

RESEARCH ARTICLE

Vitellogenin offsets oxidative costs of reproduction in female painted dragon lizards

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ABSTRACT

Vitellogenesis ('yolking' of follicles) is a bioenergetically costly stage of reproduction requiring enlargement of the liver to produce vitellogenin (VTG) yolk precursor proteins, which are transported and deposited at the ovary. VTG may, however, serve non-nutritive anti-oxidant functions, a hypothesis supported by empirical work on aging and other life-history transitions in several taxa. We test this hypothesis in female painted dragon lizards (*Ctenophorus pictus*) by examining covariation in VTG with the ovarian cycle, and relative to reactive oxygen species (ROS) including baseline superoxide (bSO). Plasma VTG decreased prior to ovulation, when VTG is deposited into follicles. VTG, however, remained elevated post-ovulation when no longer necessary for yolk provisioning and was unrelated to reproductive investment. Instead, VTG was strongly and positively predicted by prior bSO. ROS, in turn, was negatively predicted by prior VTG, while simultaneously sampled VTG was a positive predictor. These findings are consistent with the hypothesis that VTG functions as an anti-oxidant to counteract oxidative stress associated with vitellogenesis. The relationship between bSO and VTG was strongest in post-ovulatory females, indicating that its function may be largely anti-oxidant at this time. In conclusion, VTG may be under selection to offset oxidative costs of reproduction in egg-producing species.

KEY WORDS: Costs of reproduction, Life history trade-off, Lizard, Reactive oxygen species, ROS, Superoxide, Oxidative stress

INTRODUCTION

Reproductive energy expenditures are orders of magnitude higher in females than males across vertebrate and invertebrate taxa (Hayward and Gillooly, 2011), requiring dramatic increases in both basal metabolic rate and the biosynthesis of macromolecules. An imbalance in the ability to detoxify the oxidative by-products of these processes (e.g. oxidative stress) may be a leading cost mediating female life-history trade-offs between reproduction, growth and survival (Kirkwood, 1977; Monaghan et al., 2009; Metcalfe and Alonso-Alvarez, 2010; Metcalfe and Monaghan,

2013). As females increase investment into reproduction, they commonly exhibit correlated increases in oxidative stress, lending strong empirical support to the oxidative costs of reproduction hypothesis (Blount et al., 2015 and references therein). However, the transition from non-breeder to breeder can be associated with a contradictory decline in oxidative stress, even though this shift entails concomitant increases in metabolism (Speakman and Garratt, 2014; Blount et al., 2015).

Several hypotheses have been proposed to explain these paradoxical findings (reviewed in Alonso-Alvarez et al., 2017). The oxidative shielding hypothesis (Blount et al., 2015) predicts up-regulation of physiological protective mechanisms *prior* to the onset of reproduction to 'shield' both females and, crucially, developing offspring from the oxidative stress of reproduction. Alternatively, the hormesis during reproduction hypothesis (Costantini, 2014) posits that exposure to oxidative stress early in the reproductive phase should up-regulate a compensatory 'hormetic' response to offset potential costs of oxidative stress. These hypotheses rely on up-regulation of innate anti-oxidant systems or physiological suppression of reactive oxygen species (ROS), such as basal superoxide (bSO). The search for an oxidative shielding mechanism is in its infancy, although research into up-regulation of potential shielding molecules specific to lactation provides a promising way forward in mammalian species (Hyatt et al., 2017). The animal kingdom is dominated, however, by egg-producing species, almost all of which share a family of vitellogenin (VTG) yolk precursor proteins that have intriguing accessory functions in anti-oxidation and aging (reviewed in Li and Zhang, 2017).

In ectotherms, vitellogenesis, the process of 'yolking' follicles to produce viable eggs, is one of the most costly phases of female reproduction, requiring systemic changes in resource use, increases in metabolism, and associated shifts in basking behaviors necessary for thermoregulation, which expose females to increased risk of predation (Shine and Harlow, 1993; Wapstra et al., 2010). Vitellogenesis accounts for approximately 30% of the metabolic demands of female viviparous snakes (Van Dyke and Beaupre, 2011), and biomarkers of oxidative stress are highest during vitellogenesis in female iguanas (Webb et al., 2019). During vitellogenesis, gonadal estradiol (E2) production stimulates both the growth of the liver and a change in hepatic protein synthesis towards the production of large nutritive VTG proteins (Callard et al., 1990). This is not a trivial shift; the liver of a vitellogenic common lizard (*Lacerta vivipara*) is as much as 225% the size of a pre-vitellogenic female (Gavaud, 1986), and in domestic hens, VTG production accounts for more than 50% of all hepatic protein synthesis (Gruber, 1972).

The selection pressures on ectothermic females to optimize reproduction–somatic maintenance trade-offs are therefore high (Isaksson et al., 2011), and non-nutritive benefits of VTG as an anti-oxidant and immune-relevant molecule may provide a physiological

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mechanism to offset simultaneous oxidative costs related to VTG production. VTG can function directly as an anti-oxidant (Li and Zhang, 2017), largely through its metal-binding capacity (Ando and Yanagida, 1999; Nakamura et al., 1999), as well as by binding to cellular membranes to improve oxidative stress tolerance (Havukainen et al., 2013), and facilitating the increased circulation of additional anti-oxidant molecules such as carotenoids (Zheng et al., 2012). In lizards, plasma VTG increases prior to egg laying, and while it dips with each ovulatory wave that corresponds to deposition of circulating content into oocytes prior to ovulation, it remains elevated throughout the breeding phase (Gavaud, 1986; Carnevali et al., 1991). Given the possible oxidative costs of VTG production, it would be advantageous to meet VTG demand with supply (Salvante and Williams, 2002), but VTG levels are often more variable than predicted by variation in individual reproductive investments (Gavaud, 1986; Carnevali et al., 1991; Salvante and Williams, 2002). This begs the question: what explains extended VTG production?

Here we examine the role of VTG in offsetting oxidative costs of reproduction in the painted dragon lizard [*Ctenophorus pictus* (Peters) Cogger], a species in which considerable information is already in place on the importance of oxidative stress for shaping life-history evolution of the sexes and male phenotypes (Tobler et al., 2011; Ballen et al., 2012; Olsson et al., 2009c,d; 2012; 2018). While male and female painted dragon lizards do not differ in circulating concentrations of bSO (Tobler et al., 2011), females suffer higher end-of-season DNA erosion than males, indicating additional female-specific oxidative costs related to reproductive investments (Olsson et al., 2012). This is further supported by a bSO increase towards ovulation (Ballen et al., 2012), as would be predicted if vitellogenesis is a source of oxidative stress. Furthermore, despite having lower levels of superoxide dismutase (SOD) than males (Olsson et al., 2012), females better withstand the erosive effects of bSO on immune responsiveness than males (Tobler et al., 2011). Two hypotheses have been proposed to explain sex differences in SOD without concurrent differences in bSO. First, male painted dragons have a relatively more active lifestyle than females, which may drive selection for higher anti-oxidation (Olsson et al., 2012). This hypothesis is unsatisfactory in light of elevated metabolic demands of reproduction in females. Alternatively, VTG may act as an anti-oxidant and enable females to obtain similar (and possibly greater) anti-oxidation compared with males with more SOD (Olsson et al., 2009d).

