

# THERMOGENESIS IN MUSCLE

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## INTRODUCTION

Skeletal muscles are most often examined at the cellular level in relationship to their primary role in force generation. Throughout the animal kingdom, regardless of phylogeny, muscle generates heat. Exercise, shivering, and nonshivering thermogenesis provide excess heat in muscle that affords adaptive significance to a wide variety of organisms. Although there is reasonable concordance on the mechanisms involved in muscle as a force-generating cell, the physiological mechanisms for thermogenesis, biological significance, and evolutionary role of muscle as a heat-producing cell are not as clearly defined. Heat liberation resulting from contractile activity is well understood, actively studied, and has been carefully measured by muscle energeticists. Heat production during periods of nonshivering thermogenesis (NST) is not as well defined, and its existence is controversial in many species, despite compelling evidence for NST from fish, birds, and mammals. Investigators interested in the role of heat production in skeletal muscle primarily focus on the relationship between heat liberation during the contraction-relaxation cycle (1, 77, 90, 110, 128). Discussions of heat production independent of contractile activity usually focus on mammals where extensive reviews exist (44, 46, 47, 83, 86, 95). Despite major efforts to understand how muscle contributes to basal metabolic rate (BMR) and to cold-induced thermogenesis in endotherms, the mechanistic basis of such activity remains poorly defined. The fundamental mechanisms for heat generation without contractile activity have not been elucidated. This review examines skeletal muscle in light of its secondary role as a furnace and

examines the cellular basis for the role of skeletal muscle in heat production from several taxa. As with the mechanics of force generation, functional similarities in the use of muscle for thermogenesis should be apparent across phyla. The goal is to use a comparative approach to determine if common physiological and molecular pathways are utilized in muscular thermogenesis in the animal kingdom.

## THERMOGENESIS IN FISH MUSCLES

### *Thermogenic Organs Modified From Muscle*

Most fish are ectothermic with body temperatures within a degree or two of ambient water temperature, the result of respiration with a gill. Endothermy, the ability to elevate body temperature by internal heat generation occurs in one group of teleosts, the Scombroidei (20, 40). The two forms of endothermy, systemic and cranial, have linkages to oxidative phenotypes of skeletal muscle. Thus the few endothermic fish provide one of the best model systems to study the properties of skeletal muscle that are selected for heat generation.

Tunas (Scombridae: *Euthynnus*, *Auxis*, *Katsuwonus* and *Thunnus*) are systemic endotherms and raise their body temperature in a fashion similar to mammals: elevated metabolism is coupled with reduced whole body thermal conductance. Muscle, brain, and viscera temperatures in tuna are elevated above water temperature. Billfishes (Xiphiidae: *Xiphias*, and Istiophoridae: *Istiophorus*, *Makaira*, *Tetrapturus*) and a single species of mackerel from the family Scombridae, *Gasterochisma melampus*, have evolved the minimum required endothermic capacity; a brain heater that warms the central nervous system (17, 37). The thermogenic organs of billfishes and *Gasterochisma* provide a unique system for identifying the key components of muscle-based thermogenesis. In the early 1980's, Carey discovered that swordfish and other closely related species of billfishes had a specialized thermogenic organ derived from extraocular muscles beneath the brain (37). Swordfish range through daily vertical excursions in the water column and experience changes in temperature as much as 19°C in 2 hr (38, 39). During the daily fluctuations in temperature, the swordfish reduces the temperature change experienced by the brain and retina by warming these tissues with the heater organ. The evolution of cranial endothermy in fish has been linked with selection for thermal niche expansion (23).

Heater organs, modified from extraocular muscles, have been identified in ten species of fishes from three families (Xiphiidae, Istiophoridae, and Scombridae). Sequencing of the cytochrome b gene to establish phylogenetic

relationships (22, 23) indicates the billfishes share a common ancestor to the exclusion of other scombroids, which indicates that a thermogenic organ associated with the superior rectus eye muscle is a synapomorphy of these taxa. However, the heater organ is an independently acquired specialization of the butterfly mackerel, *Gasterochisma melampus*. *Gasterochisma* is more closely related to primitive mackerels and tunas, which indicates that the thermogenic cell type of *Gasterochisma* is not homologous with that of the billfishes. The heater organ of *Gasterochisma* is derived from the lateral rectus eye muscle that supports a separate evolutionary origin of the thermogenic cell type. The phylogenetic resolution of relationships among fishes with heater organs permits distinction between cellular differences that are the result of physiological adaptation from differences due to separate evolutionary histories. For example, cellular differences between the heater organs of the butterfly mackerel and billfishes can be attributed to each lineage having independently evolved the heater system. Because of the shared common ancestry in billfishes (Istiophoridae, Xiphiidae), variation in structure and function of heater organs can be attributed to adaptive changes rather than different origins of the heater phenotype. For example, the amount of muscle modified into heater cells varies between billfishes, yet the oxidative capacity of a gram of heater tissue is relatively similar between species (17, 157). Species having the largest thermal ecological range (swordfish) have a larger proportion of extraocular fibers expressing the thermogenic phenotype than fishes that are more tropical and warm temperate in their habitat (19). Morphometric data indicate that swordfish have two to three times as much heater tissue expressed as a blue marlin of similar body size (17, 19). This additional thermogenic capacity reflects the larger thermal needs of the swordfish over the blue marlin. Telemetry studies examining the ecologies of both species support the adaptive argument (23, 38, 39, 89). Blue marlin remain in the top 200 meters of the water column and range in temperature from 17–27°C. Swordfish range to depths greater than 600 meters, experience temperatures from 6–27°C, and encounter temperature changes of 19°C in daily dives.

### *Structure and Metabolism of Heater Cells*

The modified muscle cells of the fish heater organ are unique because the force-producing machinery of the cells, the contractile proteins, are few while the internal membranes that regulate  $\text{Ca}^{2+}$  ion movements in normal muscle cells are hypertrophied (17, 24). In normal muscle, intracellular  $\text{Ca}^{2+}$  is controlled by the T-tubule and sarcoplasmic reticulum (SR) membranes that trigger contraction when  $\text{Ca}^{2+}$  is released. Heater cells have a unique structure compared to all other muscle cells; foremost is the lack of organized myofibrillar elements. Monoclonal antibodies to skeletal isoforms

of actin and myosin demonstrate a loose disposition of the myofibrillar proteins in the heater cell cytoplasm. Gel electrophoresis confirms the presence of small amounts of myosin and actin in heater cells. The heater cell volume is primarily composed of mitochondria, SR, and T-tubules (17, 24). Stereological analysis of the mitochondrial fiber volume has been completed on five species of billfishes (20; B Block, unpublished results). Mean mitochondrial volumes (mt,f) range from 60–63% of the cell volume. Among four of five genera (*Xiphias*, *Makaira nigricans*, *Tetrapturus angustirostris*, *Istiophorus*) there are no statistically significant differences if the fish are compared from the same ocean basin (i.e. water temperatures influences structure and physiology). A rich network of smooth membranes, identified as the SR and T-tubule membranes by a variety of electron microscopy techniques, are situated between the mitochondria (24). The membrane systems are so extensive that free cytoplasmic space has been difficult to identify in electron micrographs. The SR is often found in pancake-like stacks, an unusual disposition of SR in normal muscle cells. Freeze-fracture has revealed a carpet of intramembrane particles on the cytoplasmic surface of the SR similar in size (18 nm) to the  $\text{Ca}^{2+}$  ATPase (24). The disposition of the SR in stacks throughout the cytoplasm most likely increases the surface area of SR and hence the available space for packing of the  $\text{Ca}^{2+}$  ATPase.

Certain skeletal muscles from other animals occasionally have a stereological profile similar to the heater cell phenotype of fish. Such muscles are commonly used for sound production and recruited for high frequency contractions over sustained periods of time (105, 145). Muscles with hypertrophied SR and high mitochondria volumes include a variety of sound-producing muscles that operate at high frequencies (90–550 Hz, e.g. toadfish swimbladder, rattlesnake tail rattle, cicada synchronous tymbal muscles). Electron microscopy and morphometry indicate the muscles have hypertrophied SR and mitochondria volumes. This structural make-up of the muscle cells used for sound production reflects selection for rapid  $\text{Ca}^{2+}$  release and reuptake, high levels of oxidative phosphorylation potential for generation of ATP, and comparatively low levels of force production (due to reduction of the myofibrillar volume). In the specialized muscles of toadfish, cicadas, and rattlesnakes, the increased SR volume is associated primarily with increased surface area for the  $\text{Ca}^{2+}$  ATPase, the rate-limiting enzyme of  $\text{Ca}^{2+}$  sequestration (4, 21). Similarly, the morphology of the heater cells, in particular the hypertrophy of the SR membrane, is a specialization for increasing the available surface area for the  $\text{Ca}^{2+}$  ATPase, the key protein in the proposed heat generation pathway of the thermogenic cells (21, 24).

Measurements of key metabolic enzyme activities indicate that heater

tissue has a high aerobic capacity (6, 157). Citrate synthase activities at saturating substrate concentrations are among the highest reported for vertebrate tissues (136–290 units per gram tissue at 25°C). High levels of hexokinase activity indicate that glucose is an important source of fuel for energy metabolism in heater tissue (6, 157). Low glycolytic capacities are indicated by the low levels of pyruvate kinase and lactate dehydrogenase. High rates for enzyme activities associated with fatty acid metabolism (3-hydroxyacyl-CoA dehydrogenase) and carnitine transport across the mitochondrial membrane (carnitine palmitoyltransferase) also demonstrate that free fatty acids (FFAs) play a key role in thermogenesis. These results, when combined with the mitochondrial respiration experiments, indicate a high capacity for substrate oxidation and ATP generation. Other indicators of above average oxidative capacity in fish thermogenic organs include measurements of myoglobin and cytochrome C. Myoglobin in blue marlin heater organs is threefold higher (377–411  $\mu\text{mol/kg}$  wet wt) than the muscles from terrestrial amniotes (21). Cytochrome C content in swordfish heater organs ( $35 \pm 3$  nmol/g) is similar to the range reported for mammalian brown fat (22–35 nmol/g), another thermogenic cell that approaches the physiological limits of mitochondrial packing (37).

The enzymatic studies and morphometric data on mitochondrial volume coincide on an important point, the similarity of the aerobic capacity of heater cells among billfishes (e.g. swordfish and blue marlin). Both studies indicate that a gram of heater tissue from either billfish taken from the same water mass (i.e. Pacific or from the Mediterranean) has the same heat-generating capacity. Interestingly, citrate synthase activities in heater organs were substantially higher in fishes that were captured from water masses with colder waters. For example, swordfish from the Mediterranean sea (sea surface temperature of 23°C and a mixed layer extending 50 meters), had heater cells with a higher oxidative capacity than swordfish from warm waters off Hawaii (sea surface temperatures 27°C, mixed layer 26°C for 100 meters). This result suggests that thermal ecology may regulate expression of the oxidative capacity of the heater phenotype. Thus to keep the brain and eyes warm over the larger thermal gradient experienced by a swordfish in comparison to blue marlin, there must be more of the heater phenotype expressed. This has been documented comparatively between the two species. Swordfish have a considerably higher number of muscle fibers expressing the heater phenotype than do blue marlin (17, 19). Biochemical data on *Gasterochisma*, a fish from the coldest thermal environments (50° S latitude 11–12° sea surface temperature), demonstrate a significantly higher aerobic capacity of the heater cell than comparable measurements from warm and cold temperate billfishes. The 63% packing of the heater cell volume with mitochondria and the high enzymatic activities of citrate synthase in billfish heater cells may not represent the limitation for either

mitochondria packing or aerobic enzymes. *Gasterochisma* has citrate synthase activities significantly higher than billfishes (290 vs 166 units of enzyme activity per gram at 25°C).

