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Is there a role for sarcolipin in avian facultative thermogenesis in extreme cold?

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Endotherms defend their body temperature in the cold by employing shivering (ST) and/or non-shivering thermogenesis (NST). Although NST is well documented in mammals, its importance to avian heat generation is unclear. Recent work points to a prominent role for the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) in muscular NST. SERCA's involvement in both ST and NST, however, posits a tradeoff between these two heat-generating mechanisms. To explore this tradeoff, we assayed pectoralis gene expression of adult songbirds exposed to chronic temperature acclimations. Counter to mammal models, we found that cold-acclimated birds downregulated the expression of sarcolipin (*SLN*), a gene coding for a peptide that promotes heat generation by uncoupling SERCA Ca^{2+} transport from ATP hydrolysis, indicating a reduced potential for muscular NST. We also found differential expression of many genes involved in Ca^{2+} cycling and muscle contraction and propose that decreased *SLN* could promote increased pectoralis contractility for ST. Moreover, *SLN* transcript abundance negatively correlated with peak oxygen consumption under cold exposure (a proxy for ST) across individuals, and higher *SLN* transcript abundance escalated an individual's risk of hypothermia in acute cold. Our results therefore suggest that SLN-mediated NST may not be an important mechanism of—and could be a hindrance to—avian thermoregulation in extreme cold.

1. Introduction

In the face of thermal stress, endotherms can protect their body temperature (T_b) by employing heat-generating processes in the form of shivering thermogenesis (ST) and/or non-shivering thermogenesis (NST). The use of NST has been extensively described in mammals, which increase NST to regulate body temperature in the cold [1]. It is suspected that birds also use NST and, indeed, some juvenile birds increase NST with cold acclimation [2–4]. Nonetheless, few studies have explored the role of NST during cold acclimatization in adult birds.

