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Body Condition: Seasonal Effects and Influence on Aggressive Behavior of Ozark Zigzag Salamanders (Plethodon Angusticlavius)

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BODY CONDITION: SEASONAL EFFECTS AND INFLUENCE ON AGGRESSIVE BEHAVIOR OF OZARK ZIGZAG SALAMANDERS (*PLETHODON ANGUSTICLAVIUS*)

A Master's Thesis
Presented to
The Graduate College of
Missouri State University

In Partial Fulfillment
Of the Requirements for the Degree
Master of Science, Biology

By
Ayesha Abdul Farooq
May 2023

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Biology

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OVERVIEW

Body condition is a measure of the size of energy reserves such as fat and protein, which can influence health and the ability to obtain resources in aggressive encounters. Individuals with better body condition ultimately experience a fitness advantage. Physiological factors such as sex, reproductive condition, and diet can influence body condition of individuals. Body condition could be affected positively or negatively after an animal is kept in captivity for extended periods of time. In the first chapter, I explore whether sex, season (fall and spring), and time in containment at the laboratory influences body condition. In the second chapter, I examined how body conditions affected aggressive behavior in staged contests between pairs. Contestants were paired in either symmetric (same body condition) or asymmetric (different body condition) pairs. The prediction based on evolutionary game theory is that symmetric contests should result in higher levels of aggressive behavior than asymmetric contests.

KEYWORDS: body condition, salamanders, aggressive behavior, seasonal effects, laboratory containment

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I dedicate this thesis to all the young girls who wish to pursue their higher education out of their comfort zones but are subdued yet strive to stand firm on their strong convictions and can independently make decisions they deem best for themselves. If I can do it, so can you. Be brave and push through.

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INTRODUCTION

Body condition is a measure of an animal's energetic (or nutritional) state, particularly with respect to the size of energy reserves such as fat and protein (Krebs & Singleton, 1993; Gosler 1996; Kaufman et al., 2007). An animal's body condition can affect fitness through a variety of physiological mechanisms that influence metabolism and behavior. In addition to having more energy stores to fuel short-term and long-term metabolic needs, individuals in good body condition can be more resistant to or tolerant of disease (e.g., Budishchak et al., 2018), can better maintain physiological responses to stress (e.g., Kitayski et al., 1999), and can have more efficient energy mobilization (e.g., Bonnet et al., 2001).

Body condition can have important consequences for numerous aspects of fitness of individuals. For example, wolf spiders, *Schizopods ocreata*, in better body condition grew better and had significantly larger tuft sizes (a male signalling trait) than those in poorer condition (Uetz, Papkei, & Kilincz, 2002). Salamanders, *Gyrinophilus porphyriticus*, in better body condition were better able to migrate to higher quality upstream habitats, while those in poorer body condition tended to migrate downstream (Lowe et al., 2006). Female Penguins, *Spheniscus magellanicus*, in better condition were more successful in aggressive contests over high-quality nest sites (Renison et al., 2003). In large aggregations of garter snakes, *Thamnophis sirtalis parietalis*, in overwintering dens, males in poorer body condition were more likely to be suffocated beneath piles of other snakes and some died of starvation (Shine et al., 2001). Predators target red squirrels, *Tamiasciurus husonicus*, in poor body condition (Wirsing et al., 2002), and hares, *Lepus granatensis*, in poorer body condition are less able to maintain escape behaviour when being pursued by predators (Alzaga et al., 2008). Body condition can influence reproductive success in a variety of ways, including egg size (Litzgus et al., 2008), laying date (Bêty et al., 2003), development of sexual ornaments (Jawor & Breitwisch, 2004), and attractiveness of males to females (Jiguet & Bretagnolle, 2014).

Salamanders (Amphibia) can be important components in the ecology of some ecosystems, contributing to the biomass, energy flow, and in regulation of their invertebrate prey communities (Semlitsch et al., 2014). However, little is known about how body condition influences various aspects of fitness in terrestrial salamanders. Burton and Likens (1975) showed that the density of lungless terrestrial salamanders (genus *Plethodon*) at the Hubbard Brook Experimental Forest was equal to that of birds during the breeding season, and Semlitsch et al. (2014) more recently reported that this number was a substantial underestimate (by a factor of 2 – 4) in some forests.

To measure body condition, direct measurements of fat reserves are considered the most accurate, but typically are invasive/lethal (Falk et al., 2017), which limits practical applications, or is prohibitively expensive or cumbersome (McGuire et al., 2018). Because of these limitations, researchers frequently use non-invasive condition indexes that are easy to measure. Most common are indexes that consider mass relative to length, which may include correction factors to account for the lack of linearity between the two variables; analysis of mass/length residuals are also sometimes used (review in Peig & Green, 2010). Thus, an individual that is heavier than predicted for its length is in good body condition and those lighter than predicted are considered in poorer condition.

Snout-Vent Length (SVL) rather than Total Length or Mass is the conventional method of measurement of body size for salamanders because tail length and mass are influenced by whether the tail has been autotomized. Tail autotomy is a defense mechanism wherein individuals sever their tails, which distracts predators during attacks; breaks can occur anywhere along the length of the tail for *Plethodon* sp. And salamanders can later regenerate the missing length (Wake & Dresner, 1967). Mass is additionally influenced in amphibians by levels of hydration and recent food intake. A third complication is that body mass specific to length in females can be affected by whether they are carrying yolked eggs (Wells, 2010). When lipids are utilized during vitellogenesis, there is a shift of mass from energy reserves to reproduction and therefore changes in nutritional status are often undetectable (Lewis & Rose, 1968; Fitzpatrick, 1973). Moreover, females lose substantial mass after oviposition, which compromises comparisons involving gravid and nongravid females (Nissen & Bendik, 2020)**.** Due to these issues, body condition indexes that are based on SVL relative to mass measurements are problematic. Instead, researchers studying plethodontid salamanders have generated body condition scores based on Tail Width (TW) relative to SVL (e.g., Guiterrez et al., 2018; Pierce & Gonzalez, 2019; Nissen & Bendik, 2020). Tail width is a reasonable surrogate for mass in condition indices because tails of plethodontid salamanders are important for energy storage (Maiorana, 1977; Yurewicz & Wilbur, 2004).

