

## Heart rate as a predictor of energy expenditure in undisturbed fasting and incubating penguins

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### SUMMARY

Heart rate ( $f_H$ ) measurement offers the possibility to monitor energy expenditure (EE) in wild animals if the EE/ $f_H$  relationship for the species, physiological stages and activities of interest is known. This relationship has been extensively studied using oxygen consumption rate ( $\dot{V}_{O_2}$ ) measurement in captive, repeatedly handled king penguins (*Aptenodytes patagonicus*). Unfortunately, the potential effects of stress on the observed relationships resulting from handling and confinement were not considered. This study is the first involving undisturbed animals, and determines the EE/ $f_H$  relationship in naturally fasting and freely incubating or captivity-acclimatized male and female king penguins. EE determination was based on (1) the measurement of body mass loss during periods of phase II fasting, and (2) the calculation of its energy equivalent from changes in body composition, i.e.  $23.9 \text{ kJ g}^{-1}$ .  $f_H$  levels in freely incubating and captivity-acclimatized birds were found to be 50–70% lower than those previously reported for resting king penguins during  $\dot{V}_{O_2}$  measurements. Significant EE/ $f_H$  relationships were found in freely incubating and captive males and females ( $R^2=0.59$  to  $0.84$ ), with no difference observed between genders. The best overall relationship was obtained by including fasting duration ( $t$ , days) in the model:  $EE=818+43.7 \times f_H+36.3t-1.4t \times f_H$  ( $R^2=0.91$ ). This equation yielded EE estimates approximately 26% higher than the previously reported 'best' predictive equation in king penguins, and even more so when  $f_H$  was low. This result suggests that stress induces a disproportionate increase of  $f_H$  vs  $O_2$  consumption, and that the use of EE/ $f_H$  relationships obtained in stressed birds could lead to underestimated EE values.

Key words: heart rate, energy expenditure, energy pulse, seabird, fasting, incubation, stress.

### INTRODUCTION

In order to understand behavioural strategies in free-living birds, especially during breeding periods, it is important to be well informed about the energy costs related to various activities such as foraging, parental care, incubation, territory defence and self-maintenance. The calculation of energy budgets from time budgets directly implies the measurement of energy expenditure (EE) associated with each specific activity. Such measurements are challenging to obtain under field conditions, which explains why the vast majority of energy budgets have been estimated from EE measured under laboratory conditions. Unfortunately, such EE estimates often differ widely from those obtained in the field (Flint and Nagy, 1984).

During the last decade, numerous studies have estimated field EE from heart rate ( $f_H$ ) measurements, especially in birds (Woakes and Butler, 1983; Nolet et al., 1992; Bevan et al., 1994; Bevan et al., 1995; Boyd et al., 1999; Ward et al., 2002; Weimerskirch et al., 2002; Green et al., 2003; Fahlman et al., 2004; Froget et al., 2004). The use of  $f_H$  as a predictor of EE relies on the relationship between the  $O_2$  consumption rate ( $\dot{V}_{O_2}$ ) and  $f_H$  as formulated in the Fick equation for  $O_2$  convection in the cardiovascular system (Dejours, 1981). The recording of  $f_H$  provides an accurate correlation of  $O_2$  consumption (Boyd et al., 1995; Butler et al., 2004), and is also a relatively non-invasive technique offering the possibility to monitor metabolism with a fine time-resolution. Precaution is however necessary, as this relationship is species-specific, and may be affected by several factors such as gender (Froget et al., 2001; Green

et al., 2001), type and level of activity (Nolet et al., 1992; Butler et al., 2000; Froget et al., 2002), physiological and nutritional state (Froget et al., 2001; Green et al., 2001) and seasonal changes (Holter et al., 1976; Speakman, 1997).

The relationship between  $O_2$  consumption and  $f_H$ , as well as the influence of various factors on this relationship have been investigated in great detail for the king penguin *Aptenodytes patagonicus* (Froget et al., 2001; Froget et al., 2002; Fahlman et al., 2004). These previous studies, carried out on regularly manipulated captive king penguins, have shown that the relationship slope (i.e. the  $O_2$  pulse, the volume of  $O_2$  used per one heart beat), which depends on body condition (Froget et al., 2001), is three times higher during walking than while resting (Froget et al., 2002). Whether the relationship is affected by gender, however, was not clearly established (Froget et al., 2001).

These studies also showed that fasting affects the  $\dot{V}_{O_2}/f_H$  relationship during exercise but not during resting periods (Fahlman et al., 2004). For  $\dot{V}_{O_2}$  measurements, birds were kept in a metabolic chamber and forced to walk on a treadmill. As stress-related  $f_H$  variations do not always correspond to metabolic variations (McPhee et al., 2003), these  $\dot{V}_{O_2}/f_H$  relationships may be affected by handling and confinement. Moreover, the 'HRDDL Loggers' used to measure  $f_H$  in previous studies (Woakes et al., 1995), have yet to be validated in king penguins, and a limited number of individuals have been used for calibration in this species. The use of previously established relationships to estimate EE from  $f_H$  in free-living king penguins

undergoing normal activities while breeding therefore remains controversial (Halsey et al., 2007).

Whereas continuous recording of  $f_H$  in free-living king penguins breeding ashore is not a major technical difficulty, estimating EE is more challenging. Doubly labelled water could be used but would be expensive given the large size of king penguins and the number of individuals required for an accurate calibration (Nagy, 1983). Alternatively, as king penguins fast ashore for weeks during the breeding season their EE can be derived from body mass ( $M_b$ ) loss measured under freely living conditions if the energy equivalent of  $M_b$  loss is known. Interestingly, the composition of  $M_b$  loss, and thus its energy equivalent, is constant in phase II fasting animals. This observation is consistent for seabirds (Groscolas, 1988; Groscolas et al., 1991) and for animals with different body composition (Cherel and Groscolas, 1999) and levels of metabolic rate (Cherel et al., 1995).

