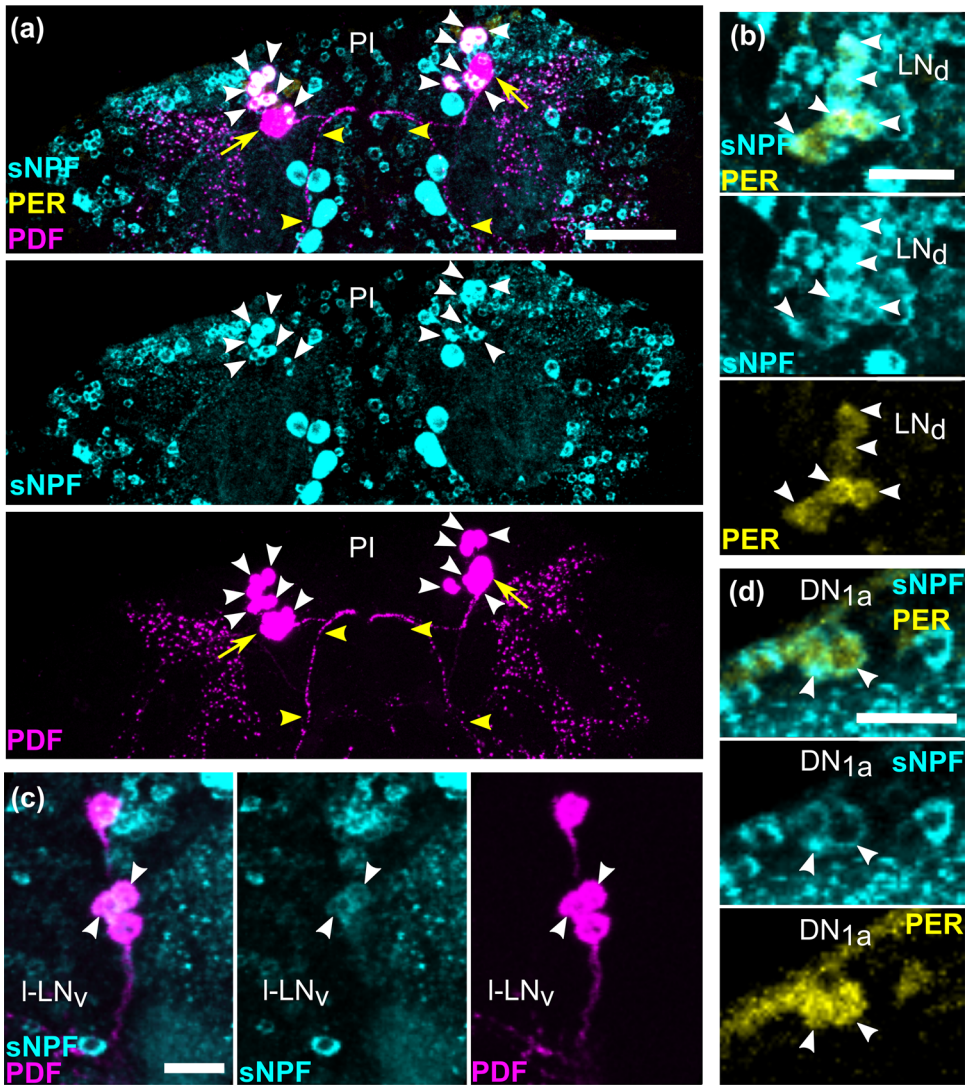

FIGURE 6 Pigment-dispersing factor (PDF) and short neuropeptide F (sNPF) immunostaining in the brain of *Drosophila littoralis*. (a) *Z* stack of 37 confocal planes. sNPF-like immunoreactivity is widely present in the dorsal brain. sNPF colocalizes with PDF in the six smaller CRZ_{1-6} neurons (white arrow heads) but not in the large CRZ_{CN} neuron (yellow arrow) and in the tract running to the pars intercerebralis (PI) and the corpora cardiaca/allata (CC/CA) complex (yellow arrowheads). (b) *Z* stack of 11 confocal planes. Colocalization of sNPF and PERIOD (PER) in most dorsolateral neuron (LN_d). (c) *Z* stack of 13 confocal planes. Faint sNPF staining in two large ventrolateral neurons (l-LN_v) (white arrowheads). (d) *Z* stack of seven confocal planes. Colocalization of PER and sNPF in the anterior dorsal neuron (DN_{1a}). Scale bars: 50 μm in panel (a); 15 μm in panels (b), (c), and (d).

Here, we identified the same clusters, and we could subdivide the dorsal neurons (DN_1s) into posterior and anterior ones, the DN_{1p}s and DN_{1a}s (Figure 4c). However, we could not detect PER in the lateral group of large ventrolateral neurons (l-LN_vs) (Figure 4b). The PER staining in the s-LN_vs was also quite weak compared to the other clock clusters (Figure 4a,b). Furthermore, we could not detect PER staining in the dorsal neurons 2 (DN_2s) (Figure 4a–d). Since all these clock neurons have been detected by the PDP1 antibody in other *virilis* species (Hermann et al., 2013), we assume that they are also present in *D. littoralis*. However, they might contain either no or very little PER, below the detection limit of the antibody. This could mean that the molecular clock in these clock neurons is very weak, which can affect circadian rhythms. In particular, the s-LN_vs are very important for maintaining circadian rhythmicity in constant darkness (Grima et al., 2004; Helfrich-Förster, 1998; Shafer & Taghert, 2009; Stoleru et al., 2004), and a weak molecular clock in them may lead to weak rhythmic

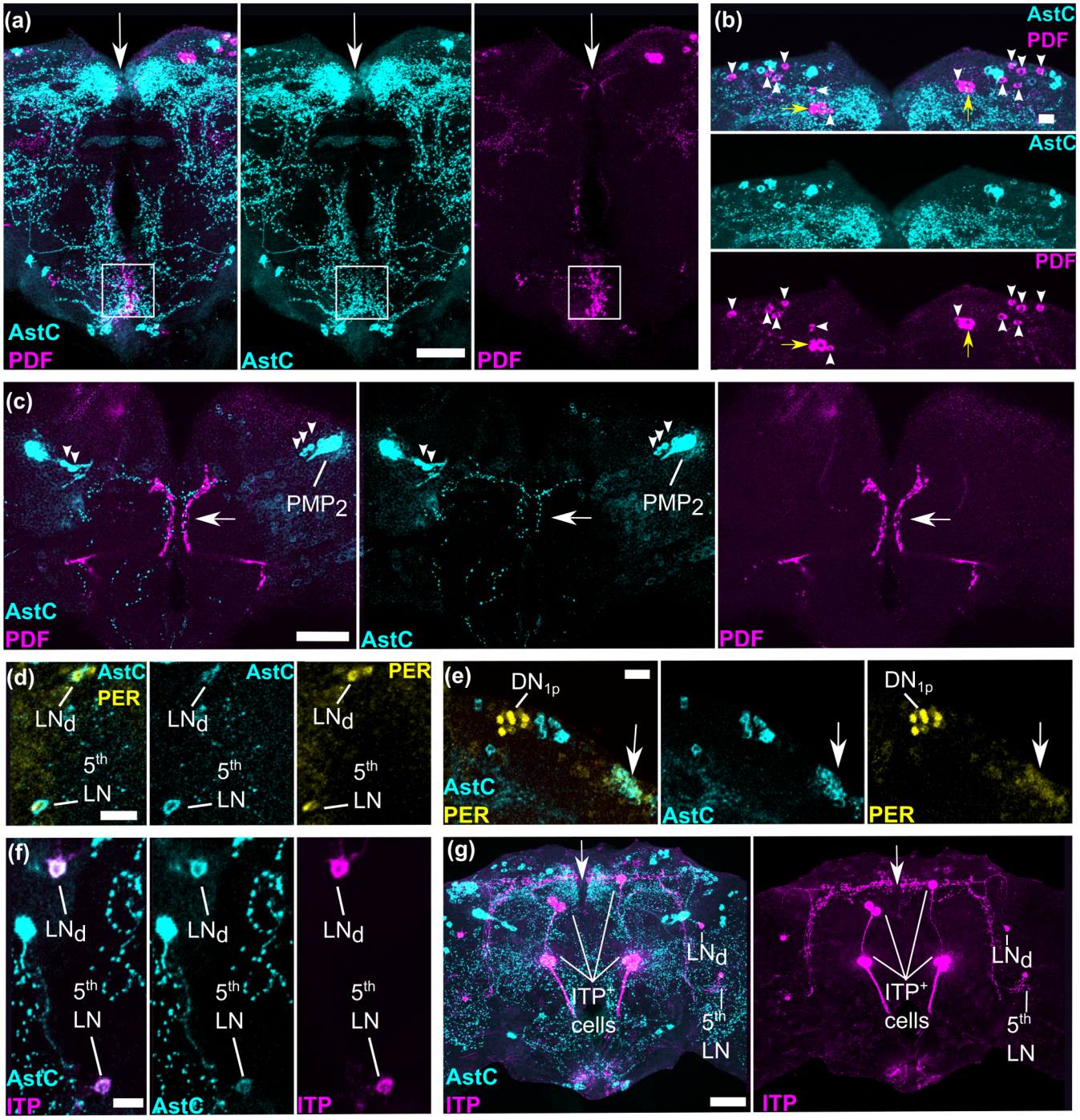

FIGURE 7 Allatostatin-C (AstC), PERIOD (PER), pigment-dispersing factor (PDF), and ion transport peptide (ITP) localization in *Drosophila littoralis* brain. (a) *Z* stack of 11 confocal planes. AstC projections to the PI (arrows). PDF and AstC terminals are in close proximity to each other in the SEZ (square). (b) *Z* stack of 18 confocal planes. The PDF/corazonin (CRZ) neurons in the pars lateralis (PL) do not express AstC (yellow arrow: large CRZ_{CN}; arrowheads: six smaller CRZ_{1-6}). (c) *Z* stack of 13 confocal planes. AstC-positive lateral posterior neuron (LPN) soma (white arrowheads) is in proximity to the large AstC-positive PMP_2 descending neuron (Diaz et al., 2019). PDF fibers stemming from the large ventrolateral neurons (l-LN_vs) are close to some AstC terminals (white arrow). (d) *Z* stack of 37 confocal planes. Colocalization of AstC and PER in one dorsolateral neuron (LN_d) and in the fifth lateral neuron (5th LN). (e) *Z* stack of 17 confocal planes. Colocalization of PER and AstC in the dorsal neurons (DN_3s) (white arrow) but not in the posterior dorsal neurons (DN_{1p}s). (f) *Z* stack of eight confocal planes. AstC and ITP colocalize in the 5th LN and in the one LN_d. (g) Z stack of 68 confocal planes. The ITP-positive clock neurons of *D. littoralis* are anatomically similar to those of *Drosophila melanogaster* and project to the PI (arrow). Additional ITP-positive non-clock neurons (ITP^+ cells) run toward the corpora cardiaca/allata (CC/CA) complex. Scale bars: 50 μm in panels (a), (c), and (g); 15 μm in panels (b) and (d–f).

