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ORIGINAL PAPER

# Reproductive trade-offs in the colorado checkered whiptail lizard (*Aspidoscelis neotesselatus*): an examination of the relationship between clutch and follicle size

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## Abstract

Life history theory predicts that there should be an inverse relationship between offspring size and number, because individuals cannot simultaneously maximize both when resources are limited. Although extensively studied in avian species, the occurrence and determinants of reproductive tradeoffs in oviparous reptiles are far less understood, particularly in parthenogenetic species. We studied this trade-off in the Colorado Checkered Whiptail, *Aspidoscelis neotesselatus*, a female-only parthenogenetic lizard. Using data previously collected in 2018 and 2019, we tested for clutch and egg size trade-offs and determined whether this relationship could be influenced by female size and aspects of physiological condition. Physiological condition included energy-mobilizing hormone (i.e. corticosterone 'CORT'), oxidative stress (i.e. reactive oxygen metabolites 'ROMs'), and innate immune function (bacterial killing ability 'BKA'). We found the effect of clutch size on follicle size was significant, but not linear. Specifically, follicle size was on average larger in females with clutches of two follicles when compared to clutches of one follicle, but smaller in females with clutches of three when compared to clutches of two. In addition, females that were larger produced larger follicles regardless of clutch size. Neither CORT nor BKA affected the relationship between follicle size and clutch size. However, ROMs did explain variability in this relationship: oxidative stress was more elevated in females that produced larger clutches and larger follicles. We conclude that clutch size and body size are key life history traits that shape follicle size, and that investments into larger clutches and follicle size come at the cost of oxidative damage.

**Keywords** Clutch size · Glucocorticoids · Immunity · Trade-off · Lizard · Oxidative damage · Reproduction · Whiptail

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## Introduction

The partitioning of finite resources across a female's reproductive life influences her investment in current and future reproductive success and her survival probability (Williams 1966). A trade-off between the total resources invested per clutch, and the number of clutches produced over reproductive life in particular, has led to the evolution of semelparous versus iteroparous life history strategies (Hughes 2017). Life-history theory further predicts there should be an inverse relationship between offspring size and offspring number because resources are limiting and females cannot simultaneously maximize both (Stearns 1992). These constraints will generally result in an inverse relationship between egg size and number per clutch (Parker and Begon 1986; Dziminski et al 2009), with selection favoring either the production of a few large eggs or many small eggs. Lack's hypothesis (1947) regarding clutch size was the first empirical test of this idea in a bird, the great tit (*Parus major*), given a female's finite egg-carrying capacity and reproductive reserve.

The offspring size vs. number trade-off (Lessels 1991; Stearns 1992) relies on the assumption that larger offspring tend to have higher adult reproductive success and greater chances of survival into adulthood (Ferguson and Fox 1984; Sinervo 1990; Roff 2002). If resources are unlimited, a female should invest more resources per offspring to enhance her own reproductive success (Lack 1947; Smith and Fretwell 1974; Grafen 1988), but if resources are limited, she can only increase the size of individual offspring at a cost to the number of offspring produced. Hence the trade-off between offspring size and number is inevitable *in natura* (Stearns 1992). Although this trade-off has been extensively studied in avian species (e.g. Rohwer 1988; Blackburn 1991; Williams 2001), we know little about the factors that may govern this relationship in oviparous lizards, apart from an acknowledgement that this trade-off is generally constrained by maternal physiology (Ljungström et al 2016). Clutch sizes vary from one to forty or more among different species of oviparous lizards, capturing a spectrum of r to K-selected species (Pianka 1970). Some species reproduce only once every second or third year, whereas others reproduce yearly and may even lay two or more clutches each year. Substantial spatial and temporal variation in clutch size also exists between species (Stearns 1992).

Aubry et al (2020) studied the trade-off between clutch size and follicle size and its determinants in the Colorado Checkered Whiptail, *Aspidoscelis neotesselatus*, a "female-only" parthenogenetic lizard. Aubry et al (2020) suggest several advantages to studying reproductive trade-offs in this species: egg cell development happens without fertilization by males and offspring are genetically identical to their mother. One can also exclude potential epigenetic effects from males, and assume that the entire investment strategy is driven by the female. In addition, energetically costly reproductive behaviors such as courtship (Sullivan and Kwiatkowski 2007) and parental care (Case 1978) are minimal in this species. Finally, one can easily and non-invasively use ultrasonography to quantify variability in follicular and egg development (Krawchuk and Brooks 1998; Gilman and Wolf 2007).

