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RESEARCH ARTICLE

Sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity during the transition to endothermy in an altricial bird

Edwin R. Price§, Tushar S. Sirsat*, Sarah K. G. Sirsat‡ and Edward M. Dzialowski

ABSTRACT

Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) is a transmembrane pump critical to muscle calcium cycling during contraction, and SERCA has also been proposed as the basis for a non-shivering thermogenesis mechanism in birds. Despite its potential importance to both shivering and non-shivering thermogenesis, the activity of this transporter has rarely been studied in altricial birds, and never during the developmental transition from ectothermy to endothermy. Here, we describe SERCA activity in the pectoralis muscle and heart ventricle of red-winged blackbird (*Agelaius phoeniceus*) nestlings, fledglings and adults. Additionally, using a diet manipulation, we tested the hypothesis that muscle SERCA activity is affected by dietary fatty acid composition, as has been shown in some previous studies. In blackbird hearts, SERCA activity increased throughout development and into adulthood, conspicuously jumping higher just prior to fledging. In pectoralis muscle, SERCA activity increased throughout the nestling period, but then declined after fledging, an effect we attribute to remodeling of the muscle from a primarily heat-generating organ to a primarily force-generating organ. SERCA activity of the pectoralis muscle was correlated with the proportion of linoleic acid in muscle phospholipids when including all ages in the control group. However, in diet-manipulated birds, there was no consistent relationship between SERCA activity and muscle membrane fatty acid composition at any tested age (5–9 days old). It is unclear whether SERCA might be affected by developmental changes in fatty acid composition at younger ages.

KEY WORDS: Linoleic acid, Docosahexaenoic acid, Maximal metabolic rate, *Agelaius phoeniceus*, Diet, Development

INTRODUCTION

Elevation of metabolic rate in response to cold temperatures, i.e. endothermy, is characteristic of adult birds but is not present at early developmental ages (Price and Dzialowski, 2018). As embryos, metabolism declines when eggs experience cold ambient temperatures. Birds later achieve their endothermic response to ambient cold temperatures according to their species-specific developmental timeline: precocial species usually develop endothermic responses around the time of hatching, whereas altricial species may not develop endothermy until several weeks after hatching (Starck and Ricklefs, 1998; Price and Dzialowski, 2018). Irrespective of this timeline, the endothermic response is dependent upon the maturation of multiple physiological systems, including the neural thermoregulatory centers and networks, oxygen and substrate supply pathways, and the oxidative and contractile machinery of skeletal muscles (reviewed by Price and Dzialowski, 2018).

Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) is a membrane-bound transporter that pumps calcium ions from the cytosol into the lumen of the sarcoplasmic reticulum (SR) following muscle contraction. High SERCA activity is crucial for rapid contraction rates in muscles (Rome and Lindstedt, 1998), as the transporter restores both cytosolic and SR calcium levels that are necessary for signaling contraction. Maturation of SERCA activity could therefore be important for developing the muscle's capacity for shivering. Additionally, SERCA has been implicated in a mechanism for non-shivering thermogenesis, especially in birds (de Meis, 1998, 2001; Bicudo et al., 2002; Kjelstrup et al., 2008; Bal et al., 2012; Rowland et al., 2015): a modulator of SERCA, sarcolipin, can bind to SERCA and uncouple the ATPase activity from calcium pumping, thereby enabling a futile cycle in skeletal muscle (Bal et al., 2012; Rowland et al., 2015). Thus, SERCA may be a key protein for both shivering and non-shivering thermogenic pathways.

A few studies have described SERCA expression or activity in neonatal birds. For example, Muscovy ducklings increased the expression of SERCA isoform 1 in gastrocnemius muscle over several weeks post-hatching (Dumonteil et al., 1995). Ducklings kept in their thermoneutral zone showed simultaneous declines in SERCA isoform 2a, whereas cold-acclimated ducklings maintained high levels of isoform 2a in the muscle (Dumonteil et al., 1995). This elevation of SERCA protein expression, and SERCA activity (Dumonteil et al., 1993), in cold-acclimated ducklings is consistent with the hypothesized importance of SERCA to thermogenesis. However, skeletal muscle SERCA activity has never been reported for an altricial bird. Thus, a primary goal of the present work was to elucidate the pattern of SERCA activity in the pectoralis muscle of the altricial red-winged blackbird (*Agelaius phoeniceus*) during the developmental transition to endothermy. We focus on the pectoralis muscle because it is the main thermogenic muscle in blackbirds (Olson, 1994), and we predicted that SERCA activity of this muscle would increase leading up to day 7, the approximate age at which the birds attain endothermy. We also investigated the development of SERCA activity in the heart.

