SYMBIODINIACEAE DINOFLAGELLATES EXPRESS UREASE IN THREE SUBCELLULAR COMPARTMENTS AND UPREGULATE ITS EXPRESSION LEVELS IN SITU IN THREE ORGANS OF A GIANT CLAM (*TRIDACNA SQUAMOSA*) DURING ILLUMINATION

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Abstract

Giant clams harbor three genera of symbiotic dinoflagellates (*Symbiodinium, Cladocopium, Durusdinium*) as extracellular symbionts (zooxanthellae). While symbiotic dinoflagellates can synthesize amino acids to benefit the host, they are nitrogen-deficient. Hence, the host must supply them with nitrogen including urea, which can be degraded to ammonia and carbon dioxide by urease (URE). Here, we report three complete coding cDNA sequences of *URE*, one for each genus of dinoflagellate, obtained from the colorful outer mantle of the giant clam, *Tridacna squamosa.* The outer mantle had higher transcript level of *Tridacna squamosa zooxanthellae URE* (*TSZURE*) than the whitish inner mantle, foot muscle, hepatopancreas and ctenidium. TSZURE was immunolocalized strongly and atypically in the plastid, moderately in the cytoplasm, and weakly in the cell wall and plasma membrane of symbiotic dinoflagellates. In the outer mantle, illumination upregulated the protein abundance of TSZURE, which could enhance urea degradation in photosynthesizing dinoflagellates. The urea-nitrogen released could then augment syntheses of amino acids to be shared with the host for its general needs. Illumination also enhanced gene and protein expression levels of *TSZURE*/TSZURE in the inner mantle and foot muscle, which contain only small quantities of symbiotic dinoflagellate, have no iridocyte, and lack direct exposure to light. With low phototrophic potential, dinoflagellates in the inner mantle and foot muscle might need to absorb carbohydrates in order to assimilate the urea-nitrogen into amino acids. Amino acids donated by dinoflagellates to the inner mantle and the foot muscle could be used especially for syntheses of organic matrix needed for light-enhanced shell formation and muscle protein, respectively.

Key index words: Amino acid, ammonia, calcification, nitrogen, *Symbiodinium*, urea

List of abbreviations

DAPI 4′,6-diamidino-2-phenylindole

DIC differential interference contrast

n nucleus

PAGE polyacrylamide gel electrophoresis

PPFD photosynthetic photon flux density

RACE rapid amplification of cDNA ends

TPBS 0.05% Tween-20 in phosphate-buffered saline

***TSCURE*/TSCURE** *Tridacna squamosa Cladocopium* Urease

***TSDURE*/TSDURE** *Tridacna squamosa Durusdinium* Urease

***TSSURE*/TSSURE** *Tridacna squamosa Symbiodinium* Urease

***TSZrbcII*/TSZRBCII** *Tridacna squamosa* zooxanthellal form II ribulose-1,5-bisphosphate carboxylase/oxygenase II

***TSZURE*/TSZURE** *Tridacna squamosa* zooxanthellae Urease

URE urease

ZX zooxanthellae

Introduction

Giant clams are bivalves found in the nutrient-deficient tropical waters around reef ecosystems of the Indo-Pacific. They live in symbiosis with three genera of phototrophic dinoflagellates (Hernawan 2008; Weber 2009; DeBoer et al. 2012; Ikeda et al. 2017; Lim et al. 2019), namely *Symbiodinium*, *Cladocopium* and *Durusdinium* (LaJeunesse et al. 2018), belonging to Family Symbiodiniaceae. The symbiotic stage (zooxanthella) of these dinoflagellates can be found extracellularly inside a branched host tubular system (Norton et al. 1992). The lateral mantle of giant clams comprises two connected portions: outer mantle and inner mantle (Ip et al. 2017). The outer mantle is fleshy, colorful and extensible, and is peculiar to giant clams. It contains large quantities of tertiary zooxanthellal tubules and high densities of symbionts (Norton and Jones 1992). It also contains host pigments as well as iridophores consisting of iridocytes, which deflect light of relevant wavelengths to the symbionts to promote photosynthesis (Holt et al. 2014). By contrast, the inner mantle is thin and spreads across the inner surface of the shell valves, with its shell-facing epithelium being in direct contact with the extrapallial fluid where light-enhanced shell formation (calcification) occurs. For the fluted giant clam, *Tridacna squamosa*, the physical presence of symbiotic dinoflagellates in five organs (colorful outer mantle, inner mantle, ctenidium, hepatopancreas and foot muscle), with high densities of dinoflagellates located in specific regions of the inner mantle and foot muscle, have been confirmed by microscopy (Poo et al. 2020). While the outer mantle of *T. squamosa* (*TS*) has the greatest phototrophic potential as reflected by its high form II RuBisCO (*TSZrbcII*) transcript level derived from dinoflagellates, the other four organs also expressed moderate levels of *TSZrbcII*, despite the lack of iridophores and direct exposure to light (Poo et al. 2020).

To fulfill the host's metabolic needs, dinoflagellates in the outer mantle conduct photosynthesis and donate photosynthates (~95%) to the host during illumination (Fitt et al. 1986). Because of that, individuals of *T. squamosa* deprived of access to planktonic/particulate matter can survive and grow in Millipore- filtered seawater for more than 10 months with light as the sole energy source (Fitt and Trench 1981). Without feeding, the host clam needs to receive adequate supplies of energy and nutrients from its phototrophic symbionts (Klumpp and Griffiths 1994). Specifically, symbionts must provide the host with a large supply of amino acids to synthesize proteins, especially for muscles, and other nitrogenous compounds needed for growth and development. In turn, the host supplies symbionts with carbon, nitrogen and phosphorous as they have no access to the ambient seawater (Rees 1991, Furla et al. 2005). Nitrogen is the basic component of amino acids, proteins and nucleic acids, and hence crucial to living systems (Campbell 1991). Most aquatic animals excrete ammonia (Ip and Chew 2010, Chew and Ip 2014), but giant clams absorb and assimilate exogenous ammonia particularly during illumination (Wilkerson and Trench 1986, Miller and Yellowlees 1989) to support the nitrogen-deficient symbionts (Wikerson and Trench 1986). The addition of inorganic nitrogen to seawater increases the photosynthetic rate in symbionts (Summons et al. 1986) and the growth rate in the host clam (Hastie and Heslinga 1988, Hastie et al. 1992).

Seawater contains organic and inorganic nitrogen. Organic nitrogen includes urea [$CO(NH_2)_2$] and dissolved amino acids, while inorganic nitrogen consists of nitrate, nitrite and ammonium. Urea concentrations can range between 1 and 25 μmol N 1^{-1} in coastal waters, but it can be undetectable in oceanic waters (Painter et al. 2008). In reef waters, urea concentrations range from <0.2 μmol N l^{-1} to 2.0 μmol N l^{-1} (Wafar et al. 1986). Some animals excrete urea, and the majority of animals do not metabolize urea due to a lack of urease (URE), an enzyme that

catalyzes urea degradation (Kappaun et al. 2018). For organisms that possess URE, urea is a good source of nitrogen as each urea molecule contains two nitrogen atoms. URE is a metalloenzyme found pervasively in plants, algae, fungi and bacteria (Krajewska 2009, Kappaun et al. 2018). Through a one-step reaction, $(NH_2)_2CO + H_2O \rightarrow CO_2 + 2NH_3$, it catalyzes the breakdown of urea to NH_3 and CO_2 (Saumya et al. 2016). Basically, URE is a trimer or hexamer, and the number of polypeptide chains that constitutes the monomer can vary depending on its origin (Krajewska 2009, Kappaun et al. 2018). In plants and fungi, a single polypeptide chain, the α subunit, makes up this monomer (Riddles et al. 1991). In bacteria, three subunits (α, β and γ) constitute the functional monomer (Jabri et al. 1995), with the exception of *Helicobacter* (with α and β only; Ha et al. 2001). Two Ni^{2+} are present in the active site of URE, and they participate directly in substrate binding (Todd and Hausinger 1989).

While animals generally excrete urea as part of nitrogenous wastes, *T. squamosa* can absorb exogenous urea, with an uptake rate higher in light than in darkness (Chan et al. 2018). Its ctenidium expresses light-dependent urea active transporter (DUR3-like; Chan et al. 2018), and Na^+:glucose cotransporter 1-like (SGLT1-like; Chan et al. 2019) that can transport both glucose and urea. The absorbed urea is supplied to the nitrogen-deficient symbionts, which must be able to absorb and metabolize urea. However, no information is available on URE derived from Symbiodiniaceae dinoflagellates. Therefore, this study aimed to obtain the complete coding cDNA sequences of *URE* derived from the symbiotic dinoflagellates residing in the colorful outer mantle of *T. squamosa* obtained from Vietnam. As giant clams are known to harbor multiple species of *Symbiodinium*, *Cladocopium* and *Durusdinium* (LaJeunesse et al. 2018), numerous *URE* sequences were obtained. Nonetheless, only one major *URE* cDNA sequence for each of the three genera was presented in this report. They were named as *Tridacna squamosa Symbiodinium URE*

(*TSSURE*), *Tridacna squamosa Cladocopium URE* (*TSCURE*) and *Tridacna squamosa Durusdinium URE* (*TSDURE*). Phenogramic analyses were performed on the deduced amino acid sequences of TSSURE, TSCURE and TSDURE to verify their dinoflagellate origin. Based on these three URE amino acid sequences and others available in various symbiotic dinoflagellate databases, an antibody that would react comprehensively with *Tridacna squamosa* zooxanthellae URE (TSZURE) was custom-made commercially. Immunofluorescence microscopy was conducted to determine the subcellular localization of TSZURE in zooxanthella in situ. In addition, a set of genera-comprehensive quantitative real-time PCR (qPCR) primers was designed to determine the transcript levels of *TSZURE* in five organs (outer mantle, inner mantle, foot muscle, ctenidium and hepatopancreas) of *T. squamosa*. Finally, the effects of 3, 6 or 12 h of light exposure on the transcript level and protein abundance of *TSZURE*/TSZURE were examined in these five organs to test the hypothesis that they could be up-regulated by illumination to facilitate the light-enhanced release of urea-nitrogen for increased amino acid syntheses in some of these organs.

