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Long-term fasting decreases mitochondrial avian UCP-mediated oxygen consumption in hypometabolic king penguins

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Rey B, Halsey LG, Dolmazon V, Rouanet J-L, Roussel D, Handrich Y, Butler PJ, Duchamp C. Long-term fasting decreases mitochondrial avian UCP-mediated oxygen consumption in hypometabolic king penguins. *Am J Physiol Regul Integr Comp Physiol* 295: R92–R100, 2008. First published May 21, 2008; doi:10.1152/ajpregu.00271.2007.—In endotherms, regulation of the degree of mitochondrial coupling affects cell metabolic efficiency. Thus it may be a key contributor to minimizing metabolic rate during long periods of fasting. The aim of the present study was to investigate whether variation in mitochondrial avian uncoupling proteins (avUCP), as putative regulators of mitochondrial oxidative phosphorylation, may contribute to the ability of king penguins (*Aptenodytes patagonicus*) to withstand fasting for several weeks. After 20 days of fasting, king penguins showed a reduced rate of whole animal oxygen consumption ($\dot{V}O_2$; −33%) at rest, together with a reduced abundance of avUCP and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1-α) mRNA in pectoralis muscle (−54%, −36%, respectively). These parameters were restored after the birds had been refed for 3 days. Furthermore, in recently fed, but not in fasted penguins, isolated muscle mitochondria showed a guanosine diphosphate-inhibited, fatty acid plus superoxide-activated respiration, indicating the presence of a functional UCP. It was calculated that variation in mitochondrial UCP-dependent respiration in vitro may contribute to nearly 20% of the difference in resting $\dot{V}O_2$ between fed or refed penguins and fasted penguins measured in vivo. These results suggest that the lowering of avUCP activity during periods of long-term energetic restriction may contribute to the reduction in metabolic rate and hence the ability of king penguins to face prolonged periods of fasting.

metabolic rate; mitochondria; oxidative capacity

KING PENGUINS (*Aptenodytes patagonicus*) are semialtricial seabirds that successfully inhabit and breed in the southern latitudes. This is despite the energetic constraints imposed by their cold marine environment and their unusual reproductive cycle (longer than a year) (65).

On land, these constraints include prolonged periods of fasting (11, 12) coupled with high energy demands during moulting (9, 57) and chick rearing (65) and exposure to extreme operative temperatures (sometimes below the zone of thermoneutrality in adults) (4) due to cold ambient temperatures and high wind speeds (35). It may be that king penguins have some control over the energetic processes that affect metabolic rate and heat production during periods on land to spare energy reserves and so enhance breeding success. Such control could be achieved through adjusting the efficiency of mitochondrial oxidative phosphorylation by modulating, at least to some degree, the conductance of the mitochondrial inner membrane to protons.

Indeed, in endotherms, uncoupling of oxidative phosphorylation related to the leaking of mitochondrial protons is estimated to account for 20% of basal metabolic rate in rodents (49) and up to 50% of oxygen consumption in perfused rat muscle (51). It follows that a large part of oxygen consumption and energy derived from substrate oxidation does not directly contribute to anabolism or any metabolic process that requires ATP but rather is dissipated as heat (43, 50). In rodents, uncoupling oxidative phosphorylation is clearly devoted to heat production in brown adipose tissue through the tissue-specific expression of uncoupling protein-1 (UCP1) contributing to adaptive nonshivering thermogenesis (9). Hence, UCP1 increases the mitochondrial proton leak and thus contributes to adaptive processes of nonshivering thermogenesis in situations of high energy demand, such as cold exposure and thyroid hormone administration (9). In contrast, mammalian UCP1 expression is decreased a during long period of undernutrition along with a depressed metabolic rate in rats (52) but also during fasting in arctic ground squirrels (3).

Birds do not have brown adipose tissue (7, 37) but have the ability to improve the thermogenic capacity of their skeletal muscles through shivering thermogenesis and adaptive nonshivering processes after cold acclimation (5, 6, 20, 53). Among the potential thermogenic mechanisms involved in nonshivering thermogenesis by birds, is an increase in the dissipation of mitochondrial energy (18, 20, 53). This may be achieved through the upregulation of specific mitochondrial protein carriers, such as the avian homologue of mammalian UCPs (avUCP) (48). The main biological role of this protein is still unclear, but a body of experimental data suggests a link with increased energy expenditure. Indeed, avUCP mRNA is upregulated in skeletal muscles of ducks and chickens after cold exposure, or administration of thermogenic hormones, such as glucagon or thyroid hormones (13, 48, 62), which are conditions known to be associated with increased energy expenditure. Moreover, previous experiments conducted on juvenile king penguins found an increase in both avUCP expression and activity once the birds were adapted to cold sea water

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(59). Finally, it was found that adult chickens, which had been selected for high levels of food intake but low efficiency for growth, had higher levels of avUCP mRNA expression than chickens selected for low food consumption (48). This indicates a link between avUCP and energetic efficiency.

Given that expression and activity of avUCP are associated with thermogenic enhancement of energy dissipation from the skeletal muscle (16) and may be influenced by nutritional state (48), a reduction in avUCP activity may contribute to the reduction in energy metabolism observed in fasted birds including penguins (4, 17, 24, 38). The specific expression and activity of avUCP in skeletal muscle may play a significant metabolic role. Indeed, skeletal muscle contributes to a large proportion of whole animal energy expenditure [up to 30% in resting ducks (19)] on account of its relative mass and its high oxidative capacities.

Comparative approaches may help to clarify the potential roles of avUCP in fasting energy expenditure in birds. Indeed, avUCP is thought to reduce the production of reactive oxygen species (ROS) from mitochondria of short-term fasting chickens (1). Accordingly, avUCP-mediated proton leaks would allow *1*) heightened activity of the mitochondrial respiratory chain concomitant with a decrease in local oxygen tension and *2*) a decrease in the reverse electron flux through complex I of the respiratory chain by lowering the protonmotive force (56). However, such avUCP-dependent increases in proton conductance might generate an extra wastage of energy that has not been evaluated so far.

