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Antioxidant enzyme cycling over reproductive lunar cycles in *Pocillopora damicornis*

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The impacts of continued degradation of watersheds on coastal coral reefs world-wide is alarming. Action addressing anthropogenic stressors and subsequent rehabilitation of watersheds and adjacent reefs is an urgent priority. The aim of this study is to develop and improve the use of antioxidant enzymes as biomarkers in coral species. In order to fully develop such tools, it is necessary to perform sampling of coral tissues over reproductive cycles to determine variations from baseline. By developing a greater understanding of biochemical markers of stress in corals, specifically antioxidant defense enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), we have provided molecular tools that identify thresholds of stress on coral reefs. Our results suggest that the coral reproductive state is a significant factor affecting the activity of antioxidant enzymes. Specifically, CAT (65.92 mmol/min/mg protein, $p = 0.0177$) and GR (12.64 nmol/min/mg protein, $p < 0.0001$) display maximum activity during peak reproductive state. Whereas significant maximal SOD (154.92 nmol/min/mg protein, $p < 0.0454$) and Se-independent GPx (5.35 nmol/min/mg protein, $p = 0.0001$) activity was measured during off-peak reproductive cycles. Such insight into the cyclical variation of the activity of these enzymes should be applied towards differentiating the influence of natural biological activity cycling in diagnostic tests identifying the effects of different physical environmental factors and chemical pollutants on coral health. Through the development and application of these molecular biomarkers of stress, we look to improve our ability to identify problems at the sub-lethal level, when action can be taken to mitigate a/biotic impacts.

Antioxidant Enzyme Cycling over Reproductive Lunar Cycles in *Pocillopora damicornis*

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Abstract

The impacts of continued degradation of watersheds on coastal coral reefs world-wide is alarming. Action addressing anthropogenic stressors and subsequent rehabilitation of watersheds and adjacent reefs is an urgent priority. The aim of this study is to develop and improve the use of antioxidant enzymes as biomarkers in coral species. In order to fully develop such tools, it is necessary to perform sampling of coral tissues over reproductive cycles to determine variations from baseline. By developing a greater understanding of biochemical markers of stress in corals, specifically antioxidant defense enzymes: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), we have provided molecular tools that identify thresholds of stress on coral reefs. Our results suggest that the coral reproductive state is a significant factor affecting the activity of antioxidant enzymes. Specifically, CAT (65.92 mmol/min/mg protein, $p = 0.0177$) and GR (12.64 nmol/min/mg protein, $p < 0.0001$) display maximum activity during peak reproductive state. Whereas significant maximal SOD (154.92 nmol/min/mg protein, $p < 0.0454$) and Se-independent GPx (5.35 nmol/min/mg protein, $p = 0.0001$) activity was measured during off-peak reproductive cycles. Such insight into the cyclical variation of the activity of these enzymes should be applied towards differentiating the influence of natural biological activity cycling in diagnostic tests identifying the effects of different physical environmental factors and chemical pollutants on coral health. Through the development and application of these molecular biomarkers of stress, we look to improve our ability to identify problems at the sub-lethal level, when action can be taken to mitigate a/biotic impacts.

Keywords: coral, enzymes, antioxidant defense, catalase, superoxide dismutase, glutathione reductase, glutathione peroxidase, lunar cycling

Introduction

Corals are critical to the structural and biological integrity and function of coral reef ecosystems (Birkeland, 1997). Because anthropogenic stress is increasingly impacting global marine environmental health (Gattuso et al., 2015; Heron, Maynard, van Hooidonk, & Eakin, 2016; Hughes et al., 2017; Maynard et al., 2015), it is critical that techniques for evaluating coral stress [prior to reef collapse] are developed and applied (Edge, Shearer, Morgan, & Snell, 2013). Recent advances in the application of molecular analyses to facilitate sub-lethal stress evaluations in corals have been substantial (Ainsworth, Hoegh-Guldberg, Heron, Skirving, & Leggat, 2008; Barshis, Ladner, Oliver, & Palumbi, 2014; Desalvo et al., 2008; Downs et al., 2012; Edge et al., 2013; Rougée, Downs, Richmond, & Ostrander, 2006). Researchers concerned with coral health, stress, bleaching events and their total effects on reef state, need to develop new and better diagnostic tools that address coral stress prior to death and that can inform policy, improve conservation efforts, and assist in saving coral reefs as a legacy for the future.

One way that this can be accomplished is by developing tests for the evaluation of specific stress responses. Antioxidant stress enzymes, for example, are useful for the analysis of stressor impacts on the health of coral animals due to their wide applications in response to a variety of stressors (Downs, Richmond, Mendiola, Rougée, & Ostrander, 2006; Higuchi, Yuyama, & Nakamura, 2015; Vijayavel, Downs, Ostrander, & Richmond, 2012). Antioxidant enzyme presence and activity in coral tissues have the potential to be employed as metrics for evaluating stress on reefs, including gradients of stress, and pin-pointing the impacts toxicants on the health of corals (Edge et al., 2013; Rivest & Hofmann, 2014). Such molecular biomarkers can determine the degree of stress affecting specific areas of the reef or the gradient over which a pollutant source may be diffusing across a reef (Downs et al., 2006). A library of biomarkers will be valuable in identifying physiological stress prior to coral death.

Previous studies have highlighted antioxidant enzymes as useful biomarkers of the impacts of stressors such as heat, xenobiotic exposure, and high-irradiance (Downs et al., 2006; Higuchi et al., 2015; Liñán-Cabello, Flores-Ramírez, Cobo-Díaz, et al., 2010; Olsen, Ritson-Williams, Ochrietor, Paul, & Ross, 2013). However, as useful as this suite of enzymes is in providing information about coral stress responses and threat levels, many of the substrates that trigger this type of stress are naturally produced in normal homeostatic processes (Agarwal, Gupta, & Sikka, 2006; Dowling & Simmons, 2009; Fujii, Iuchi, & Okada, 2005). Adding these

biomarkers to the available tools that can be employed to evaluate reef health is important and requires knowledge of baseline levels of protein expression and activity, including over reproductive cycles. In order to have full confidence in using these enzymes as biomarkers for stress detection, it is important to take into consideration how endogenous levels of expression may change over shifting baselines. As such, prior to adopting these enzymes into our suite of diagnostic tools, we seek to characterize whether reproductive cycling has a discernable effect on the enzymatic profile of a widely distributed species of coral, *Pocillopora damicornis*.

This study of coral reproduction arose in part from previous work describing cyclical variation in defense enzyme activity during reproduction events. A study performed by Rougée, Richmond, & Collier (2014) illustrated variations in the expression and activity of xenobiotic metabolizing enzymes during reproductive cycling in the coral *P. damicornis*. Glucuronosyltransferase, glutathione-s-transferase (GST), cytochrome P450 2E1, and cytochrome P450 reductase were all found to fluctuate significantly over natural reproductive lunar cycles (Rougée et al., 2014). Additionally, research by Ramos, Bastidas, Debrot, & García (2011) provided insight into the effect of reproductive cycling on various biotransformation and antioxidant enzyme activities. In their work, activities of cytochromes P450, GST, NADPH c reductase, and catalase (CAT) were all significantly higher during reproductive peaks in the coral *Siderastrea siderea* (Ramos et al., 2011). With such evidence for the fluctuation of enzymatic activity tied to reproductive cycling, coupled with the knowledge of ROS impacts on the health of reproductive systems in other organisms, the lack of more comprehensive research into antioxidant enzyme expression over reproductive cycles in corals underlines a lack of data regarding antioxidant enzyme expression (Agarwal et al., 2006).

Reproduction is an innate source of reactive oxygen species (ROS) generation and relies heavily upon the interplay of pro-oxidants and antioxidants (Agarwal et al., 2006; Fujii et al., 2005; Halliwell & Gutteridge, 2015; Rahal et al., 2014). This interplay of ROS production and detoxification during reproduction has a critical role in both aiding and inhibiting high quality gamete production, fertilization, and embryo development (Fujii et al., 2005). Studies in systems other than those found in Cnidarians have pointed to a heightened prevalence of ROS impacting fertility, as well as being implicated in the termination of embryos and reproductive senescence during heightened levels of oxidative stress (Agarwal, Gupta, & Sharma, 2005; Agarwal et al., 2006; Carbone et al., 2003). Oxidative stress also has the potential to reduce embryo growth and

decrease fertilization rates (Agarwal et al., 2006). However, ROS are also both beneficial and detrimental to the motility and viability of sperm cells. Specifically, sulfoxidation is required for the maturation of sperm and packaging of nuclei in sperm heads, while excess ROS proliferation acting upon the axoneme of spermatozoa can inhibit motility (de Lamirande & Gagnon, 1992; Fujii et al., 2005). Rich in polyunsaturated fatty acids, spermatozoa are highly vulnerable to lipid peroxidation due to low availability of ROS-scavenging enzymes (Agarwal et al., 2006; Saleh & Agarwal, 2002). As a result, unregulated lipid peroxidation can lead to the production of spermicidal compounds, such as (E)-4-Hydroxy-2-nonenal, which at concentrations of only 50 µm, can result in irreversible motility loss (Selley, Lacey, Bartlett, Copeland, & Ardlie, 1991). Antioxidant compounds, such as glutathione, and ROS-scavenging enzymes, such as superoxide dismutase (SOD), exist to modulate the effects of ROS on egg and sperm viability and promote embryo integrity (Agarwal et al., 2006).

