


Use it and bruise it: copulation rates are associated with muscle inflammation across anole lizard species

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Introduction

In humans and mice, it is well established that skeletal muscle use leads to muscle injury and damage (Ebbeling & Clarkson, 1989; Clarkson & Hubal, 2002). As the result of this damage, muscles recruit inflammatory cells proportional to the extent of injury and use, which help to remove damaged tissue (Wernig, Irintchev, & Weisshaupt, 1990; Hesselink *et al.*, 1996). During inflammation, a combination of immune cells (neutrophils followed by macrophages) infiltrates the muscle tissue within hours of insult to remove debris and promote regeneration (Clarkson & Hubal, 2002; McLoughlin *et al.*, 2003). Immune cells have waves of infiltration, with the neutrophil population peaking early (within 24 h) and macrophages peaking later (2–7 days, depending on macrophage type and injury severity), with some macrophages lingering for weeks as part of the muscular regeneration and remodeling process (Butterfield, Best, & Merrick, 2006; Musarò, 2014). The extent of use-induced muscle damage is dependent on numerous factors, including the intensity, frequency, and duration of muscle contractions (Ebbeling & Clarkson, 1989; Clarkson & Hubal, 2002; Baker & Cutlip, 2010). While it is well studied in mammals, the generality of the relationship between muscle use and inflammation is poorly examined within and across other groups of animals.

In addition, studies of muscle use to date have focused on variation *within* a population, examining differences between sexes, across ages, or among animals with varying behavioral

Abstract

The use of skeletal muscles causes cellular damage, a process that leads to inflammation. Although this process is well studied in mammals, it is largely unexplored in other taxa, and the behavioral use of a muscle has not been linked to muscle inflammation in a comparative framework. In this study, we examined the relationship between muscle use and inflammation across 22 anole lizard species. We tested the hypothesis that inflammation in the retractor penis magnus (RPM), a muscle used only during mating, is positively associated with copulation rate and/or with hemipenis size. We measured copulation rates for each species in the field and measured RPM inflammation for both wild-caught males and, for one species, captive virgin male anoles. We found a positive relationship between RPM inflammation and copulation rate, yet there was no correlation between RPM inflammation and hemipenis size or body size. This finding supports the generality of the relationship between muscle use and inflammation within and across species, suggesting that inflammation could act as a snapshot of recent muscle activity across vertebrate taxa.

strategies (MacIntyre, Reid, & McKenzie, 1995; Clarkson & Hubal, 2002; Baker & Cutlip, 2010). While inflammation is a transient phenomenon, occurring days to weeks after muscle use, individual measurements can reflect differences in muscle use *across* populations or species. In other words, population of animals that perform a behavioral movement at varying frequencies in a given period of time should exhibit population-level variation in muscle inflammation during that period. Phylogenetically informed comparative analyses provide a tool to determine if there is an association between muscle inflammation and behavioral frequency across species.

Copulation behavior offers a particularly promising case to examine this relationship at a broader taxonomic scale. Although most behaviors involve the recruitment of a complex group of muscles, and an individual muscle can be recruited for the performance of multiple behaviors, the muscles that move intromittent organs are generally used only during copulation. In squamate reptiles (lizards and snakes), movement of the hemipenes, a pair of bilateral, independently controlled copulatory organs, occurs via the isolated action of two pair of muscles (Arnold, 1984; Wade, 2005). Contraction of the transversus penis causes the eversion of the hemipenis through the cloacal vent, and contraction of the retractor penis magnus (RPM) retracts the hemipenis back into the tail. Thus, any damage or inflammation in these muscles should be due to movement of the hemipenes, which occurs only during mating.

Further, copulation behavior is evolutionarily conserved across squamates. Copulation occurs when a male mounts a female, orients his pelvis to align with hers, and everts a hemipenis into her cloacal vent (Crews, 1978; Shine *et al.*, 2000). Yet even among closely related species, there is substantial variation in the frequency of copulation (Johnson *et al.*, 2014) and in the size of the hemipenes (Dowling & Savage, 1960; Arnold, 1986). Thus, squamates offer a group across which a pair of muscles contracts in a single behavioral context, but their frequency of use and the contractile force required (i.e. the size of the hemipenes) vary among species.

