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Relative sarcolipin (SLN) and sarcoplasmic reticulum Ca^{2+} ATPase (SERCA1) transcripts levels in closely related endothermic and ectothermic scombrid fishes: Implications for molecular basis of futile calcium cycle non-shivering thermogenesis (NST)

Sean Robinson [a], Nicholas C. Wegner [b], Chugey A. Sepulveda [c], Jens P.C. Franck [a,*]

[a] Department of Biology, The University of Winnipeg, Winnipeg, MB R3B 2E9, Canada
[b] Fisheries Resources Division, Southwest Fisheries Science Center, National Marine Fisheries Service (NMFS), National Oceanic and Atmospheric Administration (NOAA), La Jolla, CA 92037, USA
[c] Pfleger Institute of Environmental Research (PIER), Oceanside, CA, USA

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ABSTRACT

Regional endothermy is the ability of an animal to elevate the temperature of specific regions of the body above that of the surrounding environment and has evolved independently among several fish lineages. Sarcolipin (SLN) is a small transmembrane protein that uncouples the sarcoplasmic reticulum calcium ATPase pump (SERCA1b) resulting in futile Ca^{2+} cycling and is thought to play a role in non-shivering thermogenesis (NST) in cold-challenged mammals and possibly some fishes. This study investigated the relative expression of *sln* and *serca1* transcripts in three regionally-endothermic fishes (the skipjack, *Katsuwonus pelamis*, and yellowfin tuna, *Thunnus albacares*, both of which elevate the temperatures of their slow-twitch red skeletal muscle (RM) and extraocular muscles (EM), as well as the cranial endothermic swordfish, *Xiphias gladius*), and closely related ectothermic scombrids (the Eastern Pacific bonito, *Sarda chiliensis,* and Pacific chub mackerel, *Scomber japonicus*). Using Reverse Transcription quantitative PCR (RT-qPCR) and species-specific primers, relative *sln* expression trended higher in both the RM and EM for all four scombrid species compared to white muscle. In addition, relative *serca1* expression was found to be higher in RM of skipjack and yellowfin tuna in comparison to white muscle. However, neither *sln* nor *serca1* transcripts were higher in swordfish RM, EM or cranial heater tissue in comparison to white muscle. A key phosphorylation site in sarcolipin, threonine 5, is conserved in the swordfish, but is mutated to alanine or valine in tunas and the endothermic smalleye Pacific opah, *Lampris incognitus*, which should result in increased uncoupling of the SERCA pump. Our results support the role of potential SLN-NST in endothermic tunas and the lack thereof for swordfish.

1. Introduction

Regional endothermy is the physiological process by which organisms elevate specific tissue temperatures above ambient conditions. Most fish species function at temperatures close to environmental, however, billfishes (Istiophoridae and Xiphiidae), tunas (Scombridae), lamnid sharks (Lamnidae), opahs (Lampridae), and the common thresher shark (*Alopias vulpinus*) have physiological adaptations to produce and conserve metabolic heat to varying degrees within select tissues (Bernal and Sepulveda, 2005; Carey, 1982; Legendre and Davesne, 2020; Morrissette et al., 2003; Wegner et al., 2015). Increasing tissue temperatures is advantageous as it speeds up various metabolic

Abbreviations: avUPC1, Avian Uncoupling Protein 1; BAT, Brown Adipose Tissue; EM, Extraocular Muscle; HTR, Heater Organ; NST, Non-Shivering Thermogenesis; RM, Slow-Twitch Red Muscle; RT-qPCR, Reverse Transcription Quantitative Real-Time Polymerase Chain Reaction; RyR1a, Ryanodine Type 1a Receptor; SERCA1, Sarcoplasmic Reticulum Ca2+ ATPase Pump; Sln, Sarcolipin; SR, Sarcoplasmic Reticulum; ST, Shivering Thermogenesis; UPC1, Uncoupling Protein 1; WM, Fast-Twitch White Muscle; ef1α, Elongation Factor 1 Alpha..

