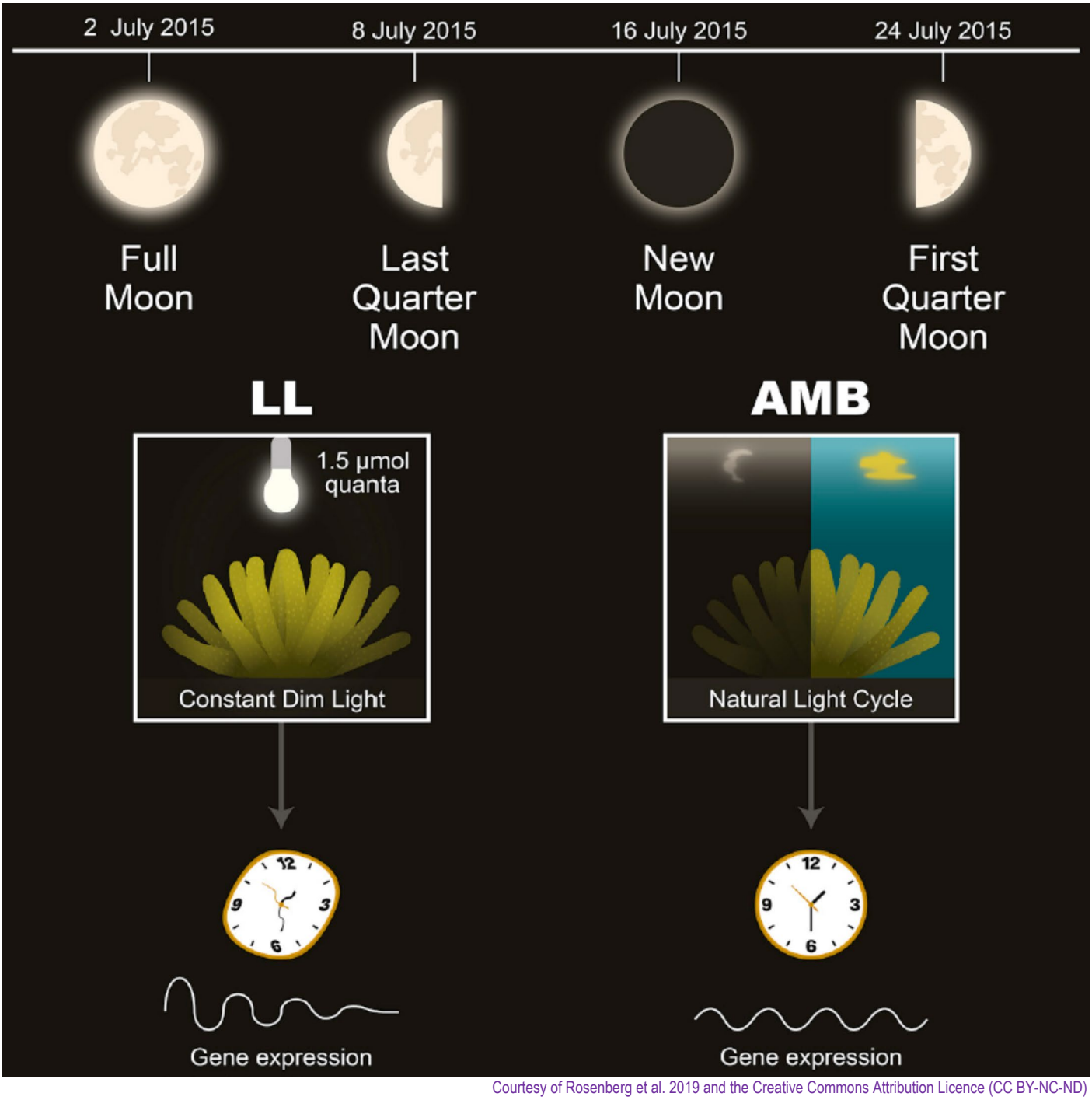

4. Coral Rhythms: Endogenous Entrained and Diurnal Light-Responses: Current Knowledge

Chris Aslett

This editorial completes our delve into what is currently known of the endogenous "clocks" of corals and their symbiotic "algae", their entrainment, and their transcriptomic and phenotypic impacts. Straightforwardness and clarity are our aim while a supplementary glossary in Appendix I introduces some unfamiliar terms. Genes are *italicised* while their proteinaceous products are sometimes UPPERCASE.

Zeitgeber (cue)-entrained circadian rhythms retain a cyclic inertia that endures for several days under fixed conditions like never-ending light (LL) or darkness (DD) which is evident in people suffering from jetlag, yet days of diurnal oscillations in photosynthetic efficiency are maintained in dimly lit saltwater unicellular algae of the genus *Lingulodinium* (Hastings et al. 1961). The pacemaker drifts and becomes "free running" in the absence of exogenous stimuli which are required to restore comparable cadences (Harmer et al. 2001). We begin by digesting the seminal study of Sorek and collaborators from 2013 who monitored photosynthesis in host-nurtured (*in-hospite*) and autonomous-cultured *Cladocopium*, *Durusdinium*, and *Symbiodinium* species of zooxanthellae whose experimental protocols are summarised in figures 1. and 3. (Sorek et al. 2013).

Stylophora pistillata is a moderate to intense light-demanding dispersed branching (ramose) small polyp stony (SPS) hermatypic coral that pervades the reef off the Interuniversity's Institute for Marine Sciences on the Gulf of Aqaba in the Israeli Red Sea. Several colonies were harvested at a depth of 10 metres (m) and were maintained ex situ in the institute's recirculating 900-litre ponds lit with 400-watt (W) Aqualine 10,000K metal halide lamps providing photosynthetically active radiation (PAR) of 200 micromoles (µmol) of photons per square metre (m^{-2}) per second (s^{-1}). Such irradiance is sufficiently saturating to induce shielding strategies,

Keywords: chronobiology; circadian; circatidal; cyclic; expression; homologue; LPS; orthologue; oscillator; rhythm; SPS; transcription.

whereas free-living zooxanthellae of the genera *Cladocopium*, *Durusdinium*, and *Symbiodinium* lit with lateral fluorescent tubes emitting 100 µmol m^{-2} s^{-1} were cultivated in half strength broth (Sorek et al. 2013).

Oxygen evolution measured using a respirometer ascertained the photosynthetic rate of captive colonies over two

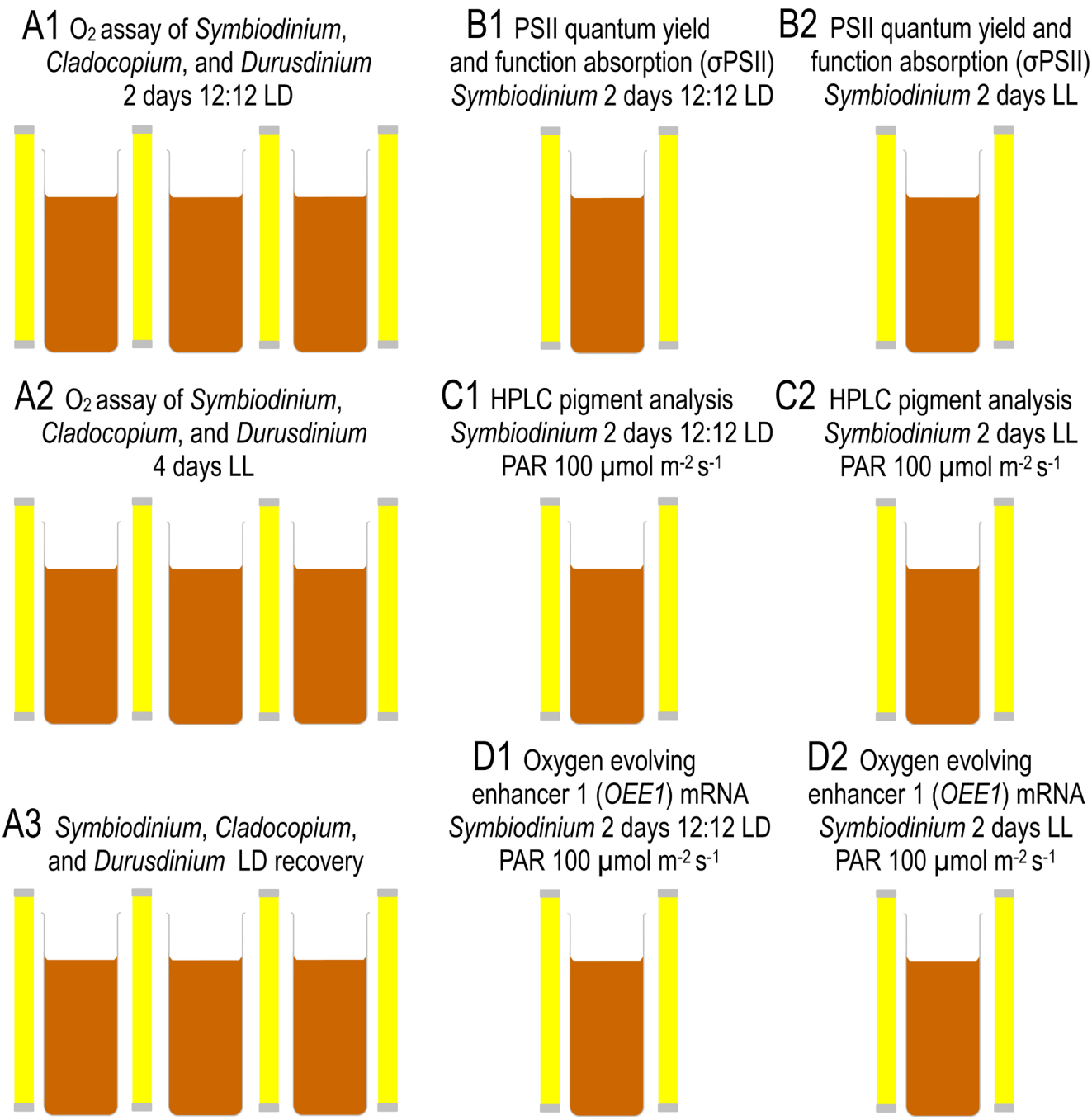

Fig 1. Illustrations of the in vitro culture experiments of Sorek and collaborators from 2013 where static phase zooxanthellae were laterally illuminated with fluorescent tubes emitting PARs of 100 µmol m^{-2} s^{-1}. *Cladocopium*'s, *Durusdinium*'s, and *Symbiodinium*'s oxygen production was measured over 12 hours of light and 12 hours of darkness (LD) for two days, four days of continuous irradiance LL, and two days of LD recovery [A1 to A3]. *Symbiodinium*'s photosystem II (PSII) maximum photochemical quantum yield ($F_V \div F_M$) and cross-sectional absorbance (σ) throughout two days of LD and two days of LL [B1 & B2]. High pressure liquid chromatography (HPLC) assays of the photoactive chemicals of *Symbiodinium*: chlorophylls *a* and *c*, peridinin, diadinoxanthin, and diatoxanthin exposed to two days of LD and two days of LL [C1 & C2]. Expression analyses of *Symbiodinium*'s oxygen evolving enhancer protein 1 encoded on *OEE1* throughout two days of LD and two days of LL [D1 & D2].

days of 14 and 10 hours of light and darkness (LD; zeitgeber time; ZT) and three days of continuous illumination (light:light; LL; circadian time; CT) when a "free running" cycle of 22.9 ± 0.4 hours was established (Figs 3. & 4.). The same measurements were performed on the in vitro static cultures of *Symbiodinium*, *Cladocopium*, and *Durusdinium* of obsolete clades A, C, and D exposed to 12 hours of light followed by 12 hours of darkness (12:12; LD; ZT; Fig 1.). Oxygen derived from zooxanthellae peaked mid-photoperiod where the former two's total rhythmic timeframes were 24.3 ± 0.23 while the latter's extended to 25.2 ± 0.3 hours. However, total periodicity became shorter when exposed to four days of continuous illumination (LL; CT) when *Symbiodinium*'s and *Cladocopium*'s became 23.5 ± 0.15 while *Durusdinium*'s shortened to 24.3 ± 0.2 hours.

Optimal and minimal oxygen evolving capacity occurred at ~14:00 (ZT 8) and during darkness when *Symbiodinium*, *Cladocopium*, and *Durusdinium* could generate up to their respective 10.24 ± 0.5, 20.37 ± 0.65, and 11.9 ± 1 milligrams per litre (mg l^{-1}) which continued under LL conditions but imprecisely with diminished amplitude. The rhythmicity of

photosynthetic O_2 appeared inversely related to PSII's quantum yield for 14:10 LD symbiosome resident and in vitro 12:12 LD zooxanthellae alike, inasmuch as the latter peaked just before dawn (06:00; ZT 0) with minima occurring ~14:00 midday (ZT 8). These patterns continued in LL for 48 hours and thus appeared to be regulated by an endogenous "clock". Conversely, PSII's cross-sectional absorbance (σ) corresponded to oxygen evolution with peaks at 14:00 (ZT 8) and 14:00 and 18:00 (CT 8 and 12; Figs 4. & 5.; Sorek et al. 2013).

Fig 2. *Stylophora pistillata* courtesy of Sorek et al. 2013 and the Creative Commons Attribution License 3.0.

Pulse-amplitude-modulation (PAM) fluorometric assays identified cyclic oscillations of photosynthetic quantum flux and rates of electron transport for *in-hospite* zooxanthellae in colonies exposed to LL, with elevated morning activities from 06:00 to 12:00 (CT 0 to 6), whereas comparable ratios of F_V to F_M were retained by LD and LL "algal" cultures and their LD recovery with minimal troughs at 18:00 and 20:00 (Fig 1. A1 to A3 & Fig 5.; Sorek et al. 2013).

High pressure liquid chromatography (HPLC) quantified the temporal oscillations in the concentrations of chlorophylls *a* and *c*, peridinin, diatoxanthin, and diadinoxanthin in the 12:12 LD cultures of *Symbiodinium* with maximum peaks occurring at 14:00 and 18:00 (ZT 8 and 12). Analogous cyclic abundances transpired within the first 24 hours of LL but with shifted maxima approaching 18:00 (CT 12; Fig 6.). ZT PAN fluorometry detected rhythmic peaks and troughs of carotenoids consistent with the surplus excitation-quenching xanthophyll cycle where diadinoxanthin is converted to diatoxanthin which reverts in darkness (Riddle 2014). Continuous illumination disrupted these transitions, but carotenoid concentrations peaked at 10:00 (ZT 4; Fig 6.).

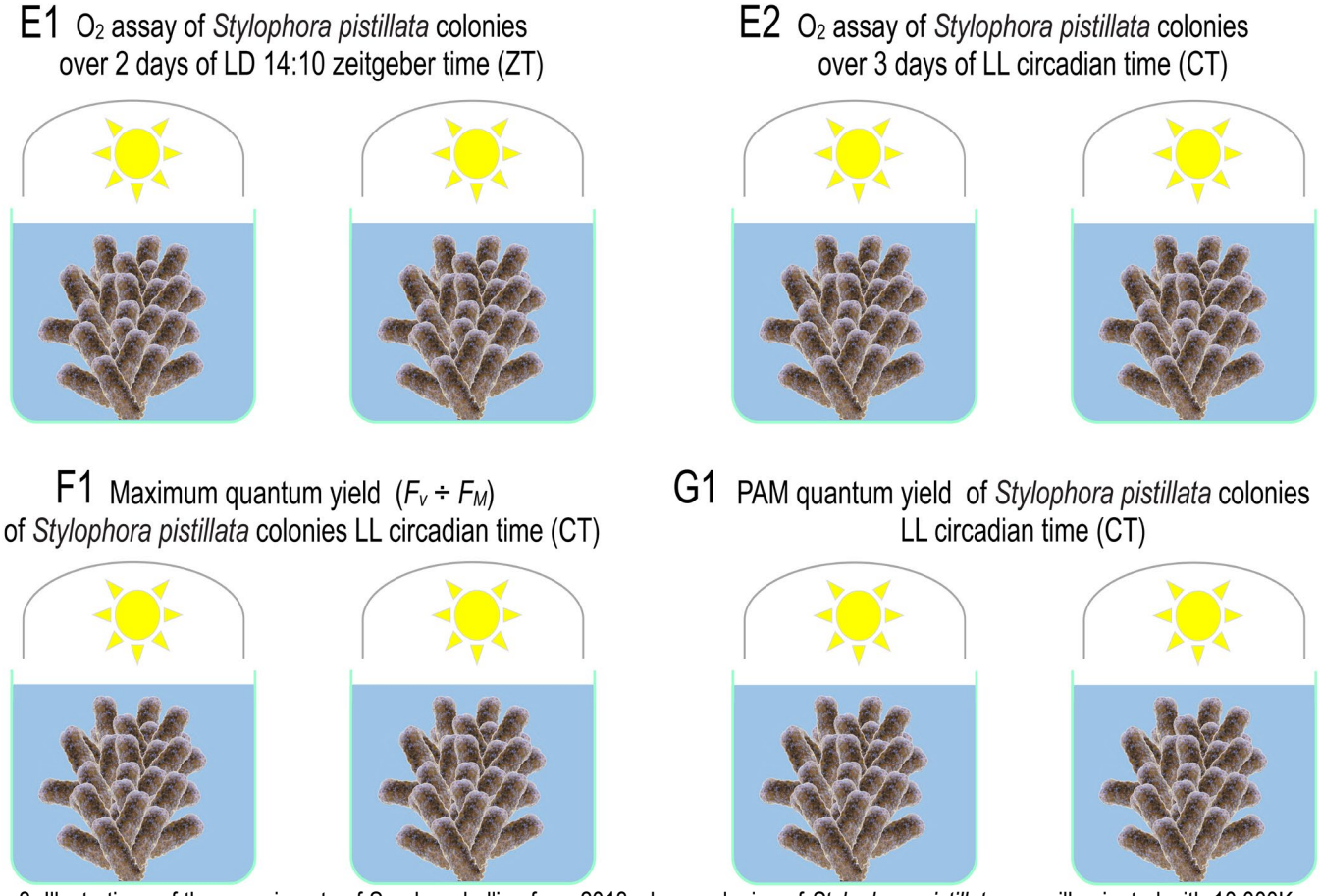

Fig 3. Illustrations of the experiments of Sorek and allies from 2013 where colonies of *Stylophora pistillata* were illuminated with 10,000K metal halide lamps emitting PARs of 200 µmol m^{-2} s^{-1}. Assays of oxygen production for colonies exposed to 14 hours of irradiance and 10 hours of darkness for two days called zeitgeber time (ZT) and three days of LL circadian time (CT) [E1 & E2]. The maximum photochemical quantum yield ($F_V \div F_M$) and pulse-amplitude-modulation (PAM) fluorimetry of colonies of *S. pistillata* throughout LL circadian time (CT) [F1 & G1].