Here, we tested the hypothesis that the frequency and intensity of muscle use is associated with muscle inflammation in a group of 22 *Anolis* (anole) lizard species that exhibit natural variation in copulation rates. To this end, we examined whether observed copulation rate in the field was positively correlated with the average RPM muscle inflammation of each species. (The orientation of the transversus penis in the tail renders it difficult to examine in histological analyses, and so we focused on the RPM in this study.) Though muscle inflammation will vary among individuals as muscles are injured and healed, we predict that species with higher copulation rates (and thus higher rates of RPM use) will exhibit higher average inflammation of the RPM. We also assessed whether hemipenis size (a proxy for contraction intensity) was associated with average inflammation, while controlling for species body size. Finally, we measured the RPM muscle inflammation of captive virgin *Anolis sagrei* males to assess whether the inflammation present in wild animals could likely be attributed to mating alone.

Materials and methods

Behavioral observation and specimen collection

To determine the natural copulation rates of a group of 22 anole species (Table 1), we conducted focal observations during the summer breeding seasons of 2004–2015 (Johnson *et al.*, 2014). For these 22 species, we conducted ~11:00 h of observation on 1200 lizards, observing a median of 37 males per species (range: 7–113) for a median of 41 h per species (range: 8.2–123.3 h).

We located undisturbed lizards by walking slowly through the habitat and observed each focal individual from a minimum distance of 5 m. We recorded all observed behaviors, with a focus on copulation events. Observations were conducted using one of three approaches. First, most populations were observed in ~500 m² study plots, in which we marked all adult lizards using unique bead tags sewn into the dorsal side of the proximal region of the tail (Fisher & Muth, 1989) or queen bee marking tags affixed to the dorsum (Johnson, 2005). These individuals were observed in a maximum of five 20-min period over 2–3 weeks (Johnson, 2007). In other populations, we conducted observations using identical procedures, except that we observed unmarked lizards in 30–60-min periods. To prevent repeated observations of these individuals, we observed lizards on perches separated by a minimum of 10 m, working in different areas of a locality each day. Finally, for

the more cryptic species that are difficult to locate in the field (*Anolis angusticeps*, *Anolis bahorucoensis*, and *Anolis occultus*), individual observations lasted up to 180 min, and when these lizards were marked, we observed no individual for more than 5 h. We calculated an average copulation rate per male and used those measures to determine the copulation rate for males per species. In species for which copulation was not observed during the formal observation periods, the copulation rate was recorded as 0.

Following observations, we collected 238 adult males from all 22 species of anoles during peak reproductive seasons (May 15–June 30). These lizards were collected from the same localities in which observations occurred, but were generally not the same lizards that were observed. Within 2–4 days after capture, lizards were transported to Michigan State University (2007–2008) or Trinity University (2009–2015). Upon arrival, we measured the snout–vent length (SVL) and mass of each male, then euthanized them by rapid decapitation. Immediately after euthanasia, males were dissected and tail tissue (including hemipenes and surrounding musculature) was removed, flash frozen on dry ice, and stored at –80°C.

To determine if muscle inflammation occurs when mating is restricted, we also sampled five adult virgin males from a captive breeding population of *A. sagrei* at the University of Virginia. These males were raised individually in plastic cages (40 × 23 × 32 cm, Lee's Critter Keeper, San Marcos, CA, USA) containing a potted plant, a carpet, a strip of fiberglass screening for a perch, and PVC pipe as a hiding place. Cages were lit with two ReptiSun 10.0 UVB bulbs (ZooMed, San Luis Obispo, CA, USA) on a 12-h L: 12-h D light cycle and maintained at 29°C with 65% relative humidity. All anoles were fed ad libitum crickets dusted once a week with Fluker's reptile vitamin and calcium supplements (Fluker's Cricket Farms, Port Allen, LA, USA) and were given water twice a day by spraying water on the cage walls. When males reached 18 months, they were euthanized, and tail tissues were collected using the protocol described above.

Quantifying hemipenis size and muscle inflammation

We sectioned frozen tail tissues at 20 μm and stained sections using hematoxylin and eosin. To estimate hemipenis size, we measured the cross-sectional area (CSA) of the hemipenis using NIS-Elements (Nikon, Tokyo, Japan) on both sides of the tail at 300 μm intervals, in four or five sections of tissue. Measures of the two sides of the tail were averaged for each individual for statistical analysis.