* Corresponding author.
E-mail address: j.franck@uwinnipeg.ca (J.P.C. Franck).
@Swm_Robinson (S. Robinson), @JensFranck55696 (J.P.C. Franck)

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processes and can enhance muscle power output, increase digestion rates, and enhance visual resolution. Ultimately these adaptations have been hypothesized to contribute to thermal niche expansion and efficient long-distance migrations (Altringham and Block, 1997; Dickson and Graham, 2004; Harding et al., 2021; Madigan et al., 2021; Wegner et al., 2015).

The development of regional endothermy in fishes has evolved independently several times. As a result, the location and mechanism of metabolic heat production and conservation is not uniform across species. Nevertheless, these fishes largely utilize similar physiological adaptions to effectively reduce heat loss. For example, regionally-endothermic fishes with elevated slow-oxidative red skeletal muscle (RM) temperatures, have RM positioned more medially and anteriorly within the body compared to closely related ectothermic species (Bernal et al., 2003; Graham and Dickson, 2001; Sepulveda et al., 2005). Internal RM positioning increases the insulation between the tissue and environment and therefore reduces convective heat loss (Graham et al., 1983). In addition, specialized counter-current heat exchangers derived of closely intertwined blood vessels (*retia mirabilia*) leaving and entering warmed regions of the body help conserve heat and minimize its loss at the gills via convection during respiration (Carey et al., 1971). Without such adaptations, endothermic fishes would not be able to maintain tissue temperatures above ambient conditions. Regional endothermy has been proposed to facilitate the expansion of both the vertical and latitudinal niche of endothermic species by enabling prolonged dives beneath the thermocline as well as long distance migrations into temperate waters (Harding et al., 2021; Legendre and Davesne, 2020).

There are two general physiological strategies employed by organisms to achieve elevated tissue temperatures: shivering thermogenesis (ST), in which heat is produced as a by-product of muscular contraction, and non-shivering thermogenesis (NST). NST is best suited for chronic heat production as it produces heat in the absence of muscle contraction, therefore reducing risk of skeletal muscle fatigue (Legendre and Davesne, 2020). The most researched NST mechanism involves mammalian brown adipose tissue (BAT) and uncoupling of the proton gradient by uncoupling protein 1 (UCP1) (Cannon and Nedergaard, 2004). However, another form of NST, fultile calcium cycling-based NST, is characterized in a broader array of taxa (Dumonteil and Meissner, 1993; Bal et al., 2012; Periasamy et al., 2017; Pani et al., 2023). Futile calcium cycling was proposed as the basis for heat production in the heater organ tissues of billfishes, classified as cranial endotherms (Morrissette et al., 2003). The heater organ, first characterized by Carey (1982) is derived from extraocular eye muscle that has lost the ability to contract due to the absence of organized myofilaments (Morrissette et al., 2003). Despite losing contractibility, the heater organ still contains extensive t-tubules with an abundant number of Ca^{2+}- ATPase transport proteins and mitochondria necessary for NST (Block and Franzini-Armstrong, 1988). The proposed NST mechanism within the heater organ involves Ca^{2+} leak from sarcoplasmic reticulum (SR), through the ryanodine calcium release receptor channel (RyR1a) (Morrissette et al., 2003). The leaky RyR1a channel, increases cytosolic Ca^{2+} concentration, causing the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA1b) pump to increase active transport to re-establish SR Ca^{2+} concentration and produce heat as a by product (Meizoso-Huesca et al., 2022; Morrissette et al., 2003; Singh et al., 2023). A similar mechanism is thought to underlie the cranial heater organ in the butterfly mackerel (*Gasterochisma melampus*) and slender tuna (*Allothunnus fallai*) (Block, 1986; Sepulveda et al., 2007).