The Ozark Zigzag Salamander, *Plethodon angusticlavius*, the subject of this study, are locally abundant and occur in the Ozarks region of Oklahoma, Arkansas, and Missouri (Highton, 1997). These salamanders spend the summers in underground burrows, thereby avoiding desiccation and overheating during these warm, dry, months (Petranka, 1998). In the fall, they emerge from their burrows and defend territories in patches of moisture under rocks and logs on the forest floor and forage in the leaf litter after periods of rain (Mathis & Britzke, 2000). Surface activity during the winter is sporadic, occurring during periods of warmer weather (Petranka, 1998). Mating activity occurs from fall to spring, and oviposition occurs in late spring or early summer (Wilkinson et al., 1993). Females begin to incorporate yolk into their ova between February and March, and ova substantially increase in size beginning in April (Wilkinson et al., 1993).

In this chapter, I explore several basic questions about body condition in Ozark Zigzag Salamanders, *Plethodon angusticlavius*. First, does carrying yolked eggs influence the body condition of females? Because I found both gravid and nongravid females during the spring, when females are producing yolk, I assume that *P. angusticlavius* in this population, like other more northern populations of small *Plethodon* (*P. serratus*: Herbeck & Semlitsch, 2000; *P. cinereus*: Sayler, 1966) are biennial, producing yolked eggs only every other year. Therefore, we are able to compare body conditions of gravid and nongravid females that were collected at the same time—during the spring. We did not find gravid females during the fall (late October), before females were producing yolk. Second, does sex (independent of gravidity condition), season (fall versus spring), and time in the laboratory (none vs 10 mo) influence body condition overall? To my knowledge, no other studies have examined effects of time in captivity on body condition of salamanders.

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METHODS

I collected adult *Plethodon angusticlavius* salamanders from Bull Shoals Field Station, Kirbyville, Missouri (n=18) in October of 2020 (n = 18) and March of 2021 (n = 37). Individuals were placed in clean, separate plastic bags containing moist leaf litter. In the lab, Salamanders were kept in individual Petri dishes (diameter = 15–23 cm) that were lined with a damp filter paper and stored in a temperature-controlled environmental chamber at 15ºC. Filter papers were changed biweekly or more frequently if needed and hydrated every 3–4 days. Salamanders were fed adult fruit flies, *Drosophila hydei,* once a week. While in the lab, salamanders were in petri dishes and consumed prey promptly.

Sexing

Most Salamander species, including *P. angusticlavius*, do not exhibit significant external sexual dimorphism. For determining sex, I used the ‘candling’ method (Gillette & Peterson, 2001), which allows non-invasive observation of gonads. Individual salamanders were placed in a plastic Ziploc bag, and the ventral surface of their abdomens was viewed while a bright light was shone through from the dorsal side. The testes in males are visible as dark lines, and ova of gravid females were visible in their ovaries. For non-gravid females, neither testes nor ovaries were visible.

Newly Collected Versus Prolonged Containment in Laboratory Conditions Experiment

Measurements and body condition indexes. Snout-Vent Length (SVL) is the conventional method of body size measurement for salamanders because tail length and mass are influenced by whether the tail has been autotomized. Tail autotomy is a defense mechanism wherein individuals sever their tails, which distracts predators during attacks; breaks can occur anywhere along the length of the tail for *Plethodon* sp. And salamanders can later regenerate the missing length (Wake & Dresner, 1967). Mass is additionally influenced by levels of hydration and recent food intake. Therefore, body condition indices that are based on length relative to mass measurements are problematic. Instead, researchers studying plethodontid salamander have generated body condition scores based on TW relative to SVL (e.g., Guiterrez et al., 2018; Pierce & Gonzalez, 2019; Nissen & Bendik, 2020). Tail width is a reasonable surrogate for mass in condition indices because tails of plethodontid salamanders are important for energy storage (Maiorana, 1977; Yurewicz & Wilbur, 2004).

In this study, the body condition score was measured as the width at the base of the tail divided by SVL. Scatter plots indicated that there was a positive linear relationship between SVL and tail width for our salamanders. To determine whether body conditions differed seasonally, we collected one set of individuals in the fall (28 October 2020; early breeding season) and a different set in the spring (24 March 2021; mid-breeding season). In the October (fall) sample, yolk was not yet visible in the ova through the body wall, whereas yolk was visible in the Spring sample, although the eggs were not as large as they would be closer to oviposition. To determine whether being held in captivity under a standard laboratory feeding regime influenced body condition, we took measurements at two separate time points, when the individuals were first collected and again 10 mo later.

Immediately prior to taking measurements, I anesthetized individuals with Benzocaine in the form of Orajel® as described by Chen & Combs (1999) and Brown et al. (2004). I applied a small amount of Orajel to the dorsal surface of the head immediately behind the eyes and left the salamander undisturbed until it stopped moving (10-15 min). I then used a pair of digital vernier calipers to measure SVL from the tip of their snout to the caudal end of the vent and TW at the base of the tail. Salamanders were positioned with the ventral surface facing up and their bodies in a straight line for accurate measurements. I rinsed the Orajel® off immediately after measuring each salamander, and normal activity was resumed within 25 min; no mortality occurred.

Statistics

A Shapiro-Wilk test confirmed that the data were normally distributed for all data sets (males and females; newly collected and lab-held: p's range between 0.07 and 0.99). Statistical analyses were conducted using SPSS v. 29.0. We addressed two questions: (1) Is there a difference in body condition among males, gravid females and nongravid females when they are first captured during the spring (no females were gravid in the fall)? The three sex classes were compared with a one-way ANOVA (SPSS v. 29.0). (2) Is there an effect of sex (male vs female), season (fall vs spring) and time (0 months vs 10 months) on body condition? A GLM was used to determine if body condition was affected by sex (male vs female), season (fall vs spring), time in the lab (hereafter "time": 0 months vs after 10 months), or an interaction between sex, season, and time.