Materials and Methods

Maintenance, experimental conditions and tissue collection

Institutional (National University of Singapore Institutional Animal Care and Use Committee) approval was not required for research on invertebrates including giant clams. Nonetheless, giant clams were anesthetized with 0.2% phenoxyethanol before killing to minimize stress. Adult individuals (520 ± 180 g with shells; *N*=28) of *T. squamosa* were purchased from Xanh Tuoi Tropical Fish, Vietnam. They were maintained in three glass tanks (length 90 cm x width 62 cm x height 60 cm) under a 12 h light:12 h dark regime for a duration of one month before exposure to various experimental conditions. Each tank contained 350 L of recirculating seawater with the following conditions: temperature, ~26°C; pH, 8.1-8.3; salinity, 30-32; hardness, 143–179 ppm; calcium, 380–420 ppm; total ammonia, <0.25 ppm; nitrate, 0 ppm; nitrite, 0 ppm, phosphate, <0.25 ppm. The shaded light intensity measured at the level of the giant clams was 120 PPFD (μmol m^{-2} s^{-1}), which is equivalent to the intensity adopted by Jantzen et al. (2008) for *T. squamosa*. Ideally, experimental giant clams should be exposed individually to the experimental conditions, but it was not feasible to do so because of the limitation of re-circulating tanks in our aquarium and the need to keep them in exactly the same environmental conditions (including salinity, water quality, and light intensity). Hence, individuals were sampled directly and randomly from the three glass tanks mentioned above at different time points. Ten individuals were killed after 12 h of darkness for the sampling of outer mantle, inner mantle, foot muscle, ctenidium and hepatopancreas for the determination of transcript levels of *TSZURE* with reference to *Tridacna squamosa zooxanthellal form II ribulose-1,5-bisphosphate carboxylase/oxygenase* (*TSZrbcII*). Another sixteen individuals were used to study the effects of illumination on *TSZURE*/TSZURE expression. Four individuals

were sacrificed at the end of 12 h of darkness for tissue sampling (control; *N*=4). The remaining twelve individuals were exposed to 3, 6 or 12 h of light (*N*=4 each). Organ samples were freeze-clamped and kept at -80°C until further analysis. For immunofluorescence microscopy, outer mantle samples were collected from another two individuals after exposure to light for 12 h (*N*=2).

Total RNA isolation and cDNA synthesis

Total RNA extraction from the outer mantle, inner mantle, foot muscle, hepatopancreas and ctenidium of *T. squamosa* and subsequent cDNA syntheses were performed according to the methods of Hiong et al. (2017).

Polymerase chain reaction (PCR), cloning and rapid amplification of cDNA ends (RACE)-PCR

Six *URE* sequences from *Symbiodinium*, *Cladocopium* and *Durusdinium* were obtained from various online symbiotic dinoflagellate databases: three from *Symbiodinium* databases (Bayer et al. 2012, Aranda et al. 2016), one from *Cladocopium* database (Shoguchi et al. 2018) and two from *Durusdinium* databases (Bayer et al. 2012, Ladner et al. 2012). These six sequences were aligned to design a set of genus-comprehensive PCR primers (Forward: 5'-GATCACACTACCACTTCATC-3' and Reverse: 5'-ACCTTAATGATGTCCGGC-3') at the homologous regions. This set of genus-comprehensive primers was used to obtain partial sequences of *URE* from the outer mantle of *T. squamosa* by PCR. The partial fragments were then cloned in pGEM-T Easy vector (Promega, Madison, WI, USA), following the methods of Hiong et al. (2017). Sixty clones were sequenced to obtain partial fragments of *TSSURE, TSCURE* and *TSDURE*, whereby the identities of the partial fragments were determined with reference to sequences available in the online symbiotic dinoflagellate databases. Then, three different sets of

genus-specific RACE primers (Forward: 5'-GCTTGCATTCAGCGGCAGTCTCC-3' and Reverse: 5'-GCTTTGGCTACCAGGCTGTGGAGA-3' for *TSSURE;* Forward: 5'-TTGCCTTCAGCGGCTTCAGAGACT-3' and Reverse: 5'-TGGTGGATGGTGTAGCAAGTCCT-3' for *TSCURE*; Forward: 5'-ATGACCAAAGCCGCCGGACTCCAC-3' and Reverse: 5'-CAGTGTCCGAAGCTCCTGCTGAG-3' for *TSDURE*) were designed to obtain the full coding sequences by RACE-PCR using SMARTer RACE cDNA amplification kit (Clontech Laboratories, Mountain View, CA, USA).

Deduced amino acid sequence and phenogramic analysis

ExPASy Proteomic server (http://web.expasy.org/translate/) was used to translate the nucleotide sequences of *TSSURE, TSCURE* and *TSDURE* into protein sequences. Subsequently, Blast protein-protein tool (BlastP; http://www.ncbi.nlm.nih.gov/BLAST), based on the National Center for Biotechnology Information (NCBI) protein database, was used to verify the deduced amino acid sequences of TSSURE, TSCURE and TSDURE.

To confirm the identity of TSSURE, TSCURE and TSDURE, a phenogramic tree was generated with URE sequences of other organisms obtained from Genbank (Supplementary Table S1). Maximum-likelihood analysis was conducted using RaxML v8.2.5 (Stamatakis 2014) with 2000 bootstraps. Through ModelGenerator v0.85 (Keane et al. 2006), the best-fitting evolutionary model under Akaike Information Criterion was determined to be LG+G (Whelan and Goldman 2001).

Antibody

A genus-comprehensive anti-TSZURE antibody was custom-made by GenScript (Piscataway, NJ, USA), based on the epitope sequence KGTRSVQKKDMILN, which corresponded to residues 820-833 of TSSURE, TSCURE and TSDURE. This epitope has high similarity with the same regions of other dinoflagellate URE sequences obtained from the online dinoflagellate databases (Supplementary Table S2).

Immunofluorescence microscopy

Samples of the outer mantle were processed for microscopy following the method of Hiong et al. (2017). The paraffin-embedded samples were sectioned (3 μm) and collected on slides. Antigen retrieval was performed by treating deparaffinized sections with 1% sodium dodecyl sulfate solution. Slides were then washed for three times with TPBS (0.05% Tween-20 in phosphate-buffered saline: 10 mmol l^{-1} Na_2HPO_4, 1.8 mmol l^{-1} KH_2PO_4, 137 mmol l^{-1} NaCl, 1.8 mmol l^{-1} KCl, pH 7.4). Sections were blocked using 1% BSA in TPBS for 30 min and then labelled with the custom-made anti-TSZURE rabbit polyclonal antibody at a dilution of 1:300 (3.33 μg ml^{-1} in blocking buffer) at 37°C for 1 h, followed with the goat anti-rabbit Alexa Fluor 488 at 1:800 dilution (2.5 μg ml^{-1}; Life Technologies Corporation) at 37°C for 1 h. After rinsing for three times with TPBS, the sections were counterstained with DAPI nuclear stain for 10 min and mounted in ProLong Gold Antifade Mountant (Life Technologies Corporation). Images were captured using an Olympus BX60 epifluorescence microscope equipped with an Olympus DP73 digital camera (Olympus Corporation, Tokyo, Japan). Symbiotic dinoflagellates were examined for red autofluorescence of their plastids using the Olympus U-MWIG Interference Green Fluorescence Filter (excitation wavelengths: 520-550 nm; emission wavelengths: 580-800 nm), and for green fluorescence of Alexa Fluor 488 using the Olympus U-WNIBA Blue Fluorescence Filter

(excitation wavelengths: 470-490 nm; emission wavelengths: 515-550 nm). The corresponding differential interference contrast (DIC) image was captured for tissue orientation. Overlaying of different channels and brightness adjustments were made using Adobe Photoshop CC.

Quantitative real-time PCR (qPCR)

qPCR was performed with a StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Carlsbad, CA, USA) for the absolute quantification of *TSZURE* and *TSZrbcII* transcripts, following the methods of Hiong et al. (2017). A set of genus-comprehensive *TSZURE* qPCR primers (Forward: 5'-TGAACTTCGGCTTCTCTGG-3'; Reverse: 5'-CCGTGTGGATGGTGATGG-3') was designed based on a conserved region of *TSSURE*, *TSCURE*, *TSDURE* and other dinoflagellate *URE* sequences from various databases (Supplementary Table S3). It had an amplification efficiency of 92%. The genus-comprehensive primer designed by Poo et al. (2020) for *TSZrbcII* was adopted in this study to quantify *TSZrbcII* transcripts. Two plasmid standard curves were constructed to calculate the absolute copy number of transcripts for *TSZURE* or *TSZrbcII*.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting

Protein extraction from the outer mantle, inner mantle and foot muscle, and SDS-PAGE were conducted according to the methods of Hiong et al. (2017), with some modifications. Protein samples were heated at 90°C for 10 min before they were separated by 8% SDS-PAGE (20 μg for the outer mantle, or 100 μg for the inner mantle and foot muscle). Separated proteins on gels were transferred electrophoretically onto nitrocellulose membranes. Western blotting was conducted using Pierce Fast Western Blot kit, SuperSignal® West Pico Substrate (Thermo Fisher Scientific).

The membranes were incubated with genus-comprehensive anti-TSZURE antibodies (2 µg ml^{-1}) with a dilution factor of 1:500 for 1 h at 25°C. Due to variable expression among different tissues, cell lines or experimental conditions, common reference proteins such as tubulin and actin do not pose as good reference proteins for studying relative expression in different organs (Thorrez et al. 2008). As the assumption of a constant expression of the reference protein in different organs could not be justified, the protein abundance of TSZURE in each of the three organs was not normalised with a reference protein but expressed as arbitrary densitometric units (a.u.) per 20 µg (for the outer mantle) or 100 (for the inner mantle or foot muscle) µg of protein.

Statistical analysis

The IBM SPSS Statistics (IBM Corp., Armonk, NY, USA) was used to analyze the data collected in this study. The values presented as ratios were compared using the non-parametric Friedman test of differences followed by a Wilcoxon Signed-Rank Test. Differences between ratios were regarded as statistically significant when the *P* value was less than 0.01 after Bonferroni adjustment. The values were presented as means + SEM and compared by One Way Analysis of Variance while the Levene's test was used to assess the equality of variance. When a significant difference was detected for the means with equal variance, this was followed by the Tukey's post-hoc test. On the other hand, when a significant difference was detected for the means with unequal variance, this was followed by the Dunnett's T3 post-hoc test. The means were considered as statistically different when the *P* value was less than 0.05.