Although corals may have different reproductive methods than vertebrates, other invertebrates, and plants, there are commonalities with respect to ROS generation and detoxification that are highly conserved across taxa and are required for optimizing reproductive integrity (Dowling & Simmons, 2009). To improve the breadth and quality of the biomarkers available, this project sought to define basal enzymatic stress levels in a major coral species with broad global application, with respect to reproductive cycling (Hoeksema, Rodgers, & Quibilan, 2014). Further, unlike other common reef-building corals, such as various *Porites* spp., *Acropora* spp., and *Montipora* spp., that utilize external fertilization and larval development through seasonal mass-broadcast spawning events following annual cycles (Harrison et al., 1984; Harrison & Wallace, 1990; Neves, 2000; Padilla-Gamiño & Gates, 2012; Stimson, 1978), the coral *P. damicornis* was chosen for study due to its monthly brooding cycles, with peak reproductive output closely tied to the first-quarter moon phase (Richmond & Jokiel, 1984). As such, reproductive shifts in antioxidant enzyme activity have been observed over monthly cycles, rather than having an annual cycling rate. This also makes the species excellent for differentiating seasonal variations and year-to-year changes in environmental stressors from basal reproductive and antioxidant enzyme activity variation (Cooper, Gilmour, & Fabricius, 2009; Harrison & Wallace, 1990; Ward, 1995). By improving baseline knowledge of inherent stress in major reef-building corals, future studies examining coral health can better differentiate stress endured by corals with respect to natural phenomena, from internal cycling.

Methods

Sample collection

Coral samples (5 cm x 2.5 cm nubbins) were collected periodically from the same colonies under Department of Land and Natural Resources – Division of Aquatic Resources coral collection permit SAP 2015-6/17 off Lilipuna Pier, Kāneʻohe, Oʻahu, Hawaiʻi (Fig 1), adjacent to the Hawaiʻi Institute of Marine Biology (HIMB).

To reduce the impact of fragmentation on the reproductive cycling or output of *P. damicornis* (Zakai, Levy, & Chadwick-Furman, 2000), colonies were not fragmented prior to the start of collections. Instead, fragments of branches were sampled from several areas on each colony to ensure reduction of microhabitat influence and intracolony stress load variation between samples. This was also done to limit variations in reproduction potential along coral branches (polyps found mid-branch retain the highest planula larvae output versus distal and central branch polyps) and sampling was also conducted during falling tides to both reduce residence time in low-flow water and match peak planula release, as it has been correlated with low tide periods (Harrison & Wallace, 1990). Colonies with minimal competition from other corals and no visible signs of disease or stress were sampled during each moon phase (New, ¼, Full, ¾ moon; n=6 colonies) during July, August. Collections also included an acute sampling period, during which corals were sampled daily for five consecutive days following the start of the peak reproductive period moon phase (¼ moon) in the month of August. This sampling period was constructed to provide finer resolution for understanding changes in antioxidant enzyme profiles following a reproductive peak.

Sampling was designed to illustrate variations in enzymatic activity within the moon phase cycle and fragments were immediately frozen in liquid nitrogen to preserve enzyme profiles and protein integrity. Samples were transported to Kewalo Marine Laboratory (KML) on dry ice and transferred to a -80°C freezer. Upon returning to KML, corals were crushed into a fine powder on liquid nitrogen and returned for storage in a VWR 5656 -80ºC freezer (Radnor, PA, USA) until further processing.

S9 protein fraction extraction and protein quantification

Prior to protein isolation, coral samples were crushed into a fine powder using liquid nitrogen and an arbor press. Following modified protocols by Lesser et al. (1990), coral S9 post-mitochondrial protein fractions and zooxanthellae were isolated from crushed coral tissue. Using 1500 mg of crushed tissue and 1500 µL of homogenization buffer per sample extraction in 50 mL tubes (0.01 M Tris-HCl buffer pH 8.0, 1 M phenylmethylsulfonyl fluoride in 1% v/v dimethyl sulfoxide), tissue was homogenized for 1 minute on ice using an Ultra-Turrax homogenizer. The homogenate was then spun for 5 minutes at 4ºC at 2000 rcf in an Eppendorf Microcentrifuge 5415D (Hauppauge, NY, USA) to separate skeleton and tissue, and the supernatant was transferred to 1.5 mL microcentrifuge tubes and spun for 20 minutes at 4ºC at 10,000 rcf. The supernatant was then aliquoted into 1.5 mL tubes and zooxanthellae pellets were saved, and frozen at -80ºC; 50 µL of each extracted sample was set aside for protein concentration analyses.

In preparation for enzymatic activity assays, protein concentrations from each sample were measured using a bicinchoninic acid (BCA) assay. The standard curve was constructed with bovine serum albumin from 0 to 1.0 mg/mL protein (25 µL/well in triplicate), 1:5 dilutions of aliquots from each extracted S9 sample fraction in double distilled water (ddH_2O) were loaded into a 96 well plate in triplicate (25 µL/well). Bicinchoninic acid development reagent (2% $Cu^{2+}SO_4$ in BCA; Sigma-Aldrich) was then added into each well (200 µL/well), and the loaded plate was incubated at 37ºC for 30 minutes. Following incubation, absorbance values were determined at $\lambda = 562$ nm in a SpectraMax M5 Micro-Plate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). To ensure triplicate absorbance variation was within acceptable experimental levels (percent coefficient of variation, %CV < 10%; coefficient of determination, $R^2 > 0.98$), data were then exported to Microsoft Excel and %CV and subsequent standard curve R^2 values were calculated. Sample protein concentration values were then interpolated from the standard curve; those extractions falling below 1 mg/mL required re-extraction of S9 post-mitochondrial protein fractions.

Enzyme activity assays

Enzyme assays were developed in-house for CAT, GR, SOD, and GPx, and chemicals for assays were sourced from Sigma-Aldrich (St Louis, MO, USA), BioVision (Zurich, Switzerland), EMD Millipore (Burilington, MA, USA), and Cayman Chemical (Ann Arbor, MI,

193 USA). Assays were analyzed using a SpectraMax M5 Multi-Plate reader and final activity values
194 calculated using SoftMax Pro and Microsoft Excel.
195 The metabolism of H_2O_2 as a marker for CAT activity was accomplished by measuring
196 the consumption of H_2O_2 over time by analyzing decreasing absorbance of H_2O_2 at $\lambda = 240$ nm.
197 Briefly, the method was performed as follows: on ice, coral protein extractions were first diluted
198 to 1 mg/mL in 50 mM potassium phosphate buffer pH 7.0 and then loaded, in triplicate (10
199 µL/well), into optically clear microtiter 96-well plates; negative controls were also run in
200 triplicate containing all assay reagents except S9 protein to correct for spontaneous H_2O_2
201 degradation during activity reads. Running buffer (50 mM potassium phosphate buffer pH 7.0)
202 was then loaded into wells (80 µL/well), and samples were incubated for 3 minutes at 25ºC. The
203 CAT activity reaction was then initiated by adding 10 µL of 120 mM H_2O_2 to each well, and
204 immediately transferring the reaction plate into the spectrophotometer to read at 10 second
205 intervals for 5 minutes. In order to dislodge O_2 bubbles created by the dismutation of H_2O_2 into
206 H_2O and O_2, the spectrophotometer was set to vibrate the 96-well plate between 10 second reads
207 for 2 seconds; this aided in preventing O_2 bubbles from obscuring the plate reader's evaluation of
208 H_2O_2 absorbance in the reaction wells.
209 To evaluate the activity of GR, the consumption of NADPH at $\lambda = 340$ nm was observed
210 over time as GR in coral samples consumed this co-factor during the reduction of the reagent
211 oxidized glutathione (GSSG). In order to account for both spontaneous degradation of NADPH
212 in reaction wells and endogenous concentrations of NADPH in coral samples, wells containing
213 no coral sample (spontaneous degradation control), and those with coral sample but no NADPH
214 (background level control), were also evaluated alongside wells containing all reagents; in place
215 of coral sample and NADPH, an extra 20 µL of 100 mM potassium phosphate buffer (pH 7.2)
216 was added to wells in the first assay step. Values for NADPH degradation obtained from these
217 controls were subtracted from overall activity following assay completion. In optically clear
218 microtiter 96-well plates, 100 mM potassium phosphate buffer was loaded into wells (130
219 µL/well, in triplicate), followed by 100 mM ethylenediaminetetraacetic acid (EDTA) in ddH_2O
220 (10 µL/well), 10 mM GSSG in ddH_2O (20 µL/well), 1.2 mM NADPH in ddH_2O (20 µL/well),
221 and 1 mg/mL coral sample (20 µL/well). Plates were then loaded into the spectrophotometer and
222 mixed using its mixing function for 5 seconds. Absorbance reads were conducted at 20 second
223 intervals for 5 minutes at 25ºC.