Several factors could affect the potential trade-off between offspring size and offspring number in *A. neotesselatus*: chief among them are a female's size and her physiological state:

(i) Female body size could affect the trade-off between offspring size and offspring number as larger females may have access to additional energetic resources, which they could then invest into follicle development. In both viviparous and oviparous populations of common lizard, larger females were able to produce larger clutches (Qualls and Shine 1998; Horváthová et al. 2013; Recknagel and Elmer 2019), and the selective benefit of larger body size led to an evolutionary increase in female size (Recknagel and Elmer 2019). In Eublepharid geckos (*Eublepharidae*), female body mass and egg mass are related isometrically, with larger females producing larger eggs (Kratochvíl and Frynta 2006). This finding contrasts with most other ectotherms, which show a negative allometry between female size and egg size rather than an isometric relationship (Bauwens and Díaz-Uriarte 1997). In *A. neotesselatus*, which produces small clutches of proportionally large eggs, it is more likely that any additional resources that are put toward egg development would increase the size of the follicle, and not increase the size of the clutch, as suggested by the fractional egg size hypothesis (Ford and Seigel 2010). For *A. neotesselatus*, investing in an additional follicle would require a much larger investment of energy according to this hypothesis, which states that changing clutch size requires a large investment of energy per additional egg (Ford and Seigel 2010).

(ii) Because reproduction is a costly activity, it is expected to decrease other key physiological functions such as immunity; likewise, initiating an immune response is costly and may use up energy that could affect reproductive success (e.g. Adamo et al 2001; French et al 2007b; Martin et al 2008; Knowles et al 2009; Cox et al 2010). Reproduction can involve energy-mobilizing hormones such as corticosterone (CORT), and can lead to the accumulation of reactive oxygen metabolites (ROMs) (Metcalfe and Alonso-Alvarez 2010). Indeed, during reproduction, levels of ROMs may exceed the capacity of antioxidant defense and repair mechanisms (i.e. oxidative stress), leading to oxidative damage of biomolecules (Metcalfe and Alonso-Alvarez 2010). Like any other organism, *A. neotesselatus* must allocate resources towards competing functions such as reproduction, growth, and maintenance (Stearns 1992). Immunity in particular is expected to limit the resources available for reproduction since immune function is an important aspect of self-maintenance (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; in birds: Hamilton and Zuk 1982; Ilmonen et al 2000; Ardia et al 2003; in mammals: Derting and Compton 2003; Graham et al 2010; and in lizards: Dunlap and Schall 1995; French et al 2007a, b; Cox et al 2010). In addition, because the stress response and corresponding glucocorticoid release can sometimes diminish immune function critical to self-maintenance (Lucas and French 2012), we may observe an increased stress response in females that produce larger clutches, larger follicles, or both. Increased physiological stress is also related to the overproduction of ROMs (Lucas and French 2012), which could be elevated in females that invest in larger clutches and (or) larger follicles. However, these relationships are often context-dependent (Costantini 2016; Smith and French 2017), hence the importance of drawing comparisons between (sub) populations that inhabit different sampling areas.

In this study, we tested whether a clutch-follicle size trade-off exists in a population of *A. neotesselatus* that has been studied at the Fort Carson Army installation. We further investigated whether the potential trade-off changes with (i) body size (snout-vent-length, SVL) or (ii) physiological parameters including energy-mobilizing hormones (CORT), innate immunity (measured with a bacterial killing assay, BKA), and oxidative stress (ROMs). We predicted larger females would not suffer as great a clutch-follicle size trade-off relative to smaller females; we further expected females investing in larger clutches and (or) larger follicles would show elevated CORT concentrations, lower BKA, and increased oxidative stress (ROMs).