Part of this discrepancy arises from uncertainty in the potential mechanism underlying avian NST. For instance, the mitochondrial uncoupling of oxidative phosphorylation from ATP synthesis is one well-characterized mechanism of mammalian NST. During this process, an uncoupling protein (UCP1) facilitates the leakage of protons across the mitochondrial membrane, which dissipates heat. In placental mammals, UCP1 is mainly expressed in brown adipose tissue (BAT) and cold acclimation is associated with BAT recruitment and an increased capacity for NST [5]. Although birds lack BAT, a role for mitochondrial uncoupling in the avian skeletal muscle has been proposed [6,7]. However, direct empirical support for a contribution of the avian UCP homologue (avUCP) to mitochondrial uncoupling is lacking [8,9].
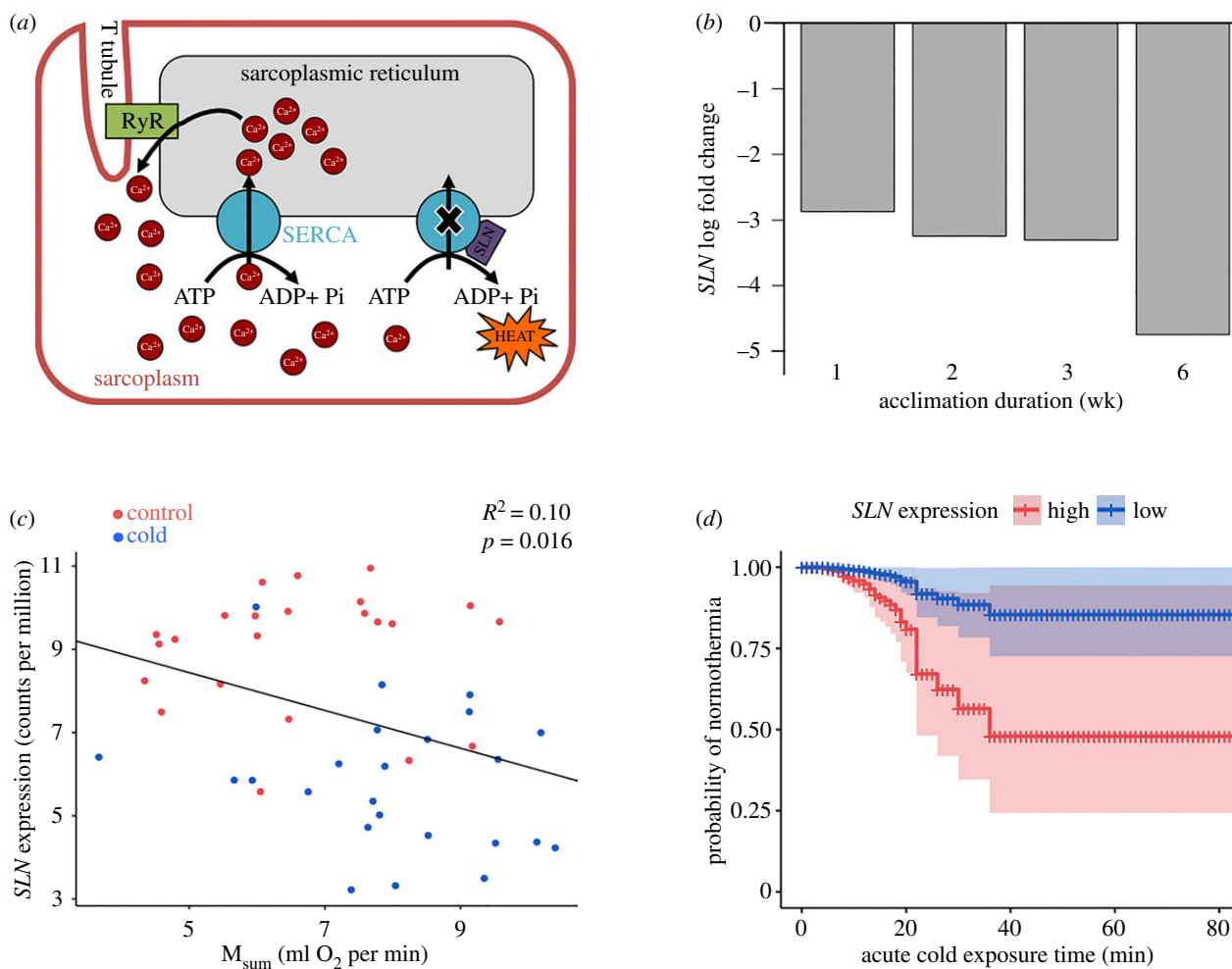
Figure 1. (*a*) Mechanism of heat generation via sarcolipin (SLN) in mammals. RyR, ryanodine receptor channel. (*b*) Magnitude of *SLN* expression change across sampling points. (*c*) Negative correlation between *SLN* transcript abundance and M_{sum}. (*d*) Effect of *SLN* expression on risk of hypothermia using best Cox proportional hazards model, with *SLN* transcript abundance represented as high or low (mean for control and cold treatments, respectively) and covariates held constant at mean values across individuals.

Instead, increasing evidence points to a role for the sarco/endoplasmic reticulum calcium ATPase (SERCA) in facilitating avian NST [10]. SERCA uses phosphate bond energy from ATP to move Ca^{2+} ions from the myocyte cytosol into the sarcoplasmic reticulum to create a Ca^{2+} gradient in resting striated muscle [11] (figure 1*a*). When present, the peptide sarcolipin (SLN) binds to SERCA and promotes uncoupling of Ca^{2+} transport from ATP hydrolysis, resulting in futile SERCA activity and heat production in mammals ([11], but see [12]). Overexpression of SLN in laboratory mice is associated with increased NST and decreased energy stores in the cold [13,14].

While SLN can enhance NST, it may also negatively impact ST. For instance, experimental increases in exogenous SLN result in reduced peak isometric force, lower rates of contraction and relaxation, and increased fatigue of the soleus in rats [15]. Because rapid muscular contractions require high Ca^{2+} cycling activity [16], SLN-associated reductions in Ca^{2+} cycling could similarly reduce shivering activity. These potentially antagonistic effects of SERCA on ST and NST therefore setup an obvious, yet unexplored tradeoff between heat-generating mechanisms.

