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RESEARCH ARTICLE

Phototransduction and clock gene expression in the troglobiont beetle *Ptomaphagus hirtus* of Mammoth cave

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SUMMARY

Obligatory cave species exhibit dramatic trait modifications such as eye reduction, loss of pigmentation and an increase in touch receptors. As molecular studies of cave adaptation have largely concentrated on vertebrate models, it is not yet possible to probe for genetic universalities underlying cave adaptation. We have therefore begun to study the strongly cave-adapted small carrion beetle *Ptomaphagus hirtus*. For over 100 years, this flightless signature inhabitant of Mammoth Cave, the world's largest known cave system, has been considered blind despite the presence of residual lens structures. By deep sequencing of the adult head transcriptome, we discovered the transcripts of all core members of the phototransduction protein machinery. Combined with the absence of transcripts of select structural photoreceptor and eye pigmentation genes, these data suggest a reduced but functional visual system in *P. hirtus*. This conclusion was corroborated by a negative phototactic response of *P. hirtus* in light/dark choice tests. We further detected the expression of the complete circadian clock gene network in *P. hirtus*, raising the possibility of a role of light sensation in the regulation of oscillating processes. We speculate that *P. hirtus* is representative of a large number of animal species with highly reduced but persisting visual capacities in the twilight zone of the subterranean realm. These can now be studied on a broad comparative scale given the efficiency of transcript discovery by next-generation sequencing.

Key words: cave adaptation, circadian clock, phototransduction, evolution.

INTRODUCTION

Caves represent one of the earth's most challenging environments to adapt to because they require organisms to subsist without sunlight as an energy source and aid in visual orientation. And yet, subterranean habitats harbor complex communities of highly adapted species that tend to exhibit a stereotypic suite of modified traits referred to as troglomorphy (Christiansen, 2005; Culver and Pipan, 2009). Species that are able to maintain permanent subterranean populations (eutroglophiles), for instance, are often characterized by decreased eye size (microphthalmy) and reduced body pigmentation. Even more dramatic, obligatory subterranean species (troglobionts) exhibit the complete loss of peripheral visual organs (anophthalmy) and body pigmentation (Sket, 2008). Molecular studies of cave adaptation were pioneered in and have subsequently focused on the Mexican cave tetra *Astyanax mexicanus* (Jeffery, 2009). Very recently, this historical vertebrate bias has been alleviated by the molecular genetic analysis of eye and pigmentation loss in the waterlouse *Asellus aquaticus* (Protas et al., 2011). Both of these systems constitute dramatic examples of recent and fast evolution to the troglobiont state. The genetic consequences of different degrees of long-term cave adaptation, ranging from eutroglophiles to troglobionts, remain poorly explored.

Coleoptera (beetles) constitute one of the most dominant invertebrate components of cave biota. The major beetle families found in caves are ground beetles (Carabidae: Trechinae), followed by small carrion beetles (Cholevidae or Cholevinae within Leiodidae) and rove beetles (Staphylinidae) (Moldovan, 2005) with large distributions in the palearctic and nearctic ecozones. In addition, a large diversity of aquatic troglobiotic diving beetles (Dytiscidae) has been discovered in Australia (Faille et al., 2010; Leys and Watts, 2008; Leys et al., 2003; Ribera et al., 2010). The Coleoptera are thus an ideal study group to explore the breadth of cave-adaptive states. A case in point is the small carrion beetle genus *Ptomaphagus*. This taxon encompasses over 50 ancestral surface-dwelling (epigean) eutroglophile and troglobiotic species in the southeast United States (Peck, 1973; Peck, 1984; Peck, 1998). One of the best-studied representatives is the small troglobiont *Ptomaphagus hirtus* (Tellkampf) (Packard, 1888; Peck, 1973; Peck, 1975; Tellkampf, 1844), which is endemic to the cave system in and around Mammoth Cave National Park (Fig. 1A,C,E) (Brucker,

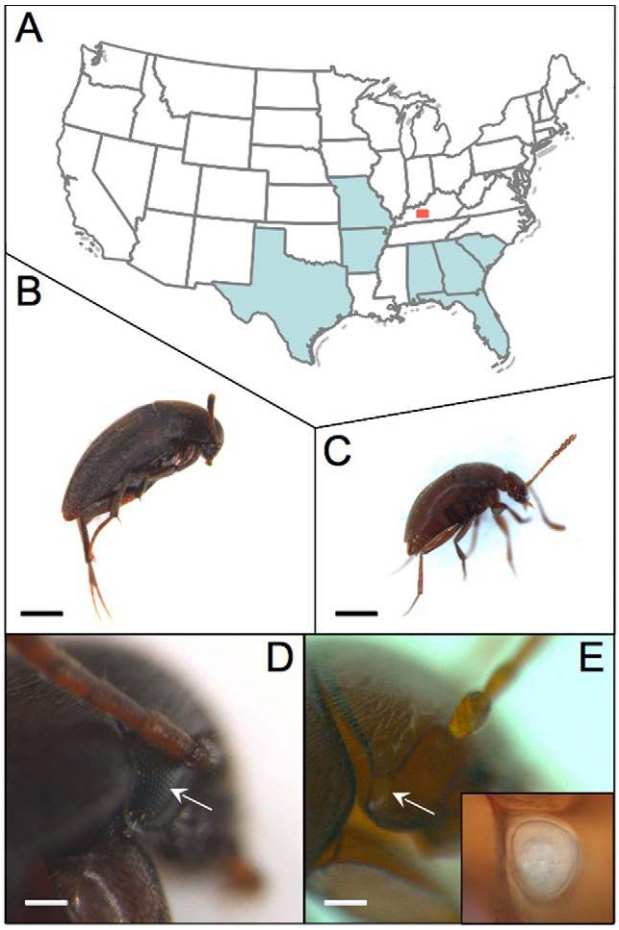

Fig. 1. Geographic distribution and morphology of the peripheral visual system in the small carrion beetle species *Ptomaphagus hirtus* and *Ptomaphagus cavernicola*. (A) Red area indicates documented occurrence of *P. hirtus* in the Mammoth cave system in Kentucky. Light blue areas identify states with frequent collection of *P. cavernicola*. Data based on (Peck, 1973). (B) Lateral view of *P. cavernicola*. (C) Lateral view of *P. hirtus*. (D) Lateral view of the adult head of *P. cavernicola*. Arrow points to compound eye. (E) Lateral view of the adult head of *P. hirtus*. Arrow points to residual lens structure. Inset shows a DIC image of the lens taken at high magnification from the dissected lateral head cuticle. Scale bars in A and C: 1 mm. Scale bars in D and E: 100 μm.

2005). *Ptomaphagus hirtus* adults, which measure between 2.0 and 2.8 mm in body length, are characterized by loss of the hindwings and the near-complete reduction of the compound eye to a non-pigmented small lens patch (Fig. 1E). This contrasts with the well-developed compound eyes of eutroglophilic representatives of this genus such as *Ptomaphagus cavernicola*, Schwarz 1989, which shows a wide geographic distribution in the southeast United States, extending into Mexico, and has been collected both in and outside caves (Fig. 1A,B,D) (Peck, 1973).

In his histological survey of North American cave animals, Packard (Packard, 1888) noted the lens-like structures in the lateral head of *P. hirtus* but was unable to detect anatomical evidence of an associated optic nerve or optic neuropiles. This led him to conclude that *P. hirtus* 'must be blind though the eyes exist in a rudimentary state'. While cursory observations in long-term culturing experiments indicated little to the contrary (Peck, 1986), more recent insights from developmental biology recommended revisiting this issue. In *Drosophila*, the specification of lens-producing cells during compound eye development is contingent on the preceding differentiation of photoreceptor cells (Wolff and Ready, 1993). As the cellular dynamics of retinal differentiation is highly conserved in other insect species (for a review, see Buschbeck and Friedrich, 2008), one has to assume that the dependence of lens formation on photoreceptor differentiation is also widely conserved. The question therefore arises as to how the presence of lateral lens structures can be reconciled with the presumed absence of photoreceptors in *P. hirtus*. One possible explanation is the life cycle-dependent elimination of photoreceptors by programmed cell death, turning the lens into a non-functional atavistic rudiment consistent with Packard's view. Alternatively, *P. hirtus* might possess a functional but extremely reduced visual system.

We adopted a molecular approach to test for visual modality in *P. hirtus*, combining deep level RNA sequencing with gene homolog-guided transcript assembly and annotation. This strategy revealed the expression not only of insect phototransduction genes but also of the circadian clock gene network. These results suggest that *P. hirtus* is photosensitive and that this modality might be used to regulate seasonally or diurnally oscillating processes. Consistent with the conservation of functional vision mediated through lateral photoreceptors, *P. hirtus* showed negative phototaxis in light/dark choice tests. Our findings support the idea that microphthalmic inhabitants of subterranean habitats represent a distinct category of cryptozoic adaptation, which is characterized by the continued use of ambient light information (Bartkowiak et al., 1991).

