Research Article

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Diurnal and circadian regulation of opsin-like transcripts in the eyeless cnidarian *Hydra*

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Abstract: Opsins play a key role in the ability to sense light both in image-forming vision and in non-visual photoreception (NVP). These modalities, in most animal phyla, share the photoreceptor protein: an opsin-based protein binding a light-sensitive chromophore by a lysine (Lys) residue. So far, visual and non-visual opsins have been discovered throughout the Metazoa phyla, including the photoresponsive *Hydra*, an eyeless cnidarian considered the evolutionary sister species to bilaterians. To verify whether light influences and modulates opsin gene expression in *Hydra*, we utilized four expression sequence tags, similar to two classic opsins (SW rhodopsin and SW blue-sensitive opsin) and two non-visual opsins (melanopsin and peropsin), in investigating the expression patterns during both diurnal and circadian time, by means of a quantitative RT-PCR. The expression levels of all four genes fluctuated along the light hours of diurnal cycle with respect to the darkness one and, in constant dark condition of the circadian cycle, they increased. The monophasic behavior in the L12:D12 cycle turned into a triphasic expression profile during the continuous darkness condition. Consequently, while the diurnal opsin-like expression revealed a close dependence on light hours, the highest transcript levels were found in darkness, leading us to novel hypothesis that in *Hydra*, an "internal" biological rhythm autonomously supplies the opsins expression during the circadian time. In conclusion, in *Hydra*, both diurnal and circadian rhythms apparently regulate the expression of the so-called visual and non-visual opsins, as already demonstrated in higher invertebrate and vertebrate species. Our data confirm that *Hydra* is a suitable model for studying ancestral precursor of both visual and NVP, providing useful hints on the evolution of visual and photosensory systems.

Keywords: visual and non-visual opsins, NVP, light regulation, circadian rhythms, *Hydra*

Introduction

Opsins play a key role in photoreception, which is the first key step in seeing in Metazoa [1,2]. Opsins are integral membrane proteins of photoreceptor cells that absorb photon energy and ultimately convert it into an electrical signal toward the central nervous system. Photoreception takes place in photoreceptor cells ("ciliary" and "rhabdomeric", respectively, in vertebrates and invertebrates) capable to sense directly ambient light [3,4]. Photoreception is phylogenetically one of the oldest sensory modalities thanks to the amazing ubiquity in all animal *phyla* of morphological, functional, and molecular systems (from simple invertebrate light-sensitive cells to more complex vertebrate eyes) that respond to environmental luminous stimuli [5,6]. Although framed in different structure-function relationships, in vertebrate and invertebrate visual cells, photoreception starts with the photoisomerization of the retinal chromophore of the photopigment, usually an opsin-based pigment. This process triggers the binding of the opsin with a G-protein that leads to an enzymatic visual cascade, culminating in a final messenger, which gates light-sensitive ion channels to modulate and shape the electric signal toward the nervous system [7,8].

In addition to conventional eyed structures, vertebrates and invertebrates have supplementary non-visual photoreception (NVP) systems for non-image-forming vision or circadian vision [9,10,11]. Photic information mediated by NVP integrates visual activity and is involved in temporal (time-of-day) and behavioral physiology of the animal (e.g., photoperiodism, timing, and photoentrainment of circadian rhythms) [12–14].

NVP cells, formerly named extraretinal or extraocular photoreceptors, are currently termed non-visual (non-image-

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forming) photosensitive cells in invertebrates, and non-rod non-cone photoreceptors in vertebrates (after the discovery of photosensitive retinal ganglion cells) [12]. NVP cells are mainly located within nervous system districts and share with retinal photoreceptors common evolutionary origin and light-sensing modalities, above all the same superfamily of opsin-based photoreceptor proteins. Therefore, NVP opsins have been identified in cells beyond the retinal photoreceptors in several vertebrates and in extraretinal tissues of some invertebrates [12,15].

Focusing the attention on both opsin-based pigments and their structure/function relationship, the search for novel opsins supplying image- and nonimage-forming photoreception is a well-established field in photosensory biology and in vision research. It provides new insights into the evolution of the visual systems and of the visual ecology.

Accordingly, for more than 30 years, we were using the eyeless freshwater *Hydra* (Cnidaria, Hydrozoa), considered the evolutionary sister to bilaterians [16], as animal model to identify the presence and the co-existence of different opsins, classified in phylogenetic higher invertebrate and vertebrate species as serving visual and non-visual functions, and to study their expression and functional role [1,17–20].

Hydra, the first metazoan in which a primitive nervous system in the form of a diffuse nerve net can be found, has no eyes or eye-like structures; nevertheless, it is photosensitive, responding to light by slow contraction and elongation movements. The photic modulation of its periodic behavior has been demonstrated [21–24] as well as the identification and the localization of opsin-based pigments responsible for its photosensitivity [16,19,25,26]. However, many questions remain unanswered due to the large number of opsin genes identified, with respect to those certainly expressed [17], and their molecular evolution [18].

Furthermore, during evolution, regular light/dark changes have had a profound impact on visual systems including the evolution of regular rhythms in biological processes ranging from behavioral to biochemical. Although a key evolutionary trait is the adaptation to day/night alternation and its impact on integrated physiology, the photic regulation of opsin gene expression remains rather understudied.

So far, evidence for diurnal and circadian regulation of opsin expression patterns has been reported in very few species of invertebrates [27–29] and vertebrates [30–33]. We directed our efforts on the possible photic regulatory effect of the diurnal and circadian regimes on the expression of selected opsin-like expressed sequence tags (ESTs) in *Hydra*, similar to visual and non-visual opsins, demonstrating that both cycles are able to tune and/or to regulate the expression of the transcripts. These results suggest that opsin rhythms are probably endogenous and that putative circadian oscillators could drive the opsin transduction. Moreover, the different opsin mRNA levels observed in the circadian regime with respect to those obtained in the diurnal one suggest that an internal mechanism could modulate those processes.

