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The neuronal clock network in the polar key species Antarctic krill (*Euphausia superba*)

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eLife Assessment

This foundational and **valuable** study expands our understanding of circadian clock work in non-model taxa in wider environmental niches, using **solid** methods for protein and RNA detection to describe the expression pattern of PDH, *cry2*, and *per* in the central nervous system of *Euphausia superba*. While the anatomical annotation is extensive, support for the identification of the clock network is **incomplete**.

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Abstract

Organisms are exposed to predictable daily and seasonal environmental oscillations. Biological clocks enable organisms to anticipate these changes and coordinate physiology and behaviour accordingly. While circadian mechanisms are well studied in terrestrial model organisms, little is known about the neuronal organisation of biological clocks in ecologically important species, especially in the marine environment. Antarctic krill (*Euphausia superba*) is central to the functioning of the Southern Ocean ecosystem and relies on precise timing to cope with the extreme, high-latitude fluctuations in photoperiod, food availability, and sea-ice cover in its habitat. Despite evidence for circadian and seasonal rhythms in krill behaviour and physiology, the neuronal architecture underlying these timing processes has remained unresolved. In this study, we use *in situ* hybridisation and antibody staining to characterise the circadian clock in the krill brain. Immunostaining with an antibody against crustacean β -Pigment-dispersing hormone (β -PDH) reveals distinct clusters of PDH-positive neurons in the optic lobes and dorsal central brain, along with an extensive PDH-positive fibre network. We further localise transcripts of the core clock genes *cryptochrome-2* (*cry2*) and *period* (*per*) in cell clusters in the optic lobes, which also include the PDH-positive neurons. More specifically, PDH-positive neurons are a subgroup of the *cry2* and *per*-positive cells. Together, these findings provide the first description of the neuronal architecture of the circadian clock in Antarctic krill and establish essential groundwork for future studies on biological timing, environmental adaptation, and the resilience of this key species in a rapidly changing Southern Ocean.

Introduction

Throughout evolution, organisms are subjected to regular environmental oscillations at daily and seasonal time scales, like changes in light intensity, temperature, humidity, and food availability. The underlying cause for these oscillations is the rotations of the Sun-Earth-Moon system, which makes these oscillations highly predictable and led to the evolution of biological clocks. Biological clocks are present across the tree of life and share a common structure: A self-sustaining

endogenous oscillator that can be synchronized by external cues (*Zeitgebers*) generates rhythms in, e.g., gene expression or neuronal activity. These rhythms coordinate the organism's physiology and behaviour with the appropriate phase of daily or seasonal cycles. Accordingly, endogenous clocks allow an organism to predict, rather than simply react to, regular environmental changes, enhancing an organism's ability to adapt to its environment, hence increasing its fitness (Horn et al., 2019 [↗](#); Ouyang et al., 1998 [↗](#)).

By far the best studied timing mechanism is the circadian clock, which generates rhythms with a period length of ~24 h and synchronizes to the daily light-dark cycle. Today, the circadian clock mechanism is known in great detail for a few terrestrial model species, which have been chosen for their genetic tractability, easy husbandry, and comparably simple neuronal architecture. These models have led to a deep understanding of circadian clocks (Beer & Helfrich-Förster, 2020 [↗](#)).

Accelerating anthropogenic pressures, such as rapid global warming, challenge organisms. Their adaptive capacity is a major determinant of species resilience to these changes. Hence, understanding the mechanisms of adaptation, including biological clocks, is crucial for predicting how species, populations, and whole ecosystems will respond to climate change. As a result, there is growing momentum to extend chronobiological research beyond traditional model organisms and to species across diverse taxa and ecosystems (Häfker et al., 2022 [↗](#); Häfker & Tessmar-Raible, 2020 [↗](#); Helm et al., 2013 [↗](#); Helm & Liedvogel, 2024 [↗](#); Helm et al., 2017 [↗](#); Kronfeld-Schor et al., 2017 [↗](#)). Due to their disproportionate importance for ecosystem function, understanding the resilience of ecological key species is essential in assessing the fate of ecosystems in the future.

Antarctic krill (*Euphausia superba*, hereafter “krill”) is an up to 6 cm long marine, pelagic crustacean that belongs to the Euphausiacea, an order within the Malacostraca. It is endemic to the Southern Ocean, a high-latitude habitat with strong seasonal fluctuations in daylength (photoperiod), food availability, and sea-ice cover. Krill efficiently makes use of the high primary productivity in the summer months and serves as an important prey for apex predators, including whales, penguins, seals, and seabirds, thus forming a crucial link between low trophic levels and higher consumers (Ballerini et al., 2014 [↗](#); Murphy et al., 2007 [↗](#)). Krill thrives in the harsh conditions of the Southern Ocean, and with estimates of >300 Mio tons, their biomass is considered the highest of any wild living single species on Earth (Atkinson et al., 2009 [↗](#); Bar-On et al., 2018 [↗](#)). Its close, rhythmic adaptation to the daily and seasonal fluctuations of the environment is thought to be key to krill's ability to sustain this biomass in such a challenging habitat (Ducklow et al., 2007 [↗](#)). Rhythmic regulation of physiology and behaviour of krill has been observed at both daily and seasonal time scales. For example, every night, large krill swarms migrate tens to hundreds of meters to the upper parts of the water column to feed on phytoplankton and micro zooplankton. With sunrise, krill return to deeper parts to hide in the dark from visually hunting predators (Bahlburg et al., 2023 [↗](#); Cisewski et al., 2010 [↗](#); Everson, 1983 [↗](#)). Due to their large biomass, this synchronized behaviour, known as diel vertical migration (DVM), has major impacts on predator-prey interactions (Annasawmy et al., 2023 [↗](#); Cox et al., 2009 [↗](#); Nichols et al., 2022 [↗](#)), the recycling of nutrients, and the efficiency of carbon export (reviewed in Cavan et al., 2019 [↗](#)). Seasonally, krill shows a pronounced regulation of physiological functions, including halted development, reduced metabolism, and altered lipid dynamics, which are closely aligned with the seasonal availability of food (Kawaguchi et al., 2007 [↗](#); Meyer et al., 2010 [↗](#)). Most importantly, the circadian clock has been shown to drive daily rhythms in swimming behaviour, related to DVM (Hüppe et al., 2025 [↗](#); Piccolin et al., 2020 [↗](#)) and suggested to be involved in the photoperiodic control of seasonal metabolism and reproduction (Höring et al., 2018 [↗](#); Piccolin, Suberg, et al., 2018; Teschke et al., 2007 [↗](#), 2008 [↗](#)).

The molecular mechanism of circadian clocks is based on transcriptional-translational feedback loops (TTFL), in which complex interactions of clock genes and their protein products result in self-sustained ~24h rhythms. In animals, TTFLs generally comprise positive elements that promote transcription and negative elements that inhibit it. This mechanism is largely conserved across taxa; however, systematic differences exist, particularly with respect to the negative elements. In vertebrates and most arthropods, PERIOD (PER) and the light-insensitive mammalian CRYPTOCHROME (mCRY or CRY2) act as transcriptional repressors, while CLOCK (CLK) and BASIC

HELIX-LOOP-HELIX ARNT-LIKE PROTEIN/CYCLE (BMAL/CYC) act as transcriptional activators (Kotwica-Rolinska, Chodakova, et al., 2022; Patke et al., 2020). Similarly, this is also the case in various crustacea (Christie et al., 2013; Christie, Yu, Pascual, et al., 2018; Hunt et al., 2019; Nesbit & Christie, 2014; O'Grady et al., 2016; Sbragaglia et al., 2015; Tilden et al., 2011), including Euphausiacea (Biscontin et al., 2017; Christie, Yu, & Pascual, 2018). In contrast, the well-known *Drosophila* clock mechanism represents a special case where only the light-sensitive *Drosophila*-type CRYPTOCHROME (dCRY or CRY1) is present, and which, by interaction with TIMELESS (TIM), resets the clock in a light-dependent manner (Emery et al., 1998). Interestingly, krill as well as several other insect and crustacean species, possess both forms of cryptochrome, representing an evolutionarily ancient molecular clock (Biscontin et al., 2017; Deppisch et al., 2022; Mazzotta et al., 2010; Yuan et al., 2007). In krill, CRY2 in combination with PER is thought to be the major repressor of CLK-BMAL/CYC activity, while binding of TIM is thought to stabilize the complex (Biscontin et al., 2017). This is similar to the mechanism described for the monarch butterfly *Danaus plexippus* (Brady et al., 2021; Zhu et al., 2008) and the marine isopod *Eurydice pulchra* (Zhang et al., 2013).

The molecular clock ticks in many tissues and cells; however, only a small number of neurons in the brain control rhythmic behaviour and coordinate the daily oscillations in other tissues (Albrecht, 2012; Tomioka et al., 2012). As known from insect studies, these clock neurons are generally organized into distinct clusters, typically found associated with the optic lobes and protocerebrum (reviewed in Helfrich-Förster et al., 1998; Numata et al., 2015). The single neurons are strongly interconnected through, e.g., synaptic and paracrine signalling within a clock network. This whole network then generates coherent rhythmicity and drives the rhythmic biology of the organism (reviewed in Helfrich-Förster & Reinhard, 2025). The best studied neuropeptide of the circadian clock is PIGMENT-DISPERSING FACTOR (PDF), which today is known to be involved in phasing and synchronizing the clock neurons and the control of rhythmic behaviour (Liang et al., 2016; Renn et al., 1999; Rojas et al., 2019; Stengl & Arendt, 2016; Vaze & Helfrich-Forster, 2021). Interestingly, modulations of the PDF neuronal network have been implicated in the ability of high latitude flies to synchronize their locomotor activity to long photoperiods (Menegazzi et al., 2017). Further, PDF has been shown to provide a link between the circadian clock and the timing of seasonal events (i.e., photoperiodism; Hasebe et al., 2022; Hidalgo et al., 2023; Kotwica-Rolinska, Damulewicz, et al., 2022; Nagy et al., 2019). PDF-like peptides have first been isolated from the eyestalks of crustaceans, and one form, termed β -PIGMENT-DISPERSING HORMONE (β -PDH), has been identified as the most common one, well-known for its effect on the dispersion of retinal and chromatophore pigments in crustaceans (Rao & Riehm, 1993). Besides their structural similarity, functional similarity has been suggested between crustacean PDH and insect PDF. For example, head extracts from several insects have been shown to be able to induce pigment dispersion in crustaceans (reviewed in Rao, 2001), while β -PDH from the intertidal crab *Cancer productus* is able to restore rhythmic activity in *Drosophila melanogaster pdf*-null mutants (Beckwith et al., 2011). Also in krill, a β -PDH sequence has been identified, but its spatial expression and function remain unknown (Hunt et al., 2017; Toullec et al., 2013).

The Southern Ocean environment experiences rapid change (Abram et al., 2025; Meredith et al., 2019; Thomalla et al., 2023). To assess krill's resilience to environmental changes, understanding the mechanisms that govern daily and seasonal timing in krill is essential. Yet, despite the ecological importance of *E. superba*, only very little is known about its neurobiology. As the relevant timing processes are orchestrated at the level of individual neurons, establishing a foundational understanding of krill neuroanatomy and the organization of its neuronal circadian system is a critical step for any deeper mechanistic insight.

In this paper, for the first time, we revealed the spatial organization of the circadian clock network in the brain of *E. superba*. First, we used immunohistochemistry and *pdh in situ* hybridization to reveal distinct clusters of PDH-positive neurons in the optic lobes and the dorsal central brain, as well as an extensive fibre network. Subsequently, we localize the transcripts of the core clock

components *cry2* and *per* and show that they partially co-localize with PDH-positive neurons. This study reveals the neuronal architecture of the krill circadian clock and thereby provides a fundamental basis for future studies of biological timing in krill.

Results

Antarctic krill has a shrimp-like habitus, which is divided into the cephalothorax and an abdomen with six segments (Figure 1a). The Cephalothorax bears eight pairs of thoracic legs, the endopodites of which six are transformed to form a feeding basket, used to filter the water for phytoplankton and microzooplankton. The abdomen bears five swimming legs (pleopods), which are the main means of propulsion (Figure 1a, white arrows). The dissected central nervous system reveals two circumesophageal (blank arrowheads), the subesophageal, eight thoracic (T1-T8), and six abdominal (A1-A5 + terminal abdominal ganglion, TAG) ganglia along the ventral nerve cord (VNC; Figure 1b and c). The supraesophageal ganglion is dominated by the large, stalked compound eyes, whose volume well exceeds that of the central brain, as well as by the nerves of the 1st antennae (AN1, Figure 1a to c), which exit the central brain anteriorly, and the 2nd antennae, which exit the posterior brain ventrally (AN2; Figure 1b).

An antibody against crab β -PDH reveals an extensive network of PDH-positive neurons throughout the krill central nervous system

To visualize potential clock neurons, we stained against the clock-related neuropeptide β -PDH, as previous studies have shown strong interspecific cross-reactivity and even a functional conservation between crabs and flies (Beckwith et al., 2011; Dirksen et al., 1987; Harzsch et al., 2009; Helfrich-Förster, 1995; Hsu et al., 2008; Mangerich et al., 1987; Nussbaum & Dirksen, 1995). Immunostainings of the dissected central nervous system with an antibody against crab β -PDH (Wilcockson et al., 2011) show extensive PDH-immunoreactivity (PDH-ir) (Figure 1d). Dense innervation is found in the supraesophageal ganglion (Figure 1d and Figure 2) and both circumesophageal ganglia (Figure 1d and e, blank arrowheads). Similarly, each ganglion along the VNC is highly innervated by PDH-ir fibres in the left and right lateral neuropile, the anterior and posterior commissures, and along the midline (Figure 1d and e). We found PDH-ir fibres running in the median neurite bundle (Figure 1e, orange arrowhead) and the two lateral connectives (Figure 1e, blank orange arrowheads), linking all single ganglia along the VNC. We also identified a pair of strongly PDH-ir cells located laterally in many, most likely all ganglia of the VNC (Figure 1e and f, white arrowheads). Additionally, we found several weakly PDH-ir cells close to the median bundle. While these cells appear to contribute to the dense fibre networks in the ganglia, they do not seem to be their only source.