Another goal of the present study was to investigate the effect of membrane fatty acid composition on SERCA activity, to determine if it might explain some previous results from our laboratory on metabolic rate (Price et al., 2018b). Manipulative experiments in mammals and fish have shown that dietary linoleic acid increases SERCA activity in the heart and skeletal muscles, whereas dietary docosahexaenoic acid decreases SERCA activity (Swanson et al., 1989; Ushio et al., 1997; Fajardo et al., 2015). These effects are thought to be mediated by alterations to sarcolemmal membrane phospholipid composition, and have been invoked to explain

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membrane remodeling in hibernating mammals as well as the effects of dietary lipids on torpor expression in hibernators (Ruf and Arnold, 2008; Giroud et al., 2013; Vuarin et al., 2016). Moreover, this effect of lipid composition on SERCA activity has been proposed to explain effects of linoleic acid (either in the diet or as a membrane component of skeletal muscles) on exercise performance (Ruf et al., 2006; Stawski et al., 2015).

In our recent study of membrane composition and metabolic rate in nestling red-winged blackbirds, we noted a developmental incorporation of both linoleic acid and docosahexaenoic acid into muscle membranes (Price et al., 2018b). These changes in membrane composition might contribute to developmental changes in membrane-bound enzyme activity, including SERCA. Moreover, using a dietary manipulation, we demonstrated that the maximal (cold-induced) metabolic rate attained by blackbirds fed linoleate-rich diets was greater than that attained by birds fed docosahexaenoate-rich diets (Price et al., 2018b). We showed that pectoralis muscle phospholipids were indeed altered by the diet treatment, and hypothesized that these membrane alterations affected SERCA activity and thereby thermogenic capacity via either shivering or non-shivering mechanisms (Price et al., 2018b). Therefore, we test this hypothesis in the present study, with the prediction that blackbirds fed linoleate-rich diets would have higher SERCA activity than those fed docosahexaenoate-rich diets.

MATERIALS AND METHODS

Animals, feeding experiment, tissue collection and fatty acid composition

Birds were collected under Texas Parks and Wildlife Department permit SPR-0214-034 and US Fish and Wildlife Service permit MB02732B-2. All procedures were approved by the University of North Texas Institutional Animal Care and Use Committee.

Animal collection and feeding were described previously for experiments that were conducted concurrently with the present work (Price et al., 2018a,b). The study was conducted at the Lewisville Aquatic Ecosystem Facility (Denton County, TX, USA), which has numerous experimental ponds that provide breeding habitat for red-winged blackbirds [*Agelaius phoeniceus* (Linnaeus 1766)]. During the breeding season, daily minimum temperatures were 60–75°C (averaged monthly May–July) while maximum temperatures were 81–95°C. Briefly, we monitored red-winged blackbird nests in the field to determine exact dates of hatching. This population of blackbirds achieves endothermic metabolic responses to cold by 7 days post-hatch (dph) (Price et al., 2018b). 'Control' nestlings were handled and weighed daily, and collected at ages 1, 3, 5, 7 and 9 dph from a total of 20 nests. Collection usually continued until reaching a sample size of six at each age, but some ages have slightly different sample sizes (provided in figures). We also caught six recently fledged birds from unknown nests; they were estimated to be 10–17 dph and were grouped with controls. In addition, we captured three adult females by mist-net at the end of the nesting season. On the day of collection, birds were transported to our laboratory for measurement of metabolic rate at thermoneutrality and during cold challenges (Price et al., 2018b; cold challenges were performed for the great majority of birds in the present study including fledglings and adults). The birds were then euthanized by decapitation under isoflurane anesthesia and dissected immediately. Pectoralis muscles and heart ventricles were flash-frozen in liquid nitrogen and stored at −80°C.

To modify the fatty acid composition of tissues, we also performed a dietary manipulation on nestlings from another 17 nests. These birds were given a once-daily oral dose of either fish oil (high ω3 and docosahexaenoate) or sunflower seed oil (high linoleate) by pipetting the oil slowly into the mouth at a dose of 30 µl per gram body mass (but we discontinued dosing for that day if the bird rejected the oil, i.e. they were not force-fed). Like the control group, these diet-manipulated nestlings were left in their natural nests in the field, such that this oil dose supplemented the regular feeding performed by the parents. Nestlings within a given nest were all provided the same dietary oil treatment. Dosing began at 1–2 dph and continued until collection, which occurred at 5, 7 or 9 dph.