In support of the latter hypothesis, offspring, and to a lesser extent female bSO, decreases with increasing relative clutch size (Olsson et al., 2009d). Circulating levels of VTG may also increase with provisioning needs of a larger clutch (Garstka et al., 1982; Han et al., 2009) and decline prior to ovulation as VTG leaves the circulation and enters the follicles (as shown by Salvante and Williams, 2002). Because both VTG and the subsequently cleaved yolk vitellin proteins can be immunoprotective and anti-oxidative (Li and Zhang, 2017), both the quantity and quality of VTG (e.g. degree of phosphorylation; Zhang et al., 2017) produced by the female may have profound consequences for the risk of oxidative stress in the offspring. Indeed, the oxidative stress response to a challenge is heritable from mothers in juvenile painted dragons (Olsson et al., 2008). Thus, potential anti-oxidative functions of VTG may protect mothers as well as 'shield' offspring from ROS onslaught.

Here we examine covariation between VTG and oxidative stress in relation to ovulatory stage (vitellogenic versus post-ovulatory), time until oviposition, and reproductive investment of female painted dragon lizards. By utilizing a temporal sampling approach, we are able to reasonably extrapolate directionality in the

relationships between our metrics of oxidative stress (bSO and ROS) and VTG. We interpret these patterns in light of the oxidative shielding and hormesis hypotheses for offsetting oxidative costs during female reproduction.

MATERIALS AND METHODS

Field methods and animal husbandry

We captured adult male and female lizards by noose or hand at Yathong Nature Reserve, New South Wales (NSW), Australia (145° 35'E; 32°35'S) during September 2015 (NSW National Parks and Wildlife Service permit no. SL100352). These animals were then transported to the University of Sydney, where they were housed individually in sand-filled 330 mm×520 mm×360 mm cages under a 12 h:12 h light:dark cycle using UVB lighting. We fitted each cage with a basking lamp and a ceramic hide, allowing for behavioral thermoregulation to preferred body temperatures. We provided mealworms and crickets three times per week at approximately 10:00 h, dusting these with calcium and multivitamins (Rep-Cal phosphorus-free calcium with vitamin D and Rep-Cal Herptivite Multivitamin), and misted each cage with water once per day. All animal care procedures were conducted in accordance with University of Sydney (AEC-2013/6050) and University of Gothenburg (5.8.18-12538/2017) ethics approval, and have been utilized repeatedly on this species with great success (e.g. Olsson et al., 2009a,b).

Lizards were kept in captivity for a minimum of 1 week prior to commencement of sampling. We continuously monitored females by palpation for onset of breeding (every fourth day) and recorded the number of follicles and eggs, and the stage of reproduction as either vitellogenic (follicles present on the ovaries) or post-ovulatory (eggs present in the oviduct). Gentle abdominal palpation provides a repeatable and accurate method for detection of ovulatory stage and the number of follicles and eggs in this and other lizard species (Sinervo et al., 1992; Olsson et al., 2001, 2012). Females were then monitored daily until egg-laying. Eggs were excavated and clutch size recorded (range 1–6 eggs, average 3.5), and clutch mass weighed to the nearest 0.1 g. We then calculated a body size-corrected metric of female reproductive investment (residual clutch number) as the residuals from a regression of the total number of clutches produced during the season on snout–vent length ($F_{1,19}=6.37$, $R^2=0.25$, $P=0.02$; females produced between one and three clutches, with an average of two).

Blood sampling

We collected blood from 21 females on 25 November 2015, and again 2 weeks later on 8 December 2015. On each date of sampling, females were measured from snout to vent to nearest 1.0 mm and weighed to nearest 0.1 g (female mean±s.e.m. body mass=12.1±0.23 g, snout–vent length=7.8±0.05 cm). We collected a maximum of 100 µl whole blood from the vena angularis, following perforation with the tip of a glass capillary tube. These blood samples were divided, with one portion treated for quantification of superoxide and ROS (see below) and the remainder processed for quantification of VTG. To this later fraction of whole blood, we added 10% total volume of 20X stock solution of phosphate inhibitor cocktail (PhosSTOP: Sigma-Aldrich, St Louis, MO, USA). After centrifugation to remove red blood cells, we treated plasma with an equal volume of quench buffer, containing triethylammonium bicarbonate and 4% w/v sodium dodecyl sulfate (Sigma-Aldrich). We heated the samples to 90°C for 5 min, after which they were immediately placed in a –80°C freezer awaiting further processing.

Quantification of superoxide and ROS by flow cytometry

We diluted whole blood with nine times the volume of phosphate-buffered saline (PBS); these samples were then stored on ice and were analysed within 4 h of sampling. Samples were processed following protocols published elsewhere (Olsson et al., 2009; Friesen et al., 2017). In short, we diluted samples 50-fold with PBS, and centrifuged them at 300 g for 5 min. We resuspended the resulting pellet of cells in 100 μ l of PBS containing either no additions (unstained control), 5 μ M MitoSOX Red (MR; Molecular Probes, Invitrogen, Carlsbad, CA, USA) or 0.1 mmol l⁻¹ dihydrorhodamine 123 (DHR; Molecular Probes). These probes detect superoxide ions (MR) and any reactive oxygen species (DHR). We incubated cell samples at 37°C for 30 min, washed them with PBS by centrifugation, and placed them on ice prior to flow cytometry; 50,000 events were acquired for all samples. We used a Becton Dickinson LSRFortessa X20 (Becton Dickinson, Sydney, NSW, Australia) to perform flow cytometry with excitation at 488 nm and emitted fluorescence collected using band-pass filters of 575 \pm 13 nm (MR) and 515 \pm 10 nm (DHR). We collected and analysed data using FACSDiva version 4.0.1 (Becton Dickinson) and FloJo (version 9.1; TreeStar Inc., Ashland, OR, USA) software. We calculated the mean fluorescence for all 50,000 cells acquired using FloJo software. Our flow cytometry technique is highly consistent based on experimental assessment of repeat sampling within individuals [$r=0.97$, $P<0.0001$; see Olsson et al. (2008) for further details].

Estradiol treatment for VTG induction

Experimental treatment with exogenous 17 β -estradiol (E2) induces vitellogenesis in lizards (Gavaud, 1986), and is effective in both males and females (Heppell et al., 1995; Brasfield et al., 2002; Rey et al., 2006). We treated one non-reproductive female and one juvenile male *C. pictus* with E2 (Sigma-Aldrich) in order to determine if we could detect VTG induction using our assay procedures. Both individuals received intraperitoneal injections of 168 ng E2 per 100 μ l reptile physiological solution (NaCl 0.07%) while two control individuals (one male, one female) received 100 μ l injections of saline only (following a protocol developed for *Podarcis sicula* by Verderame et al., 2016). Injections were given every second day for a 2-week period (as per Verderame and Limatola, 2010). We collected blood samples from both individuals prior to onset of experimentation (27 October 2017: 'pre-E2') and again at the end of treatment (10 November 2017: 'post-E2').