The most elaborate arborization pattern could be found within the supraesophageal ganglion. Widespread PDH-ir fibres innervated the central brain and the optic lobes of krill (Figure 1d and Figure 2). Since the supraesophageal ganglion was too thick for whole-mount imaging, we decided to use 200 μ m horizontal vibratome sections (Figure 2a) to investigate further the arborizations of the PDH-network within the central brain and the optic lobes (Figure 2b-f and h). Based on nuclear (Hoechst) and synapse (anti-SYNAPSIN) staining combined with autofluorescence, we could identify major brain regions, including the optic neuropiles (Figure 2a and g). As with most other malacostracan crustaceans, the krill brain can be divided into three regions: the protocerebrum, which is further subdivided into the lateral protocerebrum (hosting the optic lobe neuropiles) and the medial protocerebrum (which constitutes the anterior part of the central brain), and the deutocerebrum (which hosts the antennal neuropiles and olfactory lobes), and lastly the tritocerebrum (which constitutes the posterior central brain and the oesophageal connectives). PDH-ir fibres in the optic lobes clearly outline the optic neuropiles, including the lamina, medulla, and lobula (Figure 2b-f and h).

At the base of the eyestalk, the terminal medulla and the hemiellipsoid neuropile are found (Figure 2g). Both neuropiles are strongly innervated by PDH-ir fibres (Figure 2d and h). The fibres originate from two major cell clusters within the eye stalk: one cluster is located anteriorly between medulla and lamina and closely associated with the lamina, hence called the lamina-

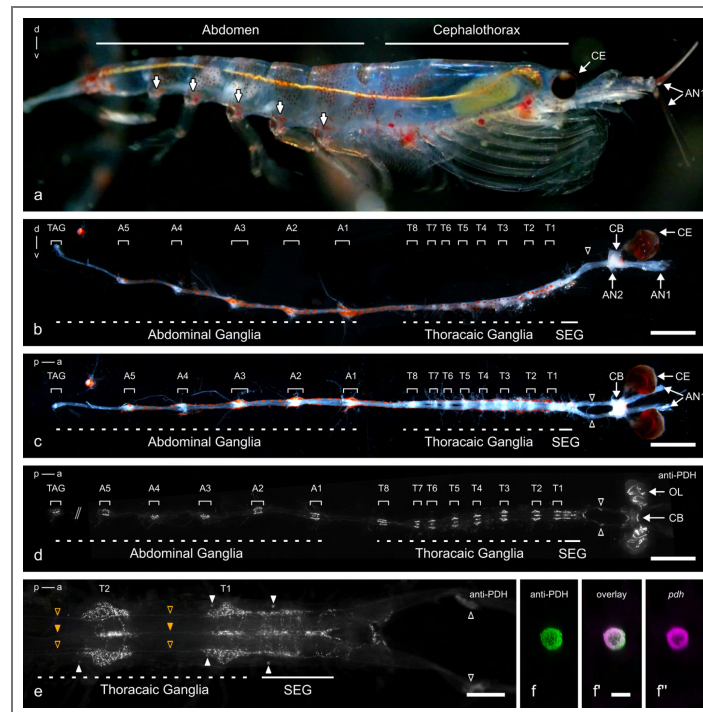


Figure 1. Gross morphology of *Euphausia superba* and its central nervous system.

a) Image of a swimming *E. superba*, facing with dorsal to the top and anterior to the right. Thick white arrows point to the five pairs of swimming legs (pleopods) on the abdomen. b, c) Lateral (b) and dorsal (c) view of the dissected central nervous system (CNS) of krill. The CNS can be divided into subesophageal (SEG), thoracic (T1-8), and abdominal (A1-A5 + TAG) ganglia. Arrows point to the compound eyes (CE), central brain (CB), and the 1st and the base of the 2nd antennae (AN1 and AN2, respectively). d) Pigment-dispersing hormone-immunoreactivity (PDH-ir) within the CNS of krill. Note that the terminal abdominal ganglion (TAG) was recorded independently of the rest of the CNS due to restrictions in dissections. e) Detailed view of the SEG and the first two thoracic ganglia (T1, T2). PDH-ir cells are labelled by white arrowheads. Fibers connecting the ganglia in the lateral connectives are labelled by blank orange arrowheads, and fibers running through the median neurite bundle by a filled orange arrowhead. f) Representative cell of one of the paired cells in the ventral nerve cord ganglia labelled by anti-PDH (green) and *pdh* in situ hybridization (magenta). Blank arrowheads label the circumesophageal ganglia. The scalebar represents 3 mm for (b-d), 250 μ m for e, and 50 μ m for (f). Abbreviations: d, dorsal; v, ventral; a, anterior; p, posterior.

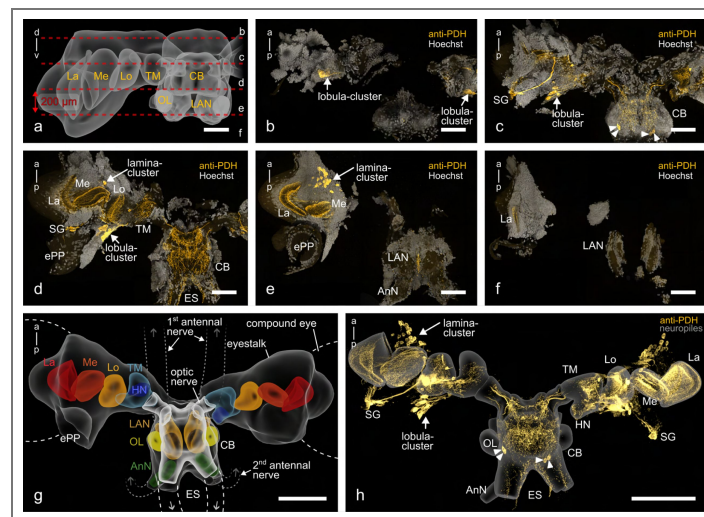


Figure 2. Overview of the PDH-network in the supraesophageal ganglion of krill.

a) Frontal view of one eyestalk and the central brain (CB) showing the position of the five horizontal 200 µm sections depicted in (b-f). b-f) Horizontal vibratome sections of the supraesophageal ganglion (left eyestalk and central brain) stained against β-PDH (orange) with a nuclear counterstaining (Hoechst, gray). The sections show extensive PDH-immunoreactivity from the dorsal (b) to ventral (f) brain and eyestalk. In the eyestalk, two prominent cell clusters (lamina-cluster, lobula-cluster) can be found. g) Reconstruction of major neuropiles found in the supraesophageal ganglion. The eyestalks contain, from lateral to medial, the lamina (La, red), medulla (Me, dark orange), the lobula complex with lobula and lobula plate (Lo, light orange), and the medulla terminalis (TM, light blue) with the hemiellipsoid neuropile (HN, dark blue). The olfactory neuropiles comprise the lateral antennal neuropile (LAN, ochre), the olfactory lobes (OL, yellow), and the antennal neuropile (AnN, green). The color scheme is based on Kenning et al. (2013) [DOI](https://doi.org/10.1002/glia.22500). Gray arrows indicate continuation of cut connectives/ nerves. Note that, due to the eyestalks' fragile connection to the central brain, the right eyestalk was rotated horizontally approximately 40° counterclockwise during processing. h) 3D projection of the whole PDH-network spanning the krill supraesophageal ganglion. The neuropiles from (g) are shown in gray for orientation. Scale bars in (a-f) represent 250 µm and 500 µm in (g) and (h). Abbreviations: ES, esophagus; SG, sinus gland; ePP, eyestalk photophore.

cluster, and the other is closely associated with the lobula, hence called the lobula-cluster. The following paragraph provides a detailed description of the clusters and their arborizations. To verify that the PDH-ir cells are indeed PDH-positive, we performed *pdh* RNA *in situ* hybridization on horizontal vibratome sections of krill brains (Figure 2-figure supplement 1). All strongly PDH-ir cells were also labelled by *pdh in situ* hybridization, independent of the cluster (Figure 1f [↗](#) and Figure 2-figure supplement 1a-j). Our data indicate that the cells that are strongly labelled by anti-β-PDH are indeed PDH-positive. However, we found in some cases weakly PDH-ir cells in the central brain and the lobula-cluster, where we could not clearly determine the colocalization of anti-PDH and *pdh in situ* labelling (Figure 2-figure supplement 1g', i' and j'; blank arrowheads). While there is the possibility that these neurons do not express *pdh* and are only labelled due to cross reactivity of the anti-β-PDH antibody with other peptides or proteins, it might well be that the expression is too low for us to detect via RNA *in situ* hybridization. The latter seems more likely since all strongly labelled cells were always colocalizing with *pdh* and could be reliably detected. The weakly stained neurons, on the other hand, were more variable and could not always be detected. We therefore focus on the cells that show both strong PDH-ir and express *pdh* in the following.

The krill optic lobes contain the vast majority of PDH-positive neurons

The lamina-cluster is the largest and most prominent of the PDH-positive cell clusters in the krill optic lobes (Figure 2b-e [↗](#) and h [↗](#), Figure 3a-f [↗](#), and Figure 2-figure supplement 2 and 3). While this cluster appears heterogeneous and likely comprises multiple subclusters, we currently lack robust characteristics to reliably separate them; it is therefore described here as a single cluster. At the proximal side of the lamina, we found several PDH-positive cells of this cluster distributed along the surface (filled arrowheads in Figure 3a-c [↗](#)). From the lamina, the cluster extends medial to the antero-ventral base of the medulla (Figure 3a-c [↗](#)). The projections of parts of the lamina-cluster neurons appear to run along the surface of the medulla before they innervate the lamina (Figure 3d [↗](#)). Some of these projections innervate the medulla, next to the lamina (blank arrowheads in Figure 3c [↗](#)). The PDH-positive fibres that run between the lamina and the medulla cross over and form the first and second optic chiasma, respectively (single and double asterisks in Figure 3a [↗](#), respectively).

The lobula-cluster lies in proximity to the lobula and can be divided into two sub-clusters (Figure 3e-g [↗](#)). Subcluster 1 lies at the posterior side of the optic lobe, and some cells of this cluster show intense staining (arrows in Figure 3e-g [↗](#)). The cell bodies of subcluster 2 lie medial to subcluster 1 and dorsal to the medulla terminalis (filled arrowheads in Figure 3e-g [↗](#)). Subcluster 2 comprises five PDH-positive cells, one of which is much larger than the other four (blank arrowhead in Figure 3h and i [↗](#)). The cells of lobula-subcluster 1 send projections towards the medulla terminalis (Figure 3e-g [↗](#)). Between the lobula and medulla terminalis, they form a dense knot, from which a thick fibre bundle projects medial and lateral (double arrowheads in Figure 3e and j [↗](#)). Lateral to the knot, the fibre bundle passes the lobula, turns anterior before it loops back posterior between the lobula and medulla, targeting the sinus gland, which is situated between the lamina and the eyestalk photophore (Figure 2d [↗](#); lines in Figure 3e and f [↗](#)). Medially of the knot, the fibre bundle innervates the medulla terminalis (Figure 3e and j [↗](#)). Parts of this bundle appear to pass the medulla terminalis and run through the eyestalk into the central brain (arrowheads in Figure 3k [↗](#)). However, due to the high number of fibres and the density of the fibre bundle and the dense arborizations in the medulla terminalis, we were unable to track the path of single fibres unambiguously.

Aside from the large cell clusters, single PDH-positive cells were found regularly in the region between the lobula and medulla terminalis, which appear to project towards the medulla terminalis (open arrowheads in Figure 3e and f [↗](#)). Due to the variations in sectioning, we could not determine their exact position yet.

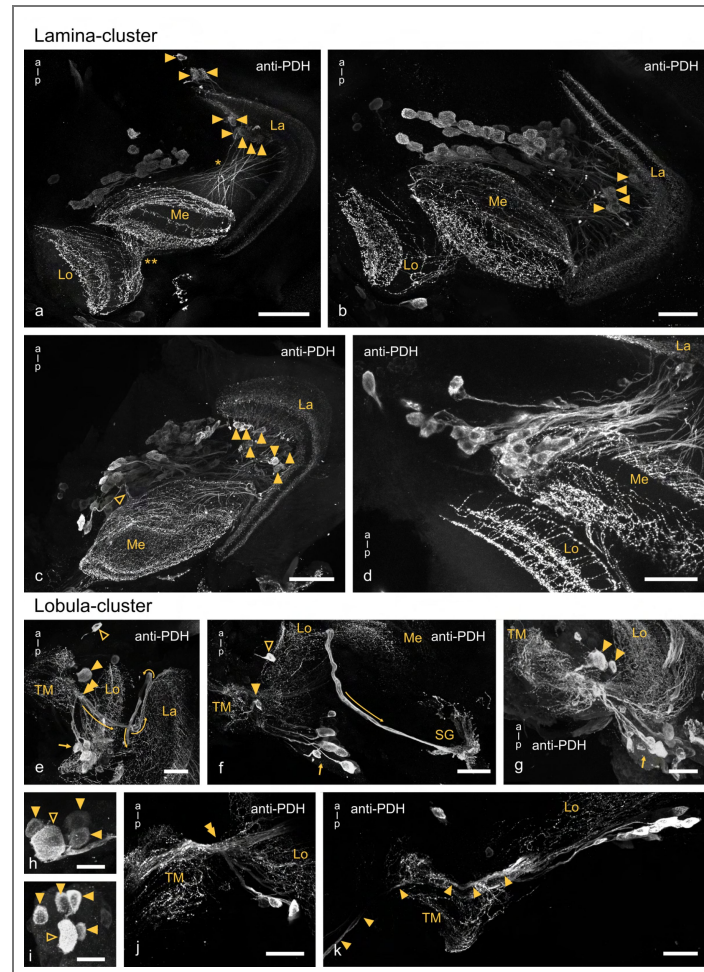


Figure 3. Detailed morphology of PDH-positive cell clusters and their projections in the krill eyestalks.

a-d) Maximum intensity projections showing PDH-positive cells and their projections in the lamina-cluster. a-c) Filled arrowheads point to cells distributed along the proximal surface of the lamina, open arrowhead in c) points to projections into the medulla. Single and double asterisks in a) indicate the first and second optic chiasma, respectively. e-k) Maximum intensity projections of PDH-positive cells and their projections in the lobula-cluster. e-g) single arrows point to lobula-subcluster 1 at the posterior side of the optic lobe and filled arrowheads point to cells of lobula-subcluster 2, projecting into the medulla terminalis. Blank arrowheads in e-f) point at single PDH-positive cells between the lobula and medulla terminalis. Double arrowheads in e) and j) point towards the dense fibre knot formed by projections of lobula-subcluster 1 cells, whereas thin lines e) and f) outline their projection lateral of the knot. i-h) Maximum intensity projection of subcluster 2 of the lobula-cluster showing five PDH-positive cells, including one characteristic large cell body (open arrowhead) and four smaller cells (filled arrowheads). k) Projections of lobula-subcluster 1 through the eyestalk towards the central brain (filled arrowheads). Scale bars represent 200 μm in a-c), and 100 μm in d-g), j), and k). Scalebars in h and i) represent 50 μm . Abbreviations: La, lamina; Me, medulla; Lo, lobula; TM, medulla terminalis; SG, sinus gland; a, anterior; p, posterior.