We explored this tradeoff using transcriptome-wide patterns of gene expression to reveal the many co-occurring processes within the skeletal muscle of dark-eyed juncos (*Junco hyemalis*) exposed to chronic temperature acclimations. Juncos winter at high latitudes across North America [17] and

we have previously shown that they increase their thermogenic performance with increasing duration of cold acclimation [18]. Here, we present the first evidence, to our knowledge, for *SLN* expression in the avian skeletal muscle. We predicted that if SLN-mediated NST is an advantageous mechanism of avian heat generation, birds should increase *SLN* expression in the cold. Alternatively, if shivering is the most important component of avian facultative thermogenesis, we expected cold-acclimated birds to decrease *SLN* expression. We further predicted that potential *SLN* differences would be accompanied by changes in the expression of genes related to ST muscle contraction, as well as whole-organism measures of thermogenic performance. Our results suggest that, if SLN-mediated NST occurs in adult birds, it has a minimal role in their acclimation to extreme cold, revealing exciting directions for future exploration of tradeoffs between these heat-generating mechanisms.

2. Methods

We have previously described our acclimation experiment and physiological assays in detail [18]. Briefly, in 2017 we exposed wild-caught, adult juncos from Missoula, MT to constant laboratory conditions for six weeks (18°C), then randomly assigned birds to cold (−8°C) or control (18°C) acclimation treatments lasting one, two, three or six weeks (electronic supplementary material, table S1). Following acclimations, we simultaneously

Table 1. Cox proportional hazards model estimates for the standardized effects of *SLN* transcript abundance and M_{sum} on the risk of hypothermia while controlling for variation in T_a ($n = 45$). Robust standard error (SE); likelihood-ratio test (LRT).

T_a			*SLN*			M_{sum}			$SLN \times M_{sum}$			
β	SE	p	β	SE	p	β	SE	p	β	SE	p	LRT
−2.45	0.53	3.5×10^{-6}										48.64
−3.23	0.68	1.8×10^{-6}	1.12	0.97	0.25							53.34
−2.56	0.54	2.4×10^{-6}				−1.16	0.48	0.02				60.23
−3.41	0.65	1.5×10^{-7}	1.21	0.87	0.16	−1.15	0.49	0.02				64.84
−4.15	0.73	1.4×10^{-8}	1.68	0.80	0.04	−1.46	0.58	0.01	−3.13	1.15	6.3×10^{-3}	81.58

assayed an individual's core T_b (using a passive-integrated transponder tag inserted into the cloaca) and peak oxygen consumption (M_{sum} [ml O_2 per min]; using open-flow respirometry) during acute cold trials (short-term exposure to temperatures below −10°C in a heliox environment). Upon trial completion, we immediately euthanized individuals and harvested the pectoralis (the principal shivering muscle for small birds [19]). We flash froze tissues and stored them at −80°C.

To assay gene expression, we isolated mRNA from left pectoralis tissue of 47 randomly selected individuals (electronic supplementary material, table S2) using TRI Reagent (Sigma-Aldrich). The UT Austin Genomic Sequencing and Analysis Facility performed TagSeq [20] library preparation and sequencing. The 47 libraries were pooled in one lane and sequenced three times on an Illumina HiSeq 2500 platform, yielding 254 million reads. We filtered raw reads in accordance with [20] using publicly available scripts (https://github.com/z0on/tag415 based_RNAseq) and trimmed reads with the FASTX-toolkit (http://hannonlab.cshl.edu/fastx_toolkit/), resulting in $\mu = 1.46$ million reads per individual. We mapped these reads to the genome of the white-throated sparrow (*Zonotrichia albicollis*, a close junco relative), using bwa mem [21], with $\mu = 816\,600$ reads per individual mapped. Finally, we generated individual-level transcript abundances using FEATURECOUNTS [22] for use in downstream analyses, which we conducted in R [23] (electronic supplementary material, table S3).

