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RESEARCH ARTICLE

# Skeletal muscle metabolism in sea-acclimatized king penguins. I. Thermogenic mechanisms

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## ABSTRACT

At fledging, king penguin juveniles undergo a major energetic challenge to overcome the intense and prolonged energy demands for thermoregulation and locomotion imposed by life in cold seas. Among other responses, sea acclimatization triggers fuel selection in skeletal muscle metabolism towards lipid oxidation *in vitro*, which is reflected by a drastic increase in lipid-induced thermogenesis *in vivo*. However, the exact nature of skeletal muscle thermogenic mechanisms (shivering and/or non-shivering thermogenesis) remains undefined. The aim of the present study was to determine *in vivo* whether the capacity for non-shivering thermogenesis was enhanced by sea acclimatization. We measured body temperature, metabolic rate, heart rate and shivering activity in fully immersed king penguins (*Aptenodytes patagonicus*) exposed to water temperatures ranging from 12 to 29°C. Results from terrestrial pre-fledging juveniles were compared with those from sea-acclimatized immature penguins (hereafter 'immatures'). The capacity for thermogenesis in water was as effective in juveniles as in immatures, while the capacity for non-shivering thermogenesis was not reinforced by sea acclimatization. This result suggests that king penguins mainly rely on skeletal muscle contraction (shivering or locomotor activity) to maintain endothermy at sea. Sea-acclimatized immature penguins also exhibited higher shivering efficiency and oxygen pulse (amount of oxygen consumed or energy expended per heartbeat) than pre-fledging juvenile birds. Such increase in shivering and cardiovascular efficiency may favor a more efficient activity–thermoregulatory heat substitution providing penguins with the aptitude to survive the tremendous energetic challenge imposed by marine life in cold circumpolar oceans.

**KEY WORDS: Endothermy, Marine birds, Metabolic rate, Non-shivering thermogenesis, Shivering**

## INTRODUCTION

King penguins (*Aptenodytes patagonicus*) are diving endotherms that spend most of their life foraging over long distances in the cold circumpolar ocean (Charrassin and Bost, 2001; Pütz, 2002; Orgeret et al., 2019). At the end of their post-hatching development, which lasts 12–14 months (Cherel et al., 2004), terrestrial chicks molt and acquire tightly packed, waterproof feathers. Thereafter, juveniles must face the high energy costs imposed by the passage from shore to marine life due to the greater specific heat capacity and thermal conductivity of water compared with these properties of air. This early life at sea is a critical period for the survival of juveniles (Orgeret et al., 2016). To survive, the development of adaptive responses to reduce heat losses, as well as to sustain the energy requirements imposed by endurance swimming and the maintenance of endothermy in cold water, represents a key step in the nutritional emancipation of juvenile penguins. At sea, heat losses and the daily metabolic costs of thermoregulation are reduced by penguins' ability to develop powerful peripheral vasoconstriction, regional hypothermia and a thick layer of subcutaneous fat (Dumonteil et al., 1994; Handrich et al., 1997; Lewden et al., 2017a; Enstipp et al., 2017). However, acute immersion in cold water induces up to a threefold increase in penguin resting metabolic rate (Barré and Roussel, 1986; Stahel and Nicol, 1988; Kooyman et al., 1992; Culik et al., 1996; Fahlman et al., 2004, 2005; Rey et al., 2016), while repeated immersions of juveniles in cold water or sea acclimatization trigger an increase in their thermogenic capacity (Barré and Roussel, 1986; Teulier et al., 2012, 2016; Rey et al., 2016). Hence, the survival of penguins in cold water also depends on their ability to sustain high levels of energy expenditure and heat production over long periods. However, the nature of their regulatory heat production in water has yet to be investigated.

Skeletal muscle contributes 70–80% of the regulatory heat production induced by cold or hormonal treatments, which involve both shivering and non-shivering processes (Hissa, 1988; Duchamp et al., 1999). On the whole, the thermogenic capacity of birds is concomitant with the development of skeletal muscle mass and increases in muscle aerobic activity, lipid oxidation capacity and mitochondrial oxidative phosphorylation intensity (Vaillancourt et al., 2005; Teulier et al., 2010, 2012; Swanson and Vézina, 2015). In penguins, the development of facultative non-shivering thermogenesis in skeletal muscle reaches its maximum capacity in response to the harsh cold winter in 6- to 8-month-old king penguin chicks (Duchamp et al., 1989) or in anticipation of marine life, as seen in terrestrial pre-fledging Adélie penguins (Dégletagne et al., 2013). Muscle non-shivering thermogenesis is regulated by fatty acids or their acyl ester derivatives (Duchamp et al., 1999; Talbot et al., 2004). These metabolites trigger heat production by enhancing intracellular membrane permeability and subsequent futile cycling of ions, such as the ATP-consuming calcium cycle across the sarcoplasmic reticulum membrane (Block, 1994; Duchamp et al., 1999) or the energy substrate-consuming proton cycle across the mitochondrial inner membrane (Skulachev and Maslov, 1960; Roussel et al., 1998; Toyomizu et al., 2002; Talbot et al., 2004; Rey et al., 2010).