Results

Nucleotide and amino acid sequences

The complete coding cDNA sequences of *TSSURE, TSCURE* and *TSDURE*, had been deposited into GenBank with accession numbers of MT017657, MT017658 and MT017659, respectively. *TSSURE, TSCURE* and *TSDURE* comprised 2613 bp, 2619 bp and 2622 bp, encoding a corresponding protein of 870 (92.9 kDa), 873 (93.3 kDa) and 874 (93.0 kDa) amino acids, respectively (Fig. 1). *TSSURE* had 100% sequence similarity with *URE* of *Symbiodinium tridacnidorum* Sh18 (ITS2 type A3; Shoguchi et al. 2018), while *TSCURE* shared the greatest resemblance (99.9%) with *URE* of *Cladocopium goreaui* (ITS2 type C1) isolated from the coral *Acropora tenuis* (Levin et al. 2016) (Table 1). Based on sequence similarity, *TSDURE* was equivalent (99.9%) to *URE* of *Durusdinium* ITS2 type D2 isolated from *Acropora hyacinthus* (Ladner et al. 2012) (Table 1).

Phenogramic analysis of the deduced amino acid sequences of TSSURE, TSCURE and TSDURE confirmed both their identity and dinoflagellate-origin (Fig. 2). A multiple alignment of TSSURE, TSCURE and TSDURE with URE sequences of *Gossypium arboretum* and *Canavalia ensiformis* identified conserved residues of Ni^{2+} binding, urea binding, proton donors and the 'mobile flap' that controls substrate entry and binding.

Immunolocalization of TSZURE in symbiotic dinoflagellates

TSZURE was strongly immunolocalized in the plastid of dinoflagellates in the outer mantle of *T. squamosa* (Fig. 3), which had not been reported before. Moderate and weak immunofluorescence were detected in the dinoflagellates' cytoplasm and cell wall cum membrane, respectively (Fig. 3).

Gene expression of TSZURE in five organs

The transcript level of *TSZURE* in the outer mantle was significantly higher than those in the other four organs (One-Way ANOVA, $F_{4,45}$=83.792, P=0.000; Fig. 4). The transcript levels of *TSZURE* in the inner mantle was comparable to those in the foot muscle and the ctenidium but significantly higher than that in the ctenidium. The ratio of *TSZURE* to *TSZrbcII* from the outer mantle was significantly higher than those from the inner mantle, foot muscle, hepatopancreas and ctenidium (Wilcoxon Signed-Rank Test, Z=-2.803, P=0.002; Table 2).

Effects of light exposure on the transcript levels of TSZURE in three organs

The transcript level of *TSZURE* in the outer mantle was high, but it remained unchanged during 12 h of exposure to light as compared to that of the control kept in darkness for 12 h (One-Way ANOVA, $F_{3,12}$=4.696 P=0.022; Fig. 5a). Although the transcript levels of *TSZURE* in the inner mantle and the foot muscle were apparently lower than that in the outer mantle, they increased significantly after 3 h of light exposure as compared to the control (inner mantle: One-Way ANOVA, $F_{3,12}$=29.819, P=0.000; Fig. 5b, foot muscle: One-Way ANOVA, $F_{3,12}$=14.564, P=0.000; Fig 5c). The transcript levels of *TSZURE* in the hepatopancreas (One-Way ANOVA, $F_{3,12}$=1.283, P=0.325; Fig. 5d) and the ctenidium (One-Way ANOVA, $F_{3,12}$=1.496, P=0.266; Fig. 5e) were low and they remained unchanged during 12 h of light exposure.

Effects of light exposure on the protein abundance of TSZURE *in three organs*

As the protein abundance of TSZURE in the outer mantle was apparently greater than those in the inner mantle and the foot muscle, different protein loads were used for Western blotting. At 100

μg of protein load, no detectable bands corresponding to the expected molecular mass of TSZURE were observed for hepatopancreas and ctenidium.

There was a significant increase (~2-fold) in the protein abundance of TSZURE in the outer mantle of *T. squamosa* exposed to light for 12 h as compared to that of the control kept in darkness for 12 h (One-Way ANOVA, $F_{3,12}$=11.164, P=0.001; Fig. 6). As for the inner mantle (One-Way ANOVA, $F_{3,12}$=5.903, P=0.010; Fig. 7) and the foot muscle (One-Way ANOVA, $F_{3,12}$=14.066, P=0.000; Fig. 8), the protein abundance of TSZURE increased significantly (by 2 to 2.5-fold) after 6 or 12 h of exposure to light as compared to that of the control.

Discussion

Free-living dinoflagellates can absorb urea, and the uptake rate of urea is faster than that of nitrate (Fan et al. 2003, Collos et al. 2007). Furthermore, urea is a better source of nitrogen than ammonia (NH_3/NH_4^+) for dinoflagellates, because high intracellular concentrations of ammonia can be cytotoxic (Leong and Taguchi 2004, Dagenais-Bellefeuille and Morse 2013). Despite urea having a lower environmental concentration than ammonia, the free-living phototrophic dinoflagellate, *Lingulodinium polyedrum*, absorbs urea at a rate 2-fold greater than that of ammonia (Kudela and Cochlan 2000). To support the nutritional need of its symbiotic dinoflagellates, *T. squamosa* conducts light-enhanced urea absorption by means of light-dependent DUR3-like (Chan et al. 2018) and SGLT1-like in its ctenidium (Chan et al. 2019). Here, we report three sequences of dinoflagellate URE obtained from the outer mantle of *T. squamosa*. *TSSURE* was derived from *Symbiodinium tridacnidorum* Sh18 (ITS2 type A3; Shoguchi et al. 2018), while *TSCURE* and *TSDURE* could originate from *Cladocopium goreaui* (ITS2 type C1) and *Durusdinium* ITS2 type D2, respectively (Table 1). In addition, we report for the first time that the expression levels of TSZURE in the outer mantle, inner mantle and foot muscle can be enhanced by light.

Molecular characterization of TSSURE, TSCURE and TSDURE

TSSURE, TSCURE and TSDURE have conserved active sites of URE, like those of *G. arboreum* and *C. ensiformis*. The active site of URE contains two Ni^{2+}. In TSSURE, TSCURE and TSDURE, Ni^{2+}(1) is coordinated by H552 and H578 (corresponding to H548 and H574 in TSSURE, H519 and H545 in *C. ensiformis*, as well as H516 and H542 in *G. arboreum*), while the Ni^{2+}(2) is coordinated by H440, H442 and D666 (corresponding to H436, H438, and D662 in TSSURE, H407, H409 and D633 in *C. ensiformis*, as well as H404, H406 and D630 in *G. arboreum*,). A

conserved K523 (corresponding to K519 in TSSURE, K490 in *C. ensiformis*, and K480 in *G. arboreum*) bridges the two Ni^{2+}, which are responsible for urea binding and render urea more electrophilic as well as susceptible to nucleophilic attack. Thiols can bind to Ni^{2+} at the active site and competitively inhibit urease activity (Mobley and Hausinger 1989). Furthermore, residue C625 (C621 in TSSURE, C592 in *C. ensiformis*, and C589 in *G. arboreum*) forms part of the 'mobile flap' of URE, which could be vital in positioning key residues in the active site into a conformation most conducive for catalysis (Maroney and Ciurli 2014).

Expression of TSZURE *is stronger in the outer mantle than the other four organs*

The outer mantle had a high transcript level of *TSZURE* by virtue of its high density of symbiotic dinoflagellates and high level of light exposure. Furthermore, it had a significantly higher *TSZURE*/*TSZrbcII* ratio than the other four organs, indicating that dinoflagellates therein might have a greater potential to assimilate urea-nitrogen with reference to their photosynthetic potential, alluding to a possible functional relationship between urea degradation and photosynthetic carbon-fixation. Nevertheless, the other four organs without direct exposure to light also expressed *TSZURE*, albeit at relatively low potentials with reference to *TSZrbcII*, indicating that urea degradation could still be essential to some physiological functions in these dinoflagellates.

TSZURE is localized atypically in the plastid, besides the cytoplasm and cell wall/membrane

In plants, URE is regarded generally as a cytoplasmic enzyme, although it is also located in the cell walls and membranes of certain plant species (Aguetoni et al. 2009). Some lichen species can secrete URE to the external medium containing urea (Legaz et al. 1986, Pérez-Urria et al. 1989), and URE can be found in their cell walls (Legaz and Vicente 1989, Molina et al. 1993, Millanes

et al. 2004). The ability to secrete URE has also been demonstrated in the vascular tank bromeliad, *Vriesea gigantean* (Inselsbacher et al. 2007), of which URE activity can be detected in the cytoplasm as well as the cell wall and membrane (Aguetoni et al. 2009). This allows *V. gigantean* to utilize exogenous urea rapidly and efficiently so that it can compete successfully with other microorganisms for the limited environmental nitrogen. As follows, the immunolocalization of TSZURE in the cytoplasm and cell wall/membrane of symbiotic dinoflagellates in the outer mantle of *T. squamosa* is not unexpected. At present, it is uncertain whether symbiotic dinoflagellates could secrete URE, but the hydrolysis of some urea along the plasma membrane might facilitate the release of ammonia close to the membrane so that it can be absorbed through ammonia transporters/channels. Cytoplasmic URE can degrade some of the absorbed urea, and the ammonia released can be used to synthesize glutamine (Sirko and Brodzik 2000), which plays a central role in the syntheses of nucleic acids (Forde and Lea 2007). Glutamine can be synthesized from NH_4^+ and glutamate with the hydrolysis of ATP catalyzed by a cytosolic glutamine synthetase (GS), while glutamate can be replenished by a reaction that involves glutamine and 2-oxoglutarate catalyzed by glutamine 2-oxoglutarate aminotransferase (GOGAT; Forde and Lea 2007). Both GS and GOGAT are known to be present in the cytosol and plastid, respectively, of symbiotic dinoflagellates in *T. squamosa* (Fam et al. 2018).