Institutional Approval

This study was approved by the Institutional Animal Care and Use Committee on 04 June 2020 and received Approval #2020-09 (See Appendix).

RESULTS

Effect of Sex Category on Body Condition

There was no significant difference among body conditions ($F_{2,36} = 1.44$; p = 0.25; Table 1; Figure 1) of males (n =17), gravid females (n =9), and non-gravid females (n =13) when they were first captured during the spring. A power analysis (SPSS v. 29.0) indicated that the effect size was moderate (partial eta-squared = 0.074). Therefore, I combined gravid and nongravid females into one group for subsequent.

Table 1. ANOVA results of body condition differences between males, gravid and non-gravid females

Source	Type III Sum of Squares	df	Mean Square	F	P
Sex category	0.001	2	0.000	1.47	0.249
Error	0.007	36	0.000		
Total	0.389	39			

Effect of Sex, Season and Time Spent in Laboratory on Body Condition

There was no significant effect of sex on body condition and no interactions between sex and either season or time in the lab (Table 2).

There was a significant interaction between season and time in the laboratory (Table 2, Figure 2). Post-hoc 2-way GLM analysis confirmed this interaction (Table 3). In the fall, individuals were in better body condition when they were first collected, losing about 25% of body condition during the 10 mo they spent in the laboratory. In contrast, individuals collected in the spring maintained virtually the same body condition after 10 mo in the laboratory.

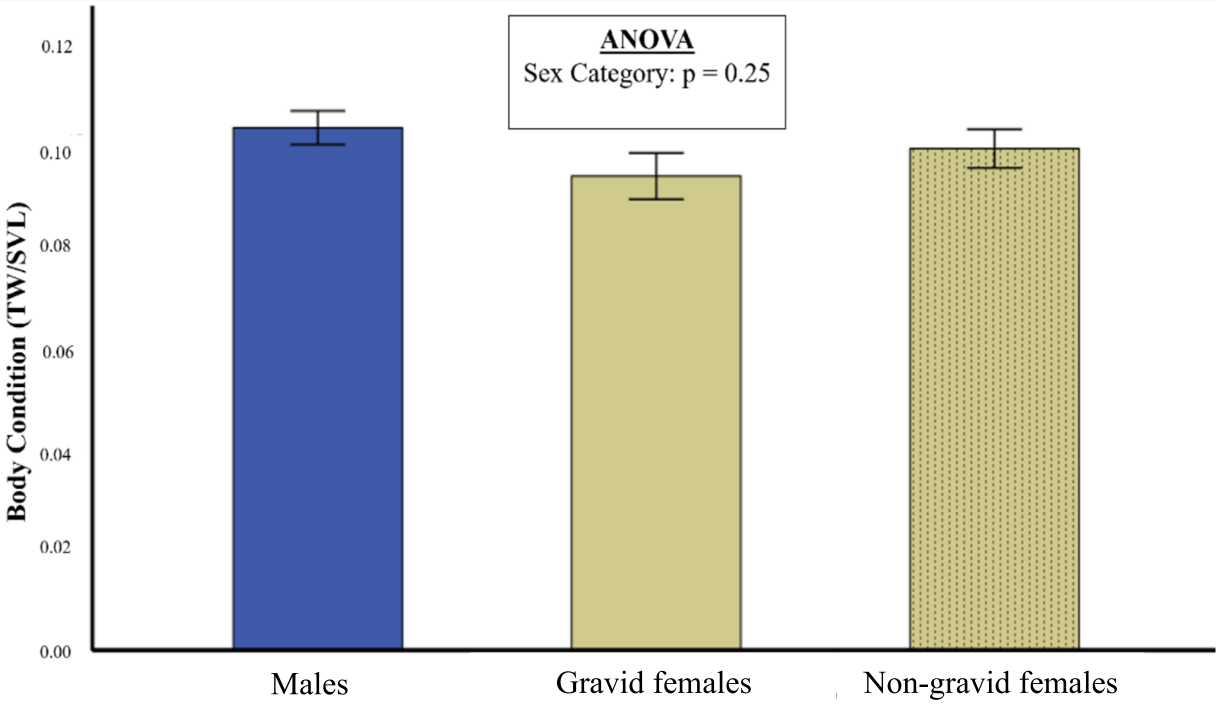

Figure 1. ANOVA results of body condition differences between males, gravid and non-gravid females. Bars are means ±1SE.

Table 2. 3-Way ANOVA to analyze the effects of sex, season and time contained in laboratory on Body Condition.

Source	Type III Sum of Squares	df	Mean Square	F	P
Sex	1.895E-5	1	1.895-5	0.089	0.766
Time	0.007	1	0.007	31.521	<0.001
Season	7.103E-6	1	7.103E-6	0.033	0.856
Sex * Time	4.005E-7	1	4.005E-7	0.002	0.966
Sex * Season	6.836E-6	1	6.836E-6	0.032	0.858
Time * Season	0.005	1	0.005	23.794	<0.001
Sex * Time * Season	0.000	1	0.000	1.817	0.181
Error	0.019	90	0.000		
Total	0.998	98			

Table 3. 2-way ANOVA table to highlight significant interaction effects of season and time on body condition.

