RESEARCH ARTICLE

A thermogenic secondary sexual character in male sea lamprey

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SUMMARY

Secondary sexual characters in animals are exaggerated ornaments or weapons for intrasexual competition. Unexpectedly, we found that a male secondary sexual character in sea lamprey (Petromyzon marinus) is a thermogenic adipose tissue that instantly increases its heat production during sexual encounters. This secondary sexual character, developed in front of the anterior dorsal fin of mature males, is a swollen dorsal ridge known as the ‘rope’ tissue. It contains nerve bundles, multivacuolar adipocytes and interstitial cells packed with small lipid droplets and mitochondria with dense and highly organized cristae. The fatty acid composition of the rope tissue is rich in unsaturated fatty acids. The cytochrome c oxidase activity is high but the ATP concentration is very low in the mitochondria of the rope tissue compared with those of the gill and muscle tissues. The rope tissue temperature immediately rose up to 0.3°C when the male encountered a conspecific. Mature males generated more heat in the rope and muscle tissues when presented with a mature female than when presented with a male (paired t-test, P<0.05). On average, the rope generated 0.027±0.013 W cm−3 more heat than the muscle in 10 min. Transcriptome analyses revealed that genes involved in fat cell differentiation are upregulated whereas those involved in oxidative-phosphorylation-coupled ATP synthesis are downregulated in the rope tissue compared with the gill and muscle tissues. Sexually mature male sea lamprey possess the only known thermogenic secondary sexual character that shows differential heat generation toward individual conspecifics.

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INTRODUCTION

Lampreys are jawless vertebrates that diverged from jawed vertebrates 560 million years ago (Kumar and Hedges, 1998; Shu et al., 1999). The sea lamprey life cycle consists of larval, parasitic juvenile and anadromous adult stages (Applegate, 1950; Hardisty, 1979; Hardisty and Potter, 1971). At the final stage of sexual maturation, adult males develop a rope-like thickening at the dorsal ridge in front of their anterior dorsal fin (Fig. 1). Field observations have shown that mature males release a pheromone that attracts ovariatory females to their nest (Li et al., 2002), and often rub their rope tissue against the female abdomen. Females sometimes rub their urogenital pore on the rope tissue in return. After this initial courtship behavior, the male attaches onto the female’s head with his oral disc and tightens a knot with his tail near the female urogenital pore. The spawning act ends with both partners thrusting vigorously to release gametes.

As the rope tissue appears to play an active role in the spawning act and is not merely an ornamental display, we examined the morphology and development of the dorsal ridge in sea lamprey. To our surprise, the morphology of the rope tissue consists of multivacuolar fat with interstitial fibroblast-like cells and collagen fibers. This finding led us to speculate that the rope tissue may be a primordial thermogenic fat. Many heat-generating organs or mechanisms have appeared along the evolution of vertebrates. Among the most well known is mammalian brown adipose tissue (BAT), which burns fatty acids to produce heat via uncoupling oxidative phosphorylation-coupled ATP production in the mitochondria (Cannon and Nedergaard, 2004). However, outside of the mammalian clade the known heat-generating tissues are all derived from muscles (Cannon and Nedergaard, 2004). Birds, the other group of homeotherms, use muscles for nonshivering thermoregulation (Mozo et al., 2005). Several groups of fishes maintain brain temperatures higher than the environment by ATP-dependent Ca2+ cycling in the sarcoplasmic reticulum of ‘heater cells’ around the brain (Block, 1987). Therefore, the finding of a possible thermogenic fat was unexpected in non-mammalian species.

We examined whether the rope tissue exhibits characteristics of thermogenic fat through histological, chemical, biochemical, molecular biological, transcriptomic and behavioral analyses. We confirm that the rope tissue showed differential thermogenic ability toward individual conspecifics.
MATERIALS AND METHODS

Collection and maintenance of animals

Adult sea lampreys, Petromyzon marinus Linnaeus (body mass 200–350 g with an average body length of 48 cm), were captured during upstream spawning migration by agents of the US Fish and Wildlife Service and the Canada Department of Fisheries and Ocean from tributaries of Lakes Huron and Michigan. They were caged in streams until they completed final sexual maturation. Animal handling procedures were approved by the Institutional Animal Care and Use Committee at Michigan State University (MSU).

Fatty acid composition analyses (GC-MS)

Sea lamprey rope tissues were collected with the skin (epidermis) and underlying muscles carefully removed. Samples were snap frozen in liquid nitrogen and stored at −80°C before extraction. Rope tissue extracts (in dichloromethane) were methanolized at 100°C for 15h. Fatty acid methyl esters were extracted with hexane and analyzed using an HP-5890A gas chromatograph with a 30 m×0.32 mm fused silica capillary column and eluted with helium at a 4°C/min increment in an oven starting at a temperature of 145°C and completed at 220°C. Chromatographic peaks were identified by comparing with a standard mixture containing 19 fatty acid methyl esters (GLC-68A, Nu-Chek Prep, Elysian, MN, USA).

Gene ontology analyses

Transcriptome expression data for sea lamprey rope, gill and muscle tissues were obtained using an Illumina Genome Analyzer II (Illumina, San Diego, CA, USA) and mRNA-Seq protocol (75-bp) at the Research Technology Support Facility at MSU. Two-way BLASTX between lamprey transcriptome expressed sequence tags and mouse protein database was performed to obtain putative orthologies. Gene ontology (GO) categories were assigned to the corresponding expressed sequence tags according to NCBI/Entrez databases (http://www.ncbi.nlm.nih.gov/). Bowtie software was used to count the number of reads (Langmead et al., 2009). The raw counts were normalized using quantile normalization. Normalized profiles were pairwise-compared using GoMiner (Zeeberg et al., 2003). Heat maps were generated using CLMiner (Weinstein et al., 1997).