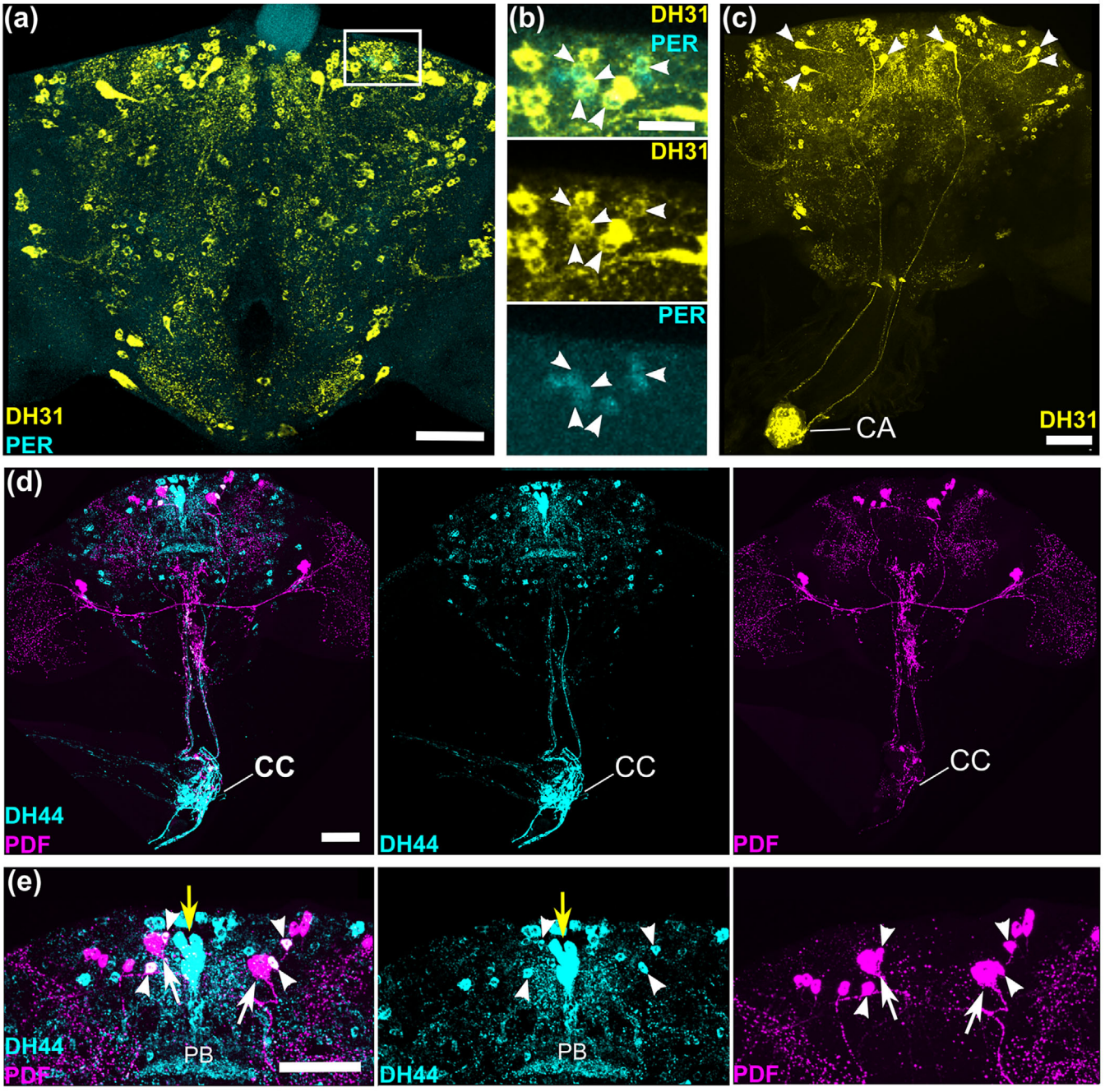

FIGURE 8 Diuretic hormone 31 (DH31) and diuretic hormone 44 (DH44) expression in *Drosophila littoralis*. (a) *Z* stack of 22 confocal planes. DH31 is widely expressed in the *D. littoralis* brain. (b) *Z* stack of six confocal planes. Inset from panel (a) shows colocalization of PER and DH31 in the posterior dorsal neurons (DN_{1p}s). (c) *Z* stack of 20 confocal planes. "DH31CA neurons" (arrowheads) that project to the corpus allatum (CA). (d) *Z* stack of 57 confocal planes. DH44 is present in neurons of the pars intercerebralis (PI) and pars lateralis (PL) that project to the corpora cardiacum (CC), where they terminate in close vicinity to the pigment-dispersing factor (PDF)-positive fibers and partly overlap with them. (e) *Z* stack of 26 confocal planes. Magnification of the PI and PL showing the large DH44-positive neurons in the PI (yellow arrow) and smaller DH44-positive cell bodies in the PL, of which two overlap with the PDF-positive six smaller CRZ_{1-6} neurons (white arrowheads). The latter are always close to the soma of the large CRZ_{CN} neuron. The small DH44 cell bodies above the large PI neurons stem most probably from neurons that arborize in the protocerebral bridge (PB) since we found weak DH44 staining in the PB. Scale bars: 50 μm in panels (a) and (c–e); 15 μm in panel (b).

behavior, which was found for *D. littoralis* and other species of the *virilis* group under constant darkness (Beauchamp et al., 2018; Bertolini et al., 2019; Kauranen et al., 2012; Menegazzi et al., 2017; Vaze & Helfrich-Förster, 2016).

Alternatively, PER in the s-LN_vs and the DN_2s might oscillate out of phase with the other clock neurons. The latter hypothesis could be especially true for the DN_2s because these were shown to cycle in opposite phase with the other clock neurons in *D. melanogaster*

larvae (Klarsfeld et al., 2004) and in adults kept for several days under constant conditions (Yoshii et al., 2009). Perhaps this is the case in *D. littoralis* already under LD cycles. Additional stainings at timepoints other than ZT1 will clarify this hypothesis in the future.

CRY was found in half of the clock neurons of *D. melanogaster* (Benito et al., 2008; Yoshii et al., 2008). Similarly, we detected CRY-like immunoreactivity in four s-LN_vs, the 5th LN, three LN_ds, six DN_{1p}s, and two DN_{1a}s in *D. littoralis* (Figure 4e), but not in the l-LN_vs (Figure 4f), which is consistent with the observations of Hermann et al. (2013) and Menegazzi et al. (2017).

As previously reported, PDF was virtually absent in the s-LN_vs of *D. littoralis*, at least at the first glance. However, upon careful inspection, we found weak PDF staining in the s-LN_vs (Figure 4f) as well as their dorsal projections that terminate close to the DN_{1p}s (Figure 4e,g). This demonstrates that PDF is present in the s-LN_vs, albeit at a much lower level compared to *D. melanogaster*. Together with the putative weak molecular clock in the s-LN_vs (see above), the extremely low levels of PDF in these neurons may be the reason for the weak circadian rhythmicity of *D. littoralis* flies.

In accordance with previous studies in different high-latitude flies (Beauchamp et al., 2018; Hermann et al., 2013; Kauranen et al., 2012; Menegazzi et al., 2017), we detected additional PDF-positive cells in the dorsal brain, close to the DN_{1p}s (Figure 4e). These cells were neither PER positive nor CRY positive (Figure 4c,g) and thus most likely not clock neurons.

3.3 | CRZ colocalizes with PDF in neurosecretory cells of the PL

As CRZ is involved in the diapause of different insects as well as in their stress responses (Shiga et al., 2003; Tsuchiya et al., 2021; Zandawala et al., 2021), we investigated its expression in the brain of *D. littoralis*. Similar to the expression pattern of *D. melanogaster*, we identified CRZ in seven "dorsolateral peptidergic neurons" per hemisphere in the PL that project into the PI and further, via median and lateral nerves, to the CC/CA complex (Figures 1b and 5a). While the six CRZ neurons had smaller somata, the seventh CRZ neuron had a larger cell body, which in *D. melanogaster* has been renamed CN neuron (Oh et al., 2019). We will maintain this nomenclature for *D. littoralis* and, henceforth, call the large CRZ neuron CRZ_{CN} and the six smaller ones CRZ_{1-6} neurons.

Surprisingly, double-labeling experiments with CRZ and PDF C7 antibodies (Table 3) revealed that the PDF neurons in the dorsal brain described above are in fact CRZ neurosecretory cells (Figure 5a). A similar colocalization of PDF in the CRZ cells of the PL was previously shown in the blow fly *Protophormia terraenovae* (Hamanaka et al., 2007), which is also strongly photoperiodic. Colocalization of CRZ and PDF was found not only in the somata of the neurons, but also in their central brain branches and their projections to the CC/CA complex (Figure 5a for *D. littoralis*). As the CC/CA complex is a neurohemal organ, PDF could be released together with CRZ into the hemolymph and target peripheral tissues.

Double labeling with CRZ and PDF antibodies allowed us to distinguish between the PDF fibers originating from the CRZ neurons and the faint PDF fibers stemming from the s-LN_vs. This enabled us to reconstruct the s-LN_v projections to the dorsal brain that terminate near the dorsal clock neurons (Figure 5b). In contrast to *D. melanogaster*, the s-LN_v projections to the dorsal brain in *D. littoralis* show more pronounced fibers forming a "dorsal horn" (Figure 5b,c). A similar projection pattern of the s-LN_v terminals was found in *Drosophila yakuba*, *Drosophila willistoni*, and *Chymomyza costata* (Bertolini et al., 2019; Hermann et al., 2013). In all these species, the s-LN_v axons terminate in the PL close to the CRZ neurons, while those of *D. melanogaster* extend medially and terminate closer to the DILP neurons in the PI and even signal to them (Nagy et al., 2019). This observation suggests that the s-LN_vs of *D. littoralis* are most likely not synaptically connected to the neurosecretory cells of the PI, but rather to those of the PL, notwithstanding the fact that they could also signal paracrine without forming any synapses (Shafer et al., 2022).

3.4 | sNPF shows a different expression pattern in the clock neurons and the neurosecretory cells of *D. littoralis* compared to *D. melanogaster*

In *D. melanogaster*, the neuropeptide sNPF is widely expressed in the brain (Nässel et al., 2008) and appears to be involved in the circadian clock as well as in the control of dormancy (Chen et al., 2013; Johard et al., 2009; Nagy et al., 2019). It is expressed in all four s-LN_vs, as well as in all four ITP cells (Kahsai et al., 2010) and all seven CRZ cells (CRZ_{CN} and CRZ_{1-6}) of the PL (Kapan et al., 2012; Figure 1b).

As seen in *D. melanogaster*, sNPF staining was widely present in the brain of *D. littoralis* (Figure 6a). In the clock network of *D. littoralis*, we found sNPF immunostaining in five of the six LN_ds (Figure 6b), in the DN_{1a}s (Figure 6d), and in some l-LN_vs at very low levels (Figure 6c). However, it was absent from the s-LN_vs (Figure 6c). Interestingly, while sNPF was present in the CRZ_{1-6} neurons (Figure 6a, white arrowheads), it could not be detected in the large CRZ_{CN} neuron (Figure 6a, yellow arrows). In *D. melanogaster*, CRZ_{CN} neuron acts as internal glucose sensor and uses sNPF to modulate glucose homeostasis via its actions on the CC and DILP-producing cells (Oh et al., 2019). Assuming that *D. littoralis* CRZ_{CN} neuron is also glucose sensing, the function of sNPF is probably served by other peptides expressed in these cells (i.e., CRZ and/or PDF).

Furthermore, the absence of sNPF from the s-LN_vs of *D. littoralis* suggests that it does not transfer time information from the clock to the DILP neurons in the PI as found for *D. melanogaster* (Nagy et al., 2019). Whether sNPF is involved in the control of diapause in *D. littoralis* needs to be investigated in future studies.

3.5 | AstC colocalizes with ITP in two clock neurons of *D. littoralis*

Similar to sNPF, the neuropeptides AstC and ITP are expressed in several *D. melanogaster* clock neurons (Diaz et al., 2019; Hermann-Luibl

et al., 2014; Johard et al., 2009; Zhang et al., 2021). AstC was described in the lateral posterior neurons (LPNs) and some DN_{1p}s and DN_3s of *D. melanogaster*, while ITP expression was shown in the 5th LN and one CRY-positive LN_d (Figure 1b).