Source	Type III Sum of Squares	df	Mean Square	F	P
Time	0.007	1	0.007	32.756	<0.001
Season	6.886E-7	1	6.886E-7	0.003	0.955
Time * Season	0.005	1	0.005	25.828	<0.001
Error	0.020	94	0.000		
Total	0.998	98			
Corrected Total	0.029	97			

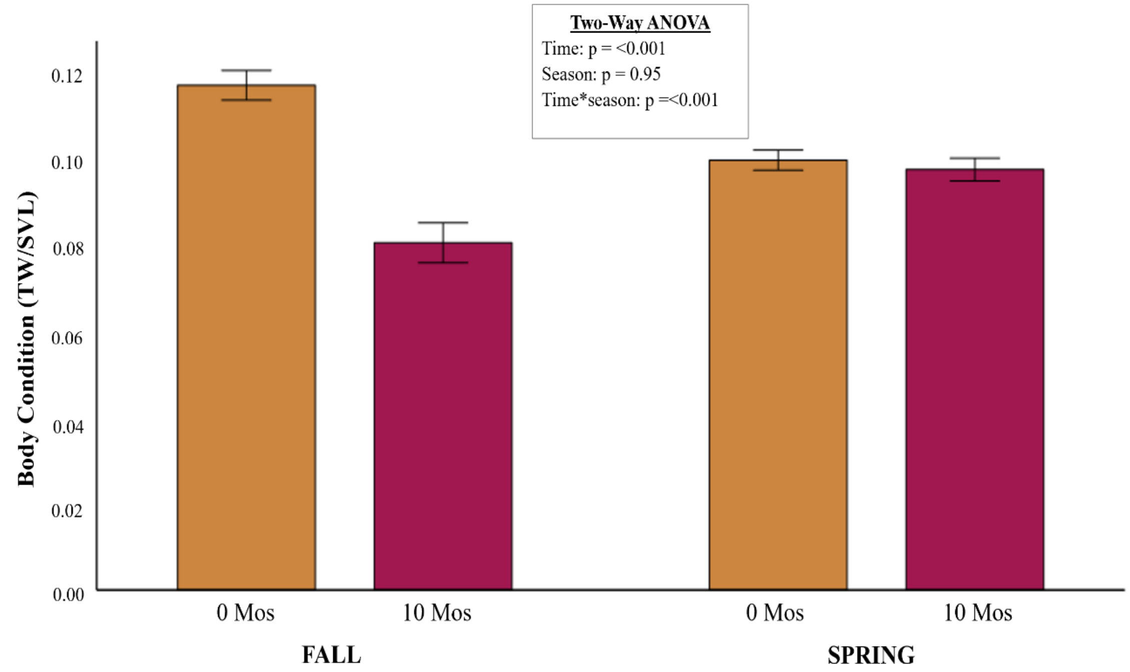

Figure 2. Two-way ANOVA depicting significant interaction effects of time and season on body condition. Bars are means ±1SE.

DISCUSSION

In my study, body condition of gravid and non-gravid females did not differ from each other or from males during the spring. In contrast, in studies using the same method of assessing body condition, non-gravid female stream salamanders, *Eurycea sosorum*, *E. tonkawae* and *E. naufragia,* were in better condition than gravid females during the breeding season (Pierce, 2022; Nissen & Bendik, 2020). One explanation for this difference could be that I collected salamanders relatively early in the spring (24 March) before the eggs were fully yolked. Although yolked eggs were visible in the abdomen currently, oviposition does not occur until late spring or early summer (Wilkinson et al., 1993). Carrying eggs may be more costly later in the breeding season, resulting in a reduction of body condition as energy in the tail becomes more fully mobilized for yolk production (Fitzpatrick, 1973; Bernardo & Agosta, 2005). A second explanation for the failure to find a difference among sex categories is that the sample size was not sufficient. Statistical power (partial eta squared) for this comparison is generally interpreted as moderate (https://www.spss-tutorials.com/effect-size/), so it might be worthwhile to repeat the comparison with a larger sample size or nearer to oviposition. In my study, sample sizes for males, gravid and non-gravid females ranged from 9–17. Nissen & Bendik (2020) studied several populations with sex categories ranging from 15–250 for *E. sosorum* and 27–596 for *E. tonkawae*, and Pierce's study included 703 unique individuals spread among the sex categories.

My second analysis considered whether body condition was affected by sex (gravid and non-gravid females combined), season (fall and spring), and time the salamanders had been maintained in the lab. Sex had no significant main effect on body condition and did not significantly interact with the other two factors to influence body condition. Whether there are sex differences in body condition of European cave salamanders (*Speleomantes* sp.) differed among species. In five hybrid populations, Lunghi et al. (2021) also found no significant differences between males and females. In contrast, females of *S. flavus* were in better body conditions than males (Lunghi et al., 2018). The authors attributed this difference to females sequestering more energy to yolk eggs, but it is not clear why there are sex differences in this species but not in the hybrid populations (Lunghi et al., 2021).

The effect of season that the animals were collected depended on how long they had been maintained in the laboratory. For salamanders that were captured during the fall, individuals whose body condition was measured immediately after capture were in better condition than those that were measured after 10 mo in the laboratory. During the warm summer months, salamanders are in underground burrows and thus do not mobilize much energy to fuel activity. Salamanders emerge during the early fall rains when prey is very abundant in the leaf litter, allowing for substantial prey consumption and storage of energy by the time I captured them in late October. While adequate for maintenance, the laboratory diet would likely not be equivalent to the high energy diet they would experience during the fall rains. Both diet quality (Haines et al., 2022) and quantity (Cornilessen & Vulink, 2015) influences body condition of individuals. For example, in summer and fall, common mudpuppies, *Necturus maculosus,* fed on invertebrates exclusively and were in poorer condition than in the in winter and spring when their diets also included fish. (Haines et al., 2022). In contrast, salamanders that I collected in the spring were in poorer body condition than those first collected in the fall and did not change in body condition even after being in the lab for 10 mo. This seasonal difference indicates that salamanders in nature are depleting the energy reserves that they gained in the fall. The laboratory diet is at least sufficient for the salamanders to sustain this level of condition.

Relative tail width as a measure of body condition is a relatively easy, noninvasive process that can be useful in both lab and field studies. However, there are some caveats to the use of this method. Invasive techniques such as lipid profiling provides a more direct measure of body condition than relative tail width, and it would be useful to compare the two techniques in future studies. Secondly, relative tail width does not appear to be an accurate measure of body condition, for at least some aquatic salamander taxa because tails of aquatic salamanders may have a higher muscle-to-fat ratio than tails of terrestrial salamanders (Delvolve et al., 1997). In addition, body condition may change seasonally due to a variety of factors including prey availability and reproductive status.