MATERIALS AND METHODS

Animals

Ptomaphagus hirtus animals used for molecular and behavioral experiments were taken from a laboratory culture that was started with 15 individuals collected in autumn 2008 from the Frozen Niagara cave entrance of Mammoth Cave (Scientific Research and Collecting Permit MAC-2008-SCI-2006). *Ptomaphagus cavernicola* animals came from a laboratory culture that was started with 84 animals collected from Warren Cave (Florida) in the spring of 2009. Both species were cultured following previously published protocols (Peck, 1975). Animals were kept in 60 mm polystyrene Petri dishes filled with ~0.5 mm deep layer of sieved soil obtained from the natural habitats. Culture Petri dishes were kept in Styrofoam boxes on a layer of moist tissue. To maintain saturated humidity, Petri dishes and paper towels were sprayed with water approximately every 3 days. *Ptomaphagus hirtus* cultures were maintained in constant dark in a coolbox incubator kept at 12°C. *Ptomaphagus cavernicola* cultures were kept in constant dark at ambient room temperature at 23–25°C. For feeding, cultures were regularly supplied with small amounts of dried Baker's yeast. In addition, we used Georgia 1 (GA 1) strain *Tribolium castaneum* animals from a culture that was maintained on yeast-enriched whole-wheat flour.

Molecular biology

Twenty-five adult *P. hirtus* heads were pooled for mRNA extraction. The head capsules were collected from CO_2-anesthetized individuals by dissection under a binocular microscope; antennae were removed. Following dissection, each head capsule was immediately transferred into a collective 1.5 ml Eppendorf tube filled with 250 μl Trizol (Invitrogen, Carlsbad, CA, USA) and homogenized using a glass–Teflon homogenizer. The homogenized tissue was stored at –70°C until total RNA extraction following the manufacturer's protocol (Invitrogen). For library construction, mRNA was purified from 20 μg of total RNA using a mRNA purification kit (Invitrogen) and libraries were generated

as described previously for sequencing with the Illumina GA II sequencing system (Daines et al., 2011).

Bioinformatic analysis

Sequenced reads were aligned to the *Tribolium* RefSeq transcript database using blastx (Thompson et al., 1994). Reads with significant hits were sorted into putative ortholog groups based on best blastx scores. The read populations of each putative ortholog group were subjected to contig assembly with Phrap compiled with the 'manyreads' option (Ewing and Green, 1998). In cases of 4000 or more individual BLAST hits for a single gene (190 occurrences), 4000 sequence reads were randomly sampled from the BLAST hits for use in contig assembly. The resulting contigs were subjected to a second round of homology search against the *Tribolium* and *Drosophila* RefSeq transcript databases. The resulting database of *P. hirtus* transcript homologs was then searched for transcripts orthologous to *Drosophila* genes with functions in biological processes of interest. In cases where there was an absence of specific orthologs in the *P. hirtus* database, the raw sequence reads were queried by blastx with the *Drosophila* gene in question. To explore the possibility that low sequence conservation precluded initial inclusion in the *P. hirtus* transcript database, we calculated the length of protein sequence alignable between *Tribolium* and *Drosophila* orthologs as a measure of expected sequence conservation between *P. hirtus* and *Drosophila* orthologs (see Tables 1–3). All sequences of contigs discussed in this study were deposited in the Gene Expression Omnibus (accession number GSE28039). None of the over 50 open reading frame-containing contigs studied in detail contained evidence of frameshift or nonsense mutations, suggesting the absence of pseudogene transcripts.

Gene tree reconstruction

Protein sequences were aligned with PRANK using default settings (Loytynoja and Goldman, 2008). Ambiguous alignment sites were filtered with Gblocks at default setting but allowing for 'gap positions within the final blocks' (Castresana, 2000). The likelihood-based phylogeny estimation algorithm Tree-Puzzle was used for gene tree reconstruction, applying the Whelan–Goldman amino acid substitution model (Schmidt et al., 2002; Whelan and Goldman, 2001).

Light/dark choice tests

Single tester animals were placed in a 9 cm diameter Petri dish with a lid that was half-covered by aluminium foil, dividing the Petri dish arena into a 31.2 cm^2 dark area (D-area) and an equally large light area (L-area). A commercial tungsten white light bulb (General Electric, 52 W, 120 V) was positioned perpendicularly 24 cm above the Petri dish arena. For each observation, a single animal was transferred onto the test arena with a moist brush and given 5 min for recovery and adjustment in complete darkness. Observations were started after switching on the light source, which, depending on the location of the animal in the arena, resulted in immediate exposure to light or exposure following appropriate repositioning of the lid, marking the first observation time point 0. Subsequently, the presence or absence of the animal in the L-area was recorded at 1 min intervals for a total duration of 10 min and averaged for a minimum of 10 different individuals.

The experiments with *P. hirtus* were performed inside the coolbox incubator at 12°C ambient temperature. A moistened paper sheet was used as the surface on the bottom of the Petri dish as preliminary observations revealed that *P. hirtus* showed uncoordinated walking and balancing problems on the bare plastic surface. Experiments with *P. cavernicola* and *T. castaneum* were performed at 23–25°C ambient temperature, on a moistened or dry paper surface, respectively. In all experiments, experimental animals were taken from continuously fed cultures.

RESULTS

The *P. hirtus* adult head transcriptome

We chose to perform RNA sequencing, which enables highly accurate qualitative and quantitative assessment of gene expression (Nagalakshmi et al., 2008; Wilhelm and Landry, 2009), on the *P. hirtus* adult head transcriptome to generate a database that could be probed for the conservation and expression of vision-related genes. Poly-A RNA was isolated from 25 dissected head capsules of CO_2-anesthetized animals of both sexes after removal of the antennae. Using the Illumina sequencing platform, we generated 27,428,409 sequence reads of 75 bp length. Close to 20% of these (5,476,803) produced significant alignments by blastx to RefSeq protein sequences of the distantly related red flour beetle *T. castaneum* (Tenebrionidae) and could be sorted into 9888 putatively ortholog-specific sequence read groups. Assembly of sequence reads in each group yielded a total of 67,079 contigs with an average length of 163.3 bp. Contig orthologies were re-investigated by reciprocal BLAST against the *T. castaneum* and *D. melanogaster* RefSeq databases. This step identified 6259 and 5649 high confidence orthologs in *T. castaneum* and *D. melanogaster*, respectively. These numbers were consistent with the closer phylogenetic relationship of *P. hirtus* to *T. castaneum*. The only moderately higher number of insect-conserved loci previously identified in genome sequence comparisons (Richards et al., 2008) validated the representational depth of the *P. hirtus* transcript database.

Expression of phototransduction genes

Using a previously established compilation of conserved insect phototransduction genes as the query sequence source (Bao and Friedrich, 2009), we searched the *P. hirtus* adult head transcript database for orthologs of insect vision-related genes. This approach revealed the presence of transcripts of all critical components of the phototransduction protein network as characterized in *Drosophila* (Table 1) (for reviews, see Katz and Minke, 2009; Wang and Montell, 2007). The first step in the phototransduction signaling cascade involves the absorption of photons by the G-protein-coupled receptor complexes (GPCRs), which consist of a member of the 7-transmembrane photopigment opsin family and the G-protein subunits $dG\alpha_q$, $G\beta_e$ and $G\gamma_e$, all of which were found in the *P. hirtus* transcript database. Most significant was the recovery of $dG\alpha_q$ and $G\beta_e$, which are photoreceptor specific in *Drosophila* (Dolph et al., 1994; Schulz et al., 1999; Scott et al., 1995; Yarfitz et al., 1991), as well as the recovery of a single opsin ortholog, which was supported by 369 single reads yielding 1008 bp of contiguous coding sequence. Gene tree analysis revealed that the *P. hirtus* singleton opsin was a member of the long wavelength (LW)-sensitive opsin subfamily (Fig. 2). Within this subfamily, the *P. hirtus* opsin was most closely related to the LW opsin of *T. castaneum*, as expected on taxonomic grounds. Interestingly, the selective conservation of the broadband-sensitive LW opsin in *P. hirtus* corresponds to the discovery of LW opsins in other species adapted to low light level ecologies such as cave crayfish (Crandall and Hillis, 1997).