The present study aims to determine the relationship between EE, as estimated from  $M_b$  loss, and  $f_H$  in undisturbed, freely incubating king penguins. Measurements were performed on both male and female incubating penguins to test for a gender effect. In order to detect a potential effect of incubation on the EE/ $f_H$  relationship, the same measurements were also carried out in naturally fasting but non-incubating males and females acclimatized to captivity. In contrast to previous studies,  $f_H$  and EE variations were not induced by potentially stressful experimental challenges, such as walking on a treadmill or exposure to various ambient temperatures when kept in a metabolic chamber. Here, EE/ $f_H$  variations were due to natural inter- and intra-individual differences in resting metabolic rate and activity levels throughout the course of natural fasting. This study can therefore be considered to provide an accurate estimate of the EE/ $f_H$  relationship free of the effect of stress.

## MATERIALS AND METHODS

Fieldwork was undertaken during the 2002–2003 and 2003–2004 breeding seasons in the king penguin colony (*Aptenodytes patagonicus* Miller 1778) (ca. 20,000 breeding pairs) of 'Baie du Marin', Possession Island, Crozet Archipelago (46°25' S; 51°52' E). The study was approved by the Ethical Committee of the Institut Polaire Français-Paul Emile Victor (IPEV) and complies with current French laws. The authorization to enter the colony and handle birds was obtained from Terres Australes et Antarctiques Françaises.

### Animals

#### Incubating birds

In the king penguin, both genders alternate shifts of incubation, with males consistently performing the first shift. Birds were therefore sexed by their breeding behaviour. Incubation lasts an average of 53 days and individual shifts last approximately 15 days (Stonehouse, 1960; Weimerskirch et al., 1992). A few days prior

to laying, experimental birds were randomly chosen from among 80 pairs then marked and banded at the flipper (plastic band). Bands were removed at the end of the study. To determine the onset of each incubation shift, banded birds were checked twice daily. A total of 51 males and 23 females were observed during one of their incubation shifts and their  $f_H$  and  $M_b$  loss were measured. To ensure that  $f_H$  and  $M_b$  loss measurements were performed while birds were in phase II fasting, these measurements started after day three of an incubation shift and stopped at least four days before its end (Le Ninan et al., 1988; Gauthier-Clerc et al., 2001; Groscolas and Robin, 2001; Robin et al., 2001). The minimum  $M_b$  measured in our study was 9.8 kg, i.e. above the 9.6 kg  $M_b$  threshold recorded in fasting, incubating king penguins at the transition between phases II and III (Cherel et al., 1988; Gauthier-Clerc et al., 2001). The mean fasting duration and  $M_b$  of incubating birds were also well within the previously reported ranges for phase II fasting king penguins [ $M_b$  is 10.3–12.9 kg for males and 9.7–11.9 kg for females (Gauthier-Clerc et al., 2001); Table 1].  $f_H$  and  $M_b$  loss were recorded over four-day periods and the same individuals were monitored for 1–3 periods. A total of 65 and 26 data points were obtained for incubating males and females, respectively.

#### Captive non-incubating birds

During the courting period, breeding penguins fasting ashore were captured and marked on the breast using a dye (Raidex GmbH, Dettingen, Germany). Gender was determined from differences in body size and singing behaviour (Jouventin, 1982). Birds were left fasting in the colony for a further 2–3 days. Then, a total of 18 males and 11 females were penned (two birds per pen), within 3 m × 3 m wooden fences located in the vicinity of the colony and under natural weather conditions. A preliminary study on king penguins showed that penning initially resulted in a  $f_H$  increase, which then returned to basal levels within five days. Thus,  $f_H$  and  $M_b$  loss measurements were started after five consecutive days of penning in order to ensure that the birds were used to captivity and had begun phase II fasting.  $M_b$  loss and  $f_H$  were measured over four-day consecutive periods, up to a maximum fasting duration of 25 days. Only data obtained before the beginning of phase III fasting were considered, the latter state being characterised by an increase in daily  $M_b$  loss (Groscolas, 1988). Mean total fasting duration and  $M_b$  during measurement periods are presented in Table 1. Depending on individuals,  $M_b$  loss and  $f_H$  were recorded over 1–6 periods, yielding a total of 102 and 32 data points for males and females, respectively. All birds were released at the end of the experiment and usually departed to feed at sea within 1–3 days.

#### $f_H$ measurements

$f_H$  was recorded using a Polar® S810  $f_H$  logger (Polar Electro Oy, Kempele, Finland) adapted for use in king penguins. This logger

Table 1. Mean fasting duration, body mass ( $M_b$ ), body mass loss and heart rate ( $f_H$ ) in incubating and non-incubating captive male and female king penguins

| Sex and status      | <i>n</i> | Fasting duration (days) | $M_b$ (kg)              | $M_b$ loss (g day <sup>-1</sup> ) | $f_H$ (beats min <sup>-1</sup> ) |
|---------------------|----------|-------------------------|-------------------------|-----------------------------------|----------------------------------|
| Males, incubating   | 65       | 18.4±0.8 <sup>a</sup>   | 11.09±0.13 <sup>a</sup> | 115±5 <sup>a</sup>                | 61.0±2.0 <sup>a</sup>            |
| Males, captive      | 102      | 12.2±0.7 <sup>b</sup>   | 12.35±0.14 <sup>b</sup> | 122±6 <sup>a</sup>                | 62.4±2.6 <sup>a</sup>            |
| Females, incubating | 26       | 6.3±1.3 <sup>c</sup>    | 11.58±0.19 <sup>c</sup> | 164±7 <sup>b</sup>                | 79.9±2.9 <sup>b</sup>            |
| Females, captive    | 32       | 8.1±1.2 <sup>c</sup>    | 10.70±0.21 <sup>a</sup> | 148±8 <sup>b</sup>                | 76.4±3.5 <sup>b</sup>            |