Although CO_2 released from urea degradation can evidently be assimilated by photosynthesis (Wilson and Walker 1988), URE is not known to be located directly in the chloroplast or the plastid. Hence, the immunolocalization of TSZURE in plastids of symbiotic dinoflagellates in *T. squamosa* is novel. There are two possible reasons and both could be related to the symbiotic nature of these dinoflagellates. Firstly, it could be an adaptation to effectively harness the urea-carbon for photosynthesis, as symbiotic dinoflagellates no longer have access to

the carbon in the ambient seawater. Despite the host's efforts to increase inorganic carbon uptake (Chew et al. 2018, Koh et al. 2018) and supply it to the symbionts through a host-mediated carbon concentration mechanism (Ip et al. 2017, 2018, Armstrong et al. 2018), it might be essential for these dinoflagellates to capture and fix the urea-carbon directly inside the plastid where photosynthesis occurs. Secondly, it could be an adaptation of symbiotic dinoflagellates to effectively capture urea-nitrogen for amino acid syntheses. In plants, amino acid synthesis occurs predominantly in the chloroplasts, where ATP and reducing power can be provided conveniently by photosynthesis (Hildebrandt et al. 2015). Hence, the degradation of urea by URE, together with the availability of fixed-carbon, inside the plastid could facilitate the synthesis of amino acids in symbiotic dinoflagellates, which, unlike free-living dinoflagellates, would need to satisfy the strong demand of amino acids by the host clam, especially for the production of muscle proteins.

While there are plastid-encoded genes that are transcribed and translated by the plastid machinery (Tiller and Bock 2014), most plastid proteins (~90%) are encoded by the nuclear genome and translated in the cytosol before import into the plastid (Keegstra and Cline 1999), and protein import into the plastid is regarded as a post-translational process (Jarvis and Robinson 2004). As URE is basically a cytosolic enzyme, the related transcription and translation processes probably occur in the cytosol of symbiotic dinoflagellates, notwithstanding the mechanism of plastid entry being unknown at present.

Light-enhanced expression of URE is uncommon in algae/plants

Light serves as an important signal to regulate development, reproduction and survival in plants and algae, and many of their genes and proteins display light-enhanced expression levels (Felitti et al. 2000). However, *URE*/URE expression in plants and algae are not known to be light-

dependent; rather, it is regulated by multiple factors, such as urea availability and nitrogen limitation. (Tolonen et al. 2006, Jing et al. 2017). When the planktonic dinoflagellate, *Prorocentrum donghaiense*, is confronted with nitrate- or urea-limitation, the *URE* transcript level increases by the same extent (Jing et al. 2017). Similarly, the transcript level of *URE* is upregulated in the marine cyanobacteria, *Prochlorococcus*, during nitrogen deprivation (Tolonen et al. 2006). Therefore, the light-induced upregulation of expression levels of *TSZURE*/TSZURE in symbiotic dinoflagellates of *T. squamosa* is unusual, and suggests some important functions in relation to the light-enhanced degradation of urea.

Light-enhanced TSZURE expression in the outer mantle could be linked to increased urea degradation for amino acid syntheses in support of the host's general need

The colourful outer mantle can be fully extended when giant clams are illuminated. It contains large quantities of tertiary zooxanthellal tubules with high densities of symbiotic dinoflagellates. It also consists of plenty of host pigments and iridocytes to facilitate photosynthesis in these symbionts. For *T. squamosa*, the outer mantle has the highest phototrophic potential, based on *TSZrbcII*, among five organs studied in (Poo et al. 2020). Notably, it also expressed the highest transcript level of *TSZURE* (~x10^3 copies per ng RNA) among these five organs. Probably because of that, the protein abundance of TSZURE in the outer mantle could be up-regulated by illumination with the transcript level remained unchanged, denoting that the regulation is at the translational level. Translational regulation would circumvent energetic requirements of transcriptional regulation related to synthesis, processing and exporting of mRNA, and allow for a faster response to external changes (Lackner and Bähler, 2008). Together with the high *TSZURE*/*TSZrbcII* ratio, it can be deduced that URE may have an important physiology role in

this organ. The increase in protein abundance of TSZURE during light exposure could probably augment the potential to release NH_3 from urea for increased amino acid synthesis in synchrony with light-induced CO_2 fixation (photosynthesis). Importantly, the expression level of TSZRBCII, which catalyzes CO_2 fixation, is also upregulated in the outer mantle during light exposure (Poo et al. 2020). Hence, it is possible that a portion of the carbon fixed by photosynthesizing symbiotic dinoflagellates in the outer mantle could be channeled to assimilate NH_3/NH_4^+ into amino acids catalyzed by glutamate dehydrogenase and various aminotransferases (Campbell 1991). Then, some of these amino acids are probably released into the tubular fluid, translocated to the hemolymph and circulated to other parts of the host's body to support general growth and development.

*Light-enhanced TSZURE/*TSZURE *expression in the inner mantle could be linked to amino acids needed for light-enhanced shell formation*

The whitish inner mantle of giant clam is in direct contact with the extrapallial fluid and participates in light-enhanced shell-formation. It lacks iridocytes and direct light exposure, yet it consists of a population of symbiotic dinoflagellates concentrated in a specific area near the valve hinge (Poo et al. 2020). In non-photosynthetic organs (tubers, roots and seeds) of plants, plastids can differentiate into colorless leucoplasts without photosynthetic pigments (Heldt and Piechulla 2011). The functions of leucoplast include the storage of starch, lipids or proteins, and syntheses of fatty acids, amino acids and hemes (Zhu et al. 2018, Sadali et al. 2019). In roots, the reduction of nitrate to ammonium in support of amino acid synthesis occurs in the leucoplasts (Heldt and Piechulla 2011). Hence, it is logical to deduce that, in giant clams, plastids of symbiotic

dinoflagellates with low light exposure and low phototrophic potential could take part in the biosyntheses of amino acids to benefit the host (Poo et al. 2020).

Shell formation requires the precipitation of $CaCO_3$ from the extrapallial fluid onto an organic matrix lining the inner surface of the shell-valve according to the equation: $Ca^{2+} + HCO_3^- \Leftrightarrow CaCO_3 + H^+$ (Wilbur and Watabe 1963, Wheeler and Sikes 1984). Despite having a low phototrophic potential (Poo et al. 2020), the inner mantle of *T. squamosa* displayed light-enhanced expression of *TSZURE*/TSZURE. Upregulation at both the transcriptional and translational levels during light exposure was required probably because of the relatively low level of *TSZURE* transcript (~$x10^2$ copies per ng RNA) in this organ. Hence, light-enhanced expression of *TSZURE*/TSZURE could be linked to light-enhanced shell formation (calcification) and there are two possible reasons. Firstly, the enhanced expression of TSZURE could lead to increased release of NH_3 from urea, and NH_3 could be translocated to the host and secreted through the shell-facing epithelial cells of the inner mantle into the extrapallial fluid. As suggested by Ip et al. (2006), NH_3 can combine with the excess H^+ produced through calcification to form NH_4^+ in the extrapallial fluid to promote light-enhanced shell formation; then, NH_4^+ is transported back to the shell-facing epithelial cells of the inner mantle for glutamine formation. Secondly, as the organic matrix for shell formation contains proteins (Wilbur and Saleuddin 1983), light-enhanced *TSZURE*/TSZURE expression in the inner mantle could increase the production of urea-nitrogen for amino acid production so as to augment organic matrix synthesis in support of light-enhanced shell formation. However, the inner mantle has much lower phototrophic potential than the outer mantle, and the expression level of its TSZRCLII remains unchanged during light exposure (Poo et al. 2020). Hence, symbiotic dinoflagellates residing in the inner mantle might have to absorb some photosynthates (carbohydrates) circulating in the hemolymph, which originate from

dinoflagellates of the outer mantle, in order to synthesize amino acids that could be dedicated for light-enhanced shell formation.

*Light-enhanced TSZURE/*TSZURE *expression in the foot muscle could be linked to amino acids needed for the production of muscle proteins*

Adult giant clams can undergo lateral displacement (Huang et al. 2007), which requires the foot to make contact with the substrate so that a force for movement can be generated by subsequent valve contraction (Stasek 1962, Huang et al. 2007). Such movement can only be achieved with substantial foot muscle mass and strength. Although the foot is shaded from light by the siphonal mantle and has a low phototrophic potential, it contains a concentrated population of symbiotic dinoflagellates at the tip of the muscle (Poo et al. 2020). As muscle mass is maintained by a balance between protein breakdown and protein synthesis (Schiaffino et al. 2013), increased availability of amino acids can augment muscle protein synthesis, leading to enhanced muscle mass and strength, as well as improved function (Wolfe 2012). Hence, light-enhanced expression of *TSZURE/*TSZURE in symbiotic dinoflagellates of the foot muscle could be an adaptation to augment the release of urea-nitrogen for increased syntheses of amino acids that are needed for the production of foot muscle proteins. Similar to those in the inner mantle, symbiotic dinoflagellates of the foot muscle (Poo et al. 2020) might have to absorb carbon substrates circulating in the hemolymph for the assimilation of urea-nitrogen, as illumination does not affect their expression level of TSZRCLII (Poo et al. 2020). The amino acids released by dinoflagellates in the foot muscle could be dedicated for muscle protein synthesis therein.

Conclusion

In giant clam-dinoflagellates associations, the host clam needs to receive adequate supplies of energy and nutrients from its phototrophic symbionts to sustain growth and to conduct light-enhanced calcification. Specifically, the host needs a large supply of amino acids from its symbionts to synthesize proteins and other nitrogenous compounds for shell formation and muscle production, besides general growth and development. Indeed, symbiotic dinoflagellates are known to be present not only in the outer mantle, but also in specific regions of the inner mantle and foot muscle (Poo et al. 2020). To produce large quantities of amino acids, symbiotic dinoflagellates must have an adequate supply of nitrogen, especially during photosynthesis when a good supply of fixed-carbon is readily available. With two nitrogen atoms per molecule, urea is a good nitrogen source. Therefore, it is advantageous for TSZURE to be located in plastids of symbiotic dinoflagellates, and for these dinoflagellates to upregulate the expression levels of TSZURE to increase urea degradation in synchrony with an increase in the supply of urea from the host during light exposure (Chan et al. 2018, 2019). By contrast, free-living algae and plants do not need to conduct light-enhanced expression of urease because they lack muscle and need only to produce sufficient amino acids and nitrogenous compounds to satisfy their own nutritional demands.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Table 1. A comparison of the nucleotide sequence of *Tridacna squamosa Symbiodinium Urease* (*TSSURE*), *Tridacna squamosa Cladocopium Urease* (*TSCURE*) and *Tridacna squamosa Durusdinium Urease* (*TSDURE*) obtained from *T. squamosa* with *URE* contigs obtained from various online symbiotic dinoflagellate databases (Bayer et al. 2012, Ladner et al. 2012, Rosic et al. 2015, Aranda et al. 2016, Levin et al. 2016, Davies et al. 2018, Shoguchi et al. 2018). Information on the database and the related reference, species/ITS2 type, contig number and the length of the sequence for comparison of each *URE* contig were presented. Comparisons were limited by the length of *URE* contigs available in the databases. FS: Full sequence.