In the present study, we investigated whether a reduction of skeletal muscle avUCP activity can contribute to the reduction of metabolic rate during long-term fasting in king penguins. We analyzed parallel changes in whole body metabolic rate and body temperature, relative muscle expression of avUCP, and mitochondrial oxygen consumption attributable to avUCP activity in relation to changes in nutritional state. Expression of peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1-α) mRNA was also measured. This is a multifunctional coactivator that plays a key role in the transcriptional program of adaptive thermogenesis, fatty acid utilization, and stimulates expression of UCP (47). The activities of antioxidant enzymes [SOD, catalase (CAT), and glutathione peroxidase (GPX)] were determined to assess muscle oxidative status between groups. The potential implications of avUCP in terms of king penguin physiology is discussed.

EXPERIMENTAL PROCEDURES

The present study was carried out on Possession Island (46°25′ S, 51°45′ E), Crozet Archipelago, during the austral summer of 2004–2005. Ethical approval for all procedures was granted by the ethics committee of the French Polar Research Institute (IPEV, programme 131) and of the Ministère de l'Environnement. The requirements of the United Kingdom (Scientific Procedures) Act 1986 were also followed. Moreover, our procedures conformed to the Code of Ethics of Animal Experimentation in Antarctic.

Animals. Experiments were conducted on nonbreeding adult king penguins of both sexes, captured during early courtship (i.e., having recently arrived at the beach after a foraging trip at sea) in the colony at Baie du Marin [~30,000 breeding pairs; (65)].

Body temperature and whole animal rate of oxygen consumption during rest ($\dot{V}O_2$) were measured in eight penguins (*group A*; mean body mass ± SE at time of capture 14.0 ± 0.2 kg). A custom-built data logger (67) was implanted into the abdominal cavity of each bird, following procedures described elsewhere (24, 27). After implantation, the penguins were placed in an open-top holding pen for ~2 wk. They were then released into the colony where they had been captured during courtship activity, and their body masses were such that they returned to sea to restore their body reserves (28). Within 24 h of returning to the colony after their foraging trip, they were recaptured and put back in the pen. At this time the birds were deemed to be in a fed state (24). The birds were left to fast for 20 ± 1 days. At the end of this fasting period, the birds were considered to be in a fasted state. They were then fed defrosted Atlantic mackerel (*Scomber vernalis*), up to 1 kg/day for 3 days, and were deemed to be in a refed state. When the birds were first recaptured (*group A*, fed), at the end of the fasting period (*group A*, fast) and 24 h after being refed (*group A*, refed), resting $\dot{V}O_2$ was measured using indirect calorimetry. The procedures for the removal of the logger and for the replacement of the bird in the colony after surgery were similar to those described for implantation (24, 27).

Molecular and biochemical studies were undertaken on three other groups of penguins (*groups B1, -2, -3*). In *group B1*, birds were fed a meal of Atlantic mackerel for three successive days starting from and including the day of capture (fed group, $n = 6$). The second set of six birds (*group B2*) was fed in the same way and then left to fast for 2 wk (fasted group). *Group B3* was treated in the same way as those in the fasted group but were then refed for 3 days (refed group, $n = 5$). At the end of each feeding protocol (mean body mass of B groups, 11.6 ± 0.2 kg) a 4–5 g biopsy was taken under isoflurane-induced anesthesia, from the superficial pectoralis muscle of each bird. A small subsample (~200 mg) was immediately frozen in liquid nitrogen and kept at −80°C for molecular analysis. The majority of the biopsy was then taken for biochemical analysis, which was conducted immediately after the bird had recovered from the procedure. After surgery, the penguins were monitored for several days and then released at the site of their capture.

Data loggers. The data loggers recorded three temperatures: *1*) upper abdominal, i.e., caudal to the heart; *2*) midabdominal; and *3*) lower abdominal, i.e., within the vicinity of the brood patch at a frequency of 0.2 Hz. After the loggers were retrieved, the temperature records for the periods when resting $\dot{V}O_2$ was obtained in each respirometer experiment (see below) were extracted.

Measurements of resting $\dot{V}O_2$ (group A). Birds were placed in a respirometer chamber connected to a gas analysis system (details given in Fahlman et al., 23) and allowed to rest for at least 2 h. During experiments, oxygen and carbon dioxide gas partial pressure, airflow through the respirometer, humidity, temperature, and pressure were processed and sampled every 2 s. All flows were corrected to standard temperature (273 K) and pressure (101.3 kPa) dry. Resting $\dot{V}O_2$ was measured as the lowest 5 min period of $\dot{V}O_2$ recorded during the respirometry experiment.

Determination of the mRNA expression of avUCP and avPCG-1α (groups B1, -2, -3). Expression of avUCP mRNA was assessed by RT-PCR following the procedure previously described for king penguins (60). Briefly, 1 μg of total RNA extracted (Trizol, Invitrogen) from 100 mg of frozen muscle sample was used for RT. This reaction was followed by a PCR performed on 2.5 μl of the resulting cDNA by adding 5 μl PCR buffer, 1.5 mM $MgCl_2$, 0.3 mM dNTP, 1 mM avUCP primers, 5′-GTGGATGCCTACAGGACCAT-3′ (sense), and 5′-ATGAACATCACCACGTTCCA-3′ (antisense), in a Hybaid thermocycler [denaturation at 94°C for 2 min followed by 28 PCR cycles (denaturation at 94°C for 45 s, annealing 59°C for 45 s, and extension at 72°C for 60 s)]. A final 72°C elongation step was performed for 10 min. β-actin was used as an internal standard and was coamplified with specific primers (sense: 5′-TGCGTGACATCAAGGAGAAG-3′ and antisense: 5′-TGCCAGGGTACATTGTGGTA-3′ primers with 24 cycles of PCR). AvPGC-1α mRNA was amplified (28 cycles of PCR, primer annealing at 60°C) using the following primers: 5′-GACTCAGGTGTCAATGGAAGTG-3′(sense) and 5′-ATCAGAAGCCCTGTGGT-3′ (antisense) (63).