Our data in *Hydra* show that a strong regulation by light on the opsin gene expression of selected opsin-like photopigments occurs and that the co-existence of classic and unconventional opsins indicates this cnidarian as a putative common precursor of both visual and non-visual photosensitive modalities, confirming the suitability of this animal model for the study of the evolution of the visual function.

Methods

Animals

Hydra vulgaris specimens were asexually cultured in *Hydra* medium (HM) (1 mM $CaCl_2$ and 0.1 mM $NaHCO_3$, pH 7) according to protocols previously reported [15,22,25]. Animals were kept in a thermostatic cabinet at 17 ± 1°C under controlled lighting conditions by a 12:12 *light–dark* (LD) cycle where lights went on at 8:00 am (ZT0) and turned off at 8:00 pm (ZT12). Circadian regime was applied keeping the animals in total darkness for 24-h cycle (dark–dark). Cultures were fed with *Artemia nauplii* daily and washed 6 h after feeding. Animals used for the experimental procedure were starved for 3 days before use to avoid any contamination by *Artemia*'s photopigments, carotenoids, and other photoreceptor proteins.

Hydra target sequence collection

Hydra ESTs of opsin-like photopigments, similar to visual and non-visual opsins, were collected from dbEST database (https://www.ncbi.nlm.nih.gov/genbank/dbest/). The sequences, showing strong similarity to photopigment cDNA sequences of other vertebrate and invertebrate organisms, were aligned at NCBI Gene (http://www.ncbi.nlm.nih.gov/gene/) and common primers were designed to obtain optimized primers for quantitative PCR analysis. In particular, the following cDNA sequences were used: DT617488 acc. (similar to putative photopigment melanopsin); CN554795 acc. (similar to rhodopsin); CN775258 acc. (similar to blue-sensitive opsin); CB073527.1 acc. (similar to visual pigment-like receptor peropsin-like); and CX055471 acc. (Beta-actin), as reference. Details of the expressed

sequences and their related forward and reverse primers are given in Tables 1 and 2.

Animal sample collection

The animal collection procedure was done in light conditions identical to the correspondent diurnal phase (housing conditions). The procedure in darkness was done by using an infrared viewer or red-light illumination for photographic development. One hundred animals were collected from a homogeneous population at each time and for each experimental light condition. The whole experimental procedure was performed twice for each light regime. Animal samples were harvested and homogenized in TRIzol Reagent (Invitrogen) for 1 day every 3 h, starting from 9:00 am, in two different light condition regimes: (1) a Zeitgeber cycle or diurnal cycle (represented by the alternation of 12 h light and 12 h darkness conditions) and (2) a circadian cycle (represented by total darkness along 24 h). Considering that T0 and T12 were fixed at 8:00 am and 8:00 pm, the ZT/CT (Zeitgeber Time/Circadian Time), numbered with 1, 4, 7, 10, 13, 16, 19, and 22, corresponded, respectively, to the harvesting made at 9:00 am, 12:00 am, 3:00 pm, 6:00 pm, 9:00 pm, 12:00 pm, 3:00 am, and 6:00 am.

RNA extraction and quantitative RT-PCR

TRIzol samples were stored at −80°C before RNA extraction. Total RNA was purified, quantified, characterized, and retrotranscribed as previously reported [34]. For all samples tested, the RNA integrity number (Bionalyzer 2100, Agilent) was greater than eight relating to a 0–10 scale. Quantitative real-time PCR (RT-PCR) was performed by an iCycler-iQ5® (Bio-Rad, Milan, Italy) in a 20 µL reaction mixture containing 10–50 ng of cDNA optimized primers for SYBR-green analysis. Assays were performed in quadruplicate (maximum ΔCt of replicate samples <0.5), and a standard curve from consecutive fivefold dilutions (100–0.16 ng) of a cDNA pool representative of all samples was included for PCR efficiency determination. Optimized primers for SYBR Green analysis and optimum annealing temperatures were designed by the Allele-Id software version 7.0 (Biosoft International, Palo Alto, CA, USA) and were synthesized (HPLC purification grade) by MWG-Biotech (Ebersberg, Germany). For each target, all sequences at NCBI Blast https://blast.ncbi.nlm.nih.gov/Blast.cgi were aligned and common primers were designed (see Tables 1 and 2). Relative expression calculation, correct for PCR efficiency and normalized with respect to reference genes β-actin, was performed by the iQ5 software. Results are expressed as fold expression, compared with control (=1) [35]. Statistical significance was evaluated by the Origin 2022 software (OriginLab Co.) performing a one-way analysis of variance (ANOVA), followed by Bonferroni's post hoc test. For the sake of clarity of figures, only the more representative differences were reported in the graphics with their significances always represented with one asterisk for both $p < 0.05$ and $p < 0.001$; for simplicity, the relative p-value was reported in the caption only if the significativity was discussed in the text, alternatively, only the fewer comparisons that resulted in not significative were indicated [36]. All the measurements were obtained from two independent experiments and values are expressed as the mean ± standard deviation.

Results

The gene sequences studied belonged to opsin-like photopigments similar to opsins serving visual and non-visual

Table 1: RT-PCR primers characteristics

Locus definition	GeneBank accession	Forward primer	Reverse primer	PCR product size (bp)
Melanopsin-like	DT617488	5′AATTCGTTTGTCATTATTACTATATTG3′	5′AGAGTCCATATTGATGTTGTTAAG 3′	221
Peropsin-like	CB073527	5′ACCATACTAGCAAAGGGAAACAC3′	5′GCAATAGCAGTTAAGGCAAGG 3′	200
Blue-sensitive opsin-like	CN775258	5′CGTTTGAGCAAGCACCTGATTC3′	5′CCGTAGCCGTTTCTTTAGTCTTATATTTAG3′	114
Rhodopsin-like	CN554795	5′CTTATTGTGCTTTGTTTGCTAAATCATC3′	5′ACTGTTGGGTTATTGAAGAGTTTCC3′	124
Beta actin	CX055471	5′CTCCGTGTTGCTCCAGAAG3′	5′AGACACCATCTCTTGAATAAAG3′	199

Gene expressed sequence tags (ESTs) nomenclature, GenBank accession number, forward and reverse primer sequences, and predicted size of the amplified product.