Histology and immunohistochemistry

Histology samples were processed in the Investigative Histopathology Laboratory, Department of Physiology and Human Pathology at MSU. Immunostaining for noradrenaline (NA) and uncoupling protein (UCP) followed methods described previously (Chung-Davidson et al., 2008). Negative controls (deprived of the NA or UCP antibody) were performed simultaneously in every immunostaining experiment. The antibody concentration used was 1:1000 for both NA (AB120, EMD Millipore, Billerica, MA, USA) and UCP-3 (U7757, reacted with an extract of rat BAT mitochondria; Sigma-Aldrich, St Louis, MO, USA).

Mitochondrial assays

Mitochondria were extracted from rope, gill and muscle tissues using a mitochondria isolation kit following the manufacturer’s instructions (BioChain Institute, Newark, CA, USA). Briefly, 100 mg of rope, muscle and gill tissues from six mature males were dissected out and washed twice with 10 ml ice-cold 0.1 mol l⁻¹ phosphate buffered saline (PBS; pH 7.4). Tissues were minced with small scissors in PBS on ice, and 1 ml 1× mitochondria isolation buffer was added. Tissues were then homogenized on ice for 20 s and held on ice for 5 s. Homogenization steps were repeated twice, and homogenates were centrifuged at 600 g at 4°C for 4 min. The supernatant was carefully transferred to a new tube and centrifuged at 12,000 g at 4°C for 15 min. The pellet was resuspended in 500 μl 1× mitochondria isolation buffer and serial centrifugation steps were repeated once. The pellet was resuspended in 100 μl isolation buffer or lysis buffer with 1× protease inhibitors. Protein concentration was measured using a microplate BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Cytochrome c oxidase assays were performed using a mitochondria activity assay kit following the manufacturer’s instructions (BioChain). Briefly, a colorimetric assay was used to measure mitochondria-specific cytochrome c oxidase activity in soluble or membrane-bound mitochondria samples as indicated by the oxidation of ferrocytochrome c to ferricytochrome c (absorbance at 550 and 565 nm, respectively). In this assay, cytochrome c was reduced with dithiothreitol and reoxidized by the active cytochrome c oxidase in the samples. The cytochrome c oxidase reaction is a first-order rate reaction with respect to the cytochrome concentration, showing an exponential decay over time. Therefore, we measured the initial fast reaction rate during the first 45 s of reaction using a kinetic program (5 s delay, 10 s interval, five readings: A₅₅, A₁₅₅, A₂₅₅, A₃₅₅ and A₄₅₅) in a spectrophotometer. A positive control (from the kit) was measured simultaneously. The integrity of the outer membrane was assessed by measuring cytochrome c oxidase activity in mitochondria membrane in the presence and absence of the detergent n-dodecyl-β-D-maltoside (from the kit). No damage of the mitochondrial outer membrane was found during our mitochondria preparation procedure.

ATP assays were performed using an ApoSENSOR ATP cell viability assay kit following the manufacturer’s instructions.
samples were then reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen) and random primers (Promega). The reactions [2 μl (5 ng μl⁻¹) cDNA + 8 μl TaqMan Universal PCR mastermix with 900 nmol l⁻¹ primers (each) and 250 nmol l⁻¹ TaqMan RGB probes] were analyzed on an ABI 7900HT real-time PCR thermal cycler with the manufacturer’s default setting (95°C for 10 min, 40 cycles of denature step: 95°C, 15 s, and anneal/extend step: 60°C, 1 min; MSU Genomics Technology Support Facility, East Lansing, MI, USA). Synthetic oligos were used as standards and run on the same 384-well plate with the samples. Standards were PCR-amplified using the primers for RT-qPCR, purified with the MinElute PCR purification kit (Qiagen, Valencia, CA, USA, and serially diluted (10-fold) into 10ⁱ⁰ to 10⁵ molecules per 2 μl solution. The sequences for standards, primers and TaqMan MGB probe for each mRNA are listed in supplementary material Appendix S1.

Phylogenetic tree analyses
The evolutionary history of the UCP proteins was inferred using the minimum evolution (ME) method. The optimal tree with the sum of branch length=5.46535823 is shown. The evolutionary distances were computed using the Jones–Taylor–Thornton (JTT) matrix-based method and are in the units of the number of amino acid substitutions per site. The ME tree was searched using the close-neighbor-interchange algorithm at a search level of 1. The neighbor-joining algorithm was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the data set (complete deletion option). There were a total of 268 positions in the final data set. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007).

Real-time quantitative PCR
Real-time quantitative PCR (RT-qPCR) was performed using the TaqMan MGB system (Life Technologies, Carlsbad, CA, USA) as described previously (Chung-Davidson et al., 2008). Briefly, total RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), treated with the TURBO DNA-free kit (Applied Biosystems, Foster City, CA, USA) and diluted to 100 ng μl⁻¹. RNA samples were then reverse-transcribed into cDNA using M-MLV reverse transcriptase (Invitrogen) and random primers (Promega). The reactions [2 μl (5 ng μl⁻¹) cDNA + 8 μl TaqMan Universal PCR mastermix with 900 nmol l⁻¹ primers (each) and 250 nmol l⁻¹ TaqMan RGB probes] were analyzed on an ABI 7900HT real-time PCR thermal cycler with the manufacturer’s default setting (95°C for 10 min, 40 cycles of denature step: 95°C, 15 s, and anneal/extend step: 60°C, 1 min; MSU Genomics Technology Support Facility, East Lansing, MI, USA). Synthetic oligos were used as standards and run on the same 384-well plate with the samples. Standards were PCR-amplified using the primers for RT-qPCR, purified with the MinElute PCR purification kit (Qiagen, Valencia, CA, USA, and serially diluted (10-fold) into 10ⁱ⁰ to 10⁵ molecules per 2 μl solution. The sequences for standards, primers and TaqMan MGB probe for each mRNA are listed in supplementary material Appendix S1.