URE from *T. squamosa*	Database (reference)	Species (ITS2 type)	Contig number	% similarity	Length of sequence compared	Nucleotide position
TSSURE	SymA (Shoguchi et al. 2018)	*Symbiodinium tridacnidorum* (A3)	comp34400 c0 seq1	100	2614 bp (FS)	1-2613
	CassKB8 (Bayer et al. 2012)	*Symbiodinium microadriaticum* (A1)	c14815	95.5	2256 bp	59-2315
	Smic (Aranda et al. 2016)	*Symbiodinium microadriaticum* (A1)	Smic27057	61.9	1597 bp	300-1897
TSCURE	TR (Levin et al. 2016)	*Cladocopium goreaui* (C1)	TR111 c0 g1 i1	99.9	2620 bp (FS)	1-2619

	Davies et al. (2018)	*Cladocopium goreaui* (C1)	comp50282 c0 seq1	99.7	939 bp	1-940
	SymC (Shoguchi et al. 2018)	*Cladocopium* C92	comp41443 c0 seq1	96.6	2620 bp (FS)	1-2619
TSDURE	GAFP (Ladner et al. 2012)	*Durusdinium* D2	GAFP01006283	99.9	1107 bp	1515-2622
	GBRR (Rosic et al. 2015)	*Durusdinium glynii* (D1)	GBRR01012083	82.7	664 bp	1515-2179

Table 2. Ratio ($x10^{-2}$) of the transcript level of *Tridacna squamosa zooxanthellal Urease* (*TSZURE*) to that of *Tridacna squamosa zooxanthellal form II ribulose-1,5-bisphosphate carboxylase/oxygenase* (*TSZrbcII*) in five organs of ten individuals of *T. squamosa* exposed to light for 3 h after 12 h of darkness. Means not sharing the same letter are significantly different ($P<0.01$ after Bonferroni adjustment).

	Ratio ($x10^{-2}$) of *TSZURE* to *TSZrbcII*				
	Outer mantle	Inner mantle	Foot muscle	Hepatopancreas	Ctenidium
Clam 1	1.17	0.18	0.19	0.36	0.56
Clam 2	1.77	0.24	0.44	0.36	0.19
Clam 3	0.49	0.29	0.39	0.49	0.25
Clam 4	1.51	0.45	0.25	0.47	0.81
Clam 5	0.68	0.30	0.36	0.30	0.48
Clam 6	1.21	0.22	0.23	0.25	0.41
Clam 7	2.35	0.52	0.44	0.39	0.68
Clam 8	1.00	0.19	0.34	0.39	0.40
Clam 9	2.16	0.28	0.34	0.71	0.31
Clam 10	0.89	0.35	0.48	0.54	0.41
Mean ± S.D.	1.32 ± 0.62^{b}	0.30 ± 0.11^{a}	0.45 ± 0.19^{a}	0.43 ± 0.13^{a}	0.35 ± 0.10^{a}

Figure 1. Molecular characterization of the complete coding amino acid sequences of *Tridacna squamosa Symbiodinium* Urease (TSSURE), *Tridacna squamosa Cladocopium* Urease (TSCURE) and *Tridacna squamosa Durusdinium* Urease (TSDURE). Multiple alignment of TSSURE, TSCURE and TSDURE with URE sequences of *Gossypium arboreum (*KHG25395.1) and *Canavalia ensiformis* (AAA83831.1). Shaded residues represent identical or highly similar amino acids. The residues involved in Ni^{2+}-binding are marked with triangles. Diamonds indicate H^{+}-donating residues. The boxed region denotes residues that partake in both Ni^{2+}-binding and H^{+}-donating. Arrows mark those residues involved in urea binding and the star marks the residue that forms the "mobile flap".

Figure 2. Phenogramic analysis of *Tridacna squamosa Symbiodinium* Urease (TSSURE), *Tridacna squamosa Cladocopium* Urease (TSCURE) and *Tridacna squamosa Durusdinium* Urease (TSDURE) from the outer mantle. Numbers shown at each branch point represent bootstrap values from 2000 replicates. URE from *Canavalia ensiformis* is used as the outgroup.

Figure 3. Immunofluorescence localization of *Tridacna squamosa* zooxanthellal Urease (TSZURE) in zooxanthellae (ZX) of the outer mantle of *T. squamosa* exposed to 12 h of light. (a) Image of differential interference contrast microscopy (DIC) merged with that of DAPI (blue, online version) staining of nuclei (n) to demonstrate ZX. (b) Auto-fluorescent images of ZX (red, online version). (c) Image of TSZURE-immunostaining (green, online version) of different cellular compartments of ZX. Arrowheads: plastids; arrows: cytoplasm; triangles: cell wall/membrane. (d) Overlaid image of DIC/DAPI, auto-fluorescence and anti-TSZURE immunofluorescence. Arrowheads: plastids; arrows: cytoplasm; triangles: cell wall/membrane. (e) Image indicating no TSZURE-immunostaining after the anti-

TSZURE antibody was treated with a peptide competition assay (PCA). Scale bar: 20 μm.

Figure 4. Transcript levels (x10^4 copies of transcript per ng total RNA) of *Tridacna squamosa zooxanthellal Urease* (*TSZURE*) in five organs of *T. squamosa.* Results represent means + S.E.M. (*N*=10). Means not sharing the same letter are significantly different (*P*<0.05).

Figure 5. Effects of light exposure on the mRNA expression level of *Tridacna squamosa zooxanthellal Urease* (*TSZURE*) in *T. squamosa*. Transcript levels (x10^4 copies of transcript per ng total RNA) of *TSZURE* in the (a) outer mantle, and transcript levels (x10^3 copies of transcript per ng total RNA) of *TSZURE* in the (b) inner mantle, (c) foot muscle, (d) hepatopancreas and (e) ctenidium of *T. squamosa* exposed to 12 h of darkness (control) or 3, 6 or 12 h of light. Results represent means + S.E.M. (*N*=4). Means not sharing the same letter are significantly different (*P*<0.05).

Figure 6. Effects of light exposure on the protein abundance of *Tridacna squamosa* zooxanthellal Urease (TSZURE) in the colorful outer mantle of *T. squamosa*. (a) A representative immunoblot of TSZURE from the outer mantle. L represents the ladder. (b) TSZURE protein abundance, expressed as arbitrary densitometric units (a.u.) per 20 μg of protein, in the outer mantle of *T. squamosa* exposed to 12 h of darkness (control) or 3, 6 or 12 h of light. Results represent means + S.E.M. (*N*=4). Means not sharing the same letter are significantly different (*P*<0.05).

Figure 7. Effects of light exposure on the protein abundance of *Tridacna squamosa* zooxanthellal Urease (TSZURE) in the whitish inner mantle of *T. squamosa.* (a) A representative immunoblot of TSZURE from the inner mantle. L represents the ladder. (b) TSZURE protein abundance, expressed as arbitrary densitometric units (a.u.) per 100 μg of protein, in the inner mantle of *T. squamosa* exposed to 12 h of

darkness (control) or 3, 6 or 12 h of light. Results represent means + S.E.M. (N=4). Means not sharing the same letter are significantly different (P<0.05).

Figure 8. Effects of light exposure on the protein abundance of *Tridacna squamosa* zooxanthellal Urease (TSZURE) in the foot muscle of *T. squamosa*. (a) A representative immunoblot of TSZURE from the foot muscle. L represents the ladder. (b) TSZURE protein abundance, expressed as arbitrary densitometric units (a.u.) per 100 µg of protein, in the foot muscle of *T. squamosa* exposed to 12 h of darkness (control) or 3, 6 or 12 h of light. Results represent means + S.E.M. (N=4). Means not sharing the same letter are significantly different (P<0.05).

Supplementary Table S1. Urease protein sequences from other organisms were obtained from Genbank and used for phenogramic analysis and their accession numbers. Maximum-likelihood analysis was applied using RaxML v8.2.5 (Stamatakis 2014) with 2000 bootstraps. Through ModelGenerator v0.85 (Keane et al. 2006), the best-fitting evolutionary model under Akaike Information Criterion was determined to be LG+G (Whelan and Goldman 2001).

Supplementary Table S2. Similarity between the amino acid sequences of dinoflagellate Urease (URE) obtained from various online dinoflagellate databases or from this study (TSSURE, TSCURE and TSDURE from *Tridacna squamosa*) and the epitope sequence used to generate the custom-made anti-TSZURE antibody. The epitope sequence KGTRSVQKKDMILN was designed at a conserved region of seven URE sequences. One *Symbiodinium* URE sequence and one *Cladocopium* URE sequence were obtained from Shoguchi et al. (2018). Two *Durusdinium* URE sequences were obtained from Ladner et al. (2012).

Supplementary Table S3. Similarity between the nucleotide sequences of zooxanthellal *Urease* (*URE*) obtained from various symbiotic dinoflagellate databases or from this study (*TSSURE*, *TSCURE* and *TSDURE* from *Tridacna squamosa*) and the comprehensive qPCR primers designed for *TSZURE*. The set of *TSZURE* genus-comprehensive qPCR primers (forward: 5'-TGAACTTCGGCTTCTCTGG-3'; reverse: 5'-CCGTGTGGATGGTGATGG-3') was designed at a conserved region of all available *URE* sequences. Two *Symbiodinium URE* sequences were retrieved from Bayer et al. (2012) and Aranda et al. (2016); two *Cladocopium URE* sequences were obtained from

Shoguchi et al. (2018); and two *Durusdinium URE* sequences were retrieved from Ladner et al. (2012).