Table 2: cDNA FASTA sequences and the relative primers sequences

EST definition	GeneBank accession no.	FASTA sequence
ACAH-aaa95g08.g1 Hydra_EST_UCI-10 Hydra vulgaris	DT617488	ACCCACGCGTCCGGCGAATGATGCTTTCTAAAGATATTACTAAAATTACATCCGTACATTATTCCATA-TGTATATTCCTTGGTATAATTATT*AATTCGTTTGTCATTATTACTATATTG*AAAAATAAAAAATTGCAAACT-ACAACAAACTTCTTCATTTTAAATTTGGCAATAGCTGATCTTTTATTTACTGTGTTTGGCATATCGGC-AATTTTTTATAAAGGCTTTGCAAAAACATTGTCGAAAGAAAACTCTATTTGTGTGGTTATAGGTTTTT-TTACATTACTTTT**CTTAACAACATCAATATGGACTCT**TGTTATGATTAGCATCAACAGATACTTAAA-TGTTGCAAAAGCCAACATTATAAAAACACTTTACACAAGAAAAAAAACTGTTTTAATTATTGCAGGC-GTTTGGATATTTTCTGTATTTATTTCAGCTCCACCTTTGATTGGTTGGAGCGAATTTAAATCAACTTC-CAGTTTTTGTACCATTAATGGCAAAAAAAATATATCGTACACAGTTTTTCTTGGTTTATTAGTTTATAT-AATACCAATGGTTTTTTTGGCAAGTTTGTACATGCGCATTTTTTTTTTTGTTAAACAAAGTCGAAAAA-GAGA AACTGAGAAGAAGTA TAAACTC
CB073527.1 taa17c02.y1 Hydra EST - II Hydra vulgaris	CB073527	GGACAGACATTTGTGTGATCAATGGCATTTGTTTTTATTATCGTATTTTTATCGTTCTTATGTGGATTT-TCTGTGATATTAAACGTCACTGTAGTCTTA*ACCATACTAGCAAAGGGAAACAC*TAAAAACACTCGAGAT-GTAATTTTGATGAGTCTTGCAATATGTGATGGCGTACAGTGCACTATAGGATATCCGGTCGAACTG-TTTGGTTATGCTAATTACAAAAATCCATCACTATCCGAAAAGTTTTGCAAACCGAGTGGTTTCATTGT-TATGTA**CCTTGCCTTAACTGCTATTGC**ACATTTGGTTTGTTTATGTATATATCGCTATTTAACTATTG-TATATCCACTAAAACTACAGATATTTCTCACAAAATCGAATTGGAGTGCATGTGGTTGTATTGCCTTT-TGCTGGATCTATGGTTTGTTTTGGTCATTAAGTCCTCTACTCGGTTGGAATGAAATAGTGCGAGAAA-ACAAGGATACCTACAAATGCTCAATTAAT
CN775258.1 tae71c10.y1 Hydra EST Darmstadt I Hydra vulgaris	CN775258	TTGAAAAGTAATATAAACTCTATTGAATATTCTGAGCAATCGGAAAATGTATCGA*CGTTTGAGCAAGC-ACCTGATTC*ATCAGTTCCACAACAAGCACGAAACGAAACACTAAACGACAAAAAAGTTACAGTTTACT-TTT**CTAAATATAAGACTAAAGAAACGGCTACGG**TAGACAGTAAATCCCCTTTAATTCAAAATAAC-TTTCTTGAAGCAGAAAAAAAGAAAGAAAAGCGTCAAATGGTCGCTATTGCAATACATTTGAAGCAA-GTTCGAGTTACAAAAATGCTTATGATTTTAGTGTTAAGCTTCTTTTTTTGTTGGACACCATTTTTTGTT-GGAGCATTGTACCACGTTTATCACAAAAAAATTAACGGTTTTCAAGTGACAACTTTTGGAATAATGT-GCGCTTGTTTAAATTGCATTCTAAATCCTTTTATTTATTCTATTATGAATCGTAGTTTTCGAAAATGCG-TGGTAAAAATGTGGAAAAACCTAATTTCATCTTANTTATTATTCAGA
CN554795.1 tae29c05.y1 Hydra EST Darmstadt I Hydra vulgaris	CN554795	CAGGATACCTACAGATGCTCAATTAATTTATATCCGGATAATGAGATAAAAAGTAGTTATTTATACG-CTCTGGCAATATTTTGTTACCTTATTCCTCTAATAATAATTATTTACTGTAGTTTAAAAGTCCGCTCAG-AACTTCGCAACATGTTAAAAATGTGCAAACAAATTTCTGGTGTTGAAGCAAATATTACAAAAGTTAC-ATATCGAATAGAAAAACAAGATTTTATATCTGTAAGTTTTATAATAGCATCATTTTTTACTGTTTGGAC-TCCATATGCCGTATGTGTTTTTTATTTGACAATTGGAAAAAAACTACCTCCTAGTTTTTTAA*CTTATTGT-GCTTTGTTTGCTAAATCATC*AACAATTCTGAACCCTATCATTTATTGTCTCATGTATAAAAAATTTCGCCA-AACATTACAAAGTAAATTTG**GGAAACTCTTCAATAACCCAACAGT**TACACCAGCTGTTTAAAAAAA-AGTTCTGATGTTTGGATACCTCAAGATTGAAAGCAAGTCGCTGCTTTGTAC
CX055471.1 tai90d12.y2 Hydra EST UCI 5 ALP Hydra vulgaris	CX055471	CAAGAGCTGTTTTCCCATCTATTGTTGGACGTCCTCGTCATCAAGGAGTCATGGTTGGTATCTTACA-GAAGGATTCCTATGTCGGTGACGAAGCTCAGAGCAAACGTGGTATCTTAACTTTGAAATACCCAAT-TGAACACGGAATTGTAACTAACTGGGATGATATGGAAAAAATTTGGCATCACACTTTTTACAATGAG-*CTCCGTGTTGCTCCAGAAG*AACACCCTGTCCTTCTTACTGAAGCTCCCCTGAATCCCAAAGCAAATCAT-GAAAAAATGACCCAAATCATGTTTGAGACATTTAACTCTCCTGCAATGTATGTTGTTATTTACGCCGT-CTTATCCTTGTATGCTTCTGGTCTTACCACTGTTATTGTA**CTTTATTCAAGAGATGGTGTCT**CTTACT-CAGTACCAATC