In contrast to the optic lobes, strong anti-PDH immunoreactivity in the central brain is restricted to 3 to 4 cells per hemisphere found dorsal in the medial protocerebrum (filled arrowheads in Figure 4a, 6i and j). They project ventrally into the anterior protocerebrum (blank arrowheads in Figure 4a), where they cross fibres that run from the eyestalks into the medial protocerebrum. Anterior to these fibres, the projections of the PDH-positive cells disappear in our preparations. The fibres from the eyestalks innervate a pair of granular, strongly PDH-ir structures, which lie dorsally in the medial protocerebrum (Figure 4a, asterisks). These structures strongly resemble the brain photoreceptor neuropile as described for the crayfish *Cherax destructor* and *Procambarus clarkii* (Sullivan et al., 2009).

An extensive network of PDH-positive fibres in the central brain reveals contralateral connections between the optic lobes, as well as ipsilateral connections between the optic lobes and the periphery

While we found only a few PDH-positive cells in the central brain of krill, complex networks of PDH-positive fibres are visible throughout most parts of it (Figure 2c-e, 4a-c, and Figure 4-figure supplement 1). In the medial protocerebrum, the most prominent structures visible are PDH-positive fibre bundles, originating from the medulla terminalis in the optic lobes, which project through the eyestalks into the central brain where they form prominent dorsal and ventral commissures (filled and blank arrowheads in Figure 4b; Figure 4-figure supplement 1). Due to the dense arborizations in the medulla terminalis, the origin of these fibres could not be identified unambiguously. Parts of the commissural fibre bundles in each hemisphere of the central brain branch and project posteriorly, forming extensive arborizations in large parts of the posterior protocerebrum and tritocerebrum (Figure 4b and c). In the tritocerebrum, several PDH-positive fibres leave the central brain via the esophageal connectives (Figure 4c). Specifically, we could trace fibres that originate in the optic lobes, run through the eyestalks into the central brain, where they project into the ventral posterior brain and leave the central brain through the esophageal connective of the respective hemisphere (filled arrowheads in Figure 4c), thus providing ipsilateral connections of the optic lobes and the periphery.

PDH co-localizes with *cry2* and *per* mRNA in the optic lobes of krill

To assess the expression of the clock genes *cry2* and *per* throughout the krill optic lobes, we used RNA *in situ* hybridization on horizontal vibratome sections of the krill brain. The *in situ* hybridization signal of both *cry2* and *per* probes shows distinct staining of two cell clusters, whose location closely resembles those of the lamina- and lobula-clusters described for PDH-positive cells above (Figure 5-figure supplement 1a-d). Negative control sections processed identically but without the addition of the RNA probes showed no detectable signal, confirming the specificity of the signal (Figure 5-figure supplement 2). Since our data show that both clock genes are expressed in the same brain regions, we next performed *cry2* and *per* *in situ* hybridization with subsequent PDH-immunolabeling. The double-labelling revealed that both *cry2* and *per* co-localize with all PDH-positive cells in the lamina- and lobula-cluster (filled arrowheads in Figure 5). Moreover, in both clusters, the co-labelling revealed additional *cry2*- and *per*-positive cells which are not PDH-positive (blank arrowheads in Figure 5), showing that the number of clock gene-expressing cells in these clusters is higher than the number of cells containing PDH.

cry2 and *per* are widely expressed but do not co-localize with PDH in the krill central brain

In contrast to the expression patterns in the optic lobes, *in situ* hybridization revealed extensive expression of *cry2* and *per* throughout large parts of the central brain (Figure 6a-h). The staining pattern of the two probes is largely similar. In the dorsal brain, *cry2* and *per*-positive cells are mainly found in the posterior brain (Figure 6a and e). Further ventrally, the *in situ* hybridization signal is mostly restricted to the lateral and posterior brain, except for two clusters of cells in a

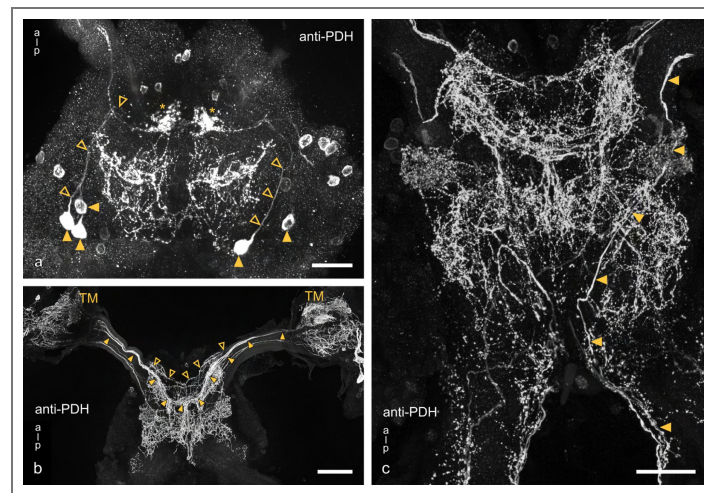


Figure 4. Detailed morphology of PDH-positive cells and projections in the krill brain

a-c) Maximum intensity projections of the PDH-positive network in dorsal (a) and medial to ventral regions of the central brain (b-c). a) Filled arrowheads point to PDH-positive cells found dorsal in the medial protocerebrum, while blank arrowheads point to their projections. Asterisks indicate a paired, granular, PDH-ir structure which is targeted by fibres coming from the optic lobes. b) PDH-ir fibres originating from the medulla terminales form dense arborizations in the medial protocerebrum. Filled and blank arrowheads indicate the paths of PDH-ir projections forming dorsal and ventral commissures, respectively. c) Filled arrowheads indicate fibres that enter the central brain through the eyestalks and exit ipsilaterally via the oesophageal connectives. The scalebar in a) represents 100 μ m, and in b and c) 200 μ m. Abbreviations: TM, medulla terminalis; a, anterior; p, posterior.

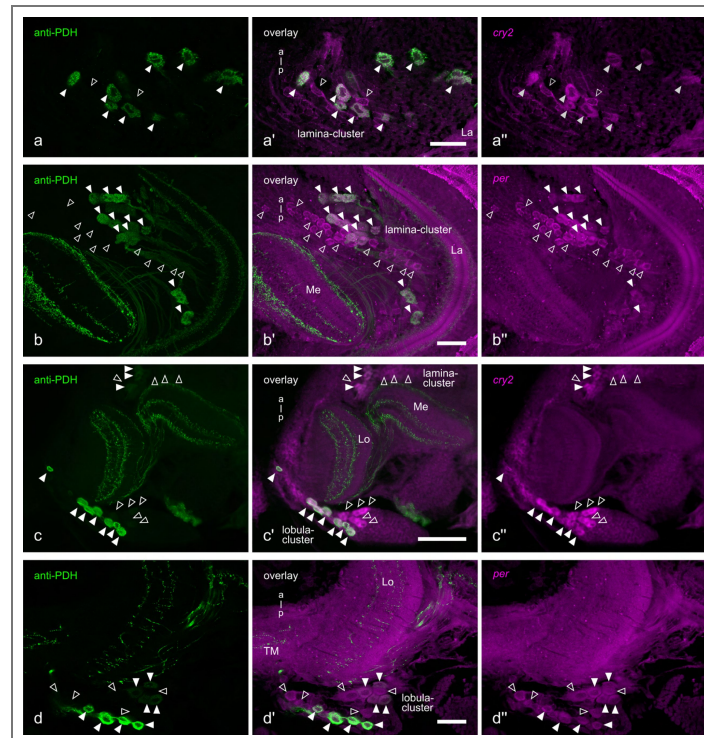


Figure 5. PDH-positive cells co-localize with *cry2* and *per* transcripts in the krill optic lobes.

a-b'') PDH-positive (green, a, b), as well as *cry2*- (magenta, a'') and *per*-positive (magenta, b'') cells in the lamina-cluster of the krill optic lobe. a' and b') show co-localization of the anti-PDH and in situ hybridization signal in a subset of *cry2* (filled arrowheads in a') and *per*-positive (filled arrowheads in b') cells. c-d'') PDH-positive (green, c, d), as well as *cry2* (magenta, c'') and *per*-positive (magenta, d'') cells in the lobula-cluster. The PDH-positive cells co-localize with a subset of the *cry2* (filled arrowheads in c') and *per*-positive (filled arrowheads in d') cells, respectively. Blank arrowheads in (a-d'') show *cry2* and *per*-expressing cells that do not colocalize with anti-PDH. Abbreviations: La, lamina; Me, medulla; Lo, lobula. Scale bars represent 100 μ m.

posterior medial position (Figure 6b and f). Even further ventral, *cry2* and *per*-expression are restricted to the most lateral parts of the central brain (Figure 6c and g), while in the most ventral sections, *cry2*- and *per*-positive cells are mainly found in the center of the brain between the prominent neuropile regions of the first and second antenna, and the olfactory lobes (Figure 6d and h). Interestingly, co-staining of both *cry2*- and *per*-hybridization probes with the β -PDH antibody showed no co-localization of the PDH-positive cells with *cry2* or *per* mRNA in the central brain (Figure 6i-j, green arrowheads).

Discussion

The rhythmic adaptation of krill to its high-latitude environment is key to its success in the Southern Ocean, which in turn represents a cornerstone for the well-being of the whole krill-centred ecosystem. To predict krill's resilience to rapid environmental changes, it is essential to understand the mechanisms that govern daily and seasonal timing in krill. However, so far, little was known about the neurobiology of krill in general, and nothing about the neuronal architecture of its circadian clock.

In this study, we provide a detailed description of the PDH-neuronal network in *Euphausia superba* and show that the PDH-positive neurons in the optic lobes also express the circadian clock genes *per* and *cry2*, strongly suggesting that they are genuine circadian clock neurons (Figure 7). This finding represents an important advance in our aim to understand the mechanistic basis of biological timing in krill.

The krill PDH-network shows similarities with those of other malacostracan crustaceans

The arborization pattern of the PDH-network has been described in various malacostracan crustaceans, including *Carcinus maenas* (Alexander et al., 2020; Mangerich & Keller, 1988; Mangerich et al., 1987), *Cancer productus* (Hsu et al., 2008), *Orconectes limosus* (de Kleijn et al., 1993; Mangerich & Keller, 1988; Mangerich et al., 1987), *Homarus americanus* (Harzsch et al., 2009), *Cherax destructor*, *Procambarus clarkii* (Sullivan et al., 2009), and *Procambarus virginalis* (Luna et al., 2010).

As nicely summarized by Meelkop & Janssen (2011), the most striking similarities found between the PDH-ir patterns in the optic lobes are clusters of PDH-ir cells between the lobula and medulla terminalis, as well as their associated fibre tracts, with the most prominent tract targeting the neurohemal sinus gland. The cell clusters between the lobula and medulla terminalis have been referred to as cluster C (Harzsch et al., 2009; Luna et al., 2010) or clusters 2, 4, and 5 (Chung & Webster, 2004) in previous malacostracan studies, and match the position of the cells of the lobula-cluster we described for *E. superba* in the present study. Earlier work reported that a subpopulation of neurons within this region forms T-shaped axons projecting laterally to the sinus gland and medially toward the terminal medulla (Harzsch et al., 2009), a pattern consistent with our observations in krill. Although we could not unambiguously trace the full projection pattern of these neurons in krill, our data indicate that the medial branches of the lobula-cluster are likely to pass through the eyestalks and continue into the medial protocerebrum. This would match the situation described in *Homarus americanus*, where cluster C projection could be traced both laterally into the sinus gland and medially into the protocerebrum (Harzsch et al., 2009; Strauß & Dirksen, 2010).

Interestingly, studies on the ontogeny of the PDH-ir network showed that cells of cluster C are present from the earliest embryonic stages on in *Carcinus maenas* and *Homarus americanus* (Chung & Webster, 2004; Harzsch et al., 2009) and are reminiscent of the patterns in adult animals by the end of embryonic development. The early establishment of the PDH-ir network in the optic lobes has been suggested to reflect its involvement in the circadian pacemaker system, although its functional significance remains unknown (Chung & Webster, 2004; Harzsch et al., 2009).

Figure 6. Distribution of *cry2* and *per* transcripts in relation to the PDH-network in the krill brain.

a-h) Stereo microscopic images of 100 μm sections of the krill central brain showing cells expressing *cry2* (a-d) and *per* (e-h) transcripts. From left to right, images show sections progressing from dorsal to ventral regions of the central brain. Areas with high *per/cry2*-expression are marked by white arrowheads. i-i'') PDH-positive neurons in the krill central brain (green arrowheads) do not colocalize with *cry2*-expressing neurons (blank arrowheads). j-j'') Similarly, PDH-positive neurons (green arrowheads) do not colocalize with *per*-expressing neurons (blank arrowheads) as revealed by RNA in situ hybridization. Abbreviations: OL, olfactory lobes; LAN, lateral antennal neuropile; AnN, antennal neuropile. The scale bars represent 200 μm in a-h) and 100 μm in i-j'').