Values are means ± s.e.m. during the pooled 4-day periods of  $f_H$  and  $M_b$  loss measurements. *n*, number of data points. Fasting duration for incubating males was calculated assuming that those recorded during shift 1 had already been fasting for two weeks at the onset of incubation. Fasting duration for non-incubating captive birds was calculated in reference to the day of penning. Within a column, values not sharing the same superscript letter are significantly different (repeated-measures ANOVA, *P*<0.05).

has a 20–235 beats per min ( $\text{beats min}^{-1}$ ) resolution range and is composed of a transmitter and a receiver/logger. The whole unit weighed 80 g. The transmitter contains a  $f_H$  processor that filters electronic signals received by the electrodes and allows the distinction of heart beats from other electric activities (e.g. muscle or electrode noises). It was supplied with two 15 cm wires to which a gold-plated safety pin electrode was attached. The  $f_H$  logger was glued in the middle of the bird's back using adhesive tape. Dorsal electrodes were placed approximately 0.5 cm under the skin. The upper electrode was placed on the upper back and the lower electrode was approximately 30 cm below, just above the tail. Loggers were programmed to record  $f_H$  every minute during a four-day period so that mean daily  $f_H$  was calculated at  $\pm 0.5 \text{ beats min}^{-1}$  from a total of 5760 measurements. The length of the recording period depended on the storage capacity of the logger and had to be long enough to allow an accurate determination of  $M_b$  loss (see below).

A calibration study was performed to validate the use of the Polar® S810 logger for  $f_H$  measurements on king penguins.  $f_H$  was simultaneously measured using a Polar® logger and a stethoscope. Forty incubating birds (males and females) were equipped with  $f_H$  loggers, and heart beats were immediately counted for 30 s using both methods in restrained birds that were either at rest or moving slightly (see Results).

#### Equipment, weighing and behavioural observations

For incubating birds, the egg was temporarily replaced by a plaster dummy egg before being transferred from the incubating position to a nearby shelter. The birds were equipped with an  $f_H$  logger and weighed on a platform scale at  $\pm 4$  g. The whole procedure lasted less than 10 min. The bird was then put back into the colony, where it continued incubating. The same procedure, without the use of a dummy egg, was used for captive birds. Thereafter, the equipped birds were left undisturbed. They behaved normally, spending most of the time resting (around 70%) or showing aggressive or comfort behaviours. Birds were recaptured four days later and weighed after switching off the logger. The durations of  $f_H$  and  $M_b$  loss measurements were therefore strictly identical. Given that  $f_H$  returned to normal levels less than half an hour after handling,  $f_H$  values obtained during the four-day recording period can be considered to be representative of undisturbed birds in over 99% of cases. When weighing was performed on a rainy day, birds were first dried using a cloth and a hairdryer to avoid any inaccuracy in  $M_b$  values. Given the accuracy of  $M_b$  measurements ( $\pm 4$  g) and the total  $M_b$  loss throughout the four-day recording period (at least 220 g), the relative error for total and daily  $M_b$  loss was estimated at less than 3.6%. No egg desertions or losses were observed in equipped incubating birds.

#### Energy equivalent of $M_b$ loss

Daily EE was calculated by multiplying daily  $M_b$  loss by its energy equivalent (the latter having been determined in a complementary study performed during the 2003–2004 breeding season). The body composition of 81 unsexed, breeding king penguins was determined from the measurement of their total body water (TBW) using tritiated water, as described and validated previously in another seabird species, the great-winged petrel *Pterodroma macroptera* (Groscolas et al., 1991). At the time of TBW measurement, penguins had been fasting ashore for 6–30 days, and their  $M_b$  (which mostly differed because of different fasting durations) ranged from 9.7 to 17.0 kg (see Fig. 1). They were therefore considered to be in phase II of fasting. Lean body mass ( $LM_b$ ) was calculated as  $TBW/0.73$ , where 0.73 is the fractional water content of  $LM_b$  (Groscolas et al., 1991).

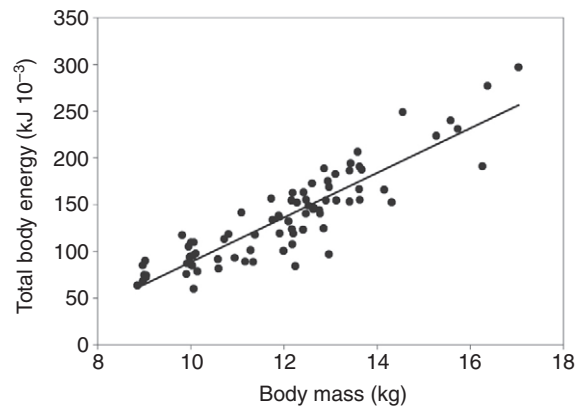


Fig. 1. Relationship between total body energy content (TBE,  $\text{kJ } 10^{-3}$ ) and body mass ( $M_b$ , kg) in phase II fasting and breeding king penguins. Each point represents an individual value. The equation is:  $TBE = -150.3 \pm 15.9 + 23.9 \pm 1.3 \times M_b$ ,  $n=81$ ,  $R^2=0.81$ ,  $P<0.0001$ .

Lipid and protein masses were calculated as  $M_b - LM_b$  and  $LM_b - TBW$ , respectively. Total body energy content (TBE) of birds was the sum of the energy contained in lipids and proteins, assuming that the energy density of lipids and proteins are  $39.3 \text{ kJ g}^{-1}$  (Schmidt-Nielsen, 1979; Groscolas et al., 1991) and  $17.8 \text{ kJ g}^{-1}$  (Schmidt-Nielsen, 1979), respectively. A significant relationship was found between TBE (kJ) and  $M_b$  (g), the best fit being linear (Fig. 1). The slope of this relationship ( $23.9 \pm 1.3 \text{ kJ g}^{-1}$ ) represents the energy equivalent of  $M_b$  loss in phase II fasting king penguins.