Further, consistent with the preservation of functional phototransduction in *P. hirtus*, we recovered orthologs of key genes specifically involved in opsin deactivation and recycling, including *Arrestin 1* (*Arr1*), *Arrestin 2* (*Arr2*), *G-protein-coupled receptor*

Table 1. Photoreceptor genes recovered from *Ptomaphagus hirtus*

Gene	Dmel gene abbreviation	Dmel orthologs	Tcas orthologs	Total alignable protein sequence	Number of Phir RNA sequence reads	Total Phir contig length (aa)	Estimated coverage (%)	Normalized abundance
*LW-opsin**	*Rh6*	NP_524368	NP_001155991	327	369	336	88.9	1.1284
*Arrestin 1**	*Arr1*	NP_476681	XP_966595	346	266	359	95.2	0.7688
Arrestin 2	*Arr2*	NP_523976	NP_001164084	383	439	394	98.5	1.1462
*Chaoptic**	*chp*	NP_524605	NP_001107810	1157	176	620	48.4	0.152
eyes shut (spacemaker)	*eys (spam)*	NP_001027571	XP_973282	1202	2	17	1.3	0.002
*G protein α-subunit 49B**	*Gα49B*	NP_725191	XP_966311	353	1129	355	100.0	3.1983
*G protein β-subunit 76C**	*Gβ76C*	NP_523720	XP_973851	344	56	200	57.6	0.1628
G protein γ-subunit 30A	*Gγ30A*	NP_524807	XP_972123	72	488	73	100.0	6.7778
G protein-coupled receptor kinase 1	*Gprk1*	NP_001036438	XP_966480	622	315	569	89.0	0.5064
inactivation no afterpotential D	*inaD*	NP_726260	XP_001810643	514	606	533	73.7	1.1790
*neither inactivation nor afterpotential A**	*ninaA*	NP_476656	XP_973192	201	–	–	–	–
*neither inactivation nor afterpotential C**	*ninaC*	NP_523503	XP_968286	1013	187	534	48.2	0.1846
*no receptor potential A; type I**	*norpA; type I*	NP_525069	XP_001812780	1044	–	–	–	–
no receptor potential A; type II	*norpA; type II*	NP_001014721	XP_001812701	1044	2029	1073	99.4	1.9435
prominin	*prom*	ABH07113	EEZ97973	333	–	–	–	–
Protein C kinase 53E; inactivation no afterpotential C	*Pkc53E; inaC*	NP_476682; NP_476863	GLEAN_03019	636	903	601	90.2	1.4198
Rab-protein 6	*Rab6*	NP_477172	XP_972453; XP_974614	204	296	211	64.8	1.4510
*retinal degeneration C**	*rdgC*	NP_788544	XP_974915	576	102	348	42.8	0.1771
*transient receptor potential**	*trp*	NP_476768	XP_968670	785	53	253	29.8	0.0675
*trp-like**	*trpl*	NP_476895	XP_968598	1013	29	153	12.6	0.0286

White background, phototransduction genes; gray background, photoreceptor membrane protein genes not associated with phototransduction. Dmel, *Drosophila melanogaster*; Phir, *Ptomaphagus hirtus*; Tcas, *Tribolium castaneum*. aa, amino acids. Asterisks indicate retina- or photoreceptor-specific genes.

kinase 1 (*Gprk1*) and the Rhodopsin phosphatase *retinal degeneration C* (*rdgC*) (Table 1). We also detected the ortholog of *Rab protein 6* (*Rab6*), which is thought to be specifically involved in the transport of rhodopsin in the Golgi complex (Shetty et al., 1998; Wang and Montell, 2007).

The second major step in *Drosophila* phototransduction involves the interaction of activated $dG\alpha_q$ with the phospholipase NO RECEPTOR POTENTIAL A (NORPA) (Bähner et al., 2000; Bloomquist et al., 1988). *Drosophila* possess two differentially spliced isoforms of *norpA*, one of which, type I, is specific to the retina while the other, type II, is found in a variety of tissues outside the retina (Bloomquist et al., 1988; Kim et al., 1995; Zhu et al., 1993). Only *norpA* type II was detected in the *P. hirtus* transcript database, although both are conserved in *Tribolium* (Table 1). Interestingly, the *Drosophila norpA* type I photoresponse phenotype can be almost completely rescued by retinal misexpression of *norpA* type II (Kim et al., 2003). It is therefore tempting to speculate that a loss of *norpA* type I was compensated by *norpA* type II in *P. hirtus*.

The activation of NORPA is followed by the transient activation of the signalplex (Montell, 1998), the core of which is formed by the Ca^{2+} channel protein TRANSIENT RECEPTOR POTENTIAL (TRP) and the scaffolding protein INACTIVATION NO AFTERPOTENTIAL D (INAD). Orthologs of both corresponding genes were present in the *P. hirtus* transcript database. In addition to TRP, *Drosophila* photoreceptor cells express the related protein channel genes *TRPL* and *TRP*γ (Niemeyer et al., 1996; Xu et al., 2000). Only the former was represented in the *P. hirtus* transcript database although *TRP*γ is conserved in *Tribolium*. Also noteworthy was the recovery of the protein kinase *neither inactivation nor afterpotential C* (*ninaC*), which is specifically required for the calmodulin (CaM)-mediated localization of the signalplex in *Drosophila* photoreceptor cells (Porter et al., 1993).

Differential preservation of structural photoreceptor gene expression

Studies in *Drosophila* have shown that the phototransduction protein machinery is localized in the highly condensed array of microvillar extensions of the photoreceptor rhabdomere, the formation of which requires specific proteins. This includes the photoreceptor cell-specific homophilic transmembrane protein CHAOPTIC (CHP), which confers adhesion between the microvilli, and the antagonistic membrane protein PROMININ (PROM) (Zelhof et al., 2006). In addition, *Drosophila* photoreceptors secrete the extracellular proteoglycan protein SPACEMAKER (SPAM), also known as EYES SHUT (EYS), which results in the creation of an inter-rhabdomeral space between the *Drosophila* photoreceptors (Husain et al., 2006; Zelhof et al., 2006). We detected

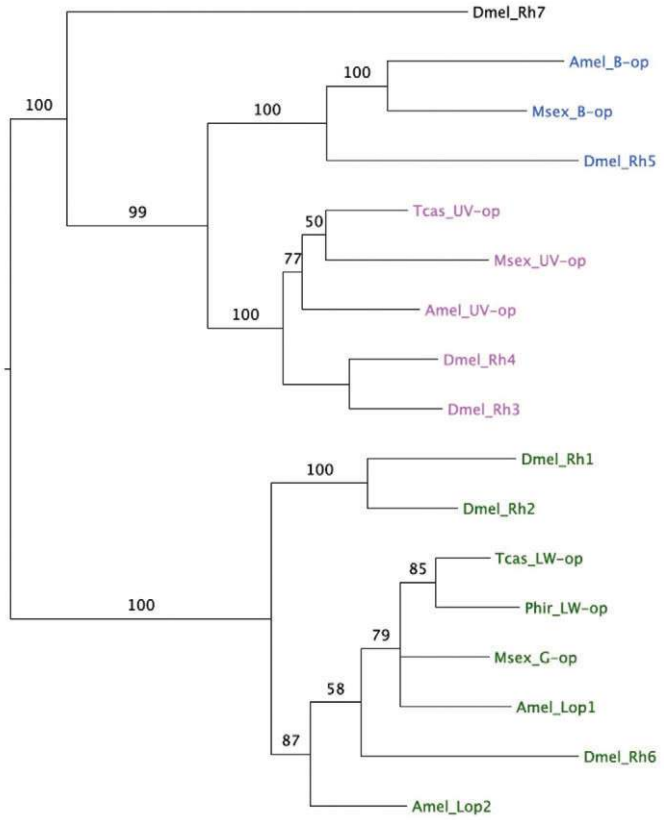

Fig. 2. Phylogenetic analysis of *P. hirtus* long wavelength (LW) opsin evolution. The putative *P. hirtus* (Phir) opsin protein sequence was aligned with the retinal opsin sequences from *Drosophila melanogaster* (Dmel), the honeybee *Apis mellifera* (Amel), the tobacco hornmoth *Manduca sexta* (Msex) and *Tribolium castaneum* (Tcas). Ambiguous regions in the resulting multiple alignment were removed, leaving 285 sites subjected to gene tree analysis. Numbers at branches indicate Tree-Puzzle branch support values.

intermediate levels of *chp* expression in *P. hirtus* (Table 1), further supporting the presence of canonical insect photoreceptor cells in the adult head of this species. We also detected a low level of *spam* expression but found no evidence of *prom* in the *P. hirtus* transcript database (Table 1). As *spam* is also involved in mechanical stress protection of sensory neurons outside the visual system (Cook et al., 2008), these findings are indicative of photoreceptors in *P. hirtus* that are not associated with inter-rhabdomeral space formation.

Differential preservation of eye pigmentation gene expression

Insect compound eyes are usually deep brown or black in appearance because of pigment granule formation in the photoreceptors and their accessory pigment cells for optical insulation between ommatidia and the regulation of light influx within ommatidia. To investigate whether the lack of overt pigmentation in the presumptive *P. hirtus* lateral eyes was correlated with a reduction or loss of eye pigmentation gene transcripts, we screened the *P. hirtus* adult head transcriptome database for the presence of *Drosophila* eye pigmentation gene orthologs. While the large majority of 25 queried genes were present in *P. hirtus* (Table 2), we failed to detect the expression of orthologs of the ABC transporter protein genes *white* (*w*), *brown* (*bw*) and *scarlet* (*st*), which are essential and specific for membrane passage of eye pigment compounds in *Drosophila* (Mackenzie et al., 2000). The lack of *bw* corresponded to the absence of this gene in *Tribolium*, which expresses only one type of eye pigment (ommochrome) (Lorenzen et al., 2002). The additional lack of *w* and *st* in the *P. hirtus* head transcriptome suggested a gene loss-associated complete reduction of eye pigmentation in this species. This conclusion was reinforced by the absence of the eye pigmentation-associated phosphoesterase *prune* (*pn*) and kynurenine 3-monooxygenase *cinnabar* (*cn*) in the *P. hirtus* transcript database, both of which are conserved in *Tribolium* (Table 2).