Fig. 1

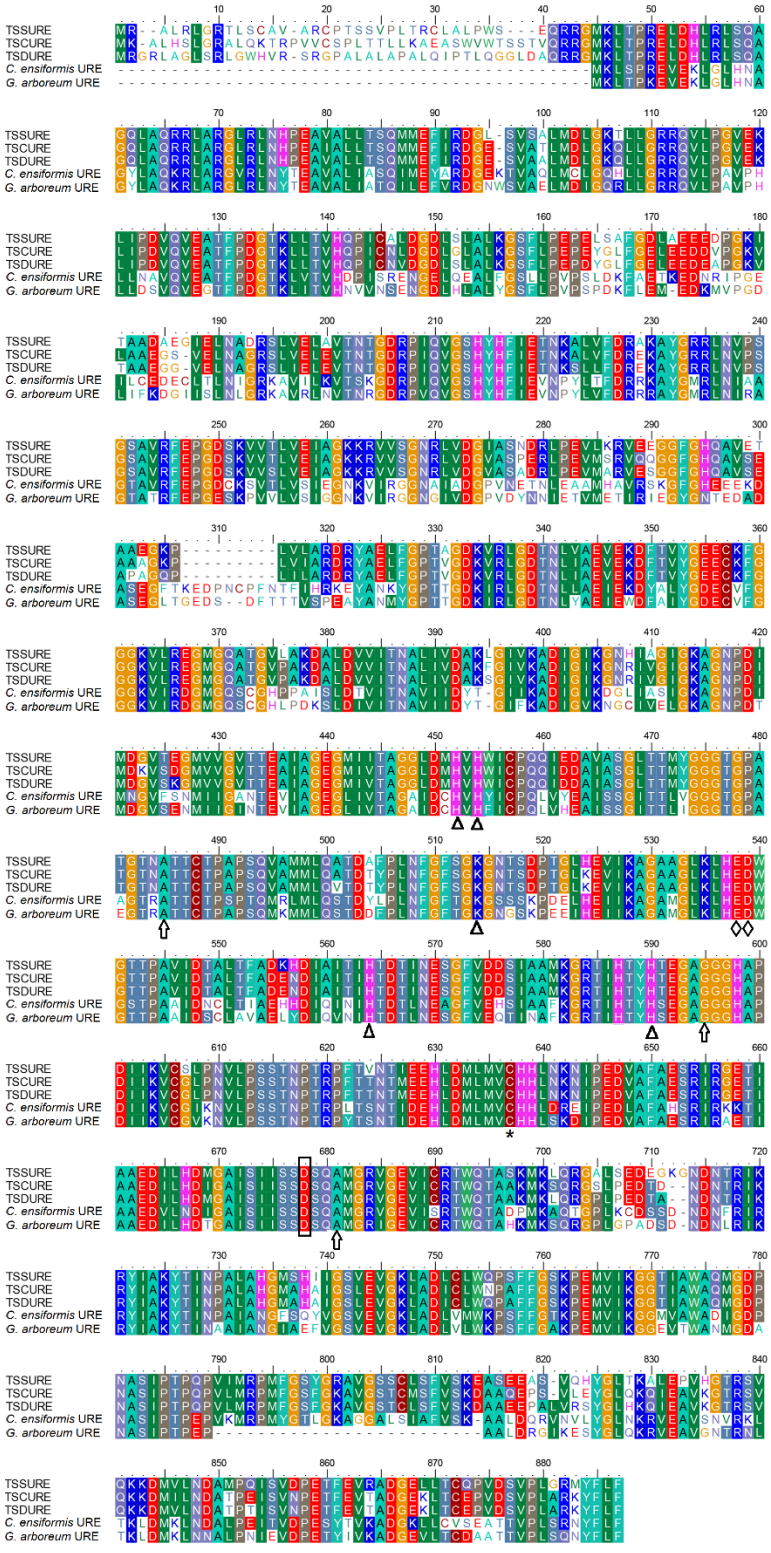
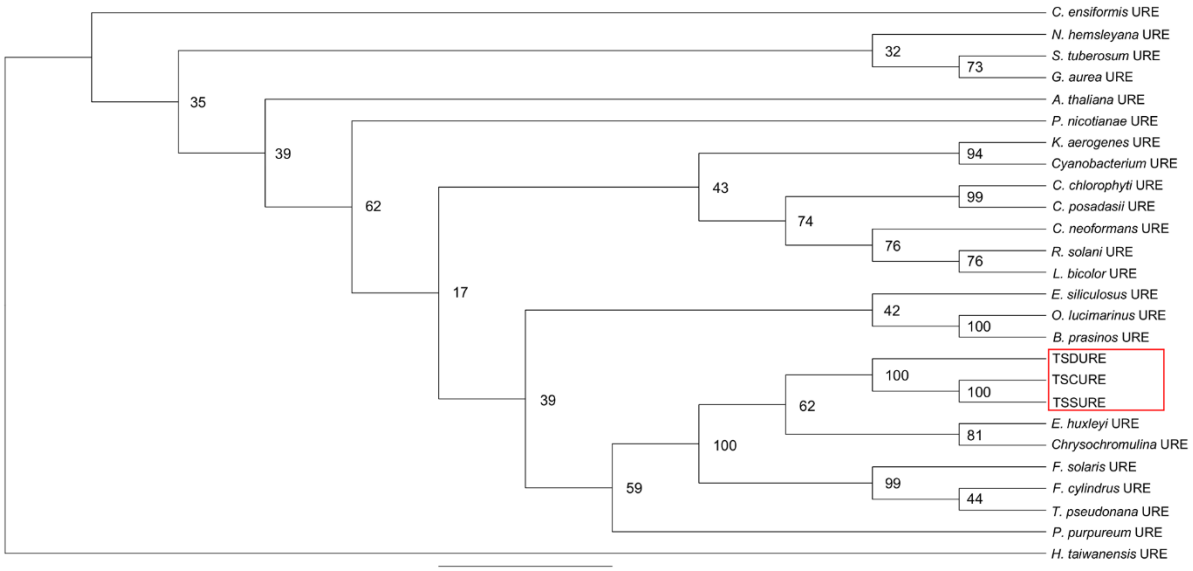


Fig. 2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N. hemsleyana URE
S. tuberosum URE
G. aurea URE
A. thaliana URE
P. nicotianae URE
K. aerogenes URE
Cyanobacterium URE
C. chlorophyti URE
C. posadasii URE
C. neoformans URE
R. solani URE
L. bicolor URE
E. siliculosus URE
O. lucimarinus URE
B. prasinos URE
TSDURE
TSCURE
TSSURE
E. huxleyi URE
Chrysochromulina URE
F. solaris URE
F. cylindrus URE
T. pseudonana URE
P. purpureum URE
H. taiwanensis URE
32
73
35
39
94
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76
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62
81
39
100
99
59
44

