

Title: Eating increases oxidative damage in a reptile

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Summary statement:

Consuming and digesting a meal affects oxidative physiology to a surprising degree, and animals that consume large or meat-based meals may be particularly susceptible to increases in oxidative damage.

Abstract

While eating has substantial benefits in terms of both nutrient and energy acquisition, there are physiological costs associated with digesting and metabolizing a meal. Frequently, these costs have been documented in the context of energy expenditure while other physiological costs have been relatively unexplored. Here, we tested whether the seemingly innocuous act of eating affects either systemic pro-oxidant (reactive oxygen metabolites: ROM) levels or antioxidant capacity of corn snakes (*Pantherophis guttatus*) by collecting plasma during absorptive (peak increase in metabolic rate due to digestion of a meal) and non-absorptive (baseline) states. When individuals were digesting a meal, there was a minimal increase in antioxidant capacity relative to baseline (4%), but a substantial increase in ROMs (nearly 155%), even when controlling for circulating nutrient levels. We report an oxidative cost of eating that is much greater than that due to long distance flight or mounting an immune response in other taxa. This result demonstrates the importance of investigating non-energetic costs associated with meal processing, and it begs future work to identify the mechanism(s) driving this increase in ROM levels. Because energetic costs associated with eating are taxonomically widespread, identifying the taxonomic breadth of eating-induced ROM increases may provide insights into the interplay between oxidative damage and life history theory.

Introduction

Animals rely upon ingesting organic material to provide the nutrients and energy necessary to survive, grow, and reproduce, but this process of digesting and absorbing nutrients comes at an energetic cost, typified by specific dynamic action (SDA; Secor, 2009). In brief, SDA incorporates the increase in metabolic rate that is commensurate with the changes in anatomy and physiology necessary to digest and metabolize a meal, such as increasing the size and activity of organs (e.g., small intestines, heart, and kidneys; Secor, 2008). This digestion-dependent increase in metabolic rate affects virtually all animals, from mollusks and arthropods, to fishes, birds, and mammals (Secor, 2009).

The energetic costs of eating are well-documented (Secor, 2008, 2009), but digesting a meal may also have non-energetic costs (e.g., oxidative damage). Reactive oxygen species (ROS) are an inadvertent by-product of oxidative phosphorylation in the mitochondria during normal metabolic processes. Increases in ROS consequently result in damage to organic molecules unless they are neutralized by an organism's antioxidant capabilities (Costantini et al., 2010). Because oxidative damage is associated with an array of biological functions (e.g., ornamentation, hibernation, immune function, and reproductive effort; Costantini et al., 2010), it may be a universal constraint in life history evolution (Dowling and Simmons, 2009; Costantini, 2014). Despite this purported importance of oxidative damage, identifying its main physiological drivers has proven challenging. While factors that increase metabolic rate have been linked to an increase in oxidative damage (e.g., immune challenges, van de Crommenacker et al., 2010; exercise, Costantini, Dell'Araccia, et al., 2008), recent work strongly suggests that increased metabolic rate *per se* is unlikely to drive increases in ROS and, thus, oxidative damage (reviewed in Speakman and Garratt, 2014; Salin et al., 2015).

While an increased metabolic rate is unlikely to directly increase oxidative damage, indirect links between metabolic rate and oxidative damage remain feasible. For example,

during periods of increased metabolic rate (e.g., due to eating or exercise), animals mobilize nutrients that are susceptible to oxidation (e.g., triglycerides, Mateos and Bravo, 2007), thus increasing the amount of oxidative damage that is detected in plasma (Sies et al., 2005; Pérez-Rodríguez et al., 2015). Because triglycerides are highly susceptible to free radical attack (Mateos and Bravo, 2007) and many commercial assay kits quantify oxidative damage using markers of lipid peroxidation, eating-induced increases in circulating triglyceride levels may result in the detection of greater amounts of lipid peroxidation (Pérez-Rodríguez et al., 2015). Indeed, work with humans has documented increases in oxidative damage after eating (Sies et al., 2005; Wallace et al., 2010), termed postprandial oxidative stress. However, a human eating three meals per day may spend up to 75% of its life in an absorptive state (Secor, 2009), making humans a poor model for investigating links between eating and oxidative damage, given that most animals likely spend significant time periods in a non-absorptive state. Thus, eating-induced variation in oxidative damage may have an underappreciated role in evaluating the extent to which oxidative damage informs life history theory.

