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SHORT COMMUNICATION

# Woodpecker drumming behavior is linked to the elevated expression of genes that encode calcium handling proteins in the neck musculature

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## ABSTRACT

Many animals perform elaborate physical displays for social communication. Identifying molecular mechanisms that co-evolve with these complex behavioral signals can therefore help reveal how forces of selection shape animal design. To study this issue, we examined gene expression profiles in select skeletal muscles that actuate woodpecker drum displays. This remarkable whole-body signal is produced when individuals rapidly hammer their bill against trees. We found that, compared with muscles that play no part in producing this behavior, the main muscle used to drum abundantly expresses two genes that encode proteins that support myocytic calcium ($Ca^{2+}$) handling dynamics – namely *parvalbumin* (*PV*) and *sarcoplasmic reticulum $Ca^{2+}$ ATPase 1* (*SERCA1*). Meanwhile, we found no such difference in the expression of another gene similarly vital to $Ca^{2+}$ handling, *ryanodine receptor 1* (*RYR1*). These differences are not present in a non-woodpecker species, which readily produce much slower drum-like movements for foraging (but not social signaling). Our data therefore point to an association between the fast drum displays of woodpeckers and muscle-specific expression of genes whose protein products enhance select aspects of myocytic $Ca^{2+}$ handling.

**KEY WORDS: Downy woodpecker, Skeletal muscle, Social display, Territorial behavior**

## INTRODUCTION

Some of the most extraordinary animal displays involve the production of elaborate body movement, including the performance of rapid gestural maneuvers (Byers et al., 2010; Mowles and Ord, 2012). Selection for these signals presumably drives changes in the neuro-motor apparatus, which allow animals to generate such specialized behavioral patterns (Fusani et al., 2014; Fuxjager and Schlinger, 2015; Girgenrath and Marsh, 2003). Identifying the physiological mechanisms that emerge to support these traits is therefore an important component of understanding phenotypic evolution in animals.

Modifications to skeletal muscle provide an avenue by which novel performance capabilities can arise, particularly the ability to generate swift appendage movements (Clifton et al., 2015; Johnson and Wade, 2010; Ota et al., 2015; Vliet, 1989). Discrete changes in myocytic calcium ($Ca^{2+}$) handling dynamics, for example, can reconfigure muscle contraction–relaxation cycling speeds (Syme and Josephson, 2002). Indeed, past studies suggest that $Ca^{2+}$ handling plays an important role in the production of rapid movements that underlie social displays (Harwood et al., 2011; Rome et al., 1996; Young et al., 2003), yet this work seldom identifies the molecular elements that underlie these fast changes in calcium transients. Accordingly, it is plausible that the skeletal muscles that actuate display movements express elevated levels of key genes that encode proteins involved in $Ca^{2+}$ handling.

Here, we study the expression profiles of three genes that regulate $Ca^{2+}$ trafficking in a muscle that governs a rapid and highly physical social display. The first of these genes is *parvalbumin* (*PV*), which encodes a protein that acts as a myoplasmic $Ca^{2+}$ buffer (Celio and Heizmann, 1982). The second gene is *sarcoplasmic reticulum $Ca^{2+}$ ATPase 1* (*SERCA1*), which encodes a protein that actively transports $Ca^{2+}$ back into the sarcoplasmic reticulum (SR) (Feher et al., 1998; Harwood et al., 2011). Indeed, both PV and SERCA1 promote rapid $Ca^{2+}$ transients, and thus allow the muscle to quickly relax by shuttling and sequestering $Ca^{2+}$ back into the SR (Syme and Josephson, 2002). The third gene is *ryanodine receptor 1* (*RYR1*), which encodes a protein that facilitates $Ca^{2+}$ release from the SR (Franzini-Armstrong, 2010). Elevated expression of the gene encoding this channel may contribute to fast muscle contraction by permitting greater quantities of $Ca^{2+}$ to leave the SR and flood the myoplasm (Hirata et al., 2007; Sobie et al., 2002). Considering the function of these genes, we hypothesize that their expression is increased in skeletal muscles that actuate especially fast display movements.