## Methods

### Field collection

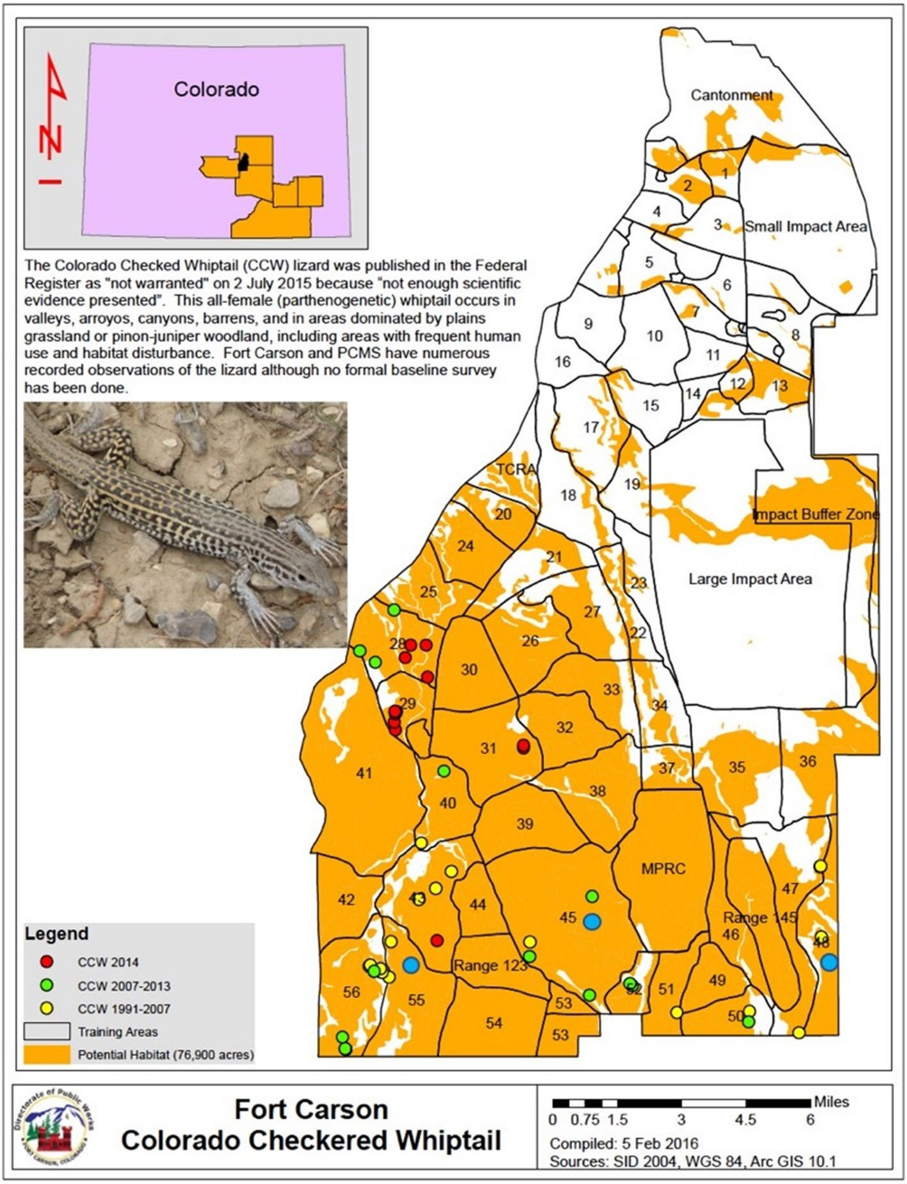
Data were collected from *A. neotesselatus* lizards (Fig. 1) living on the Fort Carson (FC) U.S. Army Installation located in unincorporated El Paso County, Colorado, near the city of Colorado Springs, USA. Fort Carson is 55,000 hectares and also extends into Pueblo and Fremont Counties. *A. neotesselatus* was sampled and surveyed in the northern edge of its range in FC. Lizards were surveyed and sampled across two reproductive seasons in 2018 and 2019. The species is known to occur in several different training areas (TAs) on Fort Carson (Fig. 1), but access to TAs was limited because of military activity. Most surveying focused on TAs 48 and 55, with some data obtained from TA45. The area surveyed across these TAs represented 0.99 ha for TA45 (38.465° N, 104.933° W), 6.12 ha for TA48 (38.467° N, 104.733° W), and 4.85 ha for TA55 (38.398° N, 104.872° W). All TAs were subject to similarly low levels of disturbance from military training activities (i.e. on-foot navigation and orientation).

The habitat for *A. neotesselatus* included Piñon Pine (*Pinus edulis*), Ponderosa Pine (*Pinus ponderosa*), and mixed oak trees (*Quercus* sp.), as well as the cactus Tree Cholla (*Cylindropuntia imbricata*) and the grass Blue Grama (*Bouteloua gracilis*) which mostly dominated TA45. In TA48, the majority of *A. neotesselatus* were found in a dry creek bed and along its banks. The shrubs found in TA48 included Shadscale (*Atriplex confertifolia*), Four-wing Saltbush (*Atriplex canescens*), James' Seaheath (*Frankenia jamesii*) and Rubber Rabbitbrush (*Chrysothamnus nauseosus*). The secondary vegetation type was One-seeded Juniper (*Juniperus monosperma*) and mixed grassland, located around the edges of the sample area. TA55 was similar to TA48, but with more Juniper trees rather than shrubs dominating the landscape.

Transect surveys were conducted during the morning and early afternoon hours (0900–1300) to seek out and capture individuals. Once caught using a snaring method, lizards were kept in mesh fabric bags in the shade to later be processed. Processing included measuring SVL (mm), unique toe clipping (for new captures), marking a unique color code with non-toxic paint pens, and performing ultrasounds on adults. Ultrasounds were performed in order to assess gravidity, clutch size, and follicular/egg size of adult females. Note that we use the term follicle throughout the manuscript as an inclusive term that encompasses anything from the smallest detectable follicle that could be measured, to the largest clearly defined egg, which ranged from 0.17 to 1.05 cm. Follicle size measurement, i.e. the length along the longest axis, was done using a Sonosite Turbo ultrasound unit with an external linear probe (Sonosite Turbo ultrasound, FUJIFILM SonoSite Inc., Bothell, Washington, USA), and each follicle in a clutch was our sampling unit. Once processed, lizards were released in the same location they were captured.

### Blood sample collection

Each captured adult lizard had blood samples taken within the first 5 min. of being pursued for capture, a validated window of time to measure baseline physiological activity in lizards



The Colorado Checkered Whiptail (CCW) lizard was published in the Federal Register as "not warranted" on 2 July 2015 because "not enough scientific evidence presented". This all-female (parthenogenetic) whiptail occurs in valleys, arroyos, canyons, barrens, and in areas dominated by plains grassland or pinon-juniper woodland, including areas with frequent human use and habitat disturbance. Fort Carson and PCMS have numerous recorded observations of the lizard although no formal baseline survey has been done.