In addition to RyR1a Ca^{2+} leak, sarcolipin (SLN), a short transmembrane protein, has been suggested to contribute to RM endothermy (Bal and Periasamy, 2020). Increased cytoplasmic Ca^{2+} concentration results in muscle contraction. To terminate muscle contraction, the SERCA1b pump, driven by ATP hydrolysis, actively transports cytosolic Ca^{2+} into the SR, lowering cytosolic concentration and terminating muscle contraction (Periasamy et al., 2017; Stammers et al., 2015). When SLN is present, the protein competes with Ca^{2+} to Serca1b and causes Ca^{2+} to 'slip' from the SR to the cytosol (Sahoo et al., 2013). Similar to the RyR1a Ca^{2+} leak, the depletion of SR Ca^{2+} concentration leads to increased Serca1b activity in an attempt to restore the SR Ca^{2+} concentration gradient, increasing ATP hydrolysis and the production of excess catabolic heat (Periasamy et al., 2017). Higher expression of SLN has been observed in cold-challenged mice and mice with ablated BAT that are fed high caloric diets (Bal et al., 2012, 2016). In fish, SLN-mediated NST has been suggested for the smalleye Pacific opah (*Lampris incognitus*) dark red pectoral muscle, which showed higher *sln* transcript levels when compared to lighter red pectoral muscle used in continuous swimming, and the glycolytic fast-twitch white muscle (WM), a tissue type typically not associated with endothermy (Franck et al., 2019). More recently, higher *sln* and *serca1* transcript levels were observed in cold-challenged ectothermic Japanese medaka (*Oryzias latipes*) (Robinson et al., 2024). Taken together, increased *sln* transcripts from cold-challenged and high caloric dieted mice, Japanese medaka, and smalleye Pacific opah RM, suggests SLN plays a role in maintaining basal metabolic rates and NST (SLN-NST).

The objective of this study was to investigate SLN as a component of NST in regionally endothermic skipjack (*Katsuwonus pelamis*) and yellowfin tuna (*Thunnus albacares)*, closely related ectothermic Eastern Pacific bonito (*Sarda chiliensis*) and Pacific chub mackerel (*Scomber japonicus*), as well as the cranially-endothermic swordfish (*Xiphias gladius*). To do so, tissues with elevated temperatures, slow-twitch oxidative red skeletal muscle, extraocular eye muscle, and heater organ (exclusive to swordfish), were compared to fast-twitch glycolytic white skeletal muscle. Relative transcript expression of *sln* and *serca1* by RT-qPCR was used to determine whether *sln* and *serca1* expression has greater expression in endothermic tissue types.

2. Materials and methods

2.1. Tissue collection and handing

Tissues were sampled from fish caught within the Pacific Ocean offshore of Southern California, flash frozen and stored at −70 °C until time of use. Fish capture and tissue sampling was done under approved procedures of the Southwest Fisheries Science Center Animal Care and Use Committee (Protocol #SW1201). Samples were taken from five yellowfin tuna (59.0–86.5 cm fork length (FL), 3.8–11.5 kg), four skipjack tuna (61.0–63.0 cm FL, 4.8–6.1 kg), two swordfish (153.7–185.0 cm lower jaw fork length), four Eastern Pacific bonito (30.5—33.8 cm FL, 380–565 g) and five Pacific chub mackerel (18.3–24.2 cm FL, 55–165 g). From each fish, red skeletal muscle, white skeletal muscle, and eye muscle was extracted. Additionally, swordfish cranial heater organ tissue was sampled from two swordfish. Swordfish tissue samples were collected during research and fishery development trials aboard the R/V *Malolo* (California Department of Fish and Wildlife Scientific Collection; permit #SCP-2471).

2.2. RNA extraction and first stand cDNA synthesis

Total RNA extraction was performed using TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). RNA was assessed using agarose gel electrophoresis and Nanodrop One spectrophotometer (Thermo Scientific). First strand DNA was reverse transcribed from a uniform amount of total RNA (64 ng) using Bio-Rad iScript cDNA Synthesis kit following manufacturer's protocol.

2.3. Primer design

Species-specific primers were designed from GenBank sequences and conserved alignments for *serca1*, *sln*, and housekeeping gene elongation factor 1 alpha (*ef1α*) (Table 1). Amplicons were sent to The Centre for Applied Genomics (TCAG) at Toronto Sick Kids Hospital DNA sequencing facility to confirm correct gene target amplification.

Table 1
Forward (F) and reverse (R) primer sequences (5′-3′) for sarcolipin (*sln*), sarcoplasmic reticulum Ca^{2+} ATPase (*serca1*) and elongation factor 1 alpha (*ef1α*).