Analysis of photoresponse behavior

The evidence of a functional visual system prompted the question of the significance of photic stimuli in the biology of *P. hirtus*. To explore the possibility of phototactic behavior in *P. hirtus*, we investigated dispersal behavior in a light (L) *versus* dark (D) choice model (Fig. 3) as detailed in Materials and methods. Strikingly, after only 2 min, less than 30% of the tested animals were recorded in the L-area. For the rest of the experiment, the proportion of animals in the L-area varied between 10 and 30%. This translated into an average phototaxis index of –0.658 or a probability of 0.17 of observing an animal in the L-area (Maria Camassa, 2001). Continuous observation of five independent animals in the same test conditions further revealed that animals tended to move faster in the L-area while resting more often in the D-area.

To evaluate how the phototactic behavior of *P. hirtus* compared with that of species with a fully developed visual system, we subjected the closely related eutroglophile *P. cavernicola* to the same L/D choice test. *Ptomaphagus cavernicola* animals withdrew on average twice as quickly into the D-area such that an average of less 30% were scored in the L-area 1 min after the start of the experiment. *Ptomaphagus cavernicola* also exhibited a stronger average negative phototaxis index (–0.889) over the subsequent 8 min of the experiment (Fig. 3A), corresponding to a probability of 0.05 of being observed in the L-area (Fig. 3B). Besides revealing a shared disposition of *P. hirtus* and *P. cavernicola* to avoid exposure to strong light, the similarity in the response of the two species was highly suggestive that *P. hirtus* utilized peripheral photoreception in phototaxis, which is a reasonable assumption for *P. cavernicola*. Continuous behavorial observation of *P. cavernicola* individuals revealed similar D-area-biased resting patterns to those in *P. hirtus* but also a faster mobility of this larger species (3–4 mm body length), contributing to the faster withdrawal into the D-area.

To explore how the phototactic behavior of the *Ptomaphagus* species compared with that of non-cave-adapted Coleoptera, we also tested *T. castaneum*, which is characterized by crepuscular digging behavior, relatively small eyes and the lack of blue-sensitive opsin (Jackowska et al., 2007). In contrast to the *Ptomaphagus* species, *T. castaneum* demonstrated no evidence of L- or D-area preference. The proportion of animals present in the L-area decreased more slowly after onset of the experiments, never dropped below 30% and averaged 49%, translating into equal probabilities of being encountered in the D- or L-area. These findings supported the notion that both *P. hirtus* and *P. cavernicola* are characterized by cave adaptation-associated enhancement of negative phototaxis.

Conservation of circadian clock gene expression

Luminosity-based measuring of day length can play a role in the entrainment of oscillating processes through the circadian clock gene network, alone or in addition to temperature or other external zeitgeber stimuli. Remarkably, eutroglophilic and troglobiotic ground beetles exhibit day length entrainment of diurnal activity rhythms (for a review, see Lamprecht and Weber, 1992). To explore the potential for a role of vision in circadian entrainment, we also

Table 2. Eye pigmentation genes recovered from *P. hirtus*

Gene	Dmel gene abbreviation	Dmel orthologs	Tcas orthologs	Total alignable protein sequence	Number of Phir RNA sequence reads	Total Phir contig length (aa)	Estimated coverage (%)	Normalized abundance
brown	*bw*	NP_523824	–	–	–	–	–	–
carmine	*cm*	NP_788873	XP_968876	415	500	416	100.0	1.205
carnation	*car*	NP_728266	XP_968688	534	82	243	40.9	0.154
cinnabar	*cn*	NP_523651	NP_001034500	376	–	–	–	–
claret	*ca*	NP_733309	GLEAN_08539 +XP_967208	741	190	421	31.7	0.256
clot	*cl*	NP_608930	XP_967120	125	297	127	100.0	2.376
deep orange	*dor*	NP_477286	XP_974055	713	599	697	69.7	0.840
Dihydropterin deaminase	*DhpD*	NP_649439	XP_972928	373	466	379	87.1	1.249
garnet	*g*	NP_524785	XP_971970	772	475	602	68.0	0.615
Henna	*Hn*	NP_523963	XP_967025	411	3957	446	96.7	9.628
karmoisin	*kar*	NP_652025	XP_969391	371	121	224	49.8	0.326
light	*lt*	NP_001036416	XP_973665	751	690	770	92.2	0.919
lightoid	*ltd*	NP_525109	XP_974043	274	77	174	46.9	0.281
maroon-like	*mal*	NP_523423	XP_967646	558	238	523	68.2	0.427
orange	*or*	NP_536793	XP_974482	191	481	192	100.0	2.518
pink	*p*	NP_649810	XP_975447	355	41	83	10.2	0.115
prune	*pn*	NP_476684	XP_974192	225	–	–	–	–
Punch	*Pu*	NP_726038	XP_967029	224	355	233	91.0	1.585
rosy	*ry*	NP_524337	XP_968229	1251	107	323	23.9	0.086
ruby	*rb*	NP_525071	XP_970593	919	287	583	58.2	0.312
scarlet	*st*	NP_524108	XP_968696	579	–	–	–	–
sepia	*se*	NP_648235	XP_967412	222	373	208	86.7	1.680
Suppressor of cytokine signaling at 36E	*Socs36E*	NP_523593	GLEAN_03596	247	208	219	48.6	0.842
vermilion	*v*	NP_511113	NP_001034499	351	222	343	88.4	0.632
white	*w*	NP_476787	NP_001034521	593	–	–	–	–

White background, metabolic genes; gray background, ABC transporter complex genes. Dmel, *Drosophila melanogaster*; Phir, *Ptomaphagus hirtus*; Tcas, *Tribolium castaneum*. aa, amino acids.

probed the *P. hirtus* transcript database for the presence of clock gene orthologs because the expression of clock genes is concentrated in neuronal cell clusters in the brain of insects (for a review, see Tomioka and Matsumoto, 2010). This search revealed the expression of all components of the insect clock gene core network that have been identified in *Tribolium* (Table 3) (Cortes et al., 2010), including the bHlH-PAS transcription factor genes *period* (*per*), *cycle* (*cyc*), *tango* (*tgo*), *clock* (*clk*) and *timeless* (*tim*) as well as interacting genes like *Par-domain protein 1* (*Pdp1*) and *vrille* (*vri*). Previous studies reported the preservation of the photolyase *chryptochrome 2* (*cry2*) but the likely ancestral absence of its paralog *cryptochrome 1* (*cry1*) in *Tribolium* (Yuan et al., 2007; Zhu et al., 2005). Consistent with this, only *cry2* was detected in the *P. hirtus* transcript database.

DISCUSSION

The molecular and behavioral data accumulated in this study revise the assumed blindness of *P. hirtus* of the past 100 years. While the anatomical localization of phototransduction in *P. hirtus* remains to be studied in detail, it is reasonable to assume that the lateral lens patches cover a photoreceptor cell population. This is predicted from the highly conserved developmental dependence of lens formation on photoreceptor development in insects (Friedrich et al., 2006; Friedrich et al., 1996). Also of potential significance in this context is the finding from a survey of North American *Ptomaphagus* that *P. hirtus* represents an extreme representative of a range of mildly to strongly microphthalmic species (Peck, 1973; Peck, 1984; Peck, 1986). This comparative evidence suggests that the *P. hirtus* lens patches were derived by gradual reduction of the compound eye. Preliminary histological analyses of the presumed lateral eyes reveal the presence of a defined, densely clustered cell population underneath a cuticular lens with remarkable similarity to the organization of the peripheral visual system in the European troglobiotic ground beetle *Typhlochoromus stolzi* (Bartkowiak et al., 1991) (M.F. and Elke Buschbeck, unpublished). Packard's difficulties with recognizing an optic nerve or optic neuropiles were very likely due to the correlated reduction of the central visual system (Packard, 1888). The visual ecology of its habitat, the loss of flight and the preservation of apparently only a single opsin gene suggest that *P. hirtus* lacks wavelength discrimination and image-forming capacity. The combination of these regressive traits is predicted to result in a similarly dramatic reduction of the central visual system as seen, for instance, in the acephalic larvae of higher dipteran species like *Drosophila* [for a detailed review see Friedrich (Friedrich, 2011)]. Functionality and neuroanatomical organization of the *P. hirtus* visual system thus represent interesting subjects of further investigation.