We test this hypothesis in downy [*Dryobates pubescens* (Linnaeus 1766)] and red-bellied [*Melanerpes carolinus* (Linnaeus 1758)] woodpeckers. Like nearly all woodpeckers, individuals of these two species defend their territories by performing drums (Miles et al., 2018; Schuppe and Fuxjager, 2018; Schuppe et al., 2016; Wilkins and Ritchison, 1999). This is a loud atonal sonation generated when an individual repeatedly hammers its bill against a tree. On average, a single drum consists of separate strikes (beats) produced at approximately 16 hits $s^{-1}$ for a downy woodpecker and 17 hits $s^{-1}$ for a red-bellied woodpecker. This means that neck muscles that control this movement must oscillate the head back and forth at roughly 50–60 ms periods (Schuppe and Fuxjager, 2018). The longus colli ventralis (LC), which originates near the second thoracic vertebra and inserts at multiple transverse processes between the 10th and third cervical vertebrae (Fig. 1A), is thought to be the primary driver of this behavior (Jenni, 1981; Kaiser, 1990). Therefore, we hypothesize that the LC itself will express higher levels of *PV*, *SERCA1* and/or *RYR1*, compared with other muscles that are not involved in drumming, such as the scapulohumeralis caudalis (SH; humeral retractor; Fig. 1A) (Dial, 1992; Dial et al., 1991). We test this hypothesis by measuring expression (mRNA levels) of these genes in both the LC and SH of male downy and red-bellied woodpeckers.

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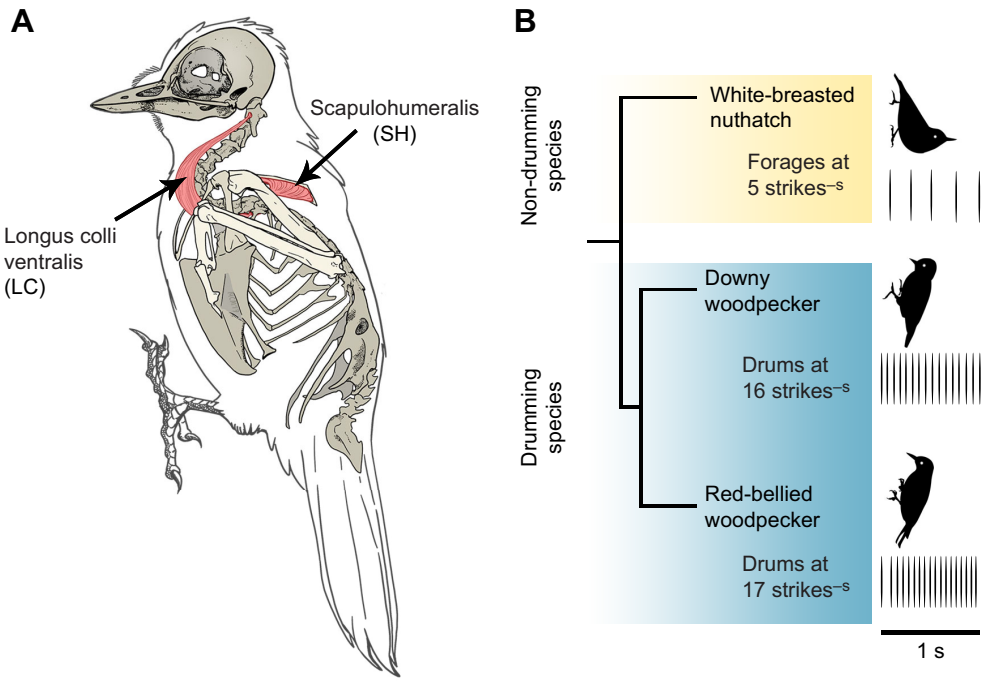


**Fig. 1. Representation of the skeletal muscle and species used for this study.** (A) The longus colli ventralis (LC) and scapulohumeralis (SH) on a downy woodpecker. Illustration by Meredith Miles. (B) Study species and their bill-hammering speed for foraging (nuthatch) or drumming (woodpeckers).