Species	Gene	Sequence 5′-3’	Amplicon Size (bp)	Accession number
		F: CGGCAAGAAGCTTGAGGATA		
	ef1α	R: GGAGAAGCTCTCCACACACAT	100	Alignment
		F: TGAAGAAGGAGTTCACACAGGA		
	serca1	R: TGGAGCACCTTTGACAAACA	119	Alignment
		F: TCGCCTAGTCTCTCCAGTCC		
Skipjack tuna	*sln*	R: CAGCAGCACGGTGATTAAGA	103	XM_044353687.1
		F: TTGACATCGCTCTGTGGAAG		
	ef1α	R: AGATACCGGCCTCAAACTCA	155	Alignment
		F: TCTCCAGAGACAGGAAGTCCA		
	serca1	R: CCTTCTGGAGCACCTTTGAC	100	XM_044336903.1
		F: AGTCCTCTCCGTCTGGGATT		
Yellowfin tuna	*sln*	R: CAGCAGCACGGTGATTAAGA	88	XM_044353687.1
		F: CGGCAAGAAGCTTGAGGATA		
	ef1α	R: GGAGAAGCTCTCCACACACAT	100	Alignment
		F: CTCCCCTACTCTCCTCCAC		
	serca1	R: ACCCCAAGGCTCTCAAGTCT	103	XM_040135509.1
		F: AGAAAGGAGCAGTCGAAAACA		
Swordfish	*sln*	R: CCTTTCCTCTTTTGGCTGTG	74	XM_040131489.1
		F: CGGCAAGAAGCTTGAGGATA		
	ef1α	R: GGAGAAGCTCTCCACACACAT	100	Alignment
		F: CTGTGTGGCAGACCTCAAAA		
	serca1	R: GTGCACCTGTCAATCACACC	75	Alignment
		F: CTCCAGTCTTCTCCGTCTGG		
Bonito	*sln*	R: CAGCAGCACGGTGATTAAGA	92	XM_044353687.1
		F: TTCCGTAATGGAGGATCAGC		
	ef1α	R: TGGGGTTATCCTCAAGCTTCT	74	Alignment
		F: TGTGCAGAGCTCACTGCTTT		
	serca1	R: CCCTGCAGGAACTCAACAAT	70	Alignment
		F: GCAGGAGCTGTTCCTCAACT		
Chub Mackerel	*sln*	R: GAGGCGTGTGTTTTCTCCAT	105	Alignment

Amplicon size is represented in base pairs (bp) and accession numbers used to acquire species-specific primers using GenBank sequences and consensus alignments (Alignment: XM_044336903.1, XM_042397348.1, XM_041029250.1, XM_039788041.1, XM_040135509.1, XM_011488036.3, XM_006639512, XM_012818387.3, XM_007245711.4, U65228.1, XM_008301307.1).

2.4. RT-qPCR

RT-qPCR reactions contained 1.5 μl of cDNA added to 13.5 μl of master mix, which contained 7.5 μl SYBR Green Master mix, 0.75 μl of both forward and reverse primers at a concentration of 333 nM, and 4.5 μl UltraPure RNase-free water (Invitrogen). Reactions were performed in 96-well 0.1 ml plates (Invitrogen) sealed with optically clear adhesive sheets (Thermo Scientific) in a QuantStudio5 RT-PCR. Thermocycling conditions were as follows: initial denaturation at 93 °C for 3 mins, 40 cycles of denaturation at 93 °C for 10 s, annealing at 57 °C for 15 s and extension at 72 °C for 20 s. After cycling, a dissociation curve was performed to ensure amplification of a single product. Primer efficiency was evaluated by preparing serial dilutions of cDNA and subjected to qPCR to the same thermocycling conditions. From the serial dilutions, a standard curve was created to ensure efficiency (E) was between 80% and 120% and R^2 values above 0.98 were deemed optimal. Analysis of qPCR data (ΔCq) was done to determine the relative expression of *sln* and *serca1* in reference to housekeeping gene *ef1α*.

2.5. Statistical analysis

Statistical analyses were performed using R-software 4.1.3 (R Core Team, 2022). Data from between-tissue effects (ΔCq: target gene – housekeeping gene) were analyzed using a Kruskal-Wallis test followed by Dunn’s post-hoc test with a Holm’s adjustment. Additionally, Dixon’s outlier test was performed, and outliers were subsequently removed. For all tests, $P < 0.05$ was considered significant.