The italic typed letters show the nucleotide sequences for forward primers while bold typed letters show those for reverse primers.

tasks in vertebrates and invertebrates, respectively, rhodopsin and blue sensitive opsins, and melanopsin and peropsin (see "Methods" section). We used partial mRNA sequences (ESTs) of these genes to investigate the expression patterns during both the *Zeitgeber* (ZT) and the circadian (CT) time by means of a quantitative RT-PCR.

In Figure 1, the relative expression of all opsin-like sequences during the ZT and CT cycles is represented. It is evident (Figure 1a) that the expression levels fluctuated along the light hours of diurnal cycle with respect to the darkness ones. In fact, they all appeared synchronized in peaking around ZT7 (3:00 pm), with DT617488 (similar to putative photopigment melanopsin) and CN554795 (similar to rhodopsin) presenting the highest levels. However, while all of them showed a monophasic cyclical alternation, a second steep rise was observed for CN554795 during the dark hours at ZT19 (Figure 1a; ANOVA one way, Bonferroni post hoc test; for statistical significance see figure caption). In addition, in the

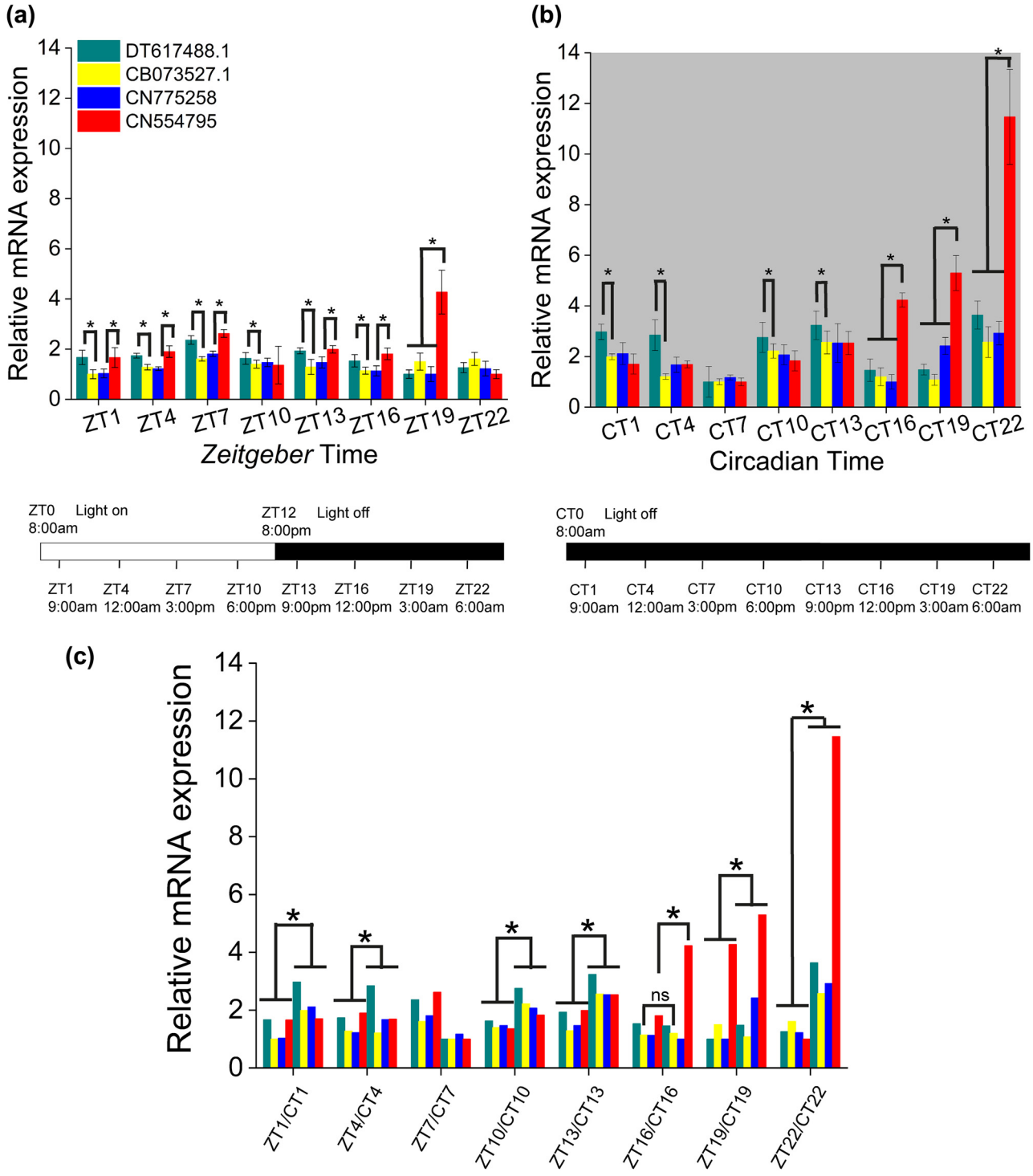

Figure 1: Relative expression level of EST sequences of opsin-like genes. (a) The relative expression level DT617488, CN554795, CN7752589, and CB073527.1 during a Zeitgeber cycle (ZT1 = 9:00 am, ZT4 = 12:00 am, ZT7 = 3:00 pm, ZT10 = 6:00 pm, ZT13 = 9:00 pm, ZT16 = 12:00 pm, ZT19 = 3:00 am, ZT22 = 6:00 am). Significativity was evaluated between the various opsins within each ZT group by means of ANOVA one way, Bonferroni post hoc. All differences were significant ($p < 0.05$; $p < 0.001$) except for.ZT1: CN77 vs CB, CN55 vs DT; ZT4: CN77 vs CB; ZT10: CN77 vs CB; ZT13: CN55 vs DT; ZT16: CN77 vs CB; ZT19: CN77 vs DT; and ZT22: CN77 vs DT. (b) The relative expression levels during a circadian cycle (CT1 = 9:00 am, CT4 = 12:00 am, CT7 = 3:00 pm, CT10 = 6:00 pm, CT13 = 9:00 pm, CT16 = 12:00 pm, CT19 = 3:00 am, and CT22 = 6:00 am). Significativity was evaluated between the various opsins-like within each CT group. Differences were significative and DT took the maximum values in CT1-4 and CT10-22 (for DT versus CB: CT1 $p = 2.6 \times 10^{-6}$, CT4 $p = 1.5 \times 10^{-12}$, CT10 $p = 1.5 \times 10^{-8}$, CT13 $p = 2.2 \times 10^{-9}$, CT16 $p = 1.68 \times 10^{-5}$, CT19 $p = 4.3 \times 10^{-8}$, and CT22 $p = 2.5 \times 10^{-9}$) and CN55 in CT16-22 (for CN55 vs DT: CT16 $p = 1.13 \times 10^{-16}$; CT19 $p = 4.8 \times 10^{-19}$, and CT22 $p = 1.2 \times 10^{-21}$). (c) Significative differences between ZT and CT cycle at each time. Differences were significative when comparing the two experimental conditions (for DT: ZT1 vs CT1 $p = 6.3 \times 10^{-7}$, ZT4 vs CT4 $p = 4.3 \times 10^{-8}$, ZT10 vs CT10 $p = 4.1 \times 10^{-9}$, ZT13 vs CT13 $p = 1.8 \times 10^{-7}$, ZT16 vs CT16 $p = 0.06$, ZT19 vs CT19 $p = 3.5 \times 10^{-4}$, and ZT22 vs CT22 $p = 5.3 \times 10^{-11}$) with CN55 presenting the maximum difference values (CN55 vs DT: ZT16 vs CT16 $p = 3.7 \times 10^{-10}$, ZT19 vs CT19 $p = 1.25 \times 10^{-7}$, and ZT22 vs CT22 $p = 1.2 \times 10^{-12}$). The bars at the base of the plots show respectively the L12:D12 and D12:D12 experimental conditions; relative quantification was carried out by normalization of the values to those of the housekeeping gene β-actin. The real-time PCRs were made in four independent replicate assays. Error bars show the standard error of the mean.