Here, we used corn snakes (*Pantherophis guttatus*), which can eat meals between 5% and 45% of their body mass (Crocker-Buta and Secor, 2014), to assess how oxidative status changes with absorptive state while controlling for the effects of circulating levels of nutrients (e.g., triglycerides, Pérez-Rodríguez et al., 2015). We chose this species because snakes generally eat relatively large meals and exhibit a robust SDA (Secor, 2009), but the five-fold increase in metabolic rate (i.e., metabolic scope ~ 5) of corn snakes (Crocker-Buta and Secor, 2014) is in the bottom third of most snakes species (Secor, 2009). Thus, while corn snakes eat larger meals and have larger metabolic scopes than many other taxa, corn snakes are not an extreme example within Serpentes. We predicted that if snakes have the ability to up-regulate antioxidant defenses in response to eating-induced oxidative stress, then

oxidative damage should change minimally between the non-absorptive and absorptive states. However, if eating results in an increase in oxidative damage beyond the animal's ability to neutralize this oxidation, then oxidative damage should be detectably higher during absorption. By investigating links between eating and oxidative physiology, we will evaluate the extent to which postprandial oxidative stress exists in non-human animals, thus informing discussions regarding how oxidative physiology interfaces with life history theory (Dowling and Simmons, 2009; Speakman and Garratt, 2014).

Materials and methods

Husbandry and experimental procedure

We used 32 captive corn snakes (14 males, 18 females), a non-venomous, medium-sized species native to the southeastern United States (Gibbons and Dorcas, 2005). All individuals were 14-16 months old and were the offspring (1st-3rd generation) of wild snakes caught in Beaufort County, South Carolina, USA. We housed snakes individually in translucent plastic enclosures (27 cm x 41 cm x 15 cm) in a room with a 12:12 light:dark cycle. Enclosures had subsurface heating elements that allowed snakes to thermoregulate along a gradient of temperatures from 24.5-33°C, which encompasses the preferred temperature range for corn snakes (Roark and Dorcas, 2000; Stahlschmidt et al., 2015). We allowed snakes *ad libitum* access to water and offered them food (frozen/thawed adult mice that were 15-20% of the snake's body weight) every two weeks throughout the 8-week study, even though snakes don't show starvation stress until > 100 days without food (McCue, 2008).

We used intra-cardiac blood draws (0.3 ml) to collect blood samples from each snake at two time points, 1 and 7 day(s) post-feeding (dpf), because the metabolic rate of *P. guttatus* peaks (approx. 5-fold greater than baseline) 1 dpf and declines back to pre-feeding levels approximately 4 dpf when fed meals of 15-20% of their body mass (Crocker-Buta and Secor, 2014). Thus, 1 dpf denotes the absorptive state while 7 dpf denotes the non-absorptive state. Corn snakes exhibit subtle post-prandial thermophily (Roark and Dorcas, 2000; Sievert et al., 2013; Stahlschmidt, unpublished) meaning snakes in the absorptive state may have had a higher body temperature (1-3°C higher) and, thus, a metabolic scope >5. To control for order effects, we randomized sampling order (i.e., half the snakes were first sampled during their absorptive state), and samples were separated by at least one full meal cycle (i.e., the duration between sampling for each snake was two weeks). To ensure treatment was not nested within blood-collection session, we sampled snakes that were either 1 or 7 dpf during

each sampling period. We stored blood samples on ice prior to centrifugation at 2350 X g for 5 min, and then stored the plasma fraction at -80°C prior to assays (see below). All procedures were approved by the Institutional Animal Care and Use Committee at Georgia Southern University (protocol #I14004).