The transcriptome data produced further clues regarding the cellular organization of the *P. hirtus* visual system. The detection of CHP transcripts, for instance, is a strong indicator of peripheral photoreceptors. The lack of transcripts of all eye-specific ABC transporter genes (*w*, *bn* and *st*), for instance, implies the presence of unpigmented photoreceptor cells, consistent with the clear appearance of the lateral eye patches (Fig. 1E). Of note, the comparison with *Tribolium* suggests that the lack of *bn* might be

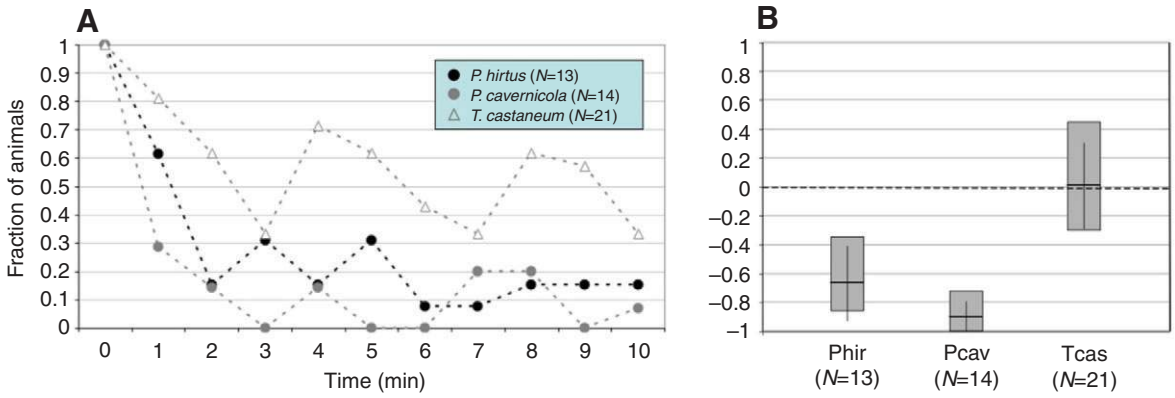

Fig. 3. Behavioral analysis of phototaxis in *P. hirtus*. (A) Time course of average presence of experimental animals in the exposed area of the light/dark (L/D) choice test arena. *X*-axis indicates time points of single observations. *Y*-axis represents fraction of test animals present in the L-area of the test plate. (B) Phototaxis index (*Y*-axis) of *P. hirtus* and *P. cavernicola* calculated from data extending from the 8–10 min time points in A. Gray box background indicates maximum range over separate time intervals. Error bar represents standard deviation. Dashed line indicates neutral expectation.

ancestral for Coleoptera. Considering the heavy pigmentation of the compound eye of *P. cavernicola* (Fig. 1B), one has therefore to assume that, at the least, *w* and *st* were lost in the *P. hirtus* lineage after separation from the *P. cavernicola*-associated lineage due to a relaxation of selection on eye pigmentation in troglobiotic species compared with eutroglophile species. The same scenario would explain the absence of the enzymatic pigmentation genes *cn* and *pn* from the *P. hirtus* transcript database. The lack of *cn* is particularly noteworthy considering the evidence for pseudogenization of this gene in multiple cases of troglobiotic Australian diving beetles (Leys et al., 2005). The large number of enzymatic eye pigmentation genes in the *P. hirtus* transcriptome is best explained by functions outside the eye. This is most obviously the case for the tryptophan oxygenase *vermilion* (*v*), which has a primary function in the tryptophan metabolism pathway (Linzen, 1974; Walker et al., 1986). It is tempting to predict that the comparison of gene repertoires between epigean and troglobiotic species can be used to identify function-specific gene groups.

Our behavioral experiments identify avoidance of light exposure as one candidate function of the *P. hirtus* visual system. The strongly negative photoresponse of *P. hirtus* and *P. cavernicola* in contrast to *T. castaneum* is in line with the idea that negative phototaxis predisposes species to cave colonization (Langecker, 1992; Romero, 1985). Visual performance and behavior, however, need to be explored in more detail, most importantly with regard to the effects of light intensity and wavelength. The negative phototaxis of *P. hirtus* in our experiments is intuitively rationalized as the effect of an upper light intensity threshold, which prevents *P. hirtus* from leaving the cave environment or presenting itself in an area populated with visual predators. However, the phototactic response of *P. hirtus* may be bimodal. A complementary low-light intensity threshold level may prevent these animals from dispersing into aphotic cave zones, which are defined by the complete absence of light and extreme limitation of organic nutrients. A bimodal photoresponse may maintain *P. hirtus* in disphotic cave areas, which are characterized by extremely low intensities of diffuse light from cave openings and a richer organic nutrient content. Such a preference has been demonstrated in the eutroglophile ground beetle *Laemostenus schreibersii* (Rusdea, 1992).

In the case of *P. hirtus*, bimodal phototaxis may be key to maintaining a dispersal pattern coincident with that of another dominant species of the Mammoth cave system: the guano-producing cave cricket *Hadenoecus subterraneus* (Hubbell and Norton, 1978). This subtroglophile forms large roosting congregations at the ceiling of cave areas that are in sufficient reach of surface links for foraging excursions, which are carried out strictly at night-time and in day-spanning intervals (Poulson, 1992; Poulson et al., 1995; Studier et al., 1986). While being attracted to a diversity of decomposing organic matter, *P. hirtus* are known to feed frequently on the cave cricket guano (Peck, 1975), which has been rated as one of three major nutrient influx sources in the Mammoth cave system (Barr and Kuehne, 1971). A shared phototactic preference for the disphotic zone may facilitate the horizontally overlapping distribution of *P. hirtus* and *H. subterraneus* despite their vertical separation.

The preservation of circadian clock gene expression points to a second trait that characterizes *P. hirtus* as potentially adapted to the disphotic zone. In *Drosophila*, the compound eyes, the larval eyes and select brain photoreceptors cooperate in the daylight cycle-contingent entrainment of the circadian clock gene network (Helfrich-Förster, 2002; Helfrich-Förster et al., 2002; Rieger et al., 2003; Veleri et al., 2007). Intriguingly, light entrainment of locomotory activity patterns is well documented in eutroglophilic as well as troglobiotic ground beetle species with moderately to strongly reduced eyes (Lamprecht and Weber, 1975; Weber et al., 1994; Rusdea, 2000). Morphologically, however, the extreme degree of eye reduction in *P. hirtus* is likely to be most similar to that of the ground beetle *T. stolzi* (Bartkowiak et al., 1991), which exhibits only very weak light entrainment (Lamprecht and Weber, 1983; Lamprecht and Weber, 1992).

While it is tempting to speculate that *P. hirtus* measures diurnal light level changes in the disphotic cave environment, the clock gene network may be environmentally adjusted by other stimuli such as temperature (Collins et al., 2004; Yoshii et al., 2009). Consistent with this last scenario, an impact of environmental temperature oscillations on circadian rhythm regulation has been documented in a number of insects including cave-adapted carabid and catopsid beetles (for reviews, see Lamprecht and Weber, 1992; Page, 1985; Rence and Loher, 1975; Tomioka and Chiba, 1989; Weber et al., 1995; Yoshii et al., 2009). Moreover, highly sensitive temperature receptors have been found in the larval antennae of troglobiotic cholevine species (Corbiere-Tichane and Loftus, 1983; Loftus and Corbiere-Tichane, 1981; Loftus and Corbiere-Tichane, 1987). Microclimates are seasonally stable in the deeper parts of the

Table 3. Circadian clock genes recovered from *P. hirtus*

Gene	Dmel gene abbreviation	Dmel orthologs	Tcas orthologs	Total alignable protein sequence	Number of Phir RNA sequence reads	Total Phir contig length (aa)	Estimated coverage (%)	Normalized abundance
clock	*clk*	NP_001014576	NP_001106937	476	251	306	52.7	0.527
clockwork orange	*cwo*	NP_524775	XP_001812240	229	111	163	39.9	0.485
c-opsin	*c-opsin*	–	NP_001138950	n.a.	–	–	–	–
cryptochrome 1	*cry 1*	NP_732407	–	–	–	–	–	–
cryptochrome 2	*cry 2*	–	NP_001076794	n.a.	6	66	12.3	n.a.
cycle	*cyc*	NP_524168	NP_001107795	371	5	44	6.5	0.013
lark	*lark*	NP_729239	XP_970745	267	399	262	73.2	1.494
no circadian temperature entrainment	*nocte*	NP_572631	GLEAN_00532	708	1137	283	14.2	1.606
PAR-domain protein 1	*Pdp1*	NP_729301	XP_967069	161	255	210	79.5	1.584
period	*per*	NP_525056	NP_001106933	677	365	604	54.2	0.539
Pigment-dispersing factor receptor	*Pdfr*	NP_570007	XP_971738	289	27	93	21.8	0.093
Rhythmically expressed gene 5	*Reg-5*	NP_477173	XP_966951	116	1082	100	47.4	9.328
slowpoke	*slo*	NP_001014659	XP_968651	1040	1446	1009	86.2	1.390
tango	*tgo*	NP_731308	XP_970422	487	98	353	53.4	0.201
timeless	*tim*	NP_722912	NP_001106934	877	188	99	8.9	0.214
vrille	*vri*	NP_477191	XP_971374	293	83	166	38.9	0.283

Dmel, *Drosophila melanogaster*; Phir, *Ptomaphagus hirtus*; Tcas, *Tribolium castaneum*. aa, amino acids.