Photosynthetic oxygen is derived from the photooxidation of water which liberates reductive electrons that are driven by light along a molecular transport chain against a thermodynamic gradient (Stryer 1995). Realtime PCR amplified and detected

sequences encoding PSII's-integral oxygen evolving enhancer protein 1 (OEE1) whose expression was consistent with oxygen availability. *OEE1*'s mRNA commenced to peak from lights-on at ZT 0 to around 14:00 midday whereafter it declined and remained minimal during darkness; nevertheless, such cyclicity continued under LL (Sorek et al. 2013). Chloroplasts have their own genome like mitochondria that encodes for *psbA* responsible for PSII's D1 protein, which was exploited as an experimental validating light-responsive reference whose expression was consistent under LD and LL (Fig 7.; Wang et al. 2005).

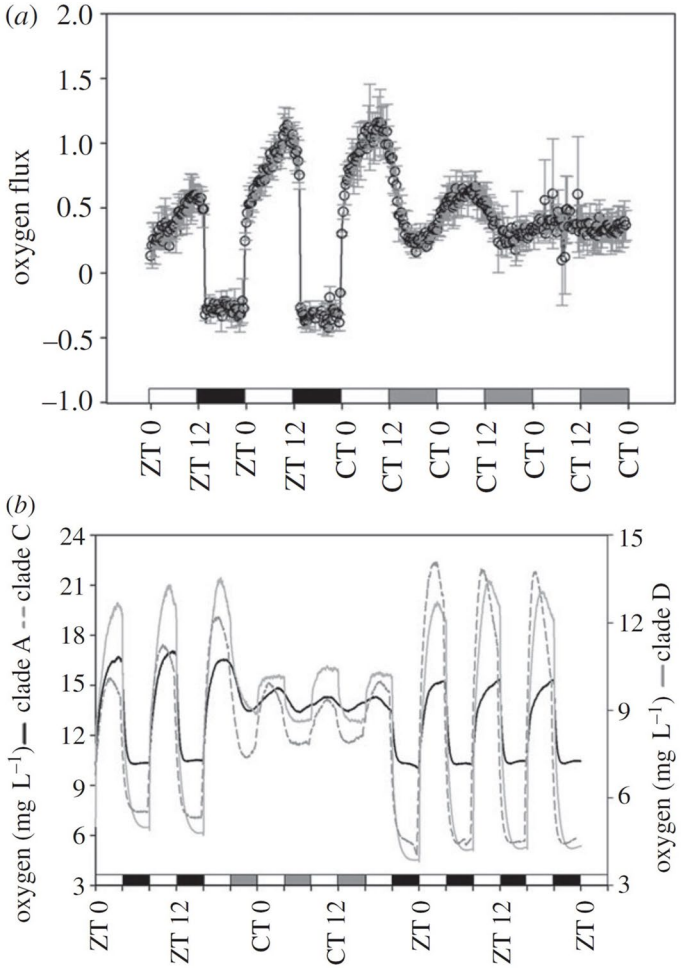

Fig 4. Oxygen generation experiments. (*a*) comparative photosynthetic oxygen flux of *S. pistillata*'s *in-hospite* zooxanthellae over 48 hours of 14:10 light:dark (LD) zeitgeber time (ZT) and 72 hours of light:light (LL) circadian time (CT) where zero and 12 represent 06:00 dawn and 18:00 evening. (*b*) mg l^{-1} of oxygen evolved by static cultures of *Symbiodinium*, *Cladocopium*, and *Durusdinium* of obsolete clades A, C, and D respectively. White and black bars indicate illumination and darkness while grey bars represent subjective nighttime during the continuous photoperiod of LL CT. Analyses and graphs courtesy of Sorek et al. 2013 and the Creative Commons Attribution Licence 3.0.

Either autonomously or in symbiosome residency, the endogenous "clocks" of zooxanthellae can be irradiance or temperature entrained which retain rhythmicity in daylight and nocturnally (Sorek & Levy 2012a).

The current study provides proof of a circadian pacemaker in mutualist "algae" that controls photosynthesis. Only static cultures were evaluated to obviate cellular division and anabolism-associated biorhythms. LL shortened the "free run" cycle of all three genera of dinoflagellates, while oscillations in the generation of photosynthetic oxygen had a periodicity approaching 24 hours. Although minor differences were observed, the core pacemaker of Symbiodiniaceae appears ubiquitous which operates independently of phylogenetic lineage and apo- or -symbiotic status (Sorek et al. 2013).

Photosynthesis exerts a meaningful impact on the REDOX status of coral tissue ranging from daytime supersaturation of oxygen to nocturnal hypoxia and plummeting pH (Kuhl et al. 1995), while the anthozoan host manipulates the proliferation and selective nurture of certain lineages of zooxanthellae (Wilkerson et al. 1988; Levy et al. 2011; Munn 2019).

Moya and collaborators observed unwavering daytime photosynthetic rates in *S. pistillata* (Moya et al. 2006), which perhaps due to Moya and allies' short period of pre-sampling entrainment, contrasts with the current study's findings (Sorek et al. 2013).

Photosynthetic quantum yield ($F_V \div F_M$) was robustly rhythmic yet diminished around midday under LD and LL which is consistent with the findings of other studies (Brown et al. 1999; Hoegh-Guldberg et al. 1999) and is akin to what occurs in *Montipora digitata* (Jones & Hoegh-Guldberg 2001). Quantum flux retained an analogous rhythmicity in both LD and LL groups, but the peaks and troughs became less pronounced in the latter on subsequent days, insofar as dark conditioning increases the photochemical competency of the reaction centres (Krause & Weis 1991).

Other antioxidant pathways exploit different carotenoids. The enzyme violaxanthin de-epoxidase sequentially converts the carotenoid violaxanthin to antheraxanthin and then to zeaxanthin, while zeaxanthin epoxidase catalyses zeaxanthin to violaxanthin but only in diminished irradiance (Frank et al. 2000; Müller-Moulé 2002; Ai-Zhen & Fang-Qing 2016). Hence chloroplasts have a violaxanthin reserve at dawn which depletes throughout the day (Hoegh-Guldberg & Jones 1999).

Quantum yield is also related to photosystem injury and host/symbiont protective strategies (Sorek et al. 2013). A decrease in F_O and the F_V to F_M ratio at ZT 0 and ZT 4 under LD is indicative of a protective role (Krause & Weis 1991). See: 3. Current Knowledge:

$$\frac{F_V}{F_M} = \frac{F_M - F_O}{F_M}$$

(Hoogenboom et al. 2012; Gong et al. 2023)

Ongoing F_O attrition in combination with a lack of violaxanthin-, D1 protein-, and PS-rejuvenating darkness, are thus implicated in LL-expedited PSII impairment (Fig 5.; Osmond et al. 1999; Sorek et al. 2013).

The rhythmic concentrations of peridinin and chlorophylls *a* and *c* peaked around midday (14:00; ZT 8) under 12:12 LD, whereas diatoxanthin and diadinoxanthin where highest at 10:00 (ZT 4). The diel oscillations of the photoreactive pigments endured in continuous irradiance but peak fortifications shifted to CT 12 on day one yet reverted to CT 8 on day two. Nevertheless, maximum xanthophyll abundance advanced from ZT 4 to CT 8 under LL (Fig 6.; Sorek et al. 2013).

Previous studies had failed to discern the periodicity of photoreactive biochemicals (Prezelin et al. 1977; Prezelin & Ley 1980), whilst zooxanthellae's circadian-controlled mitotic cell cycles conceivably prompt oscillations in pigment intensities (Ragni & D'Alcala 2007).

Xanthophylls appeared to cycle rhythmically with peaks

around midday/early afternoon while Brown and colleagues had shown that the ratios of diatoxanthin to diatoxanthin multiplied by diadinoxanthin and F_V to F_M were inversely proportional in *Goniastrea aspera* (Brown et al. 1999). However, despite the rhythmic abundances of photoreactive pigments, the xanthophyll cycle appeared disrupted by continuous light. The wealth of carotenoids escalated during the first 24 hours of LL but soon levelled out, where the compound impairment of PSII or reactive oxygen species (ROS) may have masked the circadian oscillator. Hence unlike photosynthetic quantum flux, photoactive biochemicals do not seem under the control of an endogenous "clock" (Sorek et al. 2013).

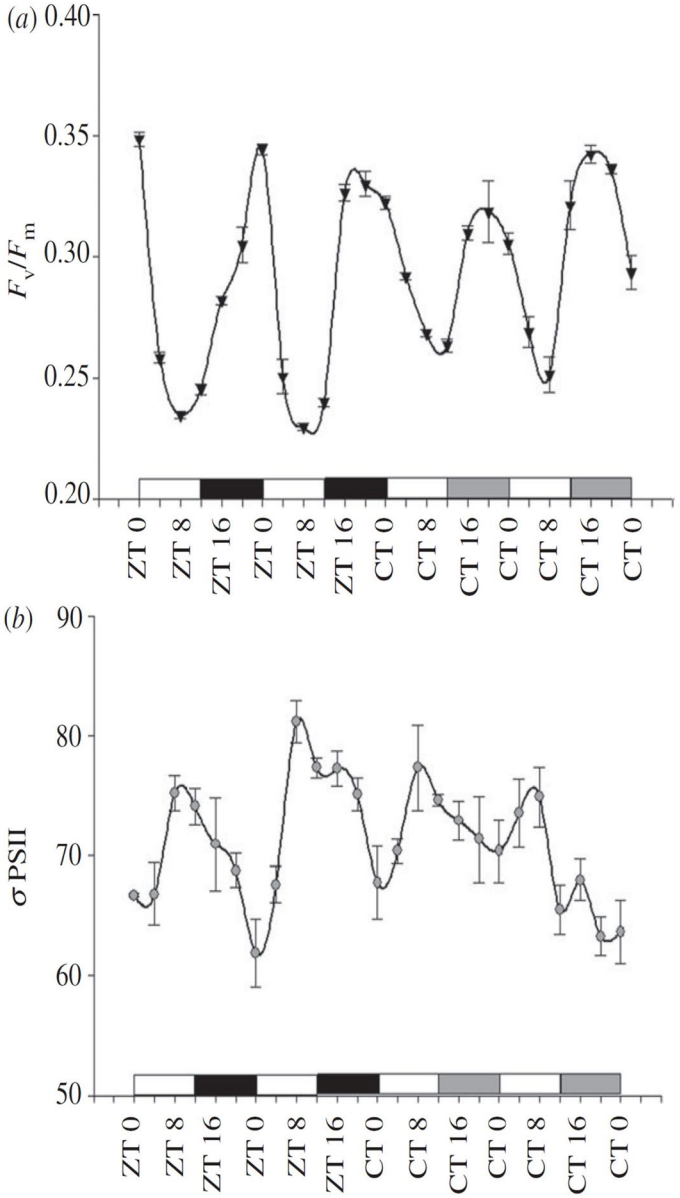

Fig 5. Chlorophyll fluorescence experiments. (*a*) the photochemical quantum yield ($F_V \div F_M$) of static cultured *Symbiodinium*'s PSIIs throughout two days of 12:12 LD (ZT) and two days of LL (CT) where zero, eight, and 16 represent 06:00 dawn, 14:00 midday, and 22:00 night. (*b*) PSII's cross-sectional absorbance (σPSII) in cultured *Symbiodinium* under alike experimental conditions. White and black bars indicate illumination and darkness while grey bars represent subjective night during the continuous photoperiod of LL CT. Analyses and graphs courtesy of Sorek et al. 2013 and the Creative Commons Attribution Licence 3.0.

Patterns of expression that expedite photosynthesis have been acknowledged in other kinds of "algae" including free-living dinoflagellates (Harmer et al. 2000; Schaffer et al. 2001; Van Dolah et al. 2007) whose genes encode proteins integral to the reaction centres of PSI and PSII like *Psad1*, or those that translate into chlorophyll-binding proteins or light harvesting complexes A and B (Giuliano et al. 1988; Harmer et al. 2000; McClung 2000).

Oxygen evolving enhancer (OEE) protein 1 encoded on *OEE1* is a key component of the OEE complex of PSII responsible for the photooxidation of water, without which zooxanthellae cannot generate oxygen (O_2) or reduce thylakoid membrane-bound manganese (Fig 7.; Murata & Miyao 1985).

OEE1 appears indispensable for photosynthetic O_2 and the integrity of the PSII complex of the "algae" *Chlamydomonas reinhardtii* (Mayfield et al. 1987) while this gene's expression is rhythmic under both LD and LL which corresponds to the liberation of O_2 in species of *Symbiodinium* (Sorek et al. 2013).

The transcriptional rhythmicity of *psbA* endures under LD and LL despite previous research highlighting its non-circadian-like expression (Wang et al. 2005). *psbA*'s exploitation appears unrelated to the oxidation of water, yet zooxanthellae's photosynthesis is controlled by an endogenous "clock" in free-living cultures and *Stylophora pistillata* (Sorek et al. 2013).

Light and lunar cycles profoundly effect marine organisms of which the previous editorials provided essential background (Aslett 2024). Although the moon's reflectance is analogous to sunlight (Johnsen et al. 2006), the photoreceptors of corals appear most sensitive to 480 nanometres (nm; Gorbunov & Falkowski 2002). They therefore detect and respond to low intensities of short wavelength blue (Rosenberg et al. 2019).

Shoguchi and collaborators unearthed seven r-opsin and three cryptochrome photoreceptor-like products encoded on the genomes of *Acropora digitifera* (Shoguchi et al. 2013) while previous studies had emphasised the influence of moonlight on *A. millepora* (Levy et al. 2007; Hoadley et al. 2011).

Several colonies of *Acropora digitifera* with diameters greater than 50 cm (19.7") were collected from a reef flat off Sesoko Island in Japan's Okinawa on 25 June 2015, which were housed in a flow-through system fed with water from the same reef, where five received natural cycles of sun- and moon-light (ambient; AMB), whereas others were exposed to continuous dim light (LL) of a PAR of 1.5 to 2 µmol m^{-2} s^{-1} emanating from a fluorescent lamp (Rosenberg et al. 2019).

All colonies were preconditioned/entrained under AMB for a week prior to sampling on each lunar quarter at 12:00 noon and 21:00 moonrise for an entire month from the full moon of 2 July 2015 which translates to four days of sampling with two timepoints on each day (fig 8.; Rosenberg et al. 2019).

Maximum quantum yield and pulse-amplitude-modulated (PAM) fluorometry ascertained photosymbiont output as a proxy for colony health, while mRNA was extracted, sequenced, and blasted against an *A. digitifera* annotated transcriptome cDNA library downloaded from the repository of the Okinawa Institute of Science and Technology (OIST), which unearthed each lunar quarter's differentially expressed genes (DEGs; Rosenberg et al. 2019).

The study compared the transcriptomes of the AMB and LL groups on each phase of the moon regardless of the time of day which were assigned moonlight-responsive genes, and at each time of day discounting the lunar cycle called light-responsive genes, while gene cluster and pathway analyses identified intracellular processes that were enriched in AMB and/or LL

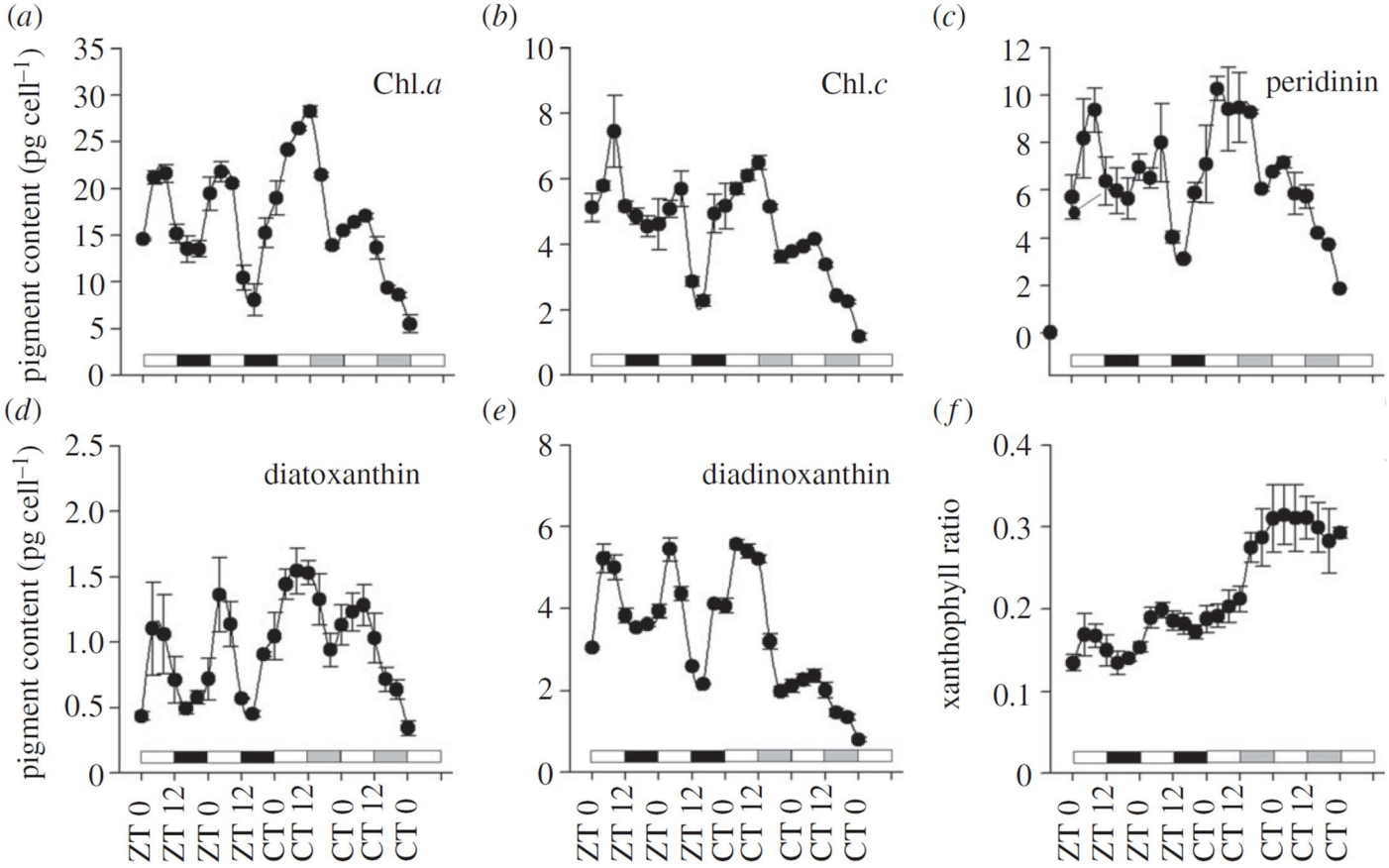

Fig 6. HPLC photoreactive pigment assays. (*a* to *e*) pigment per cell (pg cell^{-1}). (*f*) diatoxanthin ÷ (diatoxanthin + diadinoxanthin). All analyses performed on static cultured *Symbiodinium* under 12:12 LD (ZT) and LL (CT). White and black bars indicate illumination and darkness while grey bars represent subjective night during the continuous photoperiod of LL CT. Analyses and graphs courtesy of Sorek et al. 2013 and the Creative Commons Attribution Licence 3.0.