Fig. 3

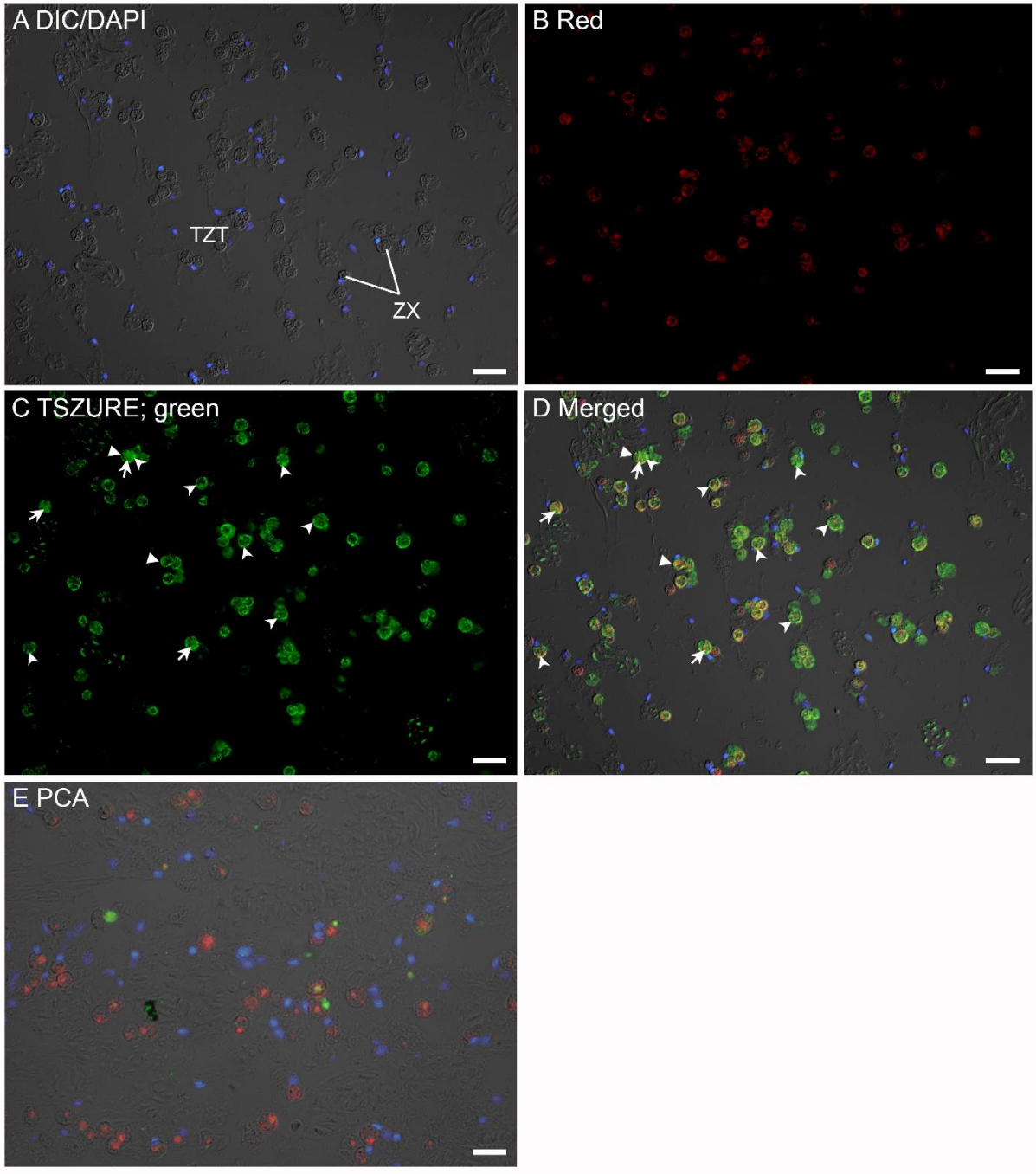


Fig. 4

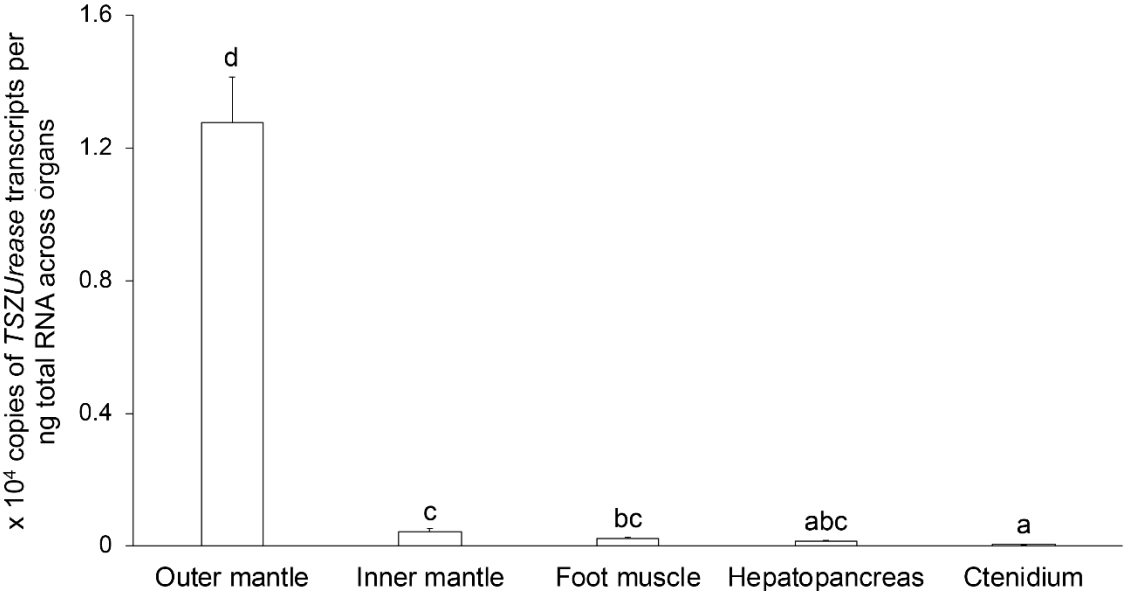

x 10⁴ copies of TSZUrease transcripts per ng total RNA across organs
1.6
1.2
0.8
0.4
0
d
c
bc
abc
a
Outer mantle
Inner mantle
Foot muscle
Hepatopancreas
Ctenidium

Fig. 5

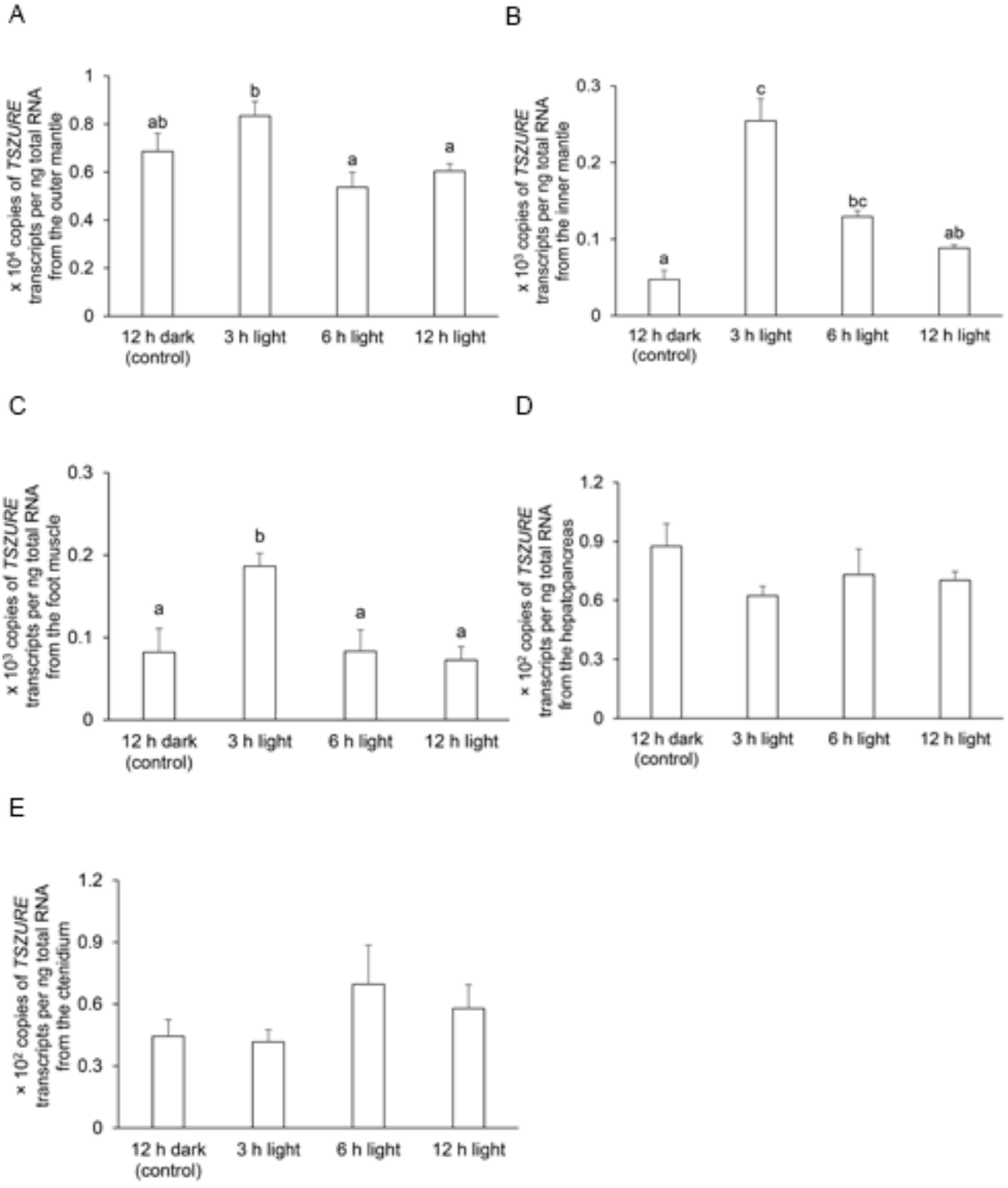

A
× 10⁴ copies of TSZURE transcripts per ng total RNA from the outer mantle
1
0.8
0.6
0.4
0.2
0
ab
b
a
a
12 h dark (control)
3 h light
6 h light
12 h light
B
× 10³ copies of TSZURE transcripts per ng total RNA from the inner mantle
0.3
0.2
0.1
0
a
c
bc
ab
12 h dark (control)
3 h light
6 h light
12 h light
C
× 10³ copies of TSZURE transcripts per ng total RNA from the foot muscle
0.3
0.2
0.1
0
a
b
a
a
12 h dark (control)
3 h light
6 h light
12 h light
D
× 10² copies of TSZURE transcripts per ng total RNA from the hepatopancreas
1.2
0.9
0.6
0.3
0
12 h dark (control)
3 h light
6 h light
12 h light
E
× 10² copies of TSZURE transcripts per ng total RNA from the ctenidium
1.2
0.9
0.6
0.3
0
12 h dark (control)
3 h light
6 h light
12 h light