3. Results and discussion

The objective of this study was to investigate SLN as a component of NST in regionally endothermic and ectothermic scombrid fishes, prompted by the discovery of higher *sln* transcript levels in dark red pectoral fin muscle in the endothermic smalleye Pacific opah (*Lampris incognitus*) (Franck et al., 2019). Specifically, we utilized *sln* and *serca1* gene-specific primers to quantify transcript levels for selected endothermic fish species, including the cranially- endothermic swordfish, a member of the Xiphiidae family, as well as yellowfin and skipjack tuna, members of the Scombridae family. Additionally, we quantified *sln* and *serca1* transcripts in two ectothermic species, chub mackerel and Eastern Pacific bonito, also from the Scombridae family. We examined gene transcript levels of *sln* and *serca1* in the slow-twitch aerobic red muscle (RM), fast-twitch white muscle (WM), extraocular eye muscles (EM) and ocular heater organ tissues (HTR: exclusive to swordfish).

3.1. Comparison of relative sln transcript expression

The expression of *sln* showed a distinct pattern across all four scombrid species examined irrespective of endothermic capacity, with EM and RM both showing elevated expression in comparison to WM. Specifically, *sln* relative transcript expression was significantly greater in EM compared to WM in skipjack tuna (Fig. 1A, Dunn: $z = 3.138$, $P = 0.005$), yellowfin tuna (Fig. 1B, Dunn: $z = 2.690$, $P = 0.0214$), bonito (Fig. 1C, Dunn: $z = 2.942$, $P = 0.0003$), and chub mackerel (Fig. 1D, Dunn: $z = 3.138$, $P = 0.005$), and significantly higher in RM compared with WM in yellowfin tuna (Fig. 1B, Dunn: $z = 2.268$, $P = 0.047$), although the other scombrid species showed a similar trend (Fig. 1). In contrast, swordfish *sln* transcript expression showed no significant difference between tissue types (Fig. 1E). SLN has been documented to increase metabolic production in mammalian skeletal muscle and has been proposed as the potential molecular basis for heat production in the smalleye Pacific opah deep red skeletal muscle (Bal et al., 2012; Bal and Periasamy, 2020; Franck et al., 2019). The presence of SLN induces skeletal muscle NST by competitively binding to the transmembrane

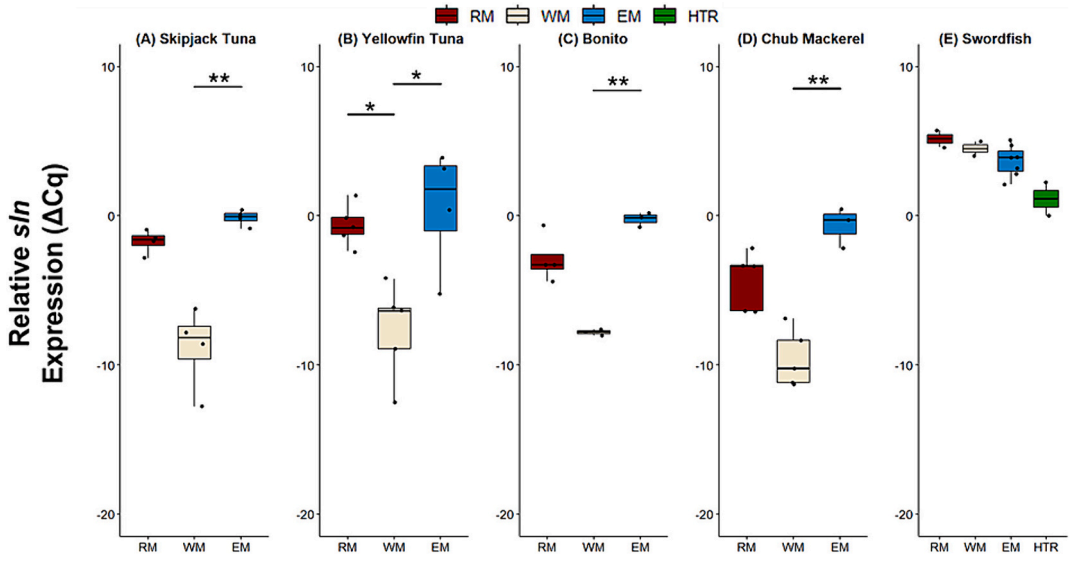

Fig. 1. *Sln* transcript levels measured relative to housekeeping gene *ef1α* (ΔCq: housekeeping –target gene), for slow-oxidative red skeletal muscle (RM), fast-glycolytic white skeletal muscle (WM), extraocular eye muscle (EM) and heater organ (HTR; exclusive to swordfish) in skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*), Eastern Pacific bonito (*Sarda chilinesis*), Pacific chub mackerel (*Scomber japonicus*) and swordfish *(Xiphias gladius*). $^{*}P < 0.05$, $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