Sea acclimatization in immature king penguins has been shown to be characterized by the fuel selection of skeletal muscle towards lipid oxidation and the increased thermogenic effect of lipids *in vivo* (Teulier et al., 2012) and of skeletal muscle mitochondria *in vitro* (Rey et al., 2017). This adaptive lipid-related thermogenesis has been linked with the high capacity of muscle mitochondria to oxidize lipid substrates (Teulier et al., 2012), an increase in

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mitochondrial content in skeletal muscle (Rey et al., 2016), and a greater mitochondrial fatty acid-dependent proton conductance mediated by both avian adenine nucleotide translocase (avANT) and avian uncoupling protein (avUCP) (Talbot et al., 2004). These thermogenic mechanisms are essentially sustained by aerobic metabolism and lipid oxidation. However, penguins must also develop an effective diving capacity to capture sufficient prey at sea, which requires the careful management of oxygen stores and the capacity for anaerobic metabolism (Ponganis and Kooyman, 2000; Williams et al., 2011; Teulier et al., 2012). Hence the fact that oxygen availability is repeatedly and greatly reduced in skeletal muscles during diving (Williams et al., 2011) might not favor the maintenance of the oxidative processes required by non-shivering thermogenesis in muscles.

The aim of the present study was to determine *in vivo* whether the transition from shore to marine living enhanced the non-shivering thermogenic capacities of skeletal muscle. To define the thermoregulatory responses of penguins submerged in water, body temperature, metabolic rate, heart rate and electromyogram activity of the pectoral muscle were simultaneously measured in water temperatures ranging from 12 to 29°C. Results from pre-fledging juveniles were compared with those of sea-acclimatized immatures returning from a foraging trip.

## MATERIALS AND METHODS

### Animals

Field experiments were conducted on the Crozet archipelago (Possession Island, 46°25′S, 51°45′E) at the French Alfred Faure Station during two austral summer campaigns (from December to February, 2005–2006 and 2006–2007). According to the Agreed Measures for the Preservation of Antarctic and Sub-Antarctic Fauna, the project received ethical approval from the French Committee for Polar Research (IPEV; program no. 131). Pre-fledging juvenile king penguins (*Aptenodytes patagonicus* J. F. Miller 1778) of both sexes (13–16 months old) were captured on the nearby breeding colony of Baie du Marin before they had completed molting, which is a pre-requisite for departing to sea. Captured birds finished their molt in an outside enclosure near the laboratory and constituted the never-immersed group (NI). A second group of post-molting birds (immatures of 25–35 months old) of both sexes was caught at the beach after they had accomplished a pre-molt foraging trip ensuring that they had fully accomplished their acclimatization to marine life. They constituted the sea-acclimatized group (SA). All birds were kept in an outside enclosure and fasted for 10 days before experiments. This protocol tended to minimize confounding effects of potential differences in nutritional status. On completion of the study, all penguins were fed mackerel (*Scomber vernalis*), up to 0.5 kg per day, for one week and then released at the site of their capture.

### Metabolic rate, shivering and heart rate measurements

Metabolic rate was measured by indirect calorimetry using an open-circuit system (Rey et al., 2015) already used for measurement in penguins (Barré and Roussel, 1986; Teulier et al., 2012; Rey et al., 2016). Twenty-three birds were included in this protocol (13 NI juveniles and 10 SA immatures). The fully immersed penguin, with its head enclosed in a transparent respiratory hood, was placed in a thermostatic chamber consisting of an insulated 70-liter polythene tank. The respiratory hood was ventilated by a constant airflow (45 liters $min^{-1}$). The air sample was dried by passage through a cold trap and a drying agent (silica gel) before the fractional concentrations of oxygen and carbon dioxide were monitored with a Servomex 1100 paramagnetic gas analyser and a Servomex 1400 infrared gas analyser (Taylor Servomex, Crowborough, UK), respectively. The gas analysers were calibrated before and after each experiment using pure nitrogen gas, atmospheric air assuming oxygen content of 20.93%, and a mixture of 0.502% carbon dioxide in pure nitrogen gas. The standard equations used to determine metabolic rate (in W) from the rates of oxygen consumption and carbon dioxide production were calculated according to the equation of Depocas and Hart (1957) and are given elsewhere (Rey et al., 2015). Water temperature within the chamber was monitored using a digital set-point potentiometric temperature controller coupled with a heater–cooler exchanger and a water-circulating pump. Body (stomach) temperature was continuously monitored during the experiment with copper–constantan thermocouples. Shivering was determined by monitoring the electromyographic (EMG) activity of the pectoralis muscle. EMG and heart rate were recorded using two sets of three distinct monopolar electrodes insulated except for the tips (Stabilohm 110, Ni 80% and Cr 20%, 0.12 mm diameter) acutely inserted into the pectoralis muscle 10 mm apart. The EMG and heartbeat signals were collected simultaneously by a multi-modal acquisition interface (MP30B-CE, Biopac Systems Inc., Santa Barbara, CA, USA) and integrated with Biopac Student Lab Pro version 3.6.7 software (https://www.biopac.com/support/bsl-analysis-student-rsd-download/). The direct visualization of EMG signal allows us to identify and eliminate the portions of EMG in which artefacts were due to occasional movements of penguins. EMG activity was measured as the mean amplitude over long periods of 10–15 min of constant activity, as described in Barré et al. (1985). Noted that the EMG device did not work for four NI over the 13 birds included in the protocol.

During the experiments, birds were first placed unrestrained in the metabolic chamber and left to equilibrate for 1 h at 10°C in air before metabolic rate, heart rate and shivering were monitored over 15 min. The tank was then filled with water and left to equilibrate for 1 h at the highest temperature tested (29°C) before the recording procedure was repeated for 15 min. Thereafter, water temperature in the metabolic chamber was reduced by steps (to reach 27, 25, 20, 16 and 12°C), and after the 1.5 h necessary to reach thermal equilibrium and metabolic steady state, the recording procedure was repeated over 15 min.