Past studies suggest that such measures of transcript level predict aspects of muscle performance, namely sprint speed, muscle relaxation time and sustained exercise at an animal's physiological limit (James et al., 2011; Seebacher and Walter, 2012). Furthermore, we compare these data with similar measures of gene expression in a distantly related songbird, the white-breasted nuthatch (*Sitta carolinensis* Latham 1790). This species not only inhabits the same forest as downy and red-bellied woodpeckers, but it also uses the LC muscle to produce bill-taps for foraging at a much slower frequency than a drum [≈5 taps $s^{-1}$ (Hz); Fig. 1B] (Dickson et al., 1979; van der Leeuw et al., 2001). Overall, we expect that the woodpecker LC will express higher levels of *PV*, *SERCA1* and *RYR1* compared with the woodpecker SH, as well as the nuthatch LC and SH.

## MATERIALS AND METHODS

### Animals

We captured adult male downy woodpeckers ($n$=8), red-bellied woodpeckers ($n$=5) and white-breasted nuthatches ($n$=7) using mist nets during the breeding season (March–April) in the woodlands of Forsyth County, North Carolina, USA. We immediately euthanized these individuals via cervical dislocation and flash-froze the carcasses on dry ice. Specimens were stored at −80°C until further processing. All appropriate federal, state and university authorities approved of this project (Wake Forest University IACUC no. A16-188).

### Tissue processing and cDNA synthesis

We dissected LC and SH tissues from each frozen carcass. We then homogenized the samples in TRIzol Reagent™, using a rotor/stator homogenizer set to medium speed. We extracted total RNA using a Zymo Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, USA), in which we included an initial phenol-chloroform separation of RNA following the manufacturer's instructions. We treated all RNA with DNase and reverse transcribed 1 µg of the product using SuperScript IV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). This reaction occurred for 10 min at 55°C, followed by 10 min at 80°C.

With the resulting cDNA, we amplified *PV*, *SERCA1* and *RYR1* in each species using primers that were designed from highly conserved regions of each of these genes (Table S1). The PCR reactions contained 40 ng of cDNA, 0.5 µmol $l^{-1}$ of forward and reverse primer, OneTaq 2× Mastermix (New England Biolabs, Ipswich, MA, USA). The program consisted of an initial denaturization step at 95°C for 5 min, followed by 40 cycles starting with a step at 95°C for 30 s to denature the DNA, a step at 57–62°C for 30 s to anneal the primers, and an extension step at 72°C for 30 s. Reactions were completed with a final extension step at 72°C for 5 min. The resulting PCR products were analyzed on a gel to ensure that PCR fragments matched expected size, and PCR products were then purified and sequenced (Genewiz Inc., La Jolla, CA, USA).

### Quantitative real-time PCR (qPCR)

We used quantitative real-time PCR (qPCR) to measure gene expression on an Applied Biosystems 7500 Fast RealTime sequence detection system (Foster City, CA, USA), as described previously (Fuxjager et al., 2012; Mangiamele et al., 2016). In this case, we generated species-specific primers for *PV*, *SERCA1* and *RYR1* (Table S2). Each reaction was performed in duplicate and contained 100 ng of template cDNA, 0.9 µmol $l^{-1}$ of forward and reverse primer, and SYBR Green PCR Master Mix (Applied Biosystems 4309155). We ran reactions at 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Samples were run in duplicate, and we used the standard curve method to determine the relative expression of each gene of interest, as compared with the housekeeping control glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Fuxjager et al., 2012).

### Data analysis

We compared the relative expression levels of each gene using a linear mixed model, in which muscle and species were fixed factors. Individual identity was also included in each model as a random factor. We also examined the effect of individual identity as well as this term's variance in each model (Table S3). We further analyzed significant main and interaction effects with *post hoc* comparisons,

using Benjamini–Hochberg corrections to account for multiple contrasts. Analyses were performed in R (v3.3.2; https://www.r-project.org/) using the lme4 package.