SDS-PAGE VTG detection and quantification

We validated a universal method for VTG detection hitherto only used to examine presence/absence of VTG (Van Veld et al., 2005; Burgmeier et al., 2011; Currylow et al., 2013), but here modified to allow for VTG quantification. We utilized a combination of protein gel electrophoresis with a phosphoprotein stain to detect and quantify staining intensity of putative VTG bands, mass spectrometry to validate peptide homologies to known VTG proteins available on The National Center for Biotechnology Information (NCBI) database, and quantitative phosphoproteomics to determine if our measurement of VTG titre as relative phosphoprotein staining intensity reflects underlying protein concentration and degree of phosphorylation.

We initially diluted plasma at 1:1 with PhosStop buffer and further diluted 5 μ l sample aliquots with 200 μ l sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (1:40). We separated proteins using SDS-PAGE, loading each well of our 12% Bis-Tris protein gels (Bio-Rad, Hercules, CA,

USA) with a 10 μ l sample (containing 0.125 μ l plasma; 8–24 μ g total protein). The gels were run in MOPS buffer at 200 V for 1 h on a Bio-Rad Criterion Electrophoresis Cell. All gels contained an in-house phosphoprotein molecular weight standard [ribonuclease A (16.80 kDa); casein 2 (30.40 kDa); casein (312.40 kDa); ovalbumin (43.40 kDa); bovine serum albumin (64.90 kDa); beta-galactosidase (131.00 kDa)]. We stained gels using Pro-Q Diamond phosphoprotein gel stain (Invitrogen) using standard manufacturer protocols. In short, we fixed the gels overnight with 50% methanol and 10% acetic acid in ultrapure water, after which they were washed three times for 10 min with ultrapure water. We stained the gels for 90 min at room temperature, in the dark, and with gentle agitation. All gels were de-stained three times for 30 min in the dark with gentle agitation using a product-specific de-staining solution (Invitrogen). We ran samples in parallel utilizing SYPRO Ruby protein gel stain (Molecular Probes; standard protocol) for detection of total proteins and excision of gel bands for use in mass spectrometry analysis (see below).

We collected gel images using a molecular imager (Bio-Rad VersaDoc MP 4000) and calculated staining intensity in putative VTG bands with Bio-Rad Image Lab 5 software. We identified VTG based on (1) sexual dimorphic expression and molecular weight with expected size between 100 and 220 kDa (Byrne et al., 1989; Carnevali et al., 1991; Van Veld et al., 2005), and (2) presence of bands following expected up-regulation of VTG with E2 treatment (see above). All intensity measurements were taken after subtracting background staining levels of each relevant location on the gel (~116 and 175 kDa) in a pooled sample of plasma from six untreated male *C. pictus* run on each gel (negative control).

Mass spectrometry

We excised putative VTG bands from total protein (SYPRO Ruby-stained) gels and subjected these to nano-liquid chromatography (nanoLC) in order to verify protein identity. For our analysis we excised four bands: bands at 175 kDa in pooled female and male plasma samples, and 116 kDa bands excised from a single female and a single male. We de-stained gel bands in 25 mmol l⁻¹ ammonium bicarbonate in 50% acetonitrile (ACN), in-gel digested these bands by addition of 10 ng/ μ l trypsin (Pierce MS grade, Thermo Fisher Scientific, Waltham, MA, USA) in 25 mmol l⁻¹ ammonium bicarbonate and incubated overnight at 37°C. We inhibited trypsin by addition of 75% ACN in 2% trifluoroacetic acid (TFA), extracted peptides using 50% ACN in 0.2% TFA, and dried and reconstituted peptides in 0.1% formic acid and 3% ACN in water.

The peptides were analysed on an Orbitrap Fusion Tribrid or Q-Exactive mass spectrometer interfaced to an Easy-nanoLC (Thermo Fisher Scientific). We separated peptides using an in-house constructed analytical column packed with 3 μ m Reprosil-Pur C18-AQ particles (Dr Maisch, Ammerbuch-Entringen, Germany). We ran an increasing ACN gradient over 45 or 60 min in 0.2% formic acid and electrosprayed ions in positive mode. We performed MS scans over m/z range 400–1600 and performed MS/MS analysis in a data-dependent mode by collisional dissociation. We conducted database matching with the Mascot search engine (Matrix Science) in Proteome Discoverer version 1.4 (Thermo Fisher Scientific) using NCBI with a protein database generated towards animal VTG with 5095 entries (November 2018). As parameters, we selected MS peptide tolerance of 5 p.p.m., MS/MS tolerance 0.2 Da, tryptic peptides with one missed cleavage and variable modifications of methionine oxidation. The peptide identification was set to Mascot significance threshold of 99%.

Quantitative phosphoproteomics

Samples were processed using the filter-aided sample preparation method (Wiśniewski et al., 2009). Briefly, 25 µg of total protein from samples pre- and post-E2 treatment and 5 µg of a single sample each showing weak, medium and strong VTG phosphoprotein staining intensity using SDS-PAGE were reduced with 100 mmol l⁻¹ dithiothreitol, transferred to Nanosep 30K Omega filters (Pall Life Sciences, New York, NY, USA), repeatedly washed using 8 mol l⁻¹ urea, alkylated with 10 mmol l⁻¹ methyl methanethiosulfonate followed by double digestion using trypsin (trypsin:protein ratio of 1:100) at 37°C in digestion buffer (1% sodium deoxycholate in 50 mmol l⁻¹ tetraethylammonium tetrahydroborate). Samples were labeled with a 10-plex tandem isobaric mass tag labeling kit (90406, Thermo Fisher Scientific), according to the manufacturer's instructions. Samples were combined and sodium deoxycholate was removed by precipitation following acidification. The peptides were desalted by Pierce Peptide Desalting Spin Columns (Thermo Fisher Scientific) and a 1% aliquot was directly analysed by LC-MS/MS to serve as a control of protein level. The majority of the sample was split into two parts and subjected to phosphopeptide enrichment using either the High-Select Fe-NTA Phosphopeptide Enrichment Kit or the Pierce TiO₂ Phosphopeptide Enrichment and Clean-Up Kit (Thermo Fisher Scientific) according to the manufacturer's recommendations. Both phosphopeptide eluates were pooled, fractionated into 20 fractions using high pH reversed-phase chromatography (XBridge BEH C18, 3.0 mm×150 mm, 3.5 µm; Waters Corporation, Milford, MA, USA) with a gradient from 3 to 100% acetonitrile in 10 mmol l⁻¹ ammonium formate (pH 10.0) over 16 min and concatenated into 10 fractions.

