Journal of Insect Physiology 55 (2009) 441–449

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Corazonin- and PDF-immunoreactivities in the cephalic ganglia of termites

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ARTICLE INFO

Article history:
Received 26 August 2008
Received in revised form 7 November 2008
Accepted 12 November 2008

Keywords:
Castes
Circadian rhythm
Neuropeptides
Photoperiod
Pigment-dispersing hormone
Termites

ABSTRACT

Antisera against the pigment-dispersing factor (PDF) and corazonin (Crz) reacted with distinct sets of neurons in the cephalic ganglia of termites. The locations of immunoreactive cells were similar but their numbers differed among the eight species examined: PDF-ir occurred in 0–6 cells in each optic lobe and 1–2 pairs of cells in the subosophageal ganglion (SOG), and Crz-ir in 0–2 pairs of cells in the pars intecerebralis, 3–14 cells in each lateral protocerebrum, and 0–6 pairs of cells in the SOG. Staining patterns were identical in the pseudergates, soldiers, and substitutive reproductives of *Prorhinotermes simplex*. Workers and soldiers were compared in the remaining 7 species. The only caste divergence was detected in *Coptotermes formosanus*, in which the soldiers differed from the workers by lack of 4 Crz-ir perikarya in the *pars intercerebralis* and occasionally also by the absence of 2 Crz-ir perikarya in the SOG. Diurnal changes in PDF-ir and Crz-ir were examined in *P. simplex* kept under long day (18:6 h light:darkness) or short day (10:14 h) photoperiods. No circadian fluctuations in the distribution or the intensity of immunostaining were found in the pseudergates and soldiers that were sacrificed in 4 h intervals or in the male and female substitutive reproductives examined in 6 h intervals.

1. Introduction

Virtually all organisms possess endogenous circadian clocks that synchronize their life processes with the cyclic environmental fluctuations (Pittendrigh, 1981). The molecular clock mechanism has partly been elucidated, first in *Drosophila melanogaster* and subsequently in several other model organisms. The clock-type oscillators possibly reside in all body cells but are mutually synchronized and adjusted to the environmental cycles by a master clock that is localized in the central nervous system. The master clock includes an input pathway that transmits environmental signals, primarily the light, to a core oscillator (the clock *in sensu stricto*), and one or more output pathways that carry time signals from the core oscillator to the peripheral physiological systems (Lowrey and Takahashi, 2000; Williams and Sehgal, 2001). The circadian clock probably also registers annual changes of day length and delivers this information to a photoperiodic clock that integrates this and other signals and translates them into a pattern of neurohormonal and pheromonal regulations adjusting insect development and reproduction to the seasonal changes (Saunders, 2002).

The circadian and photoperiodic clocks have special significance in the social insects that must adjust to the diurnal and seasonal changes in the environment as individuals and also as a social entity, the colony. The adjustment is particularly challenging for the termites whose colonies are concealed in the soil or wood with little direct contact with the surrounding environment. In spite of this, the termites adjust their diurnal foraging cycles to the thermo- and photoperiods outside the nest (Hinze and Leuthold, 1999). They are also sensitive to the seasonal changes (Cabrera and Kamble, 2001); the over-all colony activity declines in the autumn and the development and swarming of the winged reproductives is linked to the spring or the raining season. The rhythmicity of termite colony life in relation to environmental periodicities was reviewed by Corbet (1966), and numerous studies on termite swarming in respect to seasonal changes were summarized by Nutting (1969). In general, the lower termites such as *Prorhinotermes simplex* in our study, fly under a wide range of weather conditions and stage several flights over a prolonged season, whereas the higher termites, for example, the *Macrotermes* species, *Odontotermes feae*, and *Nasutitermes costalis*, fly under more restricted conditions and produce fewer if not only one swarming per season. The timing of flight to a certain day time is of particular importance for the termites whose winged imagoes (colony

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doi:10.1016/j.jinsphys.2008.11.009

founders) are highly susceptible to the physical factors (heat and dryness) and predation.

A typical termite colony includes a royal reproductive pair, eggs, larvae (young stages without the wing pads), nymphs (older stages with wing pads), workers, and soldiers (Hanus and Šobotník, 2004). Colony activities such as foraging are subject to diurnal cycles; the diurnal rhythm of workers changes with their age (Miura and Matsumoto, 1998). In Termopsidae, Kalotermitidae, and in *P. simplex* of Rhinotermitidae, the workers are absent and their function is performed by larvae called pseudergates. They undergo stationary molts and under certain conditions produce soldiers or substitutive wingless reproductives. The royal pair (imaginal or neotenic reproductives), which does not have direct contact with the external world, exerts considerable influence over caste development that is season-dependent (Roisin, 2000). Environmental changes are obviously perceived by the workers or the pseudergates who collect food and build the nest, and by the soldiers who venture to the exterior in defending the colony.

In attempt to get some insight into the regulation of the circadian rhythms, we mapped in the cephalic ganglia of the workers/pseudergates of ten termite species (representing 6 out of 7 termite families) the distribution of immunoreactivities detectable with antisera against the neuropeptides pigment-dispersing factor (PDF) NSELINSLLSLPKNMNDAa and corazonin (Crz) pQTFQYSHGWTNa (Závodská et al., 2008). These neuropeptides were recognized as tentative components of the circadian clock output. PDF is an insect homologue of the pigment dispersing hormone that was discovered as a regulator of pigmentation in crustaceans (Rao and Riehm, 1988). A single insect species may contain several PDF isoforms (Honda et al., 2006). PDF injections into the brain of cockroaches (Petri and Stengl, 1997), and either suppression (Renn et al., 1999) or ectopic expression (Helfrich-Förster et al., 2000) of PDF in *D. melanogaster* revealed interference with the circadian clock. PDF may function as an internal pacemaker component (Petri and Stengl, 1997) and also as an output signal in the circadian control of behavior (Jackson et al., 2001). Significance of the two functions is apparently different in various insect groups.