#### Statistics

A linear regression was conducted in order to compare  $f_H$  measured with both Polar® loggers and a stethoscope, and to relate total TBE to  $M_b$ . The Student's *t*-test or a multi-way repeated-measures analysis of variance (ANOVA) followed by multiple-comparisons (LSD method) were performed to compare the difference between the means of two populations or more than two populations, respectively. After testing variables for normality with the Kolmogorov–Smirnov test, a mixed generalized linear model (GLMM) with individuals as a random factor was used to assess the relationship between  $f_H$  and EE. When necessary, variables were corrected with the appropriate transformations to meet the assumption of normality. The GLMM took into account the dependence between observations from the same bird with a compound symmetry structure. Firstly, a saturated model with all fixed effects, fasting duration and  $M_b$ , and two-way interactions was explored to examine the effect of fasting duration and  $M_b$  on the EE/ $f_H$  relationship. Secondly, using a backward procedure, fitted variables were manually dropped depending on their *P* value. Collinearity diagnostics for all analyses indicated no multicollinearity problems between variables, as the highest condition index obtained was 1.6. Multicollinearity can affect parameter estimates when condition indices are  $\geq 20$  (Belsley et al., 1980). Squared correlation coefficients explaining the amount of variation in the models include both fixed and random effects. Similar  $R^2$  values were calculated when relating EE to  $f_H$  or  $\log EE$  to  $\log f_H$ . The Williams' test was used to compare correlation coefficients of EE/ $f_H$  relationships obtained from alternative methods. This test is suggested when correlation coefficients are dependent and sample sizes are between 50 and 500 (Neill and Dunn, 1975). To compare the slopes of these relationships, the

heterogeneous regression slopes with the Johnson–Neyman technique (Huitema, 1980) was used. This technique allowed the estimation of  $f_H$  values where heterogenic slopes differed significantly. All statistical analyses were performed with SAS statistical software (SAS Institute, Cary, NC, USA; version 9.1). All values are reported  $\pm 1$  s.e.m., and the statistical significance was set at 0.05 for all statistical procedures.

## RESULTS

### Polar® logger validation

Fig. 2 shows that  $f_H$  data from the logger and the stethoscope were strongly correlated ( $R^2=0.98$ ). The data ranged from 53 to 176 beats  $\text{min}^{-1}$  and the slope and intercept were very close to 1 and 0, respectively. In incubating and captive king penguins, the range of mean daily  $f_H$  (Table 1) was close to the above range. Also, their minimum and maximum instantaneous  $f_H$  (28 and 235 beats  $\text{min}^{-1}$ , respectively) was within the Polar® logger resolution range.

### $M_b$ loss and $f_H$

When considering all data points ( $N=225$ ), daily  $M_b$  loss varied from 55 to 247 g  $\text{day}^{-1}$ , i.e. a 4.5-fold range. The corresponding range for EE was from 1315 to 5903 kJ  $\text{day}^{-1}$ . Mean daily  $f_H$  varied from 39 to 111 beats  $\text{min}^{-1}$ , i.e. a 2.8-fold range. Within a gender, the mean daily  $M_b$  loss and  $f_H$  did not differ significantly between incubating and non-incubating captive birds (Table 1). Daily  $M_b$  loss was significantly higher for females than males, by 42% in incubating birds and by 22% in captive ones (Table 1). Similarly,  $f_H$  was significantly higher in females than in males, by 31% in incubating birds and 22% in captive ones (Table 1).

### Relationships between $f_H$ and EE

EE and  $f_H$  were significantly related for each of the four groups of birds ( $R^2=0.59$  to 0.84; Table 2). Neither the slopes (mixed model repeated-measures ANOVA;  $F_{1,217}=1.05$ ,  $P=0.37$ ), nor the intercepts ( $F_{1,217}=1.20$ ,  $P=0.31$ ) of the relationships differed between groups. After pooling data from incubating and non-incubating captive birds, significant relationships were derived for both males ( $R^2=0.81$ ) and females ( $R^2=0.84$ ) (Fig. 3). Equation slopes ( $F_{1,221}=0.74$ ,  $P=0.39$ ) and intercepts ( $F_{1,221}=0.96$ ,  $P=0.33$ ) did not differ according to gender. When sexes were pooled first, significant EE/ $f_H$  relationships were found for incubating ( $R^2=0.87$ ,  $P<0.0001$ ) and non-incubating ( $R^2=0.84$ ,  $P<0.0001$ ) captive penguins. For relationships, neither the

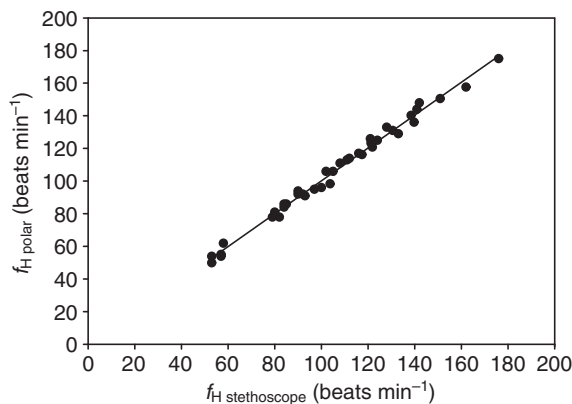


Fig. 2. Regression of heart rate ( $f_H$ , beats  $\text{min}^{-1}$ ) measured with a Polar® logger on  $f_H$  measured with a stethoscope in king penguins. The equation is:  $f_H \text{ polar}=1.006\pm 0.015\times f_H \text{ stethoscope}-0.435\pm 1.695$ ,  $n=40$ ,  $R^2=0.98$ ,  $P<0.0001$ .