## RESULTS AND DISCUSSION

### Calcium buffering

We first tested whether expression of the gene encoding parvalbumin, a myocytic $Ca^{2+}$ buffer, is elevated in the neck musculature of woodpeckers (Fig. 2A). On average, we found that the LC expressed more *PV* than the SH ($F_{1,16.86}$=84.13, $P$<0.0001) and that the three species differed markedly in terms of *PV* expression ($F_{2,17.43}$=4.72, $P$=0.02). These effects were largely driven by a robust interaction ($F_{2,16.76}$=10.61, $P$=0.001), whereby levels of *PV* mRNA differed between the two muscles in a species-specific manner. Thus, both woodpeckers had more *PV* mRNA in their LC relative to their SH (downy: $P$<0.001; red-bellied: $P$<0.001), but this difference was not detected in the nuthatch ($P$=0.13). Furthermore, both woodpecker species maintained more *PV* mRNA in their LC relative to nuthatches (downy: $P$<0.001; red-bellied: $P$=0.02). Finally, we did not detect any differences in the expression levels of *PV* in the LC muscle between woodpecker species ($P$=0.24). These data therefore indicate that the LC of the woodpecker is enriched with *PV* transcripts, whereas the muscle unrelated to drumming – the SH – is not.

The net functional effect of elevated *PV* expression in the woodpecker LC is likely a faster contraction–relaxation cycling speed (Syme and Josephson, 2002). Indeed, past work shows that increased PV in a given muscle can decrease its relaxation time by buffering large amounts of $Ca^{2+}$, which in turn allows the muscle to relax more quickly following repeated neural stimulation (Celio and Heizmann, 1982). Other experiments support this idea, showing that overexpression of *PV* can lead to a dramatic increase in muscle contraction–relaxation speeds (Müntener et al., 1995). Moreover, individual variation in aspects of locomotor performance, such as burst swim speed, are positively related to elevated muscular *PV* expression (e.g. Seebacher and Walter, 2012). In this way, $Ca^{2+}$ buffering may also be associated with the ability of woodpeckers to produce drums.

### Calcium re-uptake

We next investigated whether the woodpecker LC exhibits elevated expression of the gene encoding SERCA1, an SR $Ca^{2+}$ pump (Fig. 2B). We found that mRNA levels of this gene were, on average, greater in the LC muscle than in the SH muscle ($F_{1,16.54}$=35.41, $P$<0.001). We also found that *SERCA1* expression varied significantly across species ($F_{2,16.67}$=8.58, $P$=0.002). Again, these patterns were driven by a significant interaction ($F_{2,16.39}$=11.42, $P$<0.001). Woodpeckers expressed higher levels of *SERCA1* in the LC compared with the SH (downy: $P$<0.01; red-bellied: $P$<0.001), but this difference was not found in the nuthatch ($P$=0.96). Also, both downy ($P$<0.01) and red-bellied woodpeckers ($P$<0.001) maintain more *SERCA1* mRNA in the LC compared with nuthatches. Although these findings demonstrate that expression of *SERCA1* is dramatically upregulated in the drum-actuating LC, we also found that red-bellied woodpeckers express more *SERCA1* in their LC compared with downy woodpeckers ($P$=0.001).

In theory, elevated *SERCA1* levels in the woodpecker LC should speed up contraction–relaxation speeds cycling by accelerating the rate at which $Ca^{2+}$ is cleared from the myoplasm and moved into the SR (Feher et al., 1998). Other work links these effects to variation in adaptive performance metrics; for example, *SERCA1* levels in locomotor muscles can predict maximal swimming speed in fishes (Seebacher and Walter, 2012), whereas 'knocking out' *SERCA* can diminish aspects of motor capacity necessary for basic respiration and locomotion (Gleason et al., 2004; Pan et al., 2003). Accordingly, our data point to a clear association between processes underlying swift myoplasmic $Ca^{2+}$ clearance and drumming ability.