Crz was isolated in insects as a cardioaccelerator (Veenstra, 1989) and independently as a pigmentation regulator (Tawfik et al., 1999). Immunocytochemical investigations showed corazonin-immunoreactivity (Crz-ir) in the cerebral neuroendocrine system of various insects (Roller et al., 2003). In some cases it occurred in the neurones that are viewed as components of the clock itself (Wise et al., 2002; Qi-Mao et al., 2003). Kim et al. (2004) showed that Crz is involved in the control of ecdysis and proposed that it functions as an output signal from the circadian clock and thereby channels ecdysis to a specific diurnal time.

The present study addresses a possible dependence of PDF-ir and Crz-ir occurrence in the cephalic ganglia of termites on caste and diurnal time. To explore the relationship to caste, we compared immunoreactivities in the workers/pseudergates and soldiers of 8 termite species at the same diurnal time (middle of the photophase). The possible relationship to the clock was examined in *P. simplex* by scoring immunostaining intensity in course of a 24 h cycle in the pseudergates and the substitutive reproductives.

2. Material and methods

2.1. The termites

The list of examined species is presented in Table 1. The colonies of *P. simplex*, *Reticulitermes flavipes*, and *C. formosanus* were raised in captivity at 27 °C and natural day/night cycles (day illumination 8–10 lx) for many years. A temporary culture of *N. costalis* was established under identical conditions from specimens newly collected in Florida. One week before dissection of the cephalic ganglia, groups of at least 20 workers and soldiers were transferred from the stock colonies of these species to Petri dishes and placed to 25 °C and 12:12 h (light:darkness) photoperiodic regime. The insects were sacrificed between ZT 4 (zeitgeber time, 4 h after the start of photophase) and ZT 8. *Hodotermes mosambicus* and *Macrotermes jeanneli* were taken from the colonies maintained in the Zoological Garden of Bern (Switzerland), and *Macrotermes carbonarius* and *O. feae* were collected outdoors in Thailand. The workers and soldiers of all four species were collected in the middle of the day (ZT 5-10). Their heads were cut off and fixed and shipped in Bouin solution (ganglia were dissected and processed 3–5 days later).

Table 1
Examined termite species and their affiliation to the subfamilies and families.

Species	Subfamily	Family
Hodotermes mossambicus (Hagen)	–	Hodotermitidae
Prorhinotermes simplex (Hagen)	Prorhinotermitinae	Rhinotermitidae
Reticulitermes flavipes (Kollar)[a]	Heterotermitinae	Rhinotermitidae
Coptotermes formosanus (Shiraki)	Coptotermitinae	Rhinotermitidae
Macrotermes carbonarius (Hagen)	Macrotermitinae	Termitidae
Macrotermes jeanneli (Grassé)	Macrotermitinae	Termitidae
Odontotermes feae (Wasmann)	Macrotermitinae	Termitidae
Nasutitermes costalis (Holmgren)	Nasutitermitinae	Termitidae

[a] Introduced into Europe and reported as *R. santonensis* Feytaud (Clèment et al., 2001).

To measure the effect of circadian time, small subcolonies of *P. simplex* were established in Petri dishes supplied with fine moist sand and ca. 2 mm thick wood chops as food. The dishes were kept under standard rearing conditions for 3–4 months until the substitutive reproductives appeared (their development was induced by the absence of sexually mature individuals). The termites were then transferred to a long day (18:6 h light:darkness) or short day (10:14 h) photoperiod, and sacrificed after 10 days. The pseudergates and the soldiers were dissected in 4 h intervals through one diurnal cycle under both short and long day conditions. The substitutive reproductives were examined only in the long day culture in 6 h intervals. Dim red light (660–670 nm wavelengths) was used for handling termites during the scotophase.

2.2. Primary antibodies

Rabbit polyclonal antiserum against [His^7]-corazonin (pETFQYSHGWTNa) was produced by Wako Co. (Nagano, Japan) and characterized by Roller et al. (2003) who demonstrated cross-reactivity with [Arg^7]-Crz (Bachem AG, Bubendorf, Switzerland). The latter Crz homolog is known from cockroaches and is therefore likely to occur in the termites. Rabbit antiserum against the crustacean PDF (NSELINSILGLPKVMNDAa) was raised by Dr. H. Dircksen and shown to react in diverse insects, including cockroaches and termites (Dircksen et al., 1987; Helfrich-Förster and Homberg, 1993; Sehadová et al., 2003; Závodská et al., 2008).

2.3. Immunocytochemistry in sections

The brain-suboesophageal ganglion complexes were dissected in insect saline and fixed either overnight in Bouin–Hollande solution at 4 °C or for 2–5 days in Bouin fixative. Tests with *R. flavipes* showed that both fixatives yielded identical results. The samples were dehydrated in the standard way and embedded in paraplast. Carefully oriented serial frontal sections 8–10 μm thick were attached to glass slides and brought to water. In case of the Bouin–Hollande fixation they were treated with Lugol's iodine and 5% sodium thiosulfate to remove residual heavy metals. Following

transfer to phosphate-buffered saline supplemented with 0.3% Tween 20 (PBST), the sections were treated with 10% normal goat serum in PBST for 30 min and incubated with a primary antibody overnight at 4 °C (anti-PDF was diluted 1:10,000 and anti-Crz 1:1000 with PBST). Upon return to room temperature (rT), the sections were rinsed three times with PBST and incubated for 1 h with goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (HRP, Jackson ImmunoResearch, diluted 1:1000 in PBST). Following three 10 min washes in PBST and one in 0.05 M Tris–HCl (pH 7.4), the HRP enzymatic activity was visualized with hydrogen peroxide (0.005%) and 3,3′-diaminobenzidine tetrahydrochloride (0.25 mM in 0.05 M Tris–HCl, pH 7.4). Dehydrated sections were mounted in the DPX medium (Fluka).