Since SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2, this assay conducted an indirect evaluation of SOD activity by analyzing the degree of inhibition of the reduction of cytochrome c by O_2^-. Using the following loading protocol, reagents and samples were loaded into a 96-well microtitter plate in triplicate, loaded into and mixed using the mixing function in the spectrophotometer for 5 seconds, and analyzed for decreasing absorbance (λ = 550 nm) for 5 minutes in 20 second intervals at 25ºC. For this assay, wells were loaded in the following order: 100 mM potassium phosphate buffer (pH 7.8, 80, 74, 94 µL/well for reference, coral sample, and coral blank wells, respectively), working buffer (100 mM potassium phosphate buffer (pH 7.8), 0.2 mM EDTA in ddH_2O, 100 µM hypoxanthine in ddH_2O, and 20 µM cytochrome c in ddH_2O; 100 µL/well), coral samples diluted to 1 mg/mL in 100 mM potassium phosphate buffer (pH 7.8; 6 µL/well in coral sample and blank wells only), and reactions were initiated with the addition of 300 mU/mL of O_2^- generating xanthine oxidase in ddH_2O (20 µL/well in reference and coral sample wells only).

To evaluate the activity of GPx, assays were broken into two parts in order to determine the activity of both selenium-dependent and selenium-independent forms of this enzyme. As such, the loading protocol in place for evaluating the activity of selenium-dependent GPx employed H_2O_2 as the initiator and substrate for this reaction, including sodium azide (NaN_3) to inhibit CAT activity from consuming H_2O_2 and interfering with assay results. To evaluate the activity of selenium-independent GPx, cumene hydroperoxide (CHP) was utilized in place of H_2O_2; NaN_3 was not employed as a CAT inhibitor due to the change in substrate and buffer amounts were adjusted to bring the final reaction volume to 200 µL. In order to achieve the breakdown of their substrates, GPx uses reduced glutathione (GSH) as the cofactor for hydroperoxide reduction, producing oxidized glutathione as the final product (GSSG). In order to visualize this breakdown and quantify GPx activity, this assay has been adapted to measure the consumption of reduced NADPH by GR to replenish GSH from the GPx by-product, GSSG. By this method, measured decreases in NADPH is proportional to GPx activity, which is monitored at λ = 340 nm for 5 minutes at 20 second intervals in optically clear microtiter 96-well plates. Three sets of reference wells were run to account for: degradation of assay substrates over time (no coral sample with substrates), non-specific oxidation of NADPH in this assay (no H_2O_2 or CHP), and endogenous levels of substrate in coral tissue samples (coral sample with no substrate). Assay reagents consisted of: 100 mM potassium phosphate buffer (pH 7.0; 130, 140,

110, and 120 µL/well for reference, no H_2O_2, coral sample, and blank wells, respectively), 20 mM NaN_3 working solution in ddH_2O (10 µL/well), 100 mM EDTA in ddH_2O (10 µL/well), 100 mM GSH working solution in ddH_2O (20 µL/well), 100 U/mL GR working solution in ddH_2O (10 µL/well), 1 mg/mL coral samples in 100 mM potassium phosphate buffer (pH 7.0; 20 µL/well in coral sample and blank wells only), 1.25 mg/mL NADPH working solution in ddH_2O (10 µL/well), 150 mM H_2O_2 working solution (10 µL/well in reference and coral sample wells only), and 150 mM CHP working solution (10 µL/well in reference and coral sample wells only). Upon loading plates into the spectrophotometer, plates were mixed for 5 seconds, and absorbance changes immediately read.

Western immunoblotting

Materials for running Western Immunoblotting protocols were sourced in-house and through Li-Cor, and Western Blots were read using a Li-Cor C-DiGit Blot Scanner (Lincoln, NE, USA). Antibodies for SOD-1, CAT, GPx, and GR were sourced from Santa Cruz Biotechnology (Dallas, TX, USA; sc-11407 (anti-rabbit, FL-154), sc-50508 (anti-rabbit, H-300), sc-22145 (anti-goat, N-20), and sc-32886 (anti-rabbit, H-120), respectively) and preliminary testing with *P. damicornis* found all antibodies to work in this coral species.

Preliminary range-finding experiments found that coral protein concentrations of 60 ng provided adequate resolution for visualizing protein bands for all target proteins. As such, samples were appropriately diluted to 60 ng with Laemmli loading buffer (1.5 M Tris-HCl pH 6.8, 50% v/v glycerol, 25% v/v β-mercaptoethanol, 0.35 M SDS, 1% v/v 1% bromophenol blue), boiled for 5 min at 95ºC, centrifuged for 1 min at 13,000 rcf, and then loaded into 10% polyacrylamide gels along with PageRuler Plus protein ladder (ThermoFisher Scientific); gels were then electrophoresed for 1.5 hr at 80 V.

After electrophoresis, gels were transferred into transfer buffer (25 mM Tris Base, 0.192 M glycine, 20% v/v 100% methanol, in ddH_2O) and blotting cassettes were prepared for transfer, soaking blotting paper and sponges in chilled transfer buffer, while 0.2 µm polyvinylidene difluoride (PVDF) membranes were activated in 100% methanol. Transfer occurred using the immersion transfer method at 4ºC overnight at 40V. Protein transfer and loading evaluation were confirmed with Coomassie blue R-250 and Ponceau S, respectively; before primary antibody (1º ab) incubation.

For protein detection, membranes were rinsed in ddH_2O three times, and then rinsed in 1X Phosphate Buffered Saline with Tween 20 (PBST, pH 7.4, 0.14 M sodium chloride, 1.34 mM potassium chloride, 10.14 mM disodium phosphate, 1.76 mM monopotassium phosphate, 0.05% v/v Tween-20, in ddH_2O) for 5 min. Membranes were then immersed in Ponceau S solution (SigmaAldrich, St. Louis, MO, USA) for 5 min. Membranes were then de-stained in gel storage solution (5% v/v glacial acetic acid, in ddH_2O) for two 5 min washes, changing solution each time. Membranes were then blocked in 5% milk for 1 hr. Following the blocking process, membranes were washed for 15, 10, 5, and 5 min in PBST, changing the PBST each time. Primary antibodies (1º ab) were then prepared at 1:1000 dilutions, and membranes were incubated protein side up with their respective 1º ab overnight at 4ºC on a rotary table. The next day, membranes were washed in PBST for 15, 10, 5, and 5 min, changing PBST each time. Secondary antibodies (2º ab) bovine anti-goat (IgG-HRP, sc-2350) or goat anti-rabbit (IgG-HRP, sc-2004) were added (1:2000 dilution) to membrane blotting boxes, and membranes were incubated on a rotary table for 2 hr at room temperature. After 2º ab incubation, membranes were again rinsed for 15, 10, 5, and 5 min in PBST, changing PBST with each wash, and then prepared for visualization. A Li-Cor C-Digit Blot Scanner was utilized to read membranes. Using 1:1 WesternSure ECL Substrate for 1.5 min (1 part Luminol Enhancer Solution and 1 part Stable Peroxidase Solution; Li-Cor). Using forceps, membranes were then placed protein side down on the blot scanner and read for 12 min.

If being stripped for the next 1º ab, membranes were placed in strong stripping buffer (100 mM β-mercaptoethanol, 2% w/v SDS, 62.5 mM Tris-HCl pH 6.8, in ddH_2O) in an incubator set to 55 ºC for 5 to 10 min. After stripping, membranes were rinsed 3 times with ddH_2O, washed twice for 5 minutes in PBST, blocked in 5% milk for 20 min, followed by an additional 3 ddH_2O washes and subsequent 15, 10, 5, and 5 min in PBST. Membranes were then incubated overnight following the aforementioned 1º ab protocol.

Statistical analyses

Statistical data analyses were conducted using Prism 7.03 (GraphPad Software, La Jolla, CA, USA). Data normality was evaluated using the D'Agostino and Pearson Omnibus normality test. Data were then run through one-way analyses of variance (ANOVA) with Tukey's post-hoc test to elucidate significant variances between sample periods; $p < 0.05$, $\alpha = 0.05$, CI = 95%.

Waveform analyses were run to confirm sinusoidal cycling of enzyme activity with respect to moon phase and determine peaks and troughs in enzyme activity under reproductive cycling. These data elucidate biological minima and maxima for enzyme activity, where data below or above such values illustrate suppression or induction of enzyme activity by external factors ("perfect fit" of waveform models was determined by frequency = 1). Residual plots were constructed to confirm error was random.

Results

Enzymatic activity assays

Catalase enzyme kinetic assay analyses displayed a notable trend on enzyme activity cycling over moon phase cycles (Fig 2). Although significant variations in enzyme activity were only observed between July Full moon and August ¼ moon sampling periods ($p = 0.0177$, CI = 95%), CAT activity follows a general sinusoidal trend, with activity peaking during the new and ¼ moon phases. Resultant information from waveform analyses suggest that baseline reproductive CAT activity is 52.94 ± 0.23 mmol/min/mg protein (amplitude = 12.98 ± 0.33 mmol/min/mg protein, frequency = 1.117). As such, peak biological CAT activity is 65.92 mmol/min/mg protein, while minimum calculated biological CAT activity fitting this model is 3.99 mmol/min/mg protein. No significant variations in enzyme activity were observed during the acute sampling timeline ($p = 0.2374$, CI = 95%; Fig 3). This was expected, however, as there was no significant difference between August ¼ and full moon collections.