groove of SERCA1b (Sahoo et al., 2013). Bound SLN causes calcium to 'slip' from the SR lumen into the cytoplasm, resulting in SERCA1b pump to increase activity to sequester calcium. This futile calcium cycle results in excess catabolic heat production. While relative *sln* transcript expression trended higher in endothermic tissues (RM and EM) when compared to WM in endothermic skipjack and yellowfin tuna (Carey et al., 1971), the same pattern was observed in the closely related ectothermic Eastern Pacific bonito and Pacific chub mackerel. Although Eastern Pacific bonito and chub mackerel do not possess physiological adaptations to conserve metabolic heat (Dickson et al., 2002), similar to cold-challenged ectothermic medaka (Robinson et al., 2024), they appear to possess the same transcriptional architecture as their endothermic sister species. This matches other similarities between ectothermic and endothermic scombrids such as enhanced red muscle oxidative capacity (Block and Finnerty, 1994; Dickson, 1996) and reduced or reversed temperature dependence of blood-oxygen binding (Clark et al., 2010), characteristics that appear to predate the evolution of endothermy, and likely facilitated its development within the group. In contrast, the swordfish, a species that is a documented cranial endotherm (Carey, 1982) and suspected RM endotherm (Carey, 1990; Stoehr et al., 2018, 2020) showed no significant differences in *sln* transcript levels across all tissue types sampled, including the ocular heater organ tissue. Heat production from the heater organ tissue in marlin (*Makaira nigricans*) has been previously attributed to a futile calcium cycle triggered by a "leaky" RyR1a calcium channel (Morrissette et al., 2003). The absence of higher *sln* transcript levels suggests that swordfish heater organ may, in fact, rely solely on the RyR1a calcium leak.

