

Glycerol and NEFA kinetics in long-term fasting king penguins: phase II *versus* phase III

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Summary

In spontaneously fasting birds such as penguins, below a body mass threshold corresponding to the phase II–phase III transition, a metabolic and hormonal shift occurs and feeding behaviour is stimulated ('refeeding signal'). The major aim of this study was to determine whether a decrease in non-esterified fatty acid (NEFA) release from adipose tissue could be a component of this signal. Lipolytic fluxes and primary triacylglycerol:fatty acid (TAG:FA) cycling were determined *in vivo* in breeding, fasting king penguins (*Aptenodytes patagonicus*) using continuous infusions of 2-[³H]glycerol and 1-[¹⁴C]palmitate under field conditions. In phase II (after approximately 8 days of fasting, large fat stores, body protein spared, $N=8$), the rate of appearance (R_a) of glycerol and of NEFA were 5.7 ± 0.8 and $10.5 \pm 0.4 \mu\text{mol kg}^{-1} \text{min}^{-1}$, respectively, and the percentage of primary TAG:FA cycling was $41 \pm 7\%$. In phase III (after

approximately 25 days of fasting, fat stores reduced by fourfold, increased body protein catabolism, $N=9$), R_a glycerol kg^{-1} body mass remained unchanged, whereas R_a glycerol kg^{-1} fat mass and R_a NEFA kg^{-1} body mass were increased by 2.8-fold and 1.5-fold, respectively. Increased R_a glycerol kg^{-1} fat mass was possibly the result of a 3.5-fold increase in circulating glucagon, the increased R_a NEFA kg^{-1} body mass being attributable to decreased primary TAG:FA cycling. Thus, triggering of the refeeding signal that redirects the behavior of fasting, incubating penguins from incubation towards the search for food after entrance into phase III cannot be ascribed to a reduction in lipolytic fluxes and NEFA availability.

Key words: lipolysis, triacylglycerol:fatty acid cycling, fat stores, refeeding signal, bird, king penguin, *Aptenodytes patagonicus*.

Introduction

Birds and mammals adjust to prolonged fasting by mobilizing their fat stores (triacylglycerols, TAG) and sparing body proteins (Cahill, 1976; Goodman et al., 1980; Cherel et al., 1988a). The duration of this metabolic status, the so-called phase II of fasting (Goodman et al., 1980; Le Maho et al., 1981; Cherel et al., 1988a), depends on initial fat availability (Cherel and Groscolas, 1999). When a critical but still incomplete exhaustion of fat stores is attained, body proteins are no longer spared and animals enter into phase III of fasting (Goodman et al., 1980; Le Maho et al., 1981; Robin et al., 1988). Phase III is characterized by a progressive acceleration of protein catabolism and a decrease in the relative contribution of lipids to energy production (Goodman et al., 1980; Le Maho et al., 1981; Robin et al., 1988). It is reversible, provided that body proteins or lipids are not critically or fully depleted, respectively (Handrich et al., 1993; Robin et al., 1998).

A large part of our knowledge on how animals adjust to prolonged fasting, especially on the relationships between energy reserves, metabolic status and behaviour, arises from studies on birds that spontaneously fast at certain stages of their annual cycle. Among them are penguins (Spheniciforms), sea

birds living in the antarctic and subantarctic regions. Penguins feed exclusively at sea and must fast on land for periods of up to four months during breeding, especially during incubation (Groscolas, 1990). Both mates alternate at incubating (except in the emperor penguin, *Aptenodytes forsteri*) and normally it is relief by the partner that terminates the bird's fasting bouts and allows departure to sea for refeeding (Groscolas, 1990). At this stage, most penguins are still in the phase II metabolic and endocrine status, with only a small proportion of the birds at the onset of phase III (Groscolas and Robin, 2001). However, the relieving partner can be delayed, forcing the incubating bird to prolong its fast until eventually it abandons its egg and goes to sea for feeding. This abandonment has also been observed in other sea birds such as petrels (Chaurand and Weimerskirch, 1994; Ancel et al., 1998). Recently, Groscolas et al. (2000) showed that in the king penguin *Aptenodytes patagonicus* egg abandonment is preceded by a progressive decrease in egg attendance and occurs when the birds had been fasting into phase III for about one week. In fasting non-incubating emperor penguins, and as observed in fasting rodents (Koubi et al., 1991), entrance into phase III is

associated with an increase in locomotor activity (Robin et al., 1998). These observations have led to the hypothesis that a metabolic shift from preferential use of body lipids to body proteins as energy sources would trigger an endogenous refeeding signal (Robin et al., 1998; Groscolas et al., 2000). A reduction of the contribution of fatty acid oxidation to energy production could be a basic component of this signal. This suggestion is supported by the finding that in rats fed high fat diets, and thus relying heavily on fat as the main energy fuel (as do fasting penguins), a blockade of fatty acid oxidation stimulates food intake (Langhans and Scharrer, 1987). Since in penguins the metabolic shift occurs when fat stores are critically but not totally depleted, a decrease in the rate of fatty acid oxidation could result from a reduction in the rate of production of these substrates from adipose tissue (lipolysis).