Metabolic studies (6, 21, 132, 157) of the properties of mitochondria isolated from heater tissue support the findings of the enzymatic profiles of aerobic metabolism, fatty acid oxidation, and carbohydrate metabolism. These studies have examined the capacity of isolated mitochondria for coupled respiration and substrate oxidation rates, and have assessed the maximal in vitro activities of key enzymes involved in aerobic metabolic pathways. Additionally, the role of  $\text{Ca}^{2+}$  ions for stimulating mitochondrial oxidative metabolism, as well as a possible role of  $\text{Ca}^{2+}$  ions for uncoupling respiration, have been examined. Three independent studies on isolated mitochondria from blue marlin, sailfish, and swordfish heater organs indicate that respiration is tightly coupled (6, 20, 132). Maximal respiration rates of isolated heater organ mitochondria at 20 and 25°C are the highest of any vertebrate tissue (Table 1). The highest respiratory control ratios in the presence of ADP were obtained in swordfish ( $9.8 \pm 3$ ,  $n=4$ ). The isolated mitochondrial respiration measurements have established that heater cells can oxidize a wide variety of fatty acid fuels including octanoyl and palmitoyl carnitine, as well as the carnitine esters of stearic, decanoic, and hexanoic acids. Oxidation rates are also high with carbohydrate substrates.

Free cytoplasmic  $\text{Ca}^{2+}$  ions are known to act as a second messenger significantly affecting mitochondrial metabolism (123, 124). The stimulatory role of  $\text{Ca}^{2+}$  on mitochondrial metabolism is important when considering the models for thermogenesis in the fish heater organs described below.

**Table 1** Mitochondrial oxygen consumption rates in muscle

Animal	Tissue	Substrate	State-3 respiration rate ( $\text{nmol}_2/\text{min}/\text{mg}$ protein)
Swordfish <sup>6</sup>	Heater	Glutamate	$167 \pm 48$ (4) <sup>b</sup>
Swordfish <sup>6</sup>	Heater	Palmitoyl carnitine	$144 \pm 50$ (4)
Blue Marlin <sup>20</sup>	Heater	Pyruvate + malate	$125 \pm 25$ (5)
Locust <sup>152</sup>	Muscle	Pyruvate	$598 \pm 35$ (7)
Locust <sup>152</sup>	Muscle	Palmitoyl carnitine	$316 \pm 8$ (7)
Hummingbird <sup>151</sup>	Muscle	Pyruvate + malate	$159 \pm 10$ (4)
Hummingbird <sup>151</sup>	Muscle	Palmitoyl-CoA + carnitine + malate	$138 \pm 16$ (4)
Tuna <sup>128</sup>	Red muscle	Palmitoyl carnitine	$104 \pm 29$ (9)
Carp <sup>5</sup>	Red muscle	Lauroyl carnitine	$84 \pm 12$ (6)

All fish mitochondrial respiration rates are reported at 25°C. Insect respiration rates are at 30°C. Hummingbird muscle mitochondria are at 40°C. <sup>a</sup>Reference. <sup>b</sup>Number of individual preparations examined.

These models are based on stimulation of heat generation by release of  $\text{Ca}^{2+}$  from the SR membranes surrounding the heater cell mitochondria. In isolated heart mitochondria, nanomolar  $\text{Ca}^{2+}$  results in significant stimulation of state-3 respiration primarily through activation of matrix dehydrogenases (57). Nanomolar changes in cytosolic concentration of  $\text{Ca}^{2+}$  have been shown to activate several key mitochondrial dehydrogenases, the respiratory chain enzymes, and fatty acid oxidation rates in vertebrate mitochondria. Three matrix enzymes, pyruvate,  $\text{NAD}^+$ -isocitrate and 2-oxoglutarate dehydrogenases can be activated by increases in extramitochondrial concentrations of  $\text{Ca}^{2+}$  within a physiological range for vertebrate mitochondria (0.05–2  $\mu\text{mol}$ ; 123). Thus  $\text{Ca}^{2+}$  release in the cytoplasm plays a key role in altering the NADH supply to the respiratory chain, which affects the rate of oxidative metabolism and ATP production (56, 124). These findings have led to the hypothesis that  $\text{Ca}^{2+}$  is a metabolic stimulator capable of enhancing the rate of ATP supply to match demand.

In swordfish heater cells, Ballentyne et al (6) investigated the effects of  $\text{Ca}^{2+}$  on mitochondrial metabolism. The oxidation of  $\alpha$ -glycerolphosphate by isolated swordfish mitochondria could be stimulated fourfold by the addition of 1 mM  $\text{CaCl}_2$ . This addition of  $\text{Ca}^{2+}$  is far from physiological, thus the results, while of interest, are difficult to interpret. Despite the ambiguity of these results, a possible linkage was hypothesized to exist (6) between  $\text{Ca}^{2+}$  stimulation of mitochondrial respiration and the SR futile  $\text{Ca}^{2+}$  cycling hypothesis of Block (18). A potent nonshivering thermogenic mechanism is apparent if  $\text{Ca}^{2+}$  released in the heater cell cytoplasm stimulates oxidative processes. O'Brien et al (132) further investigated this question of a stimulatory induced metabolic coupling or, conversely, an uncoupling effect of  $\text{Ca}^{2+}$ , on blue marlin heater mitochondria. As in other vertebrate tissues, isolated heater mitochondria have active mechanisms for rapid  $\text{Ca}^{2+}$  uptake and a sodium-induced egress pathway. In blue marlin, P/O ratios remain relatively insensitive to free  $\text{Ca}^{2+}$  concentrations in the physiological range (132). The results for this species indicate that uncoupling by  $\text{Ca}^{2+}$  is not a significant process and that the heater mitochondria maintain a high oxidative ATP output in its presence. Further studies should test the hypothesis that  $\text{Ca}^{2+}$  in the heater cell cytoplasm elicits a stimulatory effect on oxidative process that would promote heat generation by increasing flux through oxidative metabolic pathways.

### *Specialized Membranes of the Heater Cells*

The properties of the SR membrane system in the heater cell have been intensively studied using conventional techniques developed for isolation and characterization of mammalian SR (24, 25, 26, 125). The biochemical studies have established that the isolated SR of the billfish heater organs

contains the  $\text{Ca}^{2+}$  ATPase calsequestrin and the  $\text{Ca}^{2+}$  SR release channel. Relatively low amounts of the L-type  $\text{Ca}^{2+}$  channel (DHP receptor) have been identified using [ $^3\text{H}$ ] PN200 binding in T-tubule fractions of heater cells (106). Parvalbumin, a calcium-binding protein found in other SR enriched fish skeletal muscles, is not present in a high concentration in heater cells (K Rodnick, personal communication). While normal skeletal muscle can be biochemically separated into two distinct fractions (heavy and light SR, depending upon the constituent SR proteins) by sucrose gradient centrifugation, heater tissue SR cannot be separated into distinct fractions. As discussed below, this is because heater SR has a homogeneous distribution of SR proteins throughout the SR membrane (26). It is thought that this distribution is associated with the different physiological constraints on  $\text{Ca}^{2+}$  release for contraction vs  $\text{Ca}^{2+}$  release for thermogenesis. The primary need for morphologically separated sites of release in skeletal muscle is to reduce diffusion distances and obtain a synchronous contraction throughout the muscle fiber. This is brought about by the even spacing of the triads throughout the sarcomeres of skeletal muscle fibers. Heater cells lack myofibrils and sarcomeres, and junctional triads are difficult to identify (24).

The  $\text{Ca}^{2+}$  ATPase is the most prominent protein in isolated SR of blue marlin and swordfish. A wide variety of antisera have been used, but fail to recognize the specific isoform of the  $\text{Ca}^{2+}$  ATPase expressed in scombroid fish skeletal muscle or heater tissue. However, freeze-fracture studies have revealed a dense set of particles on the cytoplasmic leaflet of the SR that correspond to the appearance of the  $\text{Ca}^{2+}$  ATPase in nonjunctional SR regions of normal skeletal muscle. Recently, A Tullis (personal communication) amplified 700 bp of the  $\text{Ca}^{2+}$  ATPase message from heater tissue and determined its isoform identity. Using amino acid substitutions found in the phosphorylation and nucleotide-binding domains as markers for the slow or fast form of the  $\text{Ca}^{2+}$  ATPase, the analysis indicates that heater cells primarily express the fast-twitch form of the  $\text{Ca}^{2+}$  ATPase. Extraocular muscle from which the heater tissue is derived also expresses the fast-twitch isoform of the pump.

Protein gels and immunological techniques have identified significant amounts of calsequestrin in billfish heater cells (23). The mobility of the calsequestrin isoform expressed in these cells is similar to the extraocular muscle calsequestrin. Due to a unique mobility of the fish eye muscle and heater calsequestrin isoform, when compared to either mammalian fast or slow-twitch skeletal isoforms, it remains difficult to assign the fish extraocular calsequestrin to one of these two classes of isoforms. Electron microscopy and light level immunofluorescence studies indicate a distribution of the calsequestrin throughout the SR membrane system. This is in striking contrast

to skeletal muscle where the calsequestrin is localized in a recognizable morphological structure, the triad. Similarly, the SR  $\text{Ca}^{2+}$  release channel also has a homogenous rather than punctate distribution in the heater cell SR. This too is in contrast to the inhomogenous distribution of the  $\text{Ca}^{2+}$  release channel in skeletal muscle. In heater cells, the SR  $\text{Ca}^{2+}$  release channel and calsequestrin co-localize and have a relatively homogenous distribution throughout the SR membrane system, which indicates their localization in the spaces between the mitochondria. Taken together the results indicate that the SR in between the mitochondria is highly enriched in the protein components required for  $\text{Ca}^{2+}$  uptake, sequestration, and release.

Immunological studies of the SR proteins involved in  $\text{Ca}^{2+}$  cycling indicate a close relationship between isoforms of SR proteins expressed in heater and the isoforms expressed in the muscle precursor, the superior rectus eye muscle (131). The most interesting case of the linkage between isoforms of SR proteins expressed in eye muscles and heater tissue is found in the SR  $\text{Ca}^{2+}$  release channel. The heater cell has a unique expression pattern of the SR  $\text{Ca}^{2+}$  release channel that is shared with the precursor eye muscle fibers. While mammalian skeletal muscles express a single isoform of the SR  $\text{Ca}^{2+}$  release channel, avian, amphibian, reptilian, and fish skeletal muscles have been shown to express two isoforms,  $\alpha$  and  $\beta$ , in the skeletal muscles (3, 112, 131, 134). Biochemical studies (112) demonstrate similarity between the two nonmammalian isoforms, co-expressed within a single muscle fiber, and the mammalian skeletal and cardiac  $\text{Ca}^{2+}$  release channels. The skeletal isoform of mammals runs similarly on SDS gels with the  $\alpha$  isoform of nonmammals. The mammalian cardiac form of the SR  $\text{Ca}^{2+}$  release channel has a similar mobility to the  $\beta$  isoform. Immunoblot analyses have recently shown that only the skeletal or  $\alpha$  isoform of the SR  $\text{Ca}^{2+}$  release channel is expressed in the extraocular muscles of fish, while two forms are found in the swimming muscles (131). SDS-PAGE gels also reveal one high molecular weight polypeptide in heater, while two polypeptides are seen in the body musculature. The fastest contracting twitch fibers in vertebrates are the extraocular muscles (5). O'Brien et al (131) have hypothesized that the  $\alpha$  isoform of the SR release  $\text{Ca}^{2+}$  channel has certain characteristics of activation or inactivation that are advantageous for high frequency contraction and speed. A predisposition toward the use of extraocular muscle fibers as the heater phenotype may be the expression of only the skeletal-like form of the SR  $\text{Ca}^{2+}$  release channel.