slopes ( $F_{1,221}=0.06$ ,  $P=0.81$ ) nor the intercepts ( $F_{1,221}=0.02$ ,  $P=0.89$ ) differed between incubating and captive birds. Pooling all data points (genders and status) showed that in king penguins fasting ashore during the breeding season, EE (kJ  $\text{day}^{-1}$ ) and  $f_H$  (beats  $\text{min}^{-1}$ ) are related according to:

$$EE = -387 + 52.5 \times f_H \quad (F_{1,133} = 19.33, R^2 = 0.85, P < 0.0001). \quad (1)$$

Expressing EE in  $\text{J min}^{-1}$  (1 day=1440 min), the equation is:

$$EE = -387 + 36.4 \times f_H. \quad (1a)$$

The slope of Eqn 1a represents the energy pulse of king penguins fasting ashore, i.e. the energy equivalent of one heart beat. It averaged  $36.4\pm 1.9$  J  $\text{beat}^{-1}$ . The mean residual error for Eqn 1, i.e. the difference between the observed EE from  $M_b$  loss (mean=3019 $\pm$ 62 kJ  $\text{day}^{-1}$ ) and the predicted value (mean=3041 $\pm$ 55 kJ  $\text{day}^{-1}$ ) from the regression model [(observed–predicted)/observed] was  $-3.0\%$ , with a range between  $-51.3$  and  $38.1\%$ , and an absolute mean error of 13.3%.

### EE/ $f_H$ relationship, fasting duration and $M_b$

When considering all data points with the simultaneous use of fasting duration ( $t$ ) and  $M_b$  in the model,  $t$  ( $F_{1,221}=4.11$ ,  $P=0.04$ ) and the interaction term  $t \times f_H$  ( $F_{1,221}=21.53$ ,  $P<0.0001$ ) explained the variability of EE but not  $M_b$  ( $F_{1,221}=0.61$ ,  $P=0.43$ ). Thus, including  $t$  (days) in the EE (kJ  $\text{day}^{-1}$ )/ $f_H$  (beats  $\text{min}^{-1}$ ) relationship improved the fit, the best general model being:

$$EE = 818 + 43.7 \times f_H + 36.3 \times t - 1.4 \times t \times f_H \quad (R^2 = 0.91). \quad (2)$$

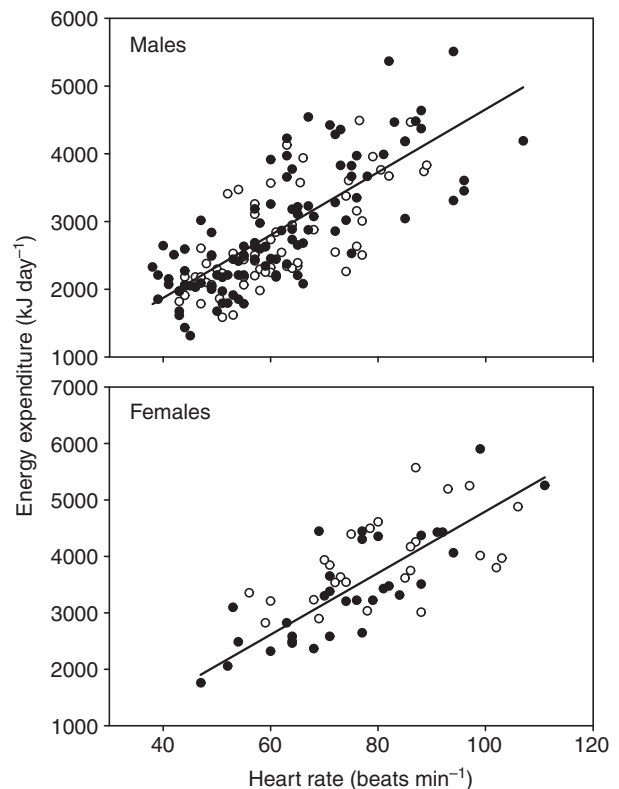


Fig. 3. Relationship between energy expenditure (EE, kJ  $\text{day}^{-1}$ ) and heart rate ( $f_H$ , beats  $\text{min}^{-1}$ ) in male and female king penguins. Open and closed symbols are for freely incubating and captive non-incubating birds, respectively. The equations are: males:  $EE=53.1\pm 3.4\times f_H-443\pm 220$ ,  $n=167$ ,  $F_{1,107}=15.45$ ,  $R^2=0.81$ ,  $P<0.0001$ ; females:  $EE=48.1\pm 5.9\times f_H-4\pm 475$ ,  $n=58$ ,  $F_{1,25}=8.16$ ,  $R^2=0.84$ ,  $P<0.0001$ .



Table 2. Regressions of daily energy expenditure ( $\text{kJ day}^{-1}$ ) on heart rate ( $\text{beats min}^{-1}$ ) in incubating and non-incubating captive male and female king penguins

| Sex and status      | <i>N</i> | <i>n</i> | Slope    | Intercept | $R^2$ | <i>P</i> |
|---------------------|----------|----------|----------|-----------|-------|----------|
| Males, incubating   | 51       | 65       | 44.8±5.4 | -6±338    | 0.59  | <0.0001  |
| Males, captive      | 18       | 102      | 54.3±4.5 | -445±300  | 0.83  | <0.0001  |
| Females, incubating | 23       | 26       | 37.1±9.9 | 949±802   | 0.84  | 0.02     |
| Females, captive    | 11       | 32       | 53.6±7.3 | -528±577  | 0.82  | <0.0001  |

*N*, number of birds; *n*, number of data points. Means ± s.e.m.