Glutathione reductase activity reflected similar trends as CAT assays, where new and ¼ moon phases harbored higher enzyme activity than comparative full and ¾ moon cycles (Fig 4). Activity of GR was significantly higher during July new and ¼ moon and August ¼ moon than those values from full and ¾ moon collection periods ($p < 0.0001$, CI = 95%). Waveform analyses defined baseline biological GR activity as 9.55 ± 1.02 nmol/min/mg protein (amplitude = 3.09 ± 1.37 nmol/min/mg protein). Maximum and minimum biological GR activity were defined as 12.64 and 6.46 nmol/min/mg protein, respectively; wave fit, however, was not strong (frequency = 4.83). Glutathione reductase activity was found to significantly decrease from day 1 to day 5 of acute sampling following the August ¼ moon ($p = 0.0189$, CI = 95%, Fig 5).

Se-independent GPx activity was found to have significant peaks in activity during both July and August ¾, and August full moon phases ($p = 0.0001$, CI = 95%, Fig 6). Inverse to the

trends of CAT and GR activity with relation to moon phase cycle, GPx was found to follow a sinusoidal activity curve, with peak activity occurring opposite to *P. damicornis* peak reproductive output. Waveform analysis defined baseline Se-independent GPx activity as 3.28 ± 0.57 nmol/min/mg protein (amplitude 2.07 ± 0.82 nmol/min/mg protein). Maximum and minimum biological Se-independent GPx activities were 5.35 and 1.21 nmol/min/mg protein, though wave fit was not strong (frequency = 1.72). When analyzing values for acute variations in GPx activity following the August ¼ moon, activity significantly increased in the days following the ¼ moon phase (Fig 7). However, only activity of August ¼ moon day 3 samples were found to be significantly different than other collection time points, having significantly higher activity than those collected on day 1 of the August ¼ moon ($p = 0.031$, CI = 95%). Interestingly, Se-dependent GPx activity was negligible or not detectable in corals from moon phase and acute collections ($p > 0.05$, CI = 95%, Figs 8 and 9, respectively). Selenium-dependent GPx did not fit waveform analyses due to multiple zeroes for enzyme activity data.

Conversely values for SOD activity, which is inversely proportional to the degree of cytochrome c oxidation over time, showed significantly higher activity during the July full moon cycle versus collection periods throughout the sampling cycle ($p = 0.0454 – 0.0002$, CI = 95%, Fig 10). This would set SOD activity as being highest following the reproductive peak of *P. damicornis*. Data showed good fit for waveform analysis (frequency = 0.91), defining baseline biological SOD activity as 139.50 ± 6.23 mmol/min/mg protein (amplitude = 15.42 ± 8.47 mmol/min/mg protein). Maximum and minimum biological SOD activity is 154.92 and 124.08 mmol/min/mg protein. Acute sampling analysis displayed day 5 of the August ¼ moon as having significantly higher SOD activity versus days 3 and 4 ($p = 0.0351$ and $p = 0.0004$, respectively, CI = 95%, Fig 11). However, day 5 SOD activity was not significantly different than measured activity from day 1 and day 2 ($p > 0.05$, CI = 95%). What is more, day 2 activity was significantly higher than that calculated during day 4 ($p = 0.0224$, CI = 95%), suggesting that SOD activity dropped significantly before increasing again between moon phases.

Western immunoblotting

Western blots evaluating for the presence of this suite of enzymes in coral samples confirmed the presence of SOD throughout time points, though band intensity did not appear to qualitatively vary between datasets (Fig 10 and 11). These results were not replicated for the

additional three enzymes of interest. Due to batch-to-batch variability in the polyclonal antibodies employed to evaluate CAT, GR, and GPx presence/absence in coral samples, chosen primary antibodies that worked in *P. damicornis* samples during pilot analyses did not work during final Western blot protein analyses.

Discussion

In order to develop rapid and efficient tools for detecting sub-lethal levels of stress in corals, first there is a need to define foundational changes in coral protein expression and activity patterns across normal homeostatic processes (Downs et al., 2012; Rougée et al., 2014). Without such definition, there is potential to mistake significant variations in coral health for responses to stress exposure, rather than those due in-part to normal biological processes based on the timing of coral sampling with respect to baseline (Rougée et al., 2006). For example, there would be great importance and versatility in the use of CAT, GR, SOD, and GPx for defining ROS-induced stress in coral animals, which aids in better defining such information as seasonal stress variations, xenobiotic impacts, and thermal stress limitations in corals (Downs & Downs, 2007; Griffin, Bhagooli, & Weil, 2006; Higuchi, Fujimura, Arakaki, & Oomori, 2008; Lesser, 1996; Liñán-Cabello, Flores-Ramírez, Zenteno-Savin, et al., 2010). Work to characterize potential basal levels of activity of these enzymes will benefit the coral conservation biology community and aid in improving experimental design by accounting for background levels of stress flux due to innate biological processes. Further, results from this study help bolster this effort to assess reproductive baselines in antioxidant enzyme capacity, as this study found activity values significantly varied in relation to reproductive cycling for CAT, GR, SOD, and GPx. Effectively, this study contributes to reproductive biology as well as conservation and toxicology.

Similarly to the findings of Ramos et al. (2011) in *S. siderea*, a significant peak in CAT activity was observed with relation to peak reproduction in *P. damicornis* (Fig 2). Though acute sampling did not detect day-to-day changes in activity values following peak planulation in August (Fig 3), August ¼ moon (peak reproductive output) CAT activity was significantly higher than that of July full moon (off-peak reproductive output) activity values ($p = 0.0177$, CI = 95%). Greater, waveform analysis data points to well-defined cyclical activity based on the lunar phase, with maximum biological CAT activity occurring during the ¼ moon cycle. Unfortunately, batch-to-batch variability in the specificity of polyclonal antibodies obscured any

ability to evaluate for CAT presence through western blotting. Antibodies that were found to work in *P. damicornis* during the preliminary study data failed to work in corals the following year using fresh but different batches of antibodies from the commercial supplier. This issue is one arising from the lack of coral-specific antibodies and our reliance on cross-species reactivity between common epitopes, rather than a coral-targeted epitope. Additionally, inherent variations in batches during the development of polyclonal antibodies may have caused non-detection. Nonetheless, the implications of these findings for CAT activity are in accordance to what has been observed in other coral species and organisms: reproduction is a process during which endogenously generated ROS are produced in *P. damicornis*. Further, it must be considered that there exist biological minima and maxima for CAT activity relevant to the understanding of induction or suppression of this enzyme. Based upon the reported results, investigators are advised that only detected activity outside the range of 39.96 – 65.92 mmol/min/mg protein likely constitute as responses to external stimuli. Hence consideration of reproductive cycling, when surveying and comparing coral populations for variability in antioxidant enzyme activity is necessary to prevent confounding data (Agarwal et al., 2005, 2006; Fujii et al., 2005; Ramos et al., 2011; Rougée et al., 2014).

The finding that significant increases in GR activity are associated with reproductive peaks ($p = 0.0001$, CI = 95%, Fig 4) provides further evidence for the need to consider reproductive time points when using antioxidant enzymes as biomarkers for oxidative stress evaluation. In conjunction with these findings, results illustrating significant decreases in GR activity over the 5-day acute sampling period provide better resolution in identifying the rate at which antioxidant enzyme activities can significantly change over natural *P. damicornis* brooding cycles ($p = 0.0162$, CI = 95%, Fig 5). Unfortunately, there again was no clear pattern observed through western blotting, as band intensity was poor in all samples, making relative quantification difficult. Despite this, results of GR activity assays provide useful insight into the replenishment of the powerful antioxidant, reduced-glutathione, under reproductive pressures. Although wave-fit of GR activity data was not strong, peak and trough data for enzyme activity help guide future analyses towards understanding general trends in naturally cycling activity levels in this coral species. These findings suggest that under reproductive peaks, enzymes are utilizing glutathione to reduce ROS to less reactive forms at a more rapid pace. Such evidence

would propose that enzymes, such as GPx would display peaks in activity during reproduction, accordingly. However, this was not observed in sampled colonies.