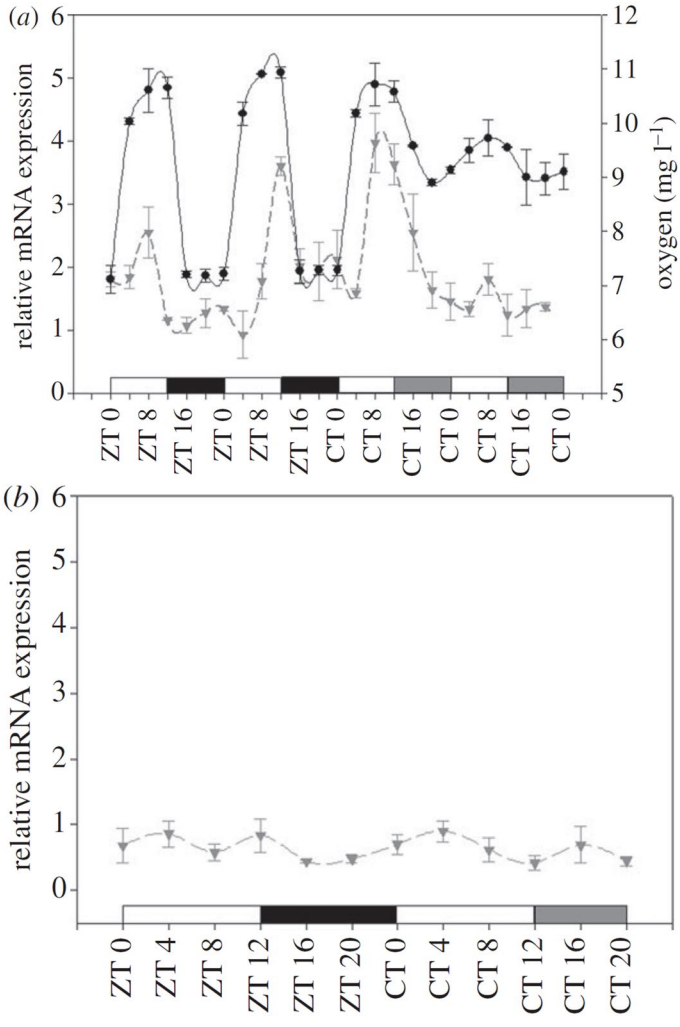

Fig 7. qPCR assays. (*a*) the relative expression of *OEE1* encoding oxygen-evolving enhancer protein 1 (grey triangles) in static cultured *Symbiodinium* over two days of 12:12 LD and two days of LL, and mg l^{-1} of dissolved oxygen (black circles). (*b*) the relative expression of the more frequently sampled reference gene, *psbA* exposed to like experimental conditions. White and black bars indicate illumination and darkness while grey bars represent subjective night during the continuous photoperiod of LL CT. Analyses and graphs courtesy of Sorek et al. 2013 and the Creative Commons Attribution Licence 3.0. (Rosenberg et al. 2019).

Comparable results were obtained by assays of maximum quantum yield and pulse-amplitude-modulated (PAM) fluorometry which confirmed that colonies and their zooxanthellae remained healthy in both AMB and LL groups throughout the study (Rosenberg et al. 2019).

MetaCycle analyses highlighted 4,472 and 4,691 genes that were up- and down-regulated in LL compared with AMB which accounted for 19 and 20 percent of the experimental transcriptome. 58 gene clusters associated with specified pathways were rhythmically expressed in synchrony in the AMB group whereas transcription remained arrhythmic in LL, where more sequences overlapped and ORFs were less unique (Fig 9.). Distinct day- and night-time expression occurred in LL on the day of the full moon, whereafter the trend vanished for 14 days until it reemerged on the new moon. Nevertheless, gene transcription in AMB and LL remained dissimilar throughout (Rosenberg et al. 2019).

Several intracellular pathway-associated gene clusters that were differentially expressed in both groups and in each phase of the moon exhibited inverse transcriptional rhythmicity. ERK/MAPK, Integrin, NGF, and CREB signalling were either

upregulated in LL in each lunar quarter or moon cyclicity had a negligible impact, while this trend was reversed under AMB. Those that were down- and up-regulated in the LL and AMB treatments comprised 3-phosphoinositide degradation, BTG protein family cell cycle regulation, D-myo-inositol 3,4,5,6- or 1,4,5,6-tetrakisphosphate biosynthesis, G beta gamma signalling, NRF2 responsive antioxidants, p14/p19ARF-mediated tumour suppression, p53 signalling, and PPARa/RXRa activation. CD40- and GP6- or rac-, dopamine-, and interleukin (IL)-8-mediated signalling were differentially transcribed in each lunar quarter merely in the LL or AMB samples respectively. The rhythmic expression patterns of each pathway observed in the AMB collections reflected those of wild corals (Figs 9. & 10.; Rosenberg et al. 2019).

The mRNA profusions from each of *A. digitifera*'s canonical circadian orthologues were measured at each lunar quarter. *cry1*, *npas2*, and *pdp* were only marginally differentially expressed in AMB samples which were upregulated during the day, whereas the reverse trend was observed in *clock* (Fig 11.). However, lunar or diurnal phase-attuned transcription was insignificant for these orthologues in corals kept under subdued irradiance (Rosenberg et al. 2019).

Genes encoding the fibroblast growth factors (FGFs) 1, 1R, and 3R were consistently upregulated in LL corals in comparison to AMB in all lunar phases where the time of day had nil impact (Fig 12.). Further analyses revealed genes whose expression was impacted by lunar phase but diurnally unresponsive, where light-sensitive GPC receptor activations and their downstream transcriptional cascades were associated with 23 annotated genes, while extracellular matrix organisation and microtubule binding pathways remained attuned to each quarter (Rosenberg et al. 2019).

Brief nocturnal pulses of visible photons disrupt the circadian rhythms of Scleractinia and therefore nearshore artificial illumination at night (ALAN) exerts meaningful impacts (Rosenberg et al. 2019; Davies et al. 2023).

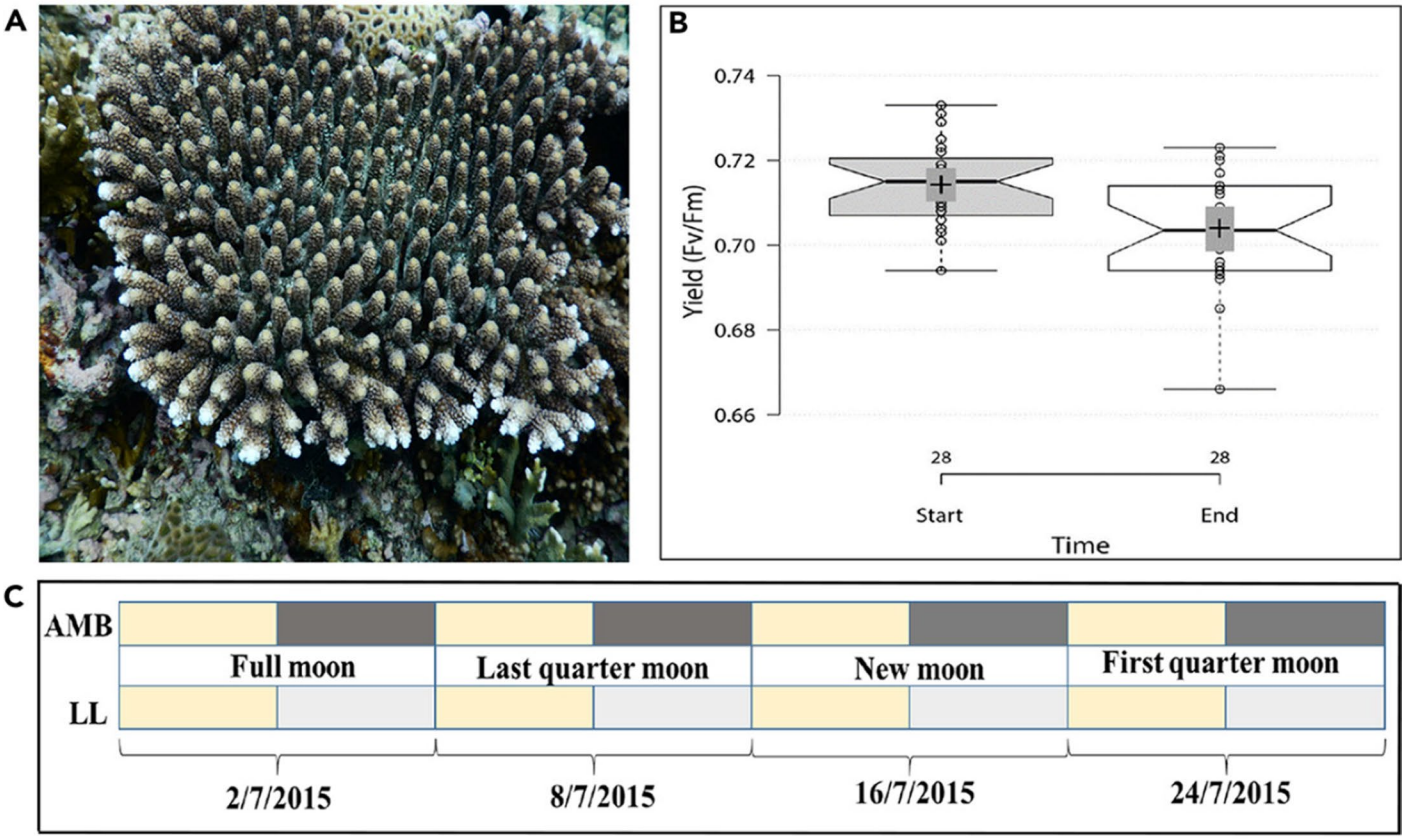

Fig 8. [A] an in situ colony of *Acropora digitifera* before relocation. [B] maximum quantum yield ($F_V \div F_M$) of the LL colonies on the first and the last day of sampling. [C] experimental design featuring sampling dates and lunar quarters where dark and white bars represent subjective nighttime. Courtesy of Rosenberg et al. 2019 and the Creative Commons Attribution Licence 4.0 (CC BY-NC-ND).

Coral transcriptomes are clearly influenced by a lunar month's changes in day- and moon-light, yet their cyclicity and periodicity may be lost in those exposed to continuous subdued irradiance (Rosenberg et al. 2019). Other studies have observed alterations in gene expression and phenotypic traits that accompany the zeitgeber of incessant softened light (Njus et al. 1977; Steinlechner et al. 2002; Granados-Fuentes 2004), inasmuch as circadian rhythms rely upon regular entrainment. The findings of the current study suggest that the endogenous oscillator of corals kept in LL conditions becomes "free running" after several days (Rosenberg et al. 2019); however, the circadian-mediated transcriptomic and behavioural activities of several organisms are maintained in fixed conditions (Griffin et al. 1999; Levy et al. 2011; Sorek & Levy 2012; Sorek et al. 2013; Peres et al. 2014; Oren et al. 2015; Chabot et al. 2016). Expression on the "first day" of the LL study that coincided with the full moon was likely under circadian control when significant differences in day- and night-time transcription were detected. The profusions of mRNA in AMB and LL corals remained dissimilar throughout, yet this "first day" trend returned on the new moon under dim irradiance when AMB-entrained transcriptional rhythmicity was comparatively elevated (Rosenberg et al. 2019). This study may have therefore substantiated an autonomous endogenous 14.8-day circasemilunar oscillator (Bünning & Müller 1961; Neumann 1978; Neumann 1988; Naylor 1996; Neumann 2014; Kaiser & Neumann 2021; Rock et al. 2022) that may be masked in wildtype milieus. See: 3. Current Knowledge.

Host cell-surface and nuclear membrane-bound receptor

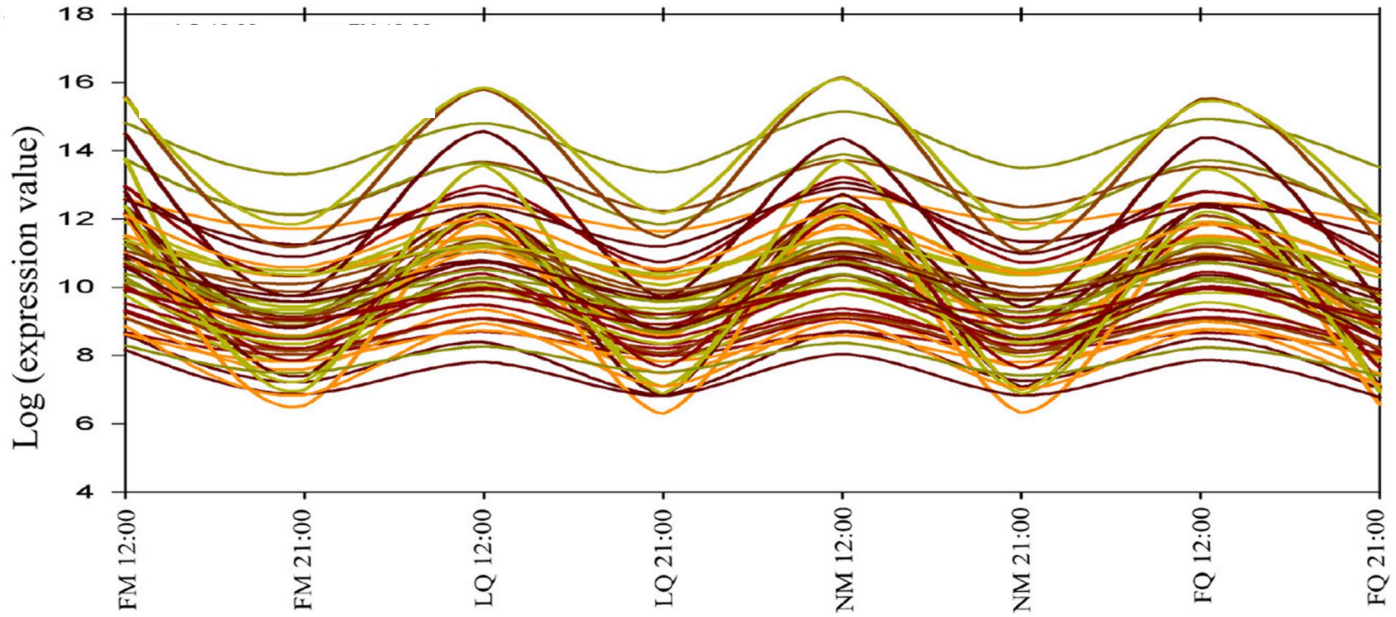

Fig 9. The rhythmic expression patterns of 58 AMB treatment-pervasive gene clusters: full moon [FM]; last quarter moon [LM], first quarter moon (FQ], and new moon [NM]. Courtesy of Rosenberg et al. 2019 and the Creative Commons Attribution Licence 4.0 (CC BY-NC-ND).

activation resulting from an exogenous molecule and ERK/MAPK signal transduction, induces mechanisms associated with cellular proliferation, differentiation, and development (Seger & Krebs 1995). This pathway was up- or down-regulated in tissues of colonies exposed to LL or AMB lighting protocols. The signalling cascades of integrin and nerve growth factor (NGF) are implicated in cellular anabolism and division (Howe et al. 1998, Niederhauser et al. 2000; Howe et al. 2001; Schwartz 2001; Howe & Mobley 2003) which were promoted in the tissues of colonies exposed to LL conditions. It was postulated that dim light entrainment disrupts the stringent intracellular mechanisms that hold the cell cycle in check (Armbrust et al. 1989; Jacquet et al. 2001; Matsuo et al. 2003; Hunt & Sassone-Corsi 2007; Rosenberg et al. 2019) which may predispose the cell to unrestricted proliferation, oncogenic insult, and neoplasia.

Cyclic-AMP (cAMP) response element binding protein (CREB) is a transcription factor that binds to upstream cAMP response elements (CRE) and thus either represses or promotes expression (Bourtchuladze et al. 1994). Nitric oxide (NO) is associated with histone tail deacetylation (epigenetic deregulation) where CREB phosphorylation remains central to pacemaker entrainment (Zhang et al. 1996; Tischkau et al. 2003; Riccio et al. 2006), while the CREB signalling pathway responds to light (Obrietan et al. 1999). Nevertheless, much of this research was investigating CREB-associated mechanisms in the suprachiasmatic nucleus integral to the hypothalamus in the brain of higher eukaryotes. Genes associated with the CREB cascade were upregulated in constant dim light but downregulated in ambient conditions which may reflect continuous induction (Rosenberg et al. 2019).

LL-reinforced CD40 and GP6 signalling pathways are key in cellular homeostasis, clotting, immunity, and phosphorylation (Jones et al. 2007; Sokol et al. 2012) while genes that were merely differentially expressed under AMB conditions remain associated with circadian control, rhythmic processes, and the dynamic equilibrium of intracellular secondary messengers like dopamine, Rac GTPases, mitogen-activated protein kinase (MAPK), and interleukin (IL)-8 (Duffield et al. 2002; Norman et al. 2005; Hermann et al. 2006; Yujnovsky et al. 2006; Beaulieu & Gainetdinov 2011; Ghasemi et al. 2011; Hwang et al. 2013). The absence of gene sets implicated in rhythmic processes in corals kept in continuous subdued light, lends credence to the zeitgeber entrainment circumvents circadian “free running” paradigm (Rosenberg et al. 2019).

The canonical circadian orthologues: *cry1*, *npas2*, *clock*, and *pdp* were differentially expressed in a diurnal pattern over an entire lunar month in the AMB samples yet insignificantly so in LL where transcription remained nominal. A loss of transcriptomic rhythmicity has been observed in several organisms in unwavering conditions (Griffin et al. 1999; Dupre & Loudon 2007; Roenneberg & Merrow 2005; Reitzel et al. 2010; Sorek et al. 2013; Sorek et al. 2014; Dominoni 2015; Chabot et al. 2016; Raible et al. 2017). A lack of cues or their masking may therefore exert profound effects on homeostasis and fecundity (Somers et al. 1998; Zheng et al. 2001; Ceriani et al. 2002; Kondratov et al. 2006; Takahashi et al. 2008).

Photoreceptors detect and react to visible photons and hence these cues cannot entrain the circadian oscillator when light receptors are absent or when they malfunction. Continuous receptor induction leads to photoactive biochemical depletion, where studies have observed concurrent upregulation of genes encoding fibroblast growth factors (FGFs; Hicks & Courtois 1988; Hicks & Courtois 1992; Campochiaro et al. 1996; Hisatomi et al. 2002; Qin et al. 2011; Hochmann et al. 2012) which belong to a sizeable family of proteins that stimulate soft tissue regeneration (Basilico & Moscatelli 1992). FGFs elicit restoration of photoreceptors under continuous irradiance (Campochiaro et al. 1996; Hisatomi et al. 2002; Qin et al. 2011; Hochmann et al. 2012) that were consistently upregulated in LL samples compared with AMB which substantiates the findings of other studies, where FGFs protect and repair photoreceptors in the face of incessant radiation (LaVail et al. 1992; Gao & Hollyfield 1996).