Notably, AstC was recently shown to be crucial for temperature sensing during the induction of dormancy in *D. melanogaster* (Meiselman et al., 2022). Therefore, we investigated its presence in the brain of *D. littoralis* (Figure 7). We found a rather dense network of AstC-positive neurites in the central brain of *D. littoralis* including the protocerebral bridge and the subesophageal ganglion, with the highest density laterally to the PI (Figure 7a, white arrow). The latter resembles the AstC staining pattern in *D. melanogaster* (Zhang et al., 2021) and might partly originate from the LPNs (Reinhard, Bertolini, et al., 2022). Indeed, we found AstC staining in the three LPNs of *D. littoralis* (Figure 7c). Additionally, we detected AstC in several DN_3s (Figure 7e), but we did not see any expression in DN_{1p}s (Figure 7e). AstC fibers partially ran in parallel to the PDF fibers originating from the CRZ neurons and the l-LN_vs, but there was no colocalization with PDF in any of the PDF-positive neurons (Figure 7a,c).

Unlike in *D. melanogaster*, AstC was present in the 5th LN and in one LN_d of *D. littoralis* (Figure 7d) colocalizing with ITP (Figure 7f). Since ITP was only present in these two clock neurons, we could determine their morphology in *D. littoralis* (Figure 7g). We found that the morphology of the ITP-positive clock neurons is highly conserved between *D. melanogaster* and *D. littoralis*. Both clock neurons have putative dendritic arborizations in the accessory medulla and project to the PI, where their arborizations overlap with the AstC-positive projections stemming from the LPNs and possibly the DN_3s (Figure 7g, white arrow). Furthermore, based on the ITP-positive LN_d and 5th LN dense arborizations in the PL and PI (Hermann-Luibl et al., 2014), it is very likely that there are additional contacts with the neurosecretory cells of the PL and PI, as already shown by Kurogi et al. (2023) for DH31-positive neurosecretory cells of the PL in *D. melanogaster*.

In addition, ITP expression is also well conserved between *D. melanogaster* and *D. littoralis* in the four neurosecretory cells in the PL that project to the CC/CA complex (Figures 1b and 7g) (Hermann et al., 2013; Kahsai et al., 2010).

In summary, we did not find differences in ITP expression between *D. melanogaster* and *D. littoralis*, but a different expression pattern of AstC within the clock neurons. While AstC is absent from the DN_{1p}s of *D. littoralis*, it is strongly expressed in the LPNs and is present in more DN_3s as compared to *D. melanogaster*. Moreover, unlike in *D. melanogaster*, AstC is also present in the ITP-positive 5th LN and LN_d of *D. littoralis*. This suggests that AstC might play an important role in the circadian system of *D. littoralis*. Since AstC in *D. melanogaster* is involved in the photoperiodic entrainment (Diaz et al., 2019) and in the induction of dormancy in response to low temperatures (Meiselman et al., 2022), it is tempting to speculate that AstC conveys diapause-inducing photoperiodic information to the PI and PL neurosecretory cells of *D. littoralis*.

3.6 | DH31 is present in some neurosecretory cells of the PL and DN_{1p} clock neurons of *D. littoralis*

The neuropeptide DH31 is present in the LPNs and in a few DN_{1p}s clock neurons of *D. melanogaster* (Goda et al., 2016; Kunst et al., 2014; Reinhard, Bertolini, et al., 2022). However, within the clock network of *D. littoralis*, we could only find it in the DN_{1p}s (Figure 8b) and LPNs.

DH31 was recently described in three neurosecretory cells in the PL that project to the CA ("DH31CA neurons"; Kurogi et al., 2023) (Figure 1b) and were linked to reproductive dormancy in *D. melanogaster* (Kurogi et al., 2023). This prompted us to investigate DH31 immunostaining in the brain of *D. littoralis*. We found that DH31 is widely expressed in *D. littoralis* brain (Figure 8a,b), including the three neurosecretory cells in the PL that project to the CA (Figure 8c). This suggests that DH31 might play a similar role in the induction of diapause in *D. littoralis* as it was found for dormancy in *D. melanogaster* (Kurogi et al., 2023).

3.7 | DH44 is present in two CRZ_{1-6} neurons of *D. littoralis*

Although it is not expressed in any of the clock neurons, DH44 plays a role in stress tolerance and was shown to be involved in the locomotor rhythmicity of *D. melanogaster* (Cabrero et al., 2002; Cavanaugh et al., 2014; King et al., 2017; Zandawala et al., 2018). Instead, it is expressed in six PI neurons that receive inputs from the clock neurons (King et al., 2017). We used the antibody against DH44 to check whether this is similar in *D. littoralis*. As true for *D. melanogaster*, in *D. littoralis*, DH44 was present in six cells of the PI (Figure 8d). DH44-positive fibers were also present in the CC/CA complex (Figure 9d) indicating that DH44 could be released into the circulation as also expected for *D. melanogaster* (Cabrero et al., 2002; Ohhara et al., 2018). The DH44-positive PI cells of *D. melanogaster* have also been shown to signal to the crop (Ohhara et al., 2018) where they are possibly involved in crop extension. The crop extension is often seen in dormant flies since they use it to store food during unfavorable conditions (Kubrak et al., 2014). The conserved expression pattern of DH44 suggests that these functions might be conserved in *D. littoralis*.

Besides the PI, DH44 signal was present in the protocerebral bridge and in two of the small CRZ_{1-6} neurons (Figure 8e). These two cells are in most cases the ones that are the closest to the CRZ_{CN} neurons, suggesting that the CRZ_{1-6} neurons could be further subclassified. Future studies are required to reveal the function of the different subclasses of CRZ neurons.

In summary, we found several striking differences in neuropeptide expression between *D. littoralis* and *D. melanogaster* that are summarized in Figure 9. Specifically, sNPF and AstC were present in more clock neurons of *D. littoralis* than of *D. melanogaster*, whereas sNPF was absent in the s-LN_vs of *D. littoralis* and the amount of PDF was greatly reduced (Figure 9a). In the CRZ-positive neurosecretory cells of

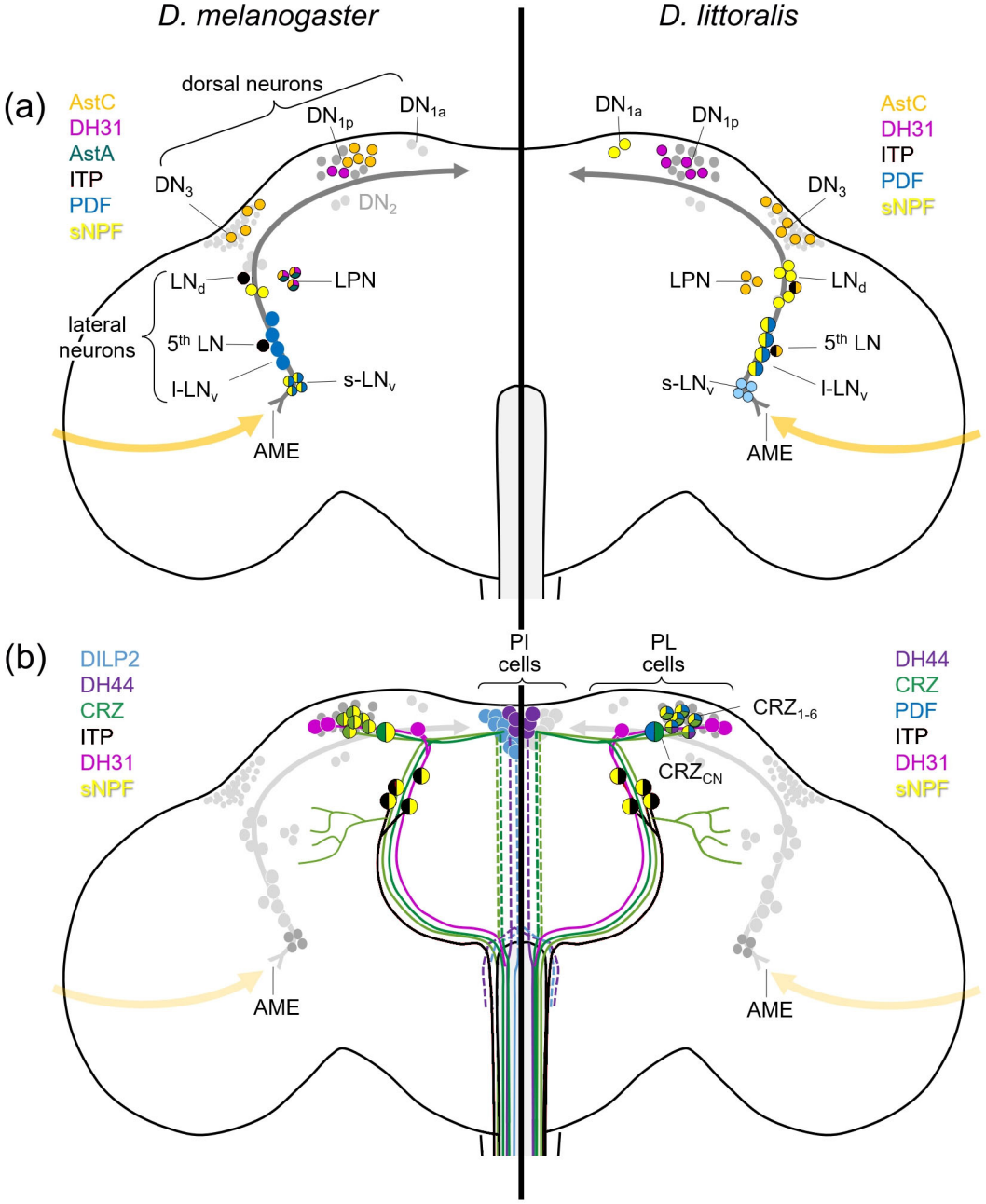

FIGURE 9 Neuropeptide expression in the clock neurons and neurosecretory cells of *Drosophila melanogaster* and *Drosophila littoralis*. (a) Neuropeptides in the clock neurons. Except for the DN_2s, all dorsal (DN), lateral (LN), and lateral posterior (LPN) clock neurons have been detected in the brain of *D. littoralis*. Major differences occur in neuropeptide expression in the small ventrolateral neurons (s-LN_vs) that express pigment-dispersing factor (PDF) and short neuropeptide F (sNPF) in *D. melanogaster* but only very little PDF (light blue) and no sNPF in *D. littoralis*. The s-LN_vs project into the dorsal protocerebrum in both species but the amount of PDF in these projections is strongly reduced in *D. littoralis*. In *D. littoralis*, sNPF is expressed in other clock neurons (large ventrolateral neurons [l-LN_vs], most dorsolateral neurons [LN_ds], and the anterior dorsal neurons [DN_{1a}s]) that do not contain sNPF in *D. melanogaster*. The ITP-positive fifth lateral neuron (5th LN) and LN_d of *D. melanogaster* contain not only ITP but additionally express AstC in *D. littoralis*. Furthermore, AstC is prominent in the dorsal neurons (DN_3s) and LPNs of *D. littoralis* (in *D. melanogaster*, the LPNs contain additionally allatostatin-A [AstA] and diuretic hormone 31 [DH31]; *D. littoralis* flies lack DH31 in the LPN and we did not test for AstA). However, AstC is absent from the posterior dorsal neurons (DN_{1p}s) in *D. littoralis*, in which DH31 appears to be more prominently expressed as compared to *D. melanogaster*. (b) Neuropeptides in the neurosecretory cells. In the pars intercerebralis (PI), we did not see any difference between the two species, but we could only stain for diuretic hormone 44 (DH44) since our *Drosophila* insulin-like peptide 2 (DILP2) antibody did not work in *D. littoralis*. The most striking differences between the two species occur in the corazonin (CRZ)-positive neurons (large CRZ_{CN} and six smaller CRZ_{1-6}) of the pars lateralis (PL), which all coexpress PDF in *D. littoralis*, while they coexpress sNPF in *D. melanogaster*. The six smaller CRZ_{1-6} of *D. littoralis* express sNPF as a third neuropeptide and two of these cells contain DH44 as a fourth peptide.

the PL, more neuropeptides were coexpressed in *D. littoralis* than in *D. melanogaster* (Figure 9b). Perhaps the most striking difference between the two species was the coexpression of PDF in all CRZ-positive neurons of *D. littoralis*.