We performed differential expression analyses using package *edgeR* [24]. We first removed lowly expressed genes that occurred in fewer than 6 individuals, resulting in 12 249 genes in our dataset (electronic supplementary material, table S4). We then normalized read counts using *calcNormFactors*, estimated dispersion using *estimateDisp* and employed a generalized linear model [25] to test for differential expression among experimental treatments using *glmFit*, with cold acclimation duration as the main effect and all control treatments combined as the reference (false discovery rate [FDR] less than 0.05). We performed functional enrichment analysis on the list of differentially expressed (DE) genes using package *gprofiler2* [26] with the 12 249 genes as our background gene set (electronic supplementary material, table S5). To help explain the pattern of increasing thermogenic performance observed across the acclimation period [18], we asked whether each DE gene also differed in its magnitude of change across the acclimation duration by regressing its log fold change (from the fitted glm) on treatment duration (in weeks) using linear regressions ($p < 0.05$).

We related normalized *SLN* transcript abundance to phenotypic measures from [18], for each individual. We tested for an association between *SLN* and M_{sum} using a linear regression. To determine if *SLN* expression influenced thermoregulatory performance, we fit T_b data from acute cold trials to Cox proportional hazards regression models with the package *Survival*

[27]. We created survival objects using an individual's hypothermic status ($T_b < 10\%$ of starting T_b) for each one-minute interval of the trial, then fit regressions using the function *coxph* with all terms clustered by individual to quantify the effects of *SLN* expression, M_{sum}, and their interaction on the risk of hypothermia. To account for variation in acute temperature stimulus among individuals, we also included ambient temperature (T_a) for each time event as a covariate (see [18] for details). We standardized each predictor variable according to [28] and removed from this analysis two individuals that ejected their T_b transponders before they became hypothermic.

Finally, we asked if cold-acclimated birds altered the expression of genes involved in skeletal muscle contraction. To do this, we mapped expression patterns onto the muscle contraction (MC) and excitation-contraction coupling (ECC) pathways identified in [29]. Pathways included multiple isoforms for many proteins and some genes were not present in the dataset (2 ECC genes) or annotated in the *Zonotrichia* genome (7 of 38 MC genes; 5 of 32 ECC), including those encoding SERCA1 and RyR1.

3. Results

We found 526 DE genes among temperature treatments (electronic supplementary material, table S6). Compared to control birds, juncos consistently upregulated 196 genes and downregulated 256 across cold groups. Fifty-seven DE genes showed patterns of increasing or decreasing fold change over the duration of cold acclimation, and the top among them was *SLN* (lowest FDR; electronic supplementary material, table S7). Normalized *SLN* transcript abundance decreased in the cold, with the magnitude of downregulation increasing with acclimation duration ($\beta = -0.37$, $p = 0.019$; figure 1*b*). *SLN* transcript abundance also negatively correlated with M_{sum} ($\beta = -0.45$, $p = 0.016$, $R^2 = 0.10$; figure 1*c*). The best model explaining risk of hypothermia in acute cold included T_a, *SLN* transcript abundance, M_{sum} and $SLN \times M_{sum}$ (table 1). A disparity in hypothermia risk emerges between high and low *SLN* expression when the other two variables are held constant, such that individuals with low expression better maintain T_b (figure 1*d*). Additionally, of the candidate skeletal muscle contraction genes present in our dataset, 5 of 31 genes in the MC pathway and 3 of 25 in the ECC pathway were DE (28% and 12% of represented proteins, respectively; figure 2; electronic supplementary material, table S8).