Biochemical assays

We quantified the amount of hydroperoxides, which are pro-oxidant intermediates of lipid peroxidation that lead to the production of oxidative damage, with the d-ROMs test (#MC002, Diacron International, Italy). This test detects reactive oxygen metabolites (ROMs) such as hydroperoxides using Fenton's reaction. We followed the directions of the kit, but amended it for smaller sample volumes *sensu* Costantini et al. (2011). In brief, we mixed 4 µl of the sample with 200 µl of kit reagent, incubated it at 37 °C for 75 minutes, and read the absorbance at 505 nm using a Tecan (Switzerland) Infinite M200Pro plate reader. We calculated the amount of ROMs relative to control blanks and the kit's calibrator, and we report values as mM H₂O₂ equivalents, with larger numbers corresponding to a greater amount of ROMs. We also quantified the antioxidant capacity of the plasma using the OXY-Adsorbent test (#MC435, Diacron International, Italy), which measures the plasma's ability to oppose the pro-oxidizing action of hypochlorous acid. Also following Costantini et al. (2011), we amended the kit's instructions by combining 5 µl of a 1:100 dilution of plasma with 200 µl of the hypochlorous acid-based oxidizing solution, incubating the plate for 10 min at 37 °C, adding 5 µl of the chromagen solution, and then measuring absorbance at 505 nm using a Tecan (Switzerland) Infinite M200Pro plate reader. We calculated antioxidant capacity relative to control blanks and the kit calibrator, and report values in mM HClO neutralized, with larger values corresponding to a more robust antioxidant barrier.

To gauge the metabolic state of the subjects, we measured levels of plasma protein and two plasma lipid markers. We quantified plasma protein concentration using the Coomassie Plus (Bradford) Assay (#23236, Thermo Scientific, IL, USA) by combining 10 μ l of a sample that was diluted 1:100 with ddH₂O with 300 μ l reagent, incubated it at room temperature for 10 minutes, and then read the absorbance at 595 nm using a Tecan (Switzerland) Infinite M200Pro plate reader. We then calculated plasma protein concentrations (μ g/ml) using a standard curve of bovine serum albumin (#23209, Thermo Scientific, IL, USA). To quantify lipid markers, we used a spectrometric endpoint assay that quantifies both plasma glycerol and triglyceride concentrations, sequentially (Fokidis et al., 2012, 2011; Neuman-Lee et al., 2015). First, we combined 5 μ l of a sample with 240 μ l of free glycerol reagent (#F6428, Sigma-Aldrich, MO, USA) and incubated it for 10 minutes at 37°C, and read the absorbance at 540 nm (primary) and 750 nm (secondary) on a Beckman-Coulter AD200 plate reader to obtain the free glycerol concentration in mM. Immediately after, we added 60 μ l of triglyceride reagent (#T2449, Sigma-Aldrich, MO, USA) and again incubated it for 10 minutes at 37°C. We read the absorbance at the same settings, and we subtracted this value from the glycerol concentration to provide the plasma levels of formed (or true) triglycerides.

Statistics

We ran the above assays in duplicate, and found that values for all assays were significantly repeatable (Lessells and Boag, 1987) within sample (d-ROMS: $P < 0.0001$, $R = 0.98$; OXY: $P = 0.0014$, $R = 0.63$; protein: $P < 0.0001$, $R = 0.56$; triglycerides: $P = 0.0006$, $R = 0.58$; glycerol: $P < 0.0001$, $R = 0.80$), so we subsequently averaged duplicate samples. We tested for an effect of digestive state, sex, and their interaction on d-ROMs, OXY, protein, glycerol, and triglyceride levels using mixed models with individual as a random effect. We also reran

the model for d-ROMs including either triglyceride, glycerol, or protein levels as a covariate to test the possibility that d-ROMs levels are proportional to circulating nutrient levels (Pérez-Rodríguez et al., 2015). For one snake (female in the absorptive state), we had enough plasma to run only the d-ROMs and OXY assays, but not to evaluate circulating nutrient levels, and there were several individuals from whom we could not successfully collect plasma in both states (five individuals during the absorptive state, one individual in the non-absorptive state). Lastly, we used Pearson correlations to test if d-ROMs and OXY levels were correlated with each other or with circulating nutrient levels within absorptive or non-absorptive states. All statistics were run using SAS 9.3 (Cary, NC), and all data will be freely available on Dryad pending acceptance for publication. Residuals from all models were normally distributed, and d-ROMs, OXY, and glycerol values were log-transformed to meet this assumption of normality. One female had very low levels of OXY, which resulted in non-normally distributed residuals for that model. Exclusion of this female resulted in normally distributed residuals, and results were qualitatively similar with or without her exclusion.