**Fig. 1** Surveys of *A. neotesselata* conducted in 1991–2007 (yellow dots), 2007–2013 (green dots), and 2014 (red dots) at Fort Carson, CO. Surveys used in this study (2018–2019) are represented by blue dots, with intensive sampling focused on TA45, TA48, and TA55

(Romero and Reed 2005). If capture attempts exceeded 5 min in duration, blood samples were not taken from that individual lizard to limit the effect of a stress response elicited by the actual pursuit of the animal. Blood samples were taken from the retro-orbital sinus area using heparinized capillary tubes immediately after capture (Maclean et al. 1973). These samples were analyzed to measure baseline levels of physiological activity (Romero and

Reed 2005; Sheriff et al 2011). After collection, blood samples were immediately stored on ice until being centrifuged at 6000 RPM for 10 min to isolate plasma, which was then separated, frozen, and stored at − 20 °C for assays at a later time. Samples were only taken during a standardized time frame in order to avoid daily circadian differences in CORT released by stimuli (Maclean et al. 1973; Dallman et al 1987; Jones and Gillham 1988; Romero and Wingfield 2001). This sampling period was 0700–1200 h, and was found to have no relationship between circulating CORT and time of day (Hudson et al 2020).

### Blood plasma analysis

*Corticosterone* In order to determine CORT concentrations, enzyme-linked immunosorbent assay (ELISA) kits were validated and used with blood plasma (10 uL), following Hudson et al (2020). This colorimetric ELISA is based on competitive binding between sheep polyclonal antibodies and plasma hormone that takes place on a donkey anti-sheep immunoglobulin (IgG) microtiter plate. We assayed each sample in duplicate across seven 96-well plates according to manufacturer guidelines (Product # ADI-901–097; Enzo Life Sciences, Farmingdale, NY). Assay sensitivity was 27 pg/ml with a mean intra-assay coefficient of variation (CV) of 1.46% and an inter-assay CV of 12.19%.

*Bacterial killing assay* To assess the inherent immune function for this species, BKA was measured using a validated volume of blood plasma (11 uL) following Hudson et al (2020). Using the protocol outlined in French and Neuman-Lee (2012), a 1:2 plasma dilution was combined with $CO_2$-independent media (plus 4 nM l-glutamine), $10^4$ colony producing units of *Escherichia coli* (EPowerTM Microorganisms #483–581-1, ATCC 8739, MicroBioLogics, St. Cloud, MN, USA), and agar broth on a 96-well microplate. Included were both negative (media and no plasma or bacteria) and positive (media and bacteria with no plasma) controls to account for potential growth and to ensure that there was no contamination. The plate was incubated for 12 h at 37 °C and absorbance per well was measured with a microplate reader at 300 nm (xMark; BioRad Benchmark, Hercules, CA, USA). Bactericidal ability was then calculated as 1 − (absorbance of sample/absorbance of positive controls) × 100. We ran 7 microplates in total with a mean intra-assay CV of 2.82% and an inter-assay CV of 7.88%.

*Reactive oxygen metabolites* Oxidative status was measured using reactive oxygen metabolites (ROMs). Circulating ROMs were quantified using a d-ROMs test kit (MC435, Diacron International, Italy) which discerns levels of hydroperoxides that oxidize an alkyl-substituted aromatic amine ($A\text{-}NH_2$). Plasma was diluted in an acidic buffered solution (5 µl: 100 µl) following "endpoint" mode manufacturer instructions that were adjusted for a 96-well microplate (Hudson et al 2020), and incubated for 90 min at 37°C. Values were calculated as absorbance change relative to the standard. The mean intra-assay CV over 8 plates was 3.26% and the inter-assay CV was 3.33%.

### Statistical analyses

All analyses and visual representation of data were performed using default packages in RStudio (version 1.2.5001, R Core Team 2019) and additional packages: 'dplyr' (Wickham 2020) and 'ggplot2' (Wickham 2016). Because we had to account for "nested" measurements of follicle size within clutches, we included a random effect of clutch identity in our models (Bolker et al 2009) and used linear mixed models with a gaussian distribution and an identity link to model the relationship between covariates of interest (e.g. SVL, CORT) and follicle size, using the 'nlme' package ("lme" function, Pinheiro et al 2021). We built models that systematically included clutch size as a categorical independent variable, and where follicle size was the dependent variable.

To address our first question, we investigated the relationship between clutch size (independent variable) and follicle size (dependent variable) while accounting for sampling month (i.e. May, June, or July). Past work (Hudson et al 2020; Aubry et al 2020) indicates that variability in clutch size is best captured by the month within which animals are sampled, with reproductive activity peaking in June and slowing down in July. Accounting for the sampling month in our analysis will help control for variability in reproductive effort, as well as various stages of investment into both clutch size and follicle size. We tested for normality of model residuals using Shapiro's test (Royston 1982) and for homogeneity of variance using Levene's test (i.e. homoscedasticity, Hines and Hines 2000).