The phosphopeptide fractions and the protein level control were analysed on an Orbitrap Fusion Tribrid mass spectrometer. Peptides were separated on a trap column (Acclaim Pepmap C18 100 µm×2 cm, 5 µm; Thermo Fisher Scientific) together with an in-house packed C18 analytical column (75 µm×32 cm, 3 µm) using an Easy-nanoLC 1200 (Thermo Fisher Scientific) with a gradient from 5 to 80% acetonitrile in 0.2% formic acid over 50 min (80 min for protein level control). MS scans were recorded at a resolution of 120,000, the most intense precursor ions were selected (top speed of 3 s) for fragmentation (collision induced dissociation 35%), and MS and MS/MS spectra were recorded in Orbitrap with an isolation window of 0.7 Da. Charge states 2 to 7 were selected for fragmentation, and dynamic exclusion was set to 60 s with 10 p.p.m. tolerance. The top five MS₂ fragment ions were selected for MS₃ fragmentation (high-energy collisional dissociation 65%) and detection in the Orbitrap at a resolution of 50,000.

Data analysis was performed using Proteome Discoverer version 2.2 (Thermo Fisher Scientific) against another Agamid lizard species, *Pogona vitticeps* (NCBI RefSeq database, version 2018-12-18, with 38,725 entries). The precursor and fragment mass tolerance were set to 5 p.p.m. and 0.2 Da. Zero missed cleavages were accepted, variable modifications of methionine oxidation, and fixed modifications of cysteine alkylation and TMT-label at N-terminal and lysine were selected. The data files from the phosphopeptide fractions were merged and database searches were performed similarly, with additional dynamic modification of serine, threonine or tyrosine phosphorylation. Reporter ion intensities were quantified in MS₃ spectra with 0.003 Da mass tolerance using S/N threshold 10 and normalized on the total protein abundance. Only values for unique peptides at a false discovery rate of 1% were used for quantification.

Statistical analysis

We first investigated sources of variation in VTG, ROS and bSO across all samples ($N=41$), combining the November and December sampling periods. Our generalized linear mixed models (GLMM; one model for ROS and bSO, two models for VTG) included all potentially influential variables: sample date, female mass and snout-vent length, bSO or ROS, VTG, ovulatory stage, number of follicles/eggs, and female reproductive investment (e.g. residual clutch number). We never analysed bSO and ROS as separate predictors in the same model as bSO is a component of ROS. We removed non-significant terms and interactions at $P>0.25$. Female identity (ID) was included as a random effect to account for repeat sampling of the same individual, although in no case did ID significantly improve model fit based on log-likelihood ratio testing (all $P>0.09$). We BoxCox-transformed bSO with optimal lambda set to -0.968 and standardized VTG, ROS and BoxCox bSO with mean set to 0 and standard deviation of 1. The resulting standardized values were normally distributed for ROS and bSO (ROS, Shapiro-Wilk $w=0.97$, $P=0.48$; bSO, $w=0.95$, $P=0.07$), but VTG was resistant to transformation and the standardized values across all 41 samples were not normally distributed ($w=0.94$, $P=0.03$). We assessed residuals from models including VTG as a predictor and they did not violate assumptions of normality or homoscedasticity. We also reinforced these analyses with accessory non-parametric Spearman rank order correlations between VTG and relevant response variables.

We subsequently examined sources of variation in VTG, ROS and bSO within November and December measurements separately (November, $N=21$; December, $N=20$) including all potentially influential variables and interactions and following a similar method of backwards predictor elimination. Our standardized values for November and December measurements of VTG, BoxCox bSO and ROS were normally distributed (all $P>0.06$) with no risk of multicollinearity between factors in our final models (all $r<0.42$ for individual factor correlations). In analysing sources of variation in VTG, we modeled the same dataset using bSO and ROS as separate predictors: we therefore adjusted our alpha values for multiple comparisons using the false discovery rate procedure (FDR; Benjamini and Hochberg, 1995). In no case did FDR correction negate significance. We performed statistics using JMP Pro 13.1.0 (SAS Institute Inc., Cary, NC, USA).

RESULTS

Our analysis follows the following axis of logic: (i) identification and verification of a quantitative VTG assay, analyses of (ii) a temporal axis of bSO and ROS variation, (iii) reproductive state-specific levels of VTG, (iv) VTG as a response to prior and current bSO and ROS, and (v) bSO and ROS as separate responses to prior and current VTG.

VTG assay validation: estradiol induction, protein quantification and mass spectrometry

We identified two dimorphic phosphoprotein bands that were variable in females and lacking in males. These are located at 175 and 116 kDa (Fig. 1) and henceforth are referred to as high molecular weight (HMW) and low molecular weight (LMW) VTG, respectively. Both HMW and LMW VTG were up-regulated via estradiol treatment: a treatment effect that was detectable with SDS-PAGE phosphoprotein staining (Fig. 1A,B) and quantitative phosphoproteomic analysis.

The proteomics analyses of our four putative VTG bands resulted in detection of strong peptide homologies to three vitellogenin-like