Temperature measurement and behavioral recording
Mature male sea lamprey were anesthetized with 0.02% MS222 (Sigma-Aldrich), wrapped with a wet cloth around the gill openings, and temporarily removed from the water for surgery. Small 5 mm incisions were cut at the center of the rope tissue and the muscle tissue on the side of the body. Miniature waterproof temperature probes (diameter 3.175 mm, ON-402-pp; Omega Engineering, Stamford, CT, USA) were sutured into the rope and muscle tissues and sealed with VetBond (3M, St Paul, MN, USA). Animals were immediately placed in a behavioral observation tank (122×122×89 cm, length × width × height) after surgery. After the lamprey recovered from the anesthesia and the rope and muscle temperatures returned from the ambient air temperature to the water temperature, individual mature females or males were placed in the same tank to observe their behavioral and temperature changes for 10 min. Temperature

![Fig. 2. Cellular morphology of the dorsal ridge of juvenile and adult sea lamprey (hematoxylin and eosin stain). The dorsal ridge in parasitic juveniles contains only white adipocytes (W); in sexually immature adults, both white adipocytes and interstitial fibroblast-like cells (arrows) are present. In mature adults, these interstitial fibroblast-like cells replace white adipose tissue in the dorsal ridge, and display robust sexual dimorphism. Note that the dorsal ridge of mature males contains large adipocytes and interstitial fibroblast-like cells, whereas the dorsal ridge of mature females contains only small interstitial fibroblast-like cells and many collagen fibers (C). F, female; M, male; Mu, muscle. Scale bars, 50 μm.](image-url)
readings were simultaneously recorded from the water, rope and muscle tissues using three handheld thermistor thermometers (ultra-high-accuracy: ±0.015°C, resolution: 0.01°C, range: −20 to ~130°C, HH41, Omega Engineering). Lamprey behaviors were recorded using a Sony HDR-CX550V video camera (New York, NY, USA) enclosed in an Ikelite custom underwater housing equipped with an Ikelite WP80 wide-angle lens (Indianapolis, IN, USA). The VetBond seal remained intact for a few hours. Once water leaked into the tissue (as determined by a temperature drop to the same level as water temperature), behavioral and temperature recordings were paused or stopped. In some cases, the behavioral and temperature recordings were resumed after reapplication of VetBond.

Fig. 3. Cellular morphology and the ultrastructure of the multivacuolar adipocytes in the rope tissue of mature male sea lamprey. (A) Toluene-Blue-stained adipocytes with multiple oil droplets (indicated by a black arrow) in the rope tissue (40× micrograph). (B) Toluene-Blue-stained adipocytes with multiple oil droplets (indicated by a black arrow) in the rope tissue (100× micrograph). (C) Toluene-Blue-stained adipocytes with multiple oil droplets (indicated by a black arrow) in the rope tissue (400× micrograph). (D) Transmission electron micrograph of the multivacuolar adipocyte in the rope tissue. Scale bar, 50 μm.

Fig. 4. The ultrastructure of the collagen fiber (A) and interstitial fibroblast-like cell (B–D) in the rope tissue. (A) Transmission electron micrograph of the collagen fibers in the rope tissue. Scale bar, 200 nm. (B) Transmission electron micrograph showing an interstitial fibroblast-like cell packed with mitochondria (arrow) with dense cristae and small lipid droplets in the rope tissue. Scale bar, 2 μm. (C) Transmission electron micrograph of the same mitochondria (arrow) shown in B at higher magnification. Scale bar, 1 μm. (D) Transmission electron micrograph showing UCP-immunogold (10 nm, indicated by arrows) in a mitochondrion of an interstitial fibroblast-like cell of the rope tissue. Scale bar, 200 nm.
Transmission electron microscopy
Fixed tissues were processed for transmission electron microscopy (TEM) in the Center for Advanced Microscopy at MSU. Ultrathin sections were blocked with normal goat serum (Vector, Burlingame, CA, USA) at room temperature for 1 h, incubated in a mixture of antibody for UCP-3 (1:1000, reacted with an extract of rat BAT mitochondria; Sigma-Aldrich) and normal goat serum (Vector) at room temperature for 1 h. Ultrathin sections were then incubated in goat-anti-rabbit IgG-10 nm gold (1:100, Sigma-Aldrich) for 1 h at room temperature. Transmission electron micrographs were taken with a JEOL100CXII (JEOL USA, Peabody, MA, USA).

RESULTS
Sexual dimorphism in the dorsal ridge of mature sea lamprey
We first examined the morphology and development of the dorsal ridge in sea lamprey at various developmental stages (Fig. 2; supplementary material Fig. S1). The dorsal ridge in parasitic juveniles contains only white adipose tissue (WAT). A structure containing interstitial fibroblast-like cells developed within the WAT in sexually immature migratory adults. The rope tissue of mature males contains large interstitial fibroblast-like cells and huge adipocytes with multiple oil droplets (Figs 2, 3), whereas the dorsal ridge of mature females contains only small interstitial fibroblast-like cells and many collagen fibers (Fig. 2, Fig. 4A). Therefore, the dorsal ridge in mature adults exhibits robust sexual dimorphism.