Relations between metabolic rate and water temperature or shivering activity and water temperature were expressed by two linear regression lines that intersect at the lower critical temperature ($T_{LC}$) or at the shivering threshold temperature ($T_{ST}$) (Barré and Roussel, 1986; Teulier et al., 2010). Therefore, $T_{LC}$ was determined as the water temperature eliciting the first significant increase in metabolic rate, while $T_{ST}$ was determined as the water temperature eliciting the first significant increase in shivering activity. Non-shivering thermogenic capacity was determined by the highest level of metabolic rate in the cold water that was not yet accompanied by shivering activity and thus calculated by the difference between metabolic rate determined at the $T_{ST}$ and the resting metabolic rate.

### Thermal conductance

Thermal conductance in water was calculated from the slope value of the regression line between metabolic rate and water temperature. Thermal conductance in air ($C_{air}$) was calculated according to the equation of Dawson and Schmidt-Nielsen (1966) [$C_{air}$=(metabolic rate−$E$)/(body temperature−$T_a$)×surface area], where metabolic rate is in W, surface area is in $m^2$, $E$ is the evaporative heat loss expressed in W and calculated from the formula of Crawford and Lasiewski

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**Table 1. Parameters for the two experimental groups**

| Parameter | Unit | Never-immersed | Sea-acclimatized |
|---|---|---|---|
| Body mass | kg | 8.7±0.3 | 9.1±0.3 |
| Resting metabolic rate | W | 32±3 | 30±3 |
| | W $kg^{-1}$ | 3.7±0.3 | 3.3±0.2 |
| Non-shivering thermogenesis | W $kg^{-1}$ | 1.0±0.3 | 1.2±0.5 |
| Lower critical temperature | °C | 28.2±0.5 | 28.5±0.5 |
| Shivering threshold temperature | °C | 25.7±0.8‡ | 25.9±0.7‡ |
| Thermal conductance | W $m^{-2}$ $°C^{-1}$ | | |
| | In air | 1.72±0.04 | 1.72±0.07 |
| | In water | 5.0±0.4‡ | 5.7±0.5‡ |
| Oxygen pulse | ml $O_2$ $beat^{-1}$ | 1.95±0.16 | 2.59±0.24* |
| Energy pulse | J $beat^{-1}$ | 39±3 | 51±5* |

Data are means±s.e.m. from $N$=8–13 never-immersed juvenile and $N$=10 sea-acclimatized immature king penguins. Resting metabolic rates were measured at thermoneutrality (10°C) in air; lower critical temperature, shivering threshold temperature, resting metabolic rate and capacity for non-shivering thermogenesis were measured in water as described in Materials and Methods. The value of the oxygen pulse is the mean of slopes calculated for each bird from the linear relationship between active metabolic rate measured in cold water (i.e. values significantly different from baseline in Fig. 1B) and heart rate (the highest points to the right in Fig. 3B). The energy pulse is calculated from oxygen pulse values assuming 19.8 kJ $l^{-1}$ for the energy value of oxygen when lipids are oxidized. *$P$<0.05, significantly different from never-immersed juveniles; ‡$P$<0.05, significantly different from lower critical temperature within the same experimental group or thermal conductance measured in air.

(1968) [$E$=0.68(body mass)$^{0.613}$], and air temperature ($T_a$) is 10°C, a temperature within the thermoneutral zone for these birds (Duchamp et al., 1989; Froget et al., 2002). The surface area (in $m^2$) was estimated by the Meeh's formula [surface area=$k$ (body mass)$^{⅔}$], with body mass in kg, and $k$=0.065 in penguins (Pinshow et al., 1976).

### Statistical analysis

Relations between integrated EMG activity and water temperature, or metabolic rate and water temperature were statistically determined as described by Teulier et al. (2010). Within experimental groups, the threshold temperatures ($T_{LC}$ or $T_{ST}$) and thermal conduction values (in air or in water) were tested using Student's paired $t$-tests (StatView version 4.5 software; https://statview.software.informer.com/4.5/). Between experimental groups, the statistical significance of observed variations was assessed using one-way ANOVA, followed by protected least significant difference tests (StatView 4.5 software). Data are presented as means±s.e.m. with significance considered at $P$<0.05.

## RESULTS

### Metabolic response to immersion in cold water

To define the thermoregulatory responses of penguins in water, body temperature ($T_b$), metabolic rate, heart rate and electromyogram (EMG) activity of the pectoral muscle were simultaneously measured for water temperature values ranging from 12 to 29°C. For the range of water temperatures studied, there were no changes in $T_b$. The mean $T_b$ in water remained at 37.9 ±0.14°C throughout the experiment in both NI juveniles and SA immatures (Fig. 1A). Fully immersed juveniles maintained a constant $T_b$ by increasing their metabolic rate below the lower critical temperature ($T_{LC}$; Fig. 1B). There were no significant differences between the two experimental groups (Table 1). Metabolic rates measured in water at 29°C were similar to the corresponding resting metabolic rates measured in air at thermoneutrality (Fig. 1B) and were not different between experimental groups (Table 1). Thermal conductance when fully immersed in water was not significantly different between the two experimental groups of birds. The thermal conductance of penguins in water was three times higher than the thermal conductance of penguins resting in air at thermoneutrality (Table 1). EMG activity increased linearly as the water temperature decreased below the shivering threshold temperature ($T_{ST}$; Fig. 1C). The $T_{ST}$ values were significantly less than the $T_{LC}$ values within the experimental groups, but were not different between experimental groups, indicating that NI juveniles displayed the same capacity for non-shivering thermogenesis in water as SA immatures (Table 1).