circadian cycle, the relative expression increased about two-fold for all opsins-like sequences and about fourfold for CN554795 (Figures 1b and c; ANOVA one way, Bonferroni post hoc test; for statistical significance see figure caption).

Figure 2 shows the fluctuations of the expression of the selected opsin-like proteins along ZT and CT cycles. The expression of each opsin-like sequence was graphed separately from each other by bar plots (Figure 2a and b) so that they could be easily compared. It clearly emerges that the monophasic behavior of DT617488, CN775258, and CB073527.1 observed during the L12:D12 cycle (Figure 2a; ANOVA one way, Bonferroni post hoc; for statistical significance see figure caption) turned in a three-phasic expression profile during the continuous darkness condition with peaks at the beginning of CT1 (9:00 am), CT10 (6:00 pm), and CT22 (6:00 am), (Figure 2b; ANOVA one way, Bonferroni post hoc test; for statistical significance see figure caption). The increase of CN554795 followed an exponential trend (Figure 2b and c) resulting well modeled by means of a one-phase exponential growth function (Figure 3):

$$\text{Relative expression } (t) = Y_0 + Ae^{Kt},$$

where Y_0 represents the initial expression level, e is the natural logarithm base, A is the expression amount, and K is the growth rate factor.

Figure 3 shows the comparison between ZT with CT for each opsin-like proteins employed. The expression of transcripts for each opsin-like was always higher during continuous darkness conditions with respect to those in the light/dark cycle (Figure 3a) except at 3:00 pm where the trend was inverted. Moreover, at 12:00 pm, DT617488, CN775258, and CB073527.1 presented approximately the same expression level both in diurnal and circadian time, which could indicate a constitutive level of the opsin expression. These considerations are better represented in Figure 3b, where each CT expression value was normalized to the corresponding ZT value.

Discussion

Nowadays, it is well established that opsin gene expression is strictly correlated with the circadian regulation of the photic input in animals. Therefore, evidence for diurnal and circadian regulation of opsin expression patterns has been reported in photoreceptors of invertebrates, *Limulus* [27,28], *Apis* [29], and (lower) vertebrates [30–33].

In recent years, light-regulated gene expression of photosensitive proteins has gained topicality due to the potential implications with development, disease onset progression, and consequent timing of therapy [37]. For example, strong correlations have been found in mice between mutated opsins with photoreceptor cell death [38], in *Drosophila* between daily blue light exposure with the onset of brain neurodegeneration [39], and in the honeybee *Apis* between temporal expression of all opsins with the maturation of the retina during pupal development [40]. Furthermore, very recently, in the seahorse *Hippocampus*, it has been shown that specific wavelengths affect opsin mRNA levels whose changes were life-state dependent, allowing interference of opsin ontogeny and of the animal development stages [41].