Using NIS-Elements, the RPM was photographed in the anterior region of the muscle, on each side of the tail. In cross section, muscle inflammation was characterized by muscle fibers invaded by mononuclear cells, as well as a dense region of cells in the connective tissue (endomysium or perimysium; Fig. 2b, c; St Pierre & Tidball, 1994). All areas of inflammation within an individual RPM (excluding the epimysium) were measured and summed. We also measured the total muscle CSA by outlining the epimysium. Finally, we calculated an inflammation ratio (inflamed area/total muscle area). One

Table 1 Samples sizes and species means for body size (snout–vent length (SVL)), hemipenis size, the ratio of retractor penis magnus (RPM) inflammation (averaged across both RPMs for each male), and copulation rate

| Species | N_{behavior} | $N_{\text{morphology}}$ | SVL (mm) | Hemipenis cross section (mm ²) | Ratio of RPM inflammation | Copulation rate per h | Collection location |
|-----------------------------|-----------------------|-------------------------|----------|--|---------------------------|-----------------------|-----------------------------------|
| <i>Anolis angusticeps</i> | 19, 10 | | 51.5 | 1.46 | 0.09 | 0.03 | Bimini, Bahamas |
| <i>Anolis bahorucoensis</i> | 10, 13 | | 44.8 | 0.81 | 0.02 | 0 | Polo, Dominican Rep. |
| <i>Anolis brevirostris</i> | 85, 12 | | 46.6 | 1.89 | 0.04 | 0.05 | La Cienaga, Dominican Rep. |
| <i>Anolis brunneus</i> | 32, 10 | | 64.2 | 2.13 | 0.05 | 0 | Crooked Island, Bahamas |
| <i>Anolis carolinensis</i> | 107, 10 | | 64.1 | 2.84 | 0.08 | 0.06 | San Antonio, Texas, United States |
| <i>Anolis chlorocyanus</i> | 22, 10 | | 70.6 | 1.67 | 0.12 | 0.21 | Bani, Dominican Rep. |
| <i>Anolis christophei</i> | 31, 9 | | 46.7 | 0.61 | 0.04 | 0.09 | Ebano Verde, Dominican Rep. |
| <i>Anolis coelestinus</i> | 87, 13 | | 65.8 | 1.72 | 0.03 | 0.03 | San Jose de Ocoa, Dominican Rep. |
| <i>Anolis cristatellus</i> | 61, 11 | | 62.4 | 1.16 | 0.05 | 0.05 | El Verde, Puerto Rico |
| <i>Anolis cybotes</i> | 113, 10 | | 67.2 | 2.52 | 0.11 | 0.05 | La Cienaga, Dominican Rep. |
| <i>Anolis distichus</i> | 37, 10 | | 51.2 | 0.77 | 0.04 | 0.07 | Bani, Dominican Rep. |
| <i>Anolis evermanni</i> | 59, 17 | | 59.5 | 1.08 | 0.09 | 0.12 | El Verde, Puerto Rico |
| <i>Anolis gundlachi</i> | 116, 10 | | 62.8 | 1.14 | 0.10 | 0.07 | El Verde, Puerto Rico |
| <i>Anolis krugi</i> | 52, 12 | | 49.8 | 0.62 | 0.10 | 0.02 | El Verde, Puerto Rico |
| <i>Anolis longitibialis</i> | 31, 10 | | 64.2 | 1.27 | 0.03 | 0 | Manuel Goya, Dominican Rep. |
| <i>Anolis occultus</i> | 7, 5 | | 38.4 | 0.58 | 0.09 | 0.25 | Cambalache, Puerto Rico |
| <i>Anolis olssoni</i> | 31, 15 | | 43.7 | 1.04 | 0.04 | 0 | La Cienaga, Dominican Republic |
| <i>Anolis poncensis</i> | 27, 10 | | 45.3 | 0.73 | 0.09 | 0.02 | Ponce, Puerto Rico |
| <i>Anolis pulchellus</i> | 42, 10 | | 44.7 | 0.46 | 0.08 | 0 | El Verde, Puerto Rico |
| <i>Anolis sagrei</i> | 19, 11 | | 51.8 | 1.57 | 0.09 | 0 | Bimini, Bahamas |
| <i>Anolis smaragdinus</i> | 19, 9 | | 58.4 | 1.26 | 0.18 | 0.12 | Bimini, Bahamas |
| <i>Anolis stratulus</i> | 50, 11 | | 46.1 | 0.87 | 0.05 | 0.03 | El Verde, Puerto Rico |

trained, blinded researcher performed all area measurements manually using NIS-Elements and ImageJ.