The fatty acid composition of the phospholipid fraction of the pectoralis muscle was determined using gas chromatography-mass spectrometry. Fatty acid compositions of the dietary oils and the phospholipid fraction of pectoralis muscles were reported previously (Price et al., 2018b), along with detailed methods for the lipid analysis.

Tissue homogenization and SERCA assay

We assayed SERCA activity similarly to previously published methods (Giroud et al., 2013). Frozen muscle samples (~100 mg) were weighed and homogenized in 2 ml of a buffer (100 mmol l^{-1} Tris, 250 mmol l^{-1} sucrose, 600 mmol l^{-1} KCl and 0.5 mmol l^{-1} dithiothreitol, pH 7) with a protease inhibitor cocktail (Sigma P8340, 15 µl per 2 ml buffer). After 10 strokes in a glass homogenizer on ice, the homogenate was transferred to a tube and centrifuged at 13,800 ***g*** for 15 min at 4°C to pellet the nuclear and mitochondrial fractions. The supernatant containing the SR was decanted and we added 200 µl glycerol before storage at −80°C. An aliquot of this homogenate was used to determine protein concentration by the Bradford method.

SERCA activity was determined by spectrophotometric measurement of the rate of ATP hydrolysis before and after addition of thapsigargin, a specific inhibitor of SERCA (Lytton et al., 1991). The 1 ml reaction cuvette contained 1 mmol l^{-1} EGTA, 0.5 mmol l^{-1} dithiothreitol, 10 mmol l^{-1} phosphoenolpyruvate, 5 mmol l^{-1} ATP, 50 mmol l^{-1} imidazole, 100 mmol l^{-1} KCl, 10 mmol l^{-1} $MgCl_2$, 10 mmol l^{-1} NaN_3, 0.96 mmol l^{-1} $CaCl_2$ (to achieve 10 µmol l^{-1} free Ca^{2+}), 5.3 units pyruvate kinase, 17.5 units lactate dehydrogenase, 2 µmol l^{-1} calcium ionophore (C7522, Sigma-Aldrich) and 300 µmol l^{-1} NADH, pH 7, 37°C. The reaction was initiated by addition of 20 µl sample homogenate (diluted as necessary), and ATP hydrolysis was determined by monitoring NADH oxidation at 340 nm. After obtaining a measurement for total ATP hydrolysis, we added 1 µl of 0.1 mmol l^{-1} thapsigargin and inverted the cuvette to mix before measuring again. The difference in hydrolysis rate with and without thapsigargin was attributed to SERCA. Samples were tested in duplicate for each pectoralis sample and single measurements for each heart sample.

Statistics

SERCA activity was calculated relative to either the amount of protein in the assay homogenate or the wet mass of tissue and is presented as U (µmol ATP hydrolyzed per minute) per milligram protein or per gram tissue. Statistical analyses were run using R version 3.2.2 (https://www.r-project.org/). Because most, but not all, individuals were given a cold challenge prior to tissue sampling, we first tested whether there was an effect of cold challenge by including this as a fixed factor in the analyses. The cold challenge procedure had no significant effect, so we then removed this factor from models before running other analyses. We used ANOVA and Tukey's HSD *post hoc* tests to compare activity among dietary treatments within an age. When comparing across ages, we used a mixed effects analysis ('lmer' function in the package 'lme4' in R; Bates et al., 2015) to account for the fact that

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some nestlings were nestmates, with age as a fixed factor and nest as a random factor. To determine significance of the age effect on SERCA activity, we used a likelihood ratio test comparing the full model with a null model that did not have the age factor. *Post hoc* analysis comparing ages was performed using the 'glht' function of the 'multcomp' package (Hothorn et al., 2008). Similarly, we determined the significance of the relationship between fatty acid proportions and SERCA activity using the 'lmer' function when comparing across ages, but we used a simple linear model when examining these relationships within an age group (in which case all individuals came from unique nests). We arcsine square root transformed fatty acid proportions prior to statistical tests. We confirmed that our data met test assumptions by visual inspection of residual plots for deviations from homoscedasticity or normality. Significance was accepted at $P<0.05$.