Cellular membrane-associated G protein-coupled receptors (GPCRs) comprise an extracellular N-terminus, seven transmembrane α-helical domains, and an intracellular C-terminus (Rehman et al. 2023). The current study unearthed genes whose expression was unresponsive to diurnal irradiance that were promoted or repressed by moonlight, many of which

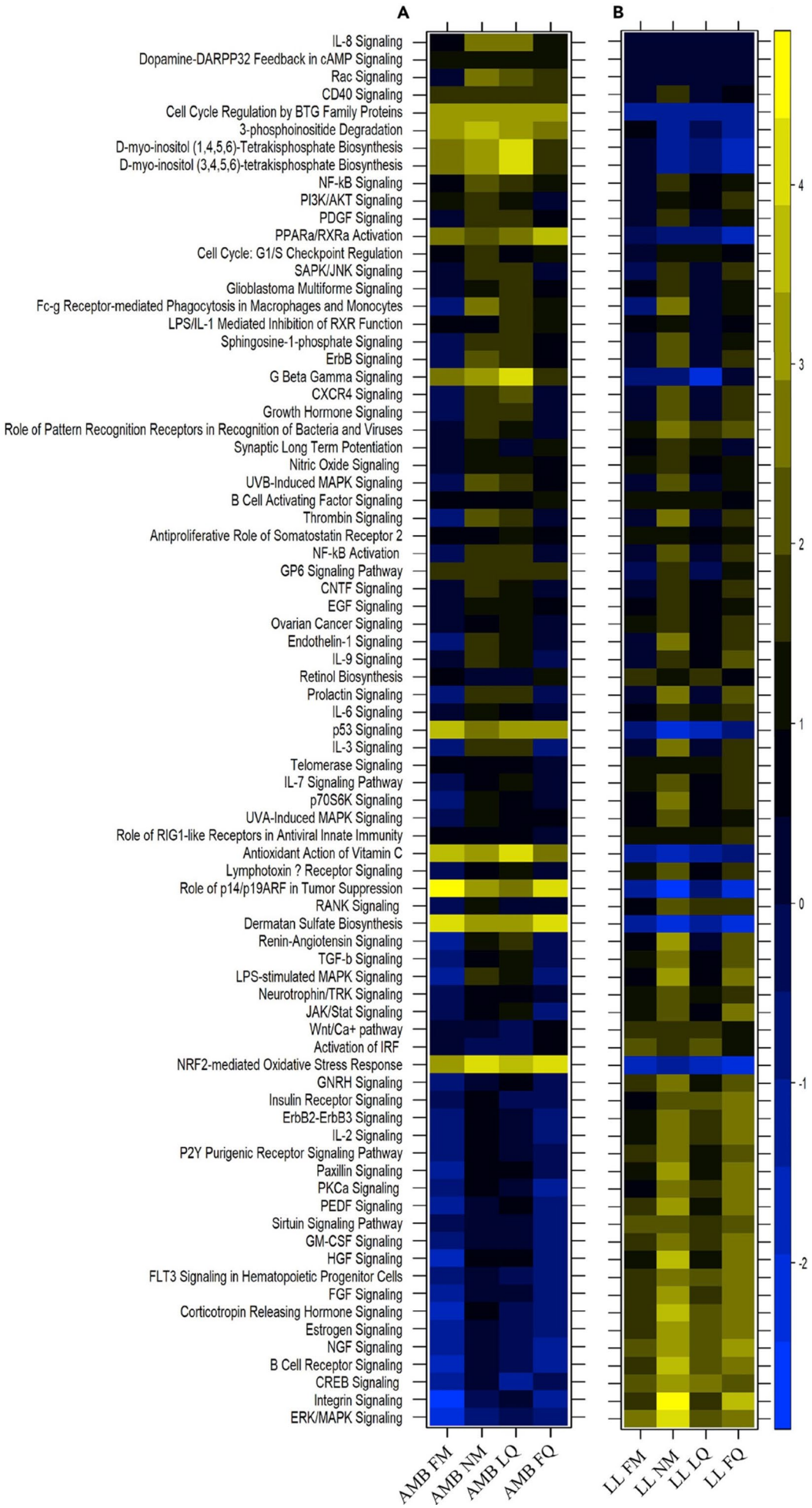

Fig 10. Heatmaps of the rhythmically enriched canonical pathways (gene ontologies; GO) in *Acropora digitifera* kept under near-natural conditions [AMB; A] and in never-ending subdued light [LL; B] where FM, NM, LQ, and FQ specify full moon, new moon, last quarter moon, and first quarter moon respectively. Courtesy of Rosenberg et al. 2019 and the Creative Commons Attribution Licence 4.0 (CC BY-NC-ND).

are recognised components of cascades activated by light-sensitive GPCRs (Rosenberg et al. 2019). Numerous genes are differentially transcribed at discrete phases of a lunar cycle (Levy et al. 2007; Fukushiro et al. 2011; Numata & Helm 2014; Brady et al. 2016) but their expression is also typically influenced by diel spectral downwelling. This study's findings further substantiate a 14.8-day circasemilunar oscillator and establish moonlight-responsive GPCR-induced ontologies implicated in the regulation, organisation, and formation of microtubule-assisted extracellular matrices.

The potentially harmful effects of nocturnal light pollution common to inshore reefs are thus emphasised which may mask or prevent entrainment of circadian and circalunar "clocks" in *A. digitifera*. Precise periods of lunar reflectance appear necessary for the synchronisation of gametogenesis (Szmant-Froelich et al. 1985; Brady et al. 2016; Rosenberg et al. 2019) while Lin and collaborators discovered the interlude of darkness which occurs on nights following the evening of the full moon, triggers spawning in *Dipsastraea speciosa*. Early moonrise inhibits spawning before sunset on evenings leading up to and on the night of the full moon (Lin et al. 2021) and thus significant light pollution will likely disrupt broadcasting where seemingly unimportant perturbations of the brief gametic fusion window could lead to reproductive failure (Rosenberg et al. 2019). Davies and collaborators proposed an hour of post-sunset diminished coastline illumination would

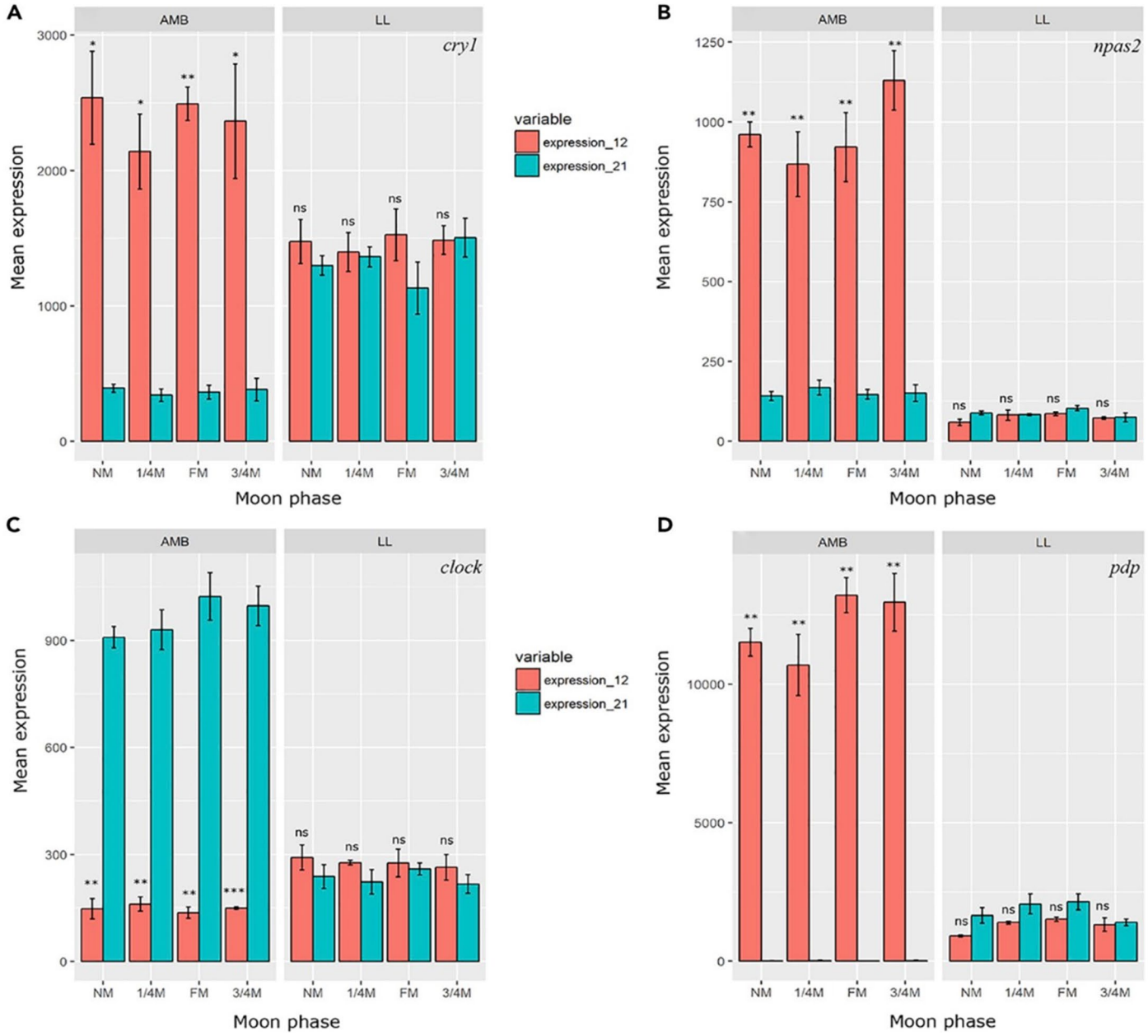

Fig 11. The mean expression of the canonical circadian orthologues: *cry1*, *npas2*, *clock*, and *pdp* in tissues of *Acropora digitifera* on a new moon [NM]; first quarter moon [1/4M]; full moon [FM], and third quarter moon [3/4M] exposed to near-natural conditions [AMB] and continuous dim light [LL] where red or blue bars indicate 12:00 or 21:00 samples and [*] or [ns] represent significant or insignificant differences. Courtesy of Rosenberg et al. 2019 and the Creative Commons Attribution Licence 4.0 (CC BY-NC-ND).

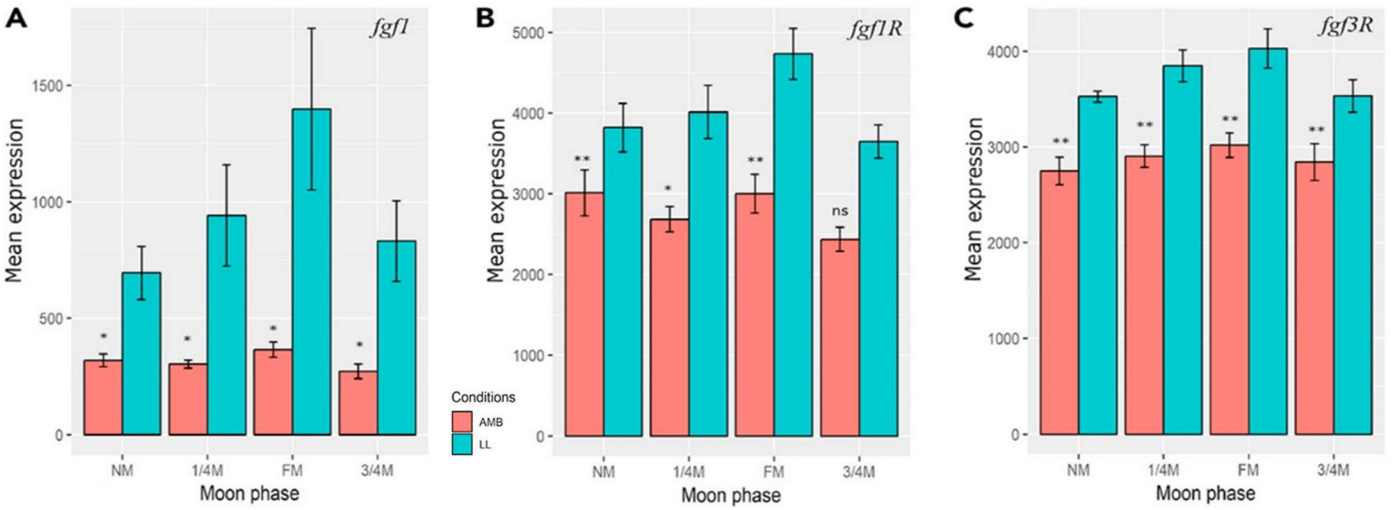

Fig 12. The mean expression of the fibroblast growth factor encoding genes: *fgf1*, *fgf1R*, and *fgf3R* in tissues of *Acropora digitifera* on a new moon [NM]; first quarter moon [1/4M]; full moon [FM], and third quarter moon [3/4M] exposed to near-natural conditions [AMB; red bars] and continuous dim light [LL; blue bars] where and [*] or [ns] represent significant or insignificant differences. Courtesy of Rosenberg et al. 2019 and the Creative Commons Attribution Licence 4.0 (CC BY-NC-ND).

emphasise the interim of darkness that triggers spawning on evenings following the night of the full moon (Davies et al. 2023).

Numerous cycles of behaviour, reproduction, and skeletogenesis are driven by light in Cnidaria (Oren et al. 2015; Artigas et al. 2018) while others are sustained in the absence of cues (Levy et al. 2007; Sorek et al. 2018).

Several homologues of the endogenous "clock" genes of Bilateria are also found in Cnidaria (Reitzel et al. 2010; Reitzel et al. 2013; Shoguchi et al. 2013), yet remarkably, their transcriptional rhythmicity is lost without recurring zeitgebers (Leach et al. 2018; Leach & Reitzel 2019; Rinsky et al. 2022). The molecular mechanisms that underpin cnidarian housekeeping, homeostasis, and behaviour thus remain enigmatic (Rinsky et al. 2023).

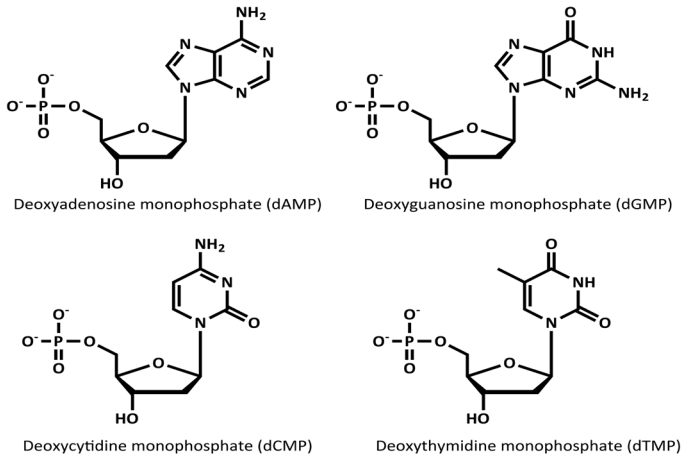

Fig 13. The four deoxynucleotides of DNA where phosphodiester bonds covalently concatenate sugars and phosphates into the "backbone" of a single strand of deoxyribonucleic acid (ssDNA). Two strands of DNA (dsDNA) form hydrogen bonds between complementary purine (top) and pyrimidine (bottom) bases which become the structurally stable and iconic double helix.

The locomotion of euryhaline azooxanthellate starlet sea anemones (*Nematostella vectensis*) is maintained in day- and night-time analogous irradiance and under consistent conditions which suggests their movement is controlled by a core circadian oscillator (Hendricks et al. 2012; Oren et al. 2015; Tarrant et al. 2019; Leach & Reitzel 2020). The *N. vectensis* genome encodes the canonical circadian orthologues: *Clock*, *Cycle*, *Timeout*, *Cry1a*, and *Cry1b* (Reitzel et al. 2010; Shoguchi et al. 2013). CLOCK:CYCLE dimers promote expression of cryptochromes in a positive feedback loop whereas PAR-leucine zippers (-bZIPs) act as repressors (Fig 31.; Reitzel et al. 2013). The CLOCK:CYCLE antagonist CIPC appears to be a core rhythm mediator (Leach & Reitzel 2020), yet despite the robust behavioural cyclicity of *N. vectensis* that prevails in the absence of cues (Hendricks et al. 2012; Oren et al. 2015; Leach & Reitzel 2020), the expression of most orthologues becomes arrhythmic in fixed environments (Leach et al. 2018; Leach & Reitzel 2019; Leach & Reitzel 2020; Rinsky et al. 2023). Rinsky and collaborators were thus motivated to create homozygous transgenic knockout mutants (*Clock*$^{-/-}$) and evaluate their transcriptomic and behavioural activities in comparison to wildtype (WT) under various lighting protocols (Rinsky et al. 2023).

N. vectensis were cultured in less than half strength seawater (1.2 %; 12 g l^{-1}) at 17°C and fed with brine shrimp nauplii (*Artemia salina*) thrice weekly. Spawning was induced by the procedure outlined in Genikhovich and Technau from 2009 where anemones were irradiated with intense white light and the temperature was raised to 25°C for 9 hours. Broadcast oocytes and sperm were mixed to optimise fertilisation and zygotic fusion (Rinsky et al. 2023), whereafter blastulae are usually washed in ~5-micron (µm) filtered seawater within two hours to circumvent ammonia spoilage. See: 3. Current Knowledge.

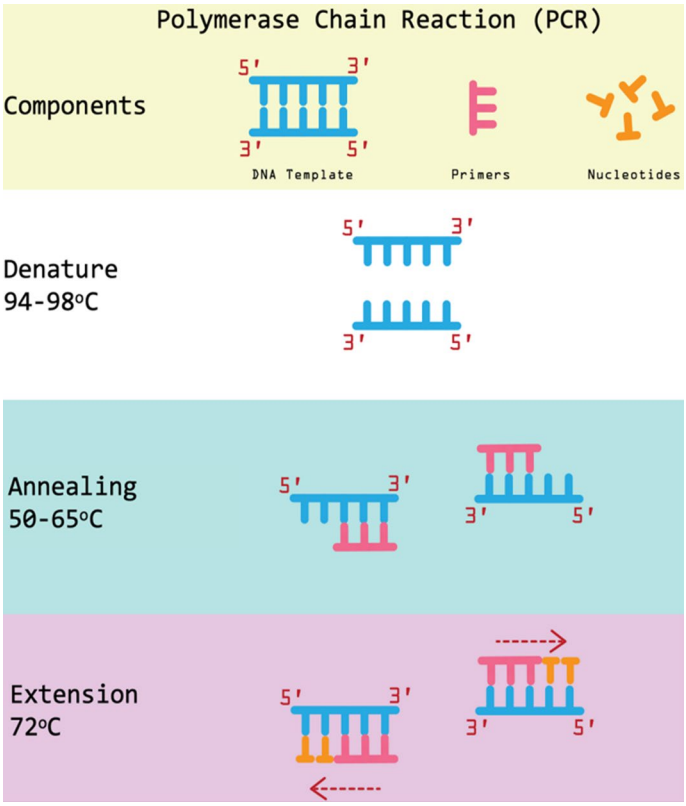

Fig 14. The polymerase chain reaction (PCR) utilises cycles of temperature and a heat-stable form of DNA polymerase (Taq polymerase) to amplify traces of nucleic acid. DNA separates [denatures] into its components strands between 94 to 98°C. Complementary RNA primers [anneal] to each template between 50 to 65°C. At 72°C a multi-subunit DNA polymerase affixes to a strand next to each primer and [extends] a complementary copy by covalently bonding the sugar/phosphate backbone of deoxyribonucleotides, where adenine pairs with thymine, and guanine with cytosine (Fig 13.).