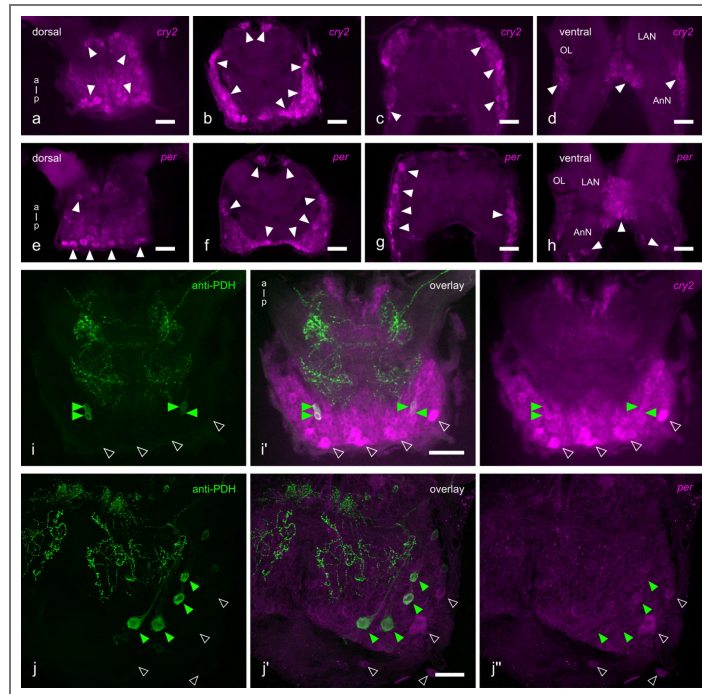
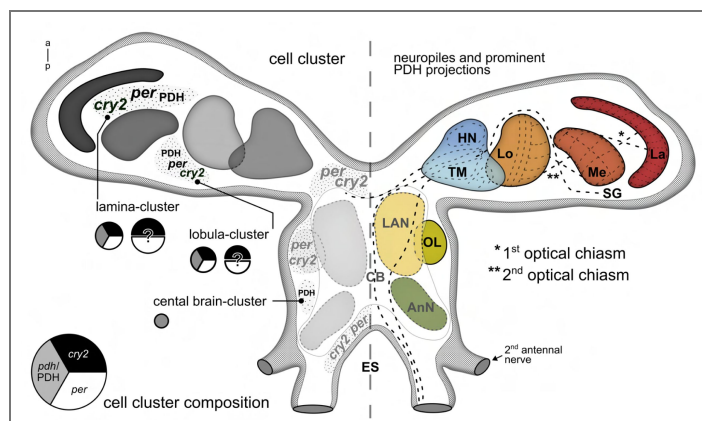


Figure 7. Location of clock neurons and neuropile regions in the supraesophageal ganglion of krill.

Left hemisphere: Schematic representation of the general location of neurons expressing *cry2* and *per* and or *pdh* (dotted areas). Cells expressing *cry2*, *per*, and *pdh* are considered clock neurons. In the optic lobe, the location and the number of *cry2* and *per*-expressing neurons suggest colocalization also in PDH-negative neurons, categorizing them as potential clock neurons. However, we could not investigate colocalization via *in situ* hybridization due to methodological limitations. *per* and *cry2* expression in the central brain was present in numerous cells, but distinct clusters could not be defined. Note that PDH-positive cells in the central brain did not colocalize with *per* or *cry2*-expressing cells. Right hemisphere: Schematic representation of the neuropiles identified in the krill brain. Black dashed lines represent major PDH-positive projections. Abbreviations: La, lamina; Me, medulla; Lo, lobula; TM, medulla terminalis; SG, sinus gland; HN, hemiellipsoid neuropile; LAN, lateral antennal neuropile; OL, olfactory lobes; AnN; antennal neuropile; ES, esophagus; CB, central brain.



In addition to the distinct PDH-positive clusters, we found single PDH-positive cells that project into the medulla terminalis. Similarly, additional single or pairs of PDH-ir cells have been identified in *Homarus americanus*, which project towards the medulla terminalis and continue through the eyestalk into the median protocerebrum (termed A and B cells in Harzsch et al., 2009). In krill, however, we lost their projections in the dense arborizations of the medulla terminalis.

Although the position and projection patterns of the lobula-cluster described here largely match those of cell clusters described for various crustacean species, the lamina-cluster is the largest, most prominent PDH-positive cluster we identified in krill. While it was difficult to follow the single fibres due to their frequent superposition, most of the lamina-cluster neurons appear to send projections into the lamina; only a few direct projections into the medulla have been identified. In other malacostracans, clusters of cells which primarily target the lamina have been documented (e.g., *Carcinus maenas* (Alexander et al., 2020; Mangerich et al., 1987), *Cancer productus* (Hsu et al., 2008), *Orconectes limosus* (de Kleijn et al., 1993), and *Procambarus virginialis* (Luna et al., 2010)). The lamina-associated clusters described in these species are characterised by a large number of small PDH-ir soma, distributed across the proximal side of the lamina. While similar PDH-positive cells have been found in krill, the lamina-cluster additionally contains many large PDH-positive cells distributed in a distinct, elongated cluster between the medulla and lamina. To our knowledge, a cluster with similar morphology has so far not been reported in malacostracan or other crustaceans.

In the central brain of krill, the most extensive PDH-ir structures appear in the median protocerebrum, but also structures in the tritocerebrum show complex immunoreactive projections, which is in agreement with other malacostracan crustaceans studied (Chung & Webster, 2004; Harzsch et al., 2009; Hsu et al., 2008; Mangerich & Keller, 1988; Sullivan et al., 2009; Wilcockson et al., 2011). In krill, several fibre bundles travel through the eyestalks, some of which form prominent commissures in the dorsal and ventral parts of the anterior median protocerebrum. Like this, they provide a direct connection between the left and right optic lobes, which is in agreement with patterns found in many malacostracan crustaceans (Chung & Webster, 2004; Harzsch et al., 2009; Hsu et al., 2008; Mangerich & Keller, 1988; Sullivan et al., 2009; Wilcockson et al., 2011) and insects (Reischig et al., 2004; Wei et al., 2010). Three to four strongly stained PDH-positive cells per hemisphere are located dorsally in the medial protocerebrum of krill and send their projections anteriorly into the median protocerebrum, where we lost their path. The position and projection pattern of these cells resemble single PDH-ir cells found in *Carcinus maenas* and *Orconectes limosus*, which send their projections into the optic neuropil located in the median protocerebrum (Mangerich & Keller, 1988), as well as in *Homarus americanus* embryos, where they have been termed group E cells, which first project anteriorly but are suggested to join axon projections from the optic lobes into the esophageal connectives (Harzsch et al., 2009).

Early studies found PDH, together with its counterpart RED PIGMENT CONCENTRATING HORMONE (RPCH), as a potent effector of pigment migration in crustaceans (Rao, 2001). In this process, PDH induces the dispersion of pigments to shield vulnerable parts of the organism against harmful radiation during the day, while RPCH reverses this process into a night-adapted state. In krill, large areas of the dorsal and lateral body are covered by erythrophores, chromatophore cells which contain red pigment granules (astaxanthin in krill), shown to rapidly expand upon light exposure or stress (Auerswald et al., 2008). Due to the rapid attenuation of light in the water column, krill are exposed to varying levels of light during their daily vertical migrations. Especially during the hours just before the morning descent, when krill are feeding typically in the upper water column, they can be exposed to elevated light levels. In addition, during midsummer, when darkness during the night is absent, but krill have a strong need to feed, they may spend extended amounts of time in illuminated waters (Cisewski et al., 2010), where protection from harmful radiation is crucial, and thus pigment dispersion during these times acts as a natural sunscreen. In turn, full contraction of the pigments will render krill almost transparent, which will reduce the risk of visual predation.

Besides chromatophore dispersion, PDH was found to induce movement of distal pigment cells in the eye, which puts it into a light-adapted state (Fernlund, 1976 [↗](#); Rao & Riehm, 1993 [↗](#); Verde et al., 2007 [↗](#)). In addition, in crayfish, PDH impacts the electrical response of photoreceptor cells to light, and this response depends on the circadian phase (Solís-Chagoyan et al., 2012 [↗](#); Verde et al., 2007 [↗](#)). Both processes can modulate the retinal light signal and are likely under circadian control. While light-adaptation mechanisms, such as those mentioned above, have not been studied systematically in *E. superba*, circadian rhythms of visual sensitivity measured by electroretinogram have been reported for the closely related arctic krill *Thysanoessa inermis* (Cohen et al., 2021 [↗](#)), suggesting that a similar regulation could be present in *E. superba*, too.

Besides its role in crustacean pigment migration, PDF, the insect homologue of PDH, is best known as an output factor of the insect circadian clock, where it functions as a neurotransmitter or neuromodulator and co-localizes with a subset of clock neurons (Helfrich-Förster, 1995 [↗](#)). Although the functional role of PDH as an output factor of the circadian clock has yet to be demonstrated in crustaceans, its direct homology to insect PDF and the general similarities in neuronal localization and projection patterns strongly suggest this. Furthermore, its involvement in regulating rhythmic physiological processes supports this interpretation. Interestingly, in contrast to insects, crustaceans have been shown to express several β -PDH isoforms, which are thought to differ in their function as neurohormone and/or neurotransmitter and thus in the regulation of the various processes described above (Alexander et al., 2020 [↗](#); Hsu et al., 2008 [↗](#); Klein et al., 1994 [↗](#); Mayer et al., 2015 [↗](#); Rao, 2001 [↗](#)). The β -PDH antibody applied here has been shown to reliably detect two isoforms (PDH-1 and PDH-3), which are thought to cover both neurohormonal and neuromodulatory roles in other crustaceans (Alexander et al., 2020 [↗](#); Hsu et al., 2008 [↗](#)). Interestingly, the PDH antibody was shown to exhibit differential reactivity to the various isoforms investigated (Alexander et al., 2020 [↗](#)), which could explain the variations in staining intensity observed in some of the PDH-ir neurons in krill. Although the presence of PDH isoforms and their specific function as neuromodulators or -hormones has not yet been studied in krill, this represents an interesting subject for future research into the krill clock and its outputs.

The circadian clock is located in the krill optic lobes

Due to its conserved sequence and the availability of potent antibodies, the location of PDH-ir neurons has been described in several crustaceans. But insights into the distribution of clock gene- and protein-expressing cells and their co-localization with putative output factors like PDH are scarce, and detailed descriptions, comparable to those of the insect clock system, are missing. However, to characterize the neuronal architecture of the circadian clock, the localization of its molecular components and their co-localization with output factors are prerequisites.

In crustaceans, most information is available from studies on crayfish using antibodies against *Drosophila melanogaster* clock proteins. In *Procambarus clarkii*, immunolabeling identified CRY-positive cells in the eyestalks and the anterior medial protocerebrum (Fanjul-Moles et al., 2004 [↗](#)). Additional work using antibodies against PER, TIM, and CLK revealed their widespread expression in anterior and lateral central brain cell clusters, as well as in the eyestalk (Escamilla-Chimal et al., 2010 [↗](#)). In agreement with that, *Cherax destructor* expresses dCRY in cells of the same anterior central brain cluster, known as cluster 6 in crustaceans (Sullivan et al., 2009 [↗](#)).

Immunocytochemical analyses in *Procambarus clarkii* detected PER in retinal photoreceptors and in cell bodies of the lamina. However, other regions were not investigated in this study (Arechiga & Rodríguez-Sosa, 1998 [↗](#)). In *Homarus americanus*, PER cycling has been reported at the tissue level in the eyestalks, with no signal detected in central brain extracts (Grabek & Chabot, 2012 [↗](#)), while immunolabeling in the crab *Cancer productus* revealed CYC/PDH co-localization in a cluster 6 cell in the protocerebrum (Beckwith et al., 2011 [↗](#)).

Outside of the Decapoda, clock genes or proteins have been localized in the marine isopods *Eurydice pulchra* and *Parhyale hawaiiensis* (Oliphant et al., 2025 [↗](#); Zhang et al., 2013 [↗](#)), and the terrestrial isopod *Armadillidium vulgare* (Fouda et al., 2010 [↗](#)). In *Eurydice pulchra* and *Parhyale hawaiiensis*, transcripts of the clock genes *cry2*, *per*, and *tim* are restricted to the protocerebrum, where distinct clusters of clock neurons appear to be responsible for the control of circatidal and

circadian rhythmicity (Oliphant et al., 2025 [↗](#)). In *Armadillidium vulgare*, few neurons expressing CLK, CYC, and PDF were localized in the optic lobes between the medulla and lobula, as well as laterally in the suboesophageal ganglion (Fouda et al., 2010 [↗](#)).

In summary, in crustaceans, clock components are most commonly found in both the optic lobes and the central brain, associated with the optic neuropils and the protocerebrum, respectively. This organization is consistent with the general architecture of the insect circadian system (reviewed in Helfrich-Förster et al., 1998 [↗](#); Numata et al., 2015 [↗](#)) and might represent the organizational principle of the clock network in arthropods in general.