RESULTS

In the pectoralis muscle, SERCA activity in the control birds varied with age when calculated either per milligram protein ($X^2_6=45$, $P<0.0001$; Fig. 1A) or per gram tissue ($X^2_6=63$, $P<0.0001$; Fig. 1B). On a per milligram protein basis, activity approximately doubled between 1 and 5 dph, when it reached a plateau at around 1.2 µmol min^{-1} mg^{-1} through 9 dph (Fig. 1A). Interestingly, SERCA activity then declined in fledglings and adults (Fig. 1A). On a per gram tissue basis, SERCA activity increased approximately 8-fold from 1 to 9 dph, when it reached 124 µmol min^{-1} g^{-1} (Fig. 1B). Again, SERCA activity then declined in fledglings and dropped to 70.5 µmol min^{-1} g^{-1} in adults (Fig. 1B). There was no effect of diet on SERCA activity at any age on either basis ($P>0.15$ for all comparisons; Fig. 1).

In the heart, SERCA activity per milligram protein was relatively constant at approximately 0.4 µmol min^{-1} mg^{-1} from 1 dph through 7 dph (Fig. 2A). SERCA activity then increased to over 0.6 µmol min^{-1} mg^{-1} in 9 dph nestlings, fledglings and adults ($X^2_6=55$, $P<0.0001$; Fig. 2A). There was no effect of diet on SERCA activity for any age ($P>0.063$ for all ages). Per gram tissue, SERCA activity increased steadily from 1 dph (20.4 µmol min^{-1} g^{-1}) to 9 dph (51.5 µmol min^{-1} g^{-1}; $X^2_6=70$, $P<0.0001$; Fig. 2B). SERCA activity increased further to 72.6 µmol min^{-1} g^{-1} in adults. Again, diet had no effect on cardiac SERCA activity on a per gram tissue basis ($P>0.332$ for all ages; Fig. 2B).

When including all ages, the proportion of linoleic acid in the pectoralis muscle had a significant positive effect on SERCA activity per milligram protein in control birds ($X^2_1=4.5$, $P=0.033$; Fig. 3). This effect was not apparent when examining only the seed oil group ($X^2_1=0.125$, $P=0.724$) or the fish oil group ($X^2_1=0.38$, $P=0.537$), or when combining all treatment groups ($X^2_1=2.38$, $P=0.123$; Fig. 3). The proportion of docosahexaenoic acid had a significant positive effect on SERCA activity when combining all treatment groups ($X^2_1=7.2$, $P=0.007$), but not when examining only control birds ($X^2_1=2.67$, $P=0.102$), the seed oil group ($X^2_1=0.33$, $P=0.57$) or the fish oil group ($X^2_1=3.2$, $P=0.074$; data not shown).

When examining any particular age, there was no consistent relationship between SERCA activity and the proportion of linoleic acid in pectoralis muscle. At 5 dph, there was no effect of linoleic acid on SERCA activity per milligram protein ($F_{1,16}=0.1385$, $P=0.71$; Fig. 4A) or per gram tissue ($F_{1,16}=1.5$, $P=0.24$) when combining all 5 dph birds, nor when considering diet or control groups individually ($P>0.23$ for all groups and calculated either per milligram protein or per gram tissue). At 7 dph, there was no effect of linoleic acid on SERCA activity per milligram protein, either when considering diet or control groups