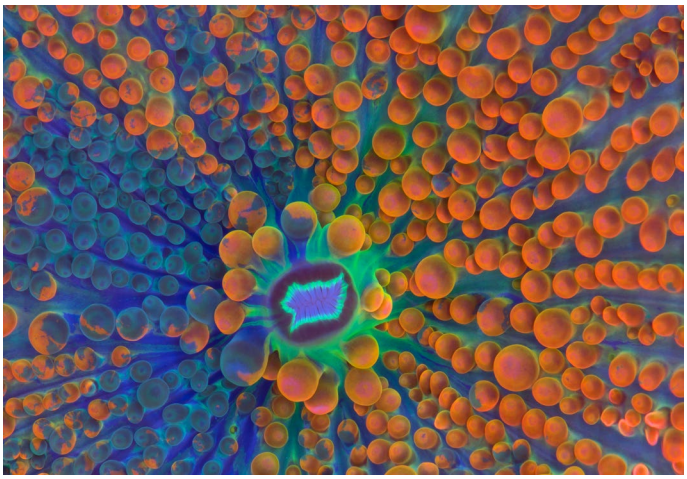

Fig 15. The mouth and tubercles of a mushroom of the genus *Ricordea* (Corallimorpharia: Ricordeidae).

The genomes of *N. vectensis*' zygotes were edited using CRISPR-Cas9 and ZiFiT software. CRISPR guide RNA (gRNA) specific for the target site upstream of the *Clock* exon was spliced into and expressed from a recombinant plasmid (pDR274). Zygotes were co-injected with the resultant CRISPR gRNA and Cas9 endonuclease with a nuclear localisation domain. F_0

homozygous (*Clock*$^{-/-}$) mutant embryos were raised in dark petri dishes at 22°C with daily water changes (Rinsky et al. 2023).

Mutations were initially confirmed using polymerase chain reaction (PCR)-based methodologies to amplify a *Clock* locus-associated 750 base pair (bp) region from dsDNA molecules extracted from seven-day old recombinant planulae and nonmutated adult colonies of *N. vectensis*, where mutagenesis was affirmed by peaks of sequence overlaps (Figs 13. & 14.). A single neoblast stem cell can regenerate an entire organism (Altincicek & Vilcinskas 2008) like *N. vectensis*' embryonic blastomeres. Most anemones including mushrooms can reproduce by pedal laceration where a trail of neoblast-comprising flesh is discarded as they journey over substratum. Remarkably, this reproductive strategy is stimulated in some anemones by prolonged periods of darkness which may benefit "ranchers" of *Ricordea* (Fig 15.). Nascent *N. vectensis* polyps develop within several days (Reitzel et al. 2007; Burton & Finnerty 2009); hence mating experiments affirmed the efficacy of gene editing and generated populations that were heterozygous (*Clock*$^{+/-}$) for wildtype (WT; *Clock*$^{+/+}$) and recombinant *Clock*$^{-/-}$.

Initially F_0 injected polyps (*Clock*$^{-/-}$) were raised to adulthood and outcrossed with (WT). These F_1 individuals were cultivated and genotyped using the methodology outlined above. Tracking of indels by DEcomposition web-tool (TIDE) computes gene editing efficiency and mutagenic composition from DNA insertions versus deletions (indels) by comparing heterogeneous post-PCR-derived genomic assortments to a definitive wildtype allele (Brinkman et al. 2014). This range of heterozygous animals were fostered to sexual maturity and outcrossed with WT. These offspring designated F_2 were cultivated to adulthood, genotyped, and crossed to produce what was termed F_3 with 25 percent heterozygosity. All anemones used in the experiments were descended from F_2 progeny carrying a mutant *Clock*^{1} allele (Fig 16.; Rinsky et al. 2023).

F_3 mutant genotypes were discerned by comparative gel

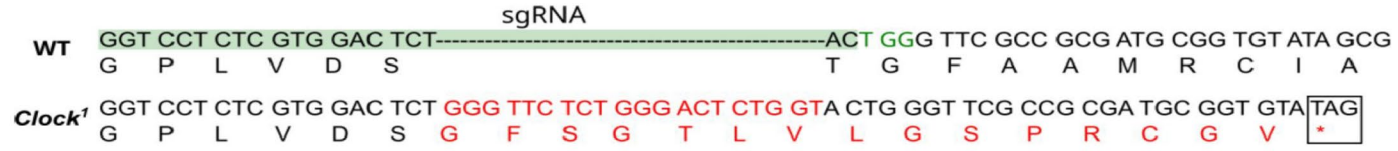

Fig 16. The CRISPR-Cas9-facilitated 20-nucleotide insertion in wildtype *Clock* [WT] to yield a single nucleotide frameshift disrupted mutant *Clock*^{1}. Featuring nucleotide triplet base pairs and translated amino acid sequences of WT (*Clock*$^{+}$) and mutant allele *Clock*^{1} (*Clock*$^{-}$). The site of CRISPR guide (g)RNA annealing, Ca9 endonuclease-editing, and the remainder of the protospacer-adjacent motif (PAM) nucleotides are highlighted in green [WT], while the frameshift-derived predicted disrupting early transcription termination sequence is boxed in black [*Clock*^{1}; *]. The anticipated DNA bases of the *Clock*^{1} polypeptide insertion, and downstream amino acid sequences are highlighted in red. DNA alkalis: adenine [A]; cytosine [C]; guanine [G], and thymine [T], and amino acids: arginine [R]; aspartic acid [D]; cysteine [C]; glycine [G]; leucine [L]; methionine [M]; phenylalanine [F]; proline [P]; serine [S]; threonine [T], and valine [V]. Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0. ATG and TATA are common promoter elements (Lee et al. 2005).

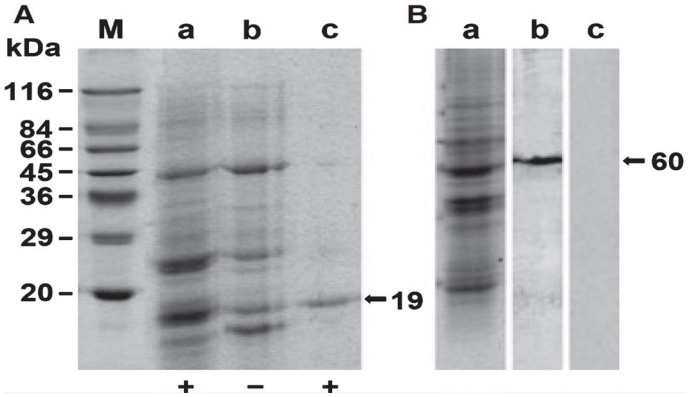

Fig 17. Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) discerning the products of several recombinant cryptochrome genes because the components of an assortment of anionic SDS/denatured (linear) protein complexes separate as discrete bands as they migrate towards the anode in proportion to their size in kilodaltons (kDa; Stryer 1995a). SDS/PAGE works on a similar principle to DNA gel electrophoresis where travel velocity is inversely proportional to $\log_{10}$ length in base pairs (bps; Stryer 1995a). Courtesy of Müller et al. 2010 and the Creative Commons Attribution Licence.

electrophoresis where PCR products are loaded into wells at one end of an even bed of polyacrylamide or agarose gel and a constant electric current separates DNA fragments according to their length and velocity, inasmuch as the speed of their electrophoretic migration is inversely proportional to the $\log_{10}$ of their base pairs (bps; Stryer 1995a). Homozygous mutant *Clock*$^{-/-}$ associated sequences were amplified into a more sizeable 120 bp oligonucleotide whereas a 100 bp amplicon was derived from homozygous wildtype (WT) *Clock*$^{+/+}$, while both oligonucleotides were present in the PCR products from heterozygous animals (*Clock*$^{+/-}$; Rinsky et al. 2023).

Early polyps were illuminated with white neon emitting 17 µmol m^{-2} s^{-1} over 12:12 and 6:6 LD cycles at 20°C throughout behavioural analyses, while the same lamps were used for the RNA sequencing experiments. All were preconditioned for 72 hours under 12:12 LD in an incubator at 18°C whereafter some remained in LD (zeitgeber time; ZT) while others were kept in continuous darkness (DD; circadian time; CT). Sampling commenced at 07:00 (ZT 0; CT 0) when three or four polyps were snap-frozen at -80°C which was repeated every four hours for a total of 24 (Fig 18.; Rinsky et al. 2023).

RNA was extracted and sequenced where an average of 17 million reads ~113 bases long were designated unique molecular identifiers (UMIs) and assembled into a library that was mapped onto the national centre for biotechnology information's (NCBI's) *N. vectensis* genome, GCA_000209225.1 (Rinsky et al. 2023).

Rhythmicity analysis incorporating non-parametric methods (RAIN) algorithm identified cyclically expressed transcripts with merely 24-hour periodicity in wildtype (WT; *Clock*$^{+/+}$) and knockout mutant (*Clock*$^{-/-}$) anemones under either LD or DD protocols. Gene ontology (GO) analyses correlated the biological processes, cellular components, and molecular functions associated with the differentially expressed genes, to determine the influence of the WT *Clock* allele on the up- and down-regulation of intracellular pathways and cascades in LD- and utter darkness-exposed anemones (Rinsky et al. 2023).

Rinsky and allies searched for gene promoters 1,000 bp upstream of start codons where DNA sequence analyses unearthed all E-box and circadian E-box motifs. *N. vectensis Myh7* encoded immediately downstream of *Clock* is expressed

in a circadian-like rhythmicity and thus was a suitable candidate for in situ hybridization chain reaction (HCR). Complementary oligonucleotide (c)DNA probes were hybridised to *Myh7* and *Clock* mRNA within cells actively expressing these genes which were amplified in situ using the chain reaction, after which *Myh7* and *Clock* amplicons were highlighted using red fluorophore-tagged monoclinal antibodies (Fig 26.). Intracellular DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) which appeared grey in microscopically examined histological wholemounts of juvenile anemones (Fig 20.; Tarnowski et al. 1991; Rinsky et al. 2023).

Phylogenetic analyses positioned *N. vectensis*' CLOCK

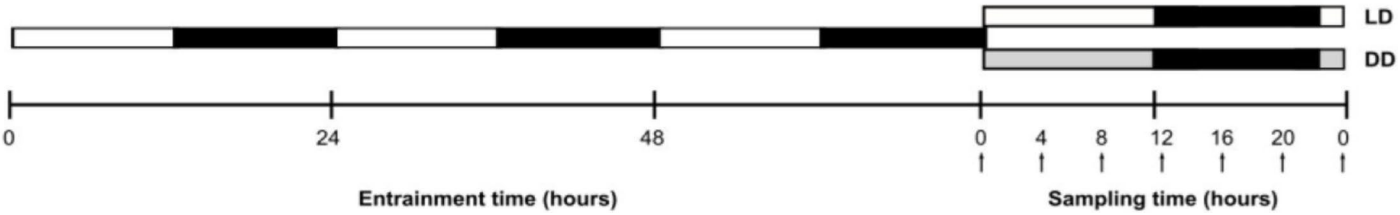

Fig 18. An experiment to evaluate the transcription of genes in the early polyps of *N. vectensis*. Anthozoa were preconditioned for 72 hours under 12:12 LD (17 µmol m^{-2} s^{-1}) at 17°C, split into two groups, and exposed to either LD (zeitgeber time; ZT) or DD (circadian time; CT) lighting protocols throughout the following 24 hours. Three to four juvenile anemones were taken from each group every four hours starting at 07:00 (ZT 0; CT 0) on the fourth day and were snap-frozen in liquid nitrogen for subsequent RNA extraction. Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

in Cnidaria (Fig 19. A; Rinsky et al. 2023). Several canonical circadian orthologues are encoded on the genome of sponges including *Clock* and *Cycle* which is a homologue of *arnt* and the mammalian orthologue of *bmal1*/*mop3* (Simionato et al. 2007; Muller et al. 2010). The CLOCK:CYCLE heterodimer regulates E-box motif (CACGTG) transcription as part of an acknowledged circadian positive feedback loop (Dunlap 1999) where *clock* and *cry1a* of *Nematostella vectensis* remain under heterodimeric control (Reitzel et al. 2010). *Cycle* and *Clock* belong to a family of transcription factors with basic (alkaline) helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) domains (Shearman et al. 2000; Hennig et al. 2009; Reitzel et al. 2010), where the latter has two co-factor PAS dimerization moieties. These regions encode for structural motifs that drive the zeitgeber-independent and perpetual intracellular pathways that underpin the circadian oscillator (Shearman et al. 2000; Hennig et al. 2009; Rinsky et al. 2023).

Nominal expression of *Clock* was disseminated throughout WT four-tentacled juvenile anemones which was moderately-enriched in the tentacle endodermis and mesenteries (Rinsky et al. 2023), somewhat akin to their planulae (Peres et al. 2014). An additional modicum of *Clock* mRNA was intracellularly focused in discrete nucleic zones (Fig 19. B), yet genetic manipulation of animalian *Clock* had not been undertaken before this study while its role in Cnidaria remained enigmatic (Shearman et al. 2000; Hennig et al. 2009; Rinsky et al. 2023).

CRISPR guide RNA (gRNA) targeted Ca9 endonuclease to a 5' start sequence-proximal region betwixt PAS2 and PAS1 in the *Clock* exons of *N. vectensis* which typically prompted 20-nucleotide disruptive insertions (Fig 16.). This F_0 generation were outcrossed to WT and their F_1 progeny were raised to adulthood and genotyped. Six alleles with frameshift mutations including a 20-bp insertion where *Clock*^{1} encoded for a knockout stop codon [*TAG] were amongst the 10 CRISPR-Ca9-generated transgenes (Fig 16.; Fig 20.). CLOCK1 was likely a PAS domain- and 459 amino acid-degenerate polypeptide comprising 203 residues (Fig 19. C). Crossing heterozygous F_1 (*Clock*$^{-/+}$) resulted in 25 percent homozygous (*Clock*$^{-/-}$) F_2 which were raised and interbred to create a genotypically identical F_3 generation of homozygous *Clock*$^{-/-}$ mutants (Rinsky et al. 2023).

The locomotion of WT and *Clock*$^{-/-}$ *N. vectensis* has an analogous periodicity of 24 hours under 12:12 LD lighting protocols (Fig 19. d & d'). WT anemones sustained slightly condensed 22-hour locomotive rhythmicity in continuous darkness or light while mutant travel was arrhythmic (Fig 19. e & e'; f & f'). All anemones were entrained for 72 hours of zeitgeber time-analogous 12:12 LD before behavioural studies commenced when some were exposed to six hours of light and darkness (6:6 LD; ZT). 22-hour weakened but sustained movements were observed in WT under 6:6 LD when mutant behaviour adopted a remarkable 12-hour rhythmicity. These findings suggest that WT locomotion is commensurate with an endogenous "clock" (Rinsky et al. 2023), but redolent of the induction of a compensative autonomous 12-hour circatidal rhythm or merely light-responsive in *Clock* disrupted mutants, insofar as halving daylength condensed the cycle by 50 percent (Fig 19. g & g'; Rinsky et al. 2023). The experimenters thus excluded circatidal influence by entraining anemones in 6:6 LD before tracking their behaviour in darkness, where WT retained their 22-hour pattern while mutant motility became arrhythmic (Fig 30.; Rinsky et al. 2023).

RAIN algorithm identified 2,261 and 2,360 genes that were rhythmically expressed in WT anemones exposed to 12:12 LD and total darkness respectively, while 1,847 and 1,314 rhythmic transcripts occurred in homozygous *Clock*^{1} mutants under the same experimental conditions (Fig 21.). 91 percent of the rhythmically expressed genes (6,200 out of 6,803) were not shared between genotypes where 55.8 (3,798) and 35.3 (2,402) percent were unique to WT and *Clock*$^{-/-}$ anemones respectively (Figs 20. & 29.). WT genes that oscillated under either LD or DD were cross-referenced to identify those that appeared under the control of a circadian pacemaker. 4.9 (220) percent were rhythmically expressed under LD and DD protocols which were assigned clock-controlled genes (CCGs). Gene ontology (GO) analyses revealed they were implicated in the regulation of a range of translational, transcriptional, and mitotic pathways where Leach and Reitzel (2019) had established these circadian-associated cascades in *N. vectensis*. 85 percent (188) of these 220 CCGs were not cyclically expressed in mutant anemones and were thus designated *Clock*-dependent CCGs. Like several canonical circadian orthologues, 28 percent had circadian-annotated E-box motifs and bHLH domains 1 kilobase upstream of their start site (Fig 22. & 23. d). 80 percent (125) of the 156 genes showing rhythmic expression in *Clock*$^{-/-}$ mutants

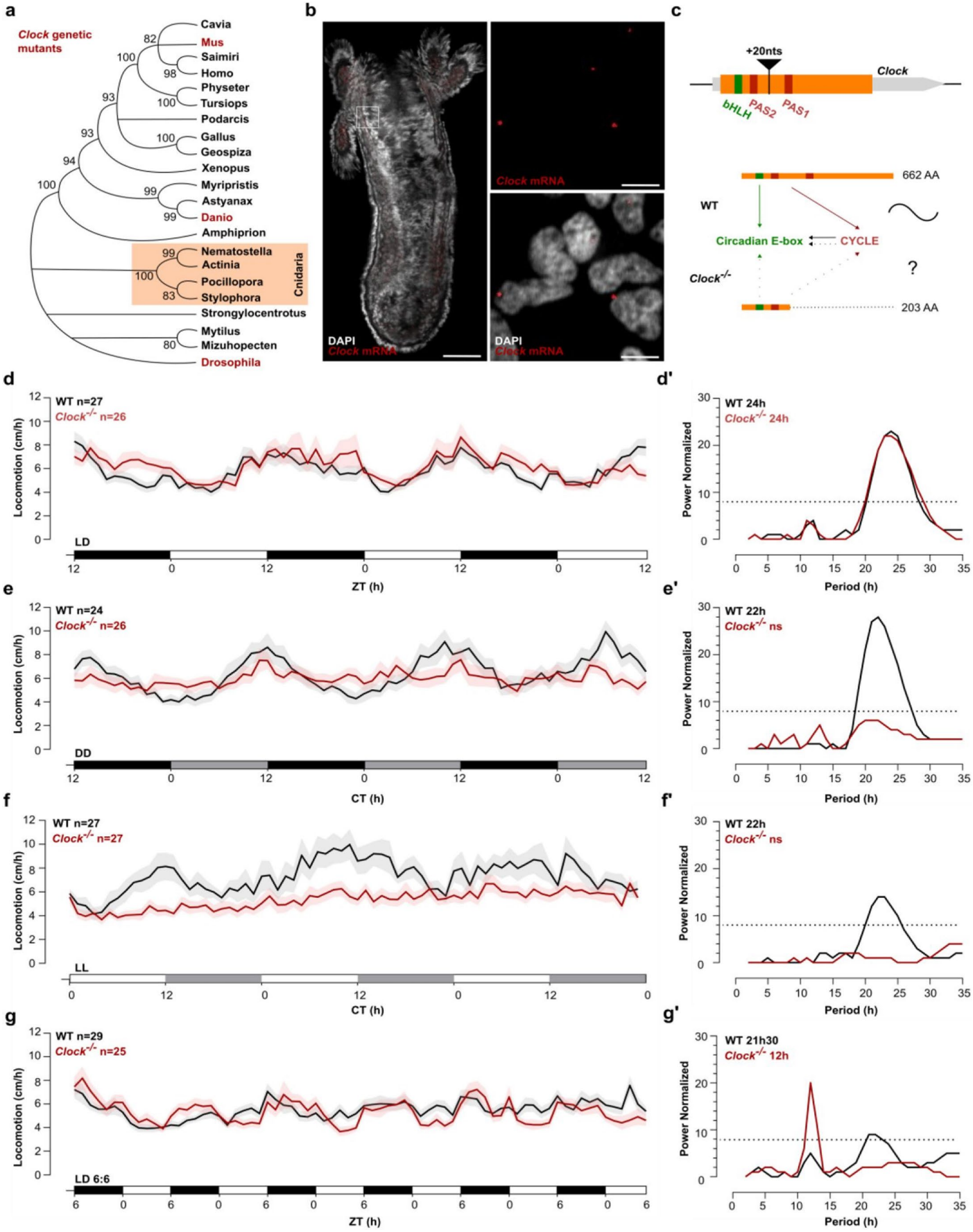

Fig 19. [a] a phylogenetic tree positioning the CLOCK of *N. vectensis* within the cnidarian branch. [b] in situ cDNA probe-hybridisation and chain reaction amplification of intracellular *Clock* mRNA emphasised with a red fluorophore-immunoglobulin-conjugate and 4',6-diamidino-2-phenylindole (DAPI; grey) within four-tentacled juvenile *N. vectensis* where bars indicate 0.1 mm and 5µm. [c] an illustration of *Clock* in grey with its wildtype (WT) open reading frame (ORF) in orange while its conserved basic helix-loop-helix (bHLH) and its Per-Arnt-Sim 1 (PAS1) and PAS2 appear in green and red respectively. The black arrowhead represents the transgene's 20-nucleotide insertion which caused a single nucleotide frameshift that encoded a downstream premature stop codon [*TAG]. [d to g] 72 hours of normalised anemone locomotion under 12:12 LD; 24 DD; 24 LL, and 6:6 LD where *Clock*$^{-/-}$ mutant and WT traces are in red and black respectively. [d' to g'] periodograms for each lighting protocol. Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