4. Discussion

Endogenous heat generation through either ST or NST can allow endotherms to maintain high T_b at low ambient
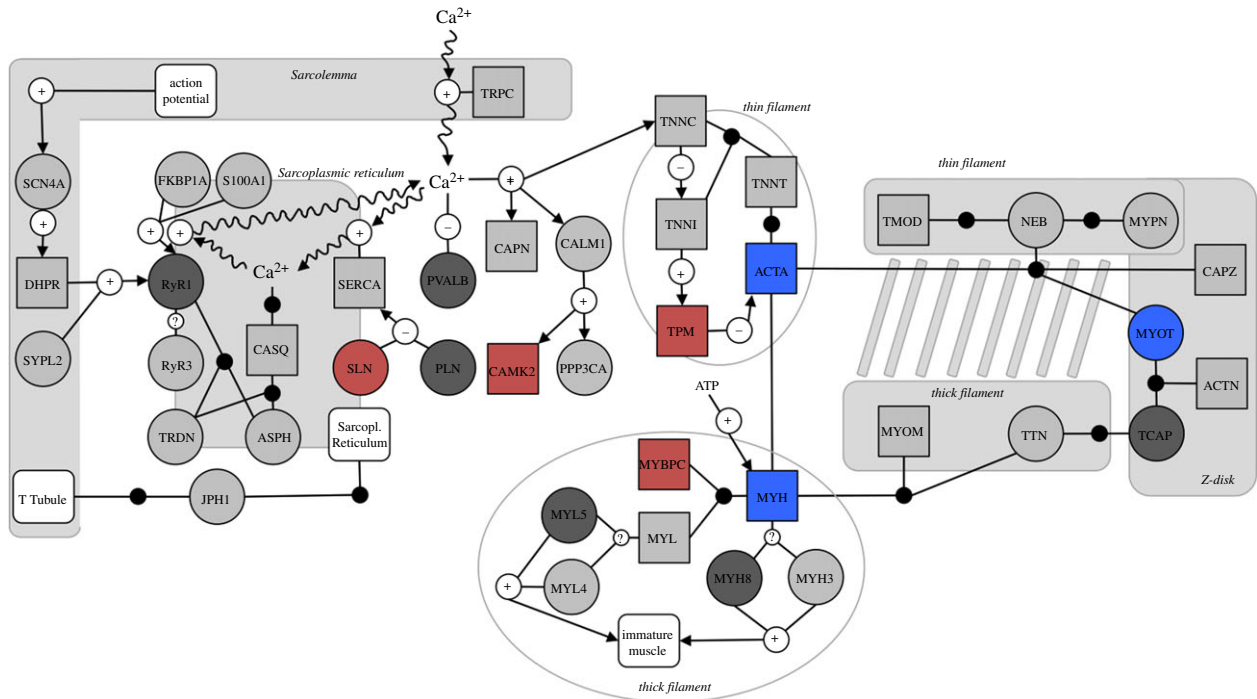
Figure 2. Expression changes in targeted skeletal muscle contraction pathways. Symbols represent genes (circles), gene complexes (squares), modules or functions (white squares), interactions (white circles; positive, negative or unknown) and binding (black circles) per [29]. Colours indicate upregulation (blue), downregulation (red) or no change (light grey) in the cold, or not present in the dataset (dark grey). See electronic supplementary material, table S7 for complete pathway details.

temperatures. Despite its established importance in mammalian thermoregulation, the adaptive significance of avian NST is difficult to determine because the evidence derives entirely from juvenile birds [30]. To address this gap, we used previously reported patterns of avian thermogenic performance to explore the use of facultative NST in wild, adult dark-eyed juncos following cold acclimation. We employed whole-transcriptome expression patterns to simultaneously examine multiple pathways related to ST and NST within the avian pectoralis. We provide novel evidence that *SLN* is expressed in adult birds; however, juncos downregulated *SLN* after acclimation to subzero temperatures, demonstrating that if SLN-mediated NST is used by birds, it is not important—and perhaps even counterproductive—to adult thermoregulation in extreme cold.