Characteristics of the rope tissue in mature male sea lamprey
To seek an explanation for the function of these male-specific interstitial cells, we investigated their ultrastructure using TEM and immunogold staining. The interstitial fibroblast-like cells in the rope tissue were packed with small oil droplets and mitochondria with highly organized cristae that spanned the entire width of the mitochondria (Fig. 4B–D). The inner membrane of the rope mitochondria (Fig. 4D) contained immunoreactivities for UCPs, which could be induced by androstenedione (Fig. 5), a sea lamprey androgen (Bryan et al., 2007). Several unmyelinated nerve bundles were also present in the rope tissue (supplementary material Fig. S2). However, enriched vasculature, a characteristic of mammalian BAT (Cannon and Nedergaard, 2004), was not observed in sea lamprey rope tissue. Enriched vasculature transfers heat away from mammalian BAT to maintain whole-body homeostasis (Cannon and Nedergaard, 2004). Because the sea lamprey is ectothermic, such vasculature is not necessary for thermoregulation of the whole body and the generated heat could be confined in the rope tissue.

We then measured the fatty acid composition of the rope tissues (Fig. 6) and found fatty acids present similar to those in mammalian BAT (Ohno et al., 2001). Interestingly, the relative abundance of docosahexaenate (C22:6), which correlates with the oxygen consumption, thermogenic activity and proliferation of BAT (12), was three times higher in the rope tissue than in mammalian BAT. Sea lamprey rope tissue also contained pentadecanoate (C15:0), hexadecadienoate (C16:2), margarate (C17:0), heptadecenoate (C17:1) and arachidate (C20:0), which had not been observed in mammalian BAT (Ohno et al., 2001).

Sea lamprey rope tissue is thermogenic
Because the rope tissue showed some morphological and biochemical hallmarks of BAT, we suspected that the rope tissue might be thermogenic. In mammalian BAT mitochondria, uncoupling protein 1 (UCP-1) is known to uncouple ATP production and dissipate proton-motive force as heat (Cannon and Nedergaard,
2004). To determine whether ATP production is low in the UCP-laden rope tissue mitochondria, we measured the cytochrome c oxidase activity and ATP concentration in the mitochondria from rope, gill and muscle tissues. Cytochrome c oxidase is the last enzyme in the electron transport chain and an indicator of respiratory activity. Gill and muscle were chosen for their enriched mitochondria and high levels of respiratory activities. The specific activity of cytochrome c oxidase in rope tissue mitochondria was 5.7- and 3.0-fold higher than in gill and muscle tissue, respectively [ANOVA, \( P < 0.01 \); Fisher’s protected least significant difference (PLSD) post hoc tests: rope versus gill, \( P < 0.005 \); rope versus muscle, \( P < 0.05 \); Fig. 7A]. In contrast, ATP concentration was much higher in the gill and muscle but minimal in the rope tissue mitochondria (one-way ANOVA for repeated measures, \( P < 0.01 \); Fisher’s PLSD post hoc tests: rope versus gill, \( P < 0.0005 \); rope versus muscle, \( P < 0.05 \); Fig. 7B). These results suggest that oxidative phosphorylation may be uncoupled from ATP production in the rope tissue. However, the actual mechanisms remain to be confirmed.

To test directly the thermogenic capacity of the rope tissue, we measured the temperature of the rope and muscle tissues of mature males (\( N = 12 \)) in the presence of conspecific individuals (mature female versus mature male). The water temperature was recorded simultaneously as a reference. Muscle tissues were chosen as a control because of their thermogenic ability. We estimated the heat generation using free convection as the primary heat transfer method (Lepría et al., 2003) and modeled the rope and muscle tissues as a long cylinder with uniform volumetric heat generation (supplementary material AppendixS2). Fig. 8 shows the recordings from a mature male responsive to different partners and a mature male non-responsive to conspecifics examined. Table 1 presents the average measures of the maximum, minimum and mean temperature difference and the heat production over 10 min, from 12 mature males exposed to different partners. The rope tissue generated 0.027±0.013 W cm\(^{-3} \) more heat than the muscle (paired \( t \)-test, \( P < 0.05 \)) in 10 min. The specific heat production of the rope tissue was higher than muscle in every situation (Table 1; supplementary material AppendixS2). Interestingly, mature males generated more heat in the rope and muscle tissues when they encountered a mature female than a mature male (paired \( t \)-test, \( P < 0.05 \)). Individual males also showed variation in the amount of heat generated in the rope and muscle tissues in response to different mature females. The specific thermogenicity per unit weight by sea lamprey rope tissue is comparable to the energy expenditure per unit weight of BAT, estimated as the metabolic difference between UCP-1 knockout and wild-type mice (McDaneld et al., 2002). The most
dramatic observation in this study was that the rope tissue temperature rose by up to 0.3°C immediately after sexual encounter (Fig. 8).

The rope tissue is under adrenergic neural control
Based on the instant heat generation by the rope tissue in response to potential mates, we reasoned that the rope tissue is under adrenergic neural control, as in mammalian BAT (Cannon and Nedergaard, 2004). We examined whether sea lamprey rope tissue contained NA. As expected, although the dorsal ridge of immature adults contained NA-immunoreactive fibers in both sexes, only mature males showed NA immunoreactivity in the rope tissue (Fig. 9). The dorsal ridge of mature females was not NA-
A thermogenic secondary sexual character

immunoreactive (Fig. 9). Our results are consistent with reports that NA fibers innervate BAT, forming a dense network within the tissue that is in contact with each brown adipocyte (Cannon and Nedergaard, 2004). The sexual dimorphism in NA immunoreactivities could also be a developmental factor responsible for the dimorphic morphology, as NA is a recruiting signal of brown adipogenesis in mammals (Cannon and Nedergaard, 2004; Farmer, 2008; Gesta et al., 2007).