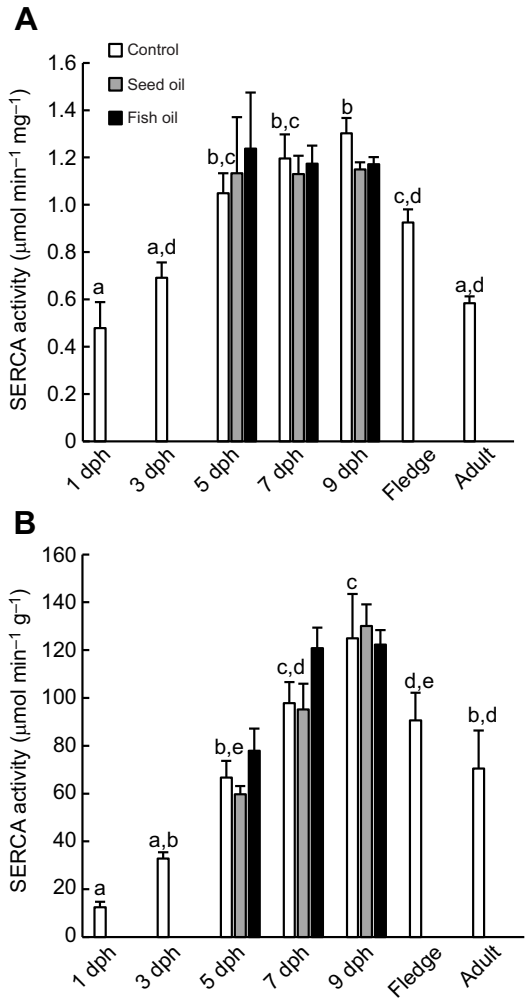

Fig. 1. Activity of sarco(endo)plasmic Ca^{2+}-ATPase (SERCA) at various ages in the pectoralis muscle of red-winged blackbirds (*Agelaius phoeniceus*). (A) SERCA activity expressed per milligram protein in the assay homogenate. (B) SERCA activity expressed per gram tissue (wet). Fledglings were estimated to be 10–17 days post-hatch (dph). Different letters above bars indicate significant differences among ages in the control group ($P<0.05$). Dietary treatment (sunflower oil or fish oil supplementation) had no significant effect on either metric of SERCA activity at any age. $N=6$ birds for each bar, except for the following: $N_{7dphControl}=7$, $N_{9dphControl}=7$, $N_{Adult}=3$. Data are means+s.e.m.

individually ($P>0.1$ for all groups), or when combining all 7 dph birds ($F_{1,17}=0.47$, $P=0.50$; Fig. 4B). There was a negative effect of linoleic acid on SERCA activity per gram tissue at 7 dph ($F_{1,17}=5$, $P=0.038$), but no significant effect within treatment groups ($P>0.08$ for all groups). At 9 dph, there was no effect of linoleic acid on SERCA activity either per milligram protein ($F_{1,17}=0.6$, $P=0.42$; Fig. 4C) or per gram tissue ($F_{1,17}=2.4$, $P=0.14$) when including all birds of this age. There was a positive effect of linoleic acid on SERCA activity per milligram protein when considering only the control 9 dph birds ($F_{1,5}=7.9$, $P=0.037$; Fig. 4C), but not when considering any other 9 dph treatment group or denominator ($P>0.16$ for all).

Similarly, docosahexaenoic acid had no consistent effects on SERCA activity within a given age. For example, there was no effect of docosahexaenoic acid on activity per milligram protein or per gram tissue at 9 dph, examining either all birds combined or by treatment group ($P>0.06$ for all, data not shown).

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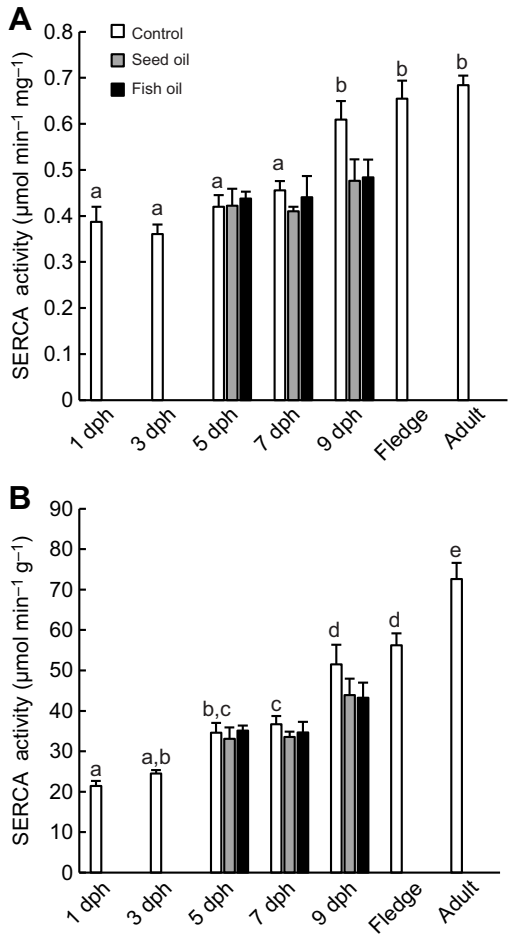

Fig. 2. Activity of SERCA at various ages in the cardiac ventricle of red-winged blackbirds. (A) SERCA activity expressed per milligram protein in the assay homogenate. (B) SERCA activity expressed per gram tissue (wet). Fledglings were estimated to be 10–17 dph. Different letters above bars indicate significant differences among ages in the control group (P<0.05). Dietary treatment (sunflower oil or fish oil supplementation) had no significant effect on either metric of SERCA activity at any age. N=6 birds for each bar, except for the following: $N_{1dphControl}$=4, $N_{7dphControl}$=7, $N_{9dphControl}$=7, N_{Adult}=3. Data are means+s.e.m.