Results

There were no differences in OXY (both $F_{1,22} < 0.26$, both $P > 0.62$), d-ROMs (both $F_{1,24} < 1.24$, both $P > 0.28$), or protein plasma (both $F_{1,23} < 2.42$, both $P > 0.13$) due to sex or the interaction of sex and digestive state. However, animals in the absorptive state had increased protein (19.8% increase; $F_{1,23} = 65.84$, $P < 0.0001$), OXY (4.3% increase; $F_{1,22} = 5.32$, $P = 0.0309$), and d-ROMs (154.9% increase; $F_{1,24} = 104.89$, $P < 0.0001$; Figure 1) levels. This effect of digestive state on d-ROMs remained when including covariates of triglyceride (165.8% increase; $F_{1,22} = 118.71$, $P < 0.0001$), protein (124.9% increase; $F_{1,22} = 37.53$, $P < 0.0001$), or glycerol (164.6% increase; $F_{1,22} = 107.59$, $P < 0.0001$) levels. Males circulated higher levels of triglycerides overall ($F_{1,23} = 8.97$, $P = 0.0065$), but there was no effect of digestive state or an interaction between sex and digestive state on circulating triglyceride levels (both $F_{1,23} < 1.15$, both $P > 0.30$), nor did sex, digestive state, or their interaction affect circulating glycerol levels (all $F_{1,23} < 2.80$; all $P > 0.11$)

During the non-absorptive state, protein levels were positively correlated with both d-ROMs ($r = 0.42$, $P = 0.0210$) and OXY ($r = 0.50$, $P = 0.0048$), and triglyceride levels were positively correlated with OXY ($r = 0.39$, $P = 0.0320$). During the absorptive state, triglyceride levels positively correlated with glycerol levels ($r = 0.43$, $P = 0.0244$), and negatively correlated with d-ROMs ($r = -0.53$, $P = 0.0043$; Figure 2). All other correlations were not statistically significant (all $r < 0.32$, all $P > 0.098$).

Discussion

The physiological consequences of eating have primarily been explored via the energetic costs of consuming, digesting, and metabolizing a meal, while oxidative costs in non-mammalian systems have been relatively unexplored. Here, we show that digesting a meal has a minimal impact on antioxidant capacity, which increased by 4% and is less than differences due to forced locomotion (Costantini, Dell'Araccia, et al., 2008), genetic selection for aggression (Costantini, Carere, et al., 2008), or natural variation among populations (Costantini et al., 2009). However, the OXY test does not capture all aspects of antioxidant status of the individual (Sepp et al., 2012), and it is possible that other antioxidant defenses were modulated in ways that we did not detect.

However, despite a minimal increase in antioxidant capacity, we detected a large increase in ROMs (Fig. 1; increases of 1.25 mM H₂O₂ equivalents, or 125-166%, depending on statistical model). This magnitude of eating-induced oxidative damage would presumably have important biological consequences, similar to an approximately 130% increase in d-ROMs due to heat stress (Costantini et al., 2012). Whereas flying 200 km or responding to an immune challenge can increase d-ROMs by approximately 0.27 (Costantini, Dell'Araccia, et al., 2008) and 0.74 (van de Crommenacker et al., 2010) mM H₂O₂ equivalents, respectively, we detected an eating-induced increase of over 1.25 mM H₂O₂ equivalents. This large increase is likely driven by increases in ROMs, rather than changes in ceruloplasmin or albumin levels that can interfere with the kit's activity (Kilk et al., 2014, but see Colombini et al., 2016), for two reasons. First, increases in albumin concentration inhibit d-ROMs activity (Kilk et al., 2014); while we quantified circulating protein levels rather than albumin levels *per se*, we detected greater (not reduced) d-ROMs levels despite increased circulating protein concentration. Secondly, while ceruloplasmin may create a false positive signal in mammals, the evidence that it affects avian samples is less robust, and its role in non-avian reptile