Transcriptional control of rope tissue adipogenesis

To further elucidate the male-specific adipogenesis in the dorsal ridge, we examined the expression of several developmental markers, PR domain containing 16 (PRDM16), peroxisome proliferator-activated receptor γ (PPARγ), peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), CCAAT-enhancer-binding protein (C/EBP), necdin and UCP in rope, gill and muscle tissues (Fig. 10). PRDM16 expression is essential for progenitor cells to

Fig. 9. Sexual dimorphism of noradrenaline (NA) immunoreactivity in sea lamprey dorsal ridge. NA immunohistochemistry (brown stain) was performed on the tissues from the dorsal ridge of lamprey in various life stages. Sexual dimorphism of NA immunoreactivity was observed in the dorsal ridge of mature adults. Sections were counterstained with hematoxylin (blue/purple nuclear stain). B, thermogenic adipose tissue; E, epidermis; F, female; M, male; W, white adipose tissue. Scale bars, 50 μm.

Fig. 10. Real-time quantitative PCR analyses of various genes in the rope tissue. (A) PRDM16, a brown adipose tissue (BAT) marker, is highly expressed in the rope tissue compared with gill and muscle. (B) PPARγ, a transcription factor, is highly expressed in muscle tissue compared with the gill and rope tissues. (C) PGC-1α, a downstream transcription factor of PPARγ, is not differentially expressed in the rope, gill or muscle tissues. (D) C/EBP, a downstream transcription factor of PGC-1α, is highly expressed in gill and muscle compared with rope tissue. (E) UCP is highly expressed in gill and muscle compared with rope tissue. (F) Necdin, an inhibitory factor for BAT development, is only expressed in mature males (SM) but not in immature males (PSM). Data represent means ± s.e.m. Sample size (N) is shown in the graphs. *Significant difference (P<0.05) from other groups.
Phylogenetic and genomic analyses of sea lamprey uncoupling protein

Our extensive search in the sea lamprey draft genomes 2.0 (Libants et al., 2009) and 7.0 (Smith et al., 2013) resulted in two homologs of UCP-2. This is consistent with the notion that UCP-2 may be the prototype of UCPs (Borecký et al., 2001; Emre et al., 2007; Jastroch et al., 2005). The sea lamprey UCP protein sequence contains segments similar to UCP energy transfer protein signatures from various species (Fig. 11). Most UCPs contain three ‘energy transfer protein signatures’ {P-x-[DE]-x-[LIVAT]-[RK]-X-[LRH]-[LIVMFY]} (Borecký et al., 2001). The first signature of sea lamprey UCP is similar to UCP1-3 (PLDTAKVRL) combined with [LIVMFY] (Borecký et al., 2001). The second signature of sea lamprey UCP is similar to UCP2 (Borecký et al., 2001), followed by two GΦG (Φ=hydrophobic amino acid residue) in all UCPs (Borecký et al., 2001; Emre et al., 2007; Jastroch et al., 2005). The sea lamprey UCP protein sequence contains segments similar to UCP energy transfer protein signatures from various species (Fig. 11). Most UCPs contain three ‘energy transfer protein signatures’ {P-x-[DE]-x-[LIVAT]-[RK]-X-[LRH]-[LIVMFY]} (Borecký et al., 2001). The first signature of sea lamprey UCP is similar to UCP1-3 (PLDTAKVRL) combined with [LIVMFY] (Borecký et al., 2001). The second signature of sea lamprey UCP is similar to UCP2 (Borecký et al., 2001), followed by two GΦG (Φ=hydrophobic amino acid residue) in all UCPs (Borecký et al., 2001). The second signature of sea lamprey UCP (PVDVVKTRYMNS) is similar to the second signature of UCP2 (Borecký et al., 2001), followed by two GΦG tripeptides. The third signature of sea lamprey UCP (PVDVVKTRYMNS) is similar to the third signature of UCP2/3 assuming the brown adipocyte lineage in mammals (Farmer, 2008; Hansen and Kristiansen, 2006; Seale et al., 2007). PPARγ, PGC-1α and C/EBP are downstream transcription factors that induce UCP expression in mammalian BAT (Seale et al., 2007). Necdin serves as an inhibitor in the process of mammalian brown adipogenesis (Seale et al., 2007). We searched for these markers in the sea lamprey draft genome 2.0 (Libants et al., 2009) and identified the full gene sequences and predicted the mRNA and protein sequences of these markers (supplementary material Appendix S1). RT-qPCR showed that PRDM16 was expressed at higher levels in rope tissue than in gill tissue (ANOVA, P<0.0005; Fisher’s LSD post hoc test: rope vs. gill, P<0.005; rope vs. muscle, P<0.0005; Fig. 10A), suggesting that it may be driving adipogenesis in sea lamprey rope tissue. PPARγ, PGC-1, C/EBP and UCP were all expressed in gill, muscle and rope tissues (Fig. 10B–E). These transcription factors and UCP-1 homologs are also found in other tissue than BAT in mammals (Borecký et al., 2001; Emre et al., 2007; Jastroch et al., 2005). Interestingly, necdin was expressed in mature but not in immature male sea lamprey (ANOVA, P<0.05; Fisher’s post hoc test: mature vs. immature males, P<0.05; Fig. 10F). This result indicates that adipogenesis may be inhibited in animals with fully developed rope tissue (mature males) but not in the immature dorsal ridge with adipogenesis in progress (immature males).
(Borecký et al., 2001). The conserved gene synteny for Ucp (supplementary material Fig. S3) was partially observed in the sea lamprey draft genome 2.0 (Libants et al., 2009) and confirmed in draft genome 7.0 (Smith et al., 2013). In addition, a steroid 5α-reductase gene is inserted between the Elmod2 and Ucp2 genes (supplementary material Fig. S3), which has not been reported in other species (Borecký et al., 2001; Emre et al., 2007; Jastroch et al., 2005). It would be interesting to look into the thermogenic activities of all UCP members because only UCP-1 has been shown to be thermogenic in mammals under physiological conditions (Cannon and Nedergaard, 2004). Further analyses of sea lamprey UCP may also shed light on UCP protein evolution.