RT-PCR analysis led to the amplification of specific fragments of the expected size (389 pb and 271 pb) according to the published partial sequence of avUCP (GenBank accession no. AY592972) and PGC1-α (GenBank accession no. AB170013).

Isolation of mitochondria and mitochondrial oxygen consumption (groups B1 -2, -3). Intermyofibrillar mitochondria were isolated from 3–4 g of pectoralis muscle by a standard extraction protocol as described previously (18, 59). This involves potter homogenization, partial protease digestion, and differential centrifugation in an ice-cold extraction buffer (100 mM sucrose, 50 mM KCl, 5 mM EDTA, 50 mM Tris base, pH 7.4). Extracted mitochondria were resuspended in a final medium (250 mM sucrose, 20 mM Tris base, and EGTA, pH 7.4) and the amount of mitochondrial protein was determined (Biuret method; see Ref. 59).

Intermyofibrillar mitochondrial oxygen consumption was measured polarographically using a 1.5-ml Clark type electrode (Hansatech). A suspension of 0.35 mg/ml of muscle mitochondria was equilibrated in an air-saturated medium (402 nmol O/ml) containing 120 mM KCl, 5 mM KH_2PO_4, 2 mM $MgCl_2$, 1 mM EGTA, 3 mM HEPES (pH 7.4). Measurements were conducted at 38°C in the presence of 5 μM rotenone, 1 μg/ml oligomycin, 0.3% fatty acid-free BSA, and 5 mM succinate as a substrate of the respiratory chain. When indicated, 50 μM xanthine and 0.01 U/2.5 ml xanthine + xanthine-oxidase (XXO) were added to generate superoxide, which is known to activate UCP in the presence of free fatty acids (21, 60). In the present study, we used 150 μM of palmitate. To quantify the contribution of avUCP in mitochondrial respiration, measurements were then performed in the presence of 1 mM guanosine diphosphate (GDP), a known UCP inhibitor (58). A typical experiment is illustrated in Fig. 1.

To test the integrity and functionality of our mitochondrial preparations, we calculated the respiratory control ratio (RCR) as an indicator of the damage to mitochondria during isolation. Briefly, mitochondria were incubated with 5 μM rotenone and 5 mM succinate in the assay medium, and respiration was measured in the presence of 100 μM ADP (phosphorylating state of respiration or *state 3*), 1 μg/ml oligomycin (nonphosphorylating state of respiration or *state 4*) and 2 μM FCCP (fully uncoupled state of respiration).

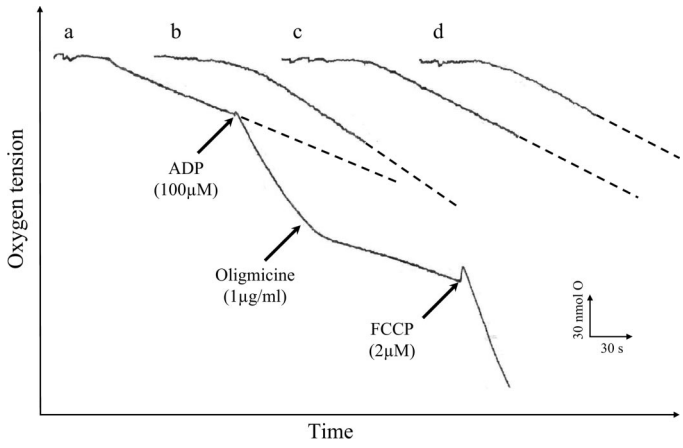

Fig. 1. Representative experimental traces of oxygen consumption by muscle mitochondria from fed penguins. All presented traces were measured in mitochondria (0.35 mg mitochondrial protein/ml) respiring on succinate (5 mM) in the presence of rotenone (5 μM); see EXPERIMENTAL PROCEDURES section for further details. *Trace a* shows the oxygen consumption procedure used to calculate the respiratory control ratio (RCR), e.g. the ratio between the phosphorylating state of respiration measured in the presence of 100 μM ADP and the nonphosphorylating state of respiration measured in the presence of 1 μg/ml oligomycin. *Traces b* and *c* show the rate of nonphosphorylating O_2 consumption in the presence of oligomycin (1 μg/ml), xanthine (50 μM), xanthine oxidase (0.01 U/2.5 ml), and palmitate (150 μM) in the absence (*trace b*) or in the presence of 1 mM GDP (*trace c*). *Trace d* shows the rate of nonphosphorylating oxygen consumption in the presence of 1 mM GDP alone. These components were successively added in the assay medium (Fig. 1, *trace a*). Respiratory control ratios were calculated as the phosphorylating (*state 3*)/nonphosphorylating (*state 4*) respiration ratio and the uncoupled respiration (FCCP)/nonphosphorylating (*state 4*) ratio.

Measurement of cytochrome c oxidase activity and determination of muscle mitochondrial content (groups B1, -2, and -3). Cytochrome *c* oxidase (COX) activity was measured polarographically at 38°C on intermyofibrillar mitochondria and tissue homogenates that were sampled during early stage of mitochondrial extraction (after elimination of the subsarcolemmal fraction) according to Barré et al. (7).

Mitochondrial content of penguin muscle (milligrams intermyofibrillar mitochondria per gram muscle) was then calculated from the intermyofibrillar mitochondria amount extracted from sampled biopsy (determined by the Biuret method, see Ref. 59) divided by the isolation yield calculated as the ratio between mitochondrial and tissue homogenate COX activity. In the following text, "mitochondrial content" will refer to the intermyofibrillar fraction of the mitochondria.

The calculated avUCP-dependent mitochondrial respiration and muscle mitochondrial content were then used to estimate the contribution of avUCP in the standard metabolic rates of penguins in different nutritional status.