### *Models for Nonshivering Thermogenesis in the Heater Cell*

Biochemical measurements of enzymatic activities along with physiological data indicate a high heat generating potential ( $250 \text{ W} \cdot \text{kg}^{-1}$ ) of the modified

billfish eye muscles (157). Although the exact molecular mechanism of thermogenesis in the heater cells has not fully been elucidated, biochemical and structural data suggest that heat production is linked to the release of  $\text{Ca}^{2+}$  from internal cytoplasmic stores. Thermogenesis is hypothesized to be associated with stimulation of catabolic processes and mitochondrial respiration via  $\text{Ca}^{2+}$ . The simplest hypothesis is that stimulation of the heater cell occurs via the cranial nerve (a unique branch of III) supplying the thermogenic portion of the extraocular muscle. Stimulation is presumed to lead to depolarization and  $\text{Ca}^{2+}$  release. The structural components for such an excitation pathway are present in the heater cells (26, 106). Changes in the concentration of intracellular  $\text{Ca}^{2+}$  are hypothesized to trigger thermogenesis. Whether this occurs because of a prolonged nervous stimulation or a series of excitatory stimulatory events (such as in a fast-twitch fiber recruited continuously) is not known. However, both mechanisms of stimulation would result in an increase flux of  $\text{Ca}^{2+}$  across the SR and a rise in cytoplasmic  $[\text{Ca}^{2+}]$ .  $\text{Ca}^{2+}$  entry into the small heater cell cytoplasmic space would occur without the usual binding to troponin or parvalbumin, effective  $\text{Ca}^{2+}$  buffers that are absent in heater cells. This suggests a rapid rise in cytoplasmic  $[\text{Ca}^{2+}]$  would occur after stimulation of the heater cell.  $\text{Ca}^{2+}$  transport by the pump that is densely spread throughout the SR would result in rapid ATP hydrolysis. Splitting of ATP would lower cytoplasmic ATP levels and increase  $[\text{ADP}]$  and  $[\text{P}_i]$ . Increased cytosolic  $[\text{ADP}]$  would subsequently stimulate the mitochondrial adenylate transporter, and ADP would be imported into the mitochondrial matrix thus increasing the ADP/ATP levels, which would result in stimulation of oxidative processes and substrate catabolism (Figure 1a). This basic pathway would be stimulated by any process that prolongs a cytoplasmic  $\text{Ca}^{2+}$  transient in the heater cell cytoplasm. A single point mutation in a gene coding for either the  $\text{Ca}^{2+}$  ATPase, the SR  $\text{Ca}^{2+}$  release channel, the T-tubule L-type  $\text{Ca}^{2+}$  channel, or any other protein involved in  $\text{Ca}^{2+}$  sequestration or release could raise sarcoplasmic  $\text{Ca}^{2+}$ , hence  $\text{Ca}^{2+}$  cycling, and stimulate thermogenesis (Figure 1b). Elevated levels of cytoplasmic  $\text{Ca}^{2+}$  would also be possible if prolonged stimulation of  $\text{Ca}^{2+}$  release occurred through a ligand or hormone that would potentiate the probability of opening of the SR release channel (Figure 1c). The SR  $\text{Ca}^{2+}$  release channels are activated by  $\text{Ca}^{2+}$  at physiological concentrations, and the opening of the channel is affected by ligands such as  $\text{Mg}^{2+}$  and adenine nucleotides. Alternatively, the SR could be the storage site for intracellular  $\text{Ca}^{2+}$ , and nervous stimulation could simply result in  $\text{Ca}^{2+}$  release, reuptake, and stimulation of mitochondrial oxidation via  $\text{Ca}^{2+}/\text{H}^+$  exchange (Figure 1d).

**EXCITATION-THERMOGENIC COUPLING** Three of the four thermogenic pathways outlined in Figure 1 involve the normal pathway for triggering  $\text{Ca}^{2+}$

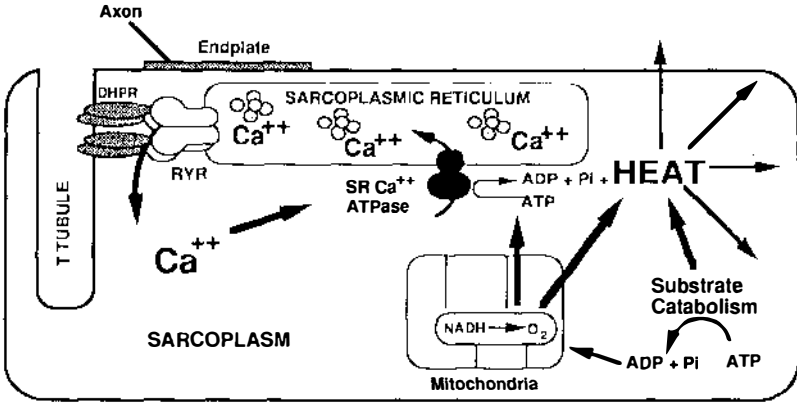
release in skeletal muscles cells. Skeletal muscles have an exquisite mechanism for depolarization-induced  $\text{Ca}^{2+}$  release, and Block (18, 20, 21) has hypothesized that such control can be combined with stimulation of oxidative phosphorylation and substrate catabolism (heat-generating processes) via  $\text{Ca}^{2+}$  release and reuptake at the SR in the heater cell. The process, called excitation-thermogenic coupling, simply implies that depolarization-induced  $\text{Ca}^{2+}$  release can trigger thermogenesis without contraction. The exact nature of the signal that leads to metabolic stimulation of heater tissue is not known but, as indicated above, structural results indicate that initiation of thermogenesis in the heater cell could occur via the normal cholinergic pathway found in skeletal muscle cells.

Such a thermogenic pathway in fish, with thermogenesis resulting from increased permeability of the heater cell SR to  $\text{Ca}^{2+}$ , has striking similarities to the proposed mechanism of thermogenesis in malignant hyperthermia (116), a condition involving abnormalities in the  $\text{Ca}^{2+}$  release channel of skeletal muscle in mammals. The condition in pigs has been genetically linked to the ryanodine receptor gene (RYR1) coding for the skeletal isoform of the SR  $\text{Ca}^{2+}$  release channel (154). A point mutation is presumed to underlie the presence of a hyperactive  $\text{Ca}^{2+}$  release channel, which is insensitive to closing. Under certain physiological stresses, such genetically predisposed pigs enter into a hyper-metabolic state that results in excessive catabolic activity in muscles and fatal levels of heat generation (154).

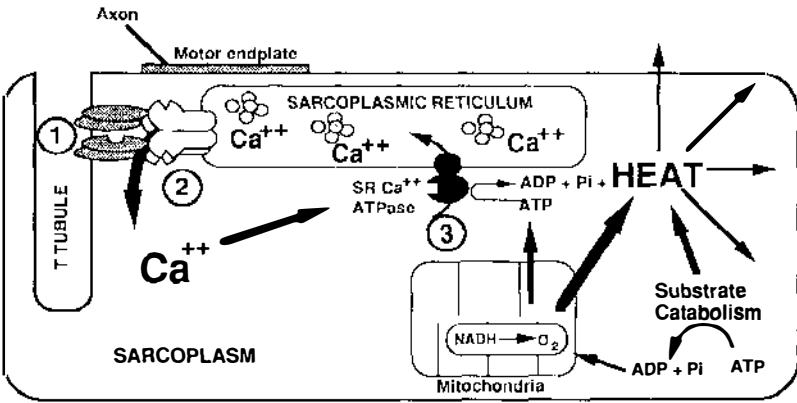
Research into the exact mechanisms of thermogenesis in heater cells has been influenced by the recent discoveries in malignant hyperthermia (MH). A current focus is to identify whether a normal molecular pathway for excitation-thermogenic coupling exists in these cells. A mutation that would result in increased SR  $\text{Ca}^{2+}$  permeability or leakiness to  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}$ ATPase, or SR  $\text{Ca}^{2+}$  release channel) or increased  $\text{Ca}^{2+}$  influx across the cell surface and T-tubule membrane (DHP receptor) could be the causative agent of the thermogenesis. The goal of current research on the heater organ is to define whether the proteins involved in this pathway are similar to mammalian junctional SR proteins, or if this unique adaptation is associated with a mutation of key proteins involved in the  $\text{Ca}^{2+}$  release pathway.

The presence of a unique expression pattern of the SR  $\text{Ca}^{2+}$  release channel in the extraocular muscles may be the predisposition required for the evolution of the unique thermogenic phenotype. One hypothesis is that fast-twitch oxidative, glycolytic (FOG) fibers of extraocular muscles in fish are enriched in the SR  $\text{Ca}^{2+}$  release channel and SR  $\text{Ca}^{2+}$  ATPase to begin with. Recent results indicate expression of only the fast, direct mechanically coupled isoform (skeletal) in extraocular muscle and heater cells. Direct sequencing of the cDNA for the fish SR  $\text{Ca}^{2+}$  release channel should reveal whether the fish have a normal extraocular isoform and release channel, or

a

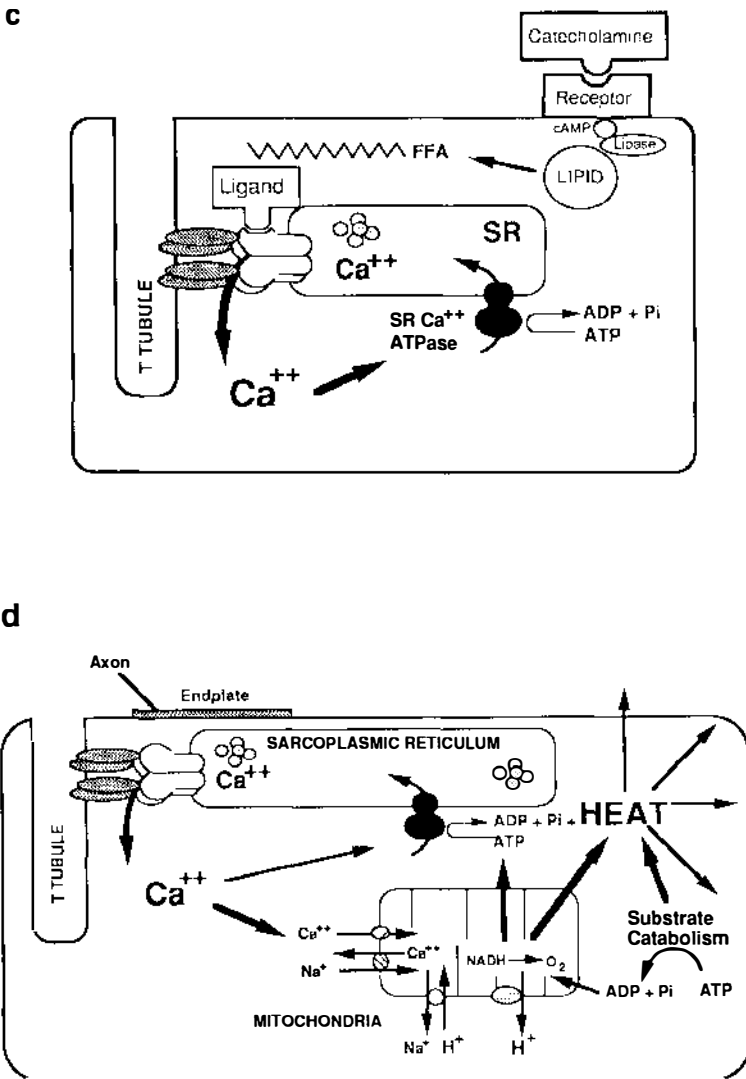


b



whether the key specialization is the high expression of the  $Ca^{2+}$  release channel throughout the heater cell SR.

As discussed above, any endogenous metabolite or ligand that increases the permeability of the SR to  $Ca^{2+}$  will increase thermogenesis according to the model (Figure 1c). Similarly, any increase in sarcoplasmic  $Ca^{2+}$  transients (due to depolarization) will stimulate thermogenesis. Evidence for pathways that increase SR permeability has been found in other endotherms and is discussed in more detail below, but the results from these studies could apply to the heater system. Ligands that prolong the open time of



**Figure 1** Models for nonshivering thermogenesis in skeletal muscle. See text for details. DHPR, dihydropyridine receptor, RYR, SR  $\text{Ca}^{2+}$  release channel.

the SR  $\text{Ca}^{2+}$  release channel, such as long chain FFAs released in the cytoplasm during lipid oxidation, could stimulate thermogenesis in the heater organ (62). Inefficiency in SR  $\text{Ca}^{2+}$  uptake caused by a reduction in the cations pumped per ATP by the SR  $\text{Ca}^{2+}$  ATPase could also be a source

of increased stimulation for thermogenesis. Finally, if the stimulatory pathway for thermogenesis in the heater organ is via the normal pathway found in skeletal muscle, it is possible that prolonging the open time of the L-type  $\text{Ca}^{2+}$  channel, for example by phosphorylation, would in turn increase sarcoplasmic  $\text{Ca}^{2+}$  transients by effectively opening the SR  $\text{Ca}^{2+}$  release channel.