Statistical analysis

We conducted statistical testing and modeling in R (v.3.4.3, <https://www.r-project.org/>). All tests were conducted in a phylogenetic framework using a fully resolved tree with branch lengths (Pyron, Burbrink, & Wiens, 2013). First, we performed phylogenetic least squares (PGLS) regressions using the function ‘corBrownian’ in the R package *geiger* (Harmon *et al.*, 2008) to test whether an increase in RPM muscle inflammation was associated with an increase in copulation frequency, \log_{10} -transformed hemipenes size, or SVL. Second, to determine the extent of muscle inflammation in the RPM in captive individuals that did not have the opportunity to mate, we compared the average ratio of inflammation of virgin individuals to the wild individuals in our study using Kruskal–Wallis tests.

Results

Our results demonstrate that the average ratio of muscle inflammation in the RPM is positively correlated with the frequency of copulation among species (Table 2, Fig. 1a). This inflammation in the muscle was not associated with differences in the size of the hemipenis moved by the muscle (a proxy for the strength of muscle contraction), as we found no relationship between RPM inflammation and relative hemipenis size (Table 2, Fig. 1b) and no relationship between muscle inflammation and body size (SVL; Table 2). Further, we found no association between copulation rate and the size of the RPM ($\lambda = 0^{1.0.05}$, $r = -0.088$, $t = -0.443$, $P = 0.667$) or the size of the hemipenes ($\lambda = 0^{0.18,1}$, $r = -0.180$, $t = -0.915$, $P = 0.372$). Finally, we confirmed that captive virgin males had less muscle inflammation than the wild-caught males in our study (Fig. 2a, $\chi^2 = 11.19$, d.f. = 1, $P < 0.001$).

Discussion

In this study, we leveraged a large data set of behavioral observations, combined with histological analysis of muscle tissues, to assess the relationship between copulation rates and RPM muscle inflammation in 22 species of anoles. Across our multispecies data set, we have shown that observed rates of a behavior in the field are correlated with inflammation in a muscle that directly underlies that behavior. We also show that muscle inflammation is virtually absent from captive virgin

male anoles, supporting our hypothesis that this inflammation is due to mating alone. To our knowledge, this is the first time that muscle inflammation has been correlated with observed behaviors in a comparative framework.

Relatively little is known about how lizard muscles respond to use or injury, although several studies have attempted to examine whether the well-established principles of muscle physiology in mammals also apply to lizards. Recent work demonstrates that *Anolis* lizard muscles respond to exercise regimens and increase their performance (Husak, Keith, & Wittry, 2015; Husak, Roy, & Lovern, 2017); however, other lizard species show no performance improvement following endurance training (Gleeson, 1979; Garland *et al.*, 1987; O’Connor *et al.*, 2011). While all of these studies focus on a trained response, muscle injury occurs acutely and inflammation is critical for muscle regeneration (Lapointe, Frémont, & Côté, 2002; Summan *et al.*, 2005; Liu *et al.*, 2017). There is some evidence that endurance training in lizards can lead to swelling of the limbs (Garland *et al.*, 1987), and edema is commonly seen alongside muscle damage and inflammation in mammals (Clarkson & Hubal, 2002), yet there has been no direct investigation of muscle inflammation in reptiles. In our study, we found exercise-induced muscle inflammation during the time frame it would be seen in mammalian muscle, suggesting a similar tissue repair dynamic.