We tested for differences in follicle size on clutches of various sizes (one to three) while accounting for female size (SVL) using linear mixed models. We then investigated the effect of CORT, BKA, and ROMs on the relationship between clutch size and follicle size with the same approach. SVL, CORT and ROMs were log-transformed to un-skew their distribution and normalize residuals.

Model assumptions of normality of residuals and homoscedasticity were tested using appropriate statistics and are reported in the result section below. We adopted a significance level alpha of 0.05 for statistical significance in all analyses.

## Results

We collected data from 454 follicles and 291 clutches. We obtained measures of CORT for 123 samples (9 from TA45, 82 from TA48, and 32 from TA55), BKA for 141 samples (11 from TA45, 96 from TA48, and 34 from TA55), and ROMs for 170 samples (16 from TA45, 102 from TA48, and 52 from TA55).

### Clutch size and follicle size trade-off

We detected an effect of clutch size on follicle size (Table 1, $n = 454$) where follicle size was on average larger in females with clutches of size two when compared to clutches of size one (*p-value* $< 0.001$, Fig. 2) or three (*p-value* $< 0.001$, Fig. 2). Although the effect of capture month was significant as well, its interaction with clutch size did not impact follicle size (Table 1). Model residuals were normally distributed (Shapiro–Wilk normality test, $W = 0.983$, *p-value* $= 0.263$) and variances were homoscedastic across clutch sizes (Bartlett test of homogeneity of variances, Bartlett's $K^2 = 0.750$, $df = 2$, *p-value* $= 0.687$).

### Effect of body size on clutch-follicle size trade-off

Females that were larger produced larger follicles regardless of clutch size (*p-value* $< 0.001$, $n = 291$, Fig. 3). Follicle size was larger in clutches of size two when compared to other clutch sizes, independently of SVL (Fig. 3). Although SVL did affect follicle size, its interaction with clutch size did not (Table 2).

**Table 1** Table of results testing for the effect of clutch size (independent variable ranging from one to three) and month of capture 'Month' (independent variable) on follicle size (dependent variable) while accounting for nested measurements of follicle size within clutch (random effect of clutch identity)

| Fixed effects | *Value* | *Std. Error* | *t-value* | *p-value* |
|---|---|---|---|---|
| Month (-Intercept) | −0.0575 | 0.0225 | −2.5518 | 0.0113 |
| Month * (Clutch size of 2) | 0.0183 | 0.0373 | 0.4909 | 0.6239 |
| Month * (Clutch size of 3) | 0.1175 | 0.1184 | 0.9926 | 0.3219 |
| Clutch size of 1 | 0.6214 | 0.0477 | 13.0314 | <0.0001 |
| Clutch size of 2 | 0.6919 | 0.0618 | 11.1871 | <0.0001 |
| Clutch size of 3 | 0.4481 | 0.2099 | 2.1345 | 0.0338 |
| Random effect | *Intercept* | *Residual* | | |
| Std. deviation (Clutch Identity) | 0.1321 | 0.0840 | | |

We report regression estimates (Value), standard error (Std. Error), t-statistic and p-value for each effect, as well as the standard deviation of the intercept and residuals of the random effect of clutch identity; degrees of freedom = 238.

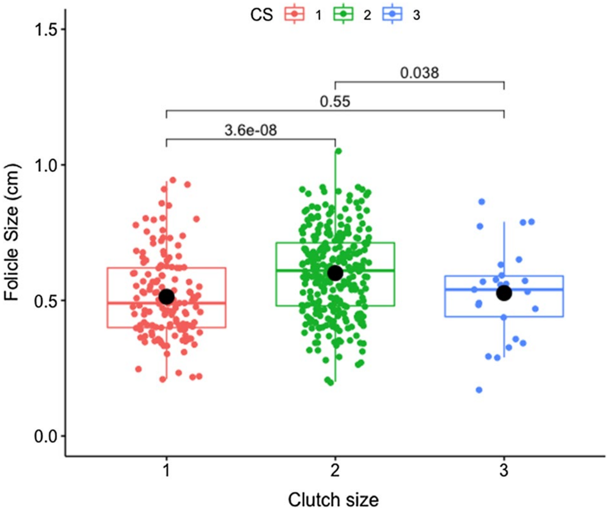


**Fig. 2** Relationship between clutch size and follicle size in *A. neotesselatus* studied at the Fort Carson Army installation near Colorado Springs, CO. Significant differences are represented by p-value for each paired comparison. The median and interquartile range are represented by the thick line and edges of the box, respectively. Whiskers represent 95% confidence intervals; thick horizontal lines show medians, while large black dots represent means

## Effect of physiological state on clutch-follicle size trade-off

The effect of CORT and its interaction with clutch size did not affect follicle size (Table 3). Similarly, the effect of BKA and its interaction with clutch size did not affect follicle size (Table 4). ROMs and its interaction with clutch size did have significant effects on follicle size (Table 5, Fig. 4). Specifically, oxidative stress was higher in females with larger clutches (two or three follicles) and follicles of larger size, when compared to females that produced a single clutch with one small follicle (Fig. 4).