2.4. Whole mount immunocytochemistry

Complexes of the brain-suboesophageal ganglion were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, at 4 °C overnight. After a rinse in PBST they were treated with colagenase (0.5 mg/ml in PBS; Sigma) for 30–60 min at rT, washed with methanol and PBST, and submerged in normal goat serum (10% in PBST) for 2 h. Then they were incubated with a primary antibody for 48 h at 4 °C, washed with PBST thrice for 10 min at rT, and incubated with the secondary antibody-HRP conjugate overnight at 4 °C. The incubation was terminated with three 10–30 min washes in PBST at rT. The enzymatic activity of HRP was detected as described above. The preparations were mounted in 80% glycerol.

2.5. Data collection and presentation

The distribution of PDF-ir and Crz-ir was first examined in the whole mounts viewed and photographed at different angles under an Olympus SZX 12 dissecting scope. The positions of immunopositive neurons and their fibers were also reconstructed (distributions of immunoreactivities in successive sections were drawn over one another) from the series of frontal sections examined under the Zeiss Axioplane 2 microscope equipped with the Nomarski (DIC) optics and CCD camera. The patterns of immunoreactivities revealed from the whole mounts and reconstructed from the sections were compared and combined in a summarizing drawing. Each such drawing and description is based on at least 6 individuals.

The intensity of immunostaining was assessed in the series of sections prepared from the ganglia processed simultaneously. The entire procedure from the tissue dissection to the staining evaluation was standardized in respect to time, temperature, and other conditions. The length of exposure to DAB was set in preliminary runs with preparations from animals sacrificed at ZT 4. The staining with DAB was checked after the first 5 min and then every 3 min in 0.05 M Tris–HCl, and the periods needed and sufficient for maximal staining were taken as the standard staining times. The standard staining time was 11 min for the antibody against Crz and 23 min for the anti-PDF antibody. Groups of 6 workers/pseudergates and 6 soldiers were taken for the comparisons of castes and 10 pseudergates, 6 soldiers, and 6 male and 6 female substitutive reproductives were fixed at each chosen time in search for diurnal changes. The immunoreactivity was initially quantified subjectively with a 3-point scale ranging from no reactivity (–) to the distinct (+) and strong reactivity (++). Since it was hard to distinguish between the + and ++ scores, we have reduced our scoring system to + (staining) and – (no staining).

3. Results

3.1. Comparison of PDF-immunoreactivity (PDF-ir) in different castes

In our previous study of the workers/pseudergates (Závodská et al., 2008) we found that 8 out of 10 termite species contained a proximal fronto-ventral (Pfv) group of 2–6 PDF-ir perikarya at the base of the optic lobe (OL) and 1–2 pairs of PDF-ir perikarya in the labial neuromere of the suboesophageal ganglion (SOG). Only *Mastotermes darwiniensis* from the most primitive termite family lacked PDF-ir cells in the SOG, and *R. flavipes*, one of the species taken from the advanced family Rhinotermitidae, had no PDF-ir cells in the OL. The present study on this and six other previously examined species revealed that the species-specific PDF-ir distribution patterns identified in the workers occur also in the soldiers (Table 2). Comparison of the PDF-ir in the soldiers with that of the workers (Závodská et al., 2008) showed that the positions of PDF-ir somata in the brain of soldiers were in some species slightly shifted due to different head shape, but cell homologies could easily be recognized from the course of their processes (Fig. 1, left columns). The soldiers of *N. costalis* lacked the eyes and their optic lobes were reduced, but the Pfv clusters of PDF-ir neurons were similar to workers. Since the soldiers lacked the medulla, they also lacked PDF-ir fibers running over the medulla surface in the workers. Our current study also included *M. carbonarius*. As in the other species, the workers and the soldiers had identical layouts of the PDF-ir system (Fig. 1). The cephalic ganglia of *M. carbonarius* resembled those of *R. flavipes* by the lack of PDF-ir perikarya in the OL, presence of two pairs of PDF-ir somata in the labial neuromere of the SOG, the distribution pattern of the PDF-ir fibers in the tritocerebrum and the SOG, and lack of PDF-ir in the frontal ganglion (FG) and the *corpora cardiaca* (CC).

Pseudergates, soldiers, and substitutive reproductives were compared in *P. simplex*. The localization of PDF-ir perikarya was very similar in all castes (Table 2). This was probably true also for

Table 2
Numbers of PDF-ir perikarya and presence of PDF-ir fibers in the cephalic ganglia of termites.

Species	Castes	Perikarya			Fibers					
		Pr	OL	SOG	Pr	De + Tr	FG	CC	CA	SOG
Hodotermes mosambicus	W + S	0	6	1	+	+	+	+	–	+
Prorhinotermes simplex	P + S + ♀ + ♂	0	3	1	+	+	+	–	–	+
Reticulitermes flavipes	W + S	0	0	2	–	+	+	–	–	+
Coptotermes formosanus	W + S	0	4	1	+	+	+	+	–	+
Macrotermes carbonarius	W + S	0	0	2	–	+	–	–	–	+
Macrotermes jeanneli	W + S	0	4	1	+	+	+	+	–	+
Odontotermes feae	W + S	0	5	1	+	+	+	+	–	+
Nasutitermes costalis	W + S	0	6	1	+	+	+	+	–	+

Numbers of perikarya are per one half of the respective ganglion. The distribution of PDF-ir in the workers (pseudergates in *P. simplex*) and soldiers were identical in all species. *W* = workers; *P* = pseudergates; *S* = soldiers; *Pr* = protocerebrum; *OL* = optic lobe; *SOG* = suboesophageal ganglion; *De* = deutocerebrum; *Tr* = tritocerebrum; *FG* = frontal ganglion; *CC* = corpora cardiaca; *CA* = corpora allata.