Elucidating the mechanism of the refeeding signal that translates alteration in energy metabolism into feeding behaviour is of interest in understanding the long-term control of energy intake and body mass. The major aim of the present study was therefore to determine whether a decrease in lipid substrate release from adipose tissue is associated with entrance into phase III in penguins. Most of our knowledge on fat mobilization and its regulation in the fasting state derives from studies in short-term fasting humans and laboratory animals, i.e. essentially during the fed state to phase II transition (Belo et al., 1976; Wolfe et al., 1987; Klein et al., 1989; Kalderon et al., 2000). Not only has lipolysis during long-term fasting rarely been examined (Armstrong et al., 1961; Steele et al., 1968; Bortz et al., 1972), but no data is available for phase III fasted animals. Since the fasting physiology of the king penguin is representative of that of other birds (Cherel et al., 1988a) and to some extent of mammals (Cherel and Groscolas, 1999), the present study may also provide useful information on whether and how the lipolytic rate and NEFA availability are affected by this fasting situation.

The rate of appearance (R_a) of non-esterified fatty acid (NEFA) and glycerol (an index of lipolysis) were measured using tracer methodology during phases II and III of fasting. Phase III birds were at the same stage of fat store depletion as incubating birds at the time of spontaneous egg abandonment. The large size and tameness of the king penguin allowed measurement of lipolytic fluxes in birds under field conditions for the first time.

Materials and methods

Animals

This study was carried out at the breeding colony of the Baie du Marin, Possession Island, Crozet Archipelago (46°25'S; 51°52'E) during the austral summers 1998–1999 and 1999–2000. It was approved by the Ethical Committee of the 'Institut Français pour la Recherche et la Technologie Polaires' and followed the 'Agreed Measures for the Conservation of Antarctic and Subantarctic Fauna'. Birds at this locality are used to seeing people almost every day. Male king penguins

Aptenodytes patagonicus J. F. Miller were identified from their song during courtship. Seventeen of them were caught while pairing, i.e. after fasting ashore for 1–3 days. They were weighed and their beak and flippers measured (body mass BM, 13.27±0.22 kg; beak length, 133±1 mm; flipper length, 322±3 mm). The two size measurements are not significantly different from data for average male king penguins (132.5±0.8 mm and 326±5 mm, $N=50$; R. Groscolas, unpublished data). Birds were then kept in an outdoor fenced area (3 m×3 m) next to the colony under natural climatic conditions.

Phase II of fasting

Eight animals were habituated to these conditions for 6 days before the experiment. This time period is known to be sufficient to suppress the confinement stress and for daily BM loss, body temperature and plasma fuel level to reach a steady state in penguins (Groscolas and Rodriguez, 1981). Thus, the fasting duration at R_a measurement was about 8 days (6 days of captivity plus 1–3 days of fasting ashore at capture), which is more than the 2–3 day duration of the transition from the fed state to phase II reported for this species (Cherel et al., 1988a). BM at R_a measurement (12 kg, Table 1) was similar to that of free-living male king penguins at the onset of the first incubation shift, i.e. after about 8–10 days of fasting (Robin et al., 2001). It was also about 2–2.5 kg higher than the 9.6–10 kg BM measured at entrance into phase III in breeding, fasting male king penguins (Cherel et al., 1988b, 1994).

Phase III of fasting

Nine animals were kept fasting and weighed every 3 days, then every day until they reached a BM close to 9 kg. This mass corresponds to that measured by Groscolas et al. (2000) in king penguins abandoning their egg to refeed at sea, and is about 0.5–1 kg lower than the BM at entrance into phase III. On average, 24±1 days of fasting in the pen were necessary for the

Table 1. *Metabolic and hormonal characteristics of king penguins during phases II and III of fasting*

	Phase II ($N=8$)	Phase III ($N=9$)
Body mass (kg)	11.99±0.25	8.94±0.04**
Fat mass (kg)	2.36±0.13	0.62±0.02**
Uric acid (mmol l ⁻¹)	0.21±0.02	0.52±0.12**
Glucose (mmol l ⁻¹)	11.47±0.30	10.51±0.27*
β-OH (mmol l ⁻¹)	2.02±0.31	1.55±0.16
TAG (mmol l ⁻¹)	0.77±0.11	0.56±0.07
Glucagon (pg ml ⁻¹)	79±11	280±32**
Insulin (i.u. ml ⁻¹)	9.6±1.0	10.7±0.7

Values are means ± S.E.M.

i.u., international unit.