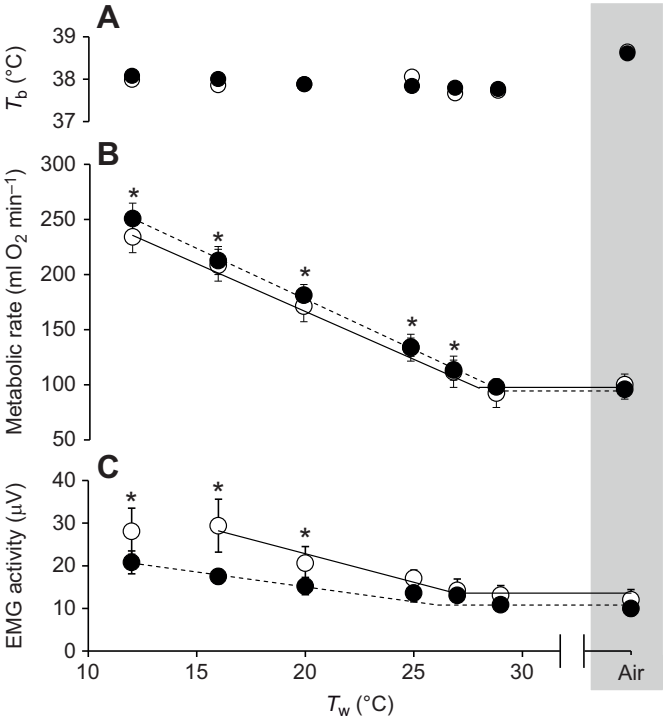


**Fig. 1. Metabolic response to cold water immersion.** (A) Body temperature ($T_b$), (B) whole-animal metabolic rate and (C) integrated muscle electrical activity (EMG) measured during immersion in water at different temperatures ($T_w$) in never-immersed pre-fledging juveniles (NI; open symbols) or sea-acclimatized immatures (SA; filled symbols). For comparison, resting values of $T_b$, metabolic rate (MR) and EMG activity measured in air at thermoneutrality (approximately 10°C) are given in the grey box. Regression lines for MR versus cold-water temperature: $MR_{water}$=−8.6$T_w$+338 ($R^2$=0.996) and in the thermoneutral zone resting MR (RMR)=98 ml $O_2$ $min^{-1}$ for NI juveniles; and $MR_{water}$=−9.0$T_w$+359 ($R^2$=0.999) and in the thermoneutral zone RMR=94 ml $O_2$ $min^{-1}$ for SA immatures. Values are means±s.e.m. from $N$=8–13 NI juveniles and $N$=10 SA immatures. *$P$<0.05, significantly different from values measured in the thermoneutral zone ($T_w$=29°C and $T_{air}$=10°C).



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### Shivering thermogenesis

The results described above indicate that penguins increase their metabolic rates as the water temperature decreases, mainly by means of shivering activity. Interestingly, Fig. 1C shows that EMG activity increased to a greater extent in NI juveniles than in SA immatures, whereas the increases in metabolic rates were similar between the two groups (Fig. 1B). Fig. 2A shows that there was a good correlation between metabolic rate and EMG activity in both experimental groups, with a steeper linear relationship in SA immatures than in NI juveniles. Indeed, the slope was significantly higher in the SA than in the NI group ($P$<0.05). This indicates that the same level of shivering activity was associated with a much larger increase in oxygen consumption, and thus heat production, in SA immatures than in NI juveniles. These values represent the aerobic heat production of shivering activity (Fig. 2B), indicating that skeletal muscle work was approximately 98% more thermogenic in SA immatures than in NI juveniles.

### Heart rate and oxygen pulse

The heart rate of penguins increased with decreasing water temperature (Fig. 3A). The resting heart rate measured at thermoneutrality in air was significantly slower in SA immatures ($f_{H,min}$=92±6 beats min$^{-1}$; range 76–126 beats min$^{-1}$) than in NI juveniles ($f_{H,min}$=112±5 beats min$^{-1}$, range 92–149 beats min$^{-1}$; $P$<0.05). In response to immersion, there was a positive relationship between heart rate and oxygen consumption in both SA immatures and NI juveniles (Fig. 3B). The mean maximal heart rates were not significantly different between groups ($f_{H,max}$=178±14 beats min$^{-1}$ in SA immatures versus $f_{H,max}$=196±10 beats min$^{-1}$ in NI juveniles). The values of oxygen pulse and energy pulse (amount of oxygen consumed and the amount of energy expended per heartbeat) were significantly higher, by 33%, in SA immatures compared with these values in NI juveniles ($P$<0.05; Table 1).

## DISCUSSION

This study showed that regulatory non-shivering thermogenesis is not reinforced by the passage from shore to marine life, indicating that king penguins mainly rely upon skeletal muscle activity (shivering or physical activity) to maintain endothermy at sea. Interestingly, pre-fledging juveniles exhibited a thermoregulatory capacity similarly effective to that of sea-acclimatized immatures, at least in the range of temperatures explored here. However, the present results also demonstrated the existence of metabolic adjustments to marine life, involving increased shivering and cardiovascular efficiency.