Thus, consistent with the above novel research frame, our study reports the first evidence of a diurnal and circadian regulation of selected opsin transcripts in the eyeless cnidarian *Hydra*, which has the first elementary nervous system in the form of a diffused net [42]. The main result that emerged from this study is that the expression level of the examined opsins showed a similar trend in the same experimental condition (ZT or CT) but not when ZT fluctuations were compared versus CT fluctuations. In fact, the opsin-like expression during the diurnal time (L12:D12) revealed a close dependence on the light hours with an increment for all opsins at 3:00 pm (ZT7) and only for SW opsin-like with a further rise at 3:00 am (ZT19).

Moreover, the condition of continuous darkness (D12:D12) unveiled that, with no external light stimuli, the expression was higher and forced to cycle three times. Therefore, the irradiance (i.e., the ambient light intensity) during the *Zeitgeber* time (ZT), acting as the main temporal indicator of the daytime, would be able to mute the circadian mechanism to better optimize the opsins expression to the variable demands of night and day. At the same time, other mechanisms, to date still unidentified, would intervene in the endogenous expression.

These data are consistent with similar findings reported in other invertebrates: e.g., the horseshoe crab *Limulus* [43], the crab *Gelasimus* [44], the aphid *Acyrthosiphon* [45], and the moth *Helicoverpa* [46]. In addition, both the light regulation of opsin expression and the diurnal rhythm have strong implications for the behavioral output [47–49]. Opposite regulation exerted by the light of opsin and clock gene expression, respectively, up-regulated for an opsin OPN4 gene and down-regulated for CLOCK and CRY clock light-sensitive genes, has been very recently reported in eyespot structures of the bivalve mollusk *Mytilus* [50].

Accordingly, the LD fluctuations observed in our study completely mirror the day/night oscillatory slope of the behavioral spectra in *Hydra* representing the diurnal variation of a biophysical parameter of the bioelectrical activity (i.e., the contraction bursts and rhythmic pulses) underlying the rhythmic body contraction and elongation which determine