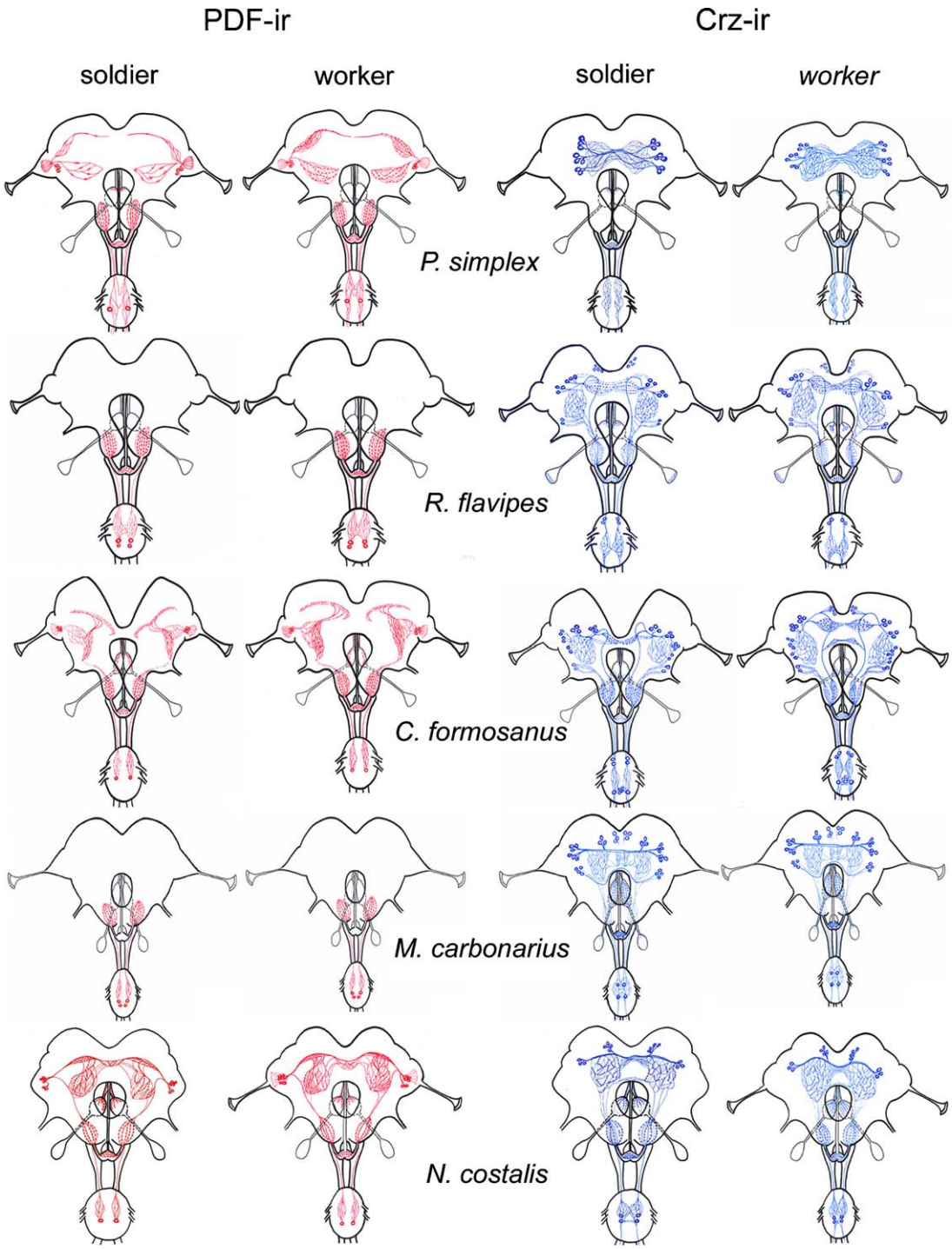

Fig. 1. Diagrams comparing the distribution of immunoreactivities in the cephalic ganglia of the soldiers and workers of *Prorhinotermes simplex* (this species uses pseudergates as workers), *Reticulitermes flavipes*, *Coptotermes formosanus*, *Macrotermes carbonarius*, and *Nasutitermes costalis*.

the tracks of PDF-ir fibers (Fig. 2A) but they could only partly be reconstructed from the sections of the cephalic ganglia in substitutive reproductives (Fig. 2B), for which total preparations were not available. A Pfv cluster of three PDF-ir somata was found in the OL of the pseudergates (Fig. 2C), soldiers (Fig. 2D), and substitutive reproductives (Fig. 2E). In both the pseudergates and soldiers, the processes of these cells formed a short common tract that produced three major branches. One of them dispersed as a fan over the frontal side of the medulla (without entering the lamina), and two others ran into the protocerebrum. One of these trajectories formed a network dorso-laterally to the mushroom bodies and then continued as a single tract above them to the brain midline. The second trajectory ramified among the neurons laying over the neuropile in the fronto-ventral part of the protocerebrum. Neither of these protocerebral networks sent distinct connections to their counterparts in the opposite brain hemisphere and to fiber arborizations in the tritocerebrum and the FG (Fig. 2F). The FG was clearly connected with the SOG that contained a pair of PDF-ir perikarya in the ventral part of the labial neuromere. The locations of these perikarya and the course of adjacent fibers were identical in the pseudergates (see Závodská et al., 2008), soldiers (Fig. 2G), and substitutive reproductives (Fig. 2H).