Though mammals and reptiles may have similar inflammatory responses following muscle use, mammals and reptiles have important anatomical and physiological differences that could influence the extent and duration of inflammation. Exercise-induced tissue damage (which initiates inflammation) has many contributing factors, such as calcium dysregulation, mitochondrial dysfunction, and reactive oxygen species (ROS) production (Gissel, 2006). Mammalian muscle has higher mitochondrial density (by volume), as well as higher total and inner membrane surface area compared to reptiles (Else & Hulbert, 1985). While ectotherm mitochondria have the potential for higher ROS production, it is potentially balanced by their higher antioxidant capacity (Treberg *et al.*, 2018). Metabolism and body temperature can also influence inflammation, two important factors when studying ectotherms. Monocytes and heterophils (the neutrophil homologue found in reptiles and birds) are seen in the early stages of tail regeneration in lizards and limb regeneration of newts (Cox, 1969; Gilbert, Payne, & Vickaryous, 2013; Godwin, Pinto, & Rosental, 2013). Both limb and tail regeneration have been shown to be body temperature dependent (Maderson & Licht, 1968; Kurup & Ramachandran, 2011; Tattersall *et al.*, 2012), as are other aspects of the lizard immune system (Sacchi *et al.*, 2017). There is clearly

Table 2 Results from a phylogenetically controlled generalized least squares regression between the average proportion of inflammation in the retractor penis magnus (RPM) muscle and copulation rate, while controlling for hemipenis size and body size [snout–vent length (SVL)]

| Trait | Predictors | λ | d.f. | Slope | t | P |
|------------------|-----------------|-----------|------|-------|-------|-------|
| RPM inflammation | Copulation rate | 0.40 | 17 | 0.48 | 2.24 | 0.038 |
| | Hemipenis size | | | -0.02 | -0.08 | 0.935 |
| | SVL | | | 0.14 | 0.59 | 0.565 |

The phylogenetic scaling parameter λ measures the level of phylogenetic dependence of the data (with 0 = low phylogenetic signal and 1 = high phylogenetic signal).

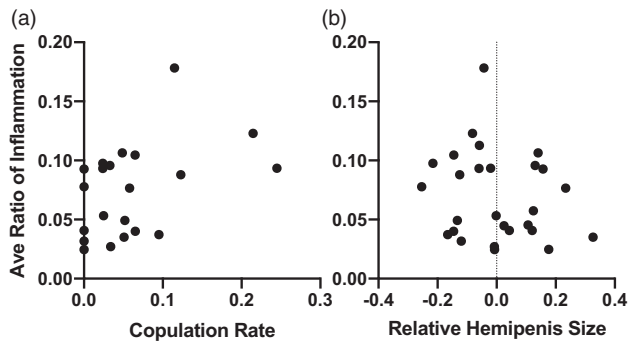


Figure 1 Retractor penis magnus (RPM) muscle inflammation (inflamed area/total RPM area) averaged for both hemipenes and (a) the observed copulation rate of each *Anolis* species in the wild and (b) relative size of the hemipenes. Relative hemipenes sizes were calculated using the residuals from the regression of \log_{10} hemipenes area on \log_{10} snout–vent length.

much more work that needs to be done to fully understand the inflammatory response to muscle injury in reptiles.

Comparative analyses of naturally occurring variation in behavior offer a valuable perspective on the ecology of diverse organisms, yet they may be limited by the variation present in natural populations. In the present study, while all individuals

sampled in each species were reproductively mature adults, our sampling likely included males of differing ages that had experienced a different number of reproductive seasons. The duration of inflammation within or across seasons is unknown; it is possible that at the beginning of each breeding season, sexually experienced and inexperienced males may be indistinguishable, or inflammation may accumulate over the life of the organism. Although differences in lizard age may have introduced within-species variation into the present data set, it is unlikely that there were systematic sampling differences in age associated with species’ size. Additionally, the virgin brown anole males used as our experimental control were all 18 months old, well within the expected age range of the reproductively mature males in the field study. Thus, individual age is unlikely to be an important confound in this data set, where species (and not individuals) was the unit of analysis.