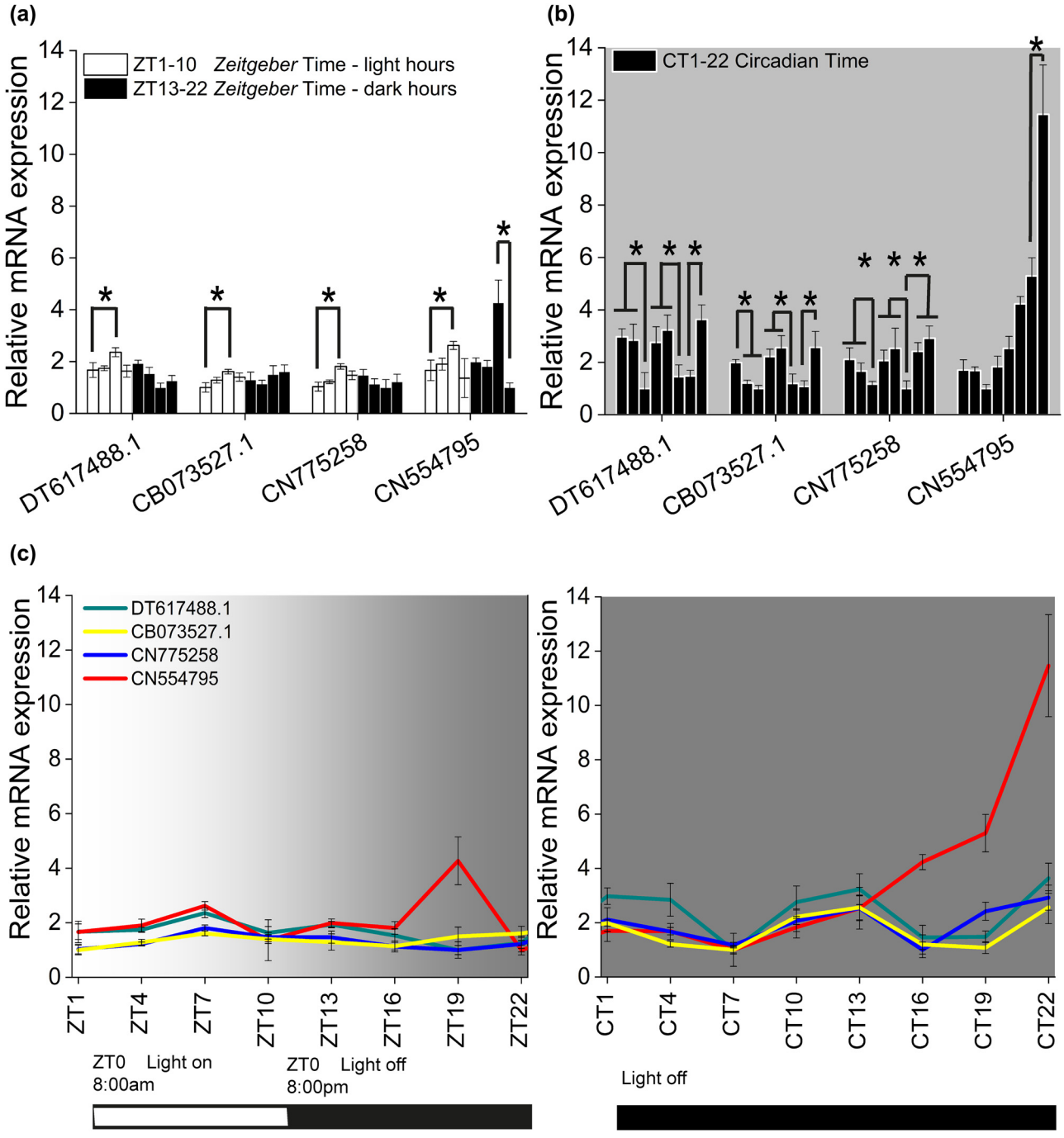

Figure 2: Opsin-like expression fluctuations along ZT and CT. The different trend of opsin-like expression respectively in (a) L12:D12 diurnal cycle, where the maximum difference values were reached at ZT7 (ZT7 vs ZT1: DT $p = 1.6 \times 10^{-4}$, CB $p = 3.4 \times 10^{-3}$, CN77 $p = 9.8 \times 10^{-6}$, CN55 $p = 7.4 \times 10^{-7}$ and ZT19 VS ZT22: CN55 $p = 1.5 \times 10^{-9}$) and in (b) D12:D12 circadian cycle, where DT, CB, CN77 peaked three times during the circadian time and CN55 exponentially rose (DT: CT1 vs CT7 $p = 5.3 \times 10^{-9}$, CT10 vs CT16 $p = 1.1 \times 10^{-9}$, CT22 vs CT19 $p = 2.4 \times 10^{-11}$; CB: CT1 vs CT7 $p = 1.4 \times 10^{-6}$, CT10 vs CT16 $p = 4.1 \times 10^{-8}$, CT22 vs CT19 $p = 1.2 \times 10^{-7}$; CN77: CT1 vs CT7 $p = 1.1 \times 10^{-6}$, CT10 vs CT16 $p = 3.2 \times 10^{-6}$, CT22 vs CT16 $p = 1.2 \times 10^{-7}$; and CN55: CT22 vs CT19 $p = 1.9 \times 10^{-12}$). (c) ZT versus CT expression fluctuations for all opsin-like. The bars at the base of the plots show respectively the L12:D12 and D12:D12 experimental conditions. Error bars show standard error of the mean.

the simple and restricted *Hydra* behaviors such as osmoregulation, locomotion, and feeding and are more elevated during the day respect to the night [51–53].

Moreover, the condition of continuous darkness in the circadian cycle unveiled that an internal mechanism autonomously expresses opsins at higher levels in the absence of a

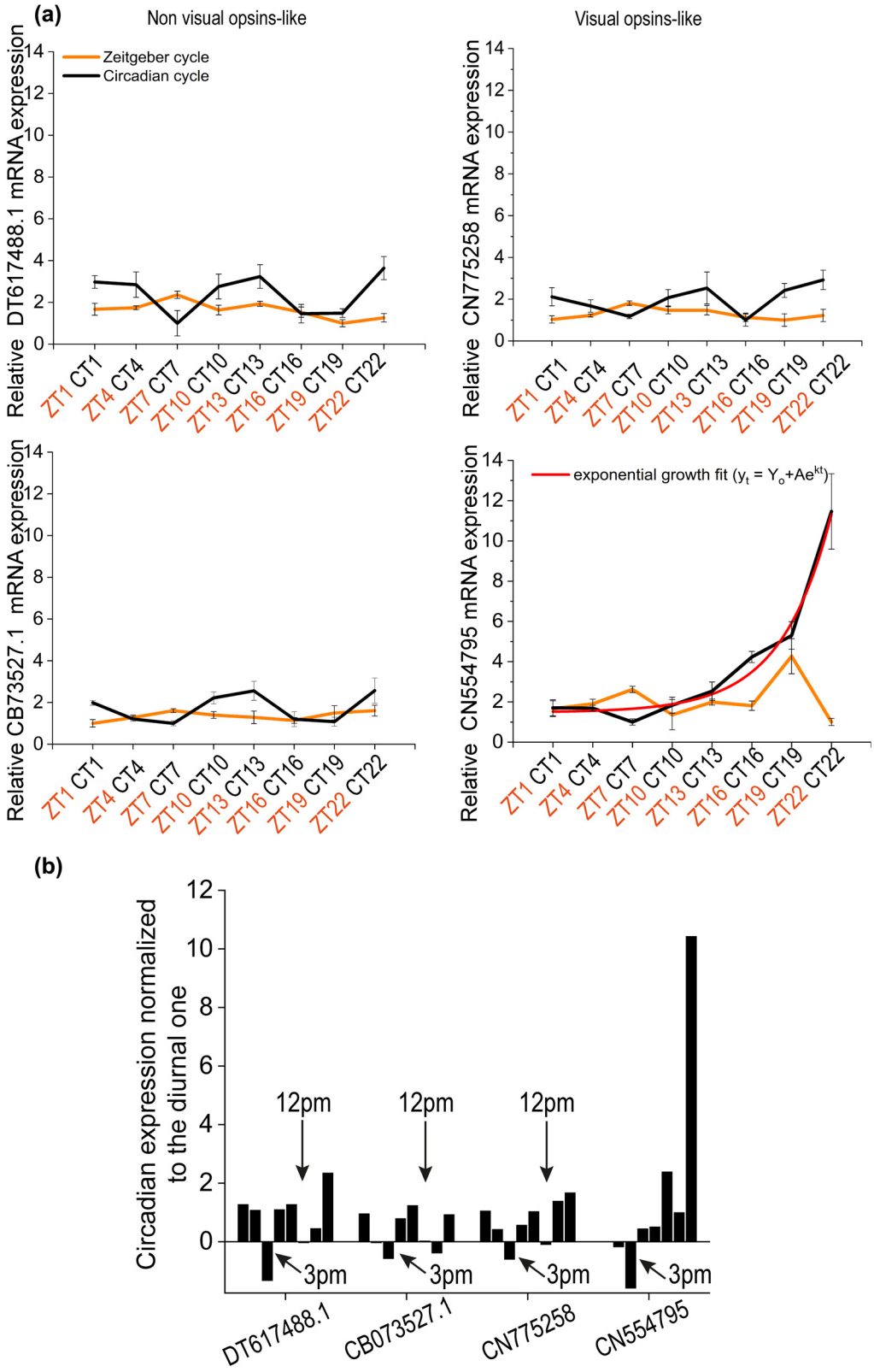

Figure 3: Comparison of ZT and CT for each opsin-like. (a) Relative mRNA expression level for Melanopsin-like (DT617488), SW Rhodopsin-like (CN554795), SW Blue sensitive opsin-like (CN7752589), and Peropsin-like (CB073527.1) in which the continuous darkness conditions was compared respect to the diurnal one. Error bars show standard error of the mean. (b) Circadian expression levels normalized to the corresponding diurnal ones for each opsin-like.