The enzyme Se-independent GPx displayed significantly greater activity during full and ¾ moon, rather than new and ¼ moon phases (Fig 6). Wave-fit data again suggest that we cannot reliably define cutoffs for internal versus environmental effect certainty. Yet, findings of activity maxima and minima during reproductive peaks and troughs will guide future study design employing Se-independent GPx in *P. damicornis* stress response detection. However, unlike Se-independent GPx, Se-dependent GPx was not significantly active, as activity assays found little to no detectable activity during the full study period (Figs 8 and 9). Further, in western blots examining Se-dependent GPx-1, bands were again poorly defined, which suggests that either Se-dependent GPx is not expressed in *P. damicornis* or that the chosen antibodies were poor in this species. This is also interesting, as preliminary pilot work utilizing heavily stressed *P. damicornis* samples displayed defined bands relating to GPx-1 presence. It is possible that associated ROS production during reproductive peaks is not a strong enough driver to elicit synthesis of the Se-dependent form of GPx, or that enzymes, such as CAT are favored as primary responders to low-levels of ROS within coral tissues. Previous studies analyzing GPx activity during stress exposure and seasonal changes in corals belonging to the genus *Pocillopora* have confirmed GPx activity and presence (Downs et al., 2012; Liñán-Cabello, Flores-Ramírez, Zenteno-Savin, et al., 2010; Vijayavel et al., 2012). These findings are perplexing, as activity patterns inversely mirror those expressed in both CAT and GR. Further, it would be expected that significant increases in GR activity would be proportional to that of GPx enzymes, as the two enzymes work in apposition. Additionally, while GPx-1 is a widely expressed GPx isozyme commonly located within cellular cytosol, mitochondria, and nucleus, it is possible that corals employ different forms of this enzyme as an adaptive function of their cellular detoxification (Margis, Dunand, Teixeira, & Margis-Pinheiro, 2008). Such variation in isozyme utilization has been described in studies of other marine invertebrates, including corals, with respect to their response to hypoxia and anaerobic respiration (Eberlee, Storey, & Storey, 1983; Fields, 1983; Fields, Eng, Ramsden, Hochachka, & Weinstein, 1980; Murphy & Richmond, 2016; Plaxton & Storey, 1982). Our findings for Se-independent GPx peak activity, are supported by the work of Rougée et al. (2014), wherein the antioxidant enzyme glutathione-s-transferase, which also utilizes reduced glutathione for pro-oxidant detoxification, and the UDP-glucuronosyltransferase

family expressed significantly higher activity 2 weeks following planulation in *P. damicornis*, rather than during peak planula release.

Building upon trends observed for GPx activity and additional antioxidant enzymes analyzed by Rougée et al. (2014), SOD activity was found to retain a small, but significant increase during the July full moon phase ($p = 0.0005$, CI = 95%, Fig 10) and acute sampling following the August ¼ moon also found activity to significantly vary ($p = 0.0009$, CI = 95%, Fig 11). This variation highlights potential acute day-to-day shifts in SOD activity unrelated to reproductive cycling. Supplementary investigations of daily shifts in SOD activity over monthly cycles would help clarifying if detected significant decreases in SOD activity are attributable to variations in environmental conditions during sampling, are primarily driven by reproductive peaks. Further, waveform analyses aided in defining strong cyclical trends in SOD activity with relation to reproductive troughs. Well-defined maximum and minimum values for SOD activity instruct future analysis focused in defining significant changes in coral health in response to external pressures. Additionally, relatively low amplitude in SOD activity cyclicity versus CAT activity suggests that the activity of SOD is more tightly regulated, such that this enzyme may serve a more specialized function during high ROS load versus that of CAT, which may have more diverse biological applications in ROS detoxification. In contrast to immunoblotting results for CAT, GR, and GPx, Western blots for SOD confirmed enzyme presence across all sampling points. This aids in validating activities of SOD obtained through enzyme assays and confirmed that assays were effectively designed to evaluate for activity in these samples.

These results highlight avenues for continuing studies, as greater investigation into the interplay of ROS generation and detoxification during coral reproduction allude to significant inherent stress thresholds in corals. Now that these data exist, demonstrating that coral in the field are undergoing cyclical variations in enzyme activity with relation to reproduction, there is a need to replicate these results in a controlled laboratory setting. This will allow us to control for environmental pressures, enhancing data resolution and expanding the potential to include additional monthly replicates. Although environmental factors were controlled as much as possible by experimental design (collection times, tides, and sampling around weather anomalies), additional validation of these findings in a controlled setting, would be a natural progression with respect to this research. This work, also highlights deficiencies in our capacity to expand our examination of coral proteomics, because limitations exist in our capability to

confirm protein presence – it is possible that High-Resolution Mass Spectrometry (such as with a Q-trap) might be a more fruitful avenue, and provide absolute mass determination. This would allow for greater flexibility to diagnose stress biomarkers through enzyme assays, independently confirmed by mass detection of enzyme proteins, that would aid in quantitatively and qualitatively evaluating enzyme variation.

Understanding the influence of reproduction on various biomarker enzymes in coral remains a poorly characterized field that merits expansion. The enzymes employed in this study have been widely applied to evaluate the effect of many abiotic stressors on coral health (Flores-Ramírez & Liñán-Cabello, 2007; Higuchi et al., 2008; Liñán-Cabello, Flores-Ramírez, Zenteno-Savin, et al., 2010; Richier et al., 2003; Verma, Mehta, & Srivastava, 2007; Yakovleva, Bhagooli, Takemura, & Hidaka, 2004). However, failing to consider reproduction as a source of inherent variation in ROS-induced stress responses presents a potential confounder for studies using these biomarkers by potential mischaracterization of natural fluctuations in antioxidant biomarkers as stress responses.

Conclusions

The findings of this study illustrate significant changes in the activities of CAT, SOD, GPx, and GR in response to reproductive cycling. These data demonstrate that peaks in the activities of these enzymes correlate with reproductive peaks and troughs over monthly planulation cycles. Due to their value as bioindicators of oxidative stress, our findings demonstrate the importance of determining endogenous cycling of oxidative enzymes tied to reproduction. Such baseline data tied to homeostasis helps eliminate confounding factors in studies analyzing the impact of oxidative stress on this species. These results also present greater impetus for future studies elucidating the effects of oxidative stress on reproduction and the overall health of other brooding, and perhaps broadcast spawning, coral species. Molecular tools such as those presented here provide critical data on cause-and-effect relationships between putative stressors and coral health which can be used to guide and evaluate the effectiveness of management and mitigation measures designed to protect coral reefs and those who depend on these magnificent ecosystems.

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Due to the location of this research and cultural ties of the first author, Hawaiian ‘oli, or chants, were integrated into the collection protocol. Without a written language, Hawaiians employed ‘oli as a means of passing down knowledge in the form of orally communicated genealogies, stories, and protocols for interacting with specific daily or ceremonial practices, among other things. Prior to each collection, “E Hō Mai” and “Nā ‘Aumākua” were chanted to ask for knowledge and permission to enter the collection site, while “‘Oli Mahalo” was chanted following each collection to both signify the end of the sampling period and give thanks for the coral taken.

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Figure 1

Site map denoting locations of the 6 *Pocillopora damicornis* colonies of interest in this study.

Sampled colonies were distributed over a 60 m transect in southern Kāneʻohe Bay, Oʻahu, Hawaiʻi. Photo credit: Google Earth.

**Note: Auto Gamma Correction was used for the image. This only affects the reviewing manuscript. See original source image if needed for review.*

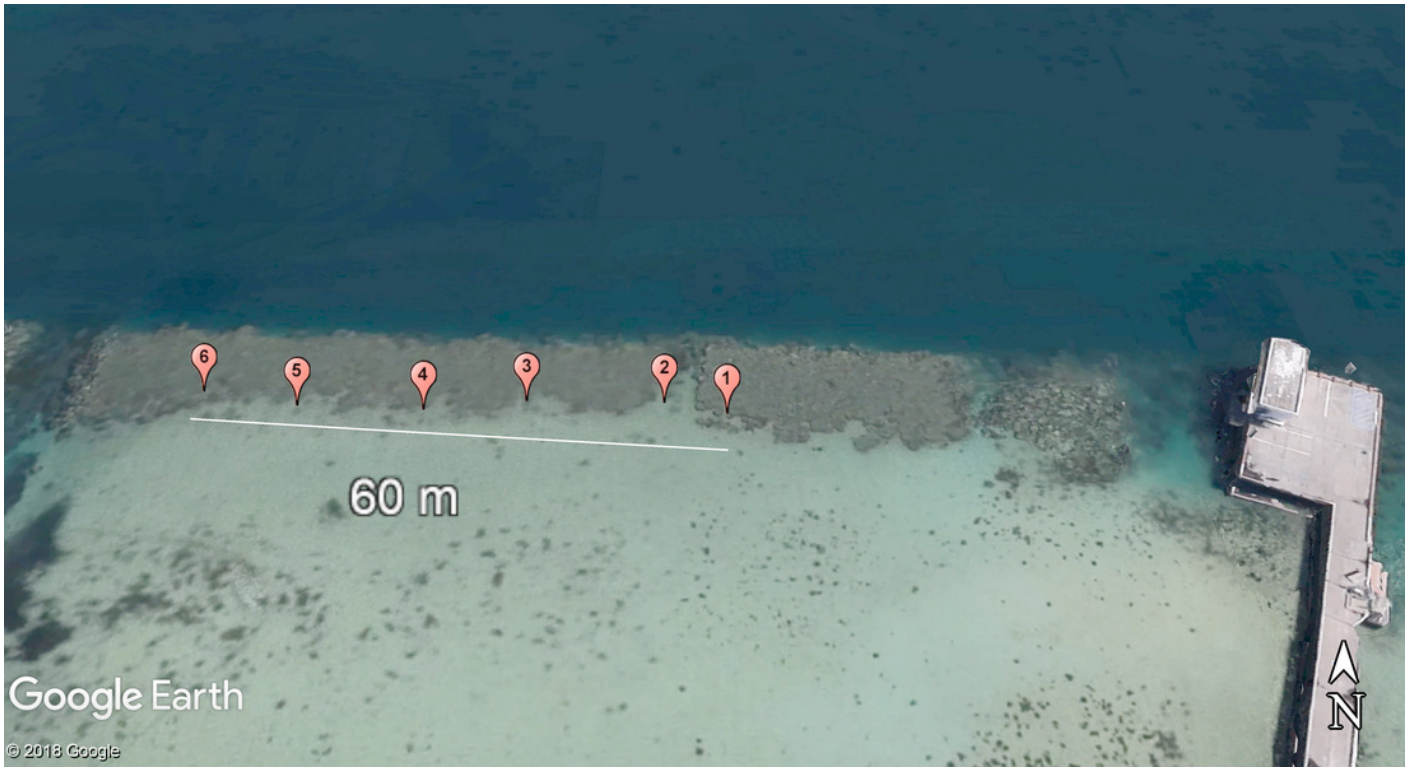

Figure 2

Catalase activity in mmol of H_2O_2 metabolized/min/mg protein versus moon phase cycle.