We attribute the pattern in *SLN* expression to the possible cost of uncoupling Ca^{2+} cycling for NST in the form of reduced muscle activity for ST. Indeed, the potential for NST to impair muscular function has been proposed as a hypothesis to explain the evolution of BAT-mediated NST in placental mammals [10,31]. It therefore follows that at truly cold temperatures, like those used here, birds should prioritize the process with the greatest heat-generating capacity. Importantly, *SLN*-mediated NST is estimated to produce only a small fraction (2%) of the heat generated during a single-muscle contraction [32]. Accordingly, we observed a tradeoff between *SLN* expression and M_{sum} across individuals. Over the course of acclimation, cold birds further decreased the expression of *SLN*, perhaps facilitating increases to ST.

In support of this idea, we found differential expression of several genes related to skeletal muscle contraction. Whether these expression differences resulted in increased muscle contractility is unknown, but several of the expression patterns we observed are consistent with this hypothesis. For instance, overexpression of β-tropomyosin (*TPM2*) in cardiac muscle is

associated with a delay in relaxation [33] and juncos accordingly downregulated *TPM2* in the cold. Many additional DE genes are involved in striated muscle Ca^{2+} cycling, such as members of the adrenergic signalling pathway (*ADCY6*, *CREB5*, *CREM*, *KCNQ1*, *PLCB1*, *PPP2R2D* and *PPP2R5A*). We also observed expression changes in transcription factors (*MEF2C*, *EGR1* and *NFATC1*) that have been implicated in heightened striated muscle performance in mice (e.g. faster relaxation, increased contractility, reduced fatigability and enhanced force) [34]. Nonetheless, while our findings indicate that juncos are simultaneously incorporating several modifications that could improve ST in the cold, quantification of shivering (e.g. using electromyographic activity [4]) is necessary to verify the thermogenic effects of these expression patterns. Moreover, although juncos did not change the expression of a biomarker for mitochondrial abundance (citrate synthase, *CS*), measures of junco mitochondrial function are needed to fully address the potential effects of SLN on muscle energetics (e.g. [14]).

Previous work has demonstrated that cold-acclimated ducklings increase SERCA activity in the gastrocnemius, and this has been cited as evidence of increased capacity for NST [2,35]. We did not measure SERCA activity, but we did not find changes in the expression of SERCA2 or SERCA3 (*ATP2A2* and *ATP2A3*) with cold acclimation. There is likely functional differentiation between SERCA isoforms, with SERCA1 being implicated in NST and SERCA2a in ST [31,36]. However, the gene that encodes SERCA1 is not annotated in our reference genome. These discrepancies are difficult to interpret but it is possible that the relative benefit of NST differs among muscles and/or across life stages in birds.

Although limited to a single muscle in a single species, our work highlights a possible discrepancy in the utilization of NST among small birds and many mammals in the cold. This difference may emerge because mammals with BAT

can compartmentalize one mechanism of NST within a specialized organ, while for birds and other organisms lacking BAT, NST is constrained by the diverse functions of the skeletal muscle. Our evidence thus suggests a potential trade-off between shivering and non-shivering heat production in birds and emphasizes the need for direct measures of avian Ca^{2+} uncoupling. These results point to fruitful avenues for further investigation regarding the evolution of avian endothermy and the use of NST in seasonal acclimatization.

References

1. Janský L. 1973 Non-shivering thermogenesis and its thermoregulatory significance. *Biol. Rev.* **48**, 85–132. (doi:10.1111/j.1469-185X.1973.tb01115.x)

2. Dumonteil E, Barre H, Meissner G. 1995 Expression of sarcoplasmic reticulum Ca^{2+} transport proteins in cold-acclimating ducklings. *Am. J. Physiol. Cell Physiol.* **269**, C955–C960. (doi:10.1152/ajpcell.1995.269.4.C955)

3. Barre H, Geloen A, Chatonnet J, Dittmar A, Rouanet JL. 1985 Potentiated muscular thermogenesis in cold-acclimated muscovy duckling. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **249**, R533–R538. (doi:10.1152/ajpregu.1985.249.5.R533)