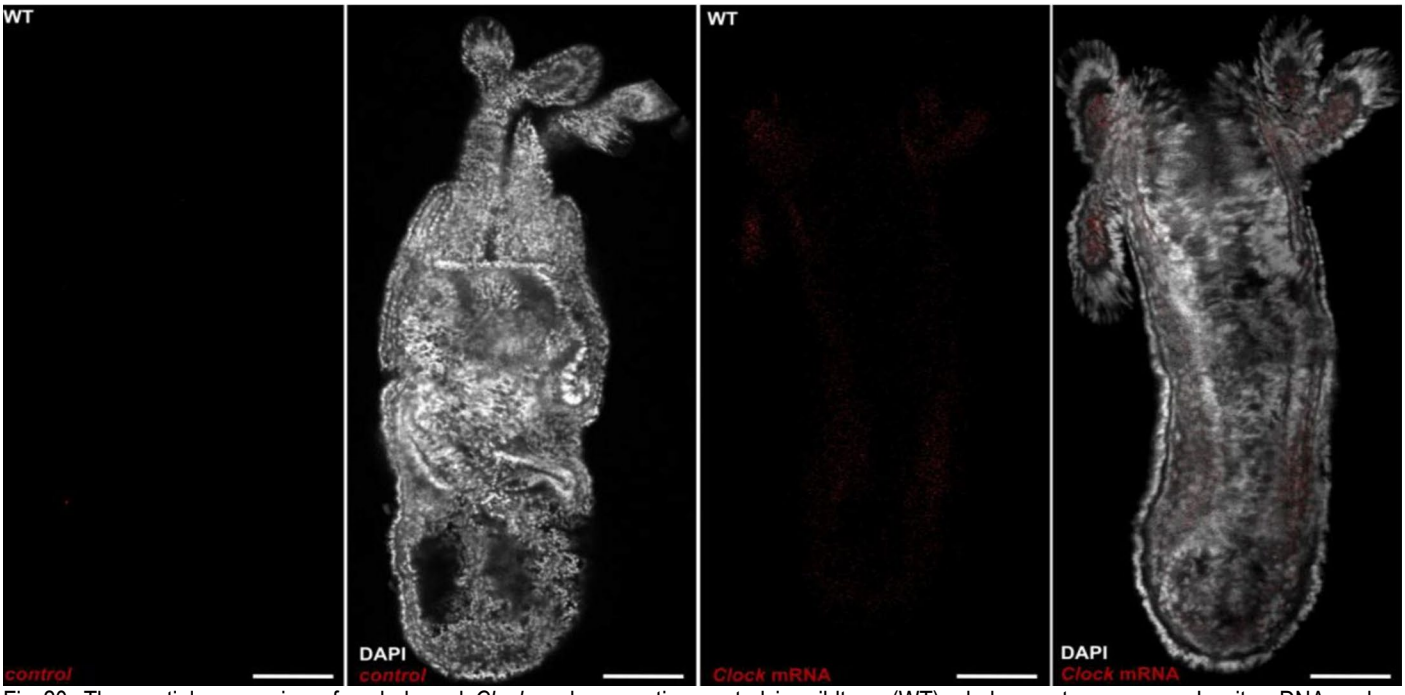

Fig 20. The spatial expression of endodermal *Clock* and a negative control in wildtype (WT) wholemount anemones. In situ cDNA probe-hybridisation and chain reaction amplification of intracellular *Clock* mRNA emphasised with a red fluorophore-immunoglobulin-conjugate in four-tentacled juveniles of *N. vectensis*. [Box 1] the *zfHcrt* negative control featuring nil fluorescence. [Box 2] *zfHcrt* negative control and DAPI stained anatomy (grey). [Box 3] signal from *Clock* mRNA-red fluorophore-immunoglobulin-conjugate. [Box 4] red fluorescence from tagged *Clock* mRNA-immunoglobulin with DAPI staining. Bars indicate 0.1 mm. Micrographs courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

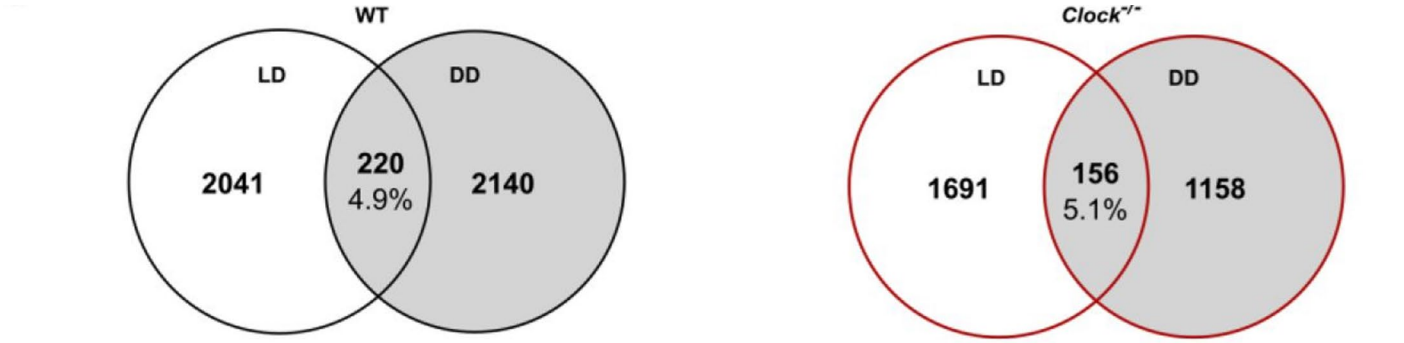

Fig 21. Venn diagrams illustrating the numbers of cyclically expressed genes in WT and *Clock*$^{-/-}$ mutant anemones exposed to LD or DD and those that were operational under both experimental conditions. Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

under both LD and DD protocols, were arrhythmic in WT and lacked circadian-controlled enriched ontologies and E-box promoters which were referred to as neoCCGs (Figs 21. to 23. & 25.; Rinsky et al. 2023).

Like other *N. vectensis* studies (Leach et al. 2018; Leach & Reitzel 2019; Rinsky et al. 2022), the transcription of eight candidate circadian genes became arrhythmic in continuous darkness: *Clock*, *Crya*, *Cryb*, *CiPC*, *PAR-bZIPa*, *PAR-bZIPc*, *PAR-bZIPd*, and *Helt*. This trend continued in *Clock*$^{-/-}$ anemones yet generalised disparities in expression occurred under LD. The expression of *Cryb* and *PAR-bZIPc* were only cyclic in WT anemones exposed to LD, while *CiPC* was the only putative circadian gene classified as a *Clock*-dependent CCG, yet its cadence was atypical (Fig 24.). E-box motifs appear plentiful in *Clock*-dependent CCGs where 16 genes out of 188 were rhythmically expressed in the current and a previous study (Fig 25.; Leach & Reitzel 2019; Rinsky et al. 2023). *Myh7* was conspicuous amongst these insofar as it is ergonomic to detect, its mRNA readily forms hybrids, it lies immediately downstream of *Clock*, and it has an analogous pattern of expression (Fig 26.; Rinsky et al. 2023). Hybrids of nucleic acid are resistant to several types of DNA- and RNA-ases (Lu et al. 2023).

Comparative analyses of the genes expressed under each lighting protocol in *Clock*$^{-/-}$ mutants and WT anemones was undertaken with a view to unearthing the intracellular processes regulated by *Clock*. 646 and 457 genes were up- and down-regulated respectively in homozygous mutants exposed to 12:12 LD in the absence of pathway enrichment, whereas 1,770 and 2,450 were promoted and repressed in *Clock*$^{-/-}$ anemones in continuous darkness. Modulation of another organism's processes, sensory perception, and ion transmembrane transport were amongst the GO terms enriched for the upregulated gene sets, whereas spermatid development, mitosis/meiosis, and ciliary/flagellar motility pathways were associated with genes that were downregulated (Figs 27. & 28.; Rinsky et al. 2023).

The *Clock* gene appears an indispensable player in the mechanisms that underpin the rhythmic motility of starlet sea anemones under constant conditions, and hence the rudimentary foundations of the circadian oscillator were present in the common animalian ancestor of Cnidaria and Bilateria (Rinsky et al. 2023).

Clock$^{-/-}$ fruit flies have demonstrated that their canonical *Clock* orthologue *Clk* (*dClk*) is non-essential for circadian

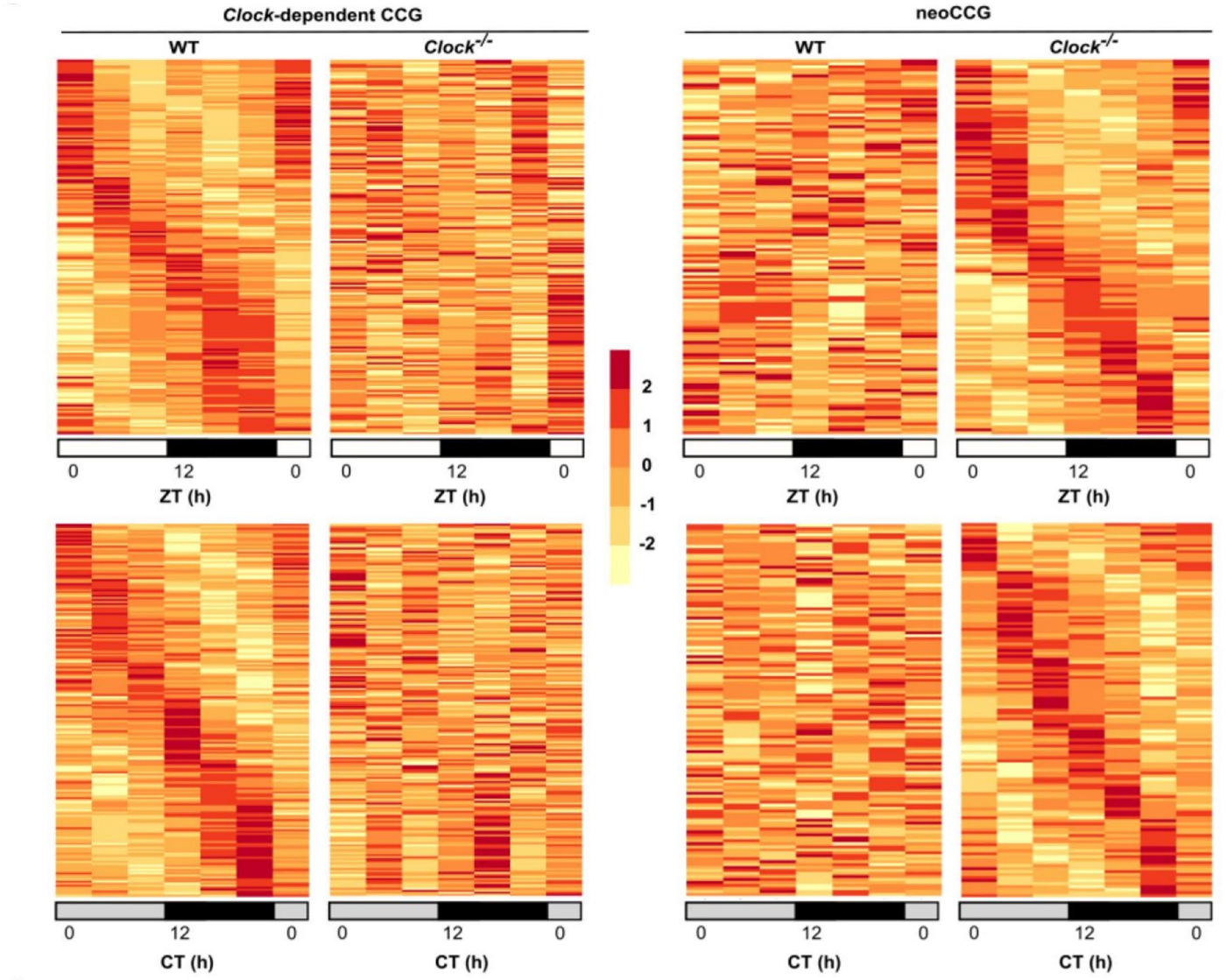

Fig 22. Heatmaps illustrating the mRNA abundances of *Clock*-dependent clock-controlled genes (CCGs; left) that were rhythmically expressed in WT anemones under both LD and DD protocols but were arrhythmic in *Clock*$^{-/-}$ mutants, while the transcription of neoCCGs oscillated in mutants exposed to LD and DD experimental conditions but were arrhythmic in WT. Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

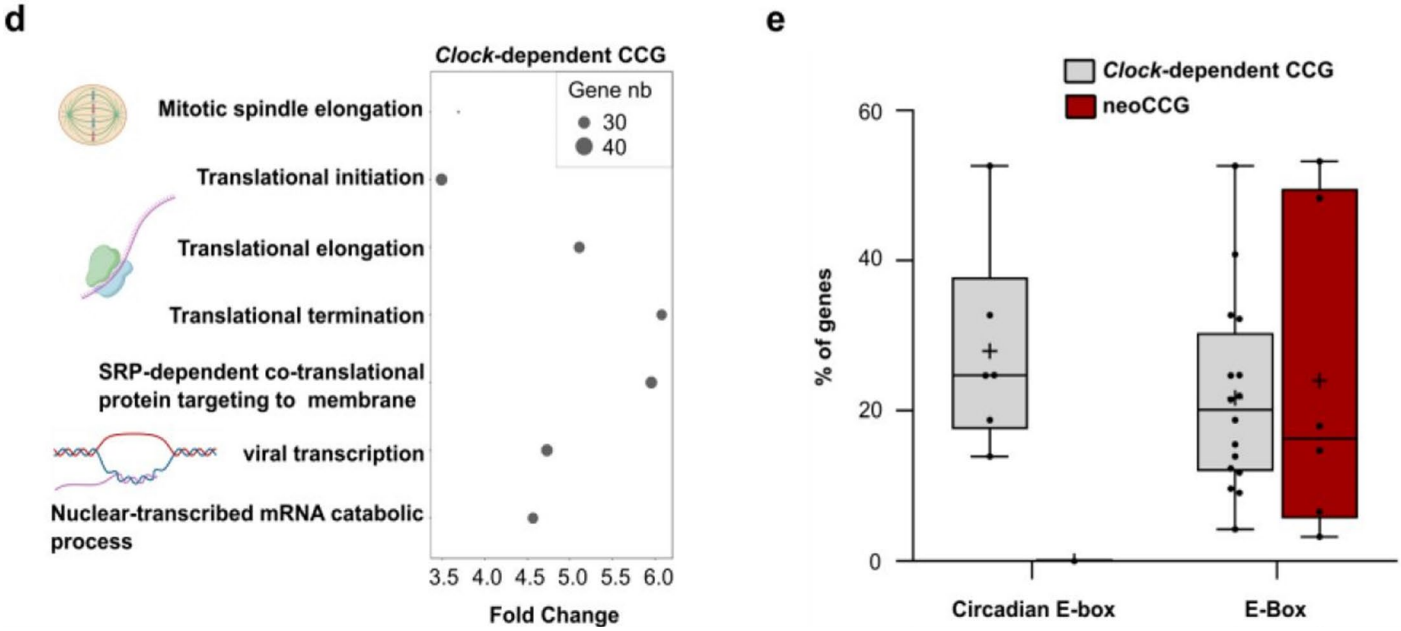

Fig 23. [d] pheatmap of gene ontologies associated with sets of *Clock*-dependent clock-controlled genes (CCGs) that were rhythmically expressed in WT anemones under both LD and DD protocols but were arrhythmic in *Clock*$^{-/-}$ mutants. [e] box plots of Homer/MEME-derived *de novo* sequence motif analyses of the transcriptional promoters found 1,000 bases upstream of the methionine-encoding ATG of *Clock*-dependent CCGs and neoCCGs, where their E-box promoters lacked circadian annotations. Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