Bars represent mean ± SD. Samples collected during Aug ¼ moon expressed significantly higher activity than those collected during the July full moon phase (p = 0.0177, CI = 95%).

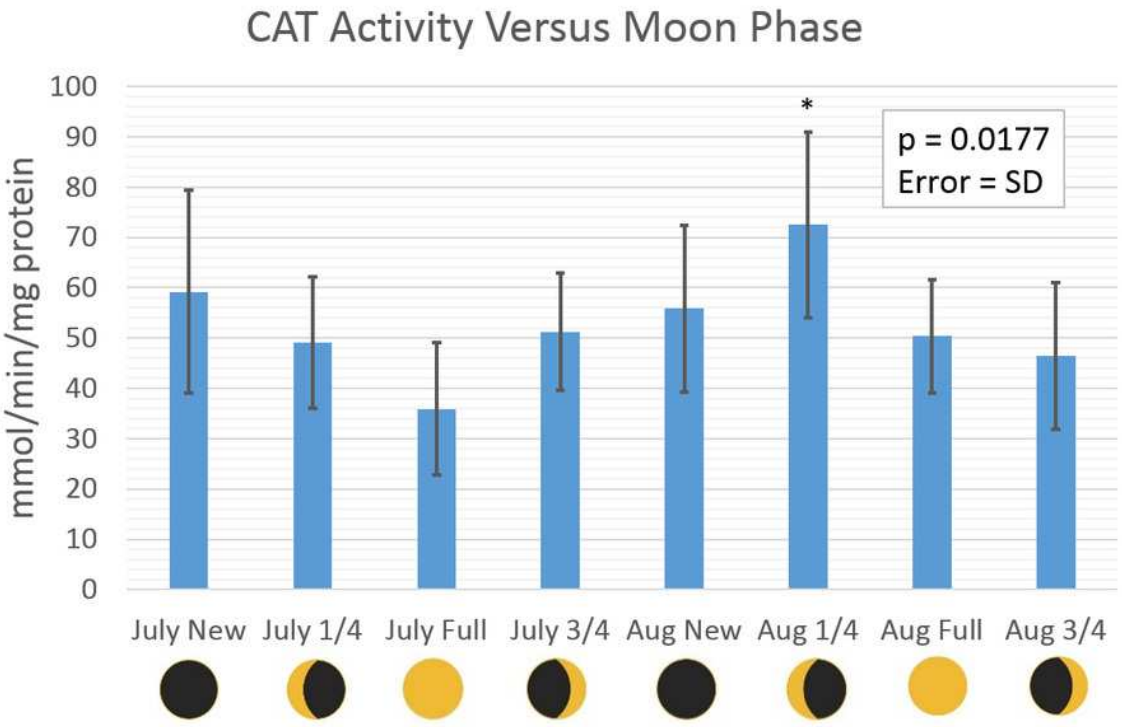

Figure 3

Acute tracking of CAT activity characterized by the consumption of H_2O_2 mmol/min/mg protein versus time following peak reproduction (¼ moon phase).

Bars represent mean ± SD (p = 0.2374, CI = 95%).

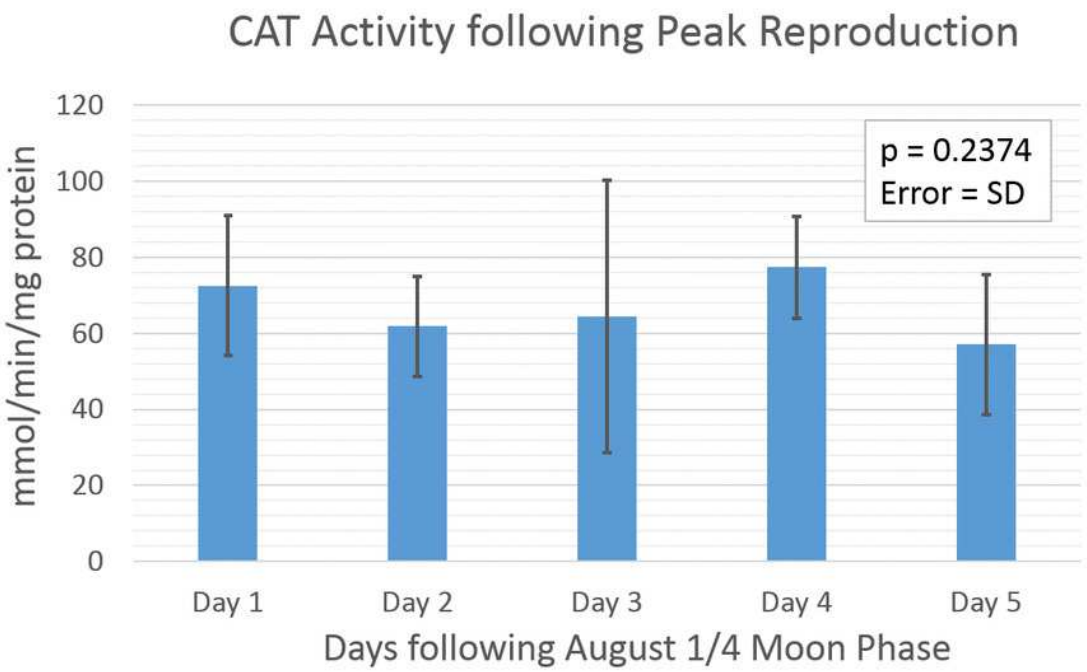

Figure 4

Glutathione reductase activity in nmol of NADPH metabolized/min/mg protein versus moon phase cycle.

Bars represent mean ± SD (p = 0.0001, CI = 95%). Samples collected during the July New, ¼ and Aug ¼ moon phases displayed significantly higher enzyme activity versus those collected during non-reproductive sampling time points.

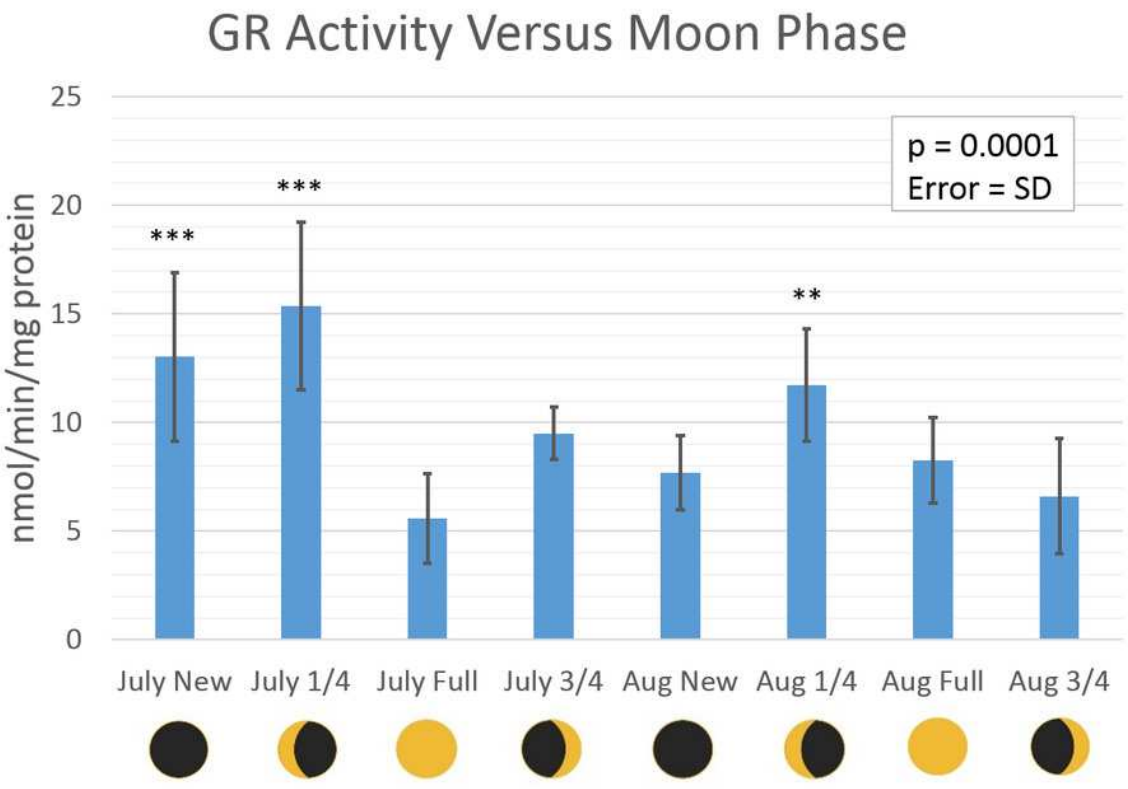

Figure 5

Acute tracking of GR activity characterized by the consumption of NADPH nmol/min/mg protein versus time following peak reproduction (¼ moon phase).