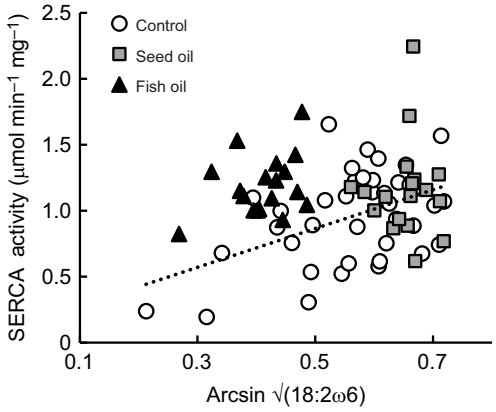

Fig. 3. Activity of SERCA (μmol min^{-1} mg^{-1} protein) as a function of the proportion of linoleic acid (arcsine square-root transformed) in the pectoralis muscle phospholipids of red-winged blackbirds, with all ages and treatments combined, excepting adults. A best-fit line is shown for the control birds only, which was the only group demonstrating a significant (P<0.05) relationship between the linoleic acid proportion and SERCA activity. $N_{Control}$=38, N_{Seed}=18, and N_{Fish}=18.

DISCUSSION

SERCA activity and the ontogeny of endothermy

Development of calcium-cycling machinery of muscles has rarely been studied during the critical transition from ectothermy to endothermy in birds. In chickens – which develop endothermic metabolic responses around 1 dph – the concentration of parvalbumin (a cytosolic calcium-binding protein) begins increasing rapidly in the thigh muscle approximately 1 day before hatching, and then continues to increase exponentially for at least a week after hatching (Le Peuch et al., 1979). Concordantly, SERCA activity increases shortly before hatching in chicken leg muscles and continues to rise until reaching a plateau around 20 dph (Boland et al., 1974). SERCA activity of chicken hearts shows a similar pattern, but cardiac SERCA activity is much lower and shows smaller developmental changes compared with the leg, consistent with the full functionality of the heart at early embryonic stages (Boland et al., 1974).

Our data on red-winged blackbirds are similar to those from chickens in that SERCA activity increased in both skeletal and cardiac muscle during post-natal development, and in that the change in SERCA activity was greater in the pectoralis muscle than the heart. Again, this latter characteristic presumably reflects the requirement that the heart must be functional at early embryonic ages, whereas skeletal muscle is poorly developed even after hatching in altricial species. Although the developmental pattern of SERCA activity was the same regardless of whether it was expressed relative to protein or tissue mass, developmental changes were greatest percentagewise when expressed per gram wet mass rather than per milligram protein. Developmental changes in SERCA activity using the latter denominator may represent increased density of SERCA molecules in the SR membrane. Changes in SERCA activity calculated relative to wet mass may represent these changes as well as increasing SR volume and surface area, and changing percent dry mass during development, which approximately doubles between 1 and 9 dph in blackbird pectoralis and hearts (Ricklefs, 1967).

One intriguing feature of our data was the decline in pectoralis muscle SERCA activity that begins after fledging and continues into adulthood. Although the developmental rise in SERCA activity may represent a general development of muscle function, this post-fledging decline may suggest a specific trade-off between thermoregulatory and locomotory roles of the muscle. Muscle volume can be divided into three major parts – myofibrils, SR and mitochondria – that compete for space and primarily promote three muscle characteristics: high force generation, rapid frequency and sustained use, respectively (Rome and Lindstedt, 1998). Comparative studies suggest that SERCA, along with other aspects of calcium-cycling machinery such as parvalbumin, help to endow muscles with high-frequency capability (Rome and Klimov, 2000; Schuppe et al., 2018), but with a trade-off to force generation or aerobic endurance (Rome and Lindstedt, 1998; Tikunov and Rome, 2009; Mead et al., 2017). SERCA activity is critical to shivering thermogenesis, and is a critical component of a proposed mechanism for non-shivering thermogenesis (Rowland et al., 2015), but neither thermogenic process should demand high levels of force generation.