Of course, we cannot definitively rule out the possibility that our observed differences in *SERCA1* are due to an increase in SR volume in the woodpecker LC, compared with the SH (Knollmann et al., 2006). We suspect that this is not the case, however, considering that we found no species differences in the expression of other genes similarly embedded in the SR – namely *RYR1* (see below). Indeed, when levels of SERCA1 co-vary with SR volume, these effects are often mirrored by similar co-variation in RYR1 (Meyer et al., 1995). Given that we found only effects of *SERCA1*, we expect that the expression of this gene is upregulated, independently of SR volume per se.

### Calcium release

Finally, we assessed whether expression of *RYR1*, a key player in the mechanism for $Ca^{2+}$ release from the SR, is elevated in the LC muscle (Fig. 2C). Although we found evidence that *RYR1* expression levels were greater in the LC across all species ($F_{1,16.90}$=9.83, $P$=0.006), we detected no difference among species in transcript levels of this gene ($F_{2,17.44}$=0.29, $P$=0.75) or an effect of a species×tissue interaction ($F_{2,16.80}$=0.0007, $P$=0.99).

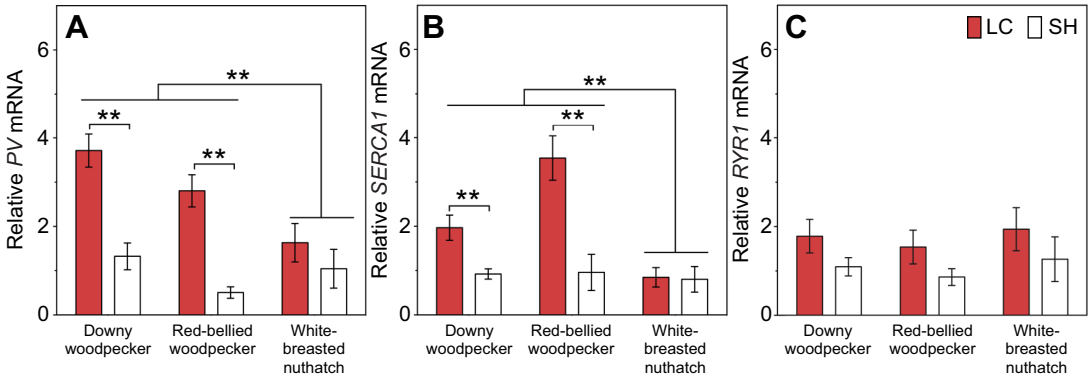


**Fig. 2. Relative mRNA expression in the longus colli ventralis (LC; red bars) and scapulohumeralis (SH; white bars).** (A) *Parvalbumin* (*PV*); (B) *sarcoplasmic reticulum calcium ATPase 1* (*SERCA1*); and (C) *ryanodine receptor 1* (*RYR1*). Species include randomly collected male downy woodpeckers ($n$=8; *Dryobates pubescens*), red-bellied woodpeckers ($n$=5; *Melanerpes carolinus*) and white-breasted nuthatches ($n$=7; *Sitta carolinensis*) during the breeding season. Each bar represents the mean level of expression in the given species' muscle, with the error bars depicting ±1 s.e.m. **$P$<0.01.

Similar to our current findings, past work shows that muscles that operate at different speeds do not always vary with respect to *RYR1* levels (Ferretti et al., 2015). Instead, differences in speed among muscles might be attributed to myoplasmic $Ca^{2+}$ buffering and reuptake (Ferretti et al., 2015). Moreover, one important aspect of these findings is that they are consistent with the idea that SR volume in the LC and SH is similar among taxa, given that *RYR1* is embedded in the SR membrane, and thus we would expect to observe differences in this gene's expression if SR volumes dramatically varied (Meyer et al., 1995).