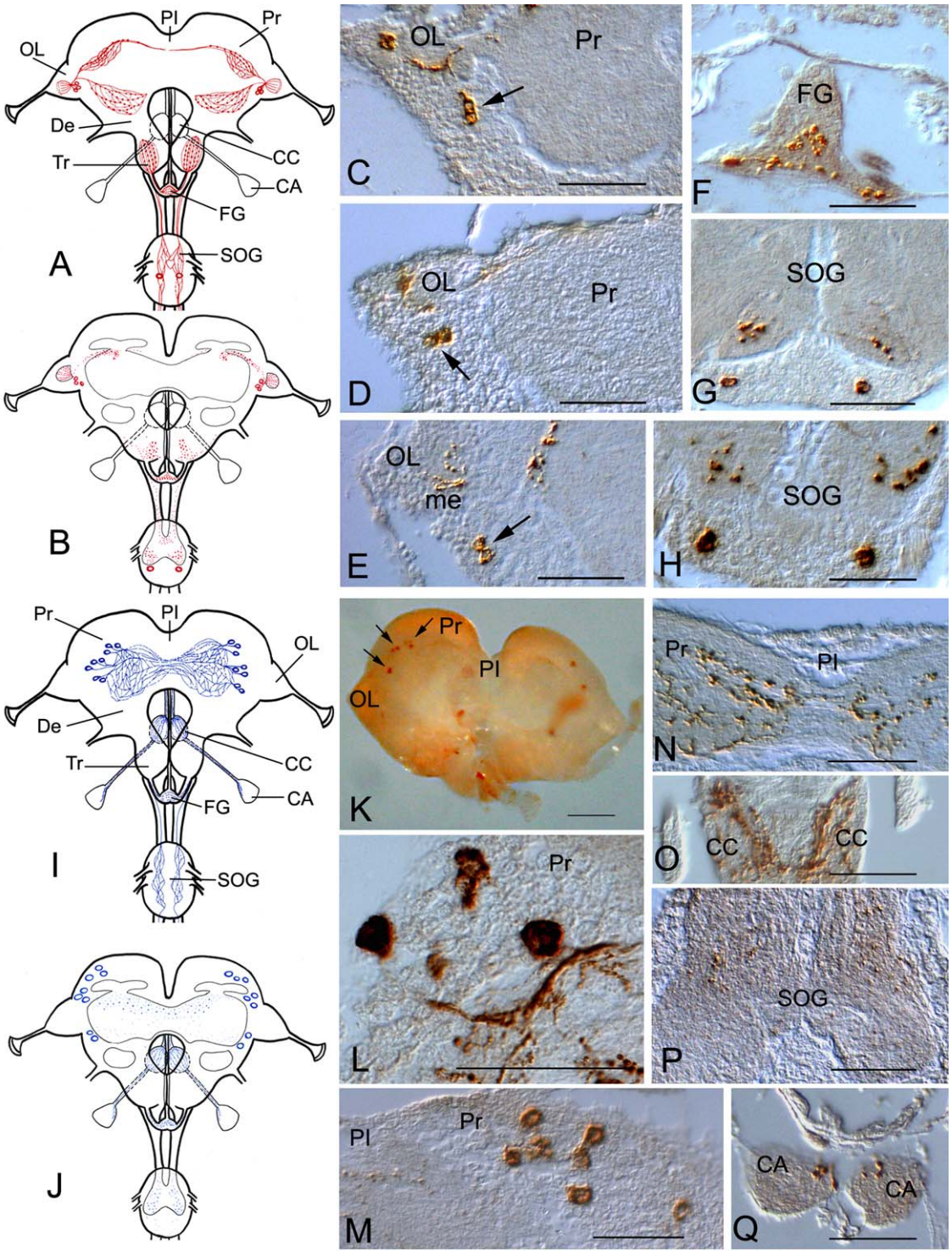

Fig. 2. Immunoreactivities in the brain (*Pl* = *pars intercerebralis*, *Pr* = protocerebrum, *OL* = optic lobe, *De* = deutocerebrum, *Tr* = tritocerebrum), retrocerebral glands (*CC* = *corpora cardiaca*, *CA* = *corpora allata*), the frontal ganglion (*FG*), and the suboesophageal ganglion (SOG) of *Prorhinotermes simplex*. (A) and (B) Diagrams illustrating the topology of PDF-ir perikarya and fibers in the pseudergates and in the substitutive male reproductives, respectively (B is based on the reconstruction from sections only). (C), (D) and (E) The Pvf cluster of perikarya (*arrows*) in the *OL* of a pseudergate, soldier, and substitutive male reproductive, respectively (*me*, medulla). (F) PDF-fibers in the *FG* and (G) PDF-ir somata in the *SOG* of soldiers. (H) PDF-ir perikarya and fibers in the *SOG* of a substitutive female reproductive. (I) Diagram of Crz-ir distribution in the pseudergates. (J) Crz-ir distribution in the male substitutive reproductives (reconstructed from sections). (K) Three groups (*arrows*) of Crz-ir somata in the fronto-lateral *Pr* of a pseudergate. (L) The Crz-ir cells in the same part of brain in a section. (M) and (N) Crz-ir neurons in the lateral *Pr* and bilateral fiber networks in the frontal *Pr* and the *PI*, respectively, of a substitutive male reproductive. (O) Crz-ir fibers permeating the *CC* of a pseudergate. (P) Axon arborization in the *SOG* of a soldier. (Q) Fibers in the *CA* of a substitutive male reproductive. Bar = 100 μm in C–H and L; 500 μm in K and M–Q. All photographs represent either a frontal section (C–E, L–Q) or the frontal view of the brain (K).

3.2. Crz-immunoreactivity (Crz-ir) in different castes

The distribution of Crz-ir in the cephalic ganglia of soldiers (Fig. 1) was similar to that of the workers in all examined species except *C. formosanus* (Table 3). Here we describe Crz-ir in *M. carbonarius* that was not included in our previous study (Závodská et al., 2008). Both the workers and the soldiers of *M. carbonarius* contained in the *pars intercerebralis* (PI) 3 Crz-ir somata whose

Table 3
Numbers of Crz-ir perikarya and presence of Crz-ir fibers in the cephalic ganglia of termites.