Using *in situ* hybridization together with immunohistochemistry, we found two distinct cell clusters in the optic lobe. While the number of *cry2/per*-expressing cells was higher than the *pdh*-expressing cells, all PDH-positive cells in the optic lobe clusters showed colocalization with either *cry2* or *per*, strongly suggesting a functional role of PDH in the krill circadian clock. In contrast to the expression in the optic lobes, extensive expression of *cry2* and *per* transcripts was detected throughout large parts of the krill central brain and did not colocalize with anti-PDH. Detailed studies from several insect species show that clock neurons in the central brain are typically organized into specific clusters, most commonly found in the dorsal protocerebrum (reviewed in Helfrich-Förster et al., 1998 [↗](#); Numata et al., 2015 [↗](#)). The widespread expression of *cry2* and *per* throughout most parts of the central brain of krill is in stark contrast to the general pattern found in insects, as well as its distinct expression pattern found in the krill optic lobes. Because of methodological limitations, we could not verify if *per* and *cry2* are co-expressed in these cells or if the transcripts are present in distinct cells. The broad expression might indicate that the clock transcripts in the central brain of krill may fulfil other or additional purposes unrelated to the circadian clock. Indeed, non-clock-related functions of clock genes have also been reported in insects (Beaver et al., 2003 [↗](#); Tobbäck et al., 2011 [↗](#); Tobbäck et al., 2012 [↗](#)).

In summary, our results support the conclusion that the clock gene-expressing neurons located in the optic lobes represent the pacemaker of the circadian clock in krill. However, due to the current lack of antibodies against krill clock proteins, our analysis of core clock components is limited to the transcript-level, which might not fully represent the protein level. In addition, methodological limitations currently prevent us from investigating daily and circadian changes in clock gene transcript levels. In an earlier study on krill, Mazzotta et al. (2010 [↗](#)) reported subtle daily changes in CRY2 levels by Western blot on extracts of whole krill heads, suggesting that CRY2 might cycle at the protein level in krill. Future studies may build on our findings to localize clock proteins and quantify their temporal changes at the cellular level.

Potential homology to the insect circadian clock network

Across the tree of life, considerable variability is observed in the presence and role of single clock genes and proteins (Kotwica-Rolinska, Chodakova, et al., 2022; Tomioka & Matsumoto, 2010 [↗](#)), the use of retinal or extraretinal entrainment pathways (Helfrich-Förster, 2019 [↗](#); Yoshii et al., 2015 [↗](#)), and the exact location and projections of clock neurons (Helfrich-Förster, 2004 [↗](#), 2017 [↗](#); Helfrich-Förster et al., 1998 [↗](#); Numata et al., 2015 [↗](#)). However, its general molecular structure and gross neuronal architecture are conserved at least across insects (Helfrich-Förster et al., 1998 [↗](#); Numata et al., 2015 [↗](#)). In terms of the neuronal architecture, a unifying feature in the distribution of the circadian clock appears to be the separation into dorsal (DN) and lateral (LN) clock neurons. While the DN are found in the dorsal central brain, typically anterior in the medial protocerebrum, the LN are closely associated with neuropils of the light signalling pathway (Helfrich-Förster et al., 1998 [↗](#); Numata et al., 2015 [↗](#)).

Due to the well-known function of PDF as a clock output factor in insects (reviewed in Hermann-Luibl & Helfrich-Förster, 2015 [↗](#); Shafer & Yao, 2014 [↗](#)) and based on the location and projection patterns of PDH-ir neurons, several studies have discussed potential homologies of PDH-ir clusters to the well-studied insect circadian clock architecture (reviewed in Strauß & Dirksen, 2010 [↗](#)). In the following, we will briefly outline similarities between clock neurons and PDH-ir structures found in krill and insects.

Although absent in flies, the most lateral PDH-ir clusters are those associated with the lamina, as found in locusts, crickets, and cockroaches (Homberg & Prakash, 1996 [↗](#); Homberg et al., 1991 [↗](#); Nässel et al., 1991 [↗](#); Wei et al., 2010 [↗](#)). In these insects, the neurons are organized in distinct ventral and dorsal clusters at the proximal side of the lamina. Cockroaches lack the light-sensitive dCRY, and entrainment of the clock depends on the compound eyes. The PDF-positive lamina neurons are thought to be involved in this pathway (reviewed in Helfrich-Förster, 2019 [↗](#); Stengl et al., 2015 [↗](#)). In krill, we identified a PDH-positive cluster of clock neurons with large somata whose projections mainly target the lamina. In addition, we observed extensive PDH-positive connections between the lamina and medulla, as well as the medulla and lobula, which could at least partly originate from cells of the lamina-cluster. In addition, we showed that the PDH-positive cells of the lamina-cluster co-localize with transcripts of *cry2* and *per*, which are thought to function as transcriptional repressors in the krill core clock (Biscontin et al., 2017 [↗](#)). Thus, the clock neurons of the lamina-cluster could function as an input pathway of the retinal light signal to the circadian clock. At the same time, they could provide a mechanism to modulate the incoming light signal in a circadian fashion. Additionally, they could also contain circadian clock neurons that control behavioural rhythms, as suggested for the cricket, *Gryllus bimaculatus* (Abe et al., 1997 [↗](#); Tomioka & Chiba, 1986 [↗](#), 1992 [↗](#)) and the New Zealand weta *Hemideina thoracica* (Waddell et al., 1990 [↗](#)), in which the main circadian pacemaker neurons were traced to neurons located between the lamina and medulla. Although up to now we can only speculate on the functional role of the lamina clock neurons in krill, the general size of their compound eyes, the restriction of putative pacemakers to the optic lobes, and their overall light-centred lifestyle collectively indicate a high priority towards light processing and signalling, which could explain the prominent lamina-cluster in krill.

Another cluster of PDH-positive clock neurons is located between the medulla and lobula in krill, which closely resembles cluster C neurons in other crustaceans (reviewed in Meelkop et al., 2011 [↗](#)). Based on their characteristic position between medulla and lobula, as well as their arborization patterns, projecting into the medial protocerebrum and the contralateral optic lobe, they have been thought to be homologues of LN found closely associated with the accessory medulla in several insects, including flies, bees, cockroaches, and aphids (Beer et al., 2018 [↗](#); Colizzi et al., 2023 [↗](#); Helfrich-Förster & Homberg, 1993 [↗](#); Wei et al., 2010 [↗](#)). The accessory medulla is a key centre of the circadian clock, which integrates photic input and the signals from various clock neurons to control rhythmic output (reviewed in Helfrich-Förster, 2019 [↗](#); Stengl et al., 2015 [↗](#)).

As shown for several decapod crustaceans, a subset of PDH-positive neurons of the lobula-cluster seems to terminate in the sinus gland, and in krill, these cells express *cry2* and *per*, strongly suggesting that they form a part of the clock network. The sinus gland is a major neurohemal centre in crustaceans, serving as the primary release site for a diverse set of regulatory hormones (reviewed in Simoes et al., 2024 [↗](#)). Neuropeptides such as PDH, RPCH, MOLT-INHIBITING HORMONE, GONAD-INHIBITING HORMONE, and members of the CRUSTACEAN HYPERGLYCEMIC HORMONE family are secreted from the sinus gland into the haemolymph, where they regulate processes such as light sensitivity and chromatophore-mediated colour change, moulting, vitellogenesis, maturation, and osmoregulation. Further, rhythmic hormone release from the sinus gland is thought to drive circadian rhythms in the response to light, which depends on PDH (Hernández-Falcón et al., 1987 [↗](#); Moreno-Sáenz et al., 1987 [↗](#); Moreno-Sáenz et al., 2005 [↗](#)). In addition, daily rhythms in PDH-immunoreactivity of the SG were found in *Cancer productus* (de la Iglesia & Hsu, 2010 [↗](#)). Like many other zooplankton species, krill regularly performs DVM in the water column, which represents an adaptation to the daily change in predation pressure, driven by the daily light-dark cycle (Bahlburg et al., 2023 [↗](#); Gastauer et al., 2021 [↗](#); Hobbs et al., 2021 [↗](#)). Supporting a role for endogenous timing mechanisms in this behaviour, we recently demonstrated that changes in krill swimming activity are under circadian clock control (Hüppe et al., 2025 [↗](#)). Although not explicitly studied in *E. superba*, adaptations of the visual system in response to the day-night cycle have been shown for other daily migrators (Cohen et al., 2021 [↗](#); Deleo & Bracken-Grissom, 2020 [↗](#)). Besides daily rhythms like DVM, krill show pronounced physiological adaptations in response to the strong seasonality of the Southern Ocean environment. This

includes active reproduction, development, and growth in summer, and metabolic reduction and lipid accumulation, as well as halted development in winter (Kawaguchi et al., 2007 [↗](#); Meyer et al., 2010 [↗](#)). Most interestingly, the sexual maturity cycle, closely linked to their seasonal reproduction, is under photoperiodic control (Höring et al., 2018 [↗](#)). Recent studies could show that the circadian clock provides daylength information to time seasonal events and that PDF plays a key role in signalling photoperiodic information to downstream neuroendocrine centres (reviewed in Helfrich-Förster, 2024 [↗](#); Hidalgo et al., 2023 [↗](#); Nagy et al., 2019 [↗](#)). In the context of clock-dependent timing of daily and seasonal physiology and behaviour, the direct connection between PDH-positive clock neurons and the sinus gland in krill thus provides a promising target for future studies on the neuronal basis of daily and seasonal adaptation in the future.

Temporal synchronization at both daily and seasonal timescales is central to the success of krill in the Southern Ocean. Over the past decade, growing evidence has highlighted the major role of endogenous timing mechanisms in shaping this temporal adaptation (Höring et al., 2018 [↗](#); Hüppe et al., 2025 [↗](#); Piccolin, Meyer, et al., 2018; Piccolin et al., 2020 [↗](#); Piccolin, Suberg, et al., 2018). At the same time, reliance on internal clocks may constrain responses under conditions of rapid environmental change (Helm et al., 2013 [↗](#); Visser & Gienapp, 2019 [↗](#)). A detailed mechanistic understanding of the flexibility of clock-based processes is therefore essential to predict krill resilience in a changing Southern Ocean. However, as in most crustaceans, chronobiological research in krill has long remained predominantly phenomenological, providing valuable descriptive insights but leaving many mechanistic questions unresolved. In this study, we present findings that offer a foundational framework for dissecting the underlying mechanisms that drive rhythmic processes in krill. By establishing this mechanistic basis, our work opens the door to a deeper understanding of how these rhythms shape physiological functions, behaviour, and ultimately the adaptive strategies of krill. Together, these advances represent an important step towards a more comprehensive and mechanistic understanding of krill biology and ecology.

Material and Methods

Animal collection and fixation

Adult krill (*E. superba*) of mixed sexes were sampled from the Atlantic sector of the Southern Ocean, north of the South Orkney Islands (60.1°S, 45°W) during austral late summer and autumn (8th February to 21st March 2022). Samples were collected at different times throughout the day-night cycle. Krill were sampled from the continuous fishing system of the krill fishing vessel Antarctic Endurance. Krill were pumped aboard via a vacuum hose attached to the cod end of a commercial trawl, while the vessel trawled at a speed of 1.5 to 2 knots. On deck, the krill were separated from the water on a metal grate, and specimens were sampled from there. The collected krill were kept in surface seawater at 1 °C in constant darkness until further processing. After sampling, sex and length were determined under a stereo microscope before decapitating and fixing their heads in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Additionally, whole krill individuals were fixed in 4% PFA in PBS for whole central nervous system preparations. Samples were stored at 4°C in PFA until further processing.

Sample dissection and preparation

PFA-fixed krill heads were washed at least three times in phosphate-buffered saline (PBS) at room temperature (RT) for ten minutes before dissection in PBS containing 0.5% Triton X-100 (PBST 0.5%). During dissection, the cuticle and muscle tissue were removed to reveal the nervous tissue of the central brain and optic lobes. To facilitate subsequent vibratome sectioning, the retina and ommatidia, especially the crystalline cones of the compound eyes, were removed. For *in situ* hybridization, either whole brains or vibratome sections were dehydrated in a methanol series (30%, 50%, 70%, 90%, and 100% methanol in PBS) for about 20 minutes each. After an overnight incubation at -20°C in 100% methanol, the samples were rehydrated by reversing the methanol series. After rehydration, the samples were kept in PBS at 4°C. For antibody staining, antigen

retrieval was carried out by incubating the brains in citrate buffer (0.01 M citric acid monohydrate, pH = 6.0 with NaOH) for 30 minutes at 80°C. Afterward, the brains were washed three times in PBST 0.5% for 10 minutes and stored at 4°C.

Finally, for both antibody staining and RNA *in situ* hybridization, the brains were embedded in 7% agarose (Sigma, A9539) and sectioned into 100–200 µm slices in ice-cold PBS, using a vibratome (Leica VT1000S; 0.225 mm/s at 50 Hz).

Immunohistochemistry

Horizontal vibratome sections of krill brains were incubated in a blocking solution (5% normal goat/donkey serum, NGS/NDS in PBST + 0.02% NaN₃) for at least three hours at room temperature (RT) or overnight at 4°C. After blocking, the samples were incubated at 4°C for two nights in a primary antibody solution containing 5% NGS/NDS in PBST 0.5%, 0.02% NaN₃, and the primary antibodies in the specified concentration. For labelling PDH-positive neurons, a polyclonal antibody raised in rabbit against synthetic *Uca pugilator* β-PDH (Dircksen et al., 1987 [↗](#); Wilcockson et al., 2011 [↗](#)) was used (1:4000). Anti-β-PDH was kindly provided by David Wilcockson (Aberystwyth University, Wales). For neuropile labelling, a monoclonal anti-SYNAPSIN (1:50) antibody (DSHB Cat# 3C11, RRID:AB_528479 [↗](#)) was used. The incubation with the primary antibody solution was followed by six washes with PBST 0.5% at RT for 20 minutes each. Then, the sections were incubated overnight in a secondary antibody solution containing 5% NGS/NDS in PBST 0.5%, 0.02% NaN₃, and the corresponding secondary antibodies at a concentration of 5 µg/mL. Highly cross-adsorbed secondary antibodies raised in either goat or donkey, and conjugated with Alexa Fluor 488, 555, or 647, were purchased from Thermo Scientific. As a reference staining, the nuclei were counterstained with Hoechst 33342 (10 µM) during the secondary antibody incubation. Vibratome sections were washed five times with PBST 0.5% for 10 minutes before embedding. The sections were arranged on an object slide and mounted in Vectashield antifade mounting medium (Vector Laboratories; H-100) under coverslips sealed with FixoGum (Marabu GmbH). The samples were stored in the dark at 4°C until imaging.