Measurement of antioxidant enzyme activity (SOD, GPX, and CAT) (groups B1, -2, and -3). Enzyme activity was determined as described previously (55). Skeletal muscle was homogenized at 4°C, in buffer containing 100 mM KH_2PO_4, 1 mM DTT, and 2 mM EDTA at pH 7.4 and centrifuged (3,000 *g* for 5 min). The supernatant was used for enzymatic assays. SOD activity was assayed by monitoring the rate of acetylated cytochrome *c* reduction by superoxide radicals generated by the xanthine-xanthine oxidase (XXO) system (25). One activity unit of SOD was defined as the amount of enzyme that inhibits the rate of acetylated cytochrome *c* reduction by 50%. To distinguish MnSOD, located in the mitochondrial matrix, from Cu,Zn-SOD, which is primarily located in the cytosol, SOD activity was determined after incubation with 1 mM NaCN. At this concentration, cyanide inhibits the Cu,Zn isoform of the enzyme but does not affect the Mn isoform (25). The assay for total activity of GPX coupled the reduction of cumene hydroperoxide to the oxidation of NADPH by glutathione reductase, and this coupled reaction was monitored at 340 nm. CAT activity was determined by the method of Aebi (2). This technique uses the first-order rate constant of the decomposition of H_2O_2 by tissue CAT at 20°C. One unit of catalase activity was calculated by using $k = (2.3/dt)(\log A1/A2)$, where k is CAT activity, dt is change in time, A1 is initial absorbance, and A2 is final absorbance. All enzyme activities are expressed in U/mg of proteins.

Statistical analysis. Values are presented as means ± SE. Statistical significance of observed variations was assessed by ANOVA for independent (B groups) or repeated values (*group A*) followed by single or paired Student's *t*-tests. The Mann-Whitney nonparametric test for independent values was performed on small data sets. Statistical significance was assumed at $P \leq 0.05$.

RESULTS

Nutritional state, bird $\dot{V}O_2$, and body temperature (group A). As expected, fasted penguins had a lower mass than fed birds (10.6 ± 0.1 vs. 13.0 ± 0.3 kg; see Table 1). Critical body mass, i.e., body mass at the beginning of *phase 3* of fasting and estimated from beak length as an index of structural size (34), was not reached by any of the experimental birds. It follows that all fasted birds were in *phase 2* of fasting (32).

Table 1 shows that resting $\dot{V}O_2$ was 33% lower ($P < 0.01$) in birds after 20 days of fasting than in fed birds. The refeeding of fasted penguins was associated with a significant increase in resting $\dot{V}O_2$ (+16%; $P < 0.01$), although the new value was still significantly lower than resting $\dot{V}O_2$ in fed birds.

Table 1. *Resting rate of oxygen consumption ($\dot{V}O_2$; ml oxygen/min, standard temperature and pressure dry), body mass (kg), and internal body temperature (°C) of king penguins in 3 nutritional states (group A)*

	Fed	Long-term Fasting	Refed
Resting $\dot{V}O_2$	124.6±8.7 (6)	83.1±4.9[a] (7)	96.1±7.8[a,c] (6)
Body mass	13.04±0.24 (8)	10.61±0.13[a] (8)	10.63±0.18[a] (8)
Lower abdominal temperature	40.9±0.8 (6)	39.3±0.7[b] (6)	39.6±0.9 (6)
Middle abdominal temperature	38.7±0.7 (7)	38.2±0.8 (7)	38.6±0.8 (7)
Upper abdominal temperature	40.9±1.0 (7)	40.5±1.0[b] (7)	40.8±1.0[d] (7)

Values are means ± SE. Values in parentheses are the number of experimental determinations. [a]$P < 0.01$ vs. fed, [b]$P < 0.05$ vs. fed, and [c]$P < 0.01$ vs. long-term fasting birds, [d]$P < 0.05$ vs. long-term fasting.

Except for one experimented bird, fasted birds exhibited slight but significant decreases in lower abdominal (across all birds: −4%; $P < 0.05$) and upper abdominal (−1%; $P < 0.05$) temperatures compared with fed birds. The decreases in lower abdominal temperature and upper abdominal temperature were reversed by refeeding, with those in refed birds not being significantly different from those in fed birds. Middle abdominal temperatures did not change with nutritional status.

Effect of long-term fasting and refeeding on penguin avUCP and PGC1-α expression (B groups). As shown in Fig. 2*A*, the relative abundance of avUCP transcript in penguin pectoralis muscle was much lower in fasted than in fed birds (−54%, $P < 0.05$). The refeeding of fasted birds for 3 days caused a return of the relative abundance of avUCP mRNA to the level of fed birds. PGC1-α mRNA showed a pattern of expression similar to avUCP, i.e., a decrease in *phase 2* of fasting (−36%, $P < 0.05$) reversed by refeeding (+102%, $P < 0.01$) (Fig. 2*B*). There was no significant difference between fed and refed birds.

COX activity and mitochondrial content in pectoralis muscle (B groups). Measurement of COX activity showed no significant difference between nutritional status either in pectoralis muscle homogenates or in intermyofibrillar mitochondria (Table 2). The calculated mitochondrial content of penguin pectoralis did not change significantly with fasting (see Table 2), suggesting that metabolic adjustments to *phase 2* of fasting do not involve major modifications on muscle mitochondrial content.

Effect of fasting and subsequent refeeding on avUCP activation by superoxide in pectoralis mitochondria (B groups). As illustrated for fed penguins in Fig. 1, we first examined the quality of our mitochondrial preparations on mitochondria respiring on succinate in the presence of ADP and FCCP, successively. Mean respiratory control ratio (RCR) was 4.21 ± 0.19 and was not significantly modified either by fasting or by refeeding ($P = 0.24$; Table 2), which is indicative of the good functional integrity of our mitochondrial preparations. Equally, the FCCP/*state 4* respiration ratio was 7.18 ± 0.57 and was not significantly altered by nutritional status ($P = 0.16$; Table 2). On the whole, these results suggest that, without stimulation of avUCP, mitochondria from all groups have a great ability to enhance their respiration in response to ADP-induced phosphorylation or FCCP-mediated uncoupling activity.

Mitochondrial $\dot{V}O_2$ was then measured after the addition of oligomycin, a known ATP synthase inhibitor. In the presence of oligomycin, mitochondrial $\dot{V}O_2$ is proportional to the rate at which protons leak across the mitochondrial inner membrane. Therefore, the energy released by succinate oxidation does not lead to ATP production but rather is dissipated as heat. Present results showed no significant difference in mitochondrial basal $\dot{V}O_2$ between different nutritional states (Fig. 3).