Species	Castes	Perikarya			Nerve fibers					
		LPr	PI	SOG	Pr	De + Tr	FG	CC	CA	SOG
Hodotermes mosambicus	W + S	3 + 3	0	0	+	+	+	+	+	–
Prorhinotermes simplex	P + S + ♀ + ♂	3 + 3 + 2	0	0	+	–	+	+	–	+
Reticulitermes flavipes	W + S	3 + 3 + 2	4	2	+	+	+	+	+	+
Coptotermes formosanus	W	3 + 3 + 6 + 2	4	2 + 1 + 2/3	+	+	+	+	–	+
Coptotermes formosanus	S	3 + 3 + 6 + 2	0	2 + 1 + 2	+	+	+	+	–	+
Macrotermes carbonarius	W + S	3 + 3 + 3 + 4	3	2	+	+	+	+	+	+
Macrotermes jeanneli	W + S	3 + 3	0	0	+	+	+	+	–	–
Odontotermes feae	W + S	3 + 3 + 2	3	0	+	+	+	+	–	+
Nasutitermes costalis	W + S	3	4	2	+	+	+	+	–	+

Numbers of perikarya are per one half of the respective ganglion. The distribution of Crz-ir in the workers (pseudergates in *P. simplex*) and soldiers were identical in all species except for *C. formosanus*. *W* = workers; *P* = pseudergates; *S* = soldiers; *LPr* = lateral protocerebrum; *PI* = pars intercerebralis; *SOG* = suboesophageal ganglion; *Pr* = protocerebrum; *De* = deutocerebrum; *Tr* = tritocerebrum; *FG* = frontal ganglion; *CC* = corpora cardiaca; *CA* = corpora allata.

processes were not detected. By contrast, two groups of 3 strongly stained cells in the fronto-lateral protocerebrum sent off a distinct fiber trajectory that passed above the mushroom body, received neurites from a fronto-medial group of 3 somata, and connected the opposite brain hemisphere through the bridge (Fig. 1). Side branches of the trajectory formed a superficial lateral and a subjacent medial fiber networks in the frontal protocerebrum. A pair of gentle fibers split off from the lateral network and descended into the deuto- and tritocerebrum. The fibers were joined by fine processes of 4 perikarya located posteriorly in the ventro-lateral protocerebrum. The major neurites of these perikarya ran as a distinct tract through the base of protocerebrum to the opposite hemisphere, with possible side branches to the CC that contained rich fiber ramifications extending to the proximal part of the *corpora allata* (CA). The fiber network in the tritocerebrum seemed to receive neurites from both the protocerebrum and the SOG that contained a pair of Crz-ir cells in the mandibular and another one in the labial neuromere. There was distinct connection between the network of varicose but weakly stained fibers in the FG and the nerves rooted in the SOG. The fibers passing through the circumoesophageal connectives arborized in the central part of SOG and continued into the ventral nerve cord.

Caste-specific differences in the Crz-ir pattern were found in *C. formosanus*. Both the workers and soldiers contained two clusters of three large and strongly stained somata anteriorly to the mushroom bodies, 6 smaller and weekly stained cells located deep in the lateral protocerebrum, and 2 perikarya in the ventro-lateral protocerebrum close to the border with the deutocerebrum. However, only workers possessed 4 Crz-ir perikarya in the PI (Fig. 1). Both castes contained in the SOG two pairs of large, anteriorly located somata in the mandibular neuromere, one pair of distinct somata in the posterior region of the labial neuromere, and a cluster of cells in the central part of the labial neuromere. The latter included 4 cells in all soldiers and half of the workers; the other half of workers had 6 central cells in the labial neuromere. In spite of these differences, the courses of Crz-ir fibers in the cephalic ganglia of soldiers and workers were similar (Fig. 1) as described by Závodská et al. (2008).

The pseudergates (Fig. 2I), soldiers (Fig. 1), and substitutive reproductives (Fig. 2J) of *P. simplex* exhibited very similar distribution of the Crz-ir. Three groups of Crz-ir somata were distributed from the dorsal to the ventral part of the frontal protocerebrum, laterally to the mushroom body (Fig. 2K). The most dorsal (Fig. 2L) and the medial group each included 3, and the ventral group consisted of 2 perikarya. The dorsal and the medial groups were close to each other in the substitutive reproductives (Fig. 2 M). The fibers of the protocerebral Crz-ir cells arborized in a dense and strongly stained network that occupied the frontal part of the protocerebrum around the mushroom body (Fig. 2N) and connected the brain halves through the central body. There was no obvious connection between this protocerebral network and the lower regions of the cephalic ganglia that might have received Crz-ir innervation from the ventral nerve cord. A single Crz-ir fiber extending through the frontal connective ramified slightly in the FG; several thin processes extended through the cardiacal nerve and arborized in the CC (Fig. 2O), with a few extensions to the CA (Fig. 2Q). No relationship of these processes to a single fiber passing through the circumoesophageal connective was evident. The SOG contained small areas of Crz-ir fiber arborizations extending in the anterio-posterior direction (Fig. 2P) but no Crz-ir somata.

3.3. Crz-ir and PDF-ir at different circadian times and under different photoperiods

P. simplex was chosen to examine possible effects of circadian time and photoperiod on PDF-ir and Crz-ir in the cephalic ganglia of different castes. To obtain the substitutive reproductives, small colonies composed of the larvae and pseudergates were kept for 3 months under standard conditions before they were transferred to the distinct long day versus short day photoperiodic regimes. All castes were examined 10 days later. The examination of pseudergates in 4 h intervals during a diurnal cycle consistently disclosed the immunoreactive perikarya in their typical positions. Fig. 3A shows persistent Crz-ir staining in the three clusters of cells in the fronto-lateral protocerebrum and Fig. 3B demonstrates equally persistent PDF-ir staining in the 3 cells of the Pvf cluster in the OL. A pair of PDF-ir perikarya in the SOG was also stained consistently in all preparations (not showed). Photoperiod (short day of 10:14 h light:darkness and long day of 18:6 h were compared) had no effect on the pattern or the intensity of the immunostaining (Table 4). Examination of the substitutive reproductives also did not disclose any variation of immunostaining in samples 6 h apart (Fig. 3C and D). Tabulated data are not presented because they look like a blueprint copy of Table 4.