The energy pulse (EE divided by  $f_H$ ) significantly decreased with fasting duration ( $F_{1,223}=81.62$ ,  $R^2=0.75$ ,  $P<0.0001$ ), namely by approximately 1% per day. Similar equations were obtained for males ( $R^2=0.88$ ) and females ( $R^2=0.92$ ), with no difference between genders (slopes:  $F_{1,220}=0.65$ ,  $P=0.42$ ; intercept:  $F_{1,220}=0.76$ ,  $P=0.38$ ). The mean residual error for Eqn 2 was -2.2%, with a range between -68.1 and 34.4%, and an absolute mean error of 10.9%.

#### Validation test

Thirty randomly selected data points were removed from the sample data, and the EE/ $f_H$  relationships were then calculated from the remaining 195 data points. The new equations were:

$$\text{EE} = -318 + 51.6 \times f_H \quad (F_{1,193} = 300.9, R^2 = 0.85, P < 0.0001) \quad (3)$$

$$\text{EE} = 899 + 42.4 \times f_H + 29.9 \times t - 1.3 \times t \times f_H \quad (R^2 = 0.90). \quad (4)$$

Using these equations, the EE values predicted for the 30 data points averaged  $2992 \pm 164$  (Eqn 3) and  $2964 \pm 156 \text{ kJ day}^{-1}$  (Eqn 4). The difference between the observed EE ( $2861 \pm 181 \text{ kJ day}^{-1}$ ) and those predicted was -7.1% (-36 to 32%) and -5.8% (-48 to 25%) for Eqns 3 and 4, respectively. These differences were not significant (Eqn 3:  $t=0.54$ ,  $P=0.60$ ; Eqn 4:  $t=0.43$ ,  $P=0.71$ ).

#### Comparison with previous predictions

Equation 1 in Fahlman et al. (Fahlman et al., 2004) is currently considered to be the most suitable equation for estimating EE from  $f_H$  in king penguins for all locomotory and nutritional states (Halsey et al., 2007). This equation [ $\log(\dot{V}_{O_2}) = -0.279 + 1.24 \times \log(f_H) + 0.0237 \times t - 0.0157 \times \log(f_H) \times t$ ], with  $\dot{V}_{O_2}$  in  $\text{ml min}^{-1}$ ,  $f_H$  in  $\text{beats min}^{-1}$  and  $t$  in days, was determined by measuring  $O_2$  consumption and  $f_H$  in captive male king penguins fasting for various durations during the breeding season. The EE predicted using eqn 1 in Fahlman et al. (Fahlman et al., 2004) were compared with those of Eqn 2, by applying each equation to the  $f_H$  and  $t$  dataset obtained in the present study ( $N=225$ ,  $f_H=38-111 \text{ beats min}^{-1}$ ,  $t=3-25$  days). Oxygen consumption was converted into EE using the energy equivalent of  $20.11 \text{ J ml}^{-1} O_2$  determined in fasting birds (Schmidt-Nielsen, 1997).

Predicted EE were related to  $f_H$  for both equations (Fig. 4) and the correlation coefficients were not significantly different ( $R^2=0.85$  for Eqn 2 in our study and  $R^2=0.84$  for eqn 1 in Fahlman et al. (Fahlman et al., 2004); Williams' test:  $t_{222}=0.134$ ,  $P=0.89$ ). For  $f_H$  values lower than  $103 \text{ beats min}^{-1}$ , predicted values differed significantly between the two equations for both the slopes ( $F_{1,207}=10.05$ ,  $P=0.0012$ ) and the intercept ( $F_{1,207}=31.12$ ,  $P<0.0001$ ). Eqn 2 from our study yielded predicted EE values on average 26% higher than eqn 1 in Fahlman et al. (Fahlman et al., 2004). The difference increased with decreasing  $f_H$ , reaching 75% ( $t_{356}=21.49$ ,  $P<0.0001$ ) for  $f_H=40 \text{ beats min}^{-1}$ . In other words, for a given EE the corresponding  $f_H$  is higher for eqn 1 in Fahlman et al. (Fahlman et al., 2004) than for our Eqn 2, and even more so when EE is low.

## DISCUSSION

This study is the first to successfully calibrate the relationship between EE and  $f_H$  using a large sample of free-ranging animals during natural and undisturbed behaviours. We determined two equations for predicting EE from  $f_H$ , with and without fasting duration. Although females had a  $f_H$  22–31% higher than males, gender did not affect these relationships. Finally, we observed that EE values predicted from  $f_H$  and fasting duration were higher than values obtained in previous studies, mostly when  $f_H$  was low.

#### Validity of $f_H$ measurements, EE estimates and EE/ $f_H$ relationships

##### $f_H$ measurements in king penguins

Numerous studies have been conducted to estimate field energetics for penguins based on  $f_H$  measurements (Bevan et al., 1994; Boyd et al., 1999; Froget et al., 2001; Froget et al., 2002; Froget et al., 2004; Green et al., 2003; Fahlman et al., 2004; Fahlman et al., 2005; Fahlman et al., 2006). Unfortunately, this huge piece of work was performed without knowing the accuracy of loggers ['HRDDL Logger' (Woakes et al., 1995)] for  $f_H$  measurements over the  $f_H$  range commonly found in free-living birds. Actually, there is evidence that this logger may overestimate  $f_H$  because some electrical spikes corresponding to muscular activity are counted as heart beats (Y.H., unpublished data). Our calibration study showed that the Polar<sup>®</sup> logger yielded highly accurate  $f_H$  measurements, within a  $f_H$  range close to that documented in free-living or captivity-acclimatized king penguins fasting ashore, for restrained birds which were either resting or moving slightly. This conclusion is therefore at least valid for birds with limited physical activity, as it is the case during incubation; incubating king penguins in fact spend most of their time resting (60–75%) and have a mean level of EE only slightly higher (approximately 14%) than their resting metabolic rate (V.V., R.G. and S.D.C., unpublished data).