### Thermal sensitivity and thermoregulatory responses in cold water

Although their movements were restricted in a metabolic tank and despite the intense cooling power of water, juvenile king penguins were as effective as immature birds at producing heat to maintain their body temperature in cold water. Interestingly, the thermal responses of penguins to cold water immersion differ slightly between a progressive cooling in water (present study) and a rapid immersion in cold water (Fahlman et al., 2005; Rey et al., 2016). When penguins were rapidly immersed in cold water, the increase in their metabolic rate was shown to be associated with a slight but significant hypothermia (Fahlman et al., 2005; Rey et al., 2016). This hypothermic response was dependent on the nutritional status of birds (Fahlman et al., 2005), but remained similar between pre-fledging juveniles and sea-acclimatized immatures (Rey et al., 2016). The lower critical temperature is a measure of the thermal sensitivity of endotherms to low temperature. Its value is dependent upon the product of the overall insulation and the basal metabolic level of an animal (Scholander et al., 1950). Sea-acclimatized immature king penguins exhibited the same thermal conductance and resting metabolic rate in air as terrestrial juveniles (Table 1). Thus, both groups exhibited the same thermal sensitivity to cold water, increasing their metabolic heat production when exposed to the same lower critical water temperature of 26–27°C or less. Such high temperatures of the lower limit of the thermoneutral zone in water have previously been reported in several penguin species (Kooyman et al., 1976; Barré and Roussel, 1986; Dumonteil et al., 1994; Teulier et al., 2016). These results clearly indicate that at the time of their first departure out to sea, juveniles are already equipped to be effective endotherms in cold water. This observation suggests that efficient thermoregulatory capacity must be selected for prior to fledging. For instance, the lengthy winter fasting period is an

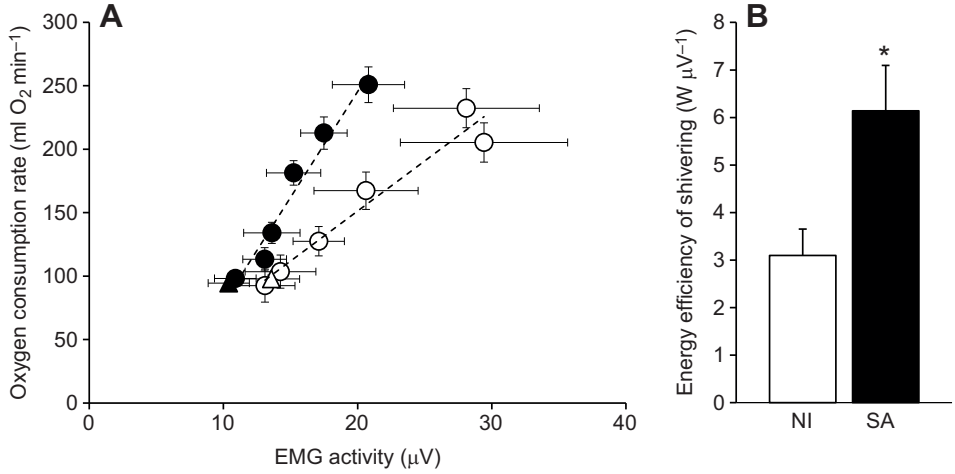


**Fig. 2. Shivering thermogenic efficiency.** (A) Linear relationship between metabolic rate and EMG activity measured in water (circles) in never-immersed pre-fledging juveniles (NI, open symbols) and sea-acclimatized immatures (SA, filled symbols). Regression lines for metabolic rate versus EMG activity in cold water: $y$=7.9$x$–6.6 ($R^2$=0.95) for NI juveniles and $y$=16.7$x$–87.9 ($R^2$=0.96) for SA immatures. For comparison, resting values measured in air at thermoneutrality (approximately 10°C) are also shown (triangles). (B) The efficiency of shivering thermogenesis was calculated from the slope of individual regression lines for metabolic rate versus EMG activity in NI juveniles and SA immatures. Values are means±s.e.m. from $N$=8 NI juveniles and $N$=10 SA immatures. *$P$<0.05, significantly different from NI juveniles.

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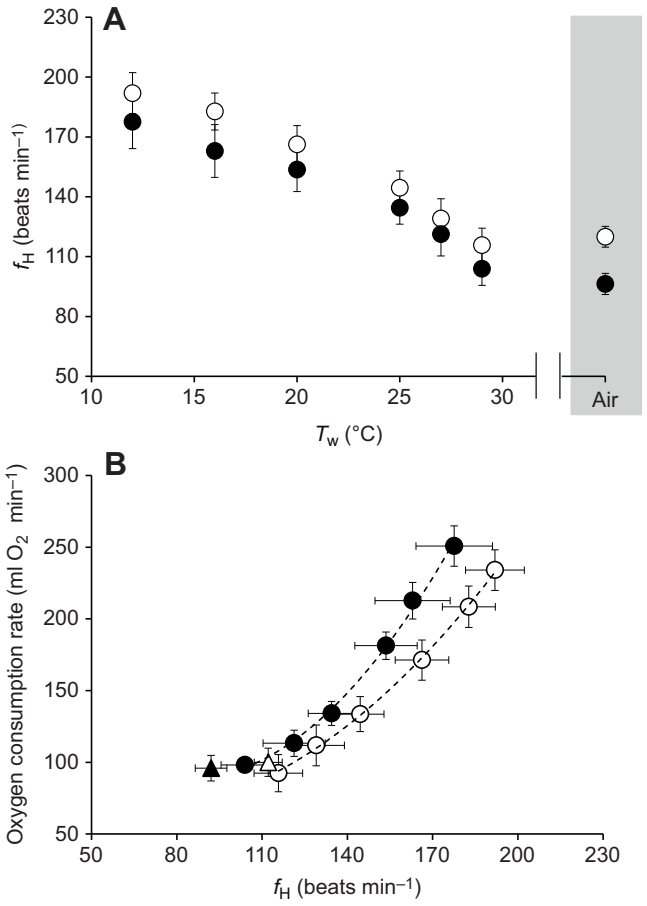


**Fig. 3. Heart rate response to cold water immersion.** Relationships between heart rate ($f_H$) and (A) water temperature ($T_w$) and (B) whole-animal metabolic rate in never-immersed pre-fledging juveniles (NI; open symbols) or sea-acclimatized immatures (SA; filled symbols). In B, lines are the best polynomial fit to the mean values (excluding basal values measured in air): $y$=0.0099$x^2$–1.24$x$+104 ($R^2$=0.999) for NI juveniles and $y$=0.0186$x^2$–3.09$x$+216 ($R^2$=0.996) for SA immatures. Values are means ±s.e.m. from $N$=13 NI juveniles and $N$=10 SA immatures.