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INTRODUCTION

Aggressive or agonistic behavior elevates the chances of procurement of quality resources. Aggressiveness facilitates the establishment of high social rank within groups, which often provides precedence to food, mates, and territories. (Holekamp & Strauss, 2016). Factors that influence agonistic behavior can be morphological, such secondary sexual characteristic, body size (either dependent or independent of age), weaponry, or color (chapters in Briffa et al., 2013). For example, aggression in crayfish, *Cambarus alleni*, size was positively correlated with a size for adult males and females (Bovbjerg, 1956). Factors not necessarily related to morphology can also play a role, including genetic strain (laboratory mouse, *Mus musculus*:(Crawley et al., 1997), season (Indochinese rhesus macaque, *Macaca mulatta*: Wilson & Boelkins, 1970), parasite load (salamanders, *Plethodon angusticlavius*:(Maksimowich & Mathis, 2000) and experience (cichlid fish, *Neolamprologus pulcher*:(Lerena et al., 2021). Body condition, another factor that may or may not be directly accessible by competitors, has received relatively little study, although condition is often discussed as a central factor in discussions of the economic defensibility (Brown, 1964). Body condition can affect an individual's fitness through various physiological mechanisms that influence metabolism and behavior. Body condition can be correlated with the characteristics listed above. For example, body condition can be positively or negatively correlated with parasite load (review:(Sánchez et al., 2018).

Individuals in good body condition can be more resistant to or tolerant of disease (Budishchak et al., 2018), are able to better maintain physiological responses to stress (Kitayski et al., 1999) and may have an increased efficiency of energy mobilization (e.g., Bonnet et al., 2001). These advantages may support higher levels of aggressive behavior (Bertram & Rook, 2012), allowing individuals in better body condition to be more effective at safeguarding and sustaining high-quality resources (Jonart et al., 2007 , Norring et al., 2019). Individuals with lower bowady condition may exhibit submissive behavior or retreat altogether (Hasegawa et al., 2014) minimizing possible injuries and even death (Poulos & McCormick, 2015) This explanation was concurrent with one of the previously wherein male house finches with lower body condition avoided males with better body condition (Hasegawa et al., 2014).

Evolutionary game theory provides a useful tool for a functional analysis of animal contest behavior (Maynard Smith, 1982). Individuals in animal populations typically vary Resource Holding Potential (RHP), which is their ability to win contests with other individuals (originally, Resource Holding Power: Parker, 1974). Characteristics that are correlated with RHP include factors such as size of body or weapons, territory ownership, experience, sex, and physiological state (hormones, hunger, condition) (overview in (Dugatkin & Reeve, 2000) When contestants differ in RHP ("asymmetric" contests), contests should be settled quickly in favor of the contestant with largest RHP (Maynard Smith & Price, 1973); individuals with the lower RHP typically retreat from the area or perform submissive behavior. Shorter contests are less likely to escalate, reducing costs of potential energy loss, injury and wasted time. For example, contests between male jumping spiders, *Euophrys parvula*, were less intense when the contestants differed in body size (Wells, 1988), and contest length for male northern elephant seals was determined by asymmetries in a combination of residency status and body size (Haley, 1994). Conversely, contests between individuals with similar RHP ("symmetric" contests) can last for longer periods of time and escalate in intensity, resulting in a large risk of injury (Jaeger 1981) energy loss (Parker 1974; Neat et al., 1998; Bortosky & Mathis, 2016), or wasted time = opportunity cost:(Parker, 2006).

Territorial salamanders of the genus *Plethodon* have proved to be good models for the study of aggressive behavior, including an understanding of the characteristics associated with aggressive behavior and success in contests. These characteristics include residency status, body size, size asymmetry between contestants, parasite load, and color polymorphism, However, as in many other taxa, the influence of body condition on territorial contests has not been studied.

The subject of this study, Ozark Zigzag Salamander, *Plethodon angusticlavius* locally and abundantly occur in the Ozarks region of Oklahoma, Arkansas, and Missouri (Highton, 1997). These salamanders spend the summers in underground burrows to avoid desiccation and overheating during the warm, dry, months (Petranka, 1998). In the fall, they emerge from their burrows and defend territories in patches of moisture under rocks and logs on the forest floor and forage in the leaf litter after periods of rain (Mathis & Britzke, 1999). Surface activity during the winter is sporadic, occurring during periods of warmer weather (Petranka, 1998). Mating activity occurs from fall to spring, and oviposition occurs in late spring or early summer (Wilkinson et al., 1993). Females begin to incorporate yolk into their ova between February and March, and ova substantially increase in size beginning in April (Wilkinson et al., 1993).

In my study, I aim to address how body condition influences the display of agonistic behavior in the Ozark zizag salamander (*Plethodon angusticlavius.*) in staged contests, where the participants are paired either asymmetrically or symmetrically according to body condition. The predicted outcome of the contests would be that symmetric pairs would have escalated contests with longer durations and asymmetric pairs will have lower levels of aggression, resulting in shorter contests.

METHODS

Collection and Maintenance

I collected adult *Plethodon angusticlavius* salamanders from Bull Shoals Field Station, Kirbyville, Missouri in October of 2020 (n = 18) and March of 2021 (n = 37). Individuals were placed in clean, separate plastic bags containing moist leaf litter. In the lab, salamanders were kept in individual Petri dishes (diameter = 15—23 cm) that were lined with damp filter paper and stored in a temperature-controlled environmental chamber at 15ºC. Filter papers were changed as needed (at least biweekly) and hydrated every 3-4 days. Salamanders were fed adult fruit flies, *Drosophila hydei,* once a week.