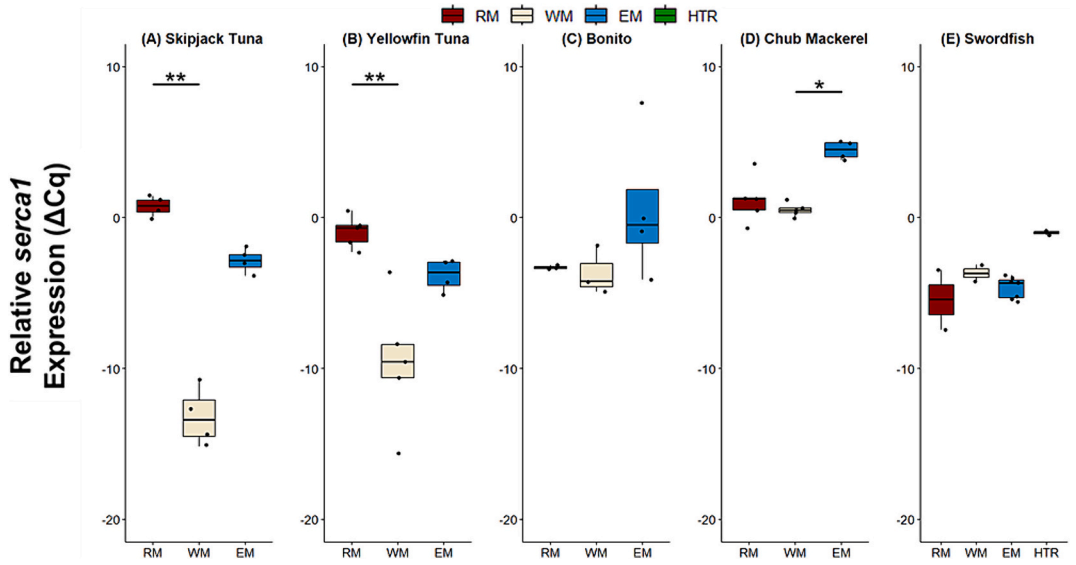

Fig. 2. *Serca1* transcript levels measured relative to housekeeping gene *ef1α* (ΔCq: housekeeping – target gene), for slow-oxidative red skeletal muscle (RM), fast-glycolytic white skeletal muscle (WM), extraocular eye muscle (EM) and heater organ (HTR; exclusive to swordfish) in skipjack tuna (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*), Eastern Pacific bonito (*Sarda chiliensis*), Pacific chub mackerel (*Scomber japonicus*) and swordfish *(Xiphias gladius*). $^{*}P < 0.05$, $^{**}P < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Comparison of relative serca1 transcript expression

Relative *serca1* transcript expression showed a generally similarly pattern to that of *sln* for the regionally-endothermic scombrids with RM *serca1* significantly higher compared to WM in both skipjack (Fig. 2A, Dunn: $z = 3.138$, $P = 0.002$) and yellowfin tuna (Fig. 2B, Dunn: $z = 3.25$, $P = 0.001$), and, while EM transcript levels trend higher for both species when compared to WM, the differences were not found to be statistically significant. In contrast, the relative expression of *serca1* in the ectothermic scombrids were generally similar between tissues (Fig. 2C and D), apart from EM *serca1* transcript expression in the Pacific chub mackerel which was significantly higher when compared to WM tissues (Fig. 2D, Dunn: $z = 2.886$, $P = 0.004$). No statistical difference was found in swordfish *serca1* transcripts in the sampled tissues (Fig. 2E).

Fish skeletal muscle is the most abundant tissue, accounting for up to 60% of total body mass in some species (Duran et al., 2021). SERCA1b, is responsible for the regulation of muscle contraction/relaxation (Li et al., 2021). Furthermore, the protein is inherently thermogenic as 18–55% of energy used by SERCA1b, is dissipated as heat (Bal and Periasamy, 2020). This study indicates endothermic tunas express greater levels of *serca1* transcripts in RM and EM compared to WM. Contrarily, ectothermic bonito and chub mackerel showed no difference in *serca1* transcript expression across all tissue types, except for chub mackerel where EM *serca1* transcript expression is greater than observed in WM. Like *sln* transcript expression, *serca1* expression is found to be elevated in tissues that have elevated temperatures compared to ambient. Previous research has indicated that SLN can inhibit a maximum 30% of SERCA1b function (Bal et al., 2018), thus, the higher transcript expression of *serca1* within the RM of skipjack and yellowfin tuna may at least in part be driven by the increase of SLN, as a greater number of SERCA1b would be necessary to maintain proper cytosolic calcium concentration for muscle relaxation. In contrast, swordfish *serca1* transcript expression was highest in the heater organ despite not seeing an increase of *sln* expression. The lack of *sln* transcripts supports an earlier study that suggests swordfish depend on a leaky RyR1a channel to establish a futile calcium cycle to generate cranial heat (Morrissette et al., 2003). The increased leak from the RyR1a channel would require higher expression of SERCA1b to compensate for the elevated cytosolic calcium levels.

3.3. Key regulatory site amino acid substitution in sarcolipin

We aligned sarcolipin amino acid sequences from representatives of Lampridae (Opah), Xiphiidae (Swordfish), and Scombridae (Fig. 3). The fifth amino acid residue, threonine (T), is substituted by a non-polar alanine (A) in the tunas and valine (V) in opah. Threonine 5 (T5) is a key regulatory site in sarcolipin. Phosphorylation of sarcolipin T5 relieves the inhibitory (uncoupling) effects of SLN on the SERCA pump (Bhupathy et al., 2009; Toyoshima et al., 2013). Additionally, rearrangement of the N-terminus upon phosphorylation of the T5 residue reduces the energy barrier for passage of ions (Cao et al., 2016). Deletion of the N terminal residues of SLN also results in the loss of the uncoupling function (Sahoo et al., 2015). Thus, the loss of the T5 phosphorylation regulatory site for the red muscle endotherms, the tunas and opah, would presumably result in SLN having a larger uncoupling effect on the SERCA pump. Conversely, the presence of the T5 regulatory site in the swordfish would reduce the uncoupling effect of SLN on SERCA1 upon phosphorylation, further suggesting that futile calcium cycling via a leaky RyR1a calcium channel as originally proposed by Morrissette et al. (2003) is the likely primary mechanism for heat production in the swordfish cranial heater organ.