Mammoth cave system but entrance areas, exposed to air currents, show variation in humidity and temperature (Barr and Kuehne, 1971). Moreover, there is evidence that the distribution of bottom-dwelling cave arthropods including *P. hirtus* is temperature and humidity dependent (Barr and Kuehne, 1971). Thus, clock gene transcript level oscillations need to be studied in relation to diurnal and seasonal light and temperature variations to elucidate the zeitgeber mechanisms in *P. hirtus*.

Examples of behavorial or metabolic rhythmicity in subterranean species include blind mole rats, the cavefish genera *Astyanax* and *Nemacheilus*, myriapods and crustaceans (Cooper et al., 1993; De La O-Martinez et al., 2004; Erckens and Martin, 1982; Goldman et al., 1997; Jegla and Poulson, 1968; Koilraj et al., 2000; Kumar Pati, 2001) (for a review, see Lamprecht and Weber, 1992). Curiously, while our data represent the first demonstration of the expression of the complete circadian gene network in a troglobiont, no evidence of oscillating behavior or metabolic activities has so far been reported for *P. hirtus*. On the contrary, previous work suggests that reproduction is season independent (Peck, 1975). Of potential significance, however, is the fact that the production of guano by *H. subterraneous* is seasonally variable with maximal deposition in the warmer months and close to zero production during winter (Barr and Kuehne, 1971). A study of cave cricket guano microcommunity variation demonstrated a tight correlation between climatic variations of guano richness and species composition (Poulson et al., 1995). The hypothesis that *P. hirtus*' life history or dispersal pattern may be seasonally adjusted therefore deserves new consideration.

In *Drosophila*, the biological clock controls processes as diverse as olfaction, neuronal physiology, detoxification, immune response, activity patterns and metabolism (for a review, see Allada and Chung, 2010). It is therefore difficult to speculate about the actual targets of the clock gene network in *P. hirtus*. We predict that transcriptomic approaches will be an efficient way not only of studying the transcriptional regulation of the clock gene network in the natural habitat but also of identifying putative target genes by virtue of correlated transcription profiles (Doherty and Kay, 2010; van der Linden et al., 2010). Our study suggests that next-generation sequencing methods will continue to serve as powerful facilitators in this endeavor. The recovery of over 5000 high confidence *Drosophila* and *Tribolium* orthologs in the adult head transcriptome of *P. hirtus* by RNA sequencing underlines the promise of next-generation sequencing for exploring the genetic organization of non-model organisms, by capitalizing on model species' genome sequences as a reference for assembly and annotation (Gibbons et al., 2009; Juan et al., 2010). The sensitivity of this approach is most impressively highlighted by the detection of circadian rhythm gene transcripts. In the insect species in which it has been studied, the expression of many circadian clock genes is restricted to small cell groups in the brain (Tomioka and Matsumoto, 2010). While the spatial expression characteristics of clock genes in *P. hirtus* remain to be determined, it is reasonable to conclude that the sensitivity of RNA sequencing has enabled the comprehensive characterization of phototransduction and clock gene expression using a very marginally cell type-enriched tissue sample. This proof-of-principle result opens the door for broad-scale comparative analyses of genetic cave adaptation.

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REFERENCES

Allada, R. and Chung, B. (2010). Circadian organization of behavior and physiology in *Drosophila*. *Annu. Rev. Physiol.* **72**, 605-624.

Bähner, M., Sander, P., Paulsen, R. and Huber, A. (2000). The visual G protein of fly photoreceptors interacts with the PDZ domain assembled INAD signaling complex via direct binding of activated Gα q to phospholipase Cβ. *J. Biol. Chem.* **275**, 2901-2904.