3.8 | CRZ and PDF levels as well as the morphology of the CRZ_{CN} neuron change in diapausing *D. littoralis*

The PL has already been shown to be important for diapause regulation in the highly photoperiodic blow fly *Protophormia terraenovae* (Shiga & Numata, 2000), and the major neuropeptides in the PL of *D. littoralis*, PDF and CRZ, are involved in diapause or reproduction in various insects (Gospocic et al., 2017; Kotwica-Rolinska et al., 2022; Nagy et al., 2019; Shiga et al., 2003; Tsuchiya et al., 2021). Therefore, we hypothesized that the CRZ_{CN} and CRZ_{1-6} neurons are important for diapause induction in *D. littoralis* and that we should see differences in the CRZ/PDF staining intensity between reproducing and diapausing flies.

To test our hypothesis, we measured CRZ/PDF immunostaining intensity in the somata of CRZ_{CN} and CRZ_{1-6} neurons and the size of the somata in diapausing and reproductive flies (Figure 10). To induce diapause, virgin females were kept under LD 08:16 at 10°C for 3 weeks, while the reproductive controls were maintained at LD 20:04 at 23°C. To assess the effect of photoperiod alone, CRZ intensity was further analyzed in diapausing (LD 08:16) and reproductive (LD 20:04) flies exposed to the same temperature of 16°C (Figure 10f). We found the size of the CRZ_{CN} soma to be very different in diapausing and reproductive flies. In diapausing flies, it had almost the same size as the small CRZ_{1-6} neurons, while under reproductive conditions, the CRZ_{CN} neuron was twice as big compared to the CRZ_{1-6} neurons and had an irregular shape (Figure 10a–c). Because of these differences, the quantification of CRZ/PDF immunostaining was performed separately in CRZ_{CN} and CRZ_{1-6} neurons (Figure 10a). Under diapausing conditions, the CRZ staining intensity was significantly higher in both neuronal types than under reproductive conditions, with the highest CRZ levels occurring in the CRZ_{CN} neuron under diapause (Figure 10d). This was true as well for the flies entrained under the same temperature but in different photoperiods (Figure 10f). In contrast, PDF staining intensity was significantly lower in diapausing flies as compared to reproductive ones and this was true for CRZ_{CN} and CRZ_{1-6} neurons (Figure 10d). In addition, the size of the CRZ_{CN} and CRZ_{1-6} somata was significantly smaller under diapausing than under reproductive conditions with a higher significance in the CRZ_{CN} neuron (Figure 10a–c). For comparison, we measured also the PDF staining intensity in the somata of l-LN_vs under the two conditions but did not see any differences (Figure 10e).

The observed differences in staining intensity could be due to a changed neuropeptide production (transcription/ translation) and/or a changed neuropeptide transport/release. In case of CRZ, the higher staining intensity under diapausing conditions might also be due to the small size of the neuron somata, because then the same amount of CRZ would be concentrated in the smaller volume of the cells. To investigate whether the differences in staining intensity were due to changes in neuropeptide production, we quantified *Crz* and *Pdf* transcripts under diapausing and reproductive conditions. As further controls, we checked the transcript levels of *Dilp2* and *Kr-h1*. *Kr-h1* transcription is controlled by the JH and, as such, serves as an indicator of JH activity, which promotes reproduction and inhibits diapause (Meiselman et al., 2017; Minakuchi et al., 2009; Zhang et al., 2021; Saunders et al., 1990). While *Dilp2* is upregulated in *D. melanogaster* under diapausing conditions (Kubrak et al., 2014), *Kr-h1* should be downregulated in the absence of JH (Zhang et al., 2021). In accordance with *D. melanogaster*, we found an upregulation of *Dilp2* and a downregulation of *Kr-h1* in diapausing *D. littoralis* females (Figure 10g). With regard to *Crz* and *Pdf*, we found that both transcripts are significantly reduced under diapausing conditions as compared to reproductive conditions (Figure 10g). This shows that CRZ/PDF neuropeptide production is reduced during diapause and therefore the size of the cell somata may shrink. In case of PDF, clearly less peptide is stored in the somata of the cells. In case of CRZ, the same amount of the peptide might be stored in the somata of the neurons than under summer conditions, perhaps to enable a quick release once it is needed (see further discussion in Section 3.9).

Since the mRNA was extracted from the whole head, we cannot be sure that the changes seen in the mRNA levels are exclusively coming from the CRZ neurons. Indeed, the l-LN_vs and partially the s-LN_vs are the additional PDF-positive neurons in the brain of *D. littoralis* (Figure 4e). It is, however, unlikely that they could influence the *Pdf* transcripts under different conditions, as no significant difference in the PDF staining intensity was detected between diapausing and reproductive flies in the l-LN_vs (Figure 10e), and the s-LN_vs showed an extremely low level of PDF (see Section 4.1). In the brain of *D. melanogaster* and *D. littoralis*, no additional neurons were found to be CRZ positive via immunohistochemistry. However, in the brain of *D. melanogaster*, *Crz* transcripts were detected in the optic lobe via in situ hybridization (Choi et al., 2005). Interestingly, the same study showed the absence of these transcripts in the optic lobe of *D. virilis*. Being part of the *virilis* group, it is likely that the same is true in *D. littoralis*; however, we cannot completely exclude that these optic lobe neurons might present *Crz* transcripts and play a role in the differences seen at the mRNA level in this species. Whether these results are specific to CRZ neurons or affect other neurons in the brain, we show here that photoperiod and temperature clearly play a role in the transcription of these genes (Figure 10g). The same is true for the soma size of the CRZ_{CN} neuron (Figure 10b,c).

3.9 | CRZ and PDF may play a crucial role in diapause regulation in *D. littoralis*

PDF appears to promote the reproductive state since its mRNA and peptide levels are significantly higher in reproductive than in diapausing flies. Most interestingly, a similar role of PDF was also proposed for *D. melanogaster*. Here, PDF from the s-LN_v terminals signals to the DILP neurons in the PI increasing their cAMP levels (Nagy et al., 2019).

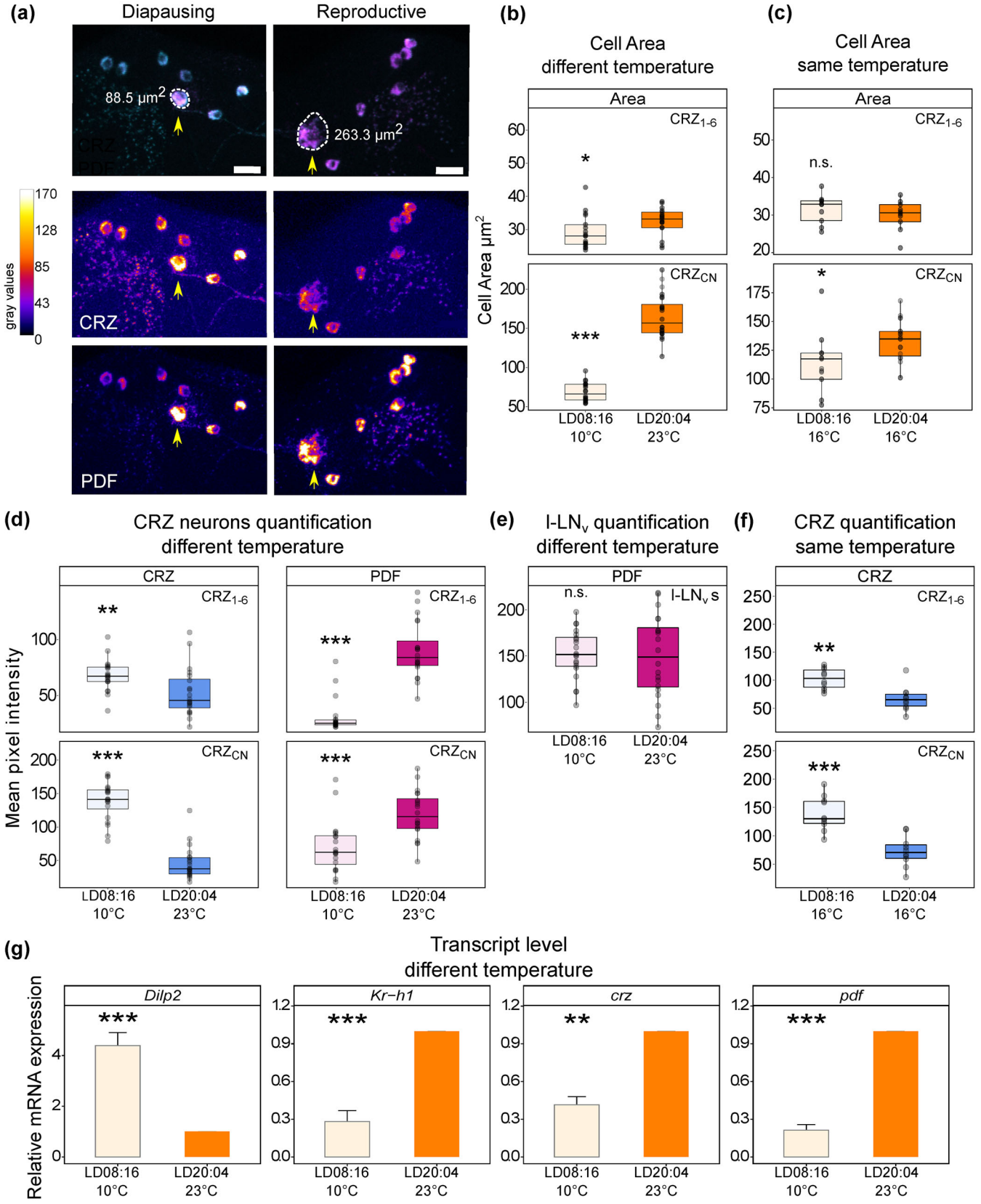

FIGURE 10 Pigment-dispersing factor (PDF) and corazonin (CRZ) staining intensity, CRZ neurons area, and reproduction-related genes transcripts in *Drosophila littoralis* under different conditions. (a) Under diapausing conditions, the PDF staining intensity is lower in CRZ neurons, but CRZ intensity is higher. Additionally, the size of CN neuron (yellow arrow) is visibly reduced compared to reproductive conditions. Z stack of 17 confocal planes for diapausing and 22 for reproductive conditions. (b) Under different temperature and photoperiodic conditions, the cell size of

(Continues)

FIGURE 10 (Continued)
diapausing flies is significantly smaller in the large CRZ_{CN} neurons as well as in the six smaller CRZ_{1-6}, although with a smaller difference. (c) Under different photoperiodic conditions but same temperature, only the CRZ_{CN} size is significantly reduced in diapausing flies. (d) CRZ (blue) intensity is significantly higher in the CRZ_{1-6} and CRZ_{CN} of diapausing flies. PDF intensity (magenta) is significantly lower in the CRZ_{1-6} and CRZ_{CN} of diapausing flies. (e) There is no significant difference between the PDF immunostaining quantification in large ventrolateral neurons (l-LN_vs) under diapausing and reproductive conditions. (f) Under diapausing condition at temperature 16°C, the CRZ intensity is higher than the one of reproductive condition at the same temperature. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ after Wilcoxon rank-sum test. (g) Relative mRNA expression (normalized to the summer condition) of genes related to reproduction of *Dilp2*, *Kr-h1*, *Crz*, and *Pdf*. There is a significant reduction in mRNA expression for *Crz*, *Kr-h1*, and *Pdf* in diapausing flies. On the contrary, there is a significant increase in the expression of *Dilp2* under diapausing conditions. $^{**}p < .01$ and $^{***}p < .001$ after Wilcoxon rank-sum test. Scale bars: 15 μm in panel (b). $n = 20$ flies in panels (b), (d), and (e); $n = 10$ flies in panels (c) and (f).