Transcriptomic analyses of sea lamprey rope tissue

The presence of the aforementioned biomarkers in the rope tissue prompted us to determine whether the rope tissue transcriptome shows characteristics expected of a thermogenic fat. We sequenced and compared the transcriptomes of rope, gill and muscle tissues of mature male sea lamprey (Fig. 12). From the gill tissue, there were 24,646,140 sequence reads (75 mers), and 58.7% of them passed the quality filter (14,478,560 reads). From the rope tissue, 24,266,464 reads were sequenced and 57.5% of them passed the quality filter (13,958,294 reads). The muscle tissues produced 24,134,527 reads, and 61.4% of them passed the quality filter (14,813,224 reads). These sequences were assembled and aligned to a total of 2766 genes, and these genes were clustered into 1575 GO categories. The transcriptomes of the rope and muscle tissues had 589 genes showing differential expression (1.414× difference) and clustered into 21 GO categories, most of which are related to electron transport chain and energy production (Fig. 7B). The transcriptomes of the rope and gill tissues had 943 genes showing differential expression and clustered into 121 GO categories (Fig. 12A). As expected, the genes involved in fat cell differentiation were upregulated [Fisher’s exact test, rope versus gill, \( P=0.01 \), false discovery rate (FDR)=0.09; Fig. 12] whereas those involved in oxidative phosphorylation (Fisher’s exact test, rope versus muscle, fisher exact test, \( P=9.4 \times 10^{-7} \), FDR=0), electron transport from NADH to ubiquinone (respiratory complex I; Fisher’s exact test, rope versus muscle, \( P=1.3 \times 10^{-5} \), FDR=0), and electron-transport-coupled ATP synthesis (Fisher’s exact test, rope versus muscle, \( P=8.3 \times 10^{-6} \), FDR=0) were downregulated in the rope tissue (Fig. 12).

**DISCUSSION**

We believe that the rope tissue found in mature male sea lamprey is a unique primordial thermogenic adipose tissue, but not BAT, based on the following reasons. (1) There is no UCP-1, a BAT marker, in the transcriptome of this adipose tissue. Instead, UCP-2, which is universal in various tissues, is present in this adipose tissue. (2) This adipose tissue contains a slightly different set of transcription factors involved in adipogenesis such as C/EBP1/2, GRIP1, RBL1, PRDM16, PPARγ and PGC-1α. Specifically, it does not contain Myf5, which dictates the cell lineage of brown adipocytes and muscles. Other factors such as BMP1, BMP3 and UCP-2 are not found in mammalian BAT. (3) The rope tissue appears only in mature male sea lamprey but not in other lamprey species. Therefore, it seems to be an isolated incidence during evolution. (4) The developmental process of the rope tissue is associated with increased NA and the male sex steroid androstenedione. (5) The rope tissue does not contain enriched blood vessels. The minute amount of heat generated is not likely to transmit through the aquatic environment or circulate throughout the body. It more likely acts via body contact and serves important functions in sea lamprey reproduction and species identity, but not in cold-induced nonshivering thermogenesis as in mammalian BAT.

One interesting feature of sea lamprey rope tissue is the bias toward downregulated respiratory complex I as shown in the transcriptome. Electrons generated from various metabolic pathways enter the electron transport system or branched electron transport chain at four separate sites that converge in the reduction of coenzyme Q (Efremov et al., 2010). Metabolism of different substrates results in electron donation to specific complexes or sites (Efremov et al., 2010). Oxidation of glutamate, malate and pyruvate provides NADH for electron entry at complex I. β-oxidation of fatty acyl-CoAs generates electrons for entry at complex I or complex II by way of acetyl-CoA metabolism through the tricarboxylic acid cycle (Efremov et al., 2010). This bias in sea lamprey rope tissue may reflect an evolved strategy of energy expenditure at a life stage when the animal has been fasting for quite some time and therefore has a tight budget for carbohydrate metabolism through the

**Fig. 12.** Heat maps showing different gene expression patterns (color scale represents the fold change) using gene ontology (GO) analyses to compare the transcriptomes of the rope versus gill (A; \( P=0.05 \), false discovery rate \( \leq 0.1 \)) or muscle tissues (B; \( P=0.05 \), false discovery rate \( \leq 0.1 \)) in mature male sea lamprey. Transcriptomes were obtained using Illumina mRNA-Seq sequencing technology. x-axis represents the GO categories and y-axis represents gene clusters (see supplementary material Appendix S1). Color scale represents the \( \log_2(\text{transcript number in rope/transcript number in gill or muscle}) \). Note that the figure only shows genes with at least 1.414× differential expression level \( [\log_2(1.414)=0.5] \).
respiratory complex I (the main entrance for carbohydrate). However, fatty acid in the rope tissue can still enter the electron transport chain through respiratory complex II.

It is curious that male sea lamprey, an ectothermic animal, develop a thermogenic secondary sexual character at the final stage of their life span. Many male vertebrates invest a large amount of energy in sexual advertisement, especially in various forms of exaggerated secondary sexual characters (Clutton-Brock, 2007; Ptacek, 2000). As in other male sexual characters, androgen treatment increases the rope size in adult male sea lamprey (Bryan et al., 2007). The rope may simply be an ornamental display, albeit very energy consuming. However, the instant heat produced in the rope by the presence of a sexually mature female and the contact between the rope and female urogenital pore suggest a more active role of the rope tissue. Sea lamprey is the only lamprey species with the rope tissue (Kott et al., 1988), and the only known species outside of the mammalian clade to possess a thermogenic fat. The origin of the rope tissue (Kott et al., 1988), and the only known species outside of the mammalian clade to possess a thermogenic fat. The origin of the rope tissue may have been driven by sexual selection, which would lead to reproductive isolation and speciation (Clutton-Brock, 2007; Ptacek, 2000). This differs from mammalian BAT, which appeared in parallel with homeothermy and nonshivering thermogenesis (Cannon and Nedergaard, 2004). These two thermogenic fats present an extraordinary example of convergent evolution, which resulted in two structures with similar thermogenicity.