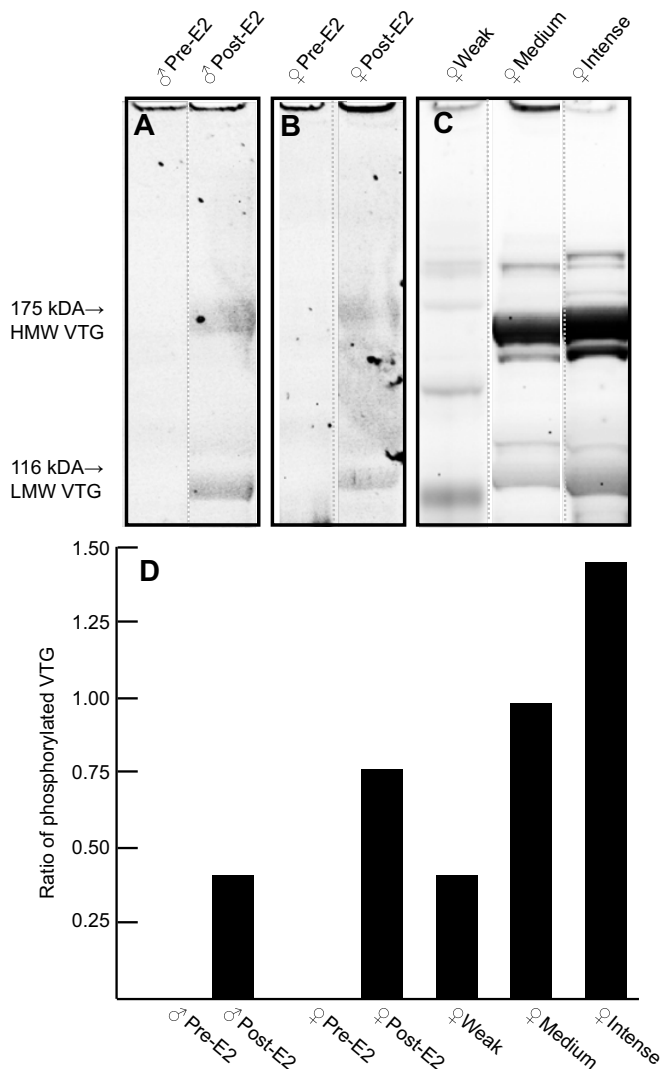


Fig. 1. Gel electrophoresis reveals two vitellogenin proteins that are up-regulated via estradiol treatment in *Ctenophorus pictus*. SDS-PAGE gel electrophoresis with Pro-Q Diamond phosphoprotein stain of plasma samples from (A) a female pre- and post-E2 treatment, (B) an E2-treated male (pre- and post-E2) and (C) three females with weak, medium and intense phosphoprotein stain intensity. Variation between sample lanes in degree of background staining and exact location of the 175 kDa (high molecular weight, HMW) and 116 kDa (low molecular weight, LMW) vitellogenin (VTG) is due to excision from three separate gels (excised lanes separated by dotted grey lines). (D) Ratio of phosphorylated versus total vitellogenin-1-like protein found in the plasma of each sample represented on the gel is shown, and indicates that Pro-Q Diamond staining intensity is an accurate measure of plasma VTG.

proteins from *Pogona vitticeps* (Table 1). We detected homologies to two VTG isoforms (VTG-1 and VTG-2), with homologies to both in our HMW and LMW VTG bands. However, the LMW band

Table 1. Mass spectrometry sequencing of high and low molecular weight vitellogenin (VTG) bands reveal significant peptide homologies to vitellogenin-like proteins in another species of Australian dragon lizard

| Protein (species) | NCBI accession no. | MW (kDa) | All samples Total unique peptides | Female no. peptides | | Male no. peptides | |
|-------------------------------------------------|--------------------|----------|--------------------------------------|---------------------|---------|-------------------|---------|
| | | | | HMW VTG | LMW VTG | HMW VTG | LMW VTG |
| Vitellogenin-1-like (<i>Pogona vitticeps</i>) | 1176330275 | 180.3 | 34 | 30 | 5 | 5 | |
| Vitellogenin-2-like (<i>Pogona vitticeps</i>) | 1176296639 | 186.5 | 38 | 32 | 25 | 4 | 2 |
| Vitellogenin-2-like (<i>Pogona vitticeps</i>) | 1176296641 | 166.5 | 5 | 3 | | | |

HMW, high molecular weight; LMW, low molecular weight. Listed are the NCBI accession numbers, protein names and the number of shared peptides present within each sex and VTG type (LMW/HMW VTG).

appears to be dominated by isoforms to VTG-2. Interestingly, our proteomics and phosphoproteomic analysis revealed the presence of VTG in both male and female samples, although phosphoprotein staining of SDS-PAGE gels was unable to detect the low levels in the male samples, largely because VTG was not phosphorylated (Fig. 1D).

Our measurement of VTG staining intensity is reflective of both the total amount of *P. vitticeps*-like VTG present in the sample, as well as the degree of VTG phosphorylation, and thus this is a reliable estimate of true sample concentration (Fig. 1C,D).

HMW and LMW VTG are strongly and positively correlated ($r_s=0.85$, $P<0.0001$, $N=41$; Fig. S1), with consistently higher staining intensity in the HMW VTG band. We ran all analyses on both HMW and LMW VTG separately, and the results did not differ substantively. We therefore present results from HMW VTG analysis only (HMW VTG is abbreviated as 'VTG' in the remainder of the paper).

Oxidative costs of vitellogenesis: cross-season sources of variation in bSO and ROS

Basal superoxide was higher in vitellogenic than post-ovulatory females (GLMM, mean±s.e.m. ovulatory stage $\beta=-0.26\pm0.12$, d.f.=31.5, $t=-2.19$, $P=0.036$), and increased over the course of the breeding season with higher concentrations in December than November (sampling period $\beta=-0.67\pm0.11$, d.f.=19.4, $t=-6.08$, $P<0.0001$). The final model describing bSO also retained the non-significant influence of female reproductive investment (residual clutch number $\beta=-0.28\pm0.18$, d.f.=19.5, $t=-1.52$, $P=0.145$).

Non-specific ROS did not vary across the season, between ovulatory stages, or with reproductive investment, but did show a positive trend with VTG (GLMM final model: retained factors VTG, $\beta=0.29\pm0.16$, d.f.=34.3, $t=1.8$, $P=0.08$; ovulatory stage, $\beta=0.21\pm0.15$, d.f.=28.2, $t=1.43$, $P=0.16$; all other $P>0.25$).

Cross-season sources of variation in VTG: time of oviposition and ovulatory stage

VTG tended to decrease in the days prior to oviposition ($r_s=-0.62$, $P=0.07$, $N=9$; Fig. 2A). Across combined November and December samples, vitellogenic females had higher VTG than post-ovulatory females (GLMM, ovulatory stage $\beta=-0.42\pm0.14$, d.f.=35, $t=-2.76$, $P=0.009$; Fig. 2B) and VTG increased with body mass ($\beta=0.29\pm0.09$, d.f.=24.9, $t=3.07$, $P=0.005$). Number of follicles or eggs, reproductive investment, sampling month, ROS and bSO did not co-vary with simultaneously sampled VTG (all $P>0.24$).

Sources of variation in successive measurements of VTG: influence of prior versus current bSO and ROS

When we analysed each sample period separately, prior but not current bSO explained variation in December VTG (final model for December VTG $R^2=0.75$; Table 2). November bSO positively predicted December VTG, and this relationship was more pronounced in post-ovulatory than vitellogenic females (Fig. 3).