To estimate UCP activity in vitro, exogenous superoxide, which was generated by the enzymatic reaction of XXO, and fatty acids (21, 60) were then applied simultaneously. Compared with fasted birds, mitochondrial $\dot{V}O_2$ was greatly increased in mitochondria from fed and refed birds in the presence of both superoxide and 150 μM fatty acid (Fig. 3). The activation by superoxide was mostly reversed by the

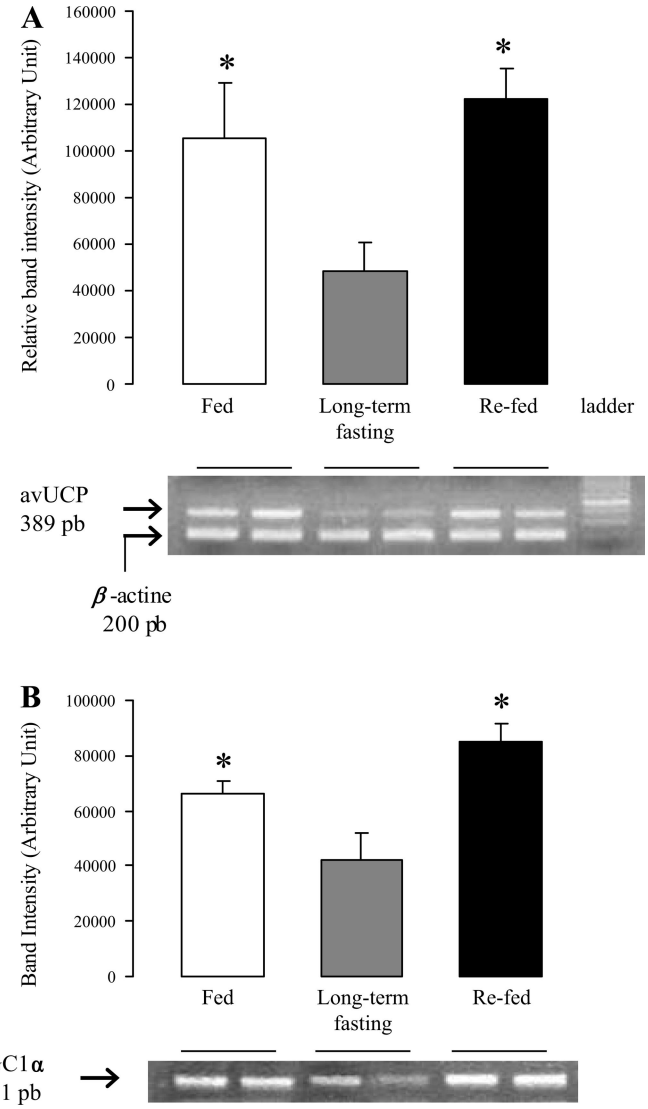

Fig. 2. Relative abundance of avian uncoupling protein (avUCP; *A*) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1-α; *B*) mRNA in king penguin pectoralis muscle assessed by RT-PCR (B groups). Data are means ± SE for 6 (fed), 5 (long-term fasting), and 6 (refed) independent preparations. A representative gel for 2 birds from each condition is shown. *Significant difference in mRNA relative abundance between long-term fasting birds vs. fed or refed birds at $P \leq 0.05$.

Table 2. *Cytochrome c oxidase (COX) activity (tissue homogenates and mitochondrial fraction), intermyofibrilar mitochondrial content, and mitochondrial parameters (respiration control ratio, FCCP/state 4 respiration rate) in pectoralis muscle from king penguins in 3 nutritional states (B groups)*

	Fed	Long-term Fasting	Refed
No. independent experimental determinations	4	3	4
COX activity measured on tissue homogenate, nmol $O \cdot mg^{-1}$ weight tissue$\cdot min^{-1}$	57.8±3.6	55.4±3.3	54.2±2.1
COX activity from isolated mitochondria, nmol $O \cdot mg^{-1}$ protein$\cdot min^{-1}$	832±67	939±100	847±58
Yield of isolated mitochondria, mg protein/g weight muscle	2.83±0.23	2.74±0.09	2.36±0.16
Calculated muscle mitochondrial content, mg protein/g muscle in vivo	65.1±7.2	61.1±7.9	64.7±6.1
Respiratory control ratio	4.41±0.17	4.43±0.15	3.67±0.62
FCCP/*state 4*	8.59±0.50	6.85±0.62	5.73±1.62

Values are means ± SE.

addition of 1 mM of GDP (Fig. 3), a UCP inhibitor (21, 60). Thus, superoxide induced a GDP-sensitive extra oxygen consumption of 44 ± 17 and 25 ± 2 nmol oxygen/min^{-1}/mg mitochondrial $protein^{-1}$ in fed and refed birds, respectively (Fig. 3). This indicates that the uncoupling activity of avUCP, when fully activated, increased the basal proton conductance of muscle mitochondria by 68% and 47% in fed and refed birds, respectively. By contrast, no significant effect of XXO was observed with mitochondria from fasted birds and the addition of GDP in the presence of superoxide did not affect the uncoupled mitochondrial respiration. As mitochondria of all experimental groups responded strongly to ADP or artificial uncouplers (FCCP), the lack of XXO effect in mitochondria from fasted birds could not be explained by a loss of ability to increase the respiration rate in this group. These results indicate the presence of a functional UCP in fed and refed penguins but not in fasted birds.

As illustrated in Fig. 1, we also examined the proton conductance properties in the presence of 1 mM GDP but in the absence of stimulation by superoxides. Results presented in Fig. 3 show no differences in basal proton conductance in the presence or absence of GDP in all groups. This indicates that UCP does not contribute to basal proton leak in our in vitro conditions.