**MITOCHONDRIAL THERMOGENESIS** The hypothesis of excitation-thermogenic coupling assumes that heat generation is stimulated by cytoplasmic processes associated with hydrolysis of ATP. This is based on two results discussed above: the presence of large amounts of the  $\text{Ca}^{2+}$  ATPase, and the coupled nature of heater cell mitochondria. Thermogenesis in any cell has two possible sources: mitochondrial uncoupling or cytoplasmic-based ATP utilization. To establish the cellular site of thermogenesis, it is necessary to compare the extent of heat generation from the changes in the mitochondrial coupling ratios, or the leak of protons across the mitochondrial membrane, to capacity for thermogenic stimulation from cytoplasmic processes (i.e. ATP hydrolysis). Both processes would generate heat and are not mutually exclusive. Cytoplasmic-based heat production requires delivery of ATP to the cytoplasm via the mitochondrial ATPase, whereas mitochondrial uncoupling does not require this step. The latter thermogenic process requires the presence of a specific uncoupler. The billfish thermogenic organ has a high density of mitochondria just as does other aerobic and thermogenic tissue [e.g mammalian brown adipose tissue (BAT)]. As in BAT, the focus of a thermogenic mechanism could be on a mitochondrial uncoupling mechanism rather than a cytoplasmically based stimulus for increasing oxidative processes in the heater cells. While numerous hypotheses for uncoupling mitochondria exist and include FFAs,  $\text{Ca}^{2+}$  ions, and differences in the efficiency of proton translocation, only the BAT uncoupling protein has been widely accepted as a designated thermogenic proton leakage pathway (130). BAT mitochondria possess a specialized uncoupling protein that is a major protein component of their inner membrane. The uncoupling protein is capable of dissipating the electrochemical gradient generated across the inner mitochondrial membrane during respiration as heat (85, 130). Immunological studies have ruled out the expression of this protein in the heater cells (18). From an evolutionary perspective there is no reason, a priori, to hypothesize that the fish thermogenic system, which evolved prior to the mammalian BAT, should have independently derived the same mechanism. However, as in questions surrounding endothermic metabolism in birds and mammals, it is important to discern if there is clear way to distinguish between the two modes of heat production.

The elucidation of some details of molecular regulation of the BAT

uncoupling protein has provided insight as to key differences in mitochondrial-based thermogenesis vs cytoplasmically based thermogenic mechanisms. One of the most interesting findings is the relationship between expression of  $F_1F_0$  ATPase and uncoupling protein in BAT mitochondria (92, 141). Stimulation of BAT via chronic cold exposure leads to an increase in expression of uncoupling protein and a simultaneous decrease in expression of  $F_1F_0$  ATPase. Thus when heat is being generated at the level of the mitochondria, ATP delivery to the cytoplasm is down-regulated. Using this paradigm for analysis of the fish thermogenic tissues is relatively easy. Examination of SDS-PAGE gels in swordfish and marlin indicate that the most intense proteins in the profile of all membranous components of the heater cell are two bands with apparent molecular weights of 55,000 and 50,100 kd. These correspond to the alpha and beta subunits of the mitochondrial  $F_1F_0$  ATPase. Although identification of these protein bands requires immunological confirmation, the results suggest that a cytoplasmically based mechanism for stimulating oxidation rates is the basis for heat production in the fish thermogenic organs.

### *Future Directions*

**THE ORIGIN OF THE HEATER PHENOTYPE** Studies of vertebrate extraocular muscles reveal an unusual and complex morphology and physiology. The muscles contain a variety of multiply and singly innervated fibers, whereas in vertebrate limb muscles, singly innervated fibers predominate. The cellular diversity of extraocular muscle has been linked in part to the physiology of the fibers. Several fiber populations have been identified via electrophysiology. Most extraocular fibers are categorized as being comparable to twitch fibers, singly innervated with action potentials (43). These fibers are capable of superfast contraction (5). Another population of fibers is associated with tonic fibers and generates graded tension, and appears to be equivalent to the tonic population of fibers in frogs. Histochemical, molecular, and immunological studies indicate a predominance of muscle fibers in fish extraocular muscles most phenotypically similar to twitch fibers (156).

Block (17) and Block & Franzini-Armstrong (24) have hypothesized that the heater phenotype is a product of one particular muscle fiber type. Rather than considering that the fiber phenotype (thermogenic) has arisen *de novo*, they suggest there is a predisposed fiber type that has a high aerobic capacity and ample SR. No study to date has unequivocally demonstrated if all extraocular muscle fibers are capable of expressing the phenotype, or if certain physiological properties of a fiber phenotype would predispose the muscle cell for becoming a heat-generating cell. Physiological arguments have been put forth for fast-twitch, slow-twitch, and tonic fibers that

emphasize aspects of calcium transients, calcium cycling abilities, and hence protein machinery for these processes and aerobic capacity. Recent work has identified the isoforms of SR proteins expressed in heater (131) and indicates linkage between the heater phenotype and fast-twitch extraocular muscle fibers (A Tullis, personal observation).

Elucidation of the nervous or hormonal control of the heater cell requires further work. In mammals, nonshivering as well as shivering thermogenesis (NST) is tightly linked to the sympathetic nervous system activity (85). A calorogenic response to catecholamines underlies the increased thermogenesis in the skeletal muscles of cold-acclimated mammals (83, 84). Similarly, in BAT, direct noradrenergic innervation of the cells causes release of norepinephrine from sympathetic nerve endings and stimulation of thermogenesis. Additionally, in mammals, chronic cold acclimation increases the sympathetic innervation to BAT, presumably increasing the ability for NST. While immunological studies have demonstrated the presence of skeletal muscle-like cholinergic nerve endings on the surface of heater cells, the potential role of sympathetic innervation should be explored in detail. It is possible that simple excitation of the muscle cell sarcolemma results in release of  $\text{Ca}^{2+}$  by the heater cell. Whether input is from the unique cranial nerve supplying this portion of the heater organ, or whether there is electrical continuity between the intact skeletal muscle portion of the extraocular muscle and the heater portion (e.g. gap junctions) remains to be determined.

In summary, two independent evolutions of a thermogenic tissue in fish demonstrate unequivocally that muscle is an important nonshivering heat source in vertebrates. In the conversion of muscle to a thermogenic organ, there has been evolutionary selection on the energetically costly pathway of  $\text{Ca}^{2+}$  cycling by the SR as the raw material for a thermogenic process, rather than harnessing the energetic potential of the cross-bridge apparatus (imagine a heater cell packed full of myosin heads). Immunological results indicate that the precursor muscle source is linked to the FOG fiber type. This fiber prior to modification is enriched in mitochondria and SR in most vertebrates. Apparently, a source of heat is readily available in all skeletal muscle fibers that cycle  $\text{Ca}^{2+}$  for continuous contraction for sustained periods of time.

### *The Role of Muscle in Tuna Endothermy*

Tunas are able to elevate muscle, viscera, brain, and eye tissue temperatures significantly above water temperature (40, 70). They lack specialized thermogenic organs and instead conserve heat in tissues with high aerobic capacities by use of vascular counter-current heat exchangers. Several studies have demonstrated that tunas have high standard metabolic rates that presumably underlie their capacity for endothermy (29, 69). While it is clear

that the metabolic costs are elevated at rest, it is difficult to explain why this is so. Most likely it reflects the numerous physiological and metabolic adaptations underlying the increased aerobic capacity of tunas (29). Aspects of tuna energetics were reviewed in a previous volume (150), thus only the contribution of the skeletal muscle to whole body metabolism and thermogenesis is considered in the following framework. (a) What contribution does skeletal muscle have in the elevation of metabolic rates of tunas? (b) Do tunas per kilogram body mass have a higher aerobic capacity in their slow oxidative (red) and fast-twitch (white) muscles than closely related ectothermic taxa? (c) Is the recruitment of both slow and fast-twitch fibers for continuous swimming occurring more frequently in tunas as a result of the numerous morphological specializations of the body plan than in ectothermic taxa and thus contributing to a higher rate of heat production from the muscles?

The metabolic rate of a tuna swimming at low speeds is significantly higher than other fishes (salmon) and may be correlated with increased aerobic costs of the tuna skeletal muscle machinery (69). Metabolic comparisons should only be made between the tuna and closely related ectothermic taxa such as the bonitos and mackerels. However, until such data exist, comparisons between the oxygen consumption rate of swimming tunas and salmonids will have to provide the basis for discussions of metabolism. In most fish, the swimming muscles are segregated into anatomically distinct zones consisting of two major fiber types, red and white, with an intermediate pink fiber often interspersed among the white fibers of many species (27). At low speeds, fish generate power only with the aerobic fibers (red), while at high speeds anaerobic fast-twitch (white) fibers are recruited (27, 142). The pink fibers are also fast-twitch fibers, but the nature of their recruitment order, especially in larger fishes such as tunas, is not clearly defined.

Endothermy in tunas is correlated with a unique positioning of the red muscle mass within the body plan of the tunas (71, 107). In contrast to all other teleosts, tunas have a more deeply situated red muscle mass along the horizontal septum and close to the axial skeleton. The deep red muscle is linked by a complex series of robust tendons to the caudal peduncle and tail. Both features, endothermy and central positioning of red muscle, also appear at the same time as a novel, more stiff-bodied swimming form, thunniform swimming (23, 71). Thunniform swimming generates forward propulsion, without the lateral undulation characteristic of many fishes, and concentrates the forces generated by contracting muscles on the tail. Thunniform swimming may reduce drag and increase the efficiency of movement through the water at higher speeds as indicated in the early metabolic measurements (69). Linkages between the novel swimming form, red muscle positioning, and endothermy have been hypothesized, but not tested (23).

Electromyograph (EMG) data on lightly anaesthetized tunas (*Katsuwonus pelamis*) have shown deep red muscle contracting at slow tail beat frequencies (140) without recruitment of the white muscle. Higher tail beat frequencies (e.g. burst activity) elicit activity from both muscle groups. Thus limited experimental evidence demonstrates that tunas recruit their slow and fast-twitch muscles in a similar fashion to other fishes. More extensive EMG recordings are required on free-swimming tunas of a wider variety of species and size before conclusions about muscle recruitment patterns can be made. One key question is whether the white muscle, in particular the FOG or pink fibers, are recruited during periods of continuous swimming.

The contribution in tunas of the deep red muscle, a highly aerobic tissue, to metabolic rate is not known, but hypothesized to be high. Microsphere measurements in nonswimming albacore tuna, *Thunnus alalunga*, provide values for the distribution of cardiac output to the muscles in a restrained tuna (163). The skeletal muscles of 10 kg albacore received 64% of the cardiac output, which indicates a key role in oxygen consumption and heat generation. Red muscle receives the highest percentage of the cardiac output (36%), although the tissue comprises only 4.5% of the total body mass in this species (163). The white muscle has the smallest relative blood flow; however, the large muscle mass as a percent of total mass (59%) results in a significant percentage of the cardiac output (28%) going to the white muscle. The microsphere studies provide the best evidence to date that the skeletal muscle of tunas, examined under basal conditions, consumes a large portion of the oxygen distributed to the whole animal and thus must be responsible for elevation of aerobic capacity as well as considerable heat generation. Further studies should follow up on these preliminary findings and methodology.