**Fig. 3** Estimates from the generalized linear model testing for a relationship between snout-vent-length, Log(SVL) (cm), and follicle size (cm) across clutch sizes (CS) of 1, 2, and 3 follicles. Shaded areas represent 95% confidence intervals around estimated slope regression coefficient for each clutch size level

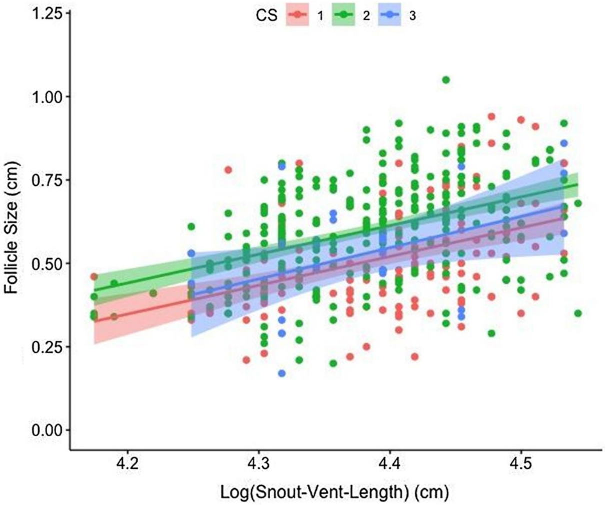


**Table 2** Linear mixed model testing for the effects of clutch size ‘ (independent variable ranging from one to three), snout-vent-length ‘SVL’ (independent variable), and their interaction on follicle size (dependent variable) while accounting for nested measurements of follicle size within clutch (random effect of clutch identity)

| Fixed effects | *Value* | *Std. Error* | *t-statistic* | *p-value* |
|---|---|---|---|---|
| log(SVL)(-Intercept) | 0.8667 | 0.1664 | 5.2086 | <0.001 |
| log(SVL) * (Clutch size of 2) | 0.1879 | 0.2231 | 0.8419 | 0.4007 |
| log(SVL) * (Clutch size of 3) | −0.1657 | 0.5593 | −0.2962 | 0.7673 |
| Clutch size of 1 | −3.2944 | 0.7297 | −4.5149 | <0.001 |
| Clutch size of 2 | −0.7106 | 0.9779 | −0.7267 | 0.4681 |
| Clutch size of 3 | 0.7760 | 2.4513 | 0.3166 | 0.7519 |
| Random effect | *Intercept* | *Residual* | | |
| Std. deviation (Clutch Identity) | 0.1099 | 0.0852 | | |

We report regression estimates (Value), standard error (*Std. Error*), *t-statistic* and *p-value* for each effect, as well as the standard deviation of the intercept and residuals of the random effect of clutch identity; degrees of freedom = 288

## Discussion

Given limited resources, theory predicts that a mother cannot simultaneously increase offspring size and offspring number, meaning that a trade-off between these two traits is inevitable because of physiological constraints (Lessels 1991; Stearns 1992). The existence of a size–number trade-off is strongly supported by both theoretical models and empirical data (Stearns 1992). Our results did not point to a trade-off between clutch size and follicle size, but rather to an optimum level of investment for clutches of size two, which also happens to be the most frequent clutch size amongst our observations. In addition, both clutch size and body size were particularly important factors in

**Table 3** Linear mixed model testing for the effects of clutch size (independent variable ranging from one to three), log(CORT) (pg/mL) (independent variable), and their interaction on follicle size (dependent variable) while accounting for nested measurements of follicle size within clutch (random effect of clutch identity)

| Fixed effects | *Value* | *Std. Error* | *t-value* | *p-value* |
|---|---|---|---|---|
| Log(CORT) (-Intercept) | 0.0087 | 0.0067 | 1.2969 | 0.2034 |
| Log(CORT) * (Clutch size of 2) | −0.0190 | 0.0134 | −1.4141 | 0.1612 |
| Log(CORT) * (Clutch size of 3) | −0.0391 | 0.2204 | −0.1776 | 0.8595 |
| Clutch size of 1 | 0.4928 | 0.0372 | 13.2438 | <0.001 |
| Clutch size of 2 | 0.6153 | 0.0422 | 14.5924 | <0.001 |
| Clutch size of 3 | 0.5318 | 0.1815 | 2.9293 | 0.0044 |
| Random effect | *Intercept* | *Residual* | | |
| Std. deviation (Clutch Identity) | 0.1410 | 0.1056 | | |

We report on regression estimates (Value), standard error (*Std. Error*), *t-statistic* and *p-value* for each effect, as well as the standard deviation of the intercept and residuals of the random effect of clutch identity; degrees of freedom = 82.