Red-winged blackbirds attain endothermic responses to cold temperatures by 9 dph (Olson, 1992; Sirsat et al., 2016), but continue to develop mechanisms that improve heat generation and retention after that age, including increases in pectoralis muscle mass and improvements to feather insulation (Olson, 1992, 2001; Price and Dzialowski, 2018). Muscle mass-specific thermogenic

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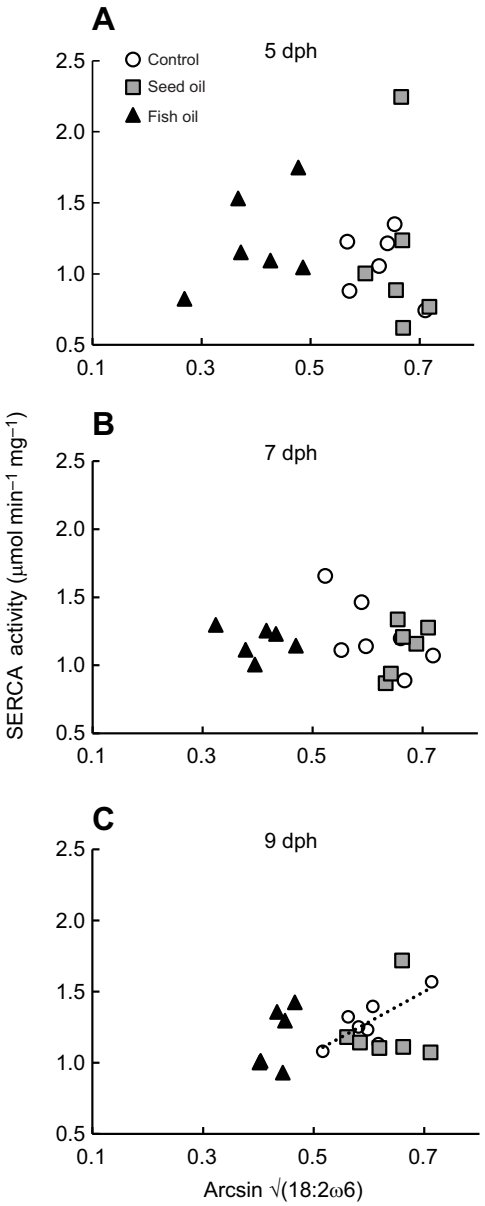

Fig. 4. Activity of SERCA (µmol min^{-1} mg^{-1} protein) as a function of the proportion of linoleic acid (arcsine square-root transformed) in the pectoralis muscle phospholipids of red-winged blackbirds, broken down by age group. The only significant (P<0.05) relationship between linoleic acid and activity was for 9 dph control birds. N=6 for all treatment groups at each age, except for 7 and 9 dph control birds, for which N=7.

capacity, including mechanisms involving SERCA, can therefore decrease after fledging without a loss of total thermogenic capacity or a loss of effective control of body temperature. In contrast to SERCA activity, pectoralis myofibril and mitochondrial volume appear to be maintained or increase as red-winged blackbirds develop into fledglings and adulthood, as evidenced by myosin ATPase activity, citrate synthase activity and mitochondrial oxidative phosphorylation capacity (Olson, 2001; Sirsat et al., 2016). Thus, the transition from nestling to fledgling includes the development of flight, with the concomitant need for sustained, high levels of force generation, but with lower requirements for mass-specific thermogenic capacity. We speculate that this drives an adaptive remodeling of the pectoralis muscle during this developmental period, resulting in lower SR volume and SERCA activity in fledglings and adults. This post-fledging decline in the pectoralis stands in contrast to the increasing SERCA activity in the heart, a muscle that is important for meeting the oxidative demands of flight but not directly involved in adaptive thermogenesis via a SERCA-mediated mechanism. Resting heart rate declines from neonates to adulthood in birds (Pearson et al., 1998), but cardiac SERCA capacity must be matched to maximal heart rate, which can reach several-fold over resting in adult birds (Berger et al., 1970; Bishop and Butler, 1995).

The effect of linoleic acid on SERCA activity

We hypothesized that the greater cold-induced maximal metabolic rate achieved by our seed oil group in a previous study (Price et al., 2018b) was due to higher SERCA activity. This was based on previously established relationships between muscle membrane linoleic acid and maximal exercise performance (Ruf et al., 2006; Stawski et al., 2015), previous observations that birds fed linoleate-rich diets can sometimes achieve higher maximal metabolic rates (Pierce et al., 2005; Price and Guglielmo, 2009), as well as the established effect of linoleic acid on SERCA activity (Swanson et al., 1989; Ushio et al., 1997). In contradiction of this hypothesis, however, our present study showed no effect of dietary treatment on SERCA activity in either the pectoralis muscle or the heart ventricle.