In situ hybridization

Probe generation

To map the expression of clock gene transcripts in krill, RNA probes targeting *E. superba cry2* and *per* mRNA were synthesized from krill cDNA.

Krill cDNA was obtained from whole head RNA extracts using krill collected from the Southern Ocean, as described above. After sampling, whole krill heads were fixed in RNAlater™ stabilizing solution (Thermo Scientific) for 24 hours at 4°C and stored at -80°C until further processing. The heads were homogenized in TRIzol™ Reagent (Thermo Scientific), and total RNA was extracted using a combination of chloroform-based phase separation followed by purification with the Direct-zol Miniprep Plus Kit (Zymo Research). The RNA quality and quantity were assessed photometrically using a NanoDrop (Thermo Scientific) and via on-chip gel electrophoresis using a 2100 Bioanalyzer (Agilent Technologies). Subsequently, cDNA was synthesized using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific).

Krill cDNA (1.3 ng/µL) was used with the Phusion™ High-Fidelity PCR Kit (New England Biolabs) and the corresponding primers for each gene (0.5 µM), as detailed in [Supplementary Table 1 \[↗\]\(#\)](#), following the manufacturer's protocol. The PCR products were validated on a 1% agarose gel using gel electrophoresis and purified using the MSB-Spin PCRapace & Fragmet Clean Up Kit (Strattec).

Next, PCR products were cloned to obtain plasmid templates. 15 ng of blunt-end amplicons were ligated into the vector (pJET1.2/blunt) using the CloneJET PCR Cloning Kit (Thermo Scientific). Vectors with inserts were then transformed into *E. coli* using Stellar™ Competent Cells. 5 µL of each ligation mixture were added to the competent cells (50 µL) and incubated on ice for 20–30 minutes. Then, the cells underwent a one-minute heat shock at 42 °C, with brief mixing halfway through. After adding 200 µL of Luria–Bertani (LB) medium, the cells were allowed to recover for

one hour at 37 °C. For each construct, the transformed cultures were plated on LB-ampicillin agar and incubated for 16–18 hours at 37 °C. After incubation, single colonies were picked and incubated with 4 mL of LB medium containing ampicillin at 37 °C for 16 hours on a shaker.

To extract the plasmid DNA from each culture, 4 mL of the culture was pelleted by centrifugation and used with the Plasmid Miniprep Kit (New England Biolabs), following the manufacturer's protocol. The subsequent plasmid DNA was validated by restriction digest (37°C, 1 h) with XbaI and XhoI (FastDigest restriction enzymes, Thermo Scientific), followed by 1 % agarose gel electrophoresis. Plasmids were quantified using a Nanodrop and verified by Sanger sequencing (Mix2Seq, Eurofins). Only constructs containing the correct insert in antisense orientation were used for probe synthesis.

The plasmids (1–2 µg) verified by sequencing were linearized with XbaI at 37 °C, 1 h, checked on a 1 % agarose gel by gel electrophoresis, and then purified using the MSB-Spin PCRapace & Fragment Clean-Up kit.

Digoxigenin (DIG)-labelled RNA probes were synthesized from 700–1000 ng of linearized plasmid using T7 RNA polymerase and a DIG RNA labelling mix (Roche) in a 20 µL reaction and incubated for two hours at 37 °C, according to the manufacturer's protocol. Reactions were terminated with 2 µL of ethylenediaminetetraacetic acid (EDTA, 0.2 µM, pH 8). The transcripts were then purified using LiCl/ethanol precipitation (2.5 µL, 4 M LiCl), followed by a 70% ethanol wash. The samples were dried and resuspended in 50µL nuclease-free water. To each resuspended probe, 200µL of hybridization buffer (Hyb) was added to dilute the probes for storage at –80 °C. Hyb contained 50% formamide, 0.1 µg/mL tRNA and 0.1 µg/mL ssDNA, 2% blocking solution for nucleic acid hybridization (Roche), and 0.05 µg/mL heparin in 5x saline-sodium citrate buffer pH 7 and 0.1% Triton-X 100.

RNA in situ hybridization procedure

Hybridized RNA probes were labelled with an anti-DIG antibody and visualized using enzymatic reactions with alkaline phosphatase (AP) with either BCIP/NBT (Thermo Scientific) or Fast Red (Sigma-Aldrich). In addition to the coloured precipitate produced by the AP reaction, Fast Red can be detected by excitation at 570 nm.

The tissue permeability and RNA accessibility were increased by incubating the vibratome sections for 10 minutes in PBST 0.3%, followed by a 10-minute treatment with proteinase K (10 µg/mL). Proteinase K activity was stopped by adding glycine (40 µg/mL) and subsequent incubation at RT for 10 minutes. The samples were refixed with 4% PFA in PBST 0.3% for one hour at RT, followed by three PBST 0.3% washes for 10 minutes each. Unless noted otherwise, all following incubation steps and reagents used during *in situ* hybridization were carried out and applied at 48 °C in a water bath. The samples were incubated for 10 minutes in a solution of 50% Hyb in PBST 0.3%. Next, the samples were prehybridized in Hyb for one hour. *Cry2* and *per* RNA probes were diluted to their final concentration with Hyb and allowed to hybridize with the samples' mRNA for at least 16 hours. Afterward, the sections were washed for 20 minutes in Hyb followed by 50% Hyb in PBST 0.3%. Finally, the samples were washed with PBST 0.3% five times for five minutes each. The RNA *in situ* hybridization was followed by antibody staining to detect the DIG-labelled RNA probes. The blocking and primary antibody incubation were performed as described for immunohistochemistry. For samples subjected to RNA *in situ* hybridization before antibody staining, the primary antibody solution additionally contained AP-conjugated anti-DIG antibody raised in sheep (1:5000; Fab-fragments; Roche). Before the incubation with the secondary antibody solution, the AP reaction was initiated. The samples were washed three times for 10 min and two times for 20 min in PBST 0.5% and then five times for 10 min with a phosphate-free AP buffer at room temperature. AP buffer contained 1.2% TRIS, 0.6% NaCl, and 0.1% MgCl₂ in water (w/v); the pH was adjusted to 9.5 with HCl. For the AP reaction, the samples were incubated in the dark in a staining solution either for BCIP/NBT or Fast Red and monitored closely until tissue coloration was observed (10 minutes to four hours). After successful detection of the RNA probes, the reaction

was stopped by at least four 10-minute washes with PBST 0.5%. Afterward, the antibody staining went on with a second blocking step followed by the incubation with the secondary antibody solution.

Image acquisition

Images were acquired with a Leica TCS SP8 confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a hybrid detector. A white light laser (Leica Microsystems, Wetzlar, Germany) was used for excitation. We used a 20-fold glycerol immersion objective (0.73 NA, HC PL APO, Leica Microsystems, Wetzlar, Germany) for scans of the vibratome sections. We obtained 8-bit or 16-bit confocal stacks with a maximal voxel size of $1.14 \times 1.14 \times 2.0 \mu\text{m}$ to $0.76 \times 0.76 \times 2 \mu\text{m}$ for overview recordings and $0.76 \times 0.76 \times 2 \mu\text{m}$ to $0.23 \times 0.23 \times 2 \mu\text{m}$ for details. The optical section thickness was always $3.12 \mu\text{m}$. Image tiles for overview images were recorded with 1024×1024 pixels and 2048×2048 pixels for detail images using LasX (3.5.7.23225) with the Navigator Plugin. Image tiles were recorded with 10% overlap, and overview images of the whole vibratome section were stitched afterward in LasX with Mosaic Merge setting “blend” to “statistic”. If the signal loss was too strong along the z-axis, a z-compensation was used by increasing the laser power with increasing imaging depth.

For widefield microscopy, a Leica M165FC fluorescent microscope (Leica Microsystems, Wetzlar, Germany) and a Nikon SMZ1270i (Nikon, Minato, Japan) were used. Images were taken with LAS AF and NIS-Elements software, respectively. If necessary, images were stitched using the Pairwise Stitching Plugin (Preibisch et al., 2009) for Fiji (Version 2.16.0; Schindelin et al., 2012). For brightfield and fluorescent scans of the total nervous system of krill, the preparation was embedded in 2% Low Melt Agarose in distilled water, using a customized silicon spacer (thickness: 1.5 mm), which was fixed with glycerol gelatine to a microscope slide. The nervous system was imaged using a Leica Inverted Microscope Thunder Imager (Leica Microsystems, Wetzlar, Germany). The microscope is equipped with Thunder deconvolution. The samples were imaged as Z-stacks (minimum voxel size: $1.3047 \times 1.3047 \times 62.6722 \mu\text{m}$). Images were only adjusted linearly for brightness and contrast, using Fiji.

3D Reconstruction

For the reconstruction of the supraesophageal ganglion, five vibratome sections were scanned as a tile scan. Each vibratome section was merged in LasX. The merged sections were opened in Fiji, and any remaining signal loss along the z-axis was corrected for using the Stack Contrast Adjustment Plugin (Capek et al., 2006). The adjusted images were opened with the Big Stitcher Plugin (Hörl et al., 2019). The vibratome sections were manually aligned using nuclear and neuropile staining as a reference. The aligned sections were exported as a single stack and the neuropiles were reconstructed using TrakEM2 (Cardona et al., 2012). Afterward, the reconstructed neuropiles were exported as labels, and Fiji 3D Viewer was used to generate 3D objects. The 3D objects were then imported into Blender (5.0; Blender Development Team, 2025), and the surfaces were smoothed to remove artefacts. For the 3D visualization of the PDH-network, the Microscopy Node Plugin for Blender (2.2.7; Gros et al., 2025) was used. For the 3D rendering, the PDH-fibre network was extracted from the background using a custom macro (Reinhard et al., 2025).

Supplementary Material

Primer	Sequence (5'-3')
Es_period 1, 5'	CCCTGTTGCTAGTCCTAATGCTGTG
Es_period 1, 3'	CGGCAATGTGTTGTGCTACAGGC
Es_cry2_a, 5'	GACTGGAGCATCATCTTGAGAGGAAAG
Es_cry2_a, 3'	CAACAGCATGACGGGCCACATTG
Es_cry2_b, 5'	GTTTCCTCACTCGGGGTGACCTTTG
Es_cry2_b, 3'	CACTCTGTAGTGAGATAGTTGTTGATAGAC

Supplementary Table 1. Primer sequences used to synthesize *in situ* hybridization probes. For *cry2* in situ hybridizations, a mix of two probes, termed Es_cry2_a and Es_cry2_b, was used.

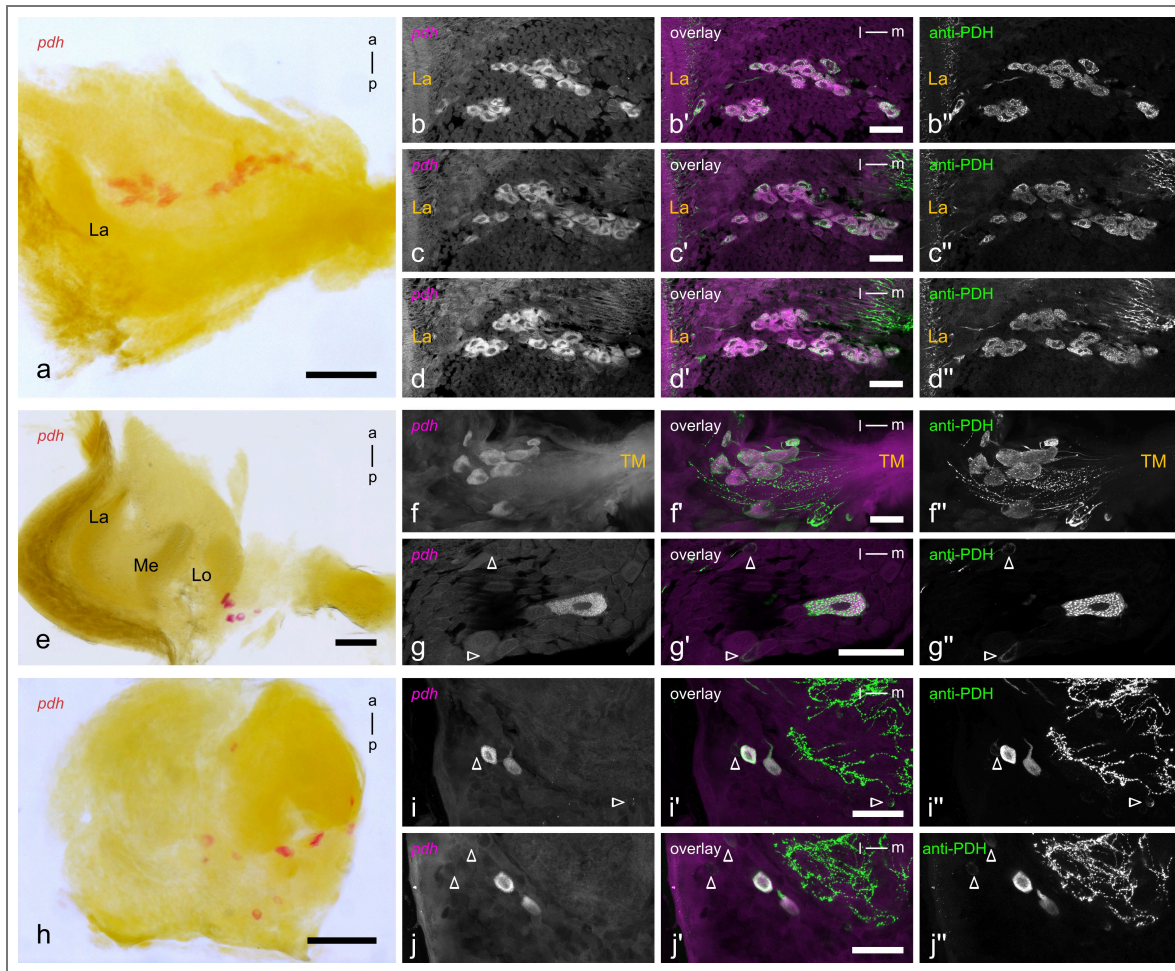


Figure 2—figure supplement 1. *pdh* in situ hybridization colocalizes with PDH-immunoreactivity.

a) *pdh* in situ hybridization (red) on a horizontal vibratome section labels a big cell cluster below the lamina (La). b-d) single focal planes through the *pdh*-expressing cell cluster (magenta) from anterior (b) to posterior (d), double labelled with anti-PDH (green), show PDH-immunoreactive (PDH-ir) cells are indeed all expressing *pdh*. e) A second cluster of *pdh*-expressing cells is found in the eyestalk, closely associated with the lobula (Lo). f, g) Double labelling with anti-PDH shows colocalization of strongly PDH-ir cells and *pdh*-expressing cells also in this cluster. Occasionally, we found weak PDH-ir cells (blank arrowheads in (g)) where we could not unambiguously identify colocalization between PDH-ir and *pdh*-expression. h) *pdh* in situ hybridization reveals several *pdh*-expressing cells in the central brain. i, j) Double labelling with anti-PDH shows, like for the other clusters, that strongly immunoreactive cells are also expressing *pdh*. While we could only reliably identify two cells per hemisphere (i, left; j, right hemisphere) labelled by in situ hybridization using fluorescence microscopy, for the chromogenic labelling, multiple cells that we might not have been able to distinguish from the strong autofluorescent background in fluorescent microscopy (compare h and i/j). Hence, we could not show colocalization for weak PDH-ir cells in the central brain (blank arrowheads in (i, j)), similar to the weakly immunoreactive cells close to the lobula. Scale bars in a, e, h represent 250 μ m and 100 μ m for the other panels. Abbreviations: Me, medulla, TM, medulla terminalis; a, anterior; p, posterior; l, lateral; m, medial.