Measurements and Body Condition Indexes

Snout-Vent Length (SVL) is the conventional body size measurement for salamanders because total length and mass are influenced by whether the tail has been autotomized. Tail autotomy is a defense mechanism whereby individuals voluntarily cast off their tails, which distracts predators during attacks; breaks can occur anywhere along the length of the tail for *Plethodon* sp., and salamanders can later regenerate the missing length (Wake and Dresner, 1967). Mass for amphibians is additionally influenced by levels of hydration, recent food intake, and, for females, number and size of yolked eggs (e.g., Hoque & Saidapur, 1994). The most common body condition indices for vertebrates are based on length relative to mass (Jakob et al., 1996), but this method would be problematic for species that can exhibit tail autotomy. Instead, researchers studying plethodontid salamanders have generated body condition indices (BCI) based on tail width (TW) relative to SVL (e.g., Guiterrez et al., 2018; Pierce & Gonzalez, 2019;

Nissen & Bendik, 2020). Tail width is a reasonable surrogate for mass in condition indices because tails of plethodontid salamanders are important for energy storage (Maiorana, 1977; Yurewicz & Wilbur, 2004).

In this study, the body condition score was measured as the width at the base of the tail divided by SVL. Scatter plots indicated that there was a positive linear relationship between SVL and tail width for our salamanders. Immediately prior to taking measurements, I anaesthetized individuals with Benzocaine in the form of Orajel® as described by Chen & Combs (1999) and Brown et al. (2004). I applied a small amount of Orajel to the dorsal surface of the head immediately behind the eyes and left the salamander undisturbed until it stopped moving (10-15 min). I then used a pair of digital vernier calipers to measure SVL from the tip of the snout to the caudal end of the vent and TW at the base of the tail (immediately behind the limbs). Salamanders were positioned with the ventral surface facing up and their bodies in a straight line for accurate measurements. I rinsed off the Orajel® immediately after measuring each salamander, and normal activity was resumed within 25 min; no mortality occurred.

Symmetric and Asymmetric Pairs

Based on the body condition indices (TW/SVL), I paired assigned salamanders to symmetric and asymmetric same sex pairs. The pairs did not vary more than 3mm in SVL for symmetric pairs, while there was more variation for asymmetric pairs. Symmetric pairs (n =10) were within 4 BCI units of each other (Mean difference = 0.008) and asymmetric pairs (n =13) were more than 4 BCI units apart. (Mean difference = 0.637)

Contests: Testing Protocol

Testing procedures followed that of Mathis & Britzke (1999). For 10 days before trial began, salamanders were maintained in separate holding chambers (24 length x 23 cm width and 1.5 cm deep) so that they could establish territorial residency by marking the substrates with chemical cues (e.g., Jaeger, 1986; Ovaska & Davis, 1992; Dalton & Mathis, 2014). For each pair, the trial was conducted in one of the randomly chosen holding chambers. So that there would not be a resident advantage, which is known to occur in this species (Mathis et al., 2000), the testing chamber was lined on either side with damp paper towel taken from each of the two test salamanders' pre-test holding chambers. This arrangement allowed each contestant to be exposed both its own substrate markings and that of its opponent during a trial. It was easy to differentiate the two individuals in asymmetric contests since the size difference was evident. However, in symmetric contests, I differentiated between the two contestants based on slight variations in color, stripe length, or other physical features.

I began the tests by keeping both contestants under separate opaque plastic dishes (8.5 cm diameter) located on their own-marked side of the chamber. After 2 min, I released them from the dishes and recorded their behavior for 10 min. Territorial salamanders of the genus *Plethodon* have proved to be good models for the study of aggressive behavior, including an understanding of the characteristics associated with aggressive behavior and success in contests. These characteristics include residency status, body size, size asymmetry between contestants, parasite load, and color polymorphism, However, as in many other taxa, the influence of body condition on territorial contests has not been studied I kept track of agonistic behaviors such as ATR (all trunk raised), Move towards, look towards, and submissive behavior such as flat on the substrate, look away. Move away and edge behavior. I also recorded frequency of nose taps which can be categorized as chemosensory behavior. For ATR, I recorded the number of seconds spent in ATR position from the time any or both contestants began displaying the ATR position to the time they stopped. I recorded the number of times conspecifics looked at each other and looked away from each other. Also, the number of times they moved away from and toward one another. Because the data were not normally distributed, I compared behavior in symmetric and asymmetric contests using non-parametric Mann-Whitney U tests (SPSS, v. 29.0), with $\alpha = 0.05$. All tests were two-tailed.

RESULTS

For behaviors considered to be aggressive, results depict that there was a significant difference in time spent in ATR (All trunk raised) behavior between asymmetric and symmetric pairs in a. ests (U = 95.00, p = 0.02). Salamanders in b. etric pairs spent significantly more time in all aggressive behaviors than salamanders in asymmetric pairs (ATR: U = 95.00, p = 0.02; Move Toward: U = 96.00, p = 0.01; Look Toward: U = 92.5, p = 0.03) (Figure 3). Although there tended to be less of the submissive posture, Flat, in symmetric contests, this behavior was highly variable and there was no significant difference between the two groups (U = 14.00, p = 0.37). There was a relatively low frequency of the other submissive behaviors, and they also did not differ significantly between treatments (Move away: U = 61.00, p = 1.0; Look away: U = 71.00, p = 0.41) (Figure 4).