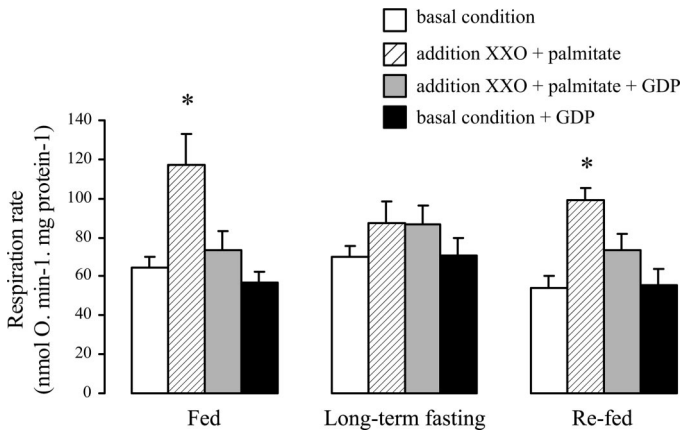

Fig. 3. Effect of UCP activators [xanthine + xanthine-oxidase (XXO)] and UCP inhibitors [guanosine diphosphate (GDP)] on mitochondrial O_2 consumption (B groups). Experiments were conducted on isolated mitochondria from pectoralis muscle in the presence of 5 μM rotenone, 1 μg/ml oligomycin, 0.3% BSA, and 5 mM succinate. In the basal condition, no significant differences were observed between groups. Mitochondrial respiration was greatly increased by the addition of both palmitate and XXO in mitochondria from fed and refed penguins, indicating the presence of a functional avUCP. This activation was prevented by the addition of a specific UCP inhibitor (1 mM GDP). Addition of GDP without stimulation by XXO did not change mitochondrial respiration. Results are means ± SE. *Significant difference ($P \leq 0.05$) of stimulated (+XXO and palmitate) compared with basal (XXO + palmitate + GDP) and basal + GDP conditions.

Antioxidant enzyme activity in pectoralis muscle (B groups). The activities of antioxidant enzymes are presented in Table 3. Two weeks of fasting resulted in a significant decrease in catalase activity ($P < 0.05$) in penguin pectoral muscle. After refeeding, the catalase activity was maintained at these significantly lower values compared with fed penguin ($P < 0.05$). By contrast, there were no detectable significant effect of fasting or refeeding in the activity of glutathione peroxidase, total SOD, Mn, and Cu,ZnSOD between groups.

DISCUSSION

The results of the present study show that the decreased rate of energy expenditure of long-term fasting penguins is associated with a reduced functional activity of an avian mitochondrial UCP that potentially decreases the metabolic activity of muscle mitochondria. These effects are rapidly reversed by refeeding.

Nutritional status, resting oxygen consumption, and body temperature. The present protocol allowed us to compare penguins in *phase 2* of fasting with birds that had recently fed for several days at sea and birds that had been refed for 3 days after a long (~20 day) fast. *Phase 2* of fasting is a period of low metabolic turnover reflected by low and steady specific daily mass losses (32). Present results showed a reduction in resting whole animal $\dot{V}O_2$ (−33%) in *phase 2*-fasted penguins compared with fed birds, confirming previous work (4, 17, 23).

Table 3. *Activities of antioxidant enzymes in pectoralis muscle of king penguin in three nutritional states (B groups)*

Enzymes	Fed	Long-term Fasting	Refed
CAT	19.0±1.4	13.4±1.5*	14.2±1.9*
GPX	8.1±1.2	9.7±1.3	8.6±1.1
SOD			
Total	8,465±3,091	9,937±2,643	7,370±1,024
Mn	1,202±246	1,727±448	1,662±376
Cu,Zn	7,263±3,033	8,210±2,427	5,707±1245

Enzyme activities were measured in pectoralis muscle from fed, long-term fasting, or refed king penguins. Catalase (CAT), glutathione peroxidase (GPX), and SOD were measured spectrophotometrically in tissue homogenates. Values are means ± SE from 6-8 animals and are expressed in U/g of protein. No significant effect of long-term fasting was detected. *$P < 0.05$ vs. fed birds.

Furthermore, the mass exponent for $\dot{V}O_2$ at rest in king penguins is considerably greater than 1 [1.89; (23)], indicating that there is a larger proportional decrease in resting $\dot{V}O_2$ than body mass during fasting. These reductions in whole animal $\dot{V}O_2$ may contribute to energy sparing and hence to enhance fasting ability. Fahlman et al. (23) suggested that the high mass exponent for $\dot{V}O_2$ is unlikely to be explained, at least entirely, by changes in body composition during fasting and speculated that decreases in body temperature might instead account for the reduced mass-specific $\dot{V}O_2$. While Fahlman et al. (24) reported no change in multiple abdominal temperatures during fasting, data in the present study show that the temperature of the lower and the upper abdominal regions were, respectively, 1.6°C and 0.4°C lower in *phase 2* fasting birds than in recently fed birds. These conflicting results underline the complexity of heterothermy previously described in penguins (36, 54); we thus cannot assume that there is always hypothermia in fasted penguins. However, marked hypothermia has been reported in long-term fasting king penguin chicks exposed to cold [−4°C (17)] and in diving king penguins (27, 36, 45, 54). Thus the reduction in local body temperatures during fasting exhibited by the birds in the present study may indicate some redistribution of blood flow and/or a reduction in metabolic rate. Such a strategy has been suggested previously for fasting king penguins (17, 61). These observations are in accordance with very recent data showing that, when fasting in the cold, emperor penguins that failed egg incubation might be able to save energy by decreasing their core temperature (measured in the abdominal cavity) (29).

The present results show that refeeding fasted birds reverses the decrease temperature in certain regions of the body and partially restores the reduction in resting $\dot{V}O_2$. Such a difference in resting $\dot{V}O_2$ between fasted penguins and fed or refed birds may be the result of an adjustment in the energy allocated to metabolic processes that dissipate heat.