Although there was a positive correlation between muscle membrane linoleate and SERCA activity when considering all ages of the control group, this correlation may be spurious and related to the developmental incorporation of both linoleic acid and docosahexaenoic acid into muscle membranes during the nestling period (Price et al., 2018b). The lack of correlation between SERCA activity and linoleic acid within the oil treatment groups could be attributed to the limited variation in linoleic acid proportion within each group; however, the lack of correlation when considering all groups combined (which includes a wide range of linoleate proportions) suggests that muscle linoleate indeed had no effect on SERCA activity. This interpretation is further supported by the lack of consistent age-specific correlations between membrane linoleate and SERCA activity (Fig. 4). Here again, one might attribute this finding to the small range of linoleate values within each diet group, or to the small sample size within each diet group. However, there were no significant correlations across groups at any age, and the non-significant correlations within groups trended negatively as much as they did positively. We therefore conclude that statistical power was not an issue; rather, there was simply no relationship between muscle linoleate and SERCA activity.

The effects of membrane lipid composition on SERCA activity have not been consistent across studies. When comparing across several fish species there is no relationship between muscle membrane fatty acid composition and SERCA activity in skeletal muscle (Gonzales et al., 2015). Manipulative experiments have not always demonstrated the diet effect on SERCA activity (Croset et al., 1989; Stubbs and Kisielewski, 1990; Kinoshita et al., 1994), although there is generally an effect on calcium uptake, usually attributed to an effect on permeability of the SR to Ca^{2+} (Croset et al., 1989; Fajardo et al., 2015). Nonetheless, many studies have reported that high dietary linoleic acid alters cardiac and skeletal muscle SR membranes and thereby increases SERCA activity, while dietary DHA has the opposite effect (Swanson et al., 1989; Vajreswari and Narayanareddy, 1992; Paige et al., 1996; Ushio et al., 1997; Sugasini and Lokesh, 2013; Fajardo et al., 2015). Thus it was unexpected that our diet manipulation did not result in altered SERCA activity, regardless of whether SERCA activity affected whole-animal metabolic rate.

Our membrane composition measurements represent total muscle phospholipids, so it is possible that we did not successfully alter SR membranes, although that seems unlikely given that previous diet manipulations have successfully done so, and that other subcellular membranes (mitochondria) were altered by our manipulation (Price et al., 2018b). Another possibility is that the effect of linoleic acid on SERCA activity can only be observed within a certain range of membrane compositions, and that our manipulation fell outside that range. Notably, all our birds had proportions of linoleic acid and docosahexaenoic acid that were relatively high compared with most previous studies in mammals and fish. Thus, although we saw no effect of the dietary manipulation on SERCA activity at a given age, this does not rule out a potential role for membrane lipid composition in controlling the ontogeny of SERCA activity in the control group, an effect that might have been present particularly at young ages. This hypothesis requires further study.

If SERCA activity was unaffected by our dietary treatment, how might we explain the previously reported (Price et al., 2018b) differences in cold-induced maximal metabolic rate among diet groups? In addition to membrane-based effects, dietary fatty acid composition has the potential to affect metabolic rates owing to differences in the mobilization and oxidation rates of different fatty acids (Price et al., 2008, 2011) and by affecting various signaling pathways (Price, 2010; Pierce and McWilliams, 2014) that can alter the activities of several metabolic enzymes (Nagahuedi et al., 2009; Dick and Guglielmo, 2019), although this does not always alter metabolic rate (Dick and Guglielmo, 2019). It is possible that maximal metabolic rates of the dietary groups differed owing to one of these still-to-be-determined mechanisms.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: E.R.P., E.M.D.; Methodology: E.R.P.; Validation: E.R.P.; Formal analysis: E.R.P.; Investigation: E.R.P., T.S.S., S.K.S., E.M.D.; Resources: E.M.D.; Writing - original draft: E.R.P.; Writing - review & editing: E.R.P., T.S.S., S.K.S., E.M.D.; Visualization: E.R.P.; Supervision: E.M.D.; Funding acquisition: E.M.D.

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