**Table 4** Linear mixed model testing for the effects of clutch size (independent variable ranging from one to three), BKA (%) (independent variable), and their interaction on follicle size (dependent variable) while accounting for nested measurements of follicle size within clutch (random effect of clutch identity)

| Fixed effects | *Value* | *Std. Error* | *t-value* | *p-value* |
|---|---|---|---|---|
| BKA (-Intercept) | −0.0006 | 0.0007 | −0.8720 | 0.3883 |
| BKA * (Clutch size of 2) | −0.0009 | 0.0011 | −0.8671 | 0.3881 |
| BKA * (Clutch size of 3) | 0.0011 | 0.0026 | 0.4214 | 0.6745 |
| Clutch size of 1 | 0.5452 | 0.0438 | 12.4496 | <0.001 |
| Clutch size of 2 | 0.6805 | 0.0463 | 14.6812 | <0.001 |
| Clutch size of 3 | 0.4868 | 0.1902 | 2.5596 | 0.0121 |
| Random effect | *Intercept* | *Residual* | | |
| Std. deviation (Clutch Identity) | 0.1268 | 0.0982 | | |

We report on regression estimates (Value), standard error (*Std. Error*), *t-statistic* and *p-value* for each effect, as well as the standard deviation of the intercept and residuals of the random effect of clutch identity; degrees of freedom = 93.

determining follicle size (Figs. 2 and 3, respectively), and a reproductive investment into larger clutches and follicle size came at the cost of oxidative damage (Fig. 4).

Specifically, our findings indicate that clutch size and follicle size were not inversely, nor linearly related in *A. neotesselatus*. Instead, follicles were larger in intermediate clutch sizes (i.e. two follicles) than those that were smaller and larger (i.e. one or three follicles). Among larger clutches of size three, we detect a trade-off between clutch size and follicle size, which suggests that this level of reproductive investment into the largest possible clutch comes at the cost of follicle quality/size, which is consistent with other reptile studies (Rowe 1994; Olsson and Shine 1997; Uller and Olsson 2009). Still,

**Table 5** Linear mixed model testing for the effects of clutch size (independent variable ranging from one to three), ROMs (mg H2O2/dL) (independent variable), and their interaction on follicle size (dependent variable) while accounting for nested measurements of follicle size within clutch (random effect of clutch identity)

| Fixed effects | *Value* | *Std. Error* | *t-value* | *p-value* |
|---|---|---|---|---|
| Log(ROMs) (-Intercept) | −0.0401 | 0.0173 | −2.3147 | 0.0244 |
| Log(ROMs) * (Clutch size of 2) | 0.0734 | 0.0267 | 2.7513 | 0.0070 |
| Log(ROMs) * (Clutch size of 3) | 0.0376 | 0.0686 | 0.5482 | 0.5847 |
| (Clutch size of 1) | 0.5859 | 0.0395 | 14.8505 | 0.0000 |
| (Clutch size of 2) | 0.5267 | 0.0459 | 11.4674 | 0.0000 |
| (Clutch size of 3) | 0.5286 | 0.1581 | 3.3423 | 0.0011 |
| Random effect | *Intercept* | *Residual* | | |
| Std. deviation (Clutch Identity) | 0.1356 | 0.0912 | | |

We report on regression estimates (Value), standard error (*Std. Error*), *t-statistic* and *p-value* for each effect, as well as the standard deviation of the intercept and residuals of the random effect of clutch identity; degrees of freedom = 108.

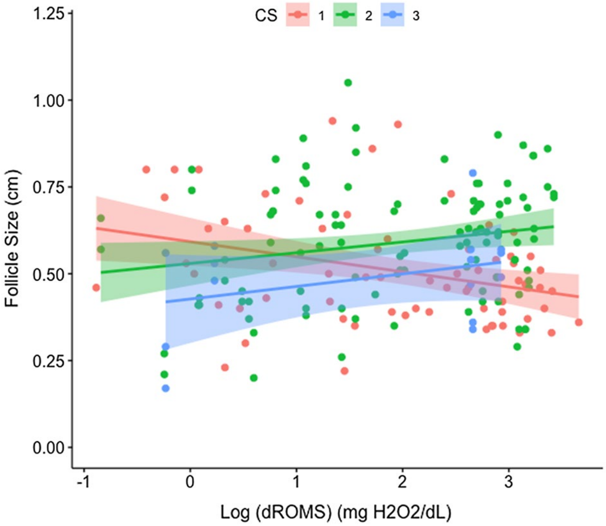


**Fig. 4** Estimates from the linear model testing for a relationship between oxidative damage (Log(ROMs) in mg H2O2/dL) and follicle size (cm) across clutch sizes (CS) of 1, 2, and 3 follicles. Shaded areas represent 95% confidence intervals around estimated slope regression coefficient for each clutch size level

the observed non-linear relationship between clutch size and follicle size is inconsistent with theoretical predictions (Lessels 1991; Stearns 1992).