Expression of avUCP mRNA and PGC1-α. Two weeks of fasting significantly reduced the mRNA level of skeletal muscle avUCP in king penguins (Fig. 2*A*), an effect that was reversed with 3 days of refeeding. It is known that mRNA expression and protein levels are not always closely related due to posttranscriptional and translational regulation (44). However, our data are in sharp contrast with reported increases in the expression of the skeletal muscle avUCP in short-term fasting birds (1, 22). Indeed, the latter studies reported a marked increase in skeletal muscle avUCP mRNA in chickens undergoing 1 or 2 days of food deprivation. Similarly, related mammalian muscle UCP3 mRNA was repeatedly found to be upregulated with short-term fasting, while mitochondrial proton conductance remained unchanged (8). One possible explanation for these discrepancies is that the duration of fasting is an important factor, with relatively short-term fasting stimulating expression of muscle avUCP and longer-duration fasting inhibiting muscle avUCP expression. Indeed, preliminary data for king penguins show that avUCP mRNA abundance is already significantly depressed after 4 days of fasting (unpublished data).

Factors controlling the lowering of avUCP mRNA after long-term fasting are unclear but may involve thyroid hormones. Indeed, a previous study reports that avUCP expression is upregulated by administration of thyroid hormones and that hypothyroid birds weakly express avUCP in their tissues (14). Moreover, a reduction in plasma T_3 hormone has been observed during periods of limited food availability and long-term fasting in a number of birds species including penguins (10, 39). Therefore, a reduction in plasma T_3 level may well contribute to lower avUCP mRNA expression in long-term fasting birds, as observed for penguins in the present study.

Although the muscle expression of avUCP and its sequence homology (73%) are close to those of mammalian UCP3, the present results also suggest that penguin avUCP could be more functionally related to brown adipose tissue UCP1. Indeed, UCP1 abundance and activity increase in response to cold exposure and decrease in fasting states in parallel with the thermogenic activity of the tissue (40). By contrast, rats fasted for 24 h showed an upregulation of skeletal muscle UCP3 mRNA (4-fold) and protein (2-fold) (8). Moreover, the observation that UCP3 knockout mice are cold tolerant (64) suggests that mammalian UCP3 may not play a significant thermogenic role in muscles.

As is apparent in Fig. 2*B*, PGC1-α expression followed the same pattern of expression as avUCP. This close correlation between UCP and PGC1-α expression is in accordance with previous studies conducted with birds and mammals (46, 63), suggesting that the transcriptional control by PGC-α might be the mechanism by which metabolic stress, such as cold exposure, induces modulation of avUCP transcript in bird skeletal muscle. In the present study, we extend these observations to birds in relation to their nutritional status. The implication of PGC1-α in mitochondrial metabolism, and to influence mitochondrial uncoupled respiration (57), in particular, suggest that it could represent an important parameter triggering the reduction in metabolism observed in fasted birds. More experimental data are required to support this hypothesis in penguins.

Contribution of avUCP activity to changes in $\dot{V}O_2$. At the subcellular level, differences in resting $\dot{V}O_2$ can be interpreted as differences in cellular respiration controlled by cell mitochondria. The present results show that the higher $\dot{V}O_2$ measured in fed and refed king penguins compared with that in fasted birds cannot be explained by changes in mitochondrial content of the muscle nor in basal mitochondrial $\dot{V}O_2$ in vitro, but can be related to modifications in expression and activity of muscle avUCP. In mice, superoxide activation of mitochondrial respiration through UCP3 in skeletal muscle mitochondria is prevented by GDP and absent in mitochondria from UCP3(−/−) mice (21). Thus the aforementioned observation in the mitochondria of penguin skeletal muscle strongly suggests that the superoxide-activated, GDP-sensitive oxygen consumption was mediated by avUCP. Present results based on activation of mitochondrial $\dot{V}O_2$ indicate that there is less functional UCP in muscle mitochondria of fasted penguins than in those of fed or refed birds, which is consistent with the reduction in the relative abundance of UCP mRNA.

Because of large differences in the partial pressure of oxygen (P_{O_2}) in in vitro experiments (P_{O_2}, ~20 kPa) compared with that in the intracellular microenvironment of mitochondria (P_{O_2} in cytosol, ~0.3–0.4 kPa, see Ref. 31), it may be inappropriate to extrapolate avUCP contribution to whole animal resting $\dot{V}O_2$ from absolute values of mitochondrial $\dot{V}O_2$ measured in vitro. However, if we assume that the percentages of variation of avUCP-related processes observed in Fig. 3 depend on avUCP expression and activity rather than on P_{O_2}, we

can estimate its consequences on variations of whole metabolic rate.

Muscle mitochondrial proton leak-related $\dot{V}O_2$ can be calculated from muscle mitochondrial content (expressed as mg protein/g^{-1} muscle; Table 2) multiplied by stimulated mitochondrial $\dot{V}O_2$ (expressed as nmol O·min^{-1}·mg $protein^{-1}$; Fig. 3; $\dot{V}O_{2mit}$ in the presence of fatty acids and superoxide minus $\dot{V}O_{2mit}$ in the presence of fatty acid, superoxide, and GDP) above basal (*state 4,* $\dot{V}O_{2mit}$). It appears that muscle mitochondrial proton leak-related $\dot{V}O_2$ was 45% lower in fasted birds compared with that in fed birds (3.9 ± 0.7 vs. 7.1 ± 1.3 μmol O·g $muscle^{-1}$·min^{-1}, respectively). Since basal $\dot{V}O_{2mit}$ rates did not differ between conditions, these changes in muscle $\dot{V}O_2$ were essentially due to changes in the activity of avUCP. If we assume that mitochondrial respiration in the presence of oligomycin (an indirect measurement of proton leak activity) accounts for up to 50% of muscle energy expenditure (51), and on the basis that skeletal muscle contributes to ~30% of the resting metabolic rate in birds (19) we can estimate that the observed decrease in mitochondrial avUCP-linked respiration would contribute to a decrease of almost 7% in whole body energy expenditure of fasted birds (0.5 × 0.45 × 0.3 = 6.75%). This represents nearly 20% of the fasting-induced reduction in metabolic rate [6.75% × $\dot{V}O_2$ fed = 20.3% × ($\dot{V}O_2$ fed − $\dot{V}O_2$ long-term fasting)].