Fig. 6

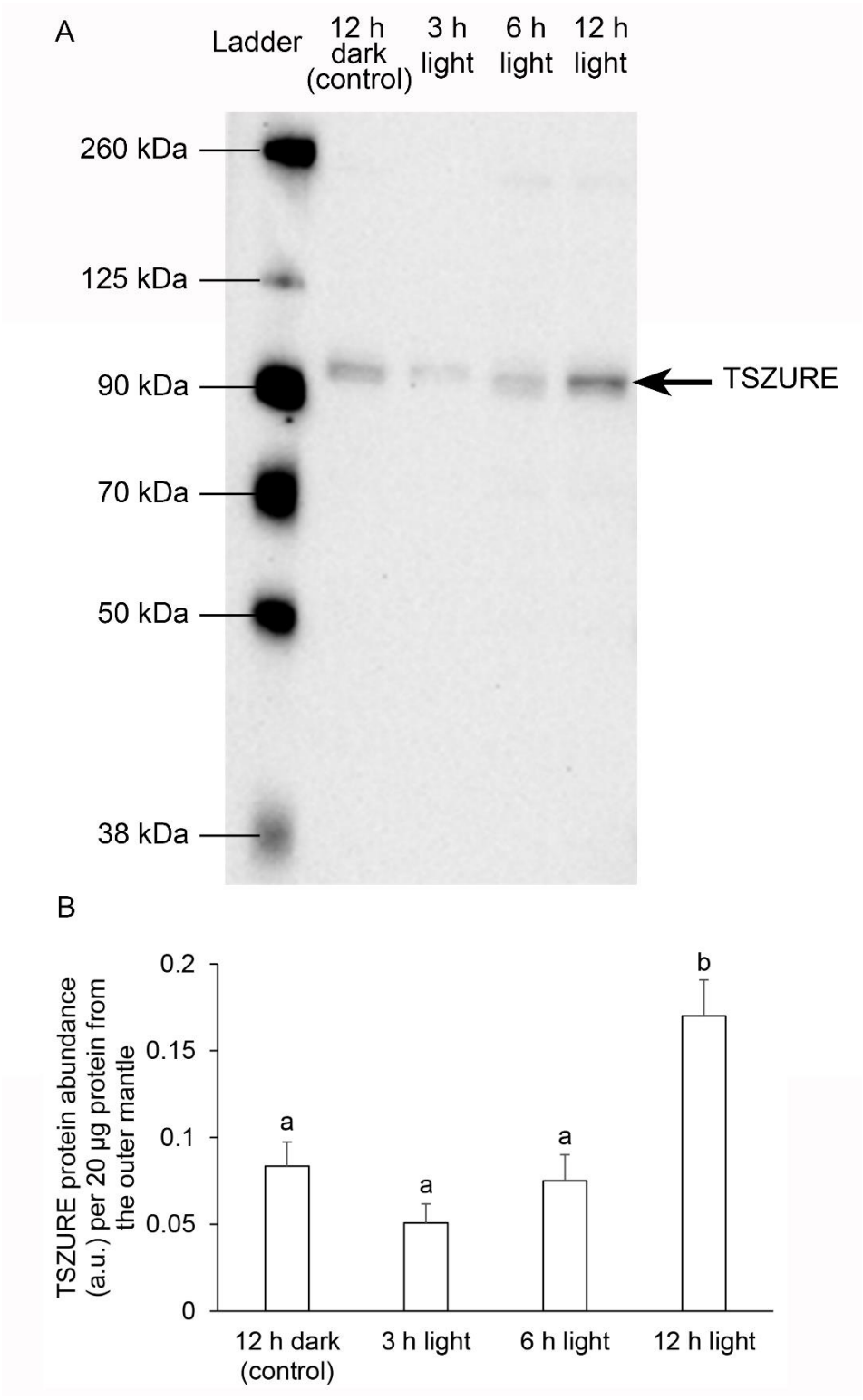

A
Ladder
12 h dark (control)
3 h light
6 h light
12 h light
260 kDa
125 kDa
90 kDa
70 kDa
50 kDa
38 kDa
TSZURE
B
TSZURE protein abundance (a.u.) per 20 µg protein from the outer mantle
0.2
0.15
0.1
0.05
0
a
a
a
b
12 h dark (control)
3 h light
6 h light
12 h light

Fig. 7

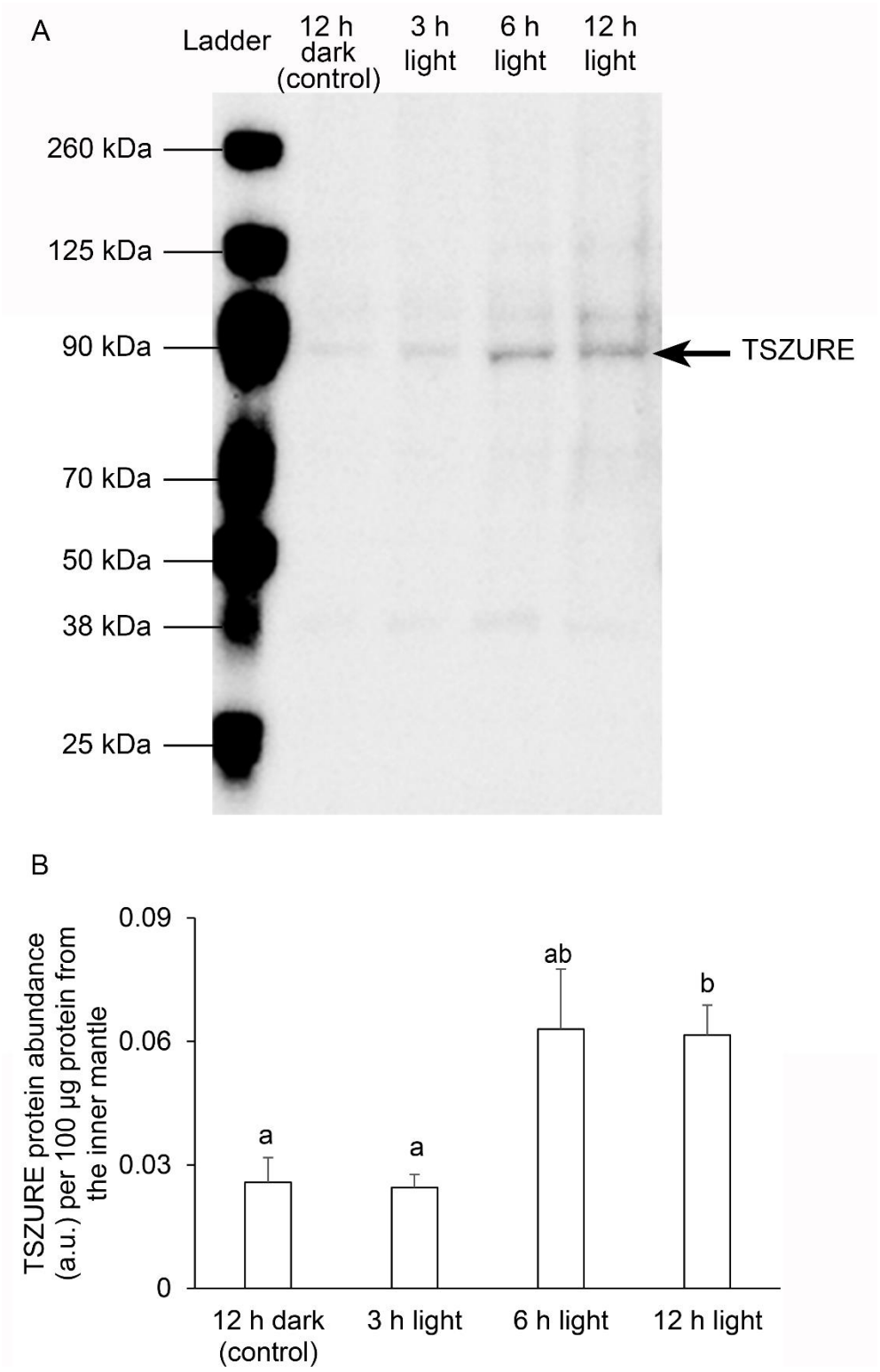
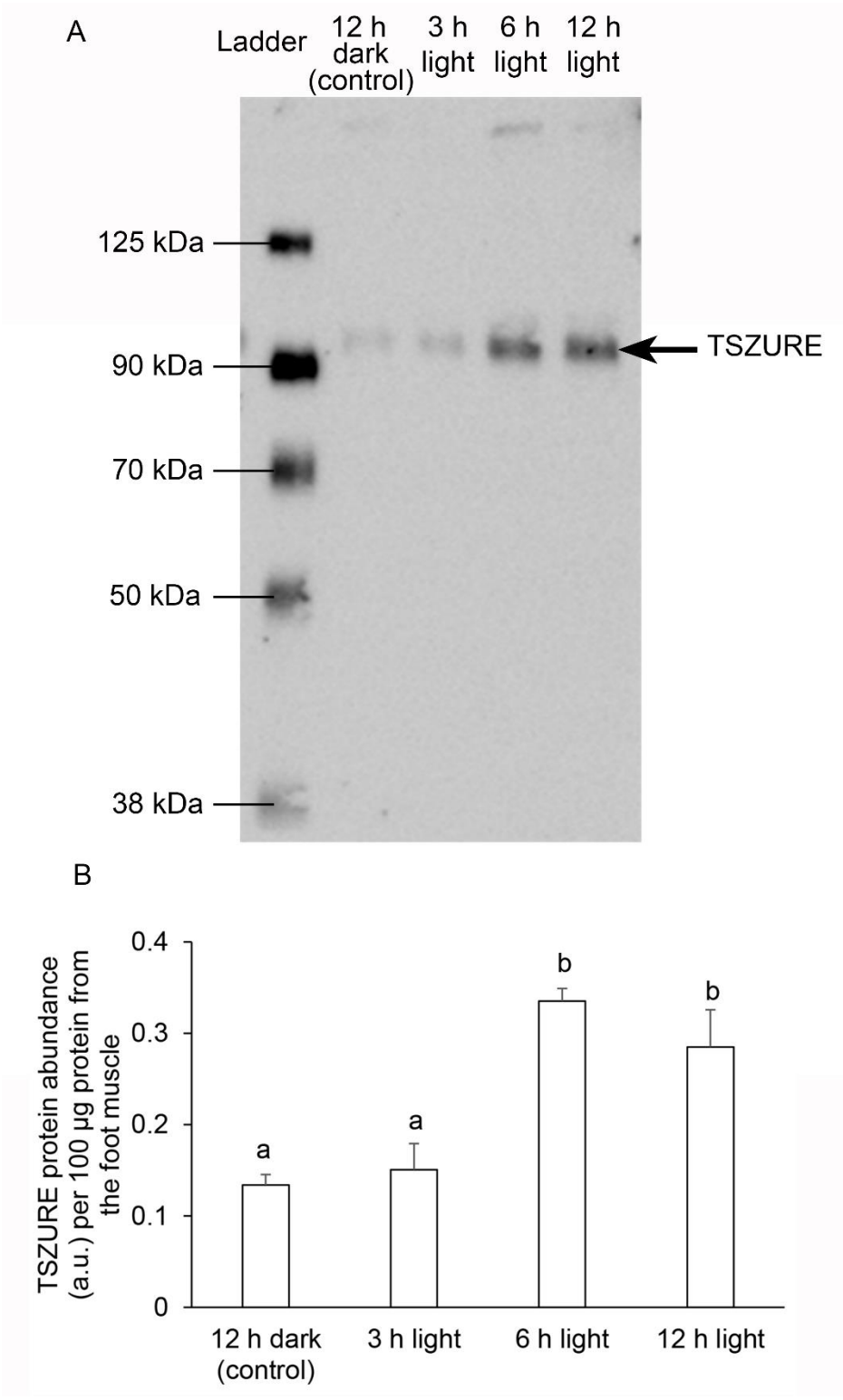

A
Ladder
12 h dark (control)
3 h light
6 h light
12 h light
260 kDa
125 kDa
90 kDa
70 kDa
50 kDa
38 kDa
25 kDa
TSZURE
B
TSZURE protein abundance (a.u.) per 100 µg protein from the inner mantle
0.09
0.06
0.03
0
a
a
ab
b
12 h dark (control)
3 h light
6 h light
12 h light

Fig. 8


A
Ladder
12 h dark (control)
3 h light
6 h light
12 h light
125 kDa
90 kDa
70 kDa
50 kDa
38 kDa
TSZURE
B
TSZURE protein abundance (a.u.) per 100 µg protein from the foot muscle
0.4
0.3
0.2
0.1
0
a
a
b
b
12 h dark (control)
3 h light
6 h light
12 h light