4. Teulier L, Rouanet J-L, Rey B, Roussel D. 2014 Ontogeny of non-shivering thermogenesis in Muscovy ducklings (*Cairina moschata*). *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **175**, 82–89. (doi:10.1016/j.cbpa.2014.05.012)

5. Cannon B, Nedergaard J. 2004 Brown adipose tissue: function and physiological significance. *Physiol. Rev.* **84**, 277–359. (doi:10.1152/physrev.00015.2003)

6. Duchamp C. 1993 Skeletal muscle as the major site of nonshivering thermogenesis in cold-acclimated ducklings. *Am. J. physiol.* **265**, R1076–R1083. (doi:10.1152/ajpregu.1993.265.5.r1076)

7. Raimbault S *et al.* 2001 An uncoupling protein homologue putatively involved in facultative muscle thermogenesis in birds. *Biochem. J.* **353**, 441–444. (doi:10.1042/bj3530441)

8. Emre Y, Hurtaud C, Ricquier D, Bouillaud F, Hughes J, Criscuolo F. 2007 Avian UCP: the killjoy in the evolution of the mitochondrial uncoupling proteins. *J. Mol. Evol.* **65**, 392–402. (doi:10.1007/s00239-007-9020-1)

9. Walter I, Seebacher F. 2009 Endothermy in birds: underlying molecular mechanisms. *J. Exp. Biol.* **212**, 2328–2336. (doi:10.1242/jeb.029009)

10. Rowland LA, Bal NC, Periasamy M. 2015 The role of skeletal-muscle-based thermogenic mechanisms in vertebrate endothermy: non-shivering thermogenic mechanisms in evolution. *Biol. Rev.* **90**, 1279–1297. (doi:10.1111/brv.12157)

11. Periasamy M, Huke S. 2001 SERCA pump level is a critical determinant of Ca^{2+} homeostasis and cardiac contractility. *J. Mol. Cell. Cardiol.* **33**, 1053–1063. (doi:10.1006/jmcc.2001.1366)

12. Mall S, Broadbridge R, Harrison SL, Gore MG, Lee AG, East JM. 2006 The presence of sarcolipin results in increased heat production by Ca^{2+}-ATPase. *J. Biol. Chem.* **281**, 36 597–36 602. (doi:10.1074/jbc.M606869200)

13. Bal NC *et al.* 2012 Sarcolipin is a newly identified regulator of muscle-based thermogenesis in mammals. *Nat. Med.* **18**, 1575–1579. (doi:10.1038/nm.2897)

14. Maurya SK, Herrera JL, Sahoo SK, Reis FCG, Vega RB, Kelly DP, Periasamy M. 2018 Sarcolipin signaling promotes mitochondrial biogenesis and oxidative metabolism in skeletal muscle. *Cell Reports* **24**, 2919–2931. (doi:10.1016/j.celrep.2018.08.036)

15. Tupling AR, Asahi M, MacLennan DH. 2002 Sarcolipin overexpression in rat slow twitch muscle inhibits sarcoplasmic reticulum Ca^{2+} uptake and impairs contractile function. *J. Biol. Chem.* **277**, 44 740–44 746. (doi:10.1074/jbc.M206171200)

16. Rome LC, Lindstedt SL. 1998 The quest for speed: muscles built for high-frequency contractions. *Physiology* **13**, 261–268. (doi:10.1152/physiologyonline.1998.13.6.261)

17. Nolan Jr V, Ketterson ED, Cristol DA, Rogers CM, Clotfelter ED, Titus RC, Schoech SJ, Snajdr E. 2002 Dark-eyed Junco (*Junco hyemalis*). *Birds N. Am.* (doi:10.2173/bna.716)

18. Stager M, Senner NR, Tobalske BW, Cheviron ZA. 2020 Body temperature maintenance acclimates in a winter-tenacious songbird. *J. Exp. Biol.* **223**, jeb221853. (doi:10.1242/jeb.221853)