plasma is unknown (Kilk et al., 2014). Regardless, the repeated-measures nature of our experimental design would require that ceruloplasmin levels would have to change within an individual between digestive states to create the pattern we detected. While data are currently lacking regarding ceruloplasmin levels in reptiles as a function of digestive state, work with chickens has shown that varying levels of food restriction do not affect circulating ceruloplasmin levels (Najafi et al., 2015). Thus, despite methodological concerns regarding the d-ROMs kit, we show that although large meals may have important benefits (e.g., energy acquisition), they can have a greater effect on oxidative damage than traditionally explored stressors. While we predict that the energetic and nutritional benefits of eating will always outweigh the oxidative costs, future work could evaluate the relative size of these costs as a function of meal size, nutrient composition, and eating interval.

There are three non-mutually exclusive proximate explanations for increased ROMs during eating. First, increases in ROMs could be due to an increased metabolic rate associated with SDA. While links between metabolic rate and oxidative damage are frequently invoked (Dowling and Simmons, 2009; Speakman and Garratt, 2014), careful consideration of the dynamic state of the mitochondrial membrane strongly suggests that increased metabolic rate *per se* is unlikely to drive variation in oxidative damage production (Speakman and Garratt, 2014; Salin et al., 2015). For example, higher temperatures increase metabolic rate in corn snakes, but they decrease (not increase) d-ROMs (Stahlschmidt et al., unpublished). Thus, while we did not experimentally test whether an increase in metabolic rate *per se* causes an increase in oxidative damage, we view this explanation as unlikely based on cellular and molecular (Speakman and Garratt, 2014) and physiological (Salin et al., 2015) evidence.

Second, increases in circulating nutrient levels, and primarily triglycerides, could increase d-ROMs levels due to either a) providing increased amounts of substrates, which

would provide a positive feedback loop of oxidizing chain reactions, or b) the food itself contains oxidized molecules, which would then be detected in circulation once they were assimilated (Pérez-Rodríguez et al., 2015). We expected to find such a positive correlation between oxidative damage and nutrient levels based on work with humans (Sies et al., 2005; Wallace et al., 2010) and non-humans (Pérez-Rodríguez et al., 2015). However, d-ROMs levels were positively correlated only to circulating protein levels and only during the non-absorptive state. During the absorptive state, the only correlation between d-ROMs and circulating nutrient levels was a *negative* correlation with triglyceride levels. Furthermore, including nutrient levels as covariates in our statistical models as suggested by Pérez-Rodríguez et al. (2015) did not account for the difference in ROMs between absorptive and non-absorptive states. In fact, inclusion of lipid-based markers as covariates yielded a stronger effect of absorptive state on ROM levels.

The third possibility is that independent of triglyceride levels, the act of digesting the meal resulted in exceptionally high levels of hydroperoxides that were subsequently detected in circulation after being absorbed by the small intestine. While digestion of lipids themselves (e.g., fish oil) does not result in hydroperoxide formation (Larsson et al., 2012), fish oil digested *in vitro* in the presence of hemoglobin does exhibit a marked increase in hydroperoxides (Larsson et al., 2012). Analogously, turkey meat digested *in vitro* shows a substantial increase in hydroperoxide production (Verzelloni et al., 2010). Thus, species consuming moderate amounts of hemoglobin- or iron-containing foods (i.e., meat) may be more likely to exhibit eating-induced increases in ROMs due to oxidation processes that take place during digestion. Thus, the substantial increase in d-ROMs that we detected due to eating may be a pattern based more on diet composition than magnitude of SDA.