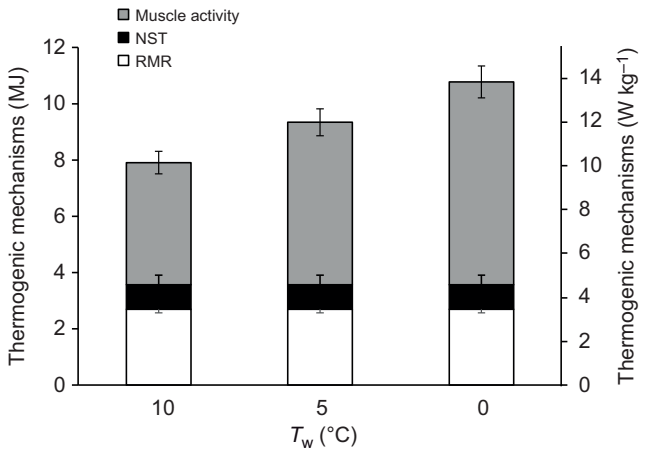


**Fig. 4. Estimated contribution of thermogenic mechanisms to heat production in water at 10, 5 or 0°C.** Contribution of resting metabolic rate (RMR), muscle contractile activity and non-shivering thermogenesis (NST) to daily energy expenditure in sea-acclimatized king penguins. Values are means±s.e.m. from 10 sea-acclimatized king penguins. Values were calculated from data shown in Fig. 1 and Table 1 (see Results for more details).

energy-challenging constraint that might act as one of the selective mechanisms for juvenile king penguins. This is supported by the fact that (i) cold-deacclimatized juveniles, i.e. juveniles kept for 2 weeks at 20°C, are less efficient thermoregulators during cold immersion than sea-acclimatized penguins (Barré and Roussel, 1986); and (ii) 10 successive immersions in cold water can improve the thermoregulatory capacity of cold-deacclimatized juveniles (Barré and Roussel, 1986) but not of juveniles kept outside in their natural environment (Teulier et al., 2016), which in turn exhibit the same thermoregulatory capacity as sea-acclimatized immatures (present study).

Fig. 4 shows the contribution of thermogenic mechanisms to energy expenditure of water-immersed immature king penguins calculated from the regression line in Fig. 1B for water temperatures of 10, 5 and 0°C. Immature king penguins would expend 12 W kg$^{-1}$ or a total energy expenditure of 9100 kJ day$^{-1}$ to maintain endothermy while foraging in 5°C water. This is more than three times the resting metabolic rate on shore and is higher than the resting metabolic rate previously measured in king penguins floating in cold water at 4–5°C (4–5 W kg$^{-1}$) (Fahlman et al., 2004, 2005; Kooyman et al., 1992; at 9°C in Culik et al., 1996). The higher energy requirements of king penguins in water observed in our study could be due the fact that the birds were in a post-molt state. The molting period is the most nutritionally and energetically challenging period of the annual cycle of king penguins, during which birds lose 50% of their initial body weight and skeletal muscle mass, as well as most of their subcutaneous fat (Cherel et al., 1994). This loss of subcutaneous fat severely impairs body insulation and greatly increases heat loss. The birds in our study weighed an average of 8.9±0.2 kg, whereas in the studies cited above, birds weighed 11–13 kg (Fahlman et al., 2004, 2005; Culik et al., 1996) and up to 16 kg in Kooyman et al. (1992). Hence, the post-molt immatures and juveniles used in our study would need to increase heat production to maintain their body temperature in cold water because of their poor subcutaneous insulation, increasing the thermoregulatory cost. The high metabolic rates measured in our study might also have been related to the long period of complete submersion (7–8 h) used in our experimental set-up, compared with the relatively short periods (less than 3 h) used in previous studies that investigated birds floating in water. Lewden et al. (2017b) reported that the resting metabolic rate of penguins was significantly higher after fasting for 4 days floating in cold water (approximately 13 W kg$^{-1}$) than at the start of the fasting period (approximately 5.8 W kg$^{-1}$). This increased metabolic rate is associated with increased peripheral-tissue temperatures (Lewden et al., 2017b), reflecting peripheral perfusion to access subcutaneous fat stores to fuel metabolism (Lewden et al., 2017a) and possibly to warm up peripheral tissues because of the reduction in fat-related thermal insulation. Finally, it must be kept in mind that the calculated daily energy cost shown in Fig. 4 can be viewed as an upper limit of the daily energy expenditure that king penguins must expend at sea. For instance, our calculation did not take into account the hypothermia and decreased metabolic rate of unstressed and free-ranging penguins during their dives. In addition, the daily energy expenditure also depends upon the nutritional and physiological status of the birds (e.g. fasted versus fed, or pre- versus post-molt states), as discussed above. Therefore, it is not surprising that the estimated energy expenditure reported in Fig. 4 is higher than the daily energy expenditure or field metabolic rate previously estimated in king penguins (Kooyman et al., 1992; Froget et al., 2004).