## General discussion

Our results suggest that woodpecker drumming behavior is associated with tissue-specific increases in the expression of select genes important for mediating myocytic $Ca^{2+}$ handling. Specifically, we found that the LC muscle, which controls the rapid movement necessary to drum (Jenni, 1981; Kaiser, 1990), exhibits a fivefold increase in *PV* and *SERCA1* expression, relative to another skeletal muscle not involved in the production of this signal. Additionally, expression of a gene encoding a key channel that governs $Ca^{2+}$ release from the SR, *RYR1*, does not differ between species. Given that prior research points to mechanisms of $Ca^{2+}$ buffering and reuptake as important mediators of rapid muscle contractility for display behavior (Appelt et al., 1991; Rome et al., 1996; Young et al., 2003), our current data provide a molecular basis for these effects.

We expect that elevated expression of *PV* and *SERCA1* supports the ability of both downy and red-bellied woodpeckers to drum, as opposed to being a by-product of drumming itself. This conclusion is rooted in the fact that downy woodpeckers drum approximately 10 times more in a given day relative to red-bellied woodpeckers (Dodenhoff, 2002; Wilkins and Ritchison, 1999). Therefore, if drumming-induced exercise determined levels of *PV* and *SERCA1* expression in the LC (Ferreira et al., 2010; Kinnunen and Mänttäri, 2012), then both genes should be expressed more in the downy woodpecker compared with the red-bellied woodpecker. However, we did not observe such a difference, as *PV* mRNA levels were indistinguishable between the two taxa, whereas *SERCA1* mRNA levels were actually higher in the red-bellied woodpecker.

With these considerations in mind, we hypothesize that increased *PV* and *SERCA1* expression in the woodpecker LC muscle is the result of sexual selection for effective drumming behavior. Faster and longer drums appear to constitute better territorial displays (Schuppe and Fuxjager, 2018; Schuppe et al., 2016), which suggests that selection should favor the evolution of mechanisms that support these signaling characteristics. Our current data therefore point to elevated expression of *PV* and *SERCA1* in the LC muscle as a mechanism by which rapid gestural signals can evolve. To this end, we also found that the nuthatch's LC and SH are indistinguishable with regard to the expression of these two genes, again suggesting that there is co-evolution between $Ca^{2+}$ handling machinery in the neck muscle and drumming displays. However, it is important to mention that we did not control for the effect of the species' relatedness in our analysis, and therefore we cannot disentangle effects of shared evolutionary history (or lack thereof) from our current findings. Future comparative work using woodpeckers that do not drum – either through secondary loss of the trait or because they represent a non-drumming ancestor – are needed to more thoroughly address this issue. In this vein, we recognize that several other factors associated with species differences in natural history may contribute to the differences in gene expression that we uncover.

Finally, there are other ways in which the woodpecker LC might be adapted for speed, particularly regarding the mechanisms of $Ca^{2+}$ handling. For example, the genetic sequences for PV, SERCA1 or even RYR1 may evolve in ways that enhance the ability of these proteins to buffer, pump or release $Ca^{2+}$ in the context of display production (Brownridge et al., 2009; Zhang et al., 1993). Studies show that even a single amino acid substitution in proteins, such as in RYR1, can cause dramatic changes in muscle performance (e.g. Zhang et al., 1993). Other genes that encode essential proteins for muscle function may similarly evolve to support LC speed, including a variety of myosin and/or troponin isoforms (Mead et al., 2017; Tikunov et al., 2001).

In summary, our data provide a robust link between specialized $Ca^{2+}$ abilities in a single muscle and the ability to perform a physical display, namely the woodpecker drum. The data therefore shed light on how performance phenotypes might evolve to support the production of elaborate behaviors, driven to extremes by potent forces of selection.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: E.R.S., M.J.F.; Methodology: E.R.S., M.J.F.; Formal analysis: E.R.S., J.O.P., M.J.F.; Investigation: E.R.S., J.O.P., M.J.F.; Resources: M.J.F.; Writing - original draft: E.R.S., M.J.F.; Writing - review & editing: E.R.S., J.O.P., M.J.F.; Supervision: M.J.F.; Project administration: M.J.F.; Funding acquisition: M.J.F.

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#### Supplementary information

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