While the current study is focused on the thermogenic role of SLN and NST, it should be noted that an increase in SLN expression is also linked to mitochondrial biogenesis and increased oxidative metabolism (Maurya et al., 2018; Bal et al., 2021). Mitochondrial biogenesis and oxidative metabolism is influenced by increased cytosolic calcium, as it initiates the CamKII/PGC1α signaling and increases CD36, CPT1-M, LPL, LCAD and HADHB, enzymes involved in fatty acid transport and oxidation (Bal et al., 2016; Maurya et al., 2018). Maurya et al. (2018) reported increased cytosolic calcium from SLN/SERCA futile calcium cycling is pertinent for maintaining mitochondrial health as well as lipid metabolism using Sln-KO mice. Furthermore, RADseq data from RM in Pacific bluefin and yellowfin tuna revealed enriched transcripts for mitochondria and lipid metabolic processes (Wu et al., 2023). Knowing this, SLN may also be responsible for sustaining adequate physiological conditions, such as increased number of mitochondria, required for constant swimming, which produces heat as a by-product, as well as potential futile calcium cycling in endothermic tunas.

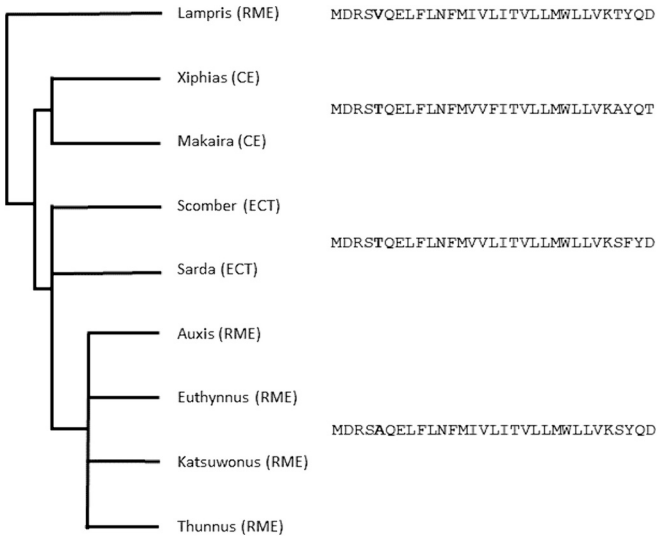

Fig. 3. Sarcolipin amino acid substitution across fishes examined in this study. The fifth amino acid residue, threonine (T), is substituted with valine (V) in opah (*Lampris*) and alanine (A) in the skipjack (*Katsuwonus*), which are both red muscle-based endotherms (RME). The swordfish (*Xiphias*), a cranial endotherm (CE), retains the threonine residue as do the ectothermic *Scomber* and *Sarda* species (ECT). Phylogeny topology adapted from Davesne et al. (2018). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Conclusion

This study showed generally higher relative *sln* gene transcript levels in red skeletal muscle and extraocular eye muscle in both the endothermic skipjack and yellowfin tuna, and ectothermic bonito and chub mackerel. In addition, significantly higher relative *serca1* gene expression was observed in red muscle in skipjack and yellowfin tuna. The higher relative quantity of *sln* and *serca1* transcripts supports their roles as potential contributors to NST though futile calcium cycling in red skeletal muscle for endothermic tunas. Conversely, the lack of a greater quantity of *sln* transcripts in swordfish heater organ supports previous research suggesting the futile calcium cycle may in fact be due to a leaky RyR1a channel and consequent increased SERCA1 activity as first proposed by Morrissette et al. (2003). The loss of the SLN T5 phosphorylation site in opah and tunas also supports a greater role of SLN in red muscle-based NST as this would result in the inability to inhibit the uncoupling mechanism of SLN on the SERCA1 pump.

CRediT authorship contribution statement

Sean Robinson: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Nicholas C. Wegner:** Writing – review & editing, Writing – original draft, Resources. **Chugey A. Sepulveda:** Writing – review & editing, Writing – original draft, Resources. **Jens P.**

C. Franck: Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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