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### Components of skeletal muscle thermogenic mechanisms at sea

In birds, skeletal muscle is the major tissue involved in metabolic adjustments in response to exposure to cold. It contributes to 70–80% of regulatory thermogenesis through increased contractile activity (shivering or locomotor activity) and in some bird species through non-shivering thermogenesis (Hissa, 1988; Duchamp et al., 1989; Duchamp and Barré, 1993). King penguin chicks develop non-shivering thermogenesis during the winter whilst living on shore (Duchamp et al., 1989). This thermogenic mechanism remains effective the following spring in pre-fledging juveniles (Teulier et al., 2016; present study). The findings of the present study suggest that endothermy in king penguins at sea would mainly be sustained by muscle activities (shivering and/or swimming). Fig. 4 shows the calculated contribution of resting metabolic rate, non-shivering thermogenesis and skeletal muscle activity (i.e. shivering and/or swimming) to the daily energy expenditure of immature king penguins immersed in water at different temperatures. The data highlight that skeletal muscle activity is the main form of thermogenesis, contributing at least to 55–67% of the daily energy needed to maintain endothermy in cold water, while the resting metabolic rate contributes around 29% (25–34%) of daily heat production. Non-shivering thermogenesis in water would contribute less than 11% of heat production, ranging from 8% at 0°C to 11% at 10°C (Fig. 4). Juveniles exhibited a similar pattern (data not shown).

Skeletal muscle non-shivering thermogenesis is closely related to fatty acid metabolism. Fatty acids are the main energy substrates and activators of heat-producing mechanisms, whether based on the futile cycling of calcium ions across the sarcoplasmic reticulum membrane or protons across the inner mitochondrial membrane (Duchamp et al., 1999). More generally, lipids are by far the main fuels used to sustain the high and prolonged metabolic needs of skeletal muscle for heat production in the cold and for energy output during long-distance flight in birds (Vaillancourt et al., 2005; Weber, 2009; Guglielmo, 2010). In penguins, lipid-induced heat production increases after repeated experimental immersions in cold water (Teulier et al., 2016) and is considerably higher in sea-acclimatized immatures than in pre-fledging juveniles (Teulier et al., 2012). In contrast, the capacity for non-shivering thermogenesis is not improved after repeated experimental immersions in cold water (Teulier et al., 2016) or after sea acclimatization (present study). Therefore, lipid-induced thermogenesis is decoupled from the capacity for non-shivering thermogenesis. This result suggests that it is the increased activity of skeletal muscle associated with shivering and/or swimming that mainly triggers the development of the thermogenic effect of lipid infusion. This hypothesis is supported by the observation of the cross-training effects of exercise and cold on lipid transport and catabolism, and on skeletal muscle mass and oxidative capacity, which also explain how exercise training can improve thermogenic performances in birds and vice versa (Schaeffer et al., 2001; Petit and Vézina, 2014; Zhang et al., 2015a, 2015b). Reinforcing this idea, king penguins forage several hundred kilometers away from their colony (Charrassin and Bost, 2001), with juveniles covering a considerably larger area than non-breeding adults during their first year at sea (Orgeret et al., 2019). Such prolonged and energetically demanding physical activity may well substitute for regulatory non-shivering thermogenesis mechanisms as a source of heat (Lovvorn, 2007; Humphries and Careau, 2011).

### Physiological adjustments to sea acclimatization

Sea acclimatization has been shown to enhance metabolic responses to cold water immersion, concomitant with an increase in mitochondrial oxidative capacity and content in skeletal muscle (Teulier et al., 2012; Rey et al., 2016, 2017). However, increased muscle metabolism must be supplied with commensurate energy substrates and oxygen delivery to be fully effective. Sea acclimatization was reported to trigger a selective up-regulation in lipid handling and oxidation (Teulier et al., 2012). The present study further showed that immature king penguins displayed a higher oxygen pulse than juvenile birds, suggesting cardiovascular adjustments towards an enhanced oxygen delivery to active tissues, including skeletal muscles. The relationship between the rate of oxygen consumption ($\dot{V}_{O_2}$) and heart rate ($f_H$) is described by the Fick equation: $\dot{V}_{O_2}=f_H\times V_s\times(C_{a,O_2}-C_{v,O_2})$, where $V_s\times(C_{a,O_2}-C_{v,O_2})$ is the oxygen pulse. A change in the oxygen pulse can therefore include changes in any of the variables in the term $V_s\times(C_{a,O_2}-C_{v,O_2})$, where $V_s$ is the cardiac stroke volume, and $C_{a,O_2}$ and $C_{v,O_2}$ are the arterial and mixed venous oxygen concentrations, respectively. Previous studies have clearly shown that exercise training as well as cold training can increase the density of blood capillaries in skeletal muscle (Butler and Turner, 1988; Duchamp et al., 1992; Mathieu-Costello et al., 1998). Furthermore, sea-acclimatization increases hematocrit and thus the capacity of the blood to transport oxygen, in both emperor (Ponganis et al., 1999) and king penguins (Rey et al., 2016), which in turn can be taken as an indirect estimate of $C_{a,O_2}$. In addition, the myoglobin content of skeletal muscles is higher in sea-acclimatized adult penguins than in chicks or pre-fledging juveniles (Weber et al., 1974; Ponganis et al., 1999, 2010; Noren et al., 2001). Taken together, these observations strongly suggest that sea acclimatization increases the capacity of penguin skeletal muscles to extract and store oxygen for enhanced oxidative catabolism.