Subsequently, PKA is activated, which phosphorylates the transcription factor Eyes absent 3 (EYA3) and leads to its degradation (Hidalgo et al., 2023). EYA3 accumulates at high levels under winter conditions and promotes dormancy in *D. melanogaster* (Abrieux et al., 2020). Since PDF levels in the s-LN_v terminals are higher under summer conditions than under winter conditions, PDF promotes EYA3 degradation in the summer, thereby keeping the flies in the reproductive state (Hidalgo et al., 2023). Future studies are needed to show whether a similar molecular mechanism works in *D. littoralis*.

In *D. littoralis*, a stronger PDF signaling to the DILP neurons may occur via the fibers of the CRZ_{CN} neurons that pass by the PI and even follow the DILP neurons toward the CC (Figures 5 and 9). Furthermore, PDF may directly work on glucagon-like adipokinetic hormone signaling in *D. littoralis*. In *D. melanogaster*, PDF stemming most likely from PDF neurons in the abdominal ganglia activates adipokinetic hormone-producing cells in the CC by increasing their cAMP levels (Braco et al., 2022). The knockdown of the PDF receptor in these cells significantly extends life span of the flies under starvation conditions (Braco et al., 2022), a situation that is comparable to reproductive dormancy. Assuming that PDF receptor is also expressed in the CC of *D. littoralis*, PDF from the CRZ_{CN} neurons might regulate both DILP and adipokinetic hormone metabolic signaling pathways.

CRZ appears to have the opposite role of PDF and provokes diapause by inhibiting egg production as was shown in the ant *Harpegnathos saltator* and the fly *D. melanogaster* (Gospocic et al., 2017). This fits to the still high levels of CRZ immunostaining in the CRZ_{CN} neurons under diapausing conditions, as egg production should be inhibited. However, CRZ is not produced and probably just stored without any release when the flies are in diapause. Moreover, *Crz* mRNA levels are high and some CRZ is present under summer conditions, especially in the CRZ_{1-6} neurons (Figure 10d), indicating that CRZ is needed even in the reproductive active state. Indeed, CRZ plays several crucial roles in fly physiology and metabolism (Kubrak et al., 2016; Zandawala et al., 2021). In the ant species *Cataglyphis nodus*, the CRZ neurons show size differences depending on developmental and behavioral states (Habenstein et al. 2021). They are smaller in freshly eclosed ants and interior workers compared to the bigger cells of foragers, supporting the idea that these neurons are important for metabolic and behavioral changes during life span. Thus, we hypothesize that CRZ is strongly elevated and released in autumn before the flies enter diapause to inhibit egg production. During diapause, CRZ is stored in the cell somata because it is not needed in flies that are barely metabolically active. However, the cells are ready to release it in spring as soon as the flies become active again, and during summer conditions, it is produced and released at levels that are essential to control metabolic activity but are not high enough to inhibit egg production.

4 | CONCLUSIONS

Here, we describe for the first time the neuropeptide composition in the circadian clock neurons and neurosecretory cells of *D. littoralis* and compare it with the expression in *D. melanogaster*. We found striking differences in the two fly species that might explain differences in their circadian rhythmic behavior and seasonal adaptation (Lankinen, 1986a; Menegazzi et al., 2017).

4.1 | The s-LN_v circadian clock neurons are less important in *D. littoralis*

The PDF- and sNPF-expressing s-LN_vs, which are important for circadian rhythmicity and keeping *D. melanogaster* flies in the reproductive summer state (Grima et al., 2004; Helfrich-Förster, 1998; Hidalgo et al., 2023; Nagy et al., 2019; Shafer & Taghert, 2009), appear to be less important in *D. littoralis*. These neurons in *D. littoralis* express only low amounts of PER and very little PDF and lack sNPF completely, which altogether suggests that their circadian clock is impaired. This can explain the weak circadian rhythmicity of the animals. In addition, it appears unlikely that the little PDF released by the s-LN_vs could activate the DILP neurons in the PI and influence the reproductive state of the animals. Thus, the s-LN_vs might neither play an important role in rhythmic behavior nor in photoperiodism. On the other hand, CRY is well conserved between the two species, suggesting that it is crucial for circadian entrainment and the detection of changing photoperiods.

4.2 | The neuropeptides PDF, CRZ, DH31, and DH44 might contribute to the control of seasonal reproduction in *D. littoralis*

In *D. littoralis*, PDF stemming from the CRZ-positive neurosecretory cells in the PL may have overtaken the role of s-LN_v-derived PDF in keeping the flies reproductively active. This PDF may act on PDF

receptors on the adipokinetic hormone-expressing neurons in the CC and increase metabolic activity (Braco et al., 2022). PDF may even be released via the CC and could target peripheral tissues that express the PDF receptor (Krupp et al., 2013; Talsma et al., 2012). PDF might also affect DILP neurons in the PI and block EYA3 action and thereby keep the flies in the summer state as was shown by Hidalgo et al. (2023) in *D. melanogaster*. Other neuropeptides expressed in the PL may then induce reproductive diapause as soon as night length exceeds a critical value in *D. littoralis*. As discussed, CRZ is a good candidate for such a role, but DH31 may also serve this function. DH31 has recently been shown to inhibit vitellogenesis by repressing JH biosynthesis in the CA of *D. melanogaster* (Kurogi et al., 2023). As we show here, DH31 expression in the PL is well conserved between *D. melanogaster* and *D. littoralis*, making it likely that it serves the same function in both species. Lastly, DH44 may contribute to diapause preparation by controlling feeding and initiating crop filling for food storage, which is essential before entering diapause. In *D. melanogaster*, DH44-positive neurons are nutrient sensing, innervate the crop, express the mechanosensory channel Piezo, and stimulate feeding (Dreyer et al., 2019; Dus et al., 2015; Oh et al., 2021). In *D. littoralis*, DH44 is not only expressed in the neurosecretory cells of the PI but additionally in two PL cells, suggesting that it may play a prominent role in diapause preparation.

4.3 | The AstC-positive clock neurons are good candidates for transferring information about night length and temperature to the neurosecretory system of *D. littoralis*

So far, it is unclear which circadian clock neurons transfer the information about day/night length to the neurosecretory cells in the PI and PL of *D. littoralis*, but the AstC-positive clock neurons are good candidates. Notably, AstC is expressed in more clock neurons of *D. littoralis* than of *D. melanogaster*. Here, we show that the AstC- and ITP-expressing 5th LN and LN_d have exactly the same morphology as the corresponding neurons in *D. melanogaster* that only express ITP. As discussed, these two neurons get light input via the accessory medulla (Li et al., 2018) and project to the PL and PI (Hermann-Luibl et al., 2014), where they may form synapses with neurosecretory neurons. Furthermore, the same ITP-positive clock neurons were shown to be postsynaptic partners of most of the clock clusters (Shafer et al., 2022). Thus, the AstC- and ITP-expressing 5th LNs are perfectly suited to transfer information about day/night length to the neurosecretory system of *D. littoralis*. In addition, the AstC-positive LPNs and DN_3s may transfer information about environmental temperature to the neurosecretory system of *D. littoralis*. The LPNs form a dense fiber network in the superior protocerebrum close to the PI in *D. melanogaster* (Reinhard, Bertolini, et al., 2022) and, as we show here, this is also true in *D. littoralis* (see Figure 8). In *D. melanogaster*, the LPNs are involved in synchronizing the daily activity of flies to temperature cycles (Reinhard, Bertolini, et al., 2022). The AstC-positive DN_3s are even more promising candidates for transferring temperature information to the neurosecretory system because they have recently been shown to be temperature sensitive and to stimulate egg production via a still unknown pathway in *D. melanogaster* (Meiselmann et al., 2022). The same study showed that the reduction in AstC under lower temperatures is conserved in *D. virilis* as well. Since our study showed that more DN_3s express AstC in *D. littoralis* compared to *D. melanogaster*, this pathway is likely valid also in *D. littoralis*.

In summary, our study lays the foundation for future investigations into the functional connection between the circadian clock and the neurosecretory system in a high-latitude fly. Future studies will have to demonstrate whether the hypotheses formulated here are true. Establishing CRISPR/Cas9 mutagenesis in *D. littoralis* will allow to unravel the proposed importance of certain clock and neuropeptide genes in the photoperiodic control of diapause.

AUTHOR CONTRIBUTIONS

Giulia Manoli performed the stainings for the anatomical characterizations, the qPCR, and the identification of the clock proteins in the genome of *D. littoralis*. Meet Zandawala retrieved the sequences of the neuropeptides from the genome of *D. littoralis*. Taishi Yoshii generated the anti-ITP antibody used in the study. Giulia Manoli analyzed the data and performed statistical analysis. Giulia Manoli and Charlotte Helfrich-Förster generated the figures. Giulia Manoli wrote the first version of the manuscript. Charlotte Helfrich-Förster improved the manuscript with the contributions of Meet Zandawala, Giulia Manoli, and Taishi Yoshii. The study was conceptualized by Charlotte Helfrich-Förster and Giulia Manoli. Charlotte Helfrich-Förster supervised the project.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available upon reasonable request from the corresponding author.

ORCID

Giulia Manoli https://orcid.org/0000-0001-5954-5919
Meet Zandawala https://orcid.org/0000-0001-6498-2208
Charlotte Helfrich-Förster https://orcid.org/0000-0002-0859-9092

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REFERENCES

Abdelsalam, S., Uemura, H., Umezaki, Y., Saifullah, A. S., Shimohigashi, M., & Tomioka, K. (2008). Characterization of PDF-immunoreactive neurons in the optic lobe and cerebral lobe of the cricket, *Gryllus bimaculatus*. *Journal of Insect Physiology*, *54*, 1205–1212. https://doi.org/10.1016/j.jinsphys.2008.05.001

Abrieux, A., Xue, Y., Cai, Y., Lewald, K. M., Nguyen, H. N., Zhang, Y., & Chiu, J. C. (2020). EYES ABSENT and TIMELESS integrate photoperiodic and temperature cues to regulate seasonal physiology in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, *117*, 15293–15304. https://doi.org/10.1073/pnas.2004262117

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Andreatta, G., Broyart, C., Borghgraef, C., Vadiwala, K., Kozin, V., Polo, A., Bileck, A., Beets, I., Schoofs, L., Gerner, C., & Raible, F. (2020). Corazonin signaling integrates energy homeostasis and lunar phase to regulate aspects of growth and sexual maturation in *Platynereis*. *Proceedings of the National Academy of Sciences of the United States of America*, *117*, 1097–1106. https://doi.org/10.1073/pnas.1910262116

Aspi, J., Lumme, J., Hoikkala, A., & Heikkinen, E. (1993). Reproductive ecology of the boreal riparian guild of *Drosophila*. *Ecography*, *16*(1), 65–72. https://doi.org/10.1111/j.1600-0587.1993.tb00059.x