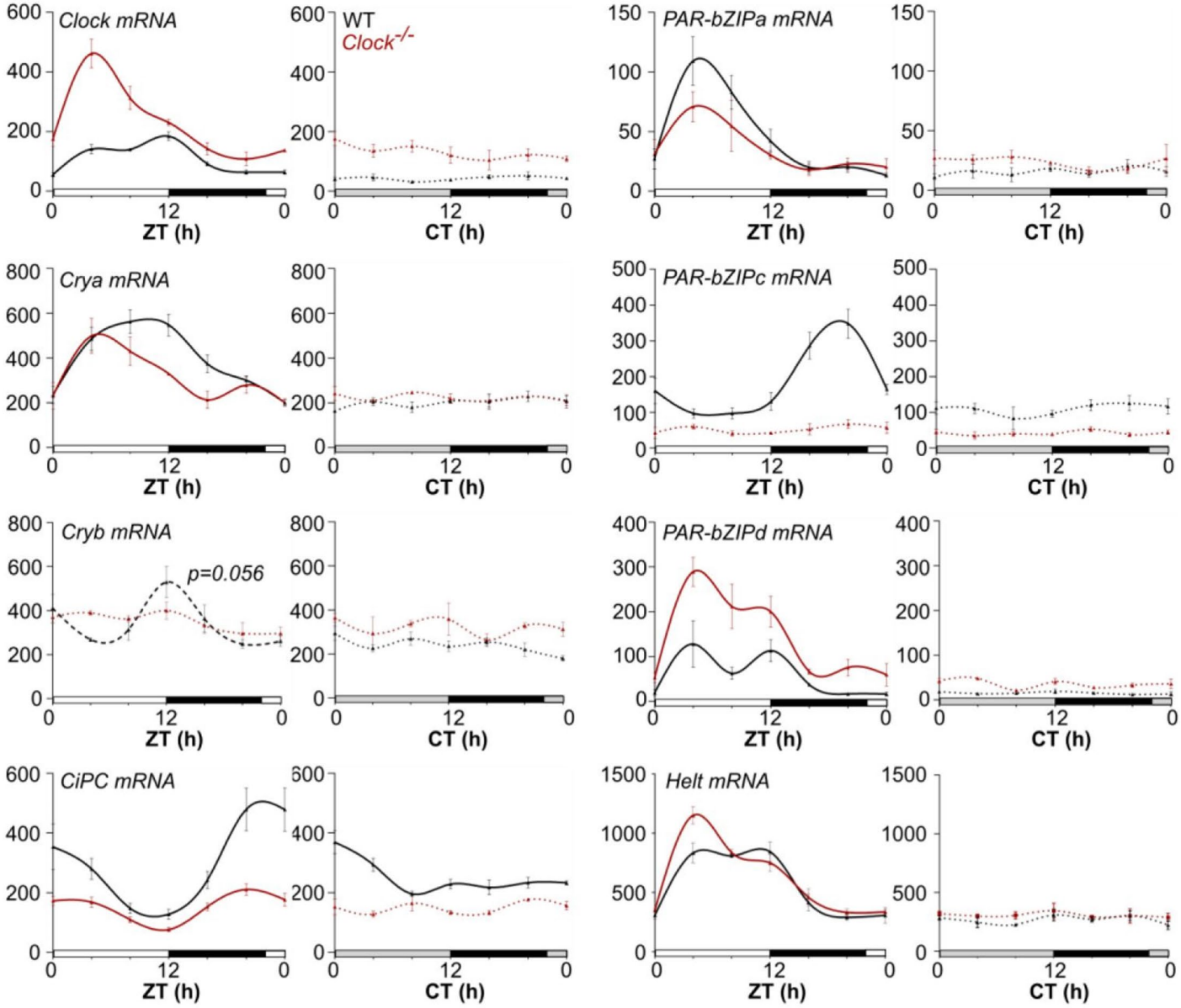

Fig 24. The expression patterns of the core clock genes: *Clock*, *Crya*, *Cryb*, *CiPC*, *PAR-bZIPa*, *PAR-bZIPc*, *PAR-bZIPd*, and *Helt* in WT (black) and *Clock*$^{-/-}$ (red) mutant anemones exposed to 12:12 LD zeitgeber time (ZT) and DD circadian time (CT), where dotted lines indicate insignificant RAIN rhythmicity while heavier dashes approach significant ($p<0.05$). Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

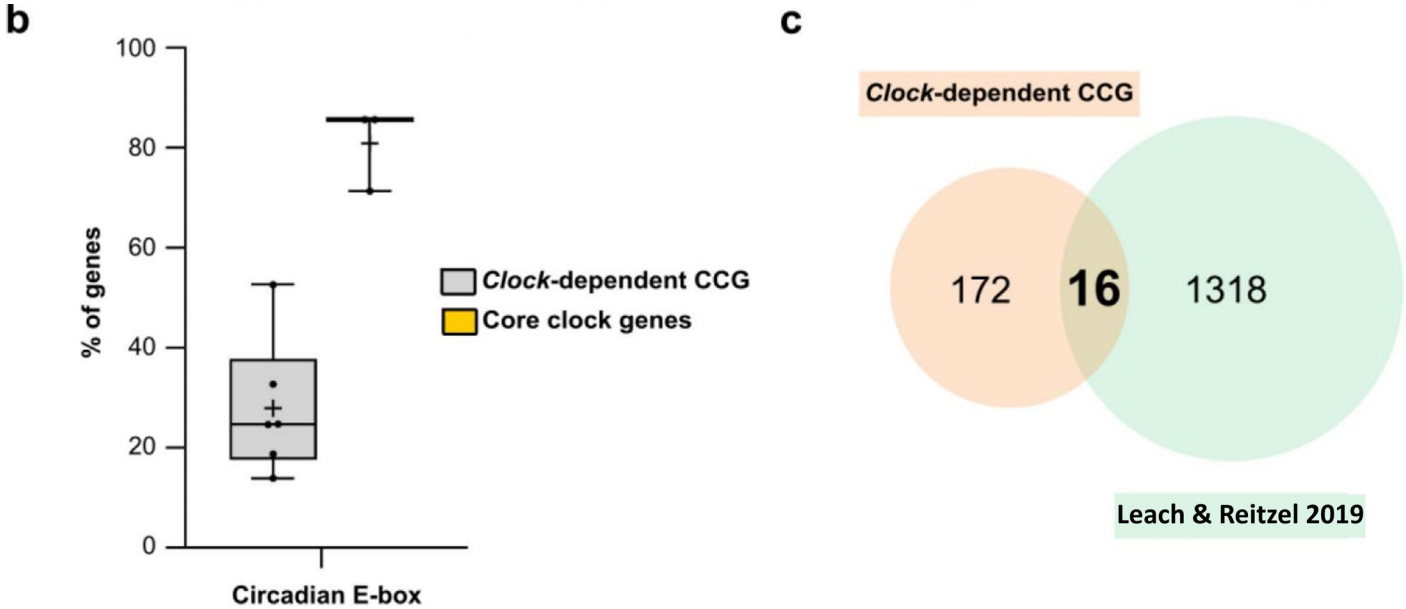

Fig 25. [b] box plots of HOMER-detected E-box promoters 1 kilobase upstream of ATG with circadian annotations. [c] a Venn diagram featuring the numbers of conserved rhythmically expressed *Clock*-dependent CCGs in WT *N. vectensis* from the current study and Leach and Reitzel (2019). Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

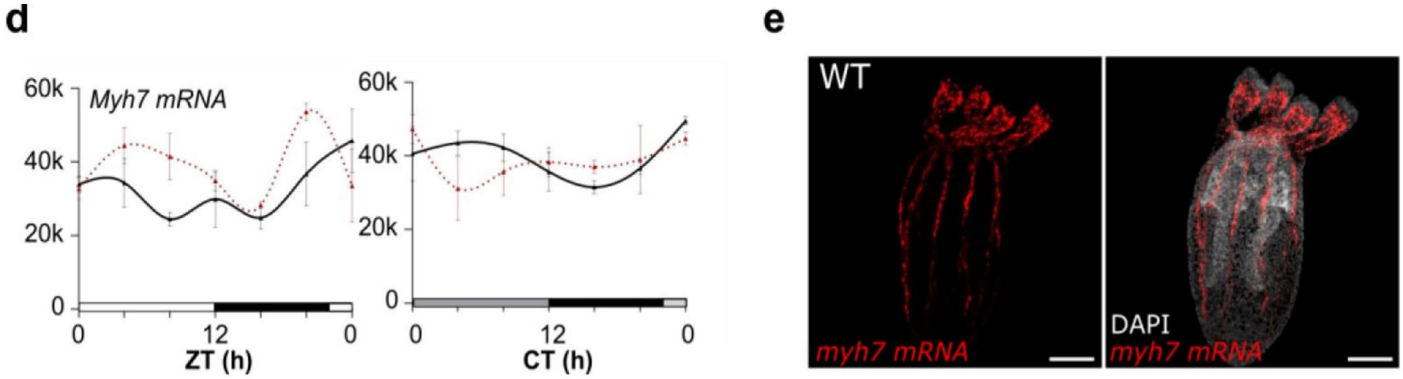

Fig 26. [d] the temporal expression pattern of the gene used as a positive control, *Myh7* found downstream of the *Clock* locus in WT (black) and *Clock*$^{-/-}$ (red) mutant anemones under LD zeitgeber time (ZT) and DD circadian time (CT) where dotted lines indicate insignificant RAIN rhythmicity. [e] in situ cDNA probe-hybridisation and chain reaction amplification of intracellular *Myh7* mRNA emphasised with a red fluorophore immunoglobulin-conjugate in wholemount WT juvenile anemones with and without grey DAPI staining. Bars represent 0.1 mm. Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

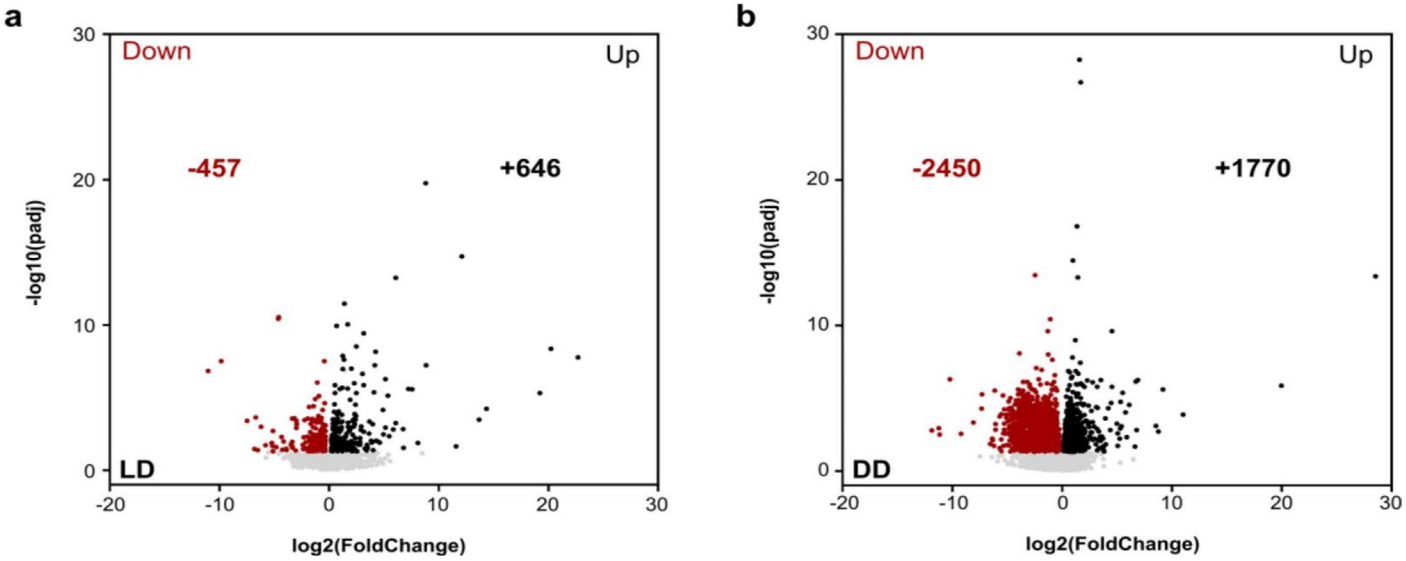

Fig 27. [a] a volcano plot featuring the upregulation (black) and downregulation (red) of genes within the tissues of *Clock*$^{-/-}$ *N. vectensis* under 12:12 LD conditions. [b] a volcano plot illustrating the intracellular promotion and repression of genes in *Clock*$^{-/-}$ anemones held in continuous darkness (DD). Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

rhythmicity but is key for light-dependent behaviour (Kim et al. 2002; Allada et al. 2003) where WT flies anticipate transitions from light to darkness and vice versa and act accordingly (Sehgal et al. 1994; Lee et al. 1998; Allada et al. 2003). All the same, murine *Clock* remains phenotypically unimportant (Debruyne et al. 2006). CLOCK has evolved to support diverse circadian and light-responsive roles in mice and flies (Rinsky et al. 2023). Likewise, *Bmal1* is currently the only mammalian circadian orthologue whose disruption leads to arrhythmic ontologies and behaviour in the absence of exogenous cues (Bunger et al. 2000, cited in Rinsky et al. 2023). Nevertheless, the operational diversity and ostensible functional redundancy conferred by multiple mammalian alleles, tends to mask the phenotypic impacts of *Clock* including *mClock* (Selby et al. 2000; Reppert & Weaver 2002; Asher 2006, cited in Rinsky et al. 2023). *N. vectensis Clock*$^{-/-}$ mutants lose all semblance of rhythmic motility which reverts under day- and night-time analogous irradiance (Leach & Reitzel 2019; Rinsky et al. 2023). The behaviour of these mutant anemones thus becomes light dependent which affirms that *Clock* is not a light receptor or is important for their downstream cascades or the entrainment of a core oscillator (Rinsky et al. 2023). *Clock* appears indispensable for sustaining zeitgeber-free circadian-associated activities; however, the products of drosophilan *Clk* and mammalian *mMop3* (orthologue: *BMAL1*) have been implicated in the light input pathway (Allada et al. 1998; Bunger et al. 2000; Kim et al. 2002; Allada et al. 2003).

Transcriptional rhythmicity is lost or diminished in numerous genes of *N. vectensis* including canonical circadian orthologues after merely 24 to 48 hours of unwavering conditions (Reitzel et al. 2010; Peres et al. 2014; Leach & Reitzel 2019). Hence *Clock* and others require cycles of light and darkness, while the diurnal behaviours and the mRNA of the current study's clock-controlled genes (CCGs) continue to cycle in WT throughout "free running". Incongruity of this kind may arise when analysing higher eukaryotes like vertebrates with complex anatomical compartmentalisation and organ-, tissue-, or cell-specific phase shifts (Albrecht 2012; Leach & Reitzel 2019). Furthermore, many of their homeostatic and housekeeping roles are modulated by hormones or cytokines (Wuitchik et al. 2019; Connelly et al. 2020). Even so, their core oscillators may be attuned by unforeseen or cryptic cues that may remain absent in vitro like those that entrain circatidal rhythm (Sehadova et al. 2009; Simoni et al. 2014). Vital stimuli may therefore be lacking in preconditioning zeitgeber time, whilst billions of years of evolution proposes phylum resolution remains reserved. In situ hybridisation and red fluorophore tagged antibodies, DAPI staining, and microscopic examination revealed the generalised dissemination of *Clock* mRNA which recommended whole animal sampling in juvenile *N. vectensis* (Rinsky et al. 2023).

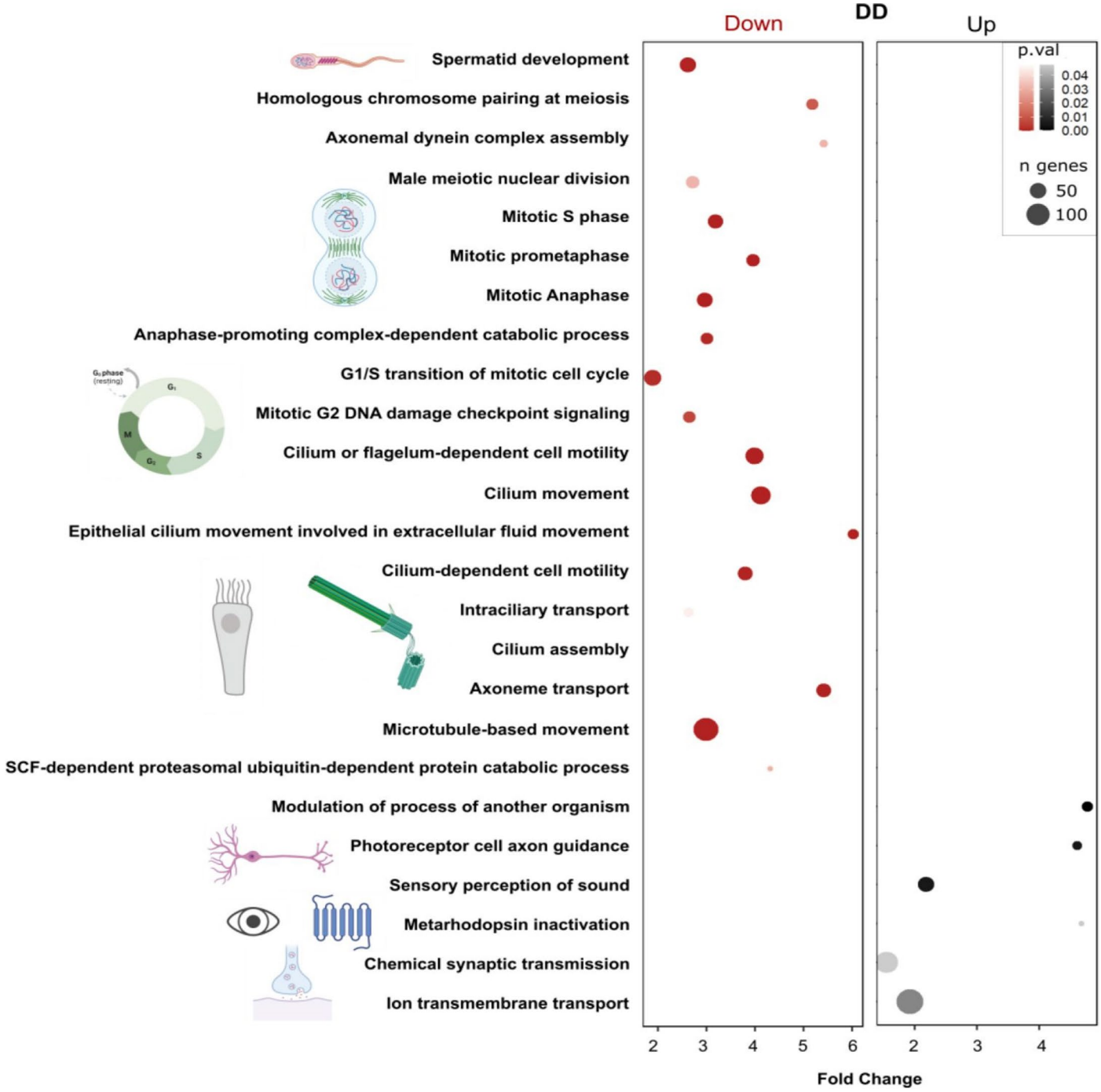

Fig 28. Pheatmaps featuring the differential expression of gene sets associated with each ontology in the cells of mutant anemones under constant darkness (DD). Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

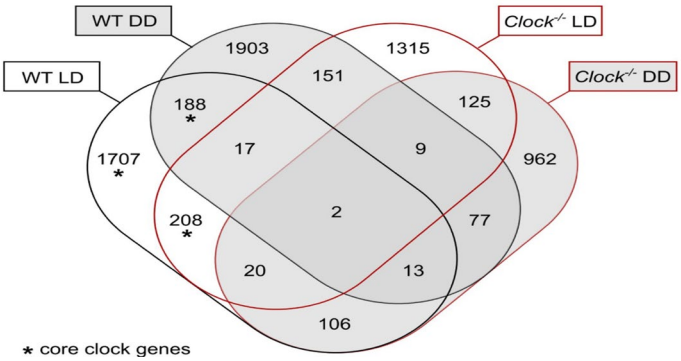

Fig 29. A Venn diagram featuring the numbers of unique and shared rhythmically expressed genes across all genotypes and experimental conditions, where merely 2 genes appear to be present in all classes. [*] indicate core circadian genes where 188 were shared between WT LD and WT DD, 208 between WT LD and *Clock*$^{-/-}$ LD, whilst 1,707 were unique to WT LD. Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

Notwithstanding light being the primary driver of rhythmic expression, *Clock*, *Crya*, *Cryb*, *CiPC*, *PAR-bZIPa*, *PAR-bZIPc*, *PAR-bZIPd*, and *Helt* were differentially expressed in homozygous *Clock* disrupted mutants compared to WT anemones, which may suggest that *Clock* attunes transcription to irradiance received in light-responsive genes (Fig 24.). Nonetheless, the impact exerted on the core pacemaker throughout circadian gene transcription remains enigmatic. 125 neoCCGs were cyclically expressed in mutants under both LD and DD experimental conditions, but they were arrhythmic in WT anemones and lacked circadian-controlled enriched ontologies and E-box promoters (Fig 23.).