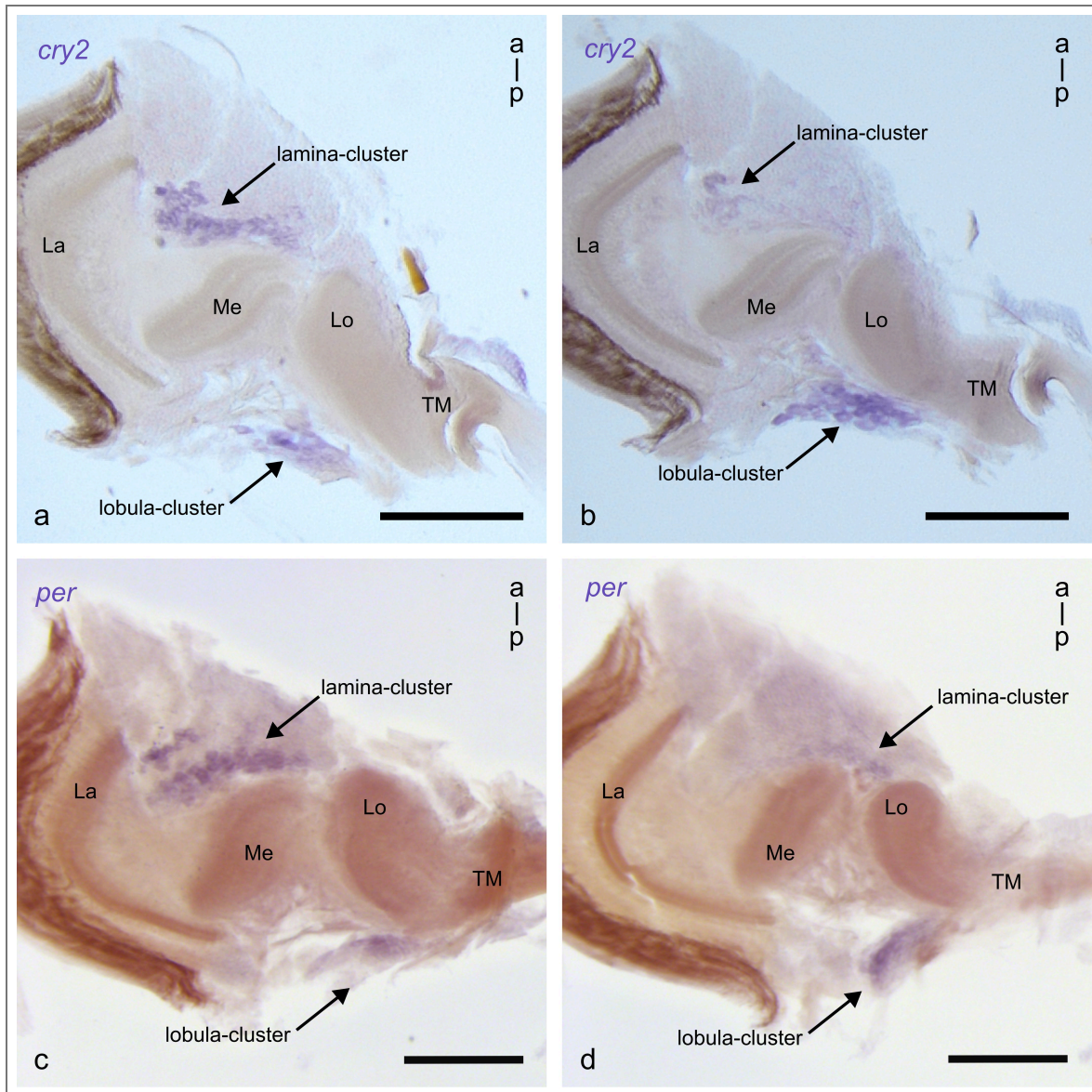


Figure 5—figure supplement 1. Chromogenic RNA *in situ* hybridization of clock gene mRNA on horizontal vibratome sections of the optic lobe.

Bright field image of the *cry2* (a, b) and *per*-expression (c, d) in the optic lobes of krill, visualized by chromogenic *in situ* hybridization (violet, arrows). Expression of *cry2* and *per* matches the location of the PDH-positive lamina- and lobula-cluster identified by antibody staining. Scale bars represent 250 μ m. Abbreviations: La, lamina; Me, medulla; Lo, lobula; TM, medulla terminalis; a, anterior; p, posterior.

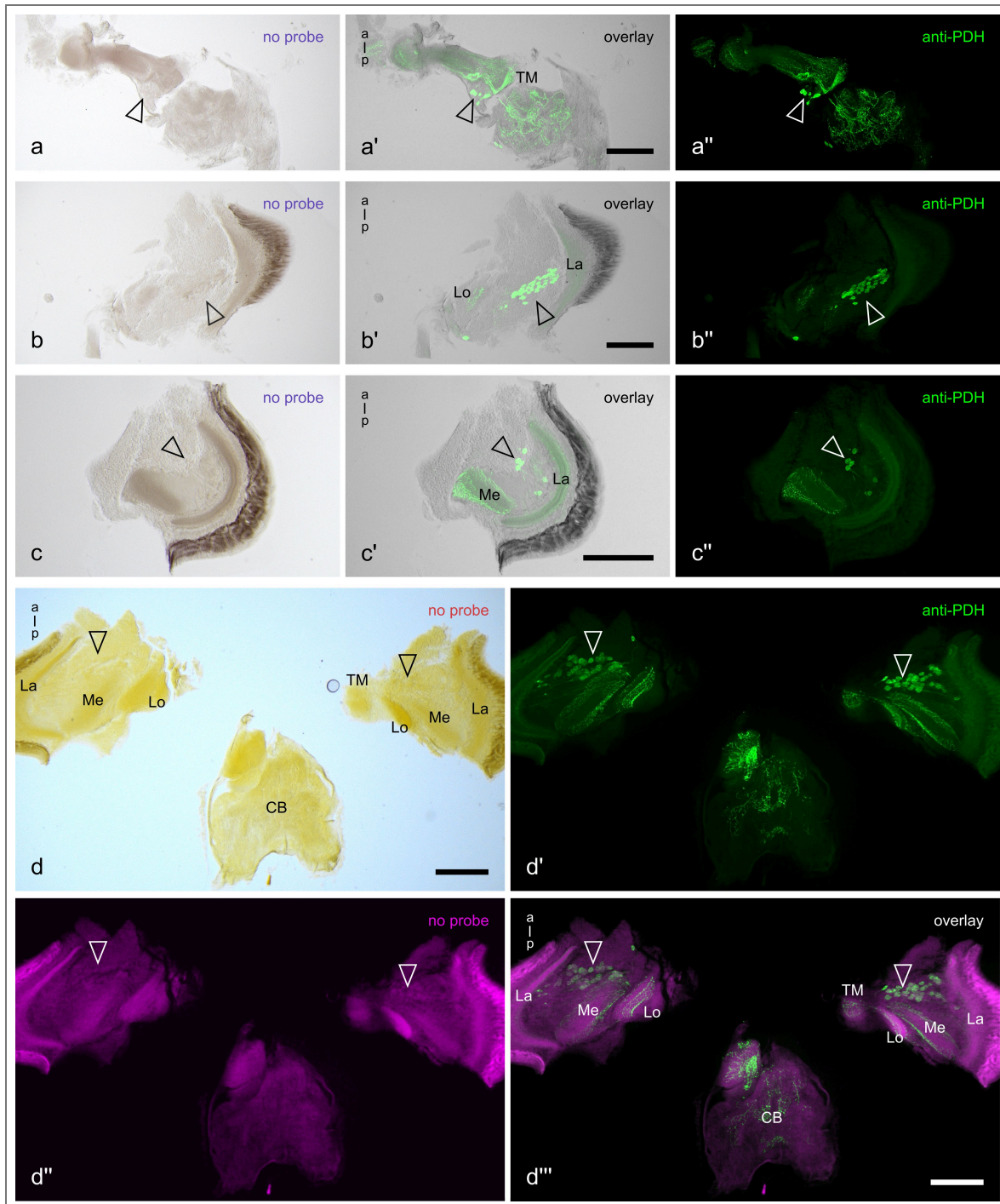


Figure 5—figure supplement 2. Negative controls for RNA *in situ* hybridization.

a-d''') Horizontal vibratome sections of krill optic lobes were used in chromogenic *in situ* RNA hybridization without RNA probes as a control for unspecific antibody binding or discoloration of the tissue. (a-c'') sections using BCIP/NBT (violet, a, b, c) co-labelled with anti-PDH (green, a'', b'', c'') do not show any *in situ* hybridization signal, hence also no colocalization with PDH-positive cells (blank arrowheads, a', b', c'). This was true for the lobula-cluster (a-a'') and for the lamina-cluster (b-b''). d-d''') Vibratome sections of krill optic lobes used in chromogenic *in situ* RNA hybridization without RNA probes, stained by Fast Red (red/ magenta, d, d'') and subsequent immunolabeling with anti-PDH (green, d', d'''), showed, similar to BCIP/NBT, no signal for the *in situ* hybridization and hence no colocalization with anti-PDH (blank arrowheads, d'''). d) brightfield micrograph. d'-d''') fluorescent micrographs. Scale bars represent 250 μ m.

Data availability

All data are included in the manuscript and supporting files.

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Additional files

Figure 2-figure supplement 2. [Video showing the 3D projection of the entire PDH-ir network.](#)

Figure 2-figure supplement 3. [Video showing single focal planes of the projection of the entire PDH-ir network.](#)

Figure 4-figure supplement 2. [Video showing the 3D projection of the PDH-ir network in the central brain.](#)

Additional information

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Peer reviews

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The authors used antibody staining against PDH across the whole central nervous system and additional in situ hybridization for cry2 and per mRNA, with a focus on the supraesophageal ganglion. There, they identified the major neuropils in the eye stalks and central brain of Antarctic krill. The resulting staining pattern aligns with the identified circadian clock network in insects and PDH-expressing networks in other crustaceans, making these neurons highly likely candidates for krill clock neurons.

Strengths:

(1) This study provides the first clues about the circadian clock architecture in a non-model organism in chronobiology, Antarctic krill, with a clear 3D reconstruction of the putative clock network.

(2) The authors effectively place their results within the extensive body of literature on arthropod circadian clock networks to argue that the neurons they describe are likely the circadian clock in krill.

Weaknesses:

(1) The data presented here are not sufficient to support the claim that the described network is the circadian clock because functional evidence is missing.

(2) Additionally, the study falls short of identifying any elements of the positive limb of the canonical circadian clock transcriptional-translational feedback loop, e.g., *clk* or *cyc*, in the PDH-expressing neurons.

(3) No sample sizes are reported, making it difficult for readers to assess the generalizability of the presented data.

<https://doi.org/10.7554/eLife.110990.1.sa2>

Reviewer #2 (Public review):

Summary:

This study advances our understanding of the neuronal basis of the circadian clock in pancrustaceans. It extends our knowledge on the pigment-dispersing hormone system and provides links to information on the expression of core clock components, cryptochrome 2, and period. The data are sound and well-documented.

Comments:

The neuronal components of the arthropod circadian clock system have been analysed extensively in insects. Much less information on this system is available on malacostraca crustacea crustaceans. However, considering that malacostracan crustaceans and insects go back to a common pancrustacean ancestor and considering that we know that the brain architecture in these two groups shares many commonalities (see, e. g., extensive reviews by N. J. Strausfeld), we have to expect that crustaceans and insects share many of the characteristics of the circadian system. This is the case, e. g., for the network of pigment-dispersing hormone-positive neurons. The authors cite these studies, although late in the paper (discussion, line 339ff), and I suggest to move this info into the introduction: "339 ff: The arborization pattern of the PDH-network has been described in various malacostracan crustaceans, including *Carcinus maenas* (Alexander et al., 2020; Mangerich & Keller, 1988; Mangerich et al., 1987), *Cancer productus* (Hsu et al., 2008), *Orconectes limosus* (de Kleijn et al., 1993; Mangerich & Keller, 1988; Mangerich et al., 1987), *Homarus americanus* (Harzsch et al., 2009), *Cherax destructor*, *Procambarus clarkii* (Sullivan et al., 2009), and *Procambarus virginalis* (Luna et al., 2010)."