Bahn, J. H., Lee, G., & Park, J. H. (2009). Comparative analysis of Pdf-mediated circadian behaviors between *Drosophila melanogaster* and *D. virilis*. *Genetics*, *181*, 965–975. https://doi.org/10.1534/genetics.108.099069

Beauchamp, M., Bertolini, E., Deppisch, P., Steubing, J., Menegazzi, P., & Helfrich-Förster, C. (2018). Closely related fruit fly species living at different latitudes diverge in their circadian clock anatomy and rhythmic behavior. *Journal of Biological Rhythms*, *33*(6), 602–613. https://doi.org/10.1177/0748730418798096

Benito, J., Houl, J. H., Roman, G. W., & Hardin, P. E. (2008). The blue-light photoreceptor CRYPTOCHROME is expressed in a subset of circadian oscillator neurons in the *Drosophila* CNS. *Journal of Biological Rhythms*, *23*(4), 296–307. https://doi.org/10.1177/0748730408318588

Bergland, A. O., Chae, H., Kim, Y.-J., & Tatar, M. (2012). Fine-scale mapping of natural variation in fly fecundity identifies neuronal domain of expression and function of an aquaporin. *PLoS Genetics*, *8*, e1002631. https://doi.org/10.1371/journal.pgen.1002631

Bertolini, E., Schubert, F. K., Zanini, D., Sehadová, H., Helfrich-Förster, C., & Menegazzi, P. (2019). Life at high latitudes does not require circadian behavioral rhythmicity under constant darkness. *Current Biology*, *29*(22), 1–9. https://doi.org/10.1016/j.cub.2019.09.032

Braco, J. T., Nelson, J. M., Saunders, C. J., & Johnsson, E. C. (2022). Modulation of metabolic hormone signaling via a circadian hormone and biogenic amine in *Drosophila melanogaster*. *International Journal of Molecular Sciences*, *23*, 4266. https://doi.org/10.3390/ijms23084266

Bünning, E. (1936). Die endonome Tagesrhythmik als Grundlage der photoperiodischen Reaktion. *Berichte der Deutschen Botanischen Gesellschaft*, *54*, 590–607.

Cabrero, P., Radford, J. C., Broderick, K. E., Costes, L., Veenstra, J. A., Spana, E. P., Davies, S. A., & Dow, J. A. (2002). The Dh gene of *Drosophila melanogaster* encodes a diuretic peptide that acts through cyclic AMP. *Journal of Experimental Biology*, *205*(24), 3799–3807. https://doi.org/10.1242/jeb.205.24.3799

Cavanaugh, D. J., Geratowski, J. D., Wooltorton, J. R., Spaethling, J. M., Hector, C. E., Zheng, X., Johnson, E. C., Eberwine, J. H., & Sehgal, A. (2014). Identification of a circadian output circuit for rest: Activity rhythms in *Drosophila*. *Cell*, *157*(3), 689–701. https://doi.org/10.1016/j.cub.2014.10.020

Chen, W., Shi, W., Li, L., Zheng, Z., Li, T., Bai, W., & Zhao, Z. (2013). Regulation of sleep by the short neuropeptide F (sNPF) in *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology*, *43*(9), 809–819. https://doi.org/10.1016/j.ibmb.2013.06.003

Choi, Y. J., Lee, G., Hall, J. C., & Park, J. H. (2005). Comparative analysis of Corazonin-encoding genes (Crz's) in *Drosophila* species and functional insights into Crz-expressing neurons. *The Journal of Comparative Neurology*, *482*, 372–385. https://doi.org/10.1002/cne.20419

Cyran, S. A., Yiannoulos, G., Buchsbaum, A. M., Saez, L., Young, M. W., & Blau, J. (2005). The double-time protein kinase regulates the subcellular localization of the *Drosophila* clock protein period. *The Journal of Neuroscience*, *25*(22), 5430–5437. https://doi.org/10.1523/JNEUROSCI.0263-05.2005

Denlinger, D. L. (2022). *Insect diapause*. Cambridge University Press.

Diaz, M. M., Schlichting, M., Abruzzi, K. C., & Rosbash, M. (2019). Allatostatin C/AstC-R2 is a novel pathway to modulate circadian activity pattern in *Drosophila*. *Current Biology*, *29*(1), 13.e3–22.e3. https://doi.org/10.1016/j.cub.2018.11.005

Dircksen, H. (2009). Insect ion transport peptides are derived from alternatively spliced genes and differentially expressed in the central and peripheral nervous system. *Journal of Experimental Biology*, *212*, 401–412. https://doi.org/10.1242/jeb.026112

Dircksen, H., Tesfai, L. K., Albus, C., & Nässel, D. R. (2008). Ion transport peptide splice forms in central and peripheral neurons throughout postembryogenesis of *Drosophila melanogaster*. *The Journal of Comparative Neurology*, *509*(1), 23–41. https://doi.org/10.1002/cne.21715

Dircksen, H., Zahnow, C., Gaus, G., Keller, R., Rao, K., & Riehm, J. P. (1987). The ultrastructure of nerve endings containing pigment-dispersing hormone (PDH) in crustacean sinus glands: Identification by an antiserum against a synthetic PDH. *Cell Tissue Research*, *250*, 377–387. https://doi.org/10.1007/BF00219082

Dreyer, A. P., Martin, M. M., Fulgham, C. V., Jabr, D. A., Bai, L., Beshel, J., & Cavanaugh, D. J. (2019). A circadian output center controlling feeding: Fasting rhythms in *Drosophila*. *PLoS Genetics*, *15*(11), e1008478. https://doi.org/10.1371/journal.pgen.1008478

Dus, M., Lai, J. S., Gunapala, K. M., Min, S., Tayler, T. D., Hergarden, A. C., Geraud, E., Joseph, C. M., & Suh, G. S. (2015). Nutrient sensor in the brain directs the action of the brain-gut axis in *Drosophila*. *Neuron*, *87*(1), 139–151. https://doi.org/10.1016/j.neuron.2015.05.032

Gáliková, M., Dircksen, H., & Nässel, D. R. (2018). The thirsty fly: Ion transport peptide (ITP) is a novel endocrine regulator of water homeostasis in *Drosophila*. *PLoS Genetics*, *14*, e1007618. https://doi.org/10.1371/journal.pgen.1007618

Goda, T., Tan, X., Umezaki, Y., Chu, M. L., Kunst, M., Nitabach, M. N., & Hamada, F. N. (2016). *Drosophila* DH31 Neuropeptide and PDF receptor regulate night-onset temperature preference. *The Journal of Neuroscience*, *36*(46), 11739–11754. https://doi.org/10.1523/JNEUROSCI.0964-16.2016

Goda, T., Umezaki, Y., Alwattari, F., Seo, H. W., & Hamada, F. N. (2019). Neuropeptides PDF and DH31 hierarchically regulate free-running rhythmicity in *Drosophila* circadian locomotor activity. *Scientific Reports*, *9*(1), 838. https://doi.org/10.1038/s41598-018-37107-3

Gospocic, J., Shields, E. J., Glastad, K. M., Lin, Y., Penick, C. A., Yan, H., Mikheyev, A. S., Linksvayer, T. A., Garcia, B. A., Berger, S. L., Liebig, J., Reinberg, D., & Bonasio, R. (2017). The neuropeptide corazonin controls social behavior and caste identity in ants. *Cell*, *170*(4), 748.e12–759.e12. https://doi.org/10.1016/j.cell.2017.07.014

Goto, S. G. (2022). Photoperiodic time measurement, photoreception, and circadian clocks in insect photoperiodism. *Applied Entomology and Zoology*, *57*, 193–212. https://doi.org/10.1007/s13355-022-00785-7

Gramates, L. S., Agapite, J., Attrill, H., Calvi, B. R., Crosby, M. A., Dos Santos, G., Goodman, J. L., Goutte-Gattat, D., Jenkins, V. K., Kaufman, T., Larkin, A., Matthews, B. B., Millburn, G., & Strelets, V. B. (2022). FlyBase: A

guided tour of highlighted features. *Genetics*, *220*(4), iyac035. https://doi.org/10.1093/genetics/iyac035

Grima, B., Chélot, E., Xia, R., & Rouyer, F. (2004). Morning and evening peaks of activity rely on different clock neurons of the *Drosophila* brain. *Nature*, *431*(7010), 869–873. https://doi.org/10.1038/nature02935

Habenstein, J., Thamm, M., & Rössler, W. (2021). Neuropeptides as potential modulators of behavioral transitions in the ant *Cataglyphis nodus*. *Journal of Comparative Neurology*, *529*(12), 3155–3170. https://doi.org/10.1002/cne.25166

Hahn, D., & Denlinger, D. L. (2011). Energetics of insect diapause. *Annual Reviews in Entomology*, *56*, 103–121. https://doi.org/10.1146/annurev-ento-112408-085436

Hamanaka, Y., Tanaka, S., Numata, H., & Shiga, S. (2007). Peptide immunocytochemistry of neurons projecting to the retrocerebral complex in the blow fly, *Protophormia terraenovae*. *Cell Tissue Research*, *329*, 581–593. https://doi.org/10.1007/s00441-007-0433-3

Helfrich-Förster, C. (1998). Robust circadian rhythmicity of *Drosophila melanogaster* requires the presence of lateral neurons: A brain-behavioral study of *disconnected* mutants. *Journal of Comparative Physiology A*, *182*, 435–453. https://doi.org/10.1007/s003590050192

Helfrich-Förster, C. (2017). The Drosophila clock system. In V. Kumar (Ed.), *Biological timekeeping: Clocks, rhythms, and behaviour* (pp. 133–176). Springer. https://doi.org/10.1007/978-81-322-3688-7_6

Helfrich-Förster, C. (2020). Light input pathways to the circadian clock of insects with an emphasis on the fruit fly *Drosophila melanogaster*. *Journal of Comparative Physiology A*, *206*(2), 259–272. https://doi.org/10.1007/s00359-019-01379-5

Helfrich-Förster, C., Bertolini, E., & Menegazzi, M. (2020). Flies as models for circadian clock adaptation to environmental challenges. *European Journal of Neuroscience*, *51*, 166–181. https://doi.org/10.1111/ejn.14180

Helfrich-Förster, C., Täuber, M., Park, J. H., Mühlig-Versen, M., Schneuwly, S., & Hofbauer, A. (2000). Ectopic expression of the neuropeptide pigment-dispersing factor alters behavioral rhythms in *Drosophila melanogaster*. *The Journal of Neuroscience*, *20*(9), 3339–3353. https://doi.org/10.1523/JNEUROSCI.20-09-03339.2000

Hermann, C., Saccon, R., Senthilan, P. R., Domnik, L., Dircksen, H., Yoshii, T., & Helfrich-Förster, C. (2013). The circadian clock network in the brain of different *Drosophila* species. *Journal of Comparative Neurology*, *521*, 367–388. https://doi.org/10.1177/0748730412471303

Hermann-Luibl, C., Yoshii, T., Senthilan, P. R., Dircksen, H., & Helfrich-Förster, C. (2014). The ion transport peptide is a new functional clock neuropeptide in the fruit fly *Drosophila melanogaster*. *The Journal of Neuroscience*, *34*, 9522–9536. https://doi.org/10.1523/JNEUROSCI.0111-14.2014

Hidalgo, S., Anguiano, M., Tabuloc, C. A., & Chiu, J. C. (2023). Seasonal cues act through the circadian clock and PDF to control EYA and downstream physiological changes. *Current Biology*, *33*(4), 675.e5–687.e5.