19. Yacoe M, Dawson W. 1983 Seasonal acclimatization in American goldfinches: the role of the pectoralis muscle. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **245**, R265–R271. (doi:10.1152/ajpregu.1983.245.2.R265)

20. Lohman BK, Weber JN, Bolnick DI. 2016 Evaluation of TagSeq, a reliable low-cost alternative for RNAseq. *Mol. Ecol. Resour.* **16**, 1315–1321. (doi:10.1111/1755-0998.12529)

21. Li H. 2013 Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. *ArXiv* 1303. (https://arxiv.org/abs/1303.3997)

22. Liao Y, Smyth GK, Shi W. 2014 FEATURECOUNTS: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**, 923–930. (doi:10.1093/bioinformatics/btt656)

23. R Core Team. 2018 *R: The R project for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. See https://www.r-project.org/.

24. Robinson MD, McCarthy DJ, Smyth GK. 2010 edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**, 139–140. (doi:10.1093/bioinformatics/btp616)

25. McCarthy DJ, Chen Y, Smyth GK. 2012 Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res.* **40**, 4288–4297. (doi:10.1093/nar/gks042)

26. Reimand J, Kolde R, Arak T. 2018 gProfileR: interface to the 'g:Profiler' toolkit. R package version 0.6.7. See https://cran.r-project.org/web/packages/gProfileR/index.html.

27. Therneau T. 2015 A package for survival analysis in S. version 2.38. See https://cran.r-project.org/web/packages/survival/citation.html.

28. Gelman A. 2008 Scaling regression inputs by dividing by two standard deviations. *Statist. Med.* **27**, 2865–2873. (doi:10.1002/sim.3107)

29. Smith LR, Meyer G, Lieber RL. 2013 Systems analysis of biological networks in skeletal muscle function. *WIREs Syst. Biol. Med.* **5**, 55–71. (doi:10.1002/wsbm.1197)

30. Hohtola E. 2002 Facultative and obligatory thermogenesis in young birds: a cautionary note. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol.* **131**, 733–739. (doi:10.1016/S1095-6433(02)00011-9)

31. Nowack J, Giroud S, Arnold W, Ruf T. 2017 Muscle non-shivering thermogenesis and its role in the evolution of endothermy. *Front. Physiol.* **8**, 889. (doi:10.3389/fphys.2017.00889)

32. Campbell KL, Dicke AA. 2018 Sarcolipin makes heat, but is it adaptive thermogenesis? *Front. Physiol.* **9**, 714. (doi:10.3389/fphys.2018.00714)

33. Muthuchamy M, Grupp IL, Grupp G, Toole BAO, Kier AB, Boivin GP, Neumann J, Wieczorek DF. 1995 Molecular and physiological effects of overexpressing striated muscle β-tropomyosin in the adult murine heart.

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J. Biol. Chem. **270**, 30593–30603. (doi:10.1074/jbc. 270.51.30593).

34. Scharf M *et al*. 2013 Mitogen-activated protein kinase-activated protein kinases 2 and 3 regulate SERCA2a expression and fiber type composition to modulate skeletal muscle and cardiomyocyte function. *Mol. Cell. Biol.* **33**, 2586–2602. (doi:10. 1128/MCB.01692-12)

35. Dumonteil E, Barre H, Meissner G. 1993 Sarcoplasmic reticulum Ca^{2+}-ATPase and ryanodine receptor in cold-acclimated ducklings and thermogenesis. *Am. J. Physiol. Cell Physiol.* **265**, C507–C513. (doi:10.1152/ajpcell.1993.265. 2.C507)

36. de Meis L, Arruda AP, Carvalho DP. 2005 Role of sarco/endoplasmic reticulum Ca^{2+}-ATPase in thermogenesis. *Biosci. Rep.* **25**, 181–190. (doi:10. 1007/s10540-005-2884-7)

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royalsocietypublishing.org/journal/rsbl *Biol. Lett.* **16**: 20200078