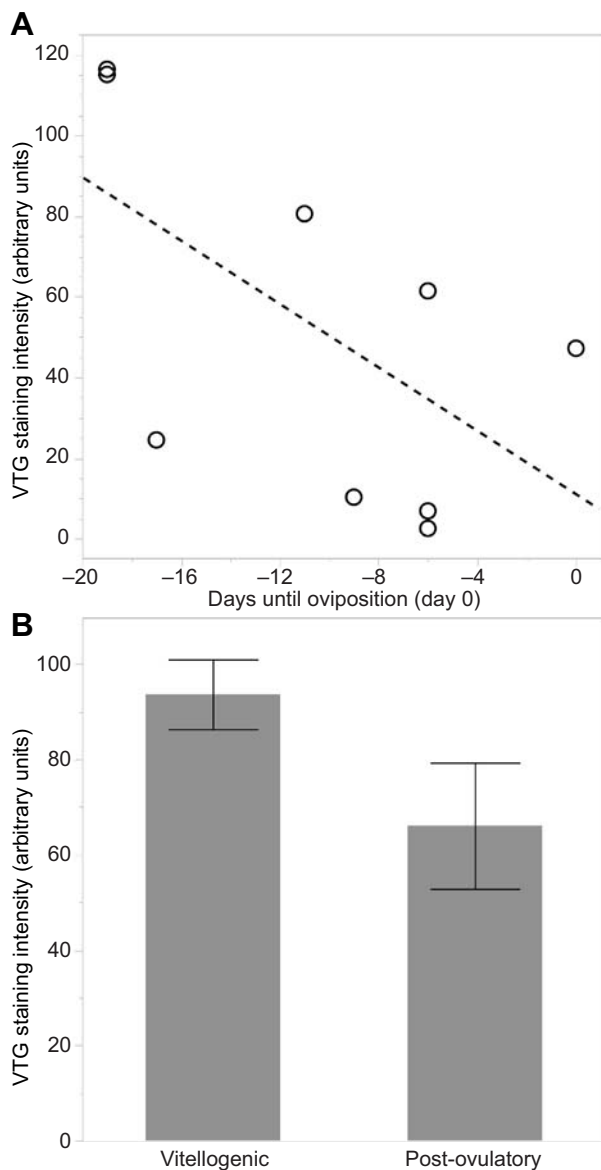


Fig. 2. Vitellogenin decreases prior to ovulation and varies with ovulatory stage. (A) As females approach ovulation, their levels of VTG tend to decline as is predicted if VTG is shunted into developing follicles and out of the blood stream ($r_s = -0.62$, $P = 0.07$, $N = 9$). (B) This is reflected by the fact that vitellogenic females who are actively yolking follicles have significantly higher levels of VTG than post-ovulatory females who no longer require VTG for yolk provisioning (but note that post-ovulatory females retain relatively elevated VTG; GLMM, ovulatory stage $\beta = -0.42 \pm 0.14$ (mean \pm s.e.m.), d.f. = 35, $t = -2.76$, $P = 0.009$; vitellogenic $N = 25$, post-ovulatory $N = 17$).

December VTG was more elevated in vitellogenic compared with post-ovulatory females, positively trended with snout–vent length, and was unrelated to the number of follicles or eggs and seasonal reproductive investment.

In contrast, December VTG was unrelated to prior ROS, while current December ROS was a positive predictor. These results derive from separate analyses with lower explanatory power than our model including bSO (model, $R^2 = 0.31$, $F_{2,17} = 3.72$, $P = 0.045$; ROS, $\beta = 0.59 \pm 0.23$, $t = 2.57$, $P = 0.019$; ovulatory stage, $\beta = -0.09 \pm 0.20$, $t = -2.14$, $P = 0.047$).

The November measurement of VTG, for which we did not have any prior metrics of oxidative stress, paralleled the cross-seasonal

analysis, with only the effects of ovulatory stage and body mass detectable (model $R^2 = 0.39$, $F_{2,18} = 5.92$, $P = 0.01$; ovulatory stage, $\beta = -0.49 \pm 0.18$, $t = -2.61$, $P = 0.018$; body mass, $\beta = 0.38 \pm 0.14$, $t = 2.61$, $P = 0.018$).

Sources of variation in successive measurements of oxidative stress: influence of prior and current VTG

November measurements of bSO were related to ovulatory stage (model $R^2 = 0.34$, $F_{3,17} = 2.97$, $P = 0.06$; ovulatory stage, $\beta = -0.43 \pm 0.19$, $t = -2.18$, $P = 0.044$; vitellogenic greater than post-ovulatory) and negatively trended with increasing reproductive investment (residual clutch size, $\beta = -0.65 \pm 0.33$, $t = -1.94$, $P = 0.069$), but were not influenced by simultaneously measured VTG (model also retained non-significant predictor; body mass, $\beta = 0.21 \pm 0.16$, $t = 1.34$, $P = 0.196$). December bSO differed similarly between the ovulatory stages, but was unrelated to any other metrics of reproductive investment or either current or prior VTG (model $R^2 = 0.21$, $F_{2,18} = 2.41$, $P = 0.118$; ovulatory stage, $\beta = -0.50 \pm 0.23$, $t = -2.16$, $P = 0.044$; November VTG was retained in the final model, $\beta = 0.30 \pm 0.23$, $t = 1.30$, $P = 0.21$).

We were unable to generate a model to predict variation in November measurements of ROS (final model $R^2 = 0.09$, $F_{1,19} = 1.92$, $P = 0.181$; with a single factor, ovulatory stage). December ROS was higher in post-ovulatory than vitellogenic females and positively co-varied with body mass (Table 3). While December VTG positively predicted simultaneously measured VTG (paralleling results from the model described above with December VTG set as the response variable; Fig. 4), prior November VTG negatively predicted December ROS. The positive covariation between December VTG and ROS was more pronounced in vitellogenic than post-ovulatory females, and while the interaction between stage and November VTG did not affect December ROS, it is apparent that the negative association in these two variables is best illustrated by examining the line of fit generated using samples from vitellogenic females (Fig. 4).

DISCUSSION

Vitellogenesis is the most metabolically costly phase of reproduction in studied reptile species (Van Dyke and Beaupre, 2011; Webb et al., 2019), and is a source of elevated bSO in painted dragon lizards. Females may overcome the oxidative costs inherent to vitellogenesis if VTG functions as an anti-oxidant. We present four lines of evidence to support this hypothesis: (1) while VTG was highest during vitellogenesis, it remained elevated post-ovulation when no longer necessary for yolking follicles, (2) VTG was unrelated to reproductive investment and thus excess VTG may be largely divorced from the demands of provisioning, (3) VTG was strongly associated with oxidative state, and specifically VTG production may be responsive to early-season elevations in bSO, and (4) end of season ROS was lower if prior VTG was high, indicating that oxidative state may, in turn, be responsive to VTG.

Plasma VTG decreases prior to egg laying, as predicted if VTG is shunted into developing follicles and out of the blood stream (Gavaud, 1986; Carnevali et al., 1991). This corresponds to an opposing increase in bSO leading up to ovulation (reported elsewhere using a much larger sample size; Olsson et al., 2012), which may reflect loss of the anti-oxidant protective mechanism (VTG). Indeed, VTG is strongly correlated with individual variation in oxidative state, a relationship that only becomes detectable when the influence of prior versus current ROS and bSO are taken into account. Late season VTG increased when prior oxidative stress was high (early-season bSO), as predicted if excess plasma VTG is an

Table 2. Prior baseline superoxide (bSO) predicts current vitellogenin

| Late season December VTG | | | | |
|------------------------------------------------|----------|--------|----------|----------|
| Model $F_{8,11}=4.18$, $P=0.016$, $R^2=0.75$ | | | | |
| | Estimate | s.e.m. | <i>t</i> | <i>P</i> |
| Current bSO, December | -0.28 | 0.17 | -1.69 | 0.119 |
| Prior bSO, November | 0.49 | 0.18 | 2.81 | 0.017 |
| Mass (g) | 0.19 | 0.12 | 1.64 | 0.129 |
| Snout-vent length (cm) | -1.13 | 0.54 | -2.11 | 0.058 |
| Number of follicles/eggs | 0.17 | 0.10 | 1.82 | 0.096 |
| Ovulatory stage (set to 'post-ovulatory') | -0.63 | 0.19 | -3.32 | 0.007 |
| Prior bSO (November)×ovulatory stage | 0.38 | 0.17 | 2.19 | 0.051 |
| Current bSO (December)×ovulatory stage | 0.22 | 0.17 | 1.27 | 0.231 |

High November bSO positively predicts December VTG, and post-ovulatory females have lower VTG than vitellogenic females. The effect of November bSO on December VTG is stronger in post-ovulatory than vitellogenic females.

anti-oxidant. The significant interaction between prior bSO and ovulatory stage on late-season VTG showed a steeper relationship between these variables when females were post-ovulatory than vitellogenic. While it is likely that circulating levels of VTG are more related to needs of oocytes during the period of vitellogenesis, their bioavailability as a maternal anti-oxidant may be highest post-ovulation.