Bars represent mean ± SD (p = 0.0189, CI = 95%). Activity was found to significantly decrease over the 5 day acute survey period, as activity in samples collected on day 5 were significantly lower than those collected during day 1 of the ¼ moon phase.

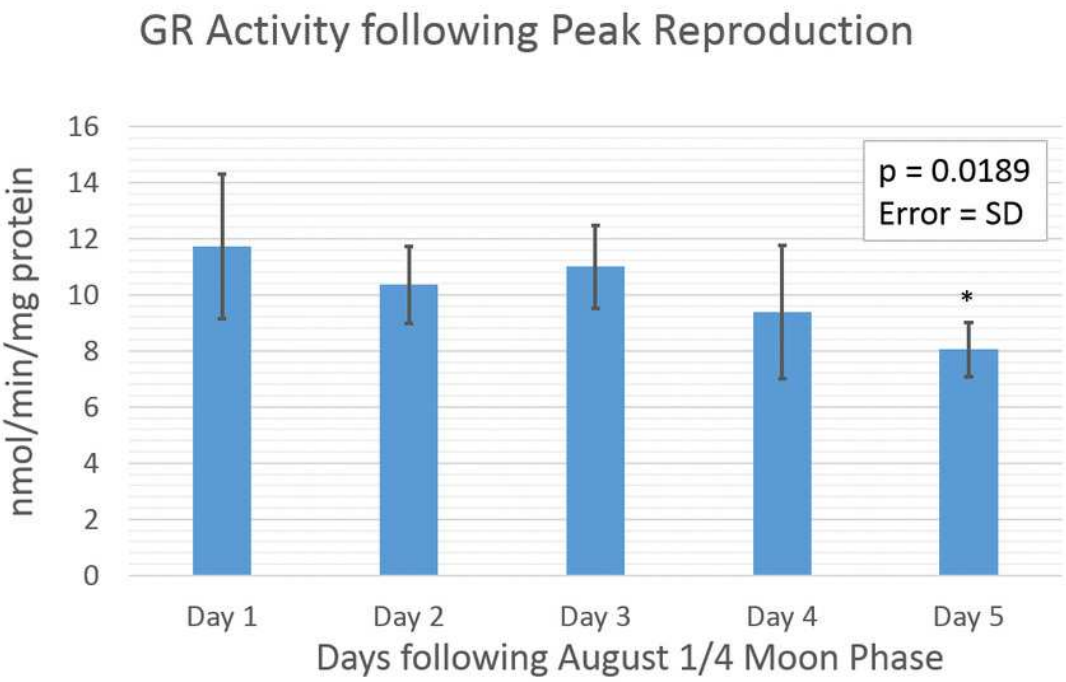

Figure 6

Se-independent GPx activity characterized by the consumption of CHP nmol/min/mg protein versus moon phase cycle.

Bars represent mean ± SD (p = 0.0001, CI=95%). Samples collected during the July ¾ and Aug full, ¾ moon phases displayed significantly higher enzyme activity versus those collected during reproductive sampling time points.

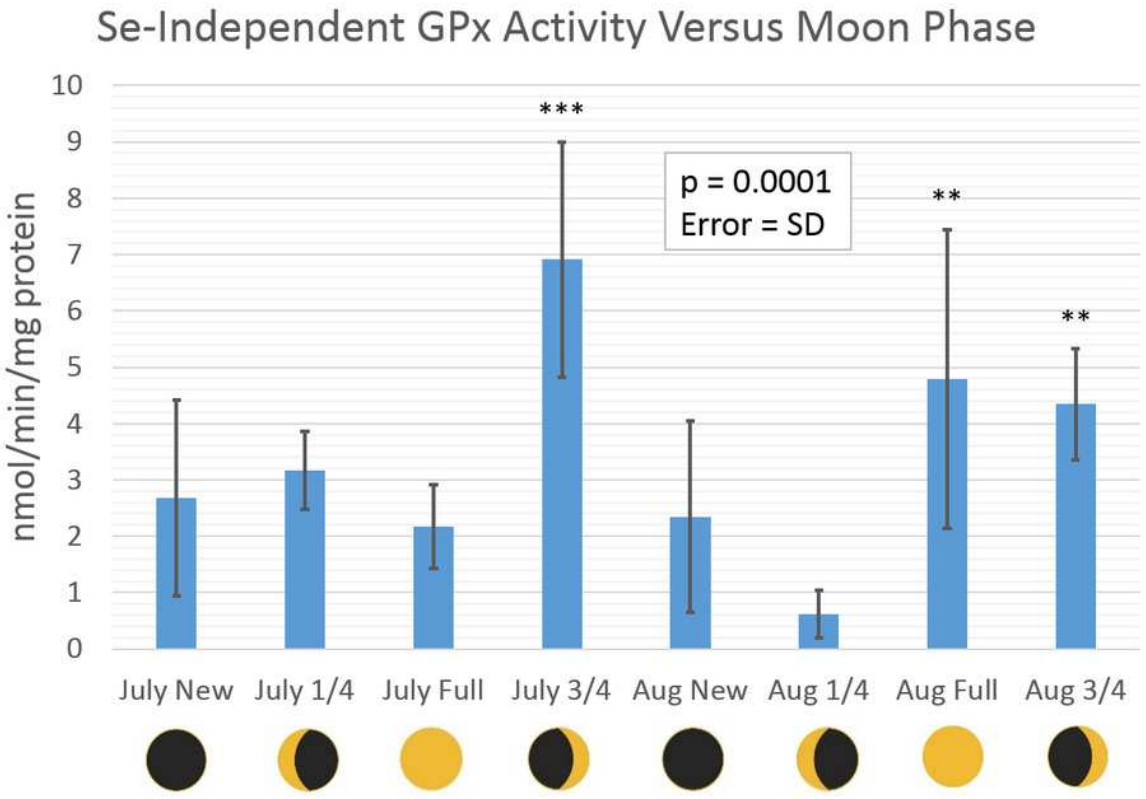

Figure 7

Acute tracking of Se-independent GPx activity characterized by the consumption of CHP nmol/min/mg protein versus time following peak reproduction (¼ moon phase).

Bars represent mean ± SD (p = 0.031, CI = 95%). Samples collected during day 1 of the ¼ moon phase were found significantly lower in activity versus those samples on day 3.

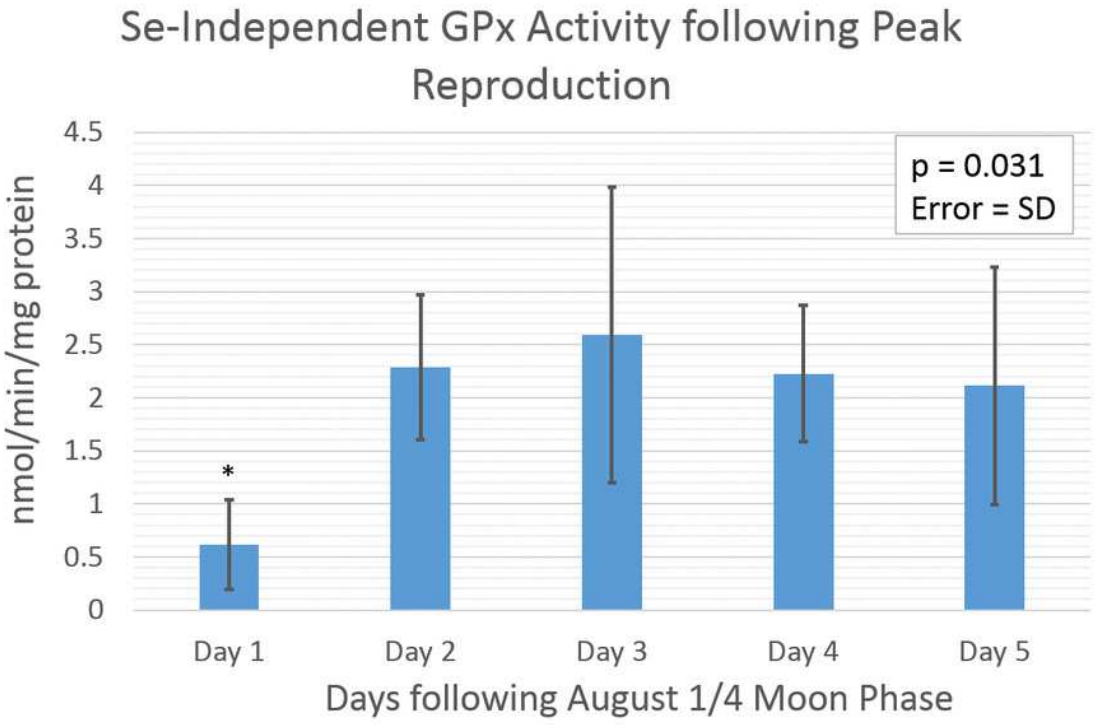

Figure 8

Se-dependent GPx activity characterized by the consumption of H2O2 nmol/min/mg protein versus moon phase cycle.