Johard, H. A., Enell, L. E., Gustafsson, E., Trifilieff, P., Veenstra, J. A., & Nässel, D. R. (2008). Intrinsic neurons of *Drosophila* mushroom bodies express short neuropeptide F: Relations to extrinsic neurons expressing different neurotransmitters. *Journal of Comparative Neurology*, *507*(4), 1479–1496. https://doi.org/10.1186/1471-2202-9-90

Johard, H. A., Yoishii, T., Dircksen, H., Cusumano, P., Rouyer, F., Helfrich-Förster, C., & Nässel, D. R. (2009). Peptidergic clock neurons in *Drosophila*: Ion transport peptide and short neuropeptide F in subsets of dorsal and ventral lateral neurons. *Journal of Comparative Neurology*, *516*(1), 59–73. https://doi.org/10.1002/cne.22099

Kahsai, L., Kapan, N., Dircksen, H., Winther, A. M., & Nässel, D. R. (2010). Metabolic stress responses in *Drosophila* are modulated by brain neurosecretory cells that produce multiple neuropeptides. *PLoS ONE*, *5*(7), 1–12. https://doi.org/10.1371/journal.pone.0015780

Kapan, N., Lushchak, O. V., Luo, J., & Nässel, D. R. (2012). Identified peptidergic neurons in the *Drosophila* brain regulate insulin-producing cells, stress responses and metabolism by coexpressed short neuropeptide F and corazonin. *Cellular and Molecular Life Sciences*, *69*(23), 4051–4266. https://doi.org/10.1007/s00018-012-1097-z

Kauranen, H., Menegazzi, P., Costa, R., Helfrich-Förster, C., Kankainen, A., & Hoikkala, A. (2012). Flies in the north: Locomotor behavior and clock neuron organization of *Drosophila montana*. *Journal of Biological Rhythms*, *27*(5), 377–387. https://doi.org/10.1177/0748730412455916

Kim, B. Y., Wang, J. R., Miller, D. E., Barmina, O., Delaney, E., Thompson, A., Comeault, A. A., Peede, D., D'Agostino, E. R. R., Pelaez, J., Aguilar, J. M., Haji, D., Matsunaga, T., Armstrong, E. E., Zych, M., Ogawa, Y., Stamenković-Radak, M., Jelić, M., Veselinović, M. S., … Petrov, D. A. (2021). Highly contiguous assemblies of 101 drosophilid genomes. *eLife*, *10*, e66405. https://doi.org/10.7554/eLife.66405

King, A. N., Barber, A. F., Smith, A. E., Dreyer, A. P., Sitaraman, D., Nitabach, M. N., Cavanaugh, D. J., & Sehgal, A. (2017). A peptidergic circuit links the circadian clock to locomotor activity. *Current Biology*, *27*(13), 1–13. https://doi.org/10.1016/j.cub.2017.05.089

Klarsfeld, A., Malpel, S., Michard-Vanhée, C., Picot, M., Chélot, E., & Rouyer, F. (2004). Novel features of cryptochrome-mediated photoreception in the brain circadian clock of *Drosophila*. *The Journal of Neuroscience*, *24*(6), 1468–1477. https://doi.org/10.1523/JNEUROSCI.3661-03.2004

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

Kotwica-Rolinska, J., Damulewicz, M., Chodakova, L., Kristofova, L., & Dolezel, D. (2022). Pigment dispersing factor is a circadian clock output and regulates photoperiodic response in the linden bug, *Pyrrhocoris apterus*. *Frontiers in Physiology*, *13*, 884909. https://doi.org/10.3389/fphys.2022.884909

Krupp, J. J., Billeter, J. C., Wong, A., Choi, C., Nitabach, M. N., & Levine, J. D. (2013). Pigment-dispersing factor modulates pheromone production in clock cells that influence mating in *Drosophila*. *Neuron*, *79*(1), 54–68. https://doi.org/10.1016/j.neuron.2013.05.019

Kubrak, O. I., Kučerová, L., Theopold, U., Nylin, S., & Nässel, D. R. (2016). Characterization of reproductive dormancy in male *Drosophila melanogaster*. *Frontiers in Physiology*, *7*, 572. https://doi.org/10.3389/fphys.2016.00572

Kubrak, O. L., Kučerová, I., Theopold, U., & Nässel, D. R. (2014). The sleeping beauty: How reproductive diapause affects hormone signaling, metabolism, immune response and somatic maintenance in *Drosophila melanogaster*. *PLoS ONE*, *9*(11), e113051. https://doi.org/10.1371/journal.pone.0113051

Kunst, M., Hughes, M. E., Raccuglia, D., Felix, M., Li, M., Barnett, G., Duah, J., & Nitabach, M. N. (2014). Calcitonin gene-related peptide neurons mediate sleep-specific circadian output in *Drosophila*. *Current Biology*, *24*(22), 2652–2664. https://doi.org/10.1016/j.cub.2014.09.077

Kurogi, Y., Imura, E., Mizuno, Y., Hoshino, R., Nouzova, M., Matsuyama, S., Mizoguchi, A., Kondo, S., Tanimoto, H., Noriega, F. G., & Niwa, R. (2023). Female reproductive dormancy in *Drosophila melanogaster* is regulated by DH31-producing neurons projecting into the corpus allatum. *Development*, *150*(10), dev201186. https://doi.org/10.1242/dev.201186

Lankinen, P. (1986a). Geographical variation in circadian eclosion rhythm and photoperiodic adult diapause in *Drosophila littoralis*. *Journal of Comparative Physiology A*, *159*, 123–142. https://doi.org/10.1007/BF00612503

Lankinen, P. (1986b). Genetic correlation between circadian eclosion rhythm and photoperiodic diapause in *Drosophila littoralis*. *Journal of Biological Rhythms*, *1*(2), 101–118. https://doi.org/10.1177/074873048600100202

Lankinen, P., & Forsman, P. (2006). Independence of genetic geographical variation between photoperiodic diapause, circadian eclosion rhythm, and Thr-Gly repeat region of the period gene in *Drosophila littoralis*. *Journal of Biological Rhythms*, *21*, 3–12. https://doi.org/10.1177/0748730405283418

Lankinen, P., Kastally, C., & Hoikkala, A. (2022). Plasticity in photoperiodism: *Drosophila montana* females have a life-long ability to switch from repro-

duction to diapause. *Journal of Biological Rhythms*, *37*, 516–527. https://doi.org/10.1177/07487304221108968

Li, M.-T., Cao, L.-H., Xiao, N., Tang, M., Deng, B., Yang, T., Yoshii, T., & Luo, D.-G. (2018). Hub-organized parallel circuits of central circadian pacemaker neurons for visual photoentrainment in *Drosophila*. *Nature Communications*, *9*(1), 4247. https://doi.org/10.1038/s41467-018-06506-5

Lumme, J., & Keränen, L. (1978). Photoperiodic diapause in *Drosophila lummei* Hackman is controlled by an X-chromosomal factor. *Hereditas*, *89*(2), 261–262. https://doi.org/10.1111/j.1601-5223.1978.tb01282.x

Lumme, J., & Oikarinen, A. (1977). The genetic basis of the geographically variable photoperiodic diapause in *Drosophila littoralis*. *Hereditas*, *86*, 129–141. https://doi.org/10.1111/j.1601-5223.1977.tb01221.x

Lumme, J., Oikarinen, A., Lakovaara, S., & Alatalo, R. (1974). The environmental regulation of adult diapause in *Drosophila littoralis*. *Journal of Insect Physiology*, *20*(10), 2023–2033. https://doi.org/10.1016/0022-1910(74)90109-7

Ma, D., Przybylski, D., Abruzzi, K. C., Schlichting, M., Li, Q., Long, X., & Rosbash, M. (2021). A transcriptomic taxonomy of *Drosophila* circadian neurons around the clock. *eLife*, *10*, e63056. https://doi.org/10.7554/eLife.63056

Meiselman, M., Lee, S. S., Tran, R. T., Dai, H., Ding, Y., Rivera-Perez, C., Wijesekera, T. P., Dauwalder, B., Noriega, F. G., & Adams, M. E. (2017). Endocrine network essential for reproductive success in *Drosophila melanogaster*. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(19), E3849–E3858. https://doi.org/10.1073/pnas.1620760114

Meiselman, M. R., Alpert, M. H., Cui, X., Shea, J., Gregg, I., Gallio, M., & Yapici, N. (2022). Recovery from cold-induced reproductive dormancy is regulated by temperature-dependent AstC signaling. *Current Biology*, *32*(6), 1362.e8–1375.e8. https://doi.org/10.1016/j.cub.2022.01.061

Menegazzi, P., Dalla Benetta, E., Beauchamp, M., Schlichting, M., Steffan-Dewenter, I., & Helfrich-Förster, C. (2017). Adaptation of circadian neuronal network to photoperiod in high-latitude European *Drosophilids*. *Current Biology*, *27*(6), 833–839. https://doi.org/10.1016/j.cub.2017.01.036

Minakuchi, C., Namiki, T., & Shinoda, T. (2009). Krüppel homolog 1, an early juvenile hormone-response gene downstream of methoprene-tolerant, mediates its anti-metamorphic action in the red flour beetle *Tribolium castaneum*. *Developmental Biology*, *325*(2), 341–350. https://doi.org/10.1016/j.ydbio.2008.10.016

Nagy, D., Andreatta, G., Bastianello, S., Martín Anduaga, A., Mazzotta, G., Kyriacou, C. P., & Costa, R. (2018). A semi-natural approach for studying seasonal diapause in *Drosophila melanogaster* reveals robust photoperiodicity. *Journal of Biological Rhythms*, *33*, 117–125. https://doi.org/10.1177/0748730417754116

Nagy, D., Cusumano, P., Andreatta, G., Anduaga, A. M., Hermann-Luibl, C., Reinhard, N., Gesto, J., Wegener, C., Mazzotta, G., Rosato, E., Kyriacou, C. P., Helfrich-Förster, C., & Costa, R. (2019). Peptidergic signaling from clock neurons regulates reproductive dormancy in *Drosophila melanogaster*. *PLoS Genetics*, *15*(6), e1008158. https://doi.org/10.1371/journal.pgen.1008158

Nässel, D. R., Enell, L. E., Santos, J. G., Wegener, C., & Johard, H. A. (2008). A large population of diverse neurons in the *Drosophila* central nervous system expresses short neuropeptide F, suggesting multiple distributed peptide functions. *BMC Neuroscience*, *9*, 1–35. https://doi.org/10.1186/1471-2202-9-90

Nässel, D. R., Kubrak, O. I., Liu, Y., Luo, J., & Lushchak, O. V. (2013). Factors that regulate insulin producing cells and their output in *Drosophila*. *Frontiers in Physiology*, *4*, 252. https://doi.org/10.3389/fphys.2013.00252

Nässel, D. R., & Vanden Broeck, J. (2016). Insulin/IGF signaling in *Drosophila* and other insects: Factors that regulate production, release and post-release action of the insulin-like peptides. *Cellular and Molecular Life Sciences*, *73*, 271–290. https://doi.org/10.1007/s00018-015-2063-3

Nässel, D. R., & Zandawala, M. (2019). Recent advances in neuropeptide signaling in Drosophila, from genes to physiology and behavior. *Progress in Neurobiology*, *179*, 101607.