The circadian-like transcriptional oscillations of neoCCG sets were not associated with ontologies and are therefore likely expressed independently of endogenous timekeeping, while their rhythmic mRNA abundances are extraordinary inasmuch as they lack circadian-annotated E-box, bHLH-PAS, or other typical

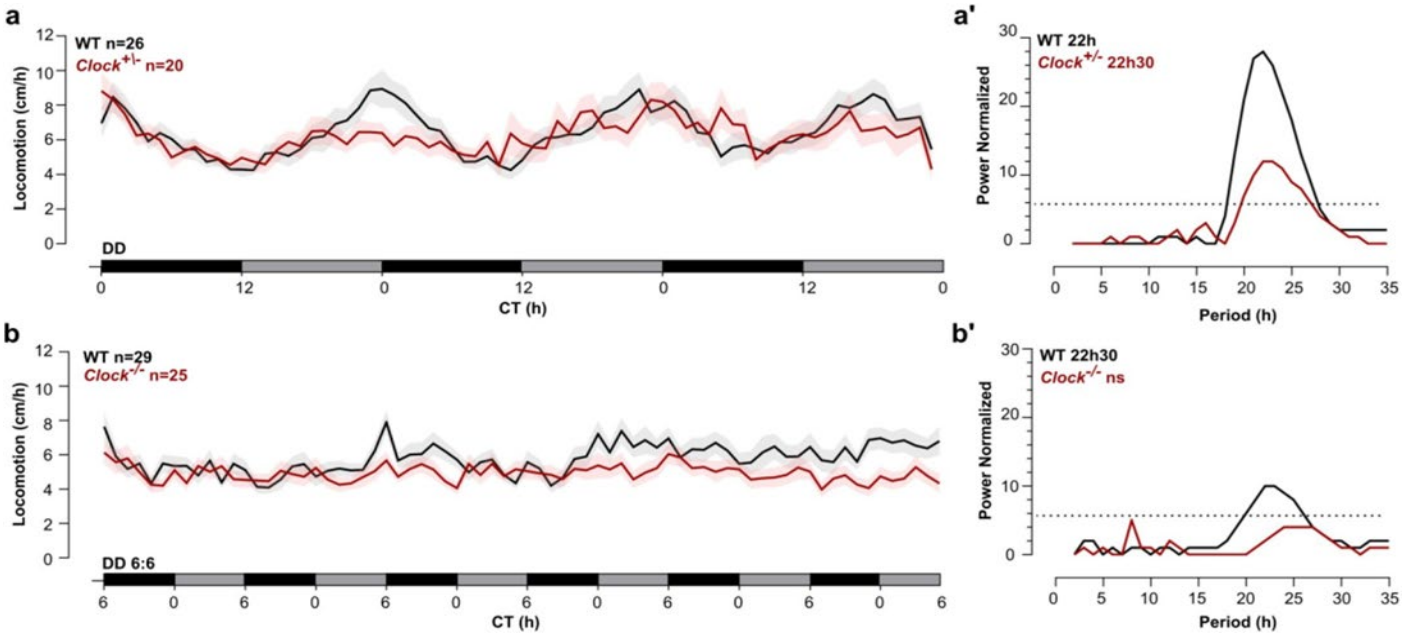

Fig 30. [a] hourly normalised anemone locomotion over 72 hours in continuous darkness after three days of 12:12 LD pre-entrainment. [b] hourly normalised anemone locomotion over 72 hours in continuous darkness after three days of pre-entrainment with 6:6 LD. Wildtype (WT) plotted in black whereas red traces represent *Clock*$^{-/-}$ mutants. [a'] a periodogram where the expression of WT and *Clock*$^{-/-}$ adopt their respective 22- and 22½-hour timeframes under "a" conditions, [b'] whereas WT and *Clock*$^{-/-}$ transcription assumes 22½- and 12-hour periodicity in constant darkness after three days of 6:6 LD preconditioning. n=x and ns represent significant and insignificant differences. Courtesy of Rinsky et al. 2023 and the Creative Commons Attribution Licence 4.0.

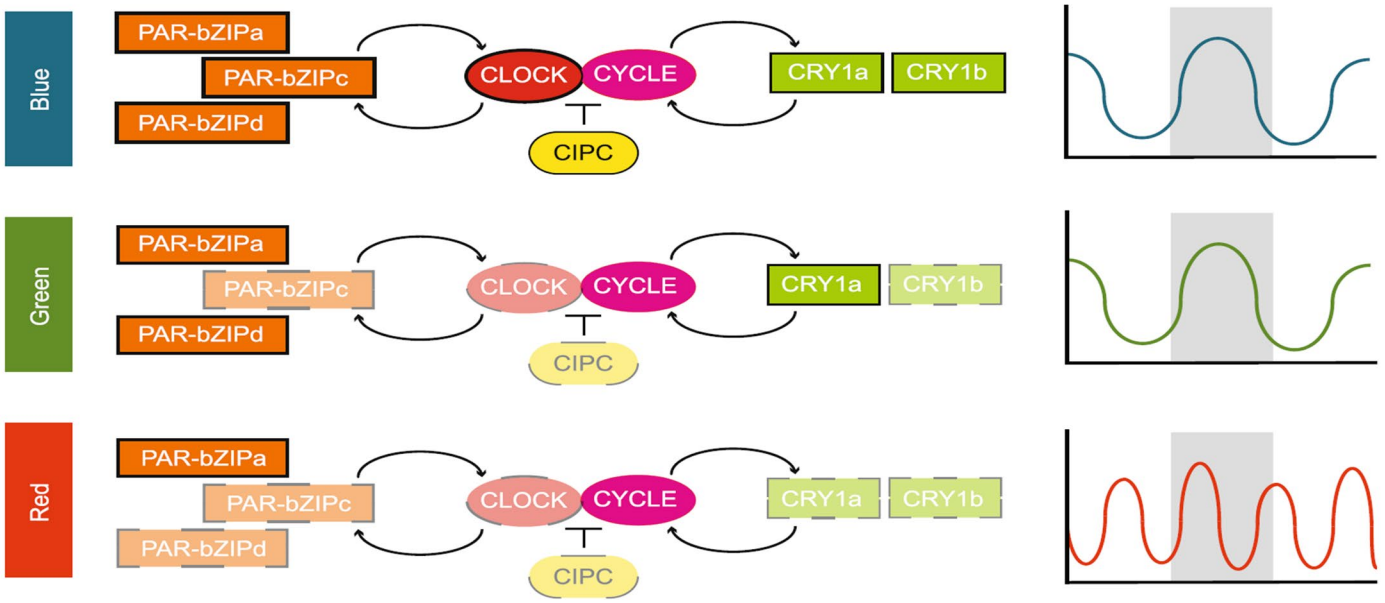

Fig 31. An illustration of *Nematostella vectensis*' gene regulatory model proposed by Reitzel and collaborators in 2013. The light-sensitive proteinaceous cryptochromes CRY1a and CRY1b are integral to a negative feedback loop that downregulates promotion by the CLOCK:CYCLE heterodimer, while PAR-bZIPa, PAR-bZIPc, and PAR-bZIPd participate in a feedforward loop that promotes or represses the transcription of *Clock* or *Cycle*, whereas CIPC acts as a CLOCK:CYCLE repressor akin to its role in fruit flies. Short, medium, and long wavelength radiation corresponding to blue, green, and red are featured to the left, while periodograms of anemone behaviour corresponding to each kind of light are indicated on the right. Remarkably, anemone motility adopts circatidal timekeeping when exposed to diurnal red light. Solid and dashed outlines represent significant and insignificant differential expression where CYCLE is not encircled due to its consistent transcription under all lighting conditions. Analyses, schematic, and hypotheses courtesy of Leach and Reitzel from 2020 and the Creative Commons Attribution Licence 4.0.

core "clock" promoter regions. Rinsky and collaborators could not rule out confounding impacts exerted by the abridged 203 amino acid CLOCK[1] polypeptide, while the genes that appeared to be under the control of an intrinsic pacemaker in both knockout mutant and WT anemones, suggests that the product of *Clock* is not integral to a positive or negative feedback loop that controls these genes in *N. vectensis* (Fig 29.). The endogenous timekeeper of these anemones clearly operates independently of *Clock*, but the core oscillator shifts expression towards a different set of genes in its absence (Rinsky et al. 2023).

The mechanisms that underpin the biorhythms of *N. vectensis* remain unclear while the absence of *Clock* has a broad impact on expression, where constant darkness downregulated genes associated with cellular division and microtubule organisation which is redolent of CLOCK's participation in circadian-independent cell cycling, homeostasis, and/or housekeeping. Likewise, the genes that were upregulated in utter darkness appeared to be involved in sensory discernment. All things considered; the product of *Clock* exerts light-dependent impacts on gene expression beyond the core oscillator which may be

pronounced at key developmental stages (Rinsky et al. 2023), as the genes of the core pacemaker are expressed arrhythmically throughout embryonation (Peres et al. 2014). Transcription-translation feedback loops appear not controlled by CLOCK in *N. vectensis*, insofar as its E-box promoter lacks circadian annotations yet it has two co-factor PAS heterodimerisation domains which implicates *Clock* in the underlying mechanisms that drive the endogenous oscillator (Shearman et al. 2000; Hennig et al. 2009; Rinsky et al. 2023). This gene may coordinate light and other exogenous cues to the core pacemaker of Anthozoa and thus expedite environmental adaptation, which warrants supplementary exploration of the phenomena outlined in Rinsky and allies' paper (2023).

That concludes our quest for Current Knowledge, while next we use our newfound insight to begin to unravel the mechanisms that control the synchrony of gameto- and embryo-genesis with a view to understanding the spawning windows of reef-forming corals.

Appendix I Glossary

Allele refers to different forms of a gene.

Amplicon is an amplified sequence of DNA (Merriam Webster 2018).

Aposymbiosis refers to when symbiotic organisms live separately.

Autotrophic organisms satisfy their nutritional requirements from simple inorganic chemicals and usually but not exclusively light.

Azooxanthellate is an organism without symbiotic "algal"-partners.

The infrakingdom **Bilateria** is a clade of bilaterally symmetrical animals with anteriors, posteriors, dorsums, flanks, and ventrums like dogs, humas, flukes, and mice.

Bootstrap replicates are equal-sized multiple sequence aligned fictional substitute nucleic acids with confidences proportional to percentage identity.

Canonical, substantiated; acknowledged.

Clade describes a class of organisms comprising all the descendants of a common ancestor.

Codons are triplet bases in mRNA that encode for one kind of amino acid.

Contig is a DNA molecule made up of smaller overlapping sequences.

CRISPR-Ca9: clustered regularly interspaced short palindromic repeats (CRISPR) and associated protein 9 (Cas9) endonuclease facilitates editing of a target genomic locus. The cellular homology-directed repair (HDR) pathway inserts DNA encoding a florescent protein alongside a plasmid-encoded transgene within the target site hence intracellular and cross-anatomy expression can be evaluated microscopically (Strohmeier et al. 2022). See **Nuclease**.

The cells of a **Diploid** organism have a chromosome from each parent (2_n), whereas the copy number of **Haploid** organisms is 1_n.

Diurnal simply means daily or daytime, yet contextually it refers to a **Diel** cycle of typical daylight followed by nighttime analogous darkness.

Endocrine refers to hormone-secreting ductless suprarenal, thymus, thyroid, parathyroid, pituitary, pancreas, ovary, and testis glands.

Endogenous factors originate from within.

Endonuclease, see **Nuclease**.

Exon is a gene integral coding sequence separated by an **Intron** (Dale 1998).

Expression, see **mRNA**.

Gonochoric organisms are female or male.

Hermatypic and **Ahermatypic Corals** - the terms hermatypic and ahermatypic have been misused. Irregularities such as sea fans possessing rigid skeletons are constructive, but they may or may not contain symbionts and do not contribute to reef substratum. Ahermatypes may have large or small polyps and do not contain zooxanthellae. Scleractinia are "true" reef builders but may be azooxanthellate or contain "algal"-symbionts. The terms Scleractinia and hermatype are not interchangeable. Hermatypic corals contain zooxanthellae and are constructive. Ahermatypes are azooxanthellate and non-constructive. This clear definition also applies to non-Scleractinian (Schuhmacher & Zibrowius 1985).

Heterotrophs cannot utilise inorganic carbon for growth so they must dine on organic plant or animal derived nourishment. Their sources include glucose, yet several microscopic taxa retain the capacity to transform inorganic compounds which are frequently used as substrates for chemical reactions.

Heterozygous refers to different forms of a gene usually originating from each parent on opposing chromosomes in a diploid (2_n) organism where each one of an individual's 1_n gametes carry one kind of allele.

Homozygous refers to the same form of a gene usually originating from each parent on opposing chromosomes in a diploid (2_n) organism where each one of an individual's 1_n gametes carry the same kind of allele.

Intron is a gene-integral interrupting (intervening) sequence which is removed by splicing (Dale 1998).

The anabolic pathways of **Mixotrophs** are both autotrophic and heterotrophic.

mRNA stands for messenger ribonucleic acid which is transcribed in the nucleus from an organism's active genes before translocation to the cytoplasm where a ribosome translates its sequence and concatenates amino acids from transfer ribonucleic acids (tRNAs) into a polypeptide that later folds into a functional protein. See: figure 3. of Pioneering Studies.

Nuclease enzymes cleave the phosphodiester bonds between the deoxy- or -ribonucleotides of the sugar phosphate backbone of DNA or RNA but usually only from 5 or 3' (prime) ends (Russell 1998). The cells and nuclei of all "living" organisms retain complex DNA proofreading, damage recognition, and repair system competencies. **Endonuclease** excises a section of ssDNA

from within a double helix (dsDNA) either side of damage or aberrations after which DNA polymerase fills in the gap and ligase seals the sugar-phosphate backbone (Russell 1998a).

Nucleic Acid is typically molecular ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) which maybe complementary (cDNA), double- or single-stranded (dsDNA or ssDNA).

Oligonucleotide is a short, typically synthetic nucleic acid.

Ontogeny/Ontogenesis describes the development of an organism from the moment of fertilisation to a mature adult.

Ontology is used contextually to describe the intra- and extra-cellular metabolic pathways associated with an organisms day-to-day housekeeping, homeostatic, and reproductive processes.

Open Reading Frames (ORFs) are the transcriptional parts of genes that lack start and stop sequences but encode for the triplet bases (codons) of mRNA which correspond to each kind of amino acid.

Orthologue refers to a sequenced and characterised gene integral to an elucidated pathway in numerous organisms: orthologues, orthologous.

Palindrome reads the same forwards or backwards.

Paralogues are two copies derived from a single ancestral gene residing at two distal loci in the same genome.

Phenotype, see **Phenotypic**.

Phenotypic Plasticity foregoes the accepted developmental and evolutionary paradigms and expedites swift morphological responses to environmental change.

Phenotypic traits are an organism's observable characteristics which include how it interacts with its environment, that originate from its epigenotype and genotype.

Photic Zones are sea surface regions extending to approximately 120 metres that receive sufficient light to support photosynthesis.

Phylogenetic Tree "is a graphic representation of the interrelations and evolutionary history of a group of organisms, indicating the relative order of successive divisions of the line of descent, coincident with speciation events" (Wallace et al. 1996a).

Phylogeny facilitates organism classification by comparing the sequences of identical or very similar genes (**Homologues**).

Planulation refers to the release of free-swimming mature larvae from brood colonies.

Plasmid is a self-replicating typically circular and mostly prokaryotic extrachromosomal molecule of ss or dsDNA. See **Nucleic Acid**.

Protospacer-adjacent motifs (PAM) are used as signals for target sites where **Cas** proteins comprise an inordinate number of PAM-interacting domains which mitigate the sequence editing or PAM-sequestering competencies of viral anti-**CRISPR**.

Spawning - countless marine organisms broadcast their sperm and eggs into the water that later fuse and ultimately transform into planktonic larvae. Corals are gonochoric or hermaphroditic where brood colonies internalise sperm released by others which fertilises oocytes (eggs) where blastulae, gastrulae, and later planulae are nurtured until free-swimming when they are released. Other corals liberate sperm and egg bundles that float leisurely to the surface where they degrade and release their cargo (Wallace et al. 1986), or colonies broadcast eggs and sperm directly that fuse and develop into zooplanktonic planulae.

Thyroid-stimulating hormone (TSH) beta is manufactured by the pituitary gland in response to low serum concentrations of thyroxine and thus regulates thyroid gland activity.

Transcription, see **mRNA**.

Transcriptome is the sum of all the messenger ribonucleic acid (**mRNA**) from an organism's active genes (≅**ORFs**; Lexico 2019).

Zooxanthellate is an organism with symbiotic dinoflagellate "algal"-partners.

Zygotes are formed when the nuclei of a sperm and egg fuse which occurs after a sperm penetrates an ovum (oocyte) whereafter additional fertilisation cannot occur.

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As the principal director of Reef Ranch Publishing Ltd (https://reefranch.co.uk/) and author of The Complete Reef Aquarist – A Conservation Manual (https://reefranch.co.uk/reef-aquarium-guide/), Chris has over 55 years of experience keeping aquatic animals with 47 of them nurturing marine species. His innate passion for system dynamics drove him from the laboratory to university where he gained a greater appreciation of biochemistry, biotechnology, epidemiology, genetics, histology, inorganic and organic chemistry, mariculture, molecular and microbiology, saltwater zoology, and the diagnosis and treatment of aquatic diseases. His dedicated marine livestock supplier, The Reef Ranch™, demanded he devise, streamline, and establish protocols for combined acclimation and prophylactic pest/parasite clearance, and innovate system design, optimisation, maintenance, and husbandry in the face of incessant influxes of hundreds of delicate marine animals. With exceptional, uncompromising, and likely the UK's most disease-free reef and fish-only facilities, losses were less than one resident every six months. 20 years hence, he has refined his expertise for digesting, authoring, editing, and publishing reef conservation-driven scientific literature (https://reefranch.co.uk/reef-research-and-aquarium-advice/), to an end of diminishing the impact the tropical marine ornamental industry exerts in the wild.

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