Bao, R. and Friedrich, M. (2009). Molecular evolution of the *Drosophila* retinome: exceptional gene gain in the higher Diptera. *Mol. Biol. Evol.* **26**, 1273-1287.
Barr, T. and Kuehne, R. (1971). Ecological studies in the Mammoth cave system of Kentucky: II. The ecosystem. *Ann. Speleol.* **26**, 47-96.
Bartkowiak, D., Tscharntke, T. and Weber, F. (1991). Effects of stabilizing selections on the regressive evolution of compound eyes in hypogean carabid beetles. *Mem. Biospeleol.* **18**, 19-24.
Bloomquist, B., Shortridge, R., Schneuwly, S., Perdew, M., Montell, C., Steller, H., Rubin, G. and Pak, W. (1988). Isolation of a putative phospholipase C gene of *Drosophila*, norpA, and its role in phototransduction. *Cell* **54**, 723-733.
Brucker, R. W. (2005). Mammoth cave system. In *Encyclopedia of Caves* (ed. D. C. Culver and W. B. White), pp. 351-355. Amsterdam: Elsevier.
Buschbeck, E. and Friedrich, M. (2008). Evolution of insect eyes: tales of ancient heritage, deconstruction, reconstruction, remodeling and recycling. *Evolution: Education and Outreach* **1**, 448-462.
Castresana, J. (2000). Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol. Biol. Evol.* **17**, 540-552.
Christiansen, K. (2005). Morphological adaptations. In *Encyclopedia of Caves* (ed. D. C. Culver and W. B. White), pp. 386-397. San Diego: Elsevier, Academic Press.
Collins, B., Rosato, E. and Kyriacou, C. (2004). Seasonal behavior in *Drosophila melanogaster* requires the photoreceptors, the circadian clock, and phospholipase C. *Proc. Natl. Acad. Sci. USA* **101**, 1945-1950.
Cook, B., Hardy, R. W., McConnaughey, W. B. and Zuker, C. S. (2008). Preserving cell shape under environmental stress. *Nature* **452**, 361-364.
Cooper, H., Herbin, M. and Nevo, E. (1993). Visual system of a naturally microphthalmic mammal: the blind mole rat, *Spalax ehrenbergi*. *J. Comp. Neurol.* **328**, 313-350.
Corbiere-Tichane, G. and Loftus, R. (1983). Antennal thermal receptors of the cave beetle, *Speophyes lucidulus* Delar. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **153**, 343-351.
Cortes, T., Ortiz Rivas, B. and Martinez Torres, D. (2010). Identification and characterization of circadian clock genes in the pea aphid *Acyrthosiphon pisum*. *Insect Mol. Biol.* **19**, 123-139.
Crandall, K. A. and Hillis, D. M. (1997). Rhodopsin evolution in the dark. *Nature* **387**, 667-668.
Culver, D. and Pipan, T. (2009). *The Biology of Caves and Other Subterranean Habitats*. New York, USA: Oxford University Press.
Daines, B., Wang, H., Wang, L., Li, Y., Han, Y., Emmert, D., Gelbart, W., Wang, X., Li, W. and Gibbs, R. (2011). The *Drosophila melanogaster* transcriptome by paired-end RNA sequencing. *Genome Res.* **21**, 315-324.
De La O-Martinez, A., Verde, M., Valadez, R., Viccon-Pale, J. and Fuentes-Pardo, B. (2004). About the existence of circadian activity in cave crayfish. *Biol. Rhythm Res.* **35**, 195-204.
Doherty, C. and Kay, S. (2010). Circadian control of global gene expression patterns. *Annu. Rev. Genet.* **44**, 419-444.
Dolph, P., Man-Son-Hing, H., Yarfitzt, S., Colley, N., Deer, J., Spencer, M., Hurley, J. and Zuker, C. (1994). An eye-specific G subunit essential for termination of the phototransduction cascade. *Nature* **370**, 59-61.
Erckens, W. and Martin, W. (1982). Exogenous and endogenous control of swimming activity in *Astyanax mexicanus* (Characidae, Pisces) by direct light response and by a circadian oscillator. II. Features of time-controlled behaviour of a cave population and their comparison to an epigean ancestral form. *Z. Naturforsch.* **37**, 1253-1265.
Ewing, B. and Green, P. (1998). Base-calling of automated sequencer traces using Phred. II. error probabilities. *Genome Res.* **8**, 186-194.
Faille, A., Ribera, I., Deharveng, L., Bourdeau, C., Garnery, L., Queinnec, E. and Deuve, T. (2010). A molecular phylogeny shows the single origin of the Pyrenean subterranean Trechini ground beetles (Coleoptera: Carabidae). *Mol. Phylogenet. Evol.* **54**, 97-106.
Friedrich, M. (2011). *Drosophila* as developmental paradigm of regressive brain evolution: proof of principle in the visual system. *Brain Behav. Evol.* **78**, 199-215.
Friedrich, M., Rambold, I. and Melzer, R. R. (1996). The early stages of ommatidial development in the flour beetle *Tribolium castaneum* (Coleoptera, Tenebrionidae). *Dev. Genes Evol.* **206**, 136-146.
Friedrich, M., Dong, Y. and Jackowska, M. (2006). Insect interordinal relationships: insights from the visual system. *Arthropod Syst. Phylogeny* **64**, 133-148.
Gibbons, J., Janson, E., Hittinger, C., Johnston, M., Abbot, P. and Rokas, A. (2009). Benchmarking next-generation transcriptome sequencing for functional and evolutionary genomics. *Mol. Biol. Evol.* **26**, 2731-2744.
Goldman, B., Goldman, S., Riccio, A. and Terkel, J. (1997). Circadian patterns of locomotor activity and body temperature in blind mole-rats, *Spalax ehrenbergi*. *J. Biol. Rhythms* **12**, 348-361.
Helfrich-Förster, C. (2002). The circadian system of *Drosophila melanogaster* and its light input pathways. *Zoology* **105**, 297-312.
Helfrich-Förster, C., Edwards, T., Yasuyama, K., Wisotzki, B., Schneuwly, S., Stanewsky, R., Meinertzhagen, I. A. and Hofbauer, A. (2002). The extraretinal eyelet of *Drosophila*: development, ultrastructure, and putative circadian function. *J. Neurosci.* **22**, 9255-9266.
Hubbell, T. H. and Norton, R. M. (1978). The systematics and biology of the cave-crickets of the North American tribe Hadenoecini (Orthoptera Saltatoria: Ensifera: Rhaphidophoridae: Dolichopodinae). *Misc. Publ. Mus. Zool. Univ. Mich.* **156**, 1-143.
Husain, N., Pellikka, M., Hong, H., Klimentova, T., Choe, K. M., Clandinin, T. R. and Tepass, U. (2006). The agrin/perlecan-related protein Eyes Shut is essential for epithelial lumen formation in the *Drosophila* retina. *Dev. Cell* **11**, 483-493.
Jackowska, M., Bao, R., Liu, Z., McDonald, E. C., Cook, T. A. and Friedrich, M. (2007). Genomic and gene regulatory signatures of cryptozoic adaptation: loss of blue sensitive photoreceptors through expansion of long wavelength-opsin expression in the red flour beetle *Tribolium castaneum*. *Front. Zool.* **4**, 24.
Jeffery, W. R. (2009). Regressive evolution in *Astyanax* cavefish. *Annu. Rev. Genet.* **43**, 25-47.
Jegla, T. and Poulson, T. (1968). Evidence of circadian rhythms in a cave crayfish. *J. Exp. Zool.* **168**, 273-282.
Juan, C., Guzik, M. T., Jaume, D. and Cooper, S. J. B. (2010). Evolution in caves: Darwin's 'wrecks of ancient life' in the molecular era. *Mol. Ecol.* **19**, 3865-3880.
Katz, B. and Minke, B. (2009). *Drosophila* photoreceptors and signaling mechanisms. *Front. Cell. Neurosci.* **3**, 2.
Kim, S., McKay, R., Miller, K. and Shortridge, R. (1995). Multiple subtypes of phospholipase C are encoded by the norpA gene of *Drosophila melanogaster*. *J. Biol. Chem.* **270**, 14376-14382.
Kim, S., Chen, D., Zavarella, K., Fourtner, C., Stark, W. and Shortridge, R. (2003). Substitution of a non-retinal phospholipase C in *Drosophila* phototransduction. *Insect Mol. Biol.* **12**, 147-153.
Koilraj, A., Sharma, V., Marimuthu, G. and Chandrashekaran, M. (2000). Presence of circadian rhythms in the locomotor activity of a cave-dwelling millipede *Glyphiulus cavernicolus sulu* (Cambalidae, Spirostreptida). *Chronobiol. Int.* **17**, 757-765.
Kumar Pati, A. (2001). Temporal organization in locomotor activity of the hypogean loach, *Nemacheilus evezardi*, and its epigean ancestor. *Environ. Biol. Fishes* **62**, 119-129.
Lamprecht, G. and Weber, F. (1975). Die Circadian-Rhythmik von drei unterschiedlich weit an ein Leben unter Höhlenbedingungen adaptierten *Laemostenus*-Arten (Coleoptera, Carabidae). *Anns. Speleol.* **30**, 471-482.
Lamprecht, G. and Weber, F. (1983). Activity control in the eyeless carabid beetle *Typhlochoromus stolzi*, an inhabitant of a superficial underground compartment. *Mem. Biospeleol.* **10**, 377-383.
Lamprecht, G. and Weber, F. (1992). Spontaneous locomotion behaviour in cavernicolous animals: the regression of the enodgenous circadian system. In *The Natural History of Biospeleology-Monographs* (ed. A. I. Camacho), pp. 225-262. Madrid: Museo Nactional de Ciencias Naturales.
Langecker, T. G. (1992). Light sensitivity of cave vertebrates. Behavioral and morphological aspects. In *The Natural History of Biospeleology-Monographs* (ed. A. I. Camacho), pp. 295-326. Madrid: Museo Nactional de Ciencias Naturales.
Leys, R. and Watts, C. (2008). Systematics and evolution of the Australian subterranean hydroporine diving beetles (Dytiscidae), with notes on *Carabhydrus*. *Invertebr. Syst.* **22**, 217-225.
Leys, R., Watts, C., Cooper, S. and Humphreys, W. (2003). Evolution of subterranean diving beetles (Coleoptera: Dytiscidae Hydroporini, Bidessini) in the arid zone of Australia. *Evolution* **57**, 2819-2834.
Leys, R., Cooper, S. J., Strecker, U. and Wilkens, H. (2005). Regressive evolution of an eye pigment gene in independently evolved eyeless subterranean diving beetles. *Biol. Lett.* **1**, 496-499.
Linzen, B. (1974). The tryptophan → ommochrome pathway in insects. *Adv. Insect Physiol.* **10**, 117-246.
Loftus, R. and Corbiere-Tichane, G. (1981). Antennal warm and cold receptors of the cave beetle, *Speophyes lucidulus* Delar., in sensilla with a lamellated dendrite. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **143**, 443-452.
Loftus, R. and Corbiere-Tichane, G. (1987). Response of antennal cold receptors of the catopid beetles, *Speophyes lucidulus* Delar. and Cholera angustata Fab. to very slowly changing temperature. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* **161**, 399-405.
Lorenzen, M., Brown, S., Denell, R. and Beeman, R. (2002). Cloning and characterization of the *Tribolium castaneum* eye-color genes encoding tryptophan oxygenase and kynurenine 3-monooxygenase. *Genetics* **160**, 225-234.
Loytynoja, A. and Goldman, N. (2008). Phylogeny-aware gap placement prevents errors in sequence alignment and evolutionary analysis. *Science* **320**, 1632-1635.
Mackenzie, S., Howells, A., Cox, G. and Ewart, G. (2000). Sub-cellular localisation of the white/scarlet ABC transporter to pigment granule membranes within the compound eye of *Drosophila melanogaster*. *Genetica* **108**, 239-252.
Maria Camassa, M. (2001). Responses to light in epigean and hypogean populations of *Gambusia affinis* (Cyprinodontiformes: Poeciliidae). *Environ. Biol. Fishes* **62**, 115-118.
Moldovan, O. (2005). Beetles. In *Encyclopedia of Caves* (ed. D. C. Culver and W. B. White), pp. 45-51. Amsterdam: Elsevier.
Montell, C. (1998). TRP trapped in fly signaling web. *Curr. Opin. Neurobiol.* **8**, 389-397.
Nagalakshmi, U., Wang, Z., Waern, K., Shou, C., Raha, D., Gerstein, M. and Snyder, M. (2008). The transcriptional landscape of the yeast genome defined by RNA sequencing. *Science* **320**, 1344-1349.
Niemeyer, B. A., Suzuki, E., Scott, K., Jalink, K. and Zuker, C. S. (1996). The *Drosophila* light-activated conductance is composed of the two channels TRP and TRPL. *Cell* **85**, 651-659.
Packard, A. S. (1888). The cave fauna of North America, with remarks in the anatomy of brain and the origin of blind species. *Mem. Natl. Acad. Sci. USA* **4**, 1-156.
Page, T. (1985). Circadian organization in cockroaches: effects of temperature cycles on locomotor activity. *J. Insect Physiol.* **31**, 235-242.
Peck, S. B. (1973). A systematic revision and evolutionary biology of the *Ptomaphagus adelops*. *Bull. Mus. Comp. Zool.* **145**, 29-162.
Peck, S. B. (1975). The life cycle of a Kentucky cave beetle, *Ptomaphagus hirtus*, (Coleoptera; Leiodidae; Catopinae). *Int. J. Speleol.* **7**, 7-17.
Peck, S. B. (1984). The distribution and evolution of cavernicolous *Ptomaphagus* beetles in the southeastern United-States (Coleoptera, Leiodidae, Cholevinae) with new species and records. *Can. J. Zool.* **62**, 730-740.
Peck, S. B. (1986). Evolution of adult morphology and life-history in cavernicolous *Ptomaphagus* beetles. *Evolution* **40**, 1021-1030.
Peck, S. B. (1998). Cladistic biogeography of cavernicolous *Ptomaphagus* beetles (Leiodidae, Cholevinae: Ptomaphagini) in the United States. In *ATTI-Phylogeny and Evolution of Subterranean and endogean Cholevidae (= Leiodidae, Cholevinae) – Proceedings of a Symposium (30 August, 1996; Florence, Italy) XX International Congress of Entomology* (ed. P. M. Giachino and S. B. Peck), pp. 235-260. Torino: Museo Regionale de Scienze Naturali.