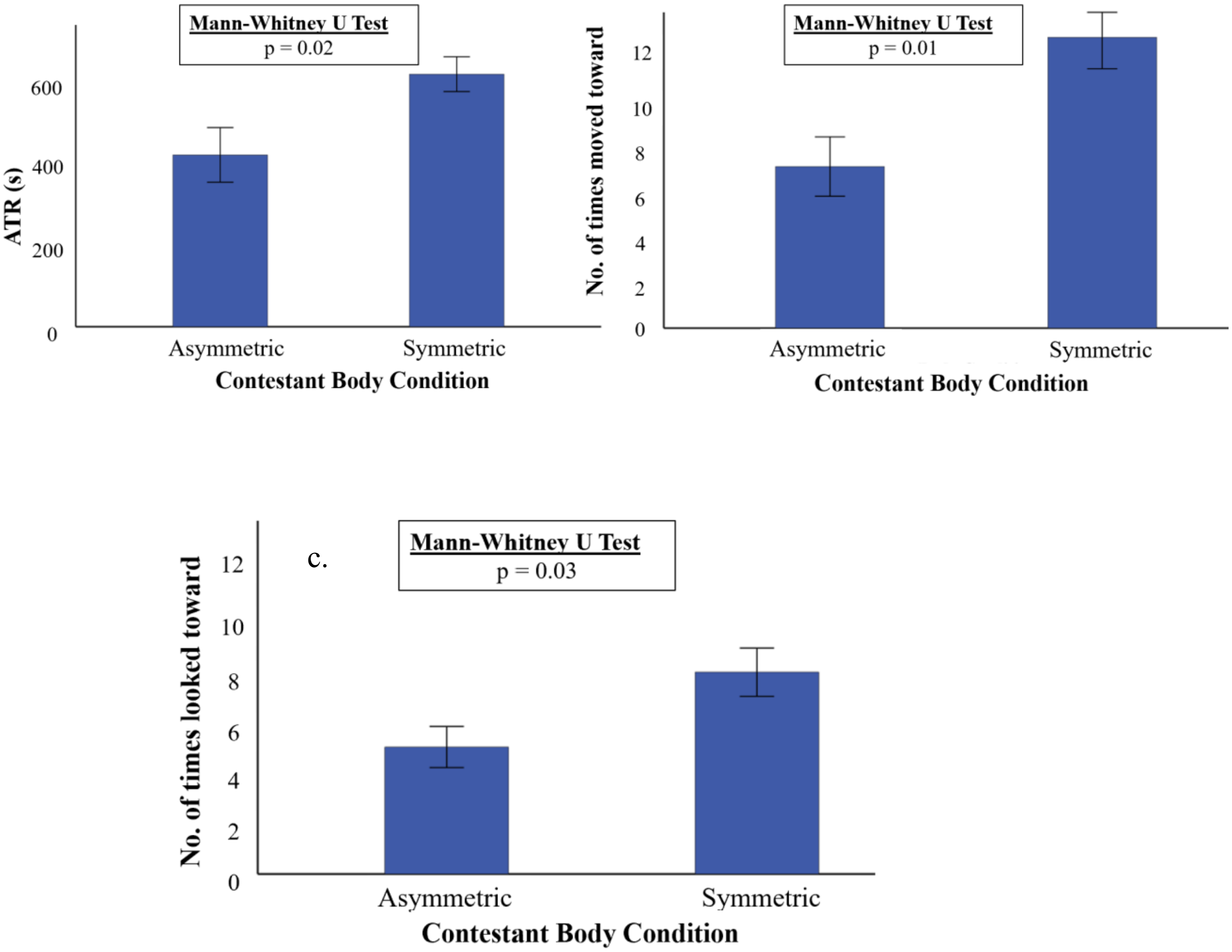

Figure 3. Aggressive behavior in symmetric vs asymmetric contests. a) ATR, b) No. of times moved towards, c) No. of times looked towards. Bars are means ±1SE