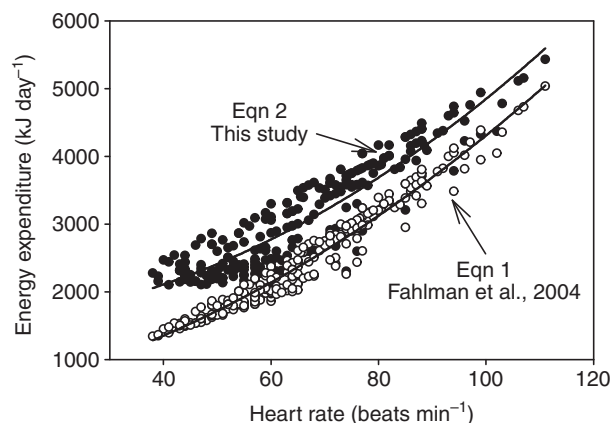


Fig. 4. Comparison of energy expenditure (EE) predicted from heart rate using Eqn 2 in our study and eqn 1 in Fahlman et al. (Fahlman et al., 2004).

### Estimating EE from $M_b$ loss

The error made for  $M_b$  loss measurements was lower than 3.6% (see Materials and methods). The validity of EE estimates therefore depended mostly on the energy equivalent of  $M_b$  loss. We found that the best fit for the relationship between total energy content and  $M_b$  was linear (Fig. 1). The constant level of the  $M_b$  loss energy equivalent throughout phase II fasting in king penguins is therefore illustrated, as previously observed in other fasting seabirds (Groscolas, 1988; Groscolas et al., 1991). The use of the same  $M_b$  loss energy equivalent to calculate EE whatever the duration of phase II fasting, as carried out in our study, was therefore appropriate. The value ( $23.9 \text{ kJ g}^{-1}$ ) calculated and used in the present study only differs by  $\pm 7\%$  from those previously determined by direct analysis of body composition in two other species of long-term fasting seabirds during phase II, the great-winged petrel [*Pterodroma macroptera*:  $22.3 \text{ kJ g}^{-1}$  (Groscolas et al., 1991)] and the emperor penguin [*Aptenodytes forsteri*:  $25.5 \text{ kJ g}^{-1}$  (Groscolas, 1988)]. Applying the latter values to the calculation of EE from  $M_b$  loss would have decreased the intercept or increased the slope of Eqn 1 by only 5.4%. The use of the  $23.9 \text{ kJ g}^{-1}$   $M_b$  loss energy equivalent can therefore be considered to have yielded a reliable estimate of EE and its relationships with  $f_H$ .

### Predicting EE from $f_H$

Our results showed that  $f_H$  explained 85 (Eqn 1) to 91% (Eqn 2) of EE variability, i.e. at least as well as for the relationships between  $f_H$  and  $\dot{V}_{O_2}$  in previous studies on penguins [46–91% (Green et al., 2001; Froget et al., 2002; Fahlman et al., 2004; Halsey et al., 2007)]. The unexplained variation in EE could be linked to inter-individual differences in stroke volume and/or tissue oxygen extraction reflecting inter-individual variations in physical fitness. Changes in the allometry of the circulatory system (McPhee et al., 2003) or differences in structural size and shape (Fahlman et al., 2006) are other potential contributors to variations in EE. Considering the accuracy of our predictive equations, it was also at least as good as those of previously reported equations obtained from the measurements of  $O_2$  consumption. For example, the mean residual error and the absolute mean error were  $-2.2$  and  $10.9\%$  for Eqn 2 from the present study, which compares with the  $3.0$  and  $19.3\%$  respective values reported for eqn 1 in Fahlman et al. (Fahlman et al., 2004). From our validation tests, we determined that the absolute mean error of the prediction was  $10.2\%$  for Eqn 2, which is slightly lower than values reported for predictive equations in king [12.6–46.1% (Halsey et al., 2007)] and macaroni [24.9% (Green et al., 2001)] penguins.

### How do fasting, incubation and gender affect the EE/ $f_H$ relationship?

#### Fasting

We found that EE and  $f_H$  decrease during fasting, and that fasting affects EE/ $f_H$  relationships. These findings are similar to those previously reported by Fahlman et al. (Fahlman et al., 2004) for king penguins, except that these authors observed an effect of fasting on the relationship for exercising (walking) but not for resting birds. Because birds in our study spent most of the time either resting or in moderate activity, we suggest that there is actually an effect of fasting for both resting and exercising penguins. This meets the conclusion of Halsey et al. (Halsey et al., 2007): an equation including fasting time and the interaction term  $f_H \times$  fasting time, as for eqn 1 in Fahlman et al. (Fahlman et al., 2004) and for our Eqn 2, is the most suitable equation to estimate EE from  $f_H$  in fasting king penguins from different activity and nutritional states. Fasting

affects the EE/ $f_H$  relationship through a decrease in the energy (or oxygen) pulse. This decrease is illustrated in Fig. 4, both for eqn 1 in Fahlman et al. (Fahlman et al., 2004) and for our Eqn 2. Indeed, the best fits for these EE/ $f_H$  relationships including the fasting duration are curvilinear, showing that for decreasing  $f_H$  (due mostly to increasing fasting duration) the relationship slope (i.e. the energy pulse) progressively decreases. As previously suggested (Froget et al., 2001; Fahlman et al., 2004), a decrease in the oxygen pulse during fasting [the product of stroke volume by the  $(Ca_{O_2} - \bar{C}_{V_{O_2}})$  difference in Fick's equation] could be due to a decrease in systolic volume linked to a decrease in heart mass and to a decrease in the capacity of tissues to extract oxygen due to inactivity. A greater decrease in heart protein mass compared with total body protein mass has indeed been observed in fasting king penguins (Cherel et al., 1994).