In contrast, simultaneously measured bSO was not related to late-season VTG, indicating directionality in the relationship between VTG and bSO with exposure to bSO probably influencing VTG production. In support of this hypothesis, a model with late-season bSO as the response variable showed no effect of either current or prior metrics of VTG (but note that the process of vitellogenesis, rather than VTG titre itself, was a positive predictor of bSO). A tight relationship between bSO and VTG production could augment total anti-oxidant capacity and thus explain how females overcome low levels of SOD (Olsson et al., 2012). Low anti-oxidant titre and anti-oxidant activity in breeding females may be a primary cost of reproduction (Alonso-Alvarez et al., 2004; Wiersma et al., 2004), and with regard to SOD, may stem from SOD toxicity (Liochev and Fridovich, 2007). However, low SOD does not necessitate poor

protection from bSO. While male painted dragon lizards have higher SOD than females, female SOD expression is more tightly linked to reductions in DNA erosion (Olsson et al., 2012), indicating that female SOD is either more effective at counteracting oxidative damage, or that co-expressed mechanisms functioning in females enable them to achieve greater protection. With regard to the former, elevated E2 in rats is associated with increased SOD activity without necessitating increased SOD concentrations (Ramara et al., 2007), and could explain the sex differences documented in this species. The function of VTG as a SOD-like molecule provides an alternative but not mutually exclusive mechanism. VTG proteins from at least two species of crustaceans contain a SOD-like domain capable of SOD activity (Kato et al., 2004; Chen et al., 2011). If taxonomically conserved, the special sensitivity of VTG to bSO helps explain how female painted dragons are more capable of immunologically overcoming high bSO than males (Tobler et al., 2011). The anti-oxidant effects of VTG may be amplified by simultaneous effects of E2 (E2 can promote anti-oxidant function; Vina et al., 2005) and experimental approaches are now needed to tease apart their relative contributions.

The relationship between VTG and non-specific ROS supports predictions for both anti-oxidant function of VTG and oxidative costs of vitellogenesis. Late-season ROS increased with simultaneously measured VTG, while decreasing with prior VTG. Therefore, while VTG may act as an anti-oxidant to offset accumulated ROS, the process of vitellogenesis probably generates oxidative stress (as also demonstrated by Webb et al., 2019). To further validate this assumption, it is during the vitellogenic phase specifically that late-season VTG and ROS positively co-vary. In terms of directionality, it is more likely that VTG production drives ROS production, then the opposite. While we do detect a negative relationship between prior VTG and ROS, this relationship is difficult to visualize when examining raw data. Moreover, the elevated ROS apparent in the post-ovulatory phase may be a byproduct of the accumulation of ROS generated during the vitellogenic period.

The co-variation between VTG and bSO documented here fits mechanistic predictions from both the oxidative shielding (Blount et al., 2015) and hormesis during reproduction (Costantini, 2014) hypotheses, which seek to explain how females avoid oxidative damage during reproduction. Where the oxidative shielding hypothesis predicts the existence of a mechanism to either preemptively reduce or offset ROS production (Blount et al., 2015), the hormesis during reproduction hypothesis instead suggests that transient exposure to oxidative stress early in the season turns on a compensatory mechanism that provides females with the ability to detoxify and gain protection against the future generation of stressors (Costantini, 2014). A unidirectional relationship between early-season bSO and levels of subsequently expressed VTG is

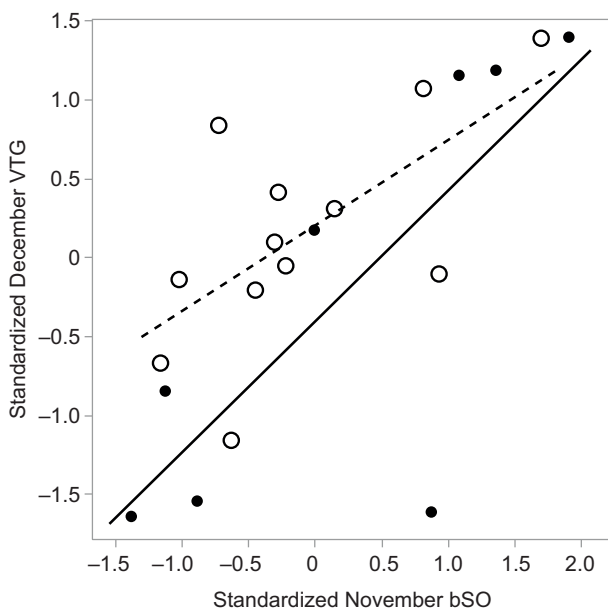


Fig. 3. December vitellogenin increases with prior baseline superoxide. The slope of the relationship between November baseline superoxide (bSO) and December VTG is steeper in post-ovulatory (continuous line, filled circles, $N=9$) than vitellogenic females (dotted line, open circles, $N=12$; GLMM, prior bSO×ovulatory stage $\beta=0.38\pm0.17$, $t=2.19$, $P=0.051$).

Table 3. Late season December reactive oxygen species (ROS) decreases with prior VTG and increases with current VTG

| Late season December ROS Model $F_{5,14}=10.76$, $P=0.0002$, $R^2=0.79$ | Estimate | s.e.m. | <i>t</i> | <i>P</i> |
|------------------------------------------------------------------------------|----------|--------|----------|----------|
| Ovulatory stage (set to 'post-ovulatory') | 0.82 | 0.16 | 5.27 | 0.0001 |
| Body mass (g) | 0.21 | 0.09 | 2.17 | 0.048 |
| Prior VTG, November | -0.57 | 0.17 | -3.26 | 0.006 |
| Current VTG, December | 0.52 | 0.14 | 3.47 | 0.004 |
| Current VTG (December)×ovulatory stage | -0.46 | 0.13 | -3.34 | 0.005 |

Post-ovulatory females had higher ROS than vitellogenic females, and ROS increases with body mass. The positive relationship between current December VTG and December ROS was more pronounced in vitellogenic than post-ovulatory females.

consistent with predictions of hormesis, yet bSO is lowest early in the season, a finding more in line with oxidative shielding. Directionality in these relationships and detailed tests of the predictions of the oxidative shielding and hormesis hypotheses with regard to the maternal environment require experimental manipulation of both VTG and ROS along with more inclusive cross-season sampling with pre- and post-breeding samples and sampling of non-reproductive individuals.