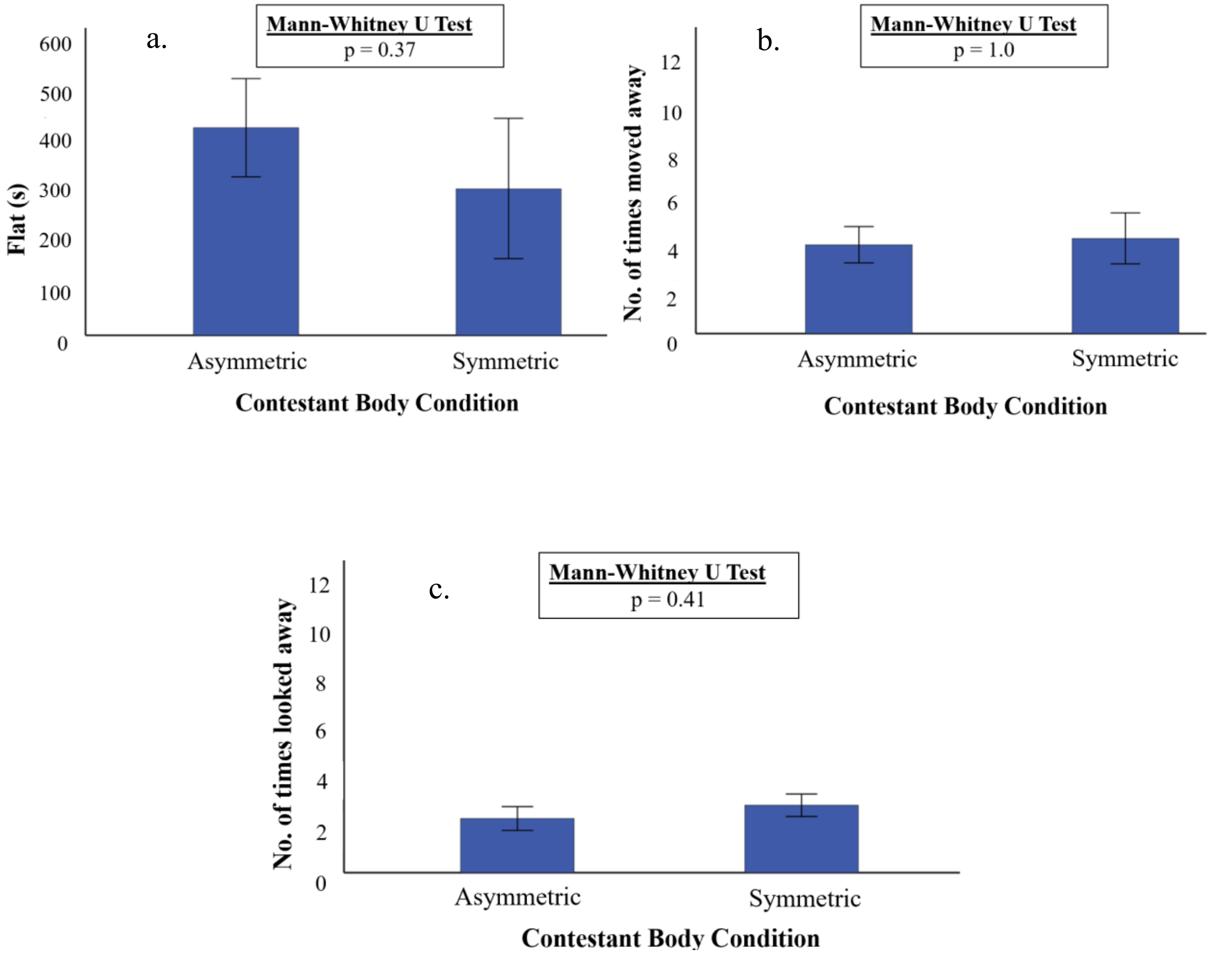

Figure 4. Submissive behavior in symmetric vs asymmetric contests. a) Flat, b) No. of times moved away, c) No. of times looked away. Bars are means ±1SE

DISCUSSION

Contests between salamanders in similar body condition resulted in higher levels of aggressive behavior than contests in which salamanders differed more substantially in body condition. The results were in accordance with the game theoretic prediction that symmetric contests should escalate to higher levels than asymmetric contests because the asymmetries influence contest costs and the probability of winning or losing (i.e., gaining the resource) for both contestants (Smith & Parker, 1976 ;Parker & Rubenstein, 1981; Maynard Smith & Harper, 2003; Riechert, 2013). In effect, the asymmetry is used to quickly settle the contest. Studies of aggressive contests measuring other characteristics correlated with Resource Holding Potential have similar outcomes, with symmetric contests escalating further than asymmetric contests (body size; shore crabs *Carcinus maenas*, Smallegage & van der Meer 2007; snow skinks, *Niveoscincus microlepidotus,* (Olsson & Shine, 2000); weaponry: tree wetas, *Hemideina crassidens*, (Kelly, 2006).

Territorial contests generally are energetically costly (Briffa et al., 2013), so it is reasonable that body condition should be correlated with Resource Holding Potential (RHP). However, tests of the effect of body condition on aggressive behavior are relatively uncommon, ranking behind body size, signals, weaponry, and age in number of studies (Kelly, 2008). Body condition generally requires two measurements (e.g., mass and length) making it more time-consuming to measure than direct measurements of size, which require only one measurement. Other measurements of condition are invasive, requiring substantial stress to the animal. These challenges may in part explain why tests of effects of correlated asymmetries more often use body size rather than condition as indicators of RHP.

How could body condition be assessed by competing salamanders? Although relative tail width is relatively subtle, it is possible that competitors could assess it visually. Alternatively, there may be other more easily detectable visual cues, such as potential differences in coloration (male damselflies, *Calopteryx maculata*, Fitzstephens & Getty, 2000) could be correlated with condition. Some animals assess RHP of their opponents during the contests using information provided during interactions, such as lateral displays in some fishes (Keeley & Grant, 1993). Although *Plethodon* salamanders have not been observed to perform lateral displays, they do walk over or under each other during territorial contests (personal observations), which could function in assessment. Assessment could be via chemical cues, since characteristics including parasite load (Maksimowich & Mathis 2001) and diet quality (Walls et al., 1989), which could be correlated with body condition can affect aggressive behavior. Note, however, that assessment of competitors is not required because individuals could base their level of aggression on assessment of RHP of self, independent of the opponent (Pinto et al., 2019)

Territorial salamanders of the genus *Plethodon* have proved to be good models for studying factors that influence aggressive behavior, partially due to the adaptability of these salamanders to laboratory conditions where variables are relatively easy to manipulate. Body size was found to be an important factor, with salamanders in symmetric contests (similar SVL's) being more aggressive than those in asymmetric contests (Mathis & Britzke, 1999). Residents are generally more aggressive and/or less submissive than intruders (e.g., Camp, 1999; Mathis et al., 2000; Jakob et al., 2004), and length of ownership is positively related to level of aggression (Nunes and Jaeger, 1989). Sex (Kohn et al., 2005; Lynn et al., 2019) and species (Anthony et al., 1997; Deitloff et al., 2009) of intruders can influence levels of agonistic behavior, and territory residents are more aggressive toward strangers than familiar neighbors

(Jaeger & Peterson, 2002). Parasite load (Maksimowich & Mathis, 2000), short-term differences in diet quality (Gabor & Jaeger, 1995) and quantity (Nunes & Jaeger, 1988), and whether the tail has been autotomized (Wise & Jaeger, 1997), all affect level of aggression in territorial contests, and these variables could be correlated with body condition (Mathis, 1990; Glass & Huntingford, 1988).

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SUMMARY

Body condition did not differ between gravid and non-gravid females, or between males and females. Season affected body condition through a significant interaction with time in the laboratory. Individuals initially collected in the fall were in the best condition of all samples, but they significantly lost mass after being in the lab for 10 mo. In contrast, individuals initially collected in the spring were in lower body condition than those collected in the fall but did not change their body condition after being in the lab for 10 mo. Body condition significantly affected aggressive behavior as predicted by game theoretic models. Salamanders were more aggressive when paired with individuals of similar condition in comparison to those paired with individuals of a substantially different condition. Although factors influencing body condition may be complex, condition can affect fitness through success in territorial aggressive interactions.

APPENDIX: Missouri State University IACUC Approval Notice

Missouri State
UNIVERSITY

June 4, 2020

RE: IACUC protocol 2020-09

Hello Dr. Mathis,

Your IACUC protocol #2020-09 entitled “Salamander Responses to Predators, Competitors, and Conspecifics: Behavioral and Physiological Effects” has been approved.

The final approval date is 6/4/2020.

Thank you and if you need anything in the future regarding this protocol please contact me either via email (janeneproctor@missouristate.edu) or at 417-836-8419.

Sincerely,

Janene Proctor