The strength of this paper is that it extends our knowledge on the PDH system and brings together neuroanatomical information on PDH-positive neurons with information on the expression of core clock components, cryptochrome 2, and period. That way, it advances our understanding of the neuronal basis of the circadian clock in pancrustaceans. The data are sound and well documented, and the authors are to be applauded for the superb dissection presented in Figure 1.

Below, please find some essential suggestions on how to further improve the paper.

(1) Framing of the study:

I know that krill is a key element of the Southern Ocean's food webs, but my sense is that discussing the current findings in a context of resilience of this species to global ocean change means largely overselling this study:

- Lines 47, 48: "and the resilience of this key species in a rapidly changing Southern Ocean."


- Lines 70 ff: "Hence, understanding the mechanisms of adaptation, including biological clocks, is crucial for predicting how species, populations, and whole ecosystems will respond to climate change."

- 154 ff: "The Southern Ocean environment experiences rapid change (Abram et al., 2025; Meredith et al., 2019; Thomalla et al., 2023). To assess krill's resilience to environmental changes, understanding the mechanisms that govern daily and seasonal timing in krill is essential."

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- 597 ff: "A detailed mechanistic understanding of the flexibility of clock-based processes is therefore essential to predict krill resilience in a changing Southern Ocean."

My understanding is that duration of day length is one of the most predictable environmental drivers, and - despite the seasonal changes of day length - nevertheless a very stable one compared to fluctuations of environmental drivers such as temperature or salinity (see, e.g. this recent review on environmental driver fluctuations on nervous system functioning in crustaceans: Stein W, Harzsch S (2021) The Neurobiology of Ocean Change - insights from decapod crustaceans. *Zoology*: 125887.

<https://www.sciencedirect.com/science/article/pii/S094420062030146X> )

I do not see how global ocean change may significantly change day length, and what this study has to do with understanding this species' resilience against ocean change. I suggest that you explain in more detail why the light day length will change in the future or strongly tone this aspect. Statements such as Line 76 ff: "Due to their disproportionate importance for ecosystem function, understanding the resilience of ecological key species is essential in assessing the fate of ecosystems in the future." are completely out of focus here and, again, trying to oversell the current study.

(2) Uncited essential studies of crustacean neuroanatomy, missing connection to contemporary crustacean neurobiology:

- Line 157: "despite the ecological importance of *E. superba*, only very little is known about its neurobiology".

- Line 329: "However, so far, little was known about the neurobiology of krill in general."

I agree that this species' brain is understudied, but this makes it even more important to cite the little information that IS available. Please consider this essential reading for any crustacean neurobiologist: "Sandeman, D.C., Scholtz, G., Sandeman, R.E., 1993. Brain evolution in decapod crustacea. *J. Exp. Zool.* 265, 112-133." to find information on the basic brain anatomy in *E. superba*.

The manuscript in many places seems to reinvent the wheel and raises the impression that our knowledge of crustacean brain morphology is close to zero. The authors in places seem to operate in a vacuum, and I find it disturbing that in a study on the crustacean brain, very few

references are provided to studies on crustacean brain anatomy, such as the following essential book chapter: "Schmidt, M., 2016. Malacostraca. In: Schmidt-Rhaesa, A., Harzsch, S., Purschke, G. (Eds.), *Structure & Evolution of Invertebrate Nervous Systems*. Oxford University Press, Oxford, pp. 529-582. <https://www.researchgate.net/publication/315366157> "

In terms of brain anatomy, I would like to know if the authors have a hypothesis on whether and how their target species' brain structure may be similar or different to the brains of other "shrimps" as described, e. g., in the following studies. If so, please elaborate in the introduction:

Krieger J, Hörnig MK, Sandeman RE, Sandeman DC, Harzsch S (2020), Masters of communication: The brain of the banded cleaner shrimp *Stenopus hispidus* (Olivier, 1811) with an emphasis on sensory processing areas. *Journal of Comparative Neurology* 528(9): 1561-1587.

Meth R, Wittfoth C, Harzsch S (2017) Brain architecture of the Pacific White Shrimp *Penaeus vannamei* Boone, 1931 (Malacostraca, Dendrobranchiata): correspondence of brain structure and sensory input? *Cell and Tissue Research* 369(2): 255-271.

(3) Lacking rigor and command of crustacean brain nomenclature


I suggest that for their brain nomenclature, the authors should rigorously stick to that laid out by Sandeman et al. 1992 (not yet cited in the ms): Sandeman, D.C., Sandeman, R.E., Derby, C.D., Schmidt, M., 1992. Morphology of the brain of crayfish, crabs, and spiny lobsters: a common nomenclature for homologous structures. *Biol. Bull.* 183, 304-326.


More specifically, in lines 41, 163, 199, 204, 207, and throughout the paper, the authors use the terms "Optic lobes" or "optic lobe neuropils". To the best of my knowledge, "optic lobe" is not a term used in crustacean neuroanatomy at all (as opposed to insects). Lamina, medulla, and lobula are collectively referred to as "visual neuropils" (see Krieger, J., Hörnig, M. K., Sandeman, R. E., Sandeman, D. C., & Harzsch, S. (2020). Masters of communication: The brain of the banded cleaner shrimp *Stenopus hispidus* (Olivier, 1811) with an emphasis on sensory processing areas. *Journal of Comparative Neurology*, 528(9), 1561-1587. <https://doi.org/10.1002/CNE.24831>). The medulla terminalis and mushroom bodies are referred to as "lateral protocerebrum". All afore-mentioned neuropils are summarized as "eyestalk neuropils" (compare nomenclature in Schmidt 2016 as referenced above).

Line 170, 172, 175 ff, and Figure 1. "abdomen", "abdominal ganglia": Contra the book chapter by Siegel 2016 "Introducing Antarctic Krill *Euphausia superba* Dana, 1850", his Fig. 1.2, the "tail" of crustaceans in most books on crustacean anatomy is not called "abdomen" but instead "pleon"; hence the name "pleopods" for the appendages of the pleon (instead of "abdomipods"). What is more, I suggest using the terms "pleon ganglia" instead of "abdominal ganglia", following the terminology suggested in "Harzsch S, Sandeman D, Chaigneau J (2012) Morphology and development of the central nervous system. In: Forest J and von Vaupel Klein JC (Eds.). *Treatise on Zoology - Anatomy, Taxonomy, Biology. The Crustacea Vol. 3*. Brill, Leiden pp. 9-236."

Line 174: "thoracic ganglia". In Figure 1, there is a labelling mistake as these ganglia are named "thoracaic ganglia".

Line 176, and throughout the paper: "supraesophageal ganglion". Following the standard nomenclature for crustaceans (see, e. g., Schmidt, M., 2016. Malacostraca. In: Schmidt-Rhaesa, A., Harzsch, S., Purschke, G. (Eds.), *Structure & Evolution of Invertebrate Nervous Systems*. Oxford University Press, Oxford, pp. 529-582. <https://www.researchgate.net/publication/315366157>), this structure (as in insects) is typically called a "brain". For terminology, also consult the following nomenclature paper: "Richter, S., Loesel, R., Purschke, G., Schmidt-Rhaesa, A., Scholtz, G., Stach, T., Vogt, L.,

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Legend, figure 2, and others, and throughout the paper: "The olfactory neuropiles comprise the lateral antennal neuropile (LAN, ochre), the olfactory lobes (OL, yellow), and the antennal neuropile (AnN, green)." This is a strange terminological mix that you should urgently revise according to the standard terminology by Sandeman et al. 1992 (as referenced above). The LAN is the lateral antenna 1 neuropil. The AnN is the antenna 2 neuropil. The AnN is NOT deutocerebral but tritocerebral.

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We thank the reviewer for recognizing the contribution of this study to advancing our understanding of clock systems in non-traditional model organisms. We acknowledge that definitive functional evidence would require the generation of null mutants of core clock components, which is currently not feasible in this species. In a revised version, we will adjust our claims to more precisely reflect the evidence presented and include sample sizes to allow the reader to better assess the representativeness of the results.

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Line 170, 172, 175 ff, and Figure 1. "abdomen", "abdominal ganglia": Contra the book chapter by Siegel 2016 "Introducing Antarctic Krill Euphausia superba Dana, 1850", his Fig. 1.2, the "tail" of crustaceans in most books on crustacean anatomy is not called "abdomen" but instead "pleon"; hence the name "pleopods" for the appendages of the pleon (instead of "abdomipods"). What is more, I suggest using the terms "pleon ganglia" instead of "abdominal ganglia", following the terminology suggested in "Harzsch S, Sandeman D, Chaigneau J (2012) Morphology and development of the central nervous system. In: Forest J and von Vaupel Klein JC (Eds.). Treatise on Zoology - Anatomy, Taxonomy, Biology. The Crustacea Vol. 3. Brill, Leiden pp. 9-236."

Line 174: "thoracic ganglia". In Figure 1, there is a labelling mistake as these ganglia are named "thoracaic ganglia".

Line 176, and throughout the paper: "supraesophageal ganglion". Following the standard nomenclature for crustaceans (see, e. g., Schmidt, M., 2016. Malacostraca. In: Schmidt-Rhaesa, A., Harzsch, S., Purschke, G. (Eds.), Structure & Evolution of Invertebrate Nervous Systems. Oxford University Press, Oxford, pp. 529-582. <https://www.researchgate.net/publication/315366157> , this structure (as in insects) is typically called a "brain". For terminology, also consult the following nomenclature paper: "Richter, S., Loesel, R., Purschke, G., Schmidt-Rhaesa, A., Scholtz, G., Stach, T., Vogt,

L., Wanninger, A., Brenneis, G., Döring, C., Faller, S., Fritsch, M., Grobe, P., Heuer, C. M., Kaul, S., Møller, O. S., Müller, C. H. G., Rieger, V., Rothe, B. H., Stegner, M., Harzsch, S. (2010). Invertebrate neurophylogeny: Suggested terms and definitions for a neuroanatomical glossary. *Frontiers in Zoology*, 7. <https://doi.org/10.1186/1742-9994-7-29> .

*Line 212, and throughout the paper - hemiellipsoid body: please refer to Harzsch Krieger 2011 and the numerous references to studies by Strausfeld cited therein in crustaceans. Strausfeld has provided compelling evidence that the crustacean hemiellipsoid body is equivalent to the insect mushroom body, so this term should be replaced. Harzsch, S., & Krieger, J. (2021). Genealogical relationships of mushroom bodies, hemiellipsoid bodies, and their afferent pathways in the brains of Pancrustacea: Recent progress and open questions. *Arthropod Structure & Development*, 65, 101100. HYPERLINK "https://doi.org/10.1016/J.ASD.2021.101100" 
<https://doi.org/10.1016/J.ASD.2021.101100> .*

Legend, figure 2, and others, and throughout the paper: "The olfactory neuropiles comprise the lateral antennal neuropile (LAN, ochre), the olfactory lobes (OL, yellow), and the antennal neuropile (AnN, green)." This is a strange terminological mix that you should urgently revise according to the standard terminology by Sandeman et al. 1992 (as referenced above). The LAN is the lateral antenna 1 neuropil. The AnN is the antenna 2 neuropil. The AnN is NOT deutocerebral but tritocerebral.

We thank the reviewer for acknowledging this paper's contribution to our understanding of the neuronal basis of the circadian clock in Pancrustaceans, as well as for the positive evaluation of the data documentation and presentation.

We would like to clarify that we are aware of the existing body of literature on crustacean neuroanatomy and did not intend to present our data as a first in this field. This study intersects multiple communities (e.g., chronobiology, crustacean neurobiology, krill ecology), and the current focus of the manuscript arose from an attempt to make the paper as accessible to these communities as possible. We acknowledge, however, that the current version falls short in its engagement with the existing literature on crustacean brain anatomy. We therefore thank the reviewer for the input on crustacean neuroanatomy and its nomenclature, which will help us improve the manuscript in these respects. In a revised version, we plan to adjust the framing of the study to more precisely reflect the data presented. This will include better situating the present findings within the existing literature on crustacean neuroanatomy and its specific nomenclature, while toning down the emphasis on ecological importance and implications.

Reviewer #3 (Public review):

Summary:

A solid and very descriptive study of gene expression of three factors in krill, PDH, per, and cry2 that are important for circadian rhythms in insects. The results reveal optic areas in which PDH colocalises with each or per and cry2, and central brain areas where it does not. The authors speculate on the functional implications of their results for biological rhythms.

Comments:

This manuscript describes a detailed anatomical study of the brain of krill in a circadian gene expression context. The results are well described, and the work is well done considering the obvious technical/practical difficulties of working with this species. Having stated that, the authors in their Methods write that the animals, after being caught, were placed in constant darkness. Is there any idea at all of when in ZT these

brains were processed? Are the representations of gene expression taken at random around the clock? Perhaps the authors might make this explicit somewhere in the ms as it is an important point.

The manuscript focuses mostly on PDH and its overlap or not with per or cry2. I found Figures 5 and 6 particularly confusing. The panels show PDH colocalising (or not-filled or unfilled arrows) with cry2 or with per. What they do not show (to me) is that per and cry2 colocalise. Now, of course, they probably do, but Figure 5 does not show this - or am I misinterpreting it? In Figure 6 again, I cannot see any panels with per and cry2 overlaid. Seems different sections were used for each probe? Is that what 'Areas with high per/cry2-expression are marked by white arrowheads' means? I see that lines 493 and 494 confirm my suspicions that per/cry were not shown to be colocalised. Perhaps the authors could make this clearer up front than halfway through the Discussion, and clarify this in their legends, which are a little misleading in this respect?

We thank the reviewer for his positive evaluation of our work, acknowledging the difficulty when working with this organism, and for the constructive comments. In a revised version of the manuscript, we will clarify the sampling time in the Methods. We will also state upfront — and in the figure legends — that *per* and *cry2* were assessed on separate sections and their direct co-localization was therefore not demonstrated. However, as both components were independently shown to co-localize with PDH, their spatial overlap is nevertheless suggested by the shared co-localization with PDH. We will make this reasoning explicit earlier in the manuscript to avoid any misleading implications.

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