Does an extraordinary oxidative capacity of red muscle underlie the capacity for tuna endothermy? The aerobic capacity of tuna red muscle has been examined in several studies (73, 129). The literature is somewhat conflicting with some authors indicating high levels of aerobic activity, whereas others recognize similarities between red muscle of tunas and ectothermic fishes (22, 58). The phylogenetically correct comparisons will be made only if aerobic activity of red muscle, determined by enzymatic data or mitochondrial oxidation rates, is compared between closely related endothermic and ectothermic taxa (22). Recent investigations (129) have systematically re-examined the aerobic capacity of the muscles in the skipjack tuna, *Katsuwonus pelamis*. This study provides evidence for some of the highest oxidative enzyme capacities ever measured in tuna red muscle (for example citrate synthase activity equals 80 units per gram tissue at 25°C). However, a more comprehensive comparison among closely related ectothermic and endothermic tunas and their relatives (58) demonstrates that

high aerobic activity is present in the red muscles of both endothermic and ectothermic scombrid fish. Thus although some tuna species have extraordinarily high aerobic capacity of the slow-twitch red muscle fibers (e.g. skipjack tuna), in other species this capacity is not significantly different from closely related ectothermic species. Block & Finnerty (22) have used parsimony analyses to demonstrate that the high oxidative capacity of red muscle of fishes is a predisposition for the evolution of thermogenesis and endothermy in these species. Fish swim continuously in a viscous medium, a constraint to locomotion that is not encountered by terrestrial animals. Differences in red muscle mitochondrial properties between fish species are modest and demonstrate that substrate oxidation rates per milligram of mitochondrial protein are relatively similar among actively swimming teleosts (129).

Morphometric analysis (121) of skipjack tuna muscle has also demonstrated a high mitochondrial volume,  $V_v$  (mt,f), on average 29–32%  $V_v$  (mt,f). Although elevated, the tuna value is similar to red muscle mitochondrial  $V_v$  reported for other fishes. Mitochondrial volumes between 20–45.5% of fiber volume have been reported in a variety of fish species including shark (109), eel (93), trout (101), and anchovy red muscle (100). The recent morphometric investigation established that the inner mitochondrial surface area (63–70  $\text{m}^2/\text{cm}^3$ ;  $S_v$  im,m) in skipjack tuna red muscle is high for vertebrate muscle tissues (one and one-half to twofold higher than for a wide variety of mammals). Skipjack tuna red muscle appears to be pushing the theoretical limit of the maximal packing of mitochondrial inner membrane,  $S_v$  (im,m) of 83  $\text{m}^2/\text{cm}^3$ , in vertebrates (129). The similarity in maximal packing of mitochondria in oxidative red muscle fibers among fish species and the high inner mitochondrial membrane values suggest that skipjack tunas have achieved one of the highest possible aerobic capacities of force-generating red muscle. The fish appear to have reached a constraint for increasing mitochondrial volume; more (40% or so) would result in myofibrillar volume reduction and loss of force-generating capacity (129).

If tuna red muscle has a higher (as in skipjack) or similar oxygen consumption as a gram of red muscle from other closely related ectothermic species, how does it play a role in thermogenesis? Do tunas have a larger volume of recruitable red muscle tissue? Do they activate red fibers more often? Does this tissue receive a higher percentage of cardiac output than in teleosts of similar size? The respiration studies and enzymatic data indicate the tissue has a relatively high rate of oxygen consumption. The microsphere study indicates that red muscle tissue of tunas during periods of low electrical stimulation receives a high amount of blood flow (163). Thus red muscle must be the site of considerable heat generation. As an obligate ram ventilator, these fish are constantly

swimming to breathe, hence standard metabolic rate can be postulated to be elevated due, in part, to the aerobic cost of propulsion required for respiration (29). Further studies will need to compare the metabolic cost of swimming between closely related endothermic and ectothermic taxa (bonitos) who swim in a comparable fashion.

One way that muscle will increase the metabolic heat output of the fish is if there is a significant increase in the volume of red muscle. Several studies have carefully examined the relative distribution of red muscle in tunas (58, 71). Three of the smaller tuna species (*Auxis*, *Euthynnus*, *Katsuwonus*) have a high red muscle mass (7–13%), but some of the larger species (*Thunnus*) have a red muscle mass similar to other fishes (4–6%). The smaller tunas have significantly higher relative red muscle masses than closely related, similarly sized ectothermic taxa such as the bonito (*Sarda chiliensis*, 4.5%) and more distantly related taxa such as mackerel (*Scomber japonicus*, 6%). In *Auxis* and *Euthynnus*, the hypothesis that the high metabolic rates of tunas are associated with greater quantities of red muscle per unit body mass, in comparison with ectothermic sister taxa, is apparently true. Among the larger tunas, which have the most wide-ranging thermal ecologies, the red muscle volume is similar to closely related ectothermic taxa. Further studies on a wider range of body sizes are needed to examine this question more carefully and with phylogenetic considerations.

The microsphere measurements indicate the white muscle receives a considerable proportion of the cardiac output due to the total mass, thus it has the potential for a significant contribution to heat production. Two properties of white muscles that may increase the total aerobic capacity of the skeletal muscles and hence heat generation are (a) higher aerobic capacity and (b) higher frequency of recruitment. Several studies have demonstrated a large potential for aerobic metabolism in white muscle of tunas (58, 129). A higher aerobic capacity in tuna white muscle could be attributed to increasing the FOG fiber contribution in this muscle (B Block, personal observation). If this is the case, such fiber types may be recruited more frequently during steady-state swimming. While electrophysiological data have shown a clear division of labor in species of fishes between the slow and fast-twitch red and white muscle fiber populations (142), data on tunas indicate a possible contribution of the white muscle fibers to a wider range of swimming activity (30). This could be associated with a high aerobic fast-twitch fiber population (FOG or pink) being present in white muscle. It remains possible that the thunniform locomotory mode results in a continuous recruitment of a higher proportion of aerobic fibers throughout the body plan (slow and fast-twitch muscle masses) at all cruising velocities.

This could be tested with EMG studies aimed at determining the frequency of red and white muscle activity at a variety of swimming speeds. Appropriate comparative data should be obtained from ectothermic mackerels or bonitos.

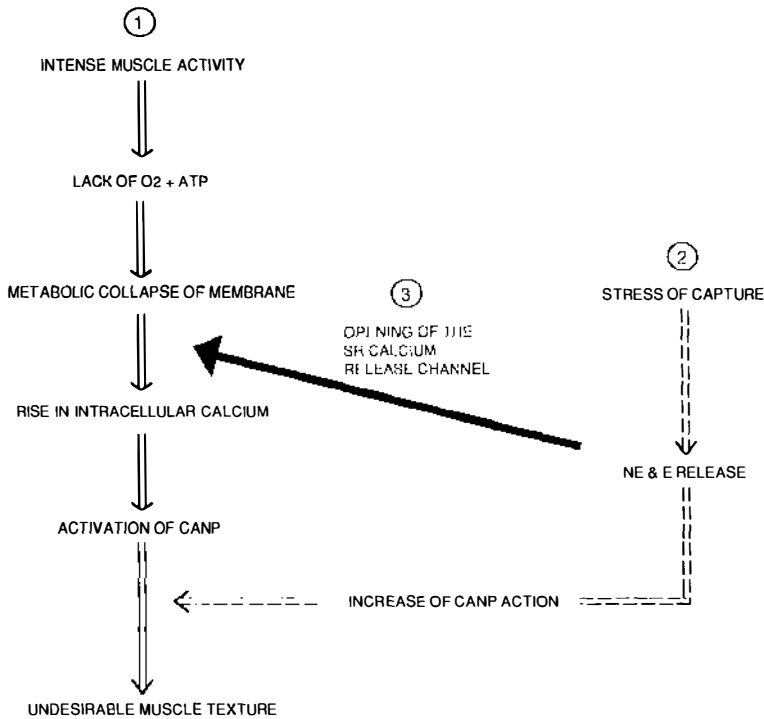
**TUNA BURN** Heat production in endothermic tunas has been linked with activity-related muscle metabolism. Tunas and their close relatives in comparison to taxa other than the Scombroidei, appear to have a higher aerobic content of muscle fibers in their body musculature (22, 58, 129). The increased aerobic capacity has been coupled to the evolution of heat retention systems that are essential for elevation of body temperatures (70). Tunas under adverse conditions of capture on hook and line often generate large amounts of heat in their muscles in a syndrome that parallels malignant hyperthermia (MH), or porcine stress syndrome (116). The highly profitable tuna fishery has commonly reported this condition known as "tuna burn," which has a dramatic effect on the quality of the tuna product that is most prized for raw consumption as sashimi. Interestingly, the literature on the etiology of tuna burn parallels MH, reported to occur with relative frequency in domestic mammals such as swine. Pigs experience the fatal syndrome, often as a reaction to the acute stress prior to slaughter. This parallels the physiological stresses for a tuna fighting capture by hook and line. However, little in the way of recent research on MH and excitation-contraction coupling has led to a cross-over of research ideas and etiology into the burnt tuna arena. This most likely is due to the relatively recent discovery of the SR  $\text{Ca}^{2+}$  release channel (111) and its only recent connection to the MH syndrome (88, 116).

Tuna muscle that has been burnt has a poor quality resulting primarily from degradation processes in the skeletal muscles that are initiated during the period of capture and continue after death. Descriptions of burnt tuna muscle are virtually identical to the descriptions of the quality of muscle after a porcine attack of MH (51). The problem in the tuna population is most often associated with certain fishing practices (hand-lines, rod and reels), which in most cases involve intense struggle for the fish. Thus as in MH in swine, a stressful situation brings about a syndrome that leads to destruction of the muscle tissue, a lower quality product for market, and economic losses. While the cause of the burnt tuna syndrome has not been unequivocally identified, most studies have suggested a variety of etiologies including low pH, high muscle temperature, lysosomal proteolytic activities, and calcium activation of proteases (reviewed in 160). Several studies have suggested that burnt tuna is associated with fish that experience the greatest stress during short-term capture proce-

dures, and it is well established that the quality of the tuna muscle can be preserved if the fishermen can pith the tuna after capture, a difficult procedure in such large-bodied fish.

Morphological studies of burnt tuna muscle indicate irregularities of the muscle cells. However, separating postmortem effects from actual causal changes related to the syndrome remains challenging for experimentation is extremely limited in these fishes. Postmortem burnt muscle provides evidence that there is a process of degradation similar in nature to reports of  $\text{Ca}^{2+}$  toxicity that occurs in the mammalian skeletal muscles. However, while this is certainly the problem associated with the decline in the quality of the meat, it is probably not the cause (51). Early hypotheses linked the burnt tuna syndrome to  $\text{Ca}^{2+}$  leakage in muscle (160). The causative agent for the leakage of  $\text{Ca}^{2+}$  in the hypothesis was low cellular ATP, which would bring about increases of sarcoplasmic  $\text{Ca}^{2+}$  from metabolic collapse and decreased cellular ATP for pumping  $\text{Ca}^{2+}$  back into the SR. This in turn was proposed to lead to a cascade of events associated with proteolytic activity of the muscles from  $\text{Ca}^{2+}$ -activated proteases. Recently, Watson et al (160) suggested that the types of postmortem changes described in the literature, which included selective destruction of Z-discs and irregularities of the SR, are associated specifically with  $\text{Ca}^{2+}$ -activated neutral proteases. The most recent hypothesis for the syndrome has added catecholamines as the key factor involved in initiation of burnt tuna. The stimulatory factor for the syndrome is stress, which leads to norepinephrine release and subsequent increase of activity of  $\text{Ca}^{2+}$ -activated neutral proteases, thus resulting in breakdown of sarcomere structure. Figure 2 takes this tuna burn hypothesis and places the model in the context of what we now understand about the EC coupling processes. The stress associated with the intense activity surrounding capture induces increased levels of circulating catecholamines that would lead to prolonged cytoplasmic  $\text{Ca}^{2+}$  transients, possibly by sympathetically mediated phosphorylation of the DHP receptor. This would in turn be associated with the increase of  $[\text{Ca}^{2+}]$  in the sarcoplasm from the prolonged opening of the SR  $\text{Ca}^{2+}$  release channel. Increased catabolism would be associated with the calcium transient. This in turn could also lead to the  $\text{Ca}^{2+}$ -activated proteolytic breakdown associated with the earlier hypotheses. Whether it is necessary to include a mutant SR  $\text{Ca}^{2+}$  release channel, as in porcine MH, remains questionable.