4. Discussion

PDF and the Crz are the only neuropeptides composed of more than five amino acid residues that have been detected in most insect orders. Their structural conservation is coupled with similar location of the cells producing them in related insect taxa. There are strong indications that both PDF and Crz play some roles in the control of circadian rhythm. For example, diurnal variation was observed in the PDF-ir accumulation in axon terminals of the small ventral (LN-vs) brain cells that are associated with the circadian clock in *D. melanogaster* (Park et al., 2000; Taghert, 2001). Staining was greatest in the early part of the day and lowest at night. The Crz

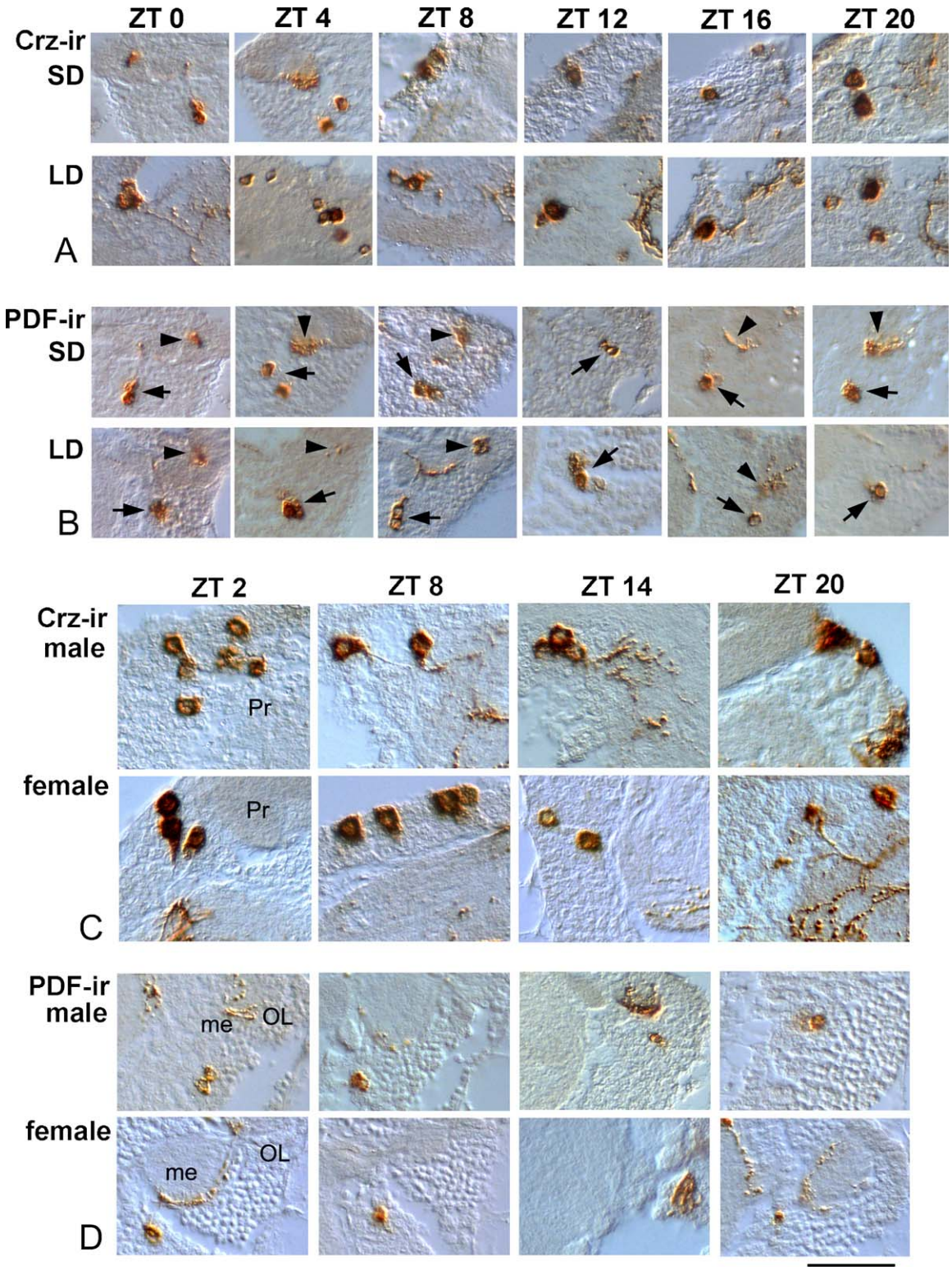

Fig. 3. (A and C) Crz-ir perikarya in the fronto-lateral protocerebrum and (B and D) the Pfv cluster of the PDF-ir cells (*arrows*) and their fibers on the frontal medulla surface (*arrowheads*) in *P. simplex*. Pseudergates were taken for the examinations in 4 h intervals from the short day (SD) and long day (LD) photoperiodic regimes (A and B, respectively), and the male and female substitutive reproductives (C and D) were examined in 6 h only in the long day culture. Zeitgeber time was counted from the lights on (ZT 0, i.e. 0 h). Bar = 100 μm applies to all micrographs. The photographs represent the frontal brain sections.

relationship to the circadian clock was first proposed for insects with a clock location in the OL. In a cockroach (Petri et al., 1995), a locust, and a grasshopper (Roller et al., 2003), Crz-ir fibers ramify in the accessory medulla of the OL where the clock protein PER is expressed (Závodská et al., 2003). In Lepidoptera, the Crz-ir occurs in 4 pairs of Ia_1 brain neurones (Hansen et al., 2001; Roller et al., 2003) that also express several clock proteins (Wise et al., 2002; Qi-Mao et al., 2003). Both the PDF-ir and the Crz-ir were found in specific neurons of the cephalic ganglia of termites (Sehadová et al., 2003; Závodská et al., 2008). This study was performed in quest of

Table 4
Lack of fluctuations in the intensity of the PDF-ir and Crz-ir staining in the cephalic ganglia of the pseudergates of *Prorhinotermes simplex* kept under short day (SD) or long day (LD) conditions and fixed at indicated circadian times.