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AUTHOR CONTRIBUTIONS

Y.-W.C.-D. and W.L. conceived the hypotheses, designed the study and prepared the manuscript. Y.-W.C.-D. performed all the experiments and data analyses. N.S.J. collected rope tissue samples for histology and electron microscopy. K.L. performed the GC-MS. C.-Y.H. helped with the mitochondrial cytochrome c oxidase and ATP assays, temperature measurements and behavioral recordings. C.O.B. helped with the temperature measurements and behavioral recordings. C.P. and J.C. provided assistance and the model for temperature measurements. K.G.N. and C.T.B. provided the computational assistance for GO analyses. M.B.B. performed the AD treatment on immature male sea lamprey. All authors discussed the results and commented on the manuscript.

COMPETING INTERESTS

No competing interests declared.

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REFERENCES


Fig. S1. General morphology of the dorsal ridge of juvenile and adult sea lamprey (H and E stain). The dorsal ridge in parasitic juveniles contains only white adipose tissue (W); however, a different structure (B) developed within the core of the white adipose tissue in sexually immature adults, captured during their upstream migration to the spawning ground. In mature adults, this structure replaces white adipose tissue in the dorsal ridge, and displays robust sexual dimorphism. B, brown adipose-like tissue; D, dermis; E, epidermis; F, female; M, male; Mu, muscle; W, white adipose tissue. Scale bars, 500 μm.

Fig. S2. Toluene-Blue-stained unmyelinated nerve bundles in the rope tissue.
Fig. S3. Gene synteny of Ucp genes in sea lamprey genome assembly 2.0 (Libants et al., 2009).
Appendix S1

Additional figure legend for Fig. 12

[missing text]
Sea lamprey rope tissue developmental markers for real-time quantitative PCR

For each marker, the following information is shown: marker name (bold) and NCBI accession number, synthetic oligo used as the standard for real-time quantitative PCR. Yellow blocks show the primer sequences (note: the reverse primer is complementary to the shown sequence). Blue block shows the sequence for TaqMan MGB probe.

**PRDM16**: PMZ\_0000429-RA

cgacgcgtcgttcaagatctggctcaacctgagcgcgaacgtgcgcacatccacaacaaggaag

**Necdin**: PMZ\_0014623-RA

cgacgcgtcgtcgttcaagatctggctcaacctgagcgcgaacgtgcgcacatccacaacaaggaag

**PPARY**: PMZ\_0011032-RA

tcacggagttccgaagtcggtatccggttacgtgctgccatcgaactaaccgctcgacagtttgcgtttgcgtgtcgtttgcgtttgcgtttgcgttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtttgcgtt
Heat Generation Calculations for Lamprey Rope Tissue

M. Cody Priess, Yu-Wen Chung-Davidson, Jongeun Choi

November 11, 2011
Heat Transfer Model

The analysis of heat generation in lamprey tissue begins with identification of a correct model. Since the anaesthetised lamprey lies reasonably still in water which is close to its body temperature, the primary method of heat transfer will be free convection. We make the following assumptions about the lamprey and experimental setup in order to make the analysis tractable:

1. The lamprey rope and body are assumed to be perfect cylinders with length much greater than their respective diameters.

2. The heat transfer between the rope tissue and body is assumed to be negligible.

3. Both the mechanical and heat-generating properties of the tissue in the rope and body are assumed to be homogeneous throughout their respective volumes.

We can then model the rope and muscle tissue as long cylinders with uniform volumetric heat generation values $Q_{\text{rope}}$ and $Q_{\text{muscle}}$ which are losing heat to the water via free convection. When measuring temperature in the rope tissue, the temperature $T_{\text{probe}}$ is assumed to be measured by the probe from the geometric center (radially) of the rope. When measuring temperature in the muscle tissue, the measured temperature $T_{\text{probe}}$ is assumed to be measured by the probe at a radial distance $r$ from the center of the body. A diagram illustrating this simplified model is shown in Figure 1.

With an approximate knowledge of the water conditions surrounding the lamprey, we can generate a mean convective coefficient $\bar{h}$ which we assume is present over the entire rope surface. This allows us to formulate the heat generation in the rope tissue as a function of the water temperature and the rope temperature. Once the average heat convection coefficient has been determined, we can use the standard temperature equations for a cylindrical body with uniform heat generation and a convective boundary condition [1] in order to relate the probe temperature to the heat generation $Q_{\text{rope}}$.

The mechanical properties of water at $14^\circ C$ are listed in Table 1. The thermal conductivity of lamprey tissue $k_f$ is estimated from data in [2] to be

$$k_f \approx 0.5 \text{ W/m} \ast \text{K}$$

Heat Transfer Analysis

The average convective heat transfer coefficient $\bar{h}$ for a long horizontal cylinder in free convection can be found via the relationship

$$\bar{h} = \frac{k_w}{D} \hat{N}_u$$  \hspace{1cm} (1)
Figure 1: The simplified heat model of the lamprey muscle and rope tissue

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Units</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\rho$</td>
<td>1000</td>
<td>$kg/m^3$</td>
</tr>
<tr>
<td>$\nu$</td>
<td>$1.2 \times 10^{-6}$</td>
<td>$m^2/s$</td>
</tr>
<tr>
<td>$c_p$</td>
<td>$4.086 \times 10^3$</td>
<td>$J/kg \times K$</td>
</tr>
<tr>
<td>$k_w$</td>
<td>0.592</td>
<td>$W/m \times K$</td>
</tr>
<tr>
<td>$Pr$</td>
<td>8.28</td>
<td></td>
</tr>
<tr>
<td>$\alpha$</td>
<td>$1.449 \times 10^{-7}$</td>
<td>$m^2/s$</td>
</tr>
<tr>
<td>$\beta$</td>
<td>$114.1 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>$g$</td>
<td>9.81</td>
<td>$m/s^2$</td>
</tr>
</tbody>
</table>