An additional factor that should be investigated in relationship to this new model is the consideration of the fiber type affected in burnt tunas vs unburnt tunas. A complication in fish is that two isoforms of the SR  $\text{Ca}^{2+}$  release channel,  $\alpha$  and  $\beta$ , are expressed in their swimming musculature (131). Recent work (B Block, unpublished observations) indicates that many



*Figure 2* Models for tuna burn. Previous explanations for the increased heat production and damage caused by tuna burn suggested involvement of  $\text{Ca}^{2+}$  (1) and catecholamines (2) as potentiators of the syndrome (159). The hypothesis presented here incorporates the SR  $\text{Ca}^{2+}$  release channel into the previous models.

large yellowfin and big-eye tunas have a large number of fast-twitch oxidative fibers mixed in with the fast-twitch glycolytic population. Both fiber types are enriched in SR and thus have a high content of the SR  $\text{Ca}^{2+}$  release channel. Such a high content of aerobic fast-twitch fibers (58) is unusual in fish and may be an important component of why and how these fish get burnt, whereas other large game fish caught under similar conditions (blue marlin) do not. The current hypothesis suggests that it is the prevalence of these fiber types, enriched with SR and the  $\text{Ca}^{2+}$  release channel that is the causative agent for the burnt tuna syndrome. An abnormality (genetic mutation) in the tuna SR  $\text{Ca}^{2+}$  release channel of skeletal muscle may account for the disruption in SR  $\text{Ca}^{2+}$  homeostasis in the tuna muscle. In this model, as suggested by earlier models, stress and the effects of catecholamines are the major cause of the syndrome. However, it remains

possible that as in the mammalian MH syndrome reviewed below, a genetic predisposition for the tuna burn syndrome involves a mutation in the SR  $\text{Ca}^{2+}$  release channel. The capacity to link polymorphisms in the skeletal SR  $\text{Ca}^{2+}$  release channel to wild populations of tunas would be an arduous task. However, amplifying and sequencing a 1 kb region around the porcine mutation might be a quick way to discern if there is a key amino acid difference in this region of the tuna SR  $\text{Ca}^{2+}$  release channel. A complication of this strategy would be the need to acquire fish sequences from normal RYRs to ensure establishing phylogenetic-specific substitutions. Initial studies could focus on the properties of the isolated SR  $\text{Ca}^{2+}$  release channel in normal and burnt tunas to establish if there are differences in the probability of the open state of the channel and ligand binding properties as was done on porcine MH.

## $\text{Ca}^{2+}$ -MEDIATED THERMOGENESIS IN MAMMALS

Long term exposure to cold temperatures has been shown to significantly increase BMR in mammals (42, 76, 161). This increase in metabolic rate of cold-acclimated mammals has been linked to nonshivering thermogenesis due in part to skeletal muscle (95). In mammals, the control of nonshivering thermogenesis has been shown to be regulated through the sympathetic nervous system, although the cellular basis for this remains unclear (95, 96, 97). For example, a calorogenic response to catecholamines in cold-acclimated mammals increases metabolic rate as much as five times the BMR (83). The first response to cold exposure in mammals is to shiver and increase heat production through muscle contractions that produce no useful work. Long-term cold exposure results in further elevation of whole body metabolism; shivering subsides and nonshivering thermogenesis is the cause for the increased energy expenditure (95, 97, 98, 126). The increased heat production from norepinephrine appears to be closely associated with the level of nonshivering thermogenesis (97). There are numerous hypotheses about the organs responsible and the mechanistic basis for nonshivering thermogenesis in mammals. Blood flow measurements have established that most nonshivering thermogenesis in small mammals is associated with brown adipose tissue (85, 130). While brown adipose tissue (BAT) elicits a large proportion of the metabolic response to cold in small mammals, calculations of the total heat production of BAT fall short of accounting for all the nonshivering thermogenesis heat production (48, 94). Additionally, BAT is less than 0.3% of the body mass of most large mammals and cannot by size alone be the only site of nonshivering thermogenesis. The controversy as to how much and by what means skeletal muscle contributes to nonshivering thermogenesis has not been settled. Long-term cold exposure

induces numerous alterations in skeletal muscles that result in an increase of oxygen demand and elevation of serum free fatty acids (31, 32, 33, 146). The large mass of muscle as a percent of mammalian body mass, and the metabolic stimulation evident in response to thyroid hormone as well as catecholamines, are suggestive of a key thermogenic role for this tissue during long-term cold exposure.

Clausen et al (47) provide an extensive review of the significance of cation transport in skeletal muscle metabolism under a variety of conditions. At rest, ATPases such as the  $\text{Ca}^{2+}$  ATPase and the  $\text{Na}^+, \text{K}^+$  ATPase consume only a small portion of the skeletal muscle resting metabolism. However, during stimulation associated with contraction, the metabolic rate of muscle rises quickly through ATP consuming processes, cross-bridge cycling, and  $\text{Ca}^{2+}$  cycling. Calculations of the costs of  $\text{Ca}^{2+}$  cycling are obtained in stretched muscle where myofilament overlap is small and the energy released is measured as heat liberated (47, 139). Activation heat measurements (heat produced at zero force) from contracting muscles indicate that 20–50% of the energetic turnover is associated with SR  $\text{Ca}^{2+}$  cycling. Muscles with a higher proportion of fast-twitch fibers, and thus SR, consume a larger fraction of the total energy from the relative contribution of  $\text{Ca}^{2+}$  cycling than do muscles composed of slow-twitch fibers with lower SR content (113). Thus a major energy consuming pathway of the skeletal muscle cell,  $\text{Ca}^{2+}$  cycling, is implicated as a mechanism for heat generation in working muscles. This is significant given that nonshivering thermogenesis, by definition, implies that the myofibrillar component (actomyosin ATPase) of a muscle cell is not involved in heat generation. However, models for nonshivering thermogenesis must reconcile the relatively low cost of maintaining ionic balance when muscle is not contracting. That is, there must be some stimulatory event that enhances cation turnover (i.e. prolonged stimulation or leak of sarcoplasmic  $\text{Ca}^{2+}$  release channel and elevation of cytoplasmic  $\text{Ca}^{2+}$  below contraction threshold) if  $\text{Ca}^{2+}$  cycling is a significant thermogenic source when muscle is not contracting.

Cold acclimation studies in mammals indicate that there is a generalized increase in the ion permeability of membranes in a variety of tissues (42). The increased active transport required to balance increases in membrane permeability could serve as one of the effectors stimulating cellular metabolism. Cold acclimation, as well as thyroxine, in mammals results in significant increases in the  $\text{Ca}^{2+}$  transport properties of skeletal muscle SR (2, 47, 148). Although such changes are suggestive of a major role for  $\text{Ca}^{2+}$  transport and cycling in skeletal muscle energy turnover and thermogenesis, doubt remains. Interestingly, the components of the muscle cell responsible for  $\text{Ca}^{2+}$  cycling, the  $\text{Ca}^{2+}$  release channel, and  $\text{Ca}^{2+}$  ATPase have homologues in liver endoplasmic reticulum, a tissue long thought to

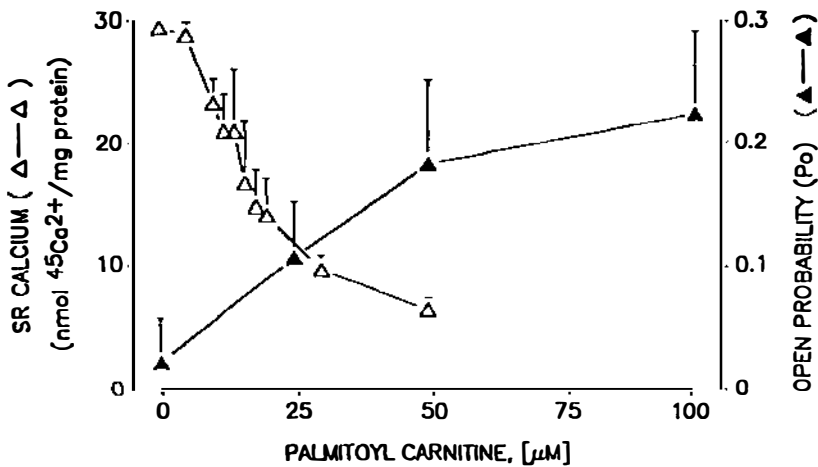
have a role in NST. The best evidence of a cellular pathway in mammalian skeletal muscle capable of extraordinary thermogenesis and associated with  $\text{Ca}^{2+}$  cycling in muscle has been found in the elucidation of the molecular cause of mammalian MH (72, 116).

**MALIGNANT HYPERTHERMIA** The role of  $\text{Ca}^{2+}$  and its stimulatory effect on muscle metabolism and thermogenesis in mammals is best illustrated in MH, where a rapid and often lethal rise in body temperature is linked to abnormalities of  $\text{Ca}^{2+}$  regulation in muscle cell cytoplasm. The proposed mechanism for thermogenesis in MH (116) bears striking resemblance to the proposed mechanism of thermogenesis in the fish heater organs (Figure 1). In humans and mammals with the genetic disorder, the syndrome is characterized by a rapid increase in muscle temperature and muscle rigidity under certain types of stresses (72). MH in humans is a syndrome that is most often triggered with volatile anesthetics. The syndrome characteristically is associated with thermogenesis in muscle leading to hyperthermia, metabolic acidosis, rhabdomyolysis, myoglobinuria, and elevated plasma creatine phosphokinase (72, 88). Altered biochemical and physiological properties of the isolated SR  $\text{Ca}^{2+}$  release channel have been detected in SR isolated from MH pigs vs normal pigs, which indicates a possible defect of the SR  $\text{Ca}^{2+}$  release channel (108, 127). Similarly, high rates of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release and increased sensitivity to caffeine were observed in human heavy SR preparations enriched in the release channel from MH patients (65). Trypsin digestion of the porcine  $\text{Ca}^{2+}$  release channel suggested an alteration in the primary sequence (108). Linkage between polymorphic markers and porcine MH led to the identification of the locus for MH pigs to chromosome 6 (115, 122). The gene encoding skeletal SR  $\text{Ca}^{2+}$  release channel, RYR1, also mapped to a related region of chromosome 19 in humans and became a suspect in the cause of MH (115). The biochemical results suggesting a hyperactive or leaky SR calcium release channel (65) and the genetic results indicating possible linkage of MH with the channel led to a large sequencing effort of porcine RYR1 cDNAs to discern whether a mutation was associated with the condition (67, 116). Porcine MH has been genetically linked to a single point mutation of RYR1. A substitution of a cysteine for arginine (i.e. substitution of cytosine for thymine at nucleotide position 1843), in the porcine SR calcium release channel is presumed to be the primary defect. Similarly, a C (nucleotide 1840) for T substitution in the human RYR1 gene was found in a few patients susceptible to MH; however, numerous human MH-susceptible patients do not show this substitution, which indicates that it is not the mutation associated with the syndrome in humans (68a). Thus hypersensitive gating of the SR  $\text{Ca}^{2+}$  release chan-

nel underlies the defect of MH responsible for thermogenesis. The discovery of the mutation and linkage to SR  $\text{Ca}^{2+}$  cycling provides clear evidence for the molecular pathway increasing energy utilization and thermogenesis in skeletal muscle.

**ROLE OF FREE FATTY ACIDS ON RYANODINE RECEPTOR** The MH syndrome in humans is also thought to be linked with other defects such as a deficiency in the enzyme, carnitine palmitoyl transferase, which catalyzes the transport of long chain acyl carnitines into the matrix of the mitochondria (158). The etiology of transferase deficiencies indicates that  $\text{Ca}^{2+}$  toxicity may be occurring in the muscle cell cytoplasm; however, until recently, a mechanistic basis for this etiology was not clearly defined. Palmitoyl carnitine and esterified long chain fatty acids (FFA) have been shown to selectively activate the SR  $\text{Ca}^{2+}$  release channel in mammals and frogs in a physiological range (62). The increase in SR permeability in response to the long chain length FFAs provides a possible pathway for myoplasmic  $\text{Ca}^{2+}$  concentrations to increase in response to a local build up of FFA in the myoplasm. El-Hayek et al (62) have proposed that palmitoyl carnitine binds to and modulates gating of the SR  $\text{Ca}^{2+}$  release channel and is capable of increasing the  $\text{Ca}^{2+}$  permeability of the skeletal muscle SR reticulum by direct interaction with a functionally important region of the molecule (Figure 3). Interestingly, only long chain acyl carnitines of a certain length (C14, C16, C18) induce SR  $\text{Ca}^{2+}$  release. This newly identified interaction of a substrate metabolite with the  $\text{Ca}^{2+}$  release channel is a strong candidate for the endogenous thermogenic factor linking  $\text{Ca}^{2+}$  cycling,  $\beta$ -oxidation, and muscle cell metabolism for heat generation. More research is required to resolve whether the interactions arise from the fatty acids interacting with the channel protein itself through an actual fatty acid binding domain, or from a partitioning of the FFAs into the membrane and a resultant alteration of the properties of the membrane surrounding the protein. Lipid metabolites have been shown to mobilize  $\text{Ca}^{2+}$  from intracellular stores and have been implicated as second messengers in a variety of signal transduction pathways involving ion channels (16, 45, 135).