Regardless of mechanism, we provide two specific insights into the role of oxidative status in life history theory (Dowling and Simmons, 2009). The first ramification is

methodological: evaluating the absorptive state of an individual may be critical in interpreting future oxidative physiology studies. Due to the sheer magnitude of the change in ROMs levels associated with eating, it is possible that accounting for this variation may reveal biologically important patterns between oxidative status and life history traits that were previously obscured by the digestive state of the animal, particularly for carnivores or species eating iron-rich foods. Second, further work is needed to evaluate the taxonomic breadth of this phenomenon. We performed our experiment with snakes because they exhibit a robust SDA (Secor, 2009). While snakes in general are well-known for having some of the largest SDA values (Secor, 2009), almost two thirds of snakes that have been studied have metabolic scopes greater than 5, demonstrating that corn snakes are not an extreme example within their suborder. Additionally, nearly 25% of anurans and 10% of fishes also have metabolic scopes greater than 5, despite eating meals that are substantially smaller (anurans: 10-15% of body mass; fishes: <10% of body mass) than those of snakes (20-25% of body mass; Secor, 2009). While birds and mammals have SDA-driven metabolic scopes that are substantially lower (e.g., between 1.3 and 2.0; Secor, 2009), the scale of our findings (a greater than two-fold increase in ROMs) encourages exploring links between digestive state and ROMs even in animals that eat proportionately smaller meals.

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Competing Interests

The authors have no competing interests.

Authors' Contributions

MWB and ZRS were responsible for the conception and design of the experiment and manuscript preparation. TJL and HBF contributed to manuscript revision. MWB, TJL, HBF, and ZRS performed the experiment. MWB carried out statistical analyses. All authors gave final approval for publication.