#### Incubation and gender

The EE/ $f_H$  relationship was similar for freely incubating and captive non-incubating birds, demonstrating that incubation *per se* does not affect this relationship. Furthermore, we did not find a gender effect on the EE/ $f_H$  relationship (Eqn 1 and 2), although  $f_H$  was 22–31% higher in females than in males. To date, contradictory results have been reported regarding a gender effect on the  $\dot{V}_{O_2}/f_H$  relationship in penguins. Gender differences have been reported in the king penguin by Froget et al. (Froget et al., 2001) but these differences were later shown to disappear when  $M_b$  and fasting duration were included in the model relating  $\dot{V}_{O_2}$  to  $f_H$  (Fahlman et al., 2004). In exercising macaroni penguins, the  $\dot{V}_{O_2}/f_H$  relationship was seen to differ greatly between males and females (Green et al., 2001). However, in this case, the comparison was between breeding males and a pool of breeding or moulting females, meaning that the difference between genders may simply be the result of moulting *vs* breeding states. Our results support the hypothesis that there is no difference in their  $\dot{V}_{O_2}/f_H$  relationship for male and female penguins, providing that they are in a similar physiological state.

#### Does stress affect the EE/ $f_H$ relationship?

Fig. 4 shows that eqn 1 in Fahlman et al. (Fahlman et al., 2004) yields predicted EE lower than those predicted from our Eqn 2. This difference could partially be due to a potential overestimation of the energy equivalent of  $M_b$  loss in our study. However, the 95% confidence interval of our estimate was small ( $\pm 1.3 \text{ kJ g}^{-1}$ , i.e.  $\pm 5.4\%$  of the  $23.9 \text{ kJ g}^{-1}$  estimate) and, as discussed above, this estimate only differed by  $\pm 7\%$  from those previously obtained in other naturally fasting seabirds (Groscolas, 1988; Groscolas et al., 1991). It is therefore unlikely that the use of a  $23.9 \text{ kJ g}^{-1}$  energy equivalent of  $M_b$  loss could have led to an average 26% overestimate of EE. Furthermore, we observed that differences between both predictions did not remain constant for the entire  $f_H$  range of measured in king penguins but increased progressively as  $f_H$  decreased. How could this discrepancy be explained?

Previous measurements of  $f_H$  (with HRDDL loggers) and  $\dot{V}_{O_2}$  in king penguins have been conducted with males placed in a respirometer or wearing a respiratory mask. Males were caught during courtship and kept fasting in a pen, in the same way as captive males in the present study. We found that for a mean  $M_b$  of  $12.3 \text{ kg}$  and a mean fasting duration of 12 days,  $f_H$  of captive and undisturbed males averaged  $62 \text{ beats min}^{-1}$ . By comparison and for similar  $M_b$  and fasting durations,  $f_H$  values averaged 93 (Froget et al., 2001) or 91 to  $107 \text{ beats min}^{-1}$  (Fahlman et al., 2004) when birds were kept resting in a respirometer. These values were 47–72% higher than for our captive undisturbed birds spending

most, but not all, of their time resting. They are also similarly higher than for freely incubating males (see Table 1). As recently recognised by Halsey et al. (Halsey et al., 2008), confinement in a respirometer and repeated handling to measure  $\dot{V}_{O_2}$  undoubtedly cause stress in individuals. Stress is known to enhance  $f_H$  (Blix et al., 1974; Wilhelm and Roth, 1998; McPhee et al., 2003), which could most probably explain the marked over-estimation of resting  $f_H$  in previous studies. Moreover, the increase in  $f_H$  during stress is generally beyond levels that can be predicted from measured oxygen uptake (Blix et al., 1974; Steptoe, 2000; McPhee et al., 2003). This 'additional  $f_H$ ' is used as an indicator of emotional activation or arousal in humans (Stromme et al., 1978; Wilhelm and Roth, 1998). We suggest that this type of disproportionate stress effect on  $f_H$  vs EE may explain why the  $f_H$  corresponding to a given EE was higher in previous studies (Fahlman et al., 2004) than in the present study. In addition, the stress-induced 'additional  $f_H$ ' could be proportionally higher when EE and  $f_H$  is low, explaining why the difference between our study and that of Fahlman et al. (Fahlman et al., 2004) is particularly marked for low EE and  $f_H$ . Lastly, confinement and walking in a respirometer (or when wearing a respirometry mask) may induce hyperthermia. Under such conditions, we recently observed a 0.5°C increase in the body temperature (stomach) of king penguins after a 15 min walk (R.G., J. Tornos and J.-L. Rouanet, unpublished data). It is known that under an extreme heat load,  $f_H$  increases to improve heat dissipation and that the oxygen pulse decreases (Brosh, 2007). Thus, heat load may also have contributed to the disproportionate increase in  $f_H$  vs  $\dot{V}_{O_2}$  in previous studies. We therefore conclude that previously calibrated EE/ $f_H$  relationships have no doubt been affected by stress (and possibly by heat load), leading to underestimations of EE from  $f_H$ , particularly at low  $f_H$  and EE levels. As a result, the use of our Eqn2 yields a more accurate estimate of EE and could therefore be an indispensable tool for the determination of EE from  $f_H$  in incubating and moderately active king penguins fasting ashore. Whether and how stress could have affected previous determinations of EE/ $f_H$  relationships in other animal species should be considered in further studies.

#### LIST OF ABBREVIATIONS

|                 |                            |
|-----------------|----------------------------|
| EE              | energy expenditure         |
| $f_H$           | heart rate                 |
| $LM_b$          | lean body mass             |
| $M_b$           | body mass                  |
| TBE             | total body energy          |
| TBW             | total body water           |
| $\dot{V}_{O_2}$ | rate of oxygen consumption |

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