Region and stained features	ZT 0		ZT 4		ZT 8		ZT 12		ZT 16		ZT 20	
	SD	LD	SD	LD	SD	LD	SD	LD	SD	LD	SD	LD
PDF-ir staining												
Pr, cells and fibers	+	+	+	+	+	+	+	+	+	+	+	+
DT, fibers	+	+	+	+	+	+	+	+	+	+	+	+
FG, fibers	+	+	+	+	+	+	+	+	+	+	+	+
CC, fibers	+	+	+	+	+	+	+	+	+	+	+	+
CA, fibers	–	–	–	–	–	–	–	–	–	–	–	–
SOG, cells and fibers	+	+	+	+	+	+	+	+	+	+	+	+
Crz-ir staining												
Pr, cells and fibers	+	+	+	+	+	+	+	+	+	+	+	+
DT, fibers	–	–	–	–	–	–	–	–	–	–	–	–
FG, fibers	+	+	+	+	+	+	+	+	+	+	+	+
CC, fibers	+	+	+	+	+	+	+	+	+	+	+	+
CA, fibers	–	–	–	–	–	–	–	–	–	–	–	–
SOG, fibers	+	+	+	+	+	+	+	+	+	+	+	+

SD = 10:14 h (light:darkness), LD = 18:6 h, ZT = zeitgeber time measured from the lights on (0 h). *Pr* = protocerebrum, *DT* = deuto- and tritocerebrum, *FG* = frontal ganglion, *CC* = corpora cardiaca, *CA* = corpora allata, *SOG* = suboesophageal ganglion.

possible dependence of immunostaining intensity on caste, photoperiod, and circadian time.

The PDF-ir resides in most insects in the OL, rarely in the protocerebrum (Závodská et al., 2003; Sehadová et al., 2003). The OL of insects belonging to the cohort Polyneoptera typically contains a proximal fronto-ventral (Pfv), a distal posterio-dorsal, and a distal posterio-ventral cluster of the PDF-ir perikarya; fibers arising from the Pfv cluster run over the frontal side of the medulla and terminate at its edge. Among the termites examined so far, only *Neotermes castaneus* was found to possess this arrangement. The great majority of termites contained only the Pfv cluster (Závodská et al., 2008) and *R. flavipes* and *M. carbonarius* had no PDF-ir perikarya in the optic lobe in either the soldier or the worker caste (Table 2). This is very exceptional; absence of PDF-ir perikarya in the OL was found only in the monarch butterfly *Danaus plexippus* (Sauman, unpublished data).

Závodská et al. (2008) reported on the presence of PDF-ir somata in the SOG of the pseudergates/workers of all examined termite species except *M. darwiniensis*, a species that contained just two PDF-ir somata in the OL. The present data confirm the occurrence of PDF-ir in the SOG of the majority of termites. For seven species we show similar distribution patterns of the PDF-ir cells in the workers and soldiers, and for *P. simplex* also in the substitutive reproductives. The PDF-ir perikarya in the SOG may supply fibers to the tritocerebrum, the FG, and even to the CC, as shown for 5 of the presently studied species. The PDF-ir innervation of the CC is rare in other insects; in Polyneoptera other than termites it was found only in the walking stick (Sehadová et al., 2003).

Two (exceptionally one) or 3 clusters of Crz-ir perikarya were found in the fronto-lateral protocerebrum of all studied pseudergates/workers; two species had additional small somata in the posterior protocerebrum, and six species contained Crz-ir cells also in the PI and/or the SOG (Závodská et al., 2008). The present study shows similar distribution of Crz-ir in the soldiers (Table 3). Only in *C. formosanus* we detected in the soldiers less Crz-ir cells than in the workers; the difference concerned perikarya in the PI and in the central SOG cluster and its functional significance is unknown. It should be noted that the presence of Crz-ir cells in the SOG is rather exceptional. Except for the termites it has been reported only for the American cockroach (Veenstra and Davis, 1993), the silverfish *Ctenolepisma lineate*, and the bug *Pyrrhocoris apterus* (Roller et al., 2003). On the other hand, the transport of Crz-ir material to the CC seems to be common (Hansen et al., 2001; Roller et al., 2003, 2006) but only in some insects such as the termites *H. mosambicus*, *M. carbonarius*, and *R. flavipes*, the Crz-ir fibers extend to the CA.

The examination of PDF-ir and Crz-ir in 4 h intervals in the pseudergates, and in 6 h intervals in the substitutive reproductives of *P. simplex* did not disclose diurnal differences. Similarly, the length of the photoperiod had no effect on either the distribution or the intensity of immunostaining. It cannot be excluded that diurnal fluctuations occur at the level of peptide release, for example, in the CC, but detection of such changes was beyond the scope of this study.

Acknowledgements

The gifts of termites from Dr. Reinhard Leuthold (Ittigen, Switzerland) and Mr. Marc Rosset (Zoological Garden, Bern, Switzerland), Dr. Jan Křeček (Ft. Lauderdale REC University of Florida, USA), and Dr. Yupa Hanboonsong (Khon Kaen University, Thailand) are gratefully acknowledged. The antiserum against Crz was kindly provided by Dr. S. Tanaka of the Institute of Agrobiological Sciences, Tsukuba, Japan, and the antiserum against PDF by Dr. H. Dircksen of Bonn University, Germany. The study was supported by institutional projects 6007665801 (R. Závodská) and Z50070508 (F. Sehnal), and by grants MSMT LC07032 (I. Sauman) and NSC 92-2313-B-002-069, NSC 93-2313-B-002-045, and NSC 2313-B-002-015 (H.-J. Lee).

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