VTG-derived yolk proteins may be a key mediator of offspring health and fitness, providing a mechanistic solution to the second prediction of the oxidative shielding hypothesis, the ability to protect offspring from maternally derived oxidative stress (Blount et al., 2015). Because conditions experienced early in life can have carry-over effects on adult fitness (Reed et al., 2008; Bouwhuis et al., 2010), selection against transference of oxidative damage to young should be strong. Maternal oxidative stress can lower

offspring survival rates (Bize et al., 2008) and shorten offspring telomere lengths (Haussmann et al., 2012), a profound effect as early-life telomere length is one of the strongest predictors of adult lifespan (Heidinger et al., 2012). In the most extreme cases, maternal oxidative stress can lead to spontaneous abortion (Gupta et al., 2007). When females are more resistant to oxidative stress, they can have bigger and more successful clutches (Bize et al., 2008) and, in painted dragon lizards, offspring capable of greater oxidative stress tolerance (Olsson et al., 2008).

The mechanisms by which females transfer oxidative stress to young can include the depletion of resources required for albumin and yolk synthesis (Blount et al., 2000) and contamination of nutrient stores through damage to immunoglobulin proteins and incorporation of toxic components in yolk (Mohiti-Asli et al., 2008). How embryos protect themselves from this oxidative damage is an interesting puzzle, and likely hinges on anti-oxidant stores deposited in the egg by the mother. These include known anti-oxidants such as vitamins A and E and carotenoids (Saino et al., 2002; Dale et al., 2017), but probably also yolk proteins. In most yolk-producing species, VTG undergoes proteolytic cleavage into lipovitellins, phosvitin (PV) and/or phosvettes (Byrne et al., 1989; Avarre et al., 2003). Like VTG, PV can act as an anti-oxidant directly (reviewed by Li and Zhang, 2017) as well as be a recognition molecule capable of identifying bacterial, viral and fungal pathogens and enhancing macrophage phagocytosis (Li et al., 2008; Wang et al., 2011). That VTG and its derivatives serve such functions has only recently become appreciated and has yet to be investigated in the context of physiological mediation of oxidative stress in reproducing females and their eggs.

Conclusions

A trade-off between reproduction and longevity is central to the theory of life-history evolution (Fisher, 1930) and is likely to be orchestrated by age- and reproductive effort-induced shifts in oxidative damage and anti-oxidant activity ('free radical theory of aging'; Harman, 1956). While intuitively satisfying, the costs of reproduction in wild animals, including lizards, remain difficult to detect (Olsson et al., 2001; Speakman et al., 2015). This may be a result of the rarity of females embarking on reproductive events outside of their optima or may stem from up-regulation of innate repair mechanisms, such as an oxidative shielding molecule, that obscure these costs. A multi-sampling approach over the course of a season and lifespan is necessary to determine how females overcome stressors to optimize both their reproductive output and the quality of their young. Here we show that VTG titre (or the underlying mechanisms for its production, E2) is related to oxidative state during reproduction, and may account for oxidative balance in breeding females. Given its necessarily transgenerational application in offspring provisioning, the immunoprotective and anti-oxidant benefits of VTG are probably felt by offspring, thus acting as a maternal shield to environmental and metabolic stress.

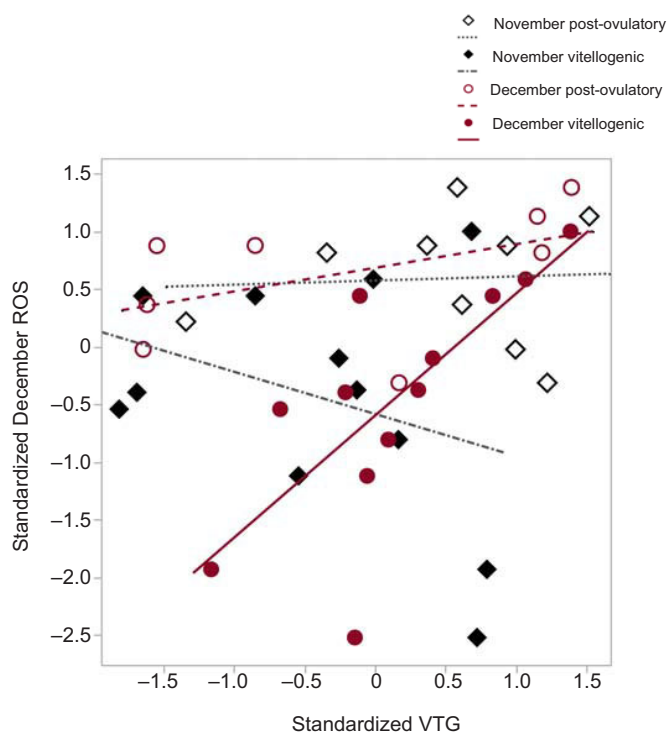


Fig. 4. December reactive oxygen species varies with ovulatory stage and both prior and current VTG. December reactive oxygen species (ROS) increases with current VTG (red lines, $N=20$; GLMM, current VTG $\beta=0.52 \pm 0.14$, $t=3.47$, $P=0.004$), and decreases with prior VTG (black lines, $N=21$; GLMM, prior VTG $\beta=-0.57 \pm 0.17$, $t=-3.26$, $P=0.006$), with a stronger relationship between variables in vitellogenic (filled symbols) than post-ovulatory females (open symbols; GLMM, current VTG×ovulatory stage $\beta=-0.46 \pm 0.13$, $t=-3.34$, $P=0.005$).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: W.R.L., C.R.F., M.O.; Methodology: W.R.L., C.R.F., C.S., J.B., E.B., M.R.W., M.O.; Software: C.S.; Validation: W.R.L., C.S., J.B., E.B., M.R.W.; Formal analysis: W.R.L., C.S., E.B.; Investigation: W.R.L., C.R.F., C.S., J.B., E.B., M.R.W.; Resources: W.R.L., C.R.F., C.S., J.B., M.R.W., M.O.; Data curation: W.R.L., C.S., J.B., E.B.; Writing - original draft: W.R.L.; Writing - review & editing: W.R.L., C.R.F., C.S., J.B., E.B., M.R.W., M.O.; Visualization: W.R.L., C.S., J.B., E.B.; Supervision: W.R.L., C.R.F., C.S., J.B.; Project administration: W.R.L., C.R.F., M.O.; Funding acquisition: C.R.F., M.O.

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Supplementary information

Supplementary information available online at <http://jeb.biologists.org/lookup/doi/10.1242/jeb.221630.supplemental>

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Figure S1. LMW VTG and HMW VTG are positively correlated. HMW and LMW VTG are strongly and positively correlated ($r_s=0.85$, $P<0.0001$, $N=41$) with consistently higher phosphoprotein staining intensity in the HMW VTG band.