Similarly, the activity of mitochondrial avUCP would lead to an increase in resting muscle rate of oxygen consumption of 24% in refed birds (5.1 ± 0.2 vs. 3.9 ± 0.7 μmol O·g $muscle^{-1}$·min^{-1}), which would slightly increase (0.5 × 0.24 × 0.3 = 3.6%) whole body energy expenditure when compared with that of fasted birds. Again, such an increase in energy expenditure represents ~20% of the refeeding rise in metabolic rate [3.6% × $\dot{V}O_2$ long-term fasting = 23% × ($\dot{V}O_2$ refed − $\dot{V}O_2$ long-term fasting)]. Thus, the present results indicate that up to 20% of the variation in resting metabolic rate of king penguins between the three nutritional conditions (fed, long-term fasting, refed) could be explained by changes in avUCP activity in skeletal muscle mitochondria. This figure might be an underestimate as mitochondrial respirations are not compared at similar membrane potentials. It has been previously shown that the activity of avUCP resulted in an increased oxygen consumption and a concomitant decrease in inner membrane potential under stimulated conditions (59, 60). For this reason, it is likely that the mitochondrial inner membrane would be decreased following the stimulation of avUCP activity, reaching lower values in fed and refed penguins than in fasting birds. This, in turn, implies that the above-calculated value for the contribution of avUCP in penguin metabolic rate is an underestimate, since mitochondria from fed and refed penguins would have to consume additional oxygen to maintain the same membrane potential as in fasting birds. On the other hand, the possibility must be considered that this figure is an overestimate as it is based on the assumption that UCP-related mechanisms would be maximally stimulated in resting conditions; however, this is unlikely to be the case. Moreover, maximal UCP stimulation was obtained by artificial exogenous addition of a supraphysiological dose of XXO that may potentially affect mitochondrial redox status, membrane integrity, and interfere with oxygen electrode measurements (15). Note however, that we did not notice any artefactual effect of the dose of XXO used on the oxygen electrode and that the observed XXO effect was reversed by GDP ruling out the possibility that it would be only artefactual. For all these reasons, the percentage of UCP contribution to global metabolic rate must therefore be considered with caution; however, our results suggest that activity of avUCP can modulate mitochondrial efficiency in muscle and has the potential to contribute significantly to changes in whole body $\dot{V}O_2$.

AvUCP acting as an antioxidant? In light of the present data and considering the concept of mild uncoupling as a line of antioxidant defense (56), a potential role of avUCP as a modulator of mitochondrial ROS production cannot be excluded. This hypothesis was first suggested by Talbot et al. in penguins (59) and received experimental support in chickens (1, 42). In the present experiment, the high metabolic rate and high substrate availability of fed or refed penguins is likely to induce oxidative stress, while long-term fasting may lower ROS production as a result of reduced metabolic rate. Consequently, the need for avUCP protein as an antioxidant defense may be reduced in fasting penguins. The reduction in catalase activity observed in fasted penguins indeed indicates a reduction in the need for antioxidant protection, thus supporting this interpretation. This is reinforced by a previous study which reported that 48-h fasting considerably reduces the antioxidant defenses in the blood of cockerels and pullets (41).

If the need for avUCP might be lowered during long-term fasting, conversely, the marked upregulation of avUCP in diving penguins (59) might therefore be linked to the metabolic stress induced by exposure to cold sea water and the resulting elevation of metabolism.

Whether the lowering of avUCP expression in fasting penguins represents an adaptive mechanism to reduce metabolic rate or a response to the decreased ROS production that might secondarily result from a decrease in the metabolic rate in fasted penguins remains unclear. Further, the activation of the protein in more physiological conditions also needs to be clarified. Nevertheless, the present study suggests that reduction of avUCP activity, whatever its primary cellular function, lowers the mitochondrial futile proton cycle providing energetic benefits that would contribute to optimizing the fasting capabilities of king penguins.

Perspectives and Significance

The reduction in both expression and functional activity of avUCP in skeletal muscle of adult king penguins in situations of low energy expenditure and its reversal by food intake, highlights a link between avUCP and energy dissipation by muscle mitochondria in agreement with previous studies (48, 59). These results provide biochemical and molecular clues to the reduction in energy expenditure observed which enables king penguins to withstand extensive periods of food deprivation.

Such an ability to reduce mitochondrial activity may also be of importance in other biological activities of king penguins, such as during foraging, being part of the explanation of their exceptional breath-holding capabilities (26). Increased duration of diving might be facilitated by reduced metabolic activity causing abdominal hypothermia in diving king penguins, with normothermia being restored when they are floating on the water surface (36). It might be postulated that diving hypothermia is facilitated by a progressive decrease in muscle metabolic activity related to a reduction in mitochondrial

proton leakage similar to that which occurs during fasting. This would reduce cellular heat dissipation and thus contribute to the observed local cooling of the abdominal cavity and pectoralis muscle (by several degrees Celsius during diving) to temperatures lower than those measured near the heart and the liver (54). Such cellular sparing of energy implies rapid variations in proton leaking and thus acute regulatory mechanisms. One can postulate that changes in cytosolic pH and/or P_{O_2} occurring in cells during dives may play a role as these factors were shown in vitro to modulate mitochondrial proton leak (30, 31, 66). For instance, a high efficiency of mitochondrial phosphorylation, together with a lowering of uncoupled respiration, were observed under hypoxic conditions (30, 31). A potential role of avUCP in these diving-related processes is also reinforced by the marked upregulation of avUCP expression after acclimation to sea-water in king penguin juveniles (59). Conversely, under adequate conditions of stimulation, activation of mitochondrial proton leaking may contribute to rewarming after diving sessions in a situation of low muscle contractions (33). Studies are clearly required to test these speculative hypotheses, although it is already apparent that exploring the mitochondrial mechanisms that control energy dissipating processes are of particular interest in understanding metabolic adaptations of living under conditions of harsh energetic constraints. With regard to avUCP, further experimental and comparative approaches are needed to clarify whether mitochondrial energy dissipation elicited by avUCP is a thermogenic adaptation or is simply a costly consequence of an antioxidant function.

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