Although progression in reproductive effort over the sampling period could confound this finding, the above relationship between clutch size and follicle size held true independently of reproductive stage. Indeed, the interaction between sampling month and clutch size did not have a significant effect on follicle size, meaning that seasonal progression did not affect the relationship that exists between clutch and follicle size. However, sampling month did have an effect on follicle size, suggesting that we did capture a change in follicle size with month of sampling. The observed non-linear relationship between follicle size and clutch size was mostly driven by samples collected in June, but there was no difference in follicle size across clutches of different sizes in July, and only a marginal difference in follicle size between clutches of size one and clutches of size two (where follicles were significantly larger in clutch of size two); results not presented herein for conciseness. Total investment per clutch, and the trade-off between follicle size and number, can shift among years within a single reptile population, depending upon a mother's provisioning rate (e.g. Olsson and Shine 1997). Additional years of data collection will help determine whether the observed bell-shaped pattern (Fig. 2) holds true, and whether intermediate clutches of size two maximize follicle size *in natura*.

We detected an effect of body size on follicle size within larger females (i.e. longer SVL) that was independent of clutch size which is consistent with other reptile studies (Ford and Siegel 1989; Qualls and Shine 1998; Horváthová et al. 2013; Recknagel and Elmer 2019). Larger females tend to have access to greater resources, and thus are more likely to allocate energy towards reproduction, resulting in larger clutches and/or larger follicles. Our findings suggest that larger *A. neotesselatus* females able to invest in larger clutches were also able to produce larger follicles, without having to compromise, suggesting that access to resources and female size are key in determining female reproductive success.

Findings regarding the effects of physiological state on the observed clutch-follicle size trade-off were mixed. We detected no relationship between CORT and follicle size, no matter the clutch size considered, indicating no difference in stress hormones between females that invested in large clutch sizes and females that invested in large follicle sizes. Aubry et al (2020) found that females who invested more energy into reproduction and produced larger clutch sizes often suffered higher CORT levels. However, focusing on a subset of the data for which we had follicle size measurements, we found that the relationship between clutch size and follicle size was not affected by energy-mobilization via CORT release.

Similarly, the effect of BKA on follicle size was not significant across clutches of various sizes, suggesting no difference in innate immunity between females that invested in large clutch sizes or large follicle size compared to other females. Aubry et al (2020) observed that an increase in reproductive investment correlated with a decrease in innate immunity, though only in lizards sampled from TA45, a difference in reproduction-immune trade-offs that is rarely documented in lizards (but see Lucas and French 2012). A decrease in immune function is seen in other animals that heavily invest in reproduction (Bonneaud et al 2003; French et al 2007a), yet the lack of a trade-off in our study suggests that more fine scale investment in clutch size and follicle size may not affect innate immunity, and vice versa.

As expected, we detect a relationship between oxidative stress and follicle size across clutches of various sizes. Aubry et al (2020) observed on a larger dataset that females with larger clutch sizes suffered greater oxidative stress. Of note, this comparison included females that were not gravid. Oxidative stress is the rate at which biomolecular oxidative damage is generated, which results from a complex interaction between compounds that oxidize (e.g. free radicals) and compounds that protect against oxidation (antioxidants) (Costantini and Verhulst 2009). Evolutionary ecologists have mainly studied the relationship between oxidative damage and life history trade-offs within the context of ageing theories, which involve compromises between current reproduction, future reproduction, and survival. A large body of work suggests that oxidative stress may be connected to reproduction, but findings have been quite mixed within and across taxa and reproductive strategies (Blount et al 2016). Our results suggest that such compromises are expressed when investigating the relationship between clutch size, follicle size, and oxidative stress (Fig. 4).

Because shifts in physiological state can affect individual reproduction and survival, which collectively drive population growth, it is important to understand the relationships that exist between physiological state and fitness components such as clutch size and egg size, which both influence reproductive success. If for example, elevations on oxidative stress markers such as reactive oxygen metabolites lead to tissue damage, then elevated physiological stress could result in decreased survival within populations (Lucas and French 2012).

With additional years of data collection on this species, we hope to bring survival probability into this equation in an effort to holistically quantify the trade-offs that exist between key fitness components such as reproduction, growth, maintenance and survival (Aubry et al 2019). A mechanistic understanding of how physiology and demographic rates are connected, along with a population-level assessment for the various sub-populations located at Fort Carson (which encompasses a large portion of the species narrow range), will help define a sound conservation plan for *A. neotesselatus.*

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**Authors' contributions** HEC, SSF and LMA conceived the study; HEC and LMA analyzed the data and wrote the manuscript; DE, HEC, field technicians and volunteers collected the data; SSF, SBH, ACW conducted the lab work; BMK and AJL facilitated field work and research activities; all authors provided feedback on earlier versions of the manuscript. This research predominantly funded by Fort Carson by way of a US Fish and Wildlife Cooperative Agreement F17AC00326. This research was also supported in part by the US Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center. The findings and conclusions in this publication have not been formally disseminated by the US Department of Agriculture and should not be construed to represent any agency determination or policy. Field methods were approved through Colorado State University IACUC 18–7772A. R code is available upon request; please contact senior author LMA.

### Declarations

**Conflict of interest** The authors declare no conflicts of interest.

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