Porter, J., Yu, M., Doberstein, S., Pollard, T. and Montell, C. (1993). Dependence of calmodulin localization in the retina on the NINAC unconventional myosin. *Science* **262**, 1038-1038.
Poulson, T. (1992). The Mammoth cave ecosystem. In *The Natural History of Biospeleology – Monographs* (ed. A. I. Camacho), pp. 569-612. Madrid: Museo Nactional de Ciencias Naturales.
Poulson, T., Lavoie, K. and Helf, K. (1995). Long-term effects of weather on the cricket (*Hadenoecus subterraneus*, Orthoptera, Rhaphidophoridae) guano community in Mammoth Cave National Park. *Am. Midl. Nat.* **134**, 226-236.
Protas, M. E., Trontelj, P. and Patel, N. H. (2011). Genetic basis of eye and pigment loss in the cave crustacean, *Asellus aquaticus*. *Proc. Natl. Acad. Sci. USA* **108**, 5702.
Rence, B. and Loher, W. (1975). Arrhythmically singing crickets: thermoperiodic reentrainment after bilobectomy. *Science* **190**, 385.
Ribera, I., Fresneda, J., Bucur, R., Izquierdo, A., Vogler, A., Salgado, J. and Cieslak, A. (2010). Ancient origin of a Western Mediterranean radiation of subterranean beetles. *BMC Evol. Biol.* **10**, 29.
Richards, S., Gibbs, R. A., Weinstock, G. M., Brown, S. J., Denell, R., Beeman, R. W., Gibbs, R., Bucher, G., Friedrich, M., Grimmelikhuijzen, C. J. et al. (2008). The genome of the model beetle and pest *Tribolium castaneum*. *Nature* **452**, 949-955.
Rieger, D., Stanewsky, R. and Helfrich-Förster, C. (2003). Cryptochrome, compound eyes, Hofbauer-Buchner eyelets, and ocelli play different roles in the entrainment and masking pathway of the locomotor activity rhythm in the fruit fly *Drosophila melanogaster*. *J. Biol. Rhythms* **18**, 377-391.
Romero, A. (1985). Ontogenetic change in phototactic responses of surface and cave populations of *Astyanax fasciatus* (Pisces: Characidae). *Copeia* **1985**, 1004-1011.
Rusdea, E. (1992). Stabilierende Selektion bei microphthalmen Höhlentieren: Untersuchungen zur tageszeitlichen Aktivitätsverteilung und Poplationsdynamik von *Laemostenus schreibersi* (Küster) (Carabidae). *Mem. Biospeleol.* **19**, 8-110.
Rusdea, E. (2000). Langlebigkeit der Adulten – ein Faktor der die Populationsgröße stabilisieren kann – Erläutert am Beispiel einer Langzeituntersuchung am Höhlenlaufkäfer *Laemostenus schreibersi* (Coleoptera, Carabidae). *Ges. Allg. Angew. Ent.* **12**, 517-521.
Schmidt, H. A., Strimmer, K., Vingron, M. and von Haeseler, A. (2002). TREE-PUZZLE: maximum likelihood phylogenetic analysis using quartets and parallel computing. *Bioinformatics* **18**, 502-504.
Schulz, S., Huber, A., Schwab, K. and Paulsen, R. (1999). A novel Gg isolated from *Drosophila* constitutes a visual G Protein g subunit of the fly compound eye. *J. Biol. Chem.* **274**, 37605-37610.
Scott, K., Becker, A., Sun, Y., Hardy, R. and Zuker, C. (1995). Gq [alpha] protein function in vivo: genetic dissection of its role in photoreceptor cell physiology. *Neuron* **15**, 919-927.
Shetty, K., Kurada, P. and O'Tousa, J. (1998). Rab6 regulation of Rhodopsin transport in *Drosophila*. *J. Biol. Chem.* **273**, 20425.
Sket, B. (2008). Can we agree on an ecological classification of subterranean animals? *J. Nat. Hist.* **42**, 1549-1563.
Studier, E. H., Lavoie, K. H., Wares, W. D., Linn, I. I. and Linn, J. A. M. (1986). Bioenergetics of the cave cricket, *Hadenoecus subterraneus*. *Comp. Biochem. Physiol.* **84**, 431-436.
Tellkampf, T. (1844). Beschreibung einiger neuer in der Mammuth-Höhle in Kentucky aufgefundener Gattungen von Gliedertieren. *Arch. Naturgesch.* **10**, 318-322.
Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673-4680.
Tomioka, K. and Chiba, Y. (1989). Photoperiodic entrainment of locomotor activity in crickets (*Gryllus bimaculatus*) lacking the optic lobe pacemaker. *J. Insect Physiol.* **35**, 827-831.
Tomioka, K. and Matsumoto, A. (2010). A comparative view of insect circadian clock systems. *Cell. Mol. Life Sci.* **67**, 1397-1406.
van der Linden, A., Beverly, M., Kadener, S., Rodriguez, J., Wasserman, S., Rosbash, M., Sengupta, P. and Schibler, U. (2010). Genome-wide analysis of light-and temperature-entrained circadian transcripts in *Caenorhabditis elegans*. *PloS Biol.* **8**, 702-715.
Veleri, S., Rieger, D., Helfrich-Förster, C. and Stanewsky, R. (2007). Hofbauer-Buchner eyelet affects circadian photosensitivity and coordinates TIM and PER expression in *Drosophila* clock neurons. *J. Biol. Rhythms* **22**, 29-42.
Walker, A. R., Howells, A. J. and Tearle, R. G. (1986). Cloning and characterization of the *vermilion* gene of *Drosophila melanogaster*. *Mol. Gen. Genet.* **202**, 102-107.
Wang, T. and Montell, C. (2007). Phototransduction and retinal degeneration in *Drosophila*. *Pflugers Arch.* **454**, 821-847.
Weber, F., Casale, A., Lamprecht, G. and Rusdea, E. (1994). Highly sensitive reactions of microphthalmic carabid beetles to light/dark cycles. In *Carabid Beetles: Ecology and Evolution*, Series Entomologica 51 (ed. K. Desender, M. Dufrêne, M. Loreau, M. L. Luff and J.-P. Maelfait), pp. 219-225. Dordrecht, The Netherlands: Kluver Academic Publishers.
Weber, F., Casale, A., Böhlke, C. and Rusdea, E. (1995). Daily temperature cycles as a Zeitgeber of the circadian locomotion rhythm of the carabid beetle *Sphodropsis ghiliani*. *Mem. Biospeleol.* **22**, 227-234.
Whelan, S. and Goldman, N. (2001). A general empirical model of protein evolution derived from multiple protein families using a maximum-likelihood approach. *Mol. Biol. Evol.* **18**, 691.
Wilhelm, B. T. and Landry, J. R. (2009). RNA-Seq-quantitative measurement of expression through massively parallel RNA-sequencing. *Methods* **48**, 249-257.
Wolff, T. and Ready, D. (1993). Pattern formation in the *Drosophila* retina. In *The Development of Drosophila melanogaster*, Vol. 2 (ed. P. Lawrence and A. M. Martinez), pp. 1277-1326. New York: Cold Spring Harbour Laboratory Press.
Xu, X. Z. S., Chien, F., Butler, A., Salkoff, L. and Montell, C. (2000). TRPγ, a *Drosophila* TRP-related subunit, forms a regulated cation channel with TRPL. *Neuron* **26**, 647-657.
Yarfitz, S., Niemi, G., McConnell, J., Fitch, C. and Hurley, J. (1991). A Gβ protein in the *Drosophila* compound eye is different from that in the brain. *Neuron* **7**, 429-438.
Yoshii, T., Vanin, S., Costa, R. and Helfrich-Förster, C. (2009). Synergic entrainment of *Drosophila*'s circadian clock by light and temperature. *J. Biol. Rhythms* **24**, 452-464.
Yuan, Q., Metterville, D., Briscoe, A. and Reppert, S. (2007). Insect cryptochromes: gene duplication and loss define diverse ways to construct insect circadian clocks. *Mol. Biol. Evol.* **24**, 948-955.
Zelhof, A. C., Hardy, R. W., Becker, A. and Zuker, C. S. (2006). Transforming the architecture of compound eyes. *Nature* **443**, 696-699.
Zhu, H., Yuan, Q., Froy, O., Casselman, A. and Reppert, S. (2005). The two CRYs of the butterfly. *Curr. Biol.* **15**, 953.
Zhu, L., McKay, R. and Shortridge, R. (1993). Tissue-specific expression of phospholipase C encoded by the norpA gene of *Drosophila melanogaster*. *J. Biol. Chem.* **268**, 15994-16001.