normal day–night cycle. A long period of darkness represents a stressful and dangerous condition for the sessile *Hydra* polyp as its behavior strictly depends on light radiation and activities slowdown in the darkness. In addition, the absence of a normal cycle would not allow the maintenance of opsin levels as *Hydra*'s cells continuously proliferate and are frequently lost by sloughing or budding. The overexpression of opsins in the continuous absence of external light stimuli would allow the animal to find itself fully reconstituted once it emerges from the unsafe darkening condition and the opsins themselves could play a role in this process. From literature, it appears clear that, depending on the context and tissue, opsins could act not only as photosensors but also as critical regulators of many biological processes thanks to their ability to interact with a plethora of molecules as polymodal sensory receptors. A major discovery is that invertebrate opsins could have sensory roles that are light independent. They can constitutively bind their downstream G-protein partner to switch or accelerate a pathway signaling [54] or alternatively work as thermosensor [55]. In this case, a significant drop in temperature could be the switch needed to detect a prolonged darkening condition and to induce overexpression.

Finally, the effect of the light regulation on behavioral patterns could depend also on the spatial distribution of the photoreceptors' expression levels, although currently the opsin expression seems to be not segregated in single animal parts but appears diffused along the whole ectodermal sheet [19,25]. Further approaches with technologies like mRNA *in situ* hybridization and antibody staining could be useful to verify possible spatial localization of expression changes.

These hints open a window on an intriguing phylogenetic scenario and should be the object of future study, as no biological clocks have as yet been molecularly identified in *Hydra* (although expression levels for 380 genes underlying diel, i.e. diurnal, behavior have been found [46]). Conversely, clock genes have been characterized in other phylogenetically close cnidarian species, the sea anemone *Nematostella* [56,57], and the coral *Acropora* [58]. Likewise, opsin photopigments have been proven to be involved in circadian clock entrainment and synchronization in *Drosophila* [59].

Furthermore, we report the first evidence of light regulation of non-visual opsin transcripts, melanopsin, and peropsin, in a lower invertebrate. Melanopsin is a vertebrate non-visual opsin even if resembles the invertebrate ones in various aspects, therefore classified as belonging to the rhabdomeric type [60]. Early identified in the inner retina of amphibian [61] and then in the fish [62], lizard [63], bird [64], mouse, and human [65] and currently widely identified in vertebrate species including humans [66], melanopsin is considered a key molecule in circadian response to light [13,67]. In the same way, peropsin is a non-visual opsin first identified from human ocular tissue and is nowadays considered a regulatory molecule of the visual physiology [68,69]. In *Hydra*, a possible role of peropsin as photoisomerase could be not excluded, supported by the presence of a similar kind of photoprotein in the jellyfish ocelli [70].

Light and circadian regulation of melanopsin has been mainly reported for vertebrate inner retina [32,62,71–73] while accounts for invertebrate extra-ocular systems are substantially lacking. Although very recently long-wavelength opsins have been found in extraocular regions of the blind shrimp *Creaseria*, they are strictly related to the synchronization of biological processes and diurnal vertical migration [74]. Therefore, our data could represent the first account of the expression of a light-regulated opsin gene similar to melanopsin in a primitive invertebrate. According to our results, the presence and the regulation of these transcripts in the lower cnidarian *Hydra* suggest that the so-called non-visual modality evolved early to allow the detection of the quantity and of the quality of the changes of light for adaptive advantage. Moreover, in the frame of the current controversial debate on cnidarian opsin phylogeny, the coexistence of visual-opsin transcripts in an eyeless cnidarian suggests that orthologs of visual opsin could have evolved prior to the evolution of animal visual systems [75]. Additionally, regarding non-visual opsins, the coexistence of non-visual and ocular expression of opsins has been already reported [76] and, for melanopsin in particular, correlation has been proved between its expression and the onset of several diseases [77,78], including those caused by the dysregulation of temporal physiological processes [79,80].

Conclusions

Overall, our study in *Hydra* can be summarized according to these key points:

i. the irradiance regulates the diurnal expression of visual and non-visual opsins, while its absence in the circadian cycle could induce an upregulation of their expression;
ii. a putative, still unidentified, circadian system autonomously drives opsin expression; and
iii. the gain of circadian transcriptional profiles is consistent with no irradiance detection.

To our knowledge, in this study, we report the first evidence of the light-regulated expression of visual and non-

visual opsin transcripts in a lower invertebrate. Therefore, we could state that the irradiance signaling system has evolved early in the Cnidaria, hypothesizing that orthologs of visual opsin would have evolved prior to the evolution of animal visual systems. In conclusion, the discovery of non-visual – extra-ocular – photoreceptor proteins (including above all opsins) and the study of their expression and of the sensory ambient modality that regulates them raise the NVP to a light-sensing signaling complementary to vision, rather than an evolutive residual modality [13,14].

Finally, in the wake of the integrated view of a visual system mutually interacting with non-visual and temporal mechanisms and processes [6], our study strongly supports the suitability of *Hydra* as an appropriate model for identifying ancestral precursors of what the current literature defines as visual and non-visual photosensitive modalities (including their irradiance signaling system and the relative phototransduction pathways), and for phylogenetic and comparative studies on the evolution of visual and circadian systems.

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Conflict of interest: Carlo Musio is a member of Biomolecular concepts' Editorial Board. The other authors state no conflict of interest.

Data availability statement: The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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