In addition to offering a first step toward understanding muscle inflammation in reptiles, our study also suggests a solution to one of the major challenges in behavioral ecology: the accurate quantification of secretive behaviors in a natural setting. A wide range of behaviors can occur cryptically, nocturnally, or at low frequencies, and measuring these behaviors in the field is a difficult and time-consuming task (Li *et al.*, 2011; Mann & Würsig, 2014). Copulation is one such behavior (Hughes, 1998; Griffith, Owens, & Thuman, 2002). The results presented here suggest that RPM inflammation could be used as a

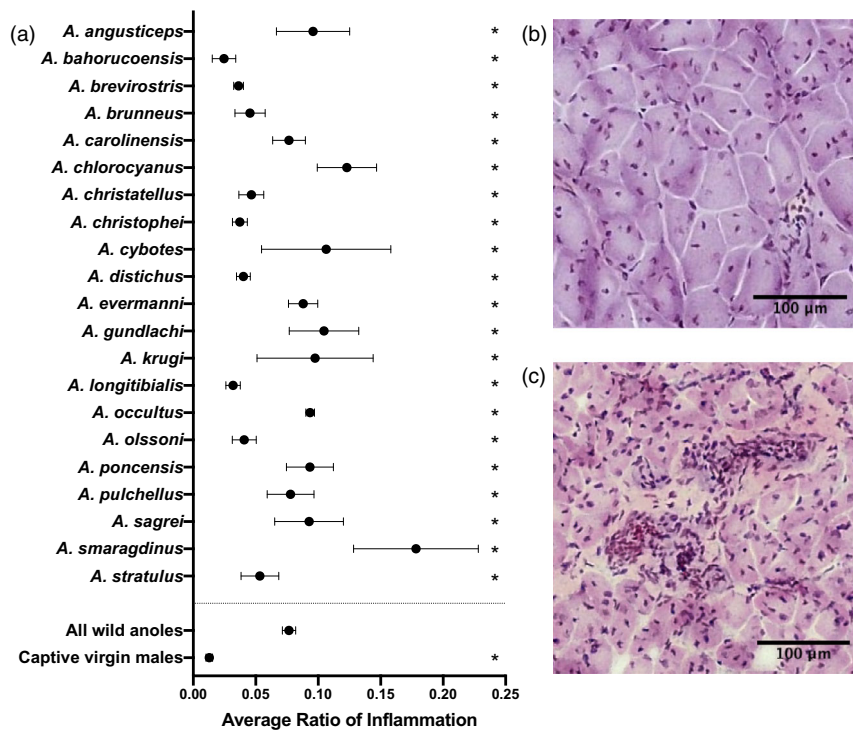


Figure 2 (a) Mean and standard error of amount of inflammation in the retractor penis magnus (RPM) muscle (inflamed area/total RPM area) averaged for each *Anolis* species. Five captive virgin male *Anolis sagrei* are plotted at the bottom. A (*) indicates samples that are significantly different from the captive virgin males using Kruskal–Wallis tests. (b) Example of RPM muscle with very little muscle inflammation (virgin male) and (c) RPM with extensive muscle inflammation. [Colour figure can be viewed at zslpublications.onlinelibrary.wiley.com.]

morphological proxy for estimating copulation rates in the wild, excluding limitations due to rare or endangered species. Support for this assessment comes from two lines of evidence: the correlation between RPM inflammation and observed copulation in the field (Fig. 1), and the minimal RPM inflammation observed in virgin males in the laboratory (Fig. 2b). Equally important, we found no relationship between hemipenis size and RPM inflammation, indicating that the inflammation was not correlated with the size of the structure moved, but the frequency of its movement. In addition, within our own behavioral data, several species had observed copulation rates of zero (Fig. 1a), yet clearly copulation occurs in these species. Likely, these species copulate more cryptically, perhaps either out of view or outside of observation periods (08:00–18:00 h; Doody, Burghardt, & Dinets, 2013). This indicates that to accurately determine the copulation rate for these species, many more hours of focal observation would be required. Thus, this morphological proxy for copulation rate could possibly eliminate the need for extensive, costly and potentially biased field observations.

In sum, in this study we extend the relationship between muscle use and inflammation to a broader scale, showing that this relationship occurs across species in a comparative context. The demonstrated relationship between copulation rate and RPM inflammation in anoles indicates that this inflammation may be useful as a proxy for copulation rate in squamates. Further, this suggests the possibility that muscle inflammation may be generally useful as a proxy for muscular activity across populations at any scale, particularly for animals or behaviors that are difficult to directly observe.

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