Table 1: Physical Properties of water at $\approx 14^\circ C$
where \( D \) is the diameter of the cylinder and \( \bar{N}_{uD} \) is the average Nusselt number over the cylinder. There are several methods for finding \( \bar{N}_{uD} \), but one which is applicable over a wide range of Rayleigh numbers [1] is

\[
\bar{N}_{uD} = \left\{ \frac{0.60 + \frac{0.387R_{aD}^{1/6}}{[1 + (0.559/Pr)^{9/16}]^{8/27}}} {2} \right\}^2,
\]

(2)

where the Rayleigh number \( R_{aD} \) is

\[
R_{aD} = \frac{g\beta(T_s - T_\infty)D^3}{\nu \alpha},
\]

(3)

and \( T_s \) and \( T_\infty \) are the temperatures at the surface of the cylinder and the temperature of the water, respectively.

**Heat Generation in Rope Tissue**

The volumetric heat generation \( Q \) of a solid cylinder with convective boundary conditions and internal thermal conductivity \( k_f \) with temperature \( T_{probe} \) measured at its radial center can be found by solving

\[
T_{probe} = \frac{Qr_0^2}{4k_f} + T_s,
\]

(4)

where \( r_0 \) is the radius of the cylinder. Since there is no direct measurement available of the surface temperature \( T_s \), it must be estimated by applying a surface energy balance to the outside of the cylinder, such that

\[
\frac{Qr_0}{2} = \bar{h}(T_s - T_\infty).
\]

(5)

Given (1)-(3), there is no analytical solution to the system of equations defined in (4) and (5), so it must be solved numerically. This was done for each measured temperature value \( (T_{probe} - T_\infty) \) by using the nonlinear function solver \textit{fsolve} in MATLAB.

**Heat Generation in Muscle Tissue**

It was desired to compare the heat generation in lamprey muscle tissue to that of the rope tissue in order to determine if the heat generated was simply a function of normal metabolic processes. The heat generation in lamprey muscle tissue was analyzed using a similar procedure to that used for the rope tissue. We again use the relations governing convective heat transfer from a long cylinder, but we no longer assume that the temperature probe is being implanted at the center of the cylinder. We re-write (4) as

\[
T_{probe} = \frac{Qr_0^2}{4k_f} \left( 1 - \frac{r^2}{r_0^2} \right) + T_s,
\]

(6)

where \( r \) is the distance from the center of the body to the location of probe implantation (measured radially), and \( r_0 \) is the diameter of the lamprey body. From here the analysis proceeds identically to that for the rope tissue.
<table>
<thead>
<tr>
<th>Lamprey</th>
<th>Rope Mean $W/cm^3$</th>
<th>Std. Deviation ($W/cm^3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$2.27 \times 10^{-3}$</td>
<td>$6.71 \times 10^{-4}$</td>
</tr>
<tr>
<td>2</td>
<td>$7.27 \times 10^{-3}$</td>
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<td>3</td>
<td>$4.17 \times 10^{-4}$</td>
<td>$3.81 \times 10^{-4}$</td>
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<tr>
<td>4</td>
<td>$1.40 \times 10^{-3}$</td>
<td>$5.84 \times 10^{-4}$</td>
</tr>
<tr>
<td>5</td>
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<td>$5.55 \times 10^{-4}$</td>
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<td>$3.09 \times 10^{-4}$</td>
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<tr>
<td>12</td>
<td>$1.63 \times 10^{-3}$</td>
<td>$4.18 \times 10^{-4}$</td>
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</table>

Table 2: Mean and standard deviation of $Q_{rope}$ in the 12 tested lamprey

<table>
<thead>
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<th>Std. Deviation ($W/cm^3$)</th>
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<td>1</td>
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<td>$6.32 \times 10^{-5}$</td>
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<tr>
<td>2</td>
<td>$2.39 \times 10^{-4}$</td>
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<td>3</td>
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<td>$3.72 \times 10^{-5}$</td>
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Table 3: Mean and standard deviation of $Q_{muscle}$ in the 12 tested lamprey

Results

Results of the analysis are shown in Figures 2-13. Values used were $D_{rope} = 7.8 \ mm$, $D_{body} = 40 \ mm$, and $r_{probe} = 16.2 \ mm$. The mean and standard deviation of the heat generation in the rope and muscle tissue are shown in Tables 2 and 3, and the ratio of the average heat generation values $Q_{rope,avg}/Q_{muscle,avg}$ for each lamprey is shown in Table 4.
Table 4: Ratio of the average heat generation $Q_{\text{rope,avg}}/Q_{\text{muscle,avg}}$ for the 12 tested lamprey

<table>
<thead>
<tr>
<th>Lamprey</th>
<th>Ratio $Q_{\text{rope}}/Q_{\text{muscle}}$</th>
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<td>19.7</td>
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Figure 2: Heat generation in Lamprey 1
Figure 3: Heat generation in Lamprey 2
Figure 4: Heat generation in Lamprey 3
Figure 5: Heat generation in Lamprey 4
Figure 6: Heat generation in Lamprey 5
Figure 7: Heat generation in Lamprey 6
Figure 8: Heat generation in Lamprey 7
Figure 9: Heat generation in Lamprey 8
Figure 10: Heat generation in Lamprey 9
Figure 11: Heat generation in Lamprey 10
Figure 12: Heat generation in Lamprey 11
Figure 13: Heat generation in Lamprey 12
Bibliography