The clear effect of FFAs on the  $\text{Ca}^{2+}$  permeability of SR suggests that an increase in acyl carnitine production during exercise or nonshivering thermogenesis could effectively increase  $\text{Ca}^{2+}$  release and muscle cell metabolic processes that are activated by  $\text{Ca}^{2+}$ . Elevation of cytoplasmic [ $\text{Ca}^{2+}$ ] is a potent stimulator of mitochondrial oxidative processes (56). Fatty acid mobilization occurs by a variety of mechanisms (epinephrine, glucagon, growth factors), and substrates can be found either in the cytoplasm or the blood. Additionally, the plasmalemma contains a rich source of phospholipids that can be released via the action of cellular phos-



**Figure 3** FFAs activate the skeletal muscle  $\text{Ca}^{2+}$  release channel in mammals. Certain long chain fatty acids prolong the open time of the SR calcium release channel in mammals. Open triangles correspond to vesicles of porcine skeletal muscle SR loaded with  $^{45}\text{Ca}^{2+}$  and exposed to solutions containing various palmitoyl carnitine concentrations at room temperature.  $\text{Ca}^{2+}$  release in the presence of  $\mu\text{M}$  palmitoyl carnitine occurs. This is indicated by the reduction of  $^{45}\text{Ca}^{2+}$  in vesicles after a filtration assay (*open triangles*). Filled triangles correspond to open time of  $\text{Ca}^{2+}$  release channels from porcine SR at the indicated palmitoyl carnitine concentrations (from 61a).

pholipases. A heat-generating cycle could commence with hydrolysis of triacylglycerol by lipases in the cell cytoplasm. Accumulation of long chain fatty acids in the cytoplasm would increase myoplasmic  $\text{Ca}^{2+}$  and generate considerable heat from futile  $\text{Ca}^{2+}$  cycling. Lipolysis could, in fact, be the stimulatory event that then leads to  $\text{Ca}^{2+}$  release and increased cycling. SR  $\text{Ca}^{2+}$  ATP turnover by the  $\text{Ca}^{2+}$  ATPase would increase cytoplasmic adenylates and stimulate mitochondrial respiration. The increased demand for FFAs from the oxidative processes within the mitochondria would feedback and stimulate cytoplasmic demand for increased lipolysis. A plausible link (or pathway) thus exists between the increase in FFAs that occurs during cold acclimation (7) to a change in the permeability of the SR membrane that leads to increased energy utilization and heat production. Lipolysis brought about by norepinephrine control is the stimulatory event in brown adipose tissue thermogenesis. Given the well demonstrated role of catecholamines in nonshivering thermogenesis, it remains possible that a FFA-mediated futile  $\text{Ca}^{2+}$  cycling pathway in skeletal muscle could be initiated with catecholamines (Figure 1c).

## NONSHIVERING THERMOGENESIS IN BIRDS: A Role for Muscle?

Endothermy in mammals and birds has many features in common including the physiological responses to cold stress (42, 52, 83). Although regulatory thermogenesis in mammals has been the subject of intense investigation for decades, the physiological responses and adaptations of birds to cold has received less study; this despite the impressive thermoregulatory abilities of small birds and in particular the model they represent for skeletal muscle-based thermogenesis. Many small birds winter in high latitudes and encounter cold temperatures that require elevations in thermogenic capacities on seasonal time scales. The thermogenic capabilities of birds of the avian subfamily Carduelinae (goldfinches, house finches redpolls) are impressive and in many cases exceed the abilities of mammals. For example, goldfinches can maintain a metabolic rate of five times standard metabolic rate for 6–8 hr during ambient temperatures of  $-70^{\circ}\text{C}$  in winter (53). The same birds in the springtime will become hypothermic after only a few minutes of exposure (53). This change occurs without any change in aerobic capacity of the skeletal muscles, which are the major thermogenic tissues in birds (35, 119).

The cold-induced metabolic scope has been examined in a few studies and has been shown to range three to eight times BMR (164). Metabolic scope during flight can range five to ten times BMR and thus exceeds maximum metabolism elicited by cold (28, 120). Rates of oxygen consumption by birds during sustained flying are among the highest reported for vertebrates. Flight muscles, particularly the pectoralis and supercoracoideus, of birds reflect this intense metabolic demand with adaptations that facilitate uptake and utilization of oxygen and energy substrates. Mass-specific aerobic capacities are among the highest in the animal kingdom (35, 119, 152). The extraordinary metabolic demands for flight in birds select for skeletal muscle fiber types with high rates of substrate utilization, and predispose the use of these muscles in birds as thermogenic organs. Histochemical studies have demonstrated that the flight muscle of birds are composed predominantly of fast glycolytic and fast-oxidative-glycolytic fiber types (35, 68, 117, 155). The latter fibers are rich in mitochondria, SR, and T-tubules and have high substrate oxidation rates (118, 119).

Birds meet the bulk of the increased thermogenic needs in response to cold stress with shivering thermogenesis (162). Electromyograms indicate shivering at all temperatures below the thermal neutrality zone. The relative importance of shivering thermogenesis vs nonshivering thermogenesis in birds, as in mammals, is still a matter of active debate (11, 50). Two questions that have been raised in the avian literature and have been well

examined are still unresolved. (a) Is there a cold-induced facultative non-shivering thermogenesis in birds? (b) Is skeletal muscle the main site of NST in birds?

Early studies by El Halawani et al (62a) demonstrated an increased capacity for nonshivering thermogenesis in cold-acclimated birds. Similarly, Barré et al (9) demonstrated a large increase in the metabolic rate of cold-acclimated muscovy ducklings that could not be attributed to shivering activity. These two studies demonstrate a nonshivering thermogenic component to cold adaptation in birds, although the site of this response remains controversial. The key role of a specialized thermogenic organ in mammals (BAT) has continually led to a search for a similar tissue in birds. The issue of whether or not BAT is present in birds has been addressed morphologically and biochemically by numerous studies (9, 114, 133, 143, 144). Although birds have been reported to have a multiocular adipose tissue, with some similarities to mammalian brown adipose tissue, it lacks the key biochemical features (presence of uncoupling protein) and thus is functionally white adipose tissue (144). From the reported morphological and biochemical evidence, one must reject the hypothesis that birds have brown adipose tissue and look elsewhere for the cold-induced increased heat production. This is expected from an evolutionary standpoint. It would be highly unlikely that birds would independently evolve the exact same specialized adipose tissues of mammals. Thus a major difference in cold defense between mammals and birds is the lack of a specialized thermogenic organ. However, as discussed above, the high oxidative capacity of the flight muscles may preclude the need for such a thermogenic organ. As in the tuna example of endothermy, it is quite clear that the presence of a highly oxidative muscle phenotype for locomotion predisposes the use of this tissue as a furnace.

### *How Do Birds Stay Warm?*

Dawson and colleagues have shown that passerines and birds of the family Carduelidae, which overwinter in cold latitudes, undergo a pronounced seasonal acclimatization involving an increased resistance to cold (55). Birds overwintering in northern latitudes have been shown to have a substantial increase in their thermogenic capacities and withstand cold exposure far longer than summer birds (55). This physiological response involves enhanced abilities for increasing heat production for sustained periods (119). Thermoregulatory stresses are more severe for many of these small birds (10–20g) because of the decreased amounts of insulation associated with their small size (34). The metabolic processes of acclimatization involve numerous biochemical changes in the flight muscles, which comprise 15–25% of body mass in adult birds (53, 54, 55, 75). Acclimatization involves

increasing energy reserves (lipid), improved mobilization of substrates to fuel thermogenesis, and a greater reliance on lipid metabolism during cold stress (119). In most cases documented, aerobic capacity as determined by marker enzymes for aerobic catabolism does not change much in flight muscles, but can be augmented in leg muscles, which contribute to shivering at low temperatures (36). The metabolic demands of flight are greater than the aerobic demands of cold exposure. The lack of change in aerobic capacity within the major muscle masses associated with thermogenesis has led to a focus on the change in energy substrate utilization during cold exposure, given the result that lipid metabolism appears to be playing a key role in the process of metabolic acclimatization (120). The specific activity of  $\beta$ -hydroxyacyl-CoA dehydrogenase in pectoralis muscles, a key enzyme in the  $\beta$ -oxidation pathway, increases 50–100% in cold-acclimated American goldfinches (120). The exact role of seasonally enhanced levels of increased  $\beta$ -oxidative capacity is not entirely clear from the existing literature, but may be involved with a role of FFA in augmenting thermogenesis. However, recent studies indicate that house finches do not undergo a seasonal change in the capacity to oxidize lipid, which complicates the interpretation of a role for lipid in stimulating thermogenesis (T O'Connor, personal communication).

The research programs on cold acclimation in overwintering birds assume that shivering thermogenesis is the major source of enhanced muscle-based heat production during winter. Nonshivering thermogenesis in winter-acclimatized birds remains subject to debate (52). Although the exact thermogenic mechanism (in wild birds) remains unclear, the seasonal changes in metabolic heat production and the well documented increased capacity for shivering thermogenesis (162) point to the flight muscles as the major source of heat. What remains to be resolved is whether the muscles are contributing via both pathways (shivering and nonshivering thermogenesis). The two are not mutually exclusive, and the basic enhancements required for energy consumption via nonshivering thermogenic pathways would indeed increase the energy consumption during shivering thermogenesis. Thus the issue is not whether muscle is involved, most research in this area would support the role of skeletal muscle involvement. What is at issue is how it is involved, and an objective view would suggest that both mechanisms are at work.

**A MECHANISM FOR THERMOGENESIS IN AVIAN SKELETAL MUSCLE** In ducklings, Barré et al (12) demonstrated that cold exposure induces the development of nonshivering thermogenesis of muscular origin. The exact pathway for this response is currently being elucidated and appears to involve free fatty acids (FFAs) and possibly futile  $\text{Ca}^{2+}$  cycling. Prolonged cold exposure in birds has long been linked to increases in FFA levels in the

plasma (13, 66, 99, 136, 159). Barré et al (12) demonstrated that FFAs increased respiration in skeletal muscle mitochondria from cold-acclimated ducklings, but did not effect membrane potential. A potent calorogenic effect of glucagon in birds, similar in scope to the norepinephrine-induced thermogenesis in mammals, has also been documented (10). Glucagon results in release of FFA in birds, and it is postulated that in response to the hormone, increases of cytoplasmic FFA would result in partial uncoupling of skeletal muscle mitochondria with a concomitant increase of respiration and heat production.