### Swimming for thermogenesis?

Sea-acclimatized immatures also consumed more oxygen per unit of EMG activity than juveniles (Fig. 2), which can be explained by a fuel selection of skeletal muscle towards lipid oxidation (Teulier et al., 2012), an increased content and oxidative activity of mitochondria (Teulier et al., 2012; Rey et al., 2016) and a higher oxygen pulse (present study) in sea-acclimatized penguins, among other remodeling processes. In turn, the lower oxygen consumption of juveniles relative to their EMG activity cannot be ascribed to a greater anaerobic metabolism, as muscle enzyme activities involved in anaerobic metabolism are similar between juveniles and sea-acclimatized immatures (Teulier et al., 2012). Notwithstanding, the higher aerobic heat production of skeletal muscle activity suggests that swimming would be more thermogenic in immatures after sea acclimatization than in pre-fledging juveniles. This hypothesis is in accordance with the increased energy cost of locomotion reported in other endotherms following long-term cold exposure, such as ducklings (Duchamp et al., 1999), short-tailed opossums (Schaeffer et al., 2005) and adult goats (Schaeffer et al., 2001).

In birds, the molecular basis of less efficient locomotion might include the over-expression of mitochondrial uncoupling proteins (avian UCP) and/or adenine nucleotide translocator (ANT). These inner mitochondrial membrane proteins are able to mediate a fatty acid-dependent increase of proton conductance in skeletal muscle mitochondria (Talbot et al., 2004; Rey et al., 2010). In king penguins, the expression and activity of these proteins increases after sea acclimatization (Talbot et al., 2004). The underlying molecular mechanisms, i.e. fatty acid-induced avian ANT/UCP-mediated basal proton conductance, could explain part of the selective increase in the thermogenic effect of lipids reported in sea-acclimatized immature king penguins, both at the whole body and skeletal muscle mitochondria levels (Teulier et al., 2012; Rey et al.,

2017). However, the thermogenic efficiency of shivering was not improved by repeated immersions of juveniles in cold water (Teulier et al., 2016), whereas the thermogenic effects of fatty acids, mediated by avian ANT/UCP in skeletal muscle mitochondria *in vitro*, was enhanced (Talbot et al., 2004). This indicates that these molecular mechanisms fully account for the higher thermogenic efficiency of shivering of sea-acclimatized immatures (present study). At the cellular level, fatty acids can also activate heat generation by increasing ATP breakdown through the futile cycling of calcium across the sarcoplasmic reticulum membrane (Duchamp et al., 1999). Although this thermogenic mechanism remains to be explored in penguins, it could be enhanced in sea-acclimatized immatures due to the increased oxidative capacity and content of mitochondria in skeletal muscle (Teulier et al., 2012; Rey et al., 2016, 2017). Another non-exclusive thermogenic mechanism can be associated with the fuel selection towards lipids oxidation and delivery to skeletal muscles reported in sea-acclimatized immatures (Teulier et al., 2012). This up-regulation of lipid metabolism would provide mitochondria with higher levels of flavin adenine dinucleotide (FAD)-linked substrates, the oxidation of which decreases mitochondrial ATP efficiency (Brand, 2005). Interestingly, the increased muscle fatty acid uptake of transgenic mice over-expressing lipoprotein lipase in their skeletal muscles enhances the thermogenic response and tolerance of mice to the cold (Jensen et al., 2008). Hence the fuel selection towards lipid oxidation and the development of a high capacity for oxidative substrates in skeletal muscle of sea-acclimatized king penguins may be key characteristics of thermogenesis in birds (Vaillancourt et al., 2005; Teulier et al., 2010, 2012; Rey et al., 2017). Potential changes in the efficiency of excitation and contraction mechanisms within muscle fibers could also be worth investigating. All these physiological and cellular adjustments to life at sea may *in fine* contribute to increase the thermogenic efficiency of shivering, and more generally, the energy cost of skeletal muscle functioning. If so, this suggests that heat generated by swimming would be an efficient substitute for the heat required for thermoregulation in king penguins during their foraging trip at sea (Lovvorn, 2007; Humphries and Careau, 2011).

## Conclusion

Pre-fledging juveniles exhibited thermoregulatory capacity as effective as sea-acclimatized immature king penguins at least in the range of temperatures investigated (12–29°C). Hence juvenile penguins that have spent their childhood ashore, and have never faced the intensive energetic challenges required to maintain a high body temperature when swimming in cold water, are already set to sustain the high energetic demands of life at sea and thus be a successful seabird endotherm. Sea-acclimatized immature penguins exhibited higher thermogenic efficiency of shivering and oxygen pulse than pre-fledging juvenile birds, indicating that thermogenic and cardiovascular adjustments do occur during their early marine life. Together with previously identified metabolic adaptations to life at sea, these physiological adjustments may favor an efficient activity–thermoregulatory heat substitution phenomenon, providing penguins with the aptitude to survive the tremendous energetic challenges imposed by a marine life in the cold circumpolar oceans.

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#### Competing interests

The authors declare no competing or financial interests.

#### Author contributions

Conceptualization: D.R., J.-L.R., C.D.; Methodology: D.R., J.-L.R.; Validation: D.R., J.-L.R.; Formal analysis: D.R., M.L.C.; Investigation: D.R., M.L.C., J.-L.R.; Writing - original draft: D.R.; Writing - review & editing: C.D.; Supervision: D.R., C.D.; Project administration: C.D.; Funding acquisition: C.D.

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