Bars represent mean ± SD (p = 0.4502, CI=95%).

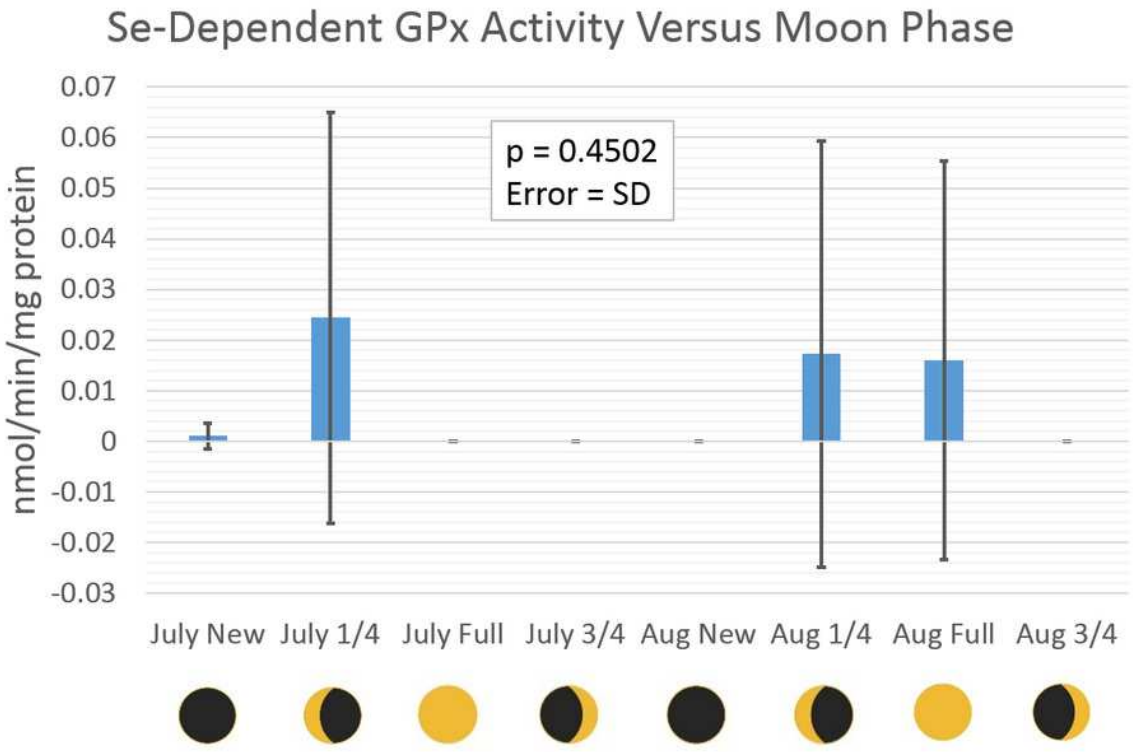

Figure 9

Acute tracking of Se-dependent GPx activity characterized by the consumption of H2O2 nmol/min/mg protein versus time following peak reproduction (¼ moon phase).

Bars represent mean ± SD (p = 0.5, CI = 95%).

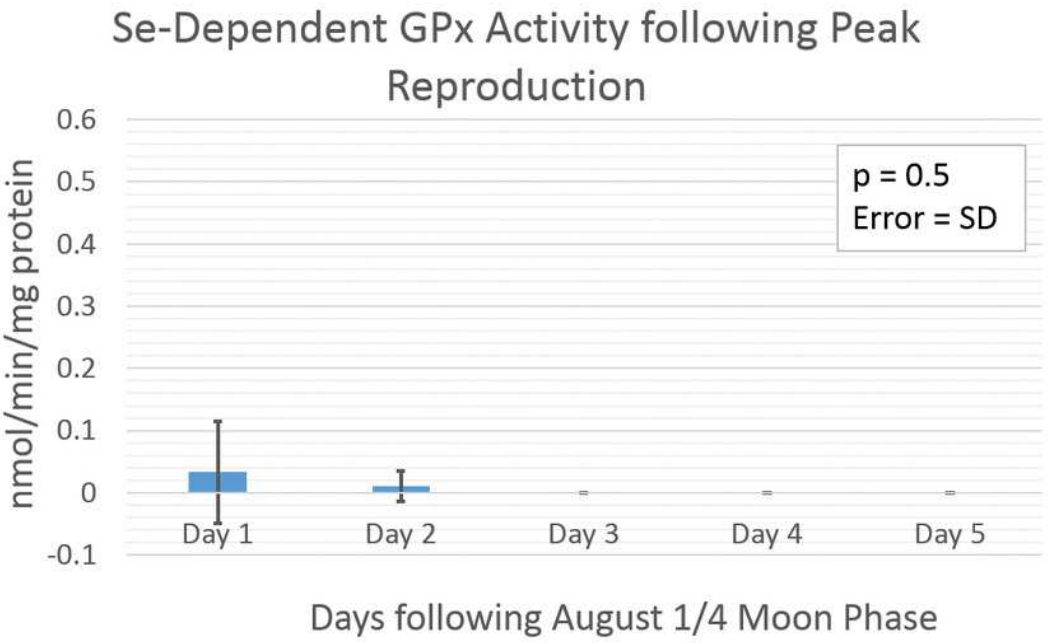

Figure 10

Superoxide dismutase activity characterized by inhibition of cytochrome c reduction mmol/min/mg protein versus moon phase cycle.

Lower cytochrome c metabolism correlating to higher SOD activity. Activity of SOD was significantly higher than July new and ¼ moon phases; bars represent mean ± SD (p = 0.0005, CI = 95%).

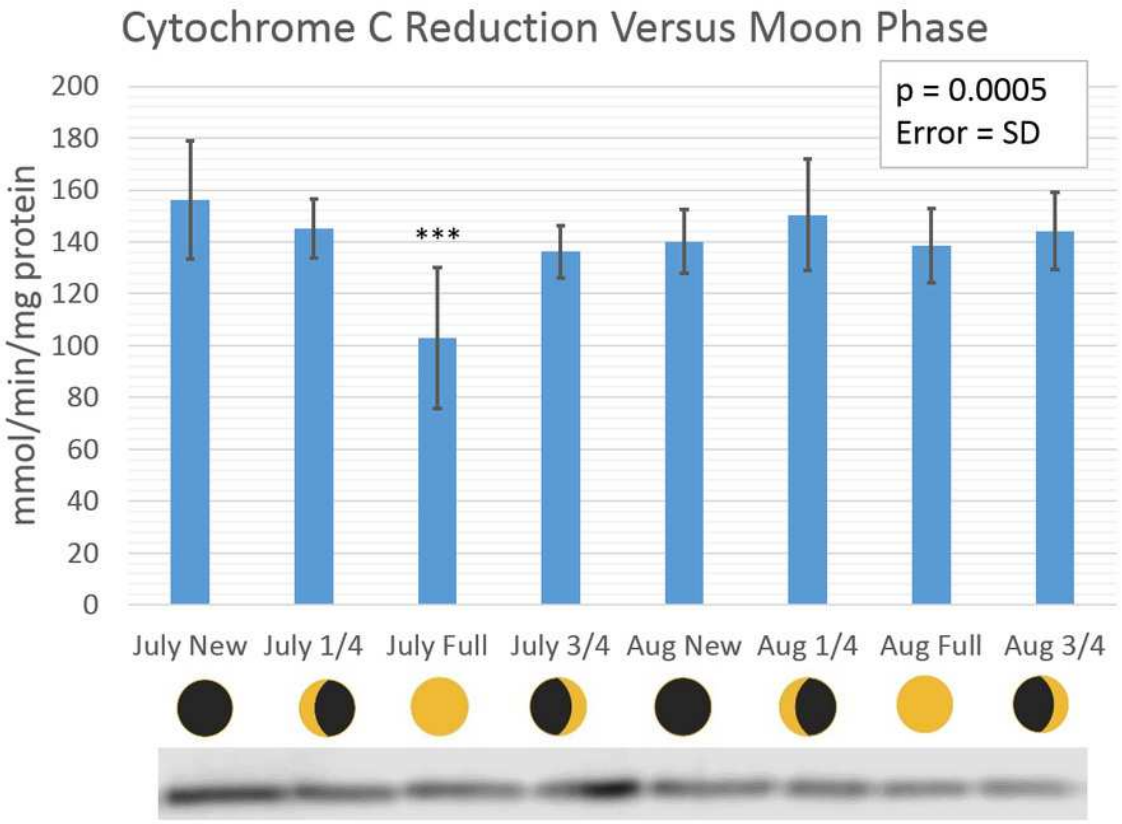

Figure 11

Acute tracking of SOD activity characterized by the inhibition of cytochrome c reduction mmol/min/mg protein versus time following peak reproduction (¼ moon phase).

Bars represent mean ± SD (p = 0.0009, CI = 95%). Samples collected during day 1 of the ¼ moon phase were found significantly lower in activity versus those samples on day 3.