Dumonteil et al (61) recently provided evidence supporting the idea that increased ATP-dependent cycling of  $\text{Ca}^{2+}$  may underlie the nonshivering thermogenesis of muscular origin in birds. The role of futile  $\text{Ca}^{2+}$  cycling of the SR in muscular nonshivering thermogenesis was determined by examining the  $\text{Ca}^{2+}$  transport activities of muscle homogenates and heavy SR microsomal fractions from thermoneutral and cold-acclimated ducklings. As in cold-acclimated mammals (2), these studies demonstrate increased  $\text{Ca}^{2+}$  loading capabilities in direct response to cold adaptation. The studies indicate that the  $\text{Ca}^{2+}$  transporting system of skeletal muscle SR undergoes a significant increase in activity associated with cold stimulation. Importantly, cold-acclimated ducklings show an increase in SR  $\text{Ca}^{2+}$  release channel content in the cold. The result is suggestive of a role of  $\text{Ca}^{2+}$  cycling in nonshivering thermogenesis of muscular origin. Future studies should decipher whether such qualitative increases in the properties of  $\text{Ca}^{2+}$  transport and release are due to changes in SR volume in specific muscle fibers or due to increased packing of the proteins within the same SR volume. Dumonteil et al (60) have also shown a potential role of FFAs for potentiating SR  $\text{Ca}^{2+}$  cycling. Palmitoyl carnitine stimulated [ $^3\text{H}$ ] ryanodine binding in cold-acclimated SR isolated from muscovy duckling muscle in a concentration-dependent manner. Palmitoyl carnitine also induced rapid  $\text{Ca}^{2+}$  release from passively loaded SR vesicles. The two results suggest that a FFA-mediated thermogenic pathway involving ATP-dependent  $\text{Ca}^{2+}$  cycling may exist between the SR and cytoplasm (as in model Figure 1c and mammals, Figure 3).

The cold acclimation response is evolutionarily conserved in vertebrates, and the results for birds as well as mammals must be examined with this in mind. Numerous vertebrates in response to cold acclimation increase the mitochondrial and SR volume, as well as  $\text{Ca}^{2+}$  ATPase content in certain muscle fiber types (2, 61, 147). For ectotherms, the cellular adaptations are hypothesized to increase the ATP delivery and relaxation components of the muscle cells and thus power output in the face of decreasing diffusion potential due to cold. In endotherms, one has to decipher whether the increased  $\text{Ca}^{2+}$  transport properties of the SR and increased mitochondrial

content of the muscle fibers in response to cold (33, 49, 61) are just an evolutionary vestige of this primitive vertebrate response. Based on the above discussion, there remains a possible link between cold acclimation in vertebrates and the increased substrate mobilization that may ultimately lead to the explanation of how nonshivering thermogenesis evolved in birds and mammals. The ancestral physiological response of certain ectothermic vertebrates to cold is to increase SR volume in certain muscle fibers and hence increase the surface area of the  $\text{Ca}^{2+}$  ATPase, the limiting step in muscle relaxation times (147). This adaptive response could have been present in the reptilian ancestors of birds and mammals. It is possible that the ectothermic vertebrate physiological response to cold (increasing the aerobic capacity of the fiber, SR volume, and  $\text{Ca}^{2+}$  release and sequestering proteins, and substrate mobilization) were modified more for wasting of energy or futile cycling, and hence heat production, rather than for the earlier function of maintaining muscle power output. This latter function would not be a problem given that birds and mammals maintain their body temperatures at elevated temperatures. The process of cold acclimation may be hardwired into the evolutionary program of some vertebrates and has since been modified (perhaps hormonally or sympathetically) to increase the level of energetic turnover, and thus thermogenesis during cold stress periods in endotherms. It remains possible that endotherms may have evolved an endogenous agonist (thyroid hormone, glucagon, epinephrine) that stimulates aerobic metabolism through the  $\text{Ca}^{2+}$  cycling pathway (i.e. opening of the SR  $\text{Ca}^{2+}$  release channel and possibly liver ryanodine receptors) in response to cold.

## HEAT GENERATION IN INSECT MUSCLES

Within the class insecta, endothermy of muscular origin is widespread. Thermogenesis occurs during a wide variety of activities including flying, running, singing, pre-flight warm up, and social activities (64, 79, 80, 82). Flying insects achieve the highest mass-specific rates of aerobic energy expenditure in the animal kingdom (14, 15) and are able to harness exogenous heat from the high rates of substrate flux occurring in their muscles during periods of activity (81). Insect muscle efficiencies range from 4–11% and thus liberate substantial heat as a byproduct of the inefficient coupling of chemical energy into mechanical work. Heat generation is substantial enough in insects that over-heating is a problem during flight, and elaborate strategies for heat dissipation have evolved (80).

The high oxidative capacity of insect flight muscles is made possible by the direct delivery of oxygen by the tracheal system. Mitochondrial morphometric measurements demonstrate higher inner mitochondrial surface

densities in bees and blowflies than in mammals of similar size (41, 91, 153). Marker enzymes for aerobic metabolism are two to threefold higher per volume of mitochondria than in vertebrate mitochondria (153). Insects are able to access oxygen and generate ATP at a rate that exceeds limits possible in a similar volume of vertebrate mitochondria. Suarez & Moyes (153) examined respiration in isolated coupled mitochondria from locust flight muscle and found oxygen consumption rates that exceed by threefold published values obtained in the most oxidative vertebrate muscle mitochondria (Table 1). The best explanation for this capability is the difference in oxygen delivery to the muscle cell. The high rates of oxygen consumption and substrate oxidation, along with the low efficiency of myofibrillar muscle (63), indicate that heat production occurs as a by-product of substrate flux stimulated by the hydrolysis of ATP, the result of cross-bridge cycling and  $\text{Ca}^{2+}$  cycling.

The muscle efficiency of insects during flight or stridulation is substantially lower than estimates for vertebrate muscle efficiency (63, 103, 151). Two types of muscle tissue power activities in insects, and they are classified as asynchronous and synchronous muscles (137) on the basis of the neural control of contraction. Synchronous and asynchronous muscles are capable of oscillating at high frequencies with asynchronous muscles reaching contraction frequencies of 1000 Hz (149) and synchronous muscles operating as high as 550 Hz during sound production in certain cicadas (105). Synchronous muscles are similar to vertebrate skeletal muscle; each action potential results in depolarization and contraction. Asynchronous muscle differs significantly from the vertebrate skeletal muscles in that rapid contraction oscillations are obtained from a single action potential. Asynchronous muscles structurally offer a paradox when considering how and why so much heat is generated. The muscle is characterized by an almost complete lack of SR and transverse T-tubules, the energetically costly (and thus thermogenic) components of most vertebrate muscles that operate at high frequencies for long periods. Morphometric analysis suggests that asynchronous muscle appears to offer the advantage of high frequency operation without the high operating costs of synchronous muscles. In synchronous muscle, high frequency operation is obtained by expanding the surface area available for the proteins that sequester and release calcium. Hypertrophy of the SR is associated with a reduction of myofibrillar volume (22% myofibril in *O. vanduzeei*). Comparison of similar frequency tymbal muscles, one asynchronous and the other synchronous, indicates that the fibrillar muscle has more myofibrillar cross-section (49% myofibril; 3% SR) by volume than the cicada using synchronous muscles (104, 105). Thus although it costs more metabolically for  $\text{Ca}^{2+}$  cycling in the synchronous muscle, cross-bridge cycling in the asynchronous muscle, because of a larger

component of myofibrils, must be significantly higher. Both types of muscles presumably have high operating costs, and this is reflected in the high mitochondria volumes of both muscle fiber types (39–42% of fiber volume). The mitochondrial volumes reflect substantial capacities for adenylate turnover, and it would be interesting to determine whether similar amounts of heat are generated as a consequence of their use.

In synchronous muscles, as in vertebrate fast-twitch oxidative muscles, a large fraction of the muscle cell volume is devoted to SR packed with the  $\text{Ca}^{2+}$  ATPase (102, B Block, personal observation). The mitochondrial volume is also large (44% of myofibril volume) as a result of the metabolic expense of cycling  $\text{Ca}^{2+}$ . The overall result is a reduction in available myofibrillar volume. Stridulation requires less tension development, and thus the reduction of contractile protein can be tolerated. Although the volume of muscle associated with cross-bridge cycling has been reduced, the energetic cost of achieving high frequency is shifted to the use of SR  $\text{Ca}^{2+}$  cycling, and the energetic expense is relatively high as evidenced by the numerous mitochondria, as discussed above. As in vertebrate FOG fibers, the cost of producing sustained high frequency contraction is elevated because of  $\text{Ca}^{2+}$  transport demands. Temperature recordings of singing Katydid indicate the large amounts of heat generated during singing when contraction frequencies are  $150 \text{ s}^{-1}$ . Such muscles, with their low myofibrillar volumes and high SR and mitochondrial content, provide a model system for studying the thermogenic output associated with  $\text{Ca}^{2+}$  cycling.

## CONCLUSION

This brief review of muscular thermogenesis in animals leads to the question as to whether there are any indications of similar thermogenic mechanisms across the animal kingdom? Other than shivering, there remains some doubt. However, a common theme is that ATP-dependent cycling of  $\text{Ca}^{2+}$  between the SR and the cytosol is emerging as a key pathway for muscular thermogenesis. The discovery of the SR  $\text{Ca}^{2+}$  release channel, and more recently the role of ligands that may alter the permeability of SR to  $\text{Ca}^{2+}$ , provide an interesting direction for future research into muscular as well as non-muscular thermogenesis. There is substantial evidence suggesting that thermogenesis in skeletal muscle may be linked to aerobic fast-twitch fiber types, changes in the permeability of SR membrane to cations and ATP utilization by the  $\text{Ca}^{2+}$  pump, and increased substrate oxidation rates in the mitochondria. As power requirements for locomotion increase with continual need for speed, a general reliance among animals on the fast-twitch, FOG fiber type (or similar phenotype such as the synchronous muscle fibers of insects) is apparent. Similarly, the use of FFA as a fuel increases. Aerobic

fiber types are predisposed for ATP production required for sustained thermogenesis. The preferential use of FFAs by such fiber types may be the endogenous thermogenic effector that links the ATP-generating potential with a stimulatory effector (Figure 1c). FFAs have often been hypothesized to be the second messenger for activation of thermogenesis. In mammalian thermogenesis studies, FFAs were originally proposed to have had uncoupling effects on mitochondria (87, 138). In mammalian BAT, norepinephrine stimulates lipolysis that results in the release of FFAs. The FFAs regulate, or act, as endogenous agents controlling the activity of the uncoupling protein, a proton translocator. Similarly, recent research is suggestive of a role of FFAs in regulating thermogenesis in cold-acclimated bird muscle via the SR  $\text{Ca}^{2+}$  release channel.

In certain vertebrates there is a generalized physiological set of responses to cold acclimation (42, 78). In cold-acclimated animals, whether they are fish or mammals, mitochondrial volume, lipid content, and sarcoplasmic reticulum volume change in a fiber-type dependent manner (8, 33, 59, 147). The morphological changes are associated in ectothermic animals with maintaining the ATP supply as temperature declines. Such changes would have less importance for endotherms maintaining a constant temperature. The increase of oxidative capacity and SR volume in endotherms may promote heat production via shivering and nonshivering thermogenesis. Thermogenic mechanisms in muscle tissue may be built upon a phylogenetically primitive physiological response in vertebrates (increase of cytoplasmic SR and mitochondrial components in response to cold) and a modification of the pathway for wasting energy as heat may be occurring in endotherms (7). Current knowledge of the role of FFAs on increasing the permeability of SR in mammals suggests that a basic thermogenic pathway is available if long chain fatty acids are, in fact, stimulating the open probability of the SR  $\text{Ca}^{2+}$  release channel. While  $\text{Ca}^{2+}$  release increases catabolic processes that may be important for delivering ATP for power, there must be a simultaneous increase in heat production. Whether there is an endogenous thermogenic effector in muscle (hormonally or sympathetically mediated) that, when released, increases the permeability of the SR via the SR  $\text{Ca}^{2+}$  release channel, thereby increasing the heat production via futile calcium cycling during periods of rest for thermogenic purposes (i.e. nonshivering thermogenesis), remains to be elucidated.

Several lines of evidence point to a common pathway of thermogenesis in skeletal muscle involving the proteins responsible for  $\text{Ca}^{2+}$  release and reuptake, lipids and mitochondria. Within the next decade, our current knowledge of the molecular channels and pumps in muscle cell  $\text{Ca}^{2+}$  homeostasis should permit the deciphering of the exact pathway for nonshivering thermogenesis in skeletal muscle. Muscles are built along a similar

plan across animals. As we define the pathways and generate hypotheses, we should use the power of the comparative method for strengthening our knowledge of the role of muscle as a furnace.

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