Nässel, D. R., & Zandawala, M. (2020). Hormonal axes in *Drosophila*: Regulation of hormone release and multiplicity of actions. *Cell and Tissue Research*, *382*, 233–266. https://doi.org/10.1007/s00441-020-03264-z

Oh, Y., Lai, J. S., Mills, H. J., Erdjument-Bromage, H., Giammarinaro, B., Saadipour, K., Wang, J. G., Abu, F., Neubert, T. A., & Suh, G. S. B. (2019). A glucose-sensing neuron pair regulates insulin and glucagon in *Drosophila*. *Nature*, *574*, 559–564. https://doi.org/10.1038/s41586-019-1675-4

Oh, Y., Lai, J. S., Min, S., Huang, H. W., Liberles, S. D., Ryoo, H. D., & Suh, G. S. B. (2021). Periphery signals generated by Piezo-mediated stomach stretch and Neuromedin-mediated glucose load regulate the *Drosophila* brain nutrient sensor. *Neuron*, *109*(12), 1979.e6–1995.e6. https://doi.org/10.1016/j.neuron.2021.04.028

Ohhara, Y., Kobayashi, S., Yamakawa-Kobayashi, K., & Yamanaka, N. (2018). Adult-specific insulin-producing neurons in *Drosophila melanogaster*. *Journal of Comparative Neurology*, *526*(8), 1351–1367. https://doi.org/10.1002/cne.24410

Ojima, N., Hara, Y., Ito, H., & Yamamoto, D. (2018). Genetic dissection of stress-induced reproductive arrest in *Drosophila melanogaster* females. *PLoS Genetics*, *14*, e1007434. https://doi.org/10.1371/journal.pgen.1007434

Park, D., Veenstra, J. A., Park, J. H., & Taghert, P. H (2008). Mapping peptidergic cells in *Drosophila*: Where DIMM fits in. *PLoS ONE*, *3*(3), e1896. https://doi.org/10.1371/journal.pone.0001896

Reddy, K. L., Wohlwill, A., Dzitoeva, S., Lin, M. H., Holbrook, S., & Storti, R. V. (2000). The Drosophila PAR domain protein 1 (Pdp1) gene encodes multiple differentially expressed mRNAs and proteins through the use of multiple enhancers and promoters. *Developmental Biology*, *224*(2), 401–114. https://doi.org/10.1006/dbio.2000.9797

Reinhard, N., Bertolini, E., Saito, A., Sekiguchi, M., Yoshii, T., Rieger, D., & Helfrich-Förster, C. (2022). The lateral posterior clock neurons (LPN) of *Drosophila melanogaster* express three neuropeptides and have multiple connections within the circadian clock network and beyond. *Journal of Comparative Neurology*, *530*, 1507–1529. https://doi.org/10.1002/cne.25294

Reinhard, N., Schubert, F., Bertolini, E., Hagedorn, N., Manoli, G., Sekiguchi, M., Yoshii, T., Rieger, D., & Helfrich-Förster, C. (2022). The neuronal circuit of the dorsal circadian clock neurons in *Drosophila melanogaster*. *Frontiers in Physiology*, *13*, 886432. https://doi.org/10.3389/fphys.2022.886432

Renn, S. C., Park, J. H., Rosbash, M., Hall, J. C., & Taghert, P. H. (1999). A pdf neuropeptide gene mutation and ablation of PDF neurons each cause severe abnormalities of behavioral circadian rhythms in *Drosophila*. *Cell*, *99*(7), 791–802. https://doi.org/10.1016/s0092-8674(00)81676-1

Salminen, T. S., Vesala, L., Laiho, A., Merisalo, M., Hoikkala, A., & Kankare, M. (2015). Seasonal gene expression kinetics between diapause phases in *Drosophila virilis* group species and overwintering differences between diapausing and non-diapausing females. *Scientific Reports*, *5*, 11197. https://doi.org/10.1038/srep11197

Saunders, D. S. (2020). Dormancy, diapause, and the role of the circadian system in insect photoperiodism. *Annual Reviews in Entomology*, *65*, 373–389. https://doi.org/10.1146/annurev-ento-011019-025116

Saunders, D. S., Henrich, V. C., & Gilbert, L. I. (1989). Induction of diapause in *Drosophila melanogaster*: Photoperiodic regulation and the impact of arrhythmic clock mutations on time measurement. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(10), 3748–3752. https://doi.org/10.1073/pnas.86.10.3748

Saunders, D. S., Richard, D. S., Applebaum, S. W., Ma, M., & Gilbert, L. I. (1990). Photoperiodic diapause in *Drosophila melanogaster* involves a block to the juvenile hormone regulation of ovarian maturation. *General and Comparative Endocrinology*, *79*(2), 174–184. https://doi.org/10.1016/0016-6480(90)90102-R

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, *9*, 676–682. https://doi.org/10.1038/nmeth.2019

Schubert, F. K., Hagedorn, N., Yoshii, T., Helfrich-Förster, C., & Rieger, D. (2018). Neuroanatomical details of the lateral neurons of *Drosophila melanogaster* support their functional role in the circadian system. *Journal of Comparative Neurology*, *526*, 1209–1231. https://doi.org/10.1002/cne.24406

Selcho, M., Millán, C., Palacios-Muñoz, A., Ruf, F., Ubillo, L., Chen, J., Bergmann, G., Ito, C., Silva, V., Wegener, C., & Ewer, J. (2017). Central and peripheral clocks are coupled by a neuropeptide pathway in *Drosophila*. *Nature Communications*, *8*, 15563. https://doi.org/10.1038/ncomms15563

Shafer, O. T., Gutierrez, G. J., Li, K., Mildenhall, A., Spira, D., Marty, J., Lazar, A. A., & Fernandez, M. P. (2022). Connectomic analysis of the *Drosophila* lateral neuron clock cells reveals the synaptic basis of functional pacemaker classes. *eLife*, *11*, e79139. https://doi.org/10.7554/eLife.79139

Shafer, O. T., & Taghert, P. (2009). RNA-interference knockdown of *Drosophila* pigment dispersing factor in neuronal subsets: The anatomical basis of a neuropeptide's circadian functions. *PLoS ONE*, *4*, e8298. https://doi.org/10.1371/journal.pone.0008298

Shafer, O. T., & Yao, Z. (2014). Pigment-dispersing factor signaling and circadian rhythms in insect locomotor activity. *Current Opinion in Insect Science*, *1*, 73–80. https://doi.org/10.1016/j.cois.2014.05.002

Shang, Y., Donelson, N. C., Vecsey, C. G., Guo, F., Rosbash, M., & Griffith, L. C. (2013). Short neuropeptide F is a sleep-promoting inhibitory modulator. *Neuron*, *80*(1), 171–183. https://doi.org/10.1016/j.neuron.2013.07.029

Shiga, S., Davis, N. T., & Hildebrand, J. G. (2003). Role of neurosecretory cells in the photoperiodic induction of pupal diapause of the tobacco hornworm *Manduca sexta*. *Journal of Comparative Neurology*, *462*(3), 275–285. https://doi.org/10.1002/cne.10683

Shiga, S., & Numata, H. (2000). The role of neurosecretory neurons in the pars intercerebralis and pars lateralis in reproductive diapause of the blowfly, *Protophormia terraenovae*. *Die Naturwissenschaften*, *87*, 125–128. https://doi.org/10.1007/s001140050689

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

Stanewsky, R., Frisch, B., Brandes, C., Hamblen-Coyle, M. J., Rosbash, M., & Hall, J. C. (1997). Temporal and spatial expression patterns of transgenes containing increasing amounts of the *Drosophila* clock gene *period* and a lacZ reporter: Mapping elements of the PER protein involved in circadian cycling. *The Journal of Neuroscience*, *17*(2), 676–696. https://doi.org/10.1523/JNEUROSCI.17-02-00676.1997

Stoleru, D., Peng, Y., Agosto, J., & Rosbash, M. (2004). Coupled oscillators control morning and evening locomotor behaviour of Drosophila. *Nature*, *431*, 862–868.

Talsma, A. D., Christov, C. P., Terriente-Felix, A., Linneweber, G. A., Perea, D., Wayland, M., Shafer, O. T., & Miguel-Aliaga, I. (2012). Remote control of renal physiology by the intestinal neuropeptide pigment-dispersing factor in *Drosophila*. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(30), 12177–12182. https://doi.org/10.1073/pnas.1200247109

Top, D., & Young, M. W. (2018). Coordination between differentially regulated circadian clocks generates rhythmic behavior. *Cold Spring Harbor Perspectives in Biology*, *10*(7), a033589. https://doi.org/10.1101/cshperspect.a033589

Tsuchiya, R., Kaneshima, A., Kobayashi, M., Yamazaki, M., Takasu, Y., Sezutsu, H., Tanaka, Y., Mizoguchi, A., & Shiomi, K. (2021). Maternal GABAergic and GnRH/corazonin pathway modulates egg diapause phenotype of the silkworm *Bombyx mori*. *Proceedings of the National Academy of Sciences of the United States of America*, *118*(1), e2020028118. https://doi.org/10.1073/pnas.2020028118

Vaze, K., & Helfrich-Förster, C. (2016). *Drosophila ezoana* uses an hour-glass based or a highly dampened circadian clock for measuring night-length and inducing diapause. *Physiological Entomology*, *41*(4), 378–389. https://doi.org/10.1111/phen.12165

Veenstra, J. A. (2021). Identification of cells expressing Calcitonins A and B, PDF and ACP in *Locusta migratoria* using cross-reacting antisera and in situ hybridization. *Peptides*, *146*, 170667. https://doi.org/10.1016/j.peptides.2021.170667

Veenstra, J. A., Agricola, H. J., & Sellami, A. (2008). Regulatory peptides in fruit fly midgut. *Cell and Tissue Research*, *334*(3), 499–516. https://doi.org/10.1007/s00441-008-0708-3

Veenstra, J. A., & Davis, N. T. (1993). Localization of corazonin in the nervous system of the cockroach *Periplaneta americana*. *Cell and Tissue Research*, *274*(1), 57–64. https://doi.org/10.1007/BF00327985

Vesala, L., & Hoikkala, A. (2011). Effects of photoperiodically induced reproductive diapause and cold hardening on the cold tolerance of *Drosophila montana*. *Journal Insect Physiology*, *57*(1), 46–51. https://doi.org/10.1016/j.jinsphys.2010.09.007

Wickham, H. (2016). *Ggplot2: Elegant graphics for data analysis*. Springer-Verlag. https://ggplot2-book.org/

Yoshii, T., Todo, T., Wülbeck, C., Stanewsky, R., & Helfrich-Förster, C. (2008). Cryptochrome is present in the compound eyes and a subset of *Drosophila's* clock neurons. *Journal of Comparative Neurology*, *508*, 952–966. https://doi.org/10.1002/cne.21702

Yoshii, T., Vanin, S., Costa, R., & Helfrich-Förster, C. (2009). Synergic entrainment of *Drosophila's* circadian clock by light and temperature. *Journal of Biological Rhythms*, *24*, 452–464. https://doi.org/10.1177/0748730409348551

Yusuf, L. H., Tyukmaeva, V., Hoikkala, A., & Ritchie, M. G. (2022). Divergence and introgression among the *virilis* group of *Drosophila*. *Evolution Letters*, *6*(6), 537–551. https://doi.org/10.1002/evl3.301

Zandawala, M., Marley, R., Davies, S. A., & Nässel, D. R. (2018). Characterization of a set of abdominal neuroendocrine cells that regulate stress physiology using colocalized diuretic peptides in *Drosophila*. *Cellular and Molecular Life Sciences*, *75*(6), 1099–1115.

Zandawala, M., Nguyen, T., Balanyà Segura, M., Johard, H. A. D., Amcoff, M., Wegener, C., Paluzzi, J. P., & Nässel, D. R. (2021). A neuroendocrine pathway modulating osmotic stress in *Drosophila*. *PLoS Genetics*, *17*(3), e1009425. https://doi.org/10.1371/journal.pgen.1009425

Zhang, C., Daubnerova, I., Jang, Y.-H., Kondo, S., Žitňan, D., & Kim, Y.-J. (2021). The neuropeptide allatostatin C from clock-associated DN1p neurons generates the circadian rhythm for oogenesis. *Proceedings of the National Academy of Sciences of the United States of America*, *118*, e2016878118. https://doi.org/10.1073/pnas.2016878118

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