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Figures

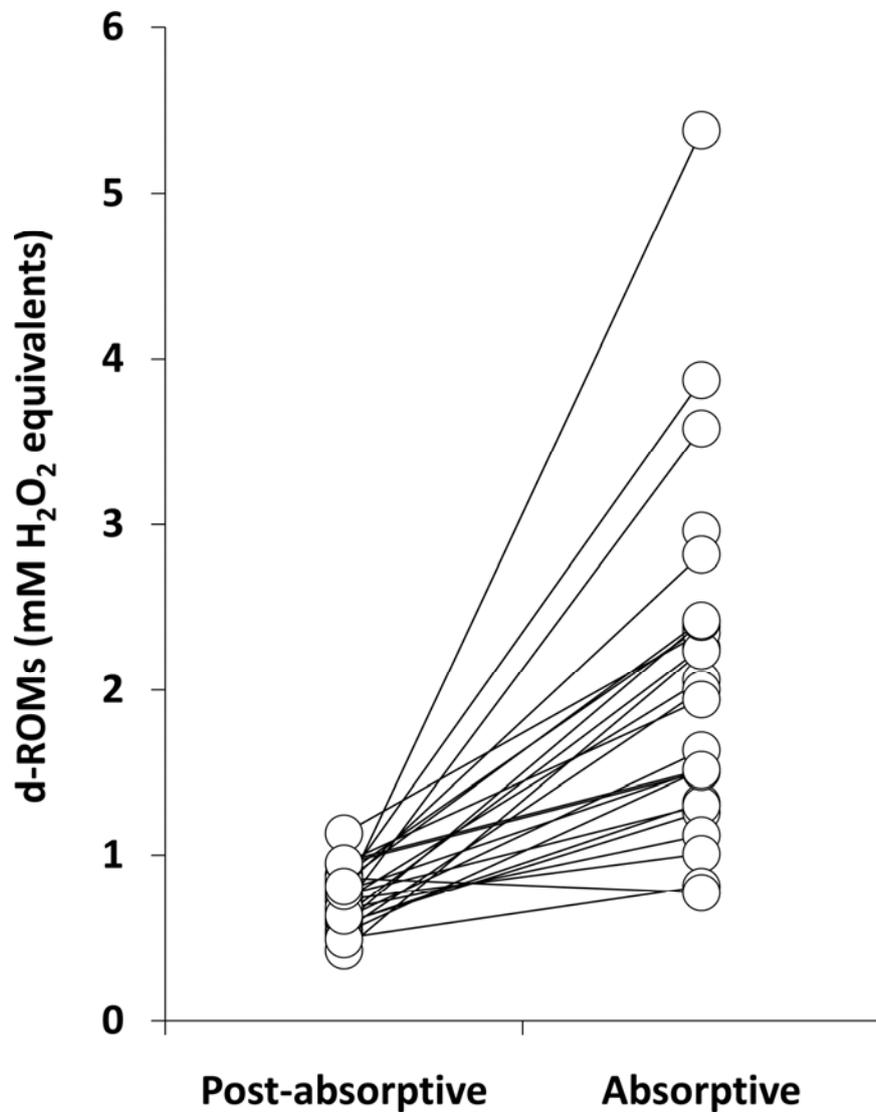


Figure 1. Oxidative damage in the plasma was approximately 155% greater ($F_{1,24} = 104.89$, $P < 0.0001$; mixed model) during the absorptive state ($N = 27$) than the non-absorptive ($N = 31$) state in corn snakes. Raw data are plotted, with lines connecting the same individual in both states. Statistics were performed using the log-transformation of d-ROMs values to meet the assumption of normality. Figure template from Weissgerber et al. (2015).

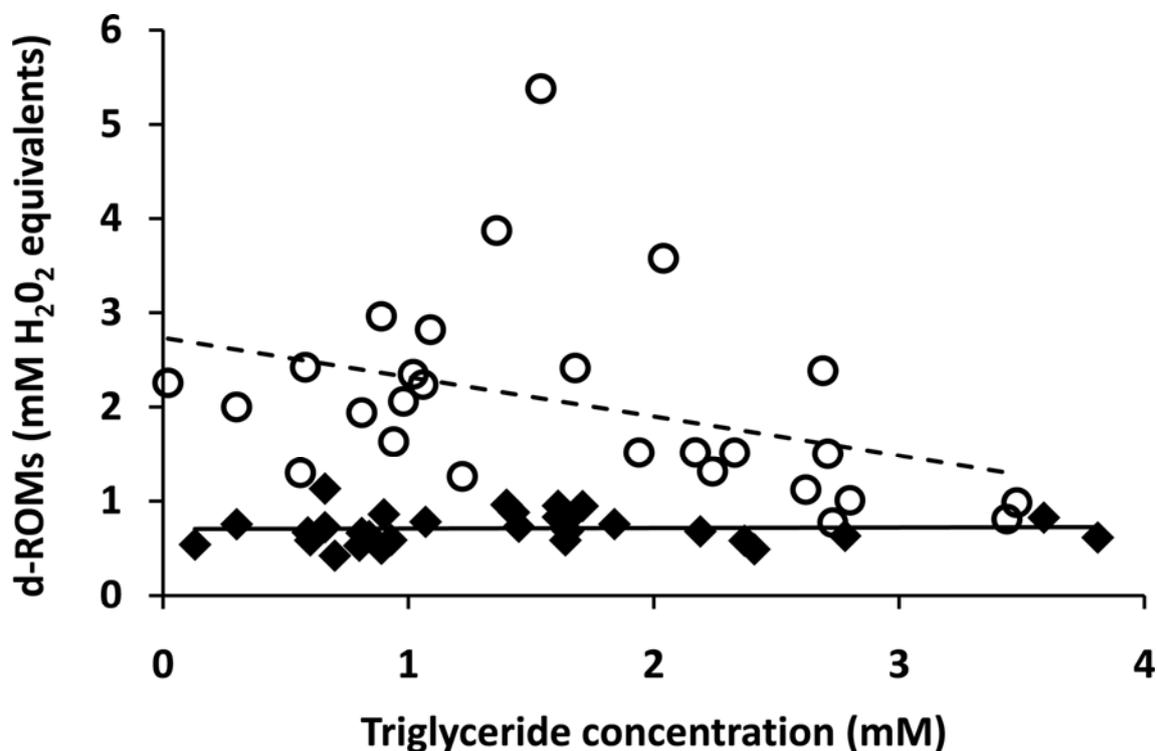


Figure 2. Oxidative damage was not correlated with triglyceride concentration during the non-absorptive state (solid diamonds, solid line; $N = 27$; Pearson correlation coefficient = 0.06, $P = 0.75$), but it was negatively correlated with triglyceride concentration during the absorptive state (open circles, dashed line; $N = 30$; Pearson correlation coefficient = -0.53, $P = 0.0043$). Raw data are plotted, but statistics were performed using the log-transformation of d-ROMs values. Each snake was sampled at both time points, although limited plasma from several individuals during the absorptive state resulted in a lower sample size during that period.