Asterisks indicate a significant difference between fasting phases (* $P<0.05$; ** $P<0.001$).

β-OH, β-hydroxybutyrate; TAG, triacylglycerols.

animals to reach the 9 kg BM. Based on the previously reported rate of daily BM loss (Cherel et al., 1988b), it can be estimated that birds had been in the phase III fasting situation for 5–7 days when R_a measurements were performed.

Catheterization and experimental setup

The day before the experiment, the nonanesthetized bird was cannulated with a polyethylene catheter (length, 50 mm; external diameter, 1.1 mm) inserted percutaneously into the marginal vein of each flipper and extended with a 2 m long prolongator filled with saline. Catheterization of an artery for blood sampling could not be securely performed in field conditions. We assumed that any particular metabolism of the flipper (essentially feathers, bones and tendons) is low and that flipper venous blood reflects whole body metabolism. Until the experiment, catheters were kept patent by continuous infusion of 0.9% NaCl (12.5 ml day⁻¹) using a small peristaltic pump. After catheterization, the birds were allowed to habituate to the experimental setup for 24 h. This setup was installed in the fenced area and consisted of a wooden pen (70 cm × 70 cm) with one wall high enough for us not to be seen by the bird. Catheter prolongators were placed into a balance lever system to avoid damaging the prolongators or tearing out catheters. It also allowed the bird to move freely inside the pen (a few steps) and even to lie on its belly or sleep with the bill under the shoulder, as was regularly observed during tracer infusion. The free ends of catheter prolongators used for infusion and blood sampling were brought outside the pen. From a distance these two operations could then be performed without disturbing the animal. Once the animal was in the experimental setup, particular care was taken to avoid any intervention or noise. During isotope infusion, the air temperature ranged from 10 to 15 °C, i.e. was within the thermoneutral range for the species (Groscolas and Robin, 2001), and the penguins were protected against wind but not against rain. Birds were de-equipped the day after R_a measurement, marked on the chest with Nyanzol dye to allow resighting and released in the colony next to the beach. All the birds used in the study were resighted during the following weeks, caught and weighed. All had restored their BM, which indicates that they had been successfully feeding at sea and that the experiments had no impact on their health.

Continuous isotope infusions

Infusate preparation

The infusate was prepared daily according to Wolfe (1992) and Turcotte et al. (1992) using 2-[³H]glycerol (Amersham, 40.7 Gbq mmol⁻¹) and 1-[¹⁴C]palmitate (Amersham, 2.04 Gbq mmol⁻¹) as tracers. Palmitate is one of the most commonly used fatty acids for measuring NEFA kinetics in mammals (Bonadonna et al., 1990). Besides its ready commercial availability, palmitate is the second most abundant NEFA in mammals and its percentage contribution to NEFA shows low interindividual variability. This was also observed in king penguins in phase II and phase III (data not shown), suggesting that labeled palmitate is as appropriate an indicator

of NEFA kinetics in king penguins as in mammals. 1-[¹⁴C]palmitate was supplied commercially in toluene. A subsample in a sterile flask was evaporated to dryness and immediately resuspended in ethanol to obtain a solution of 37 kBq µl⁻¹. NaOH (2 mmol l⁻¹ in ethanol) was added to excess and the mixture was evaporated to dryness. The water-soluble 1-[¹⁴C]palmitate sodium salt was dissolved in heated sterile saline. The solution was cooled to about 30 °C and 3.5% delipidated bovine albumin solution was added so that the palmitate/albumin molar ratio was 0.5. 2-[³H]glycerol was then added and the infusate volume was adjusted with saline. Infusion of the isotope mixture was performed using a calibrated syringe pump at 7 ml h⁻¹. 2-[³H]glycerol and 1-[¹⁴C]palmitate infusion rates were 224.9 ± 5.1 × 10³ and 109.8 ± 3.6 × 10³ d.p.m. kg⁻¹ min⁻¹ (N=17), respectively, or less than 0.1 nmol glycerol kg⁻¹ min⁻¹ and less than 1.0 nmol palmitate kg⁻¹ min⁻¹. This corresponded to trace amounts of <0.002% of R_a glycerol and <0.03% of R_a palmitate.

Infusion protocol and blood sampling

To decrease the time necessary to reach the isotopic steady state, we injected a priming dose. It was immediately followed by a 180-min continuous infusion. The first blood sample was not taken until at least 120 min after the beginning of the infusion, to ensure that the steady state was reached (see Results). During the last hour of infusion, 3–4 blood samples of 5 ml each were drawn. Immediately after sampling, the blood was centrifuged and the plasma separated. Plasma was stored at -20 °C until analysis.

Analytical procedures

A portion of plasma (1 ml) was mixed with 25 ml chloroform:methanol (2:1, v:v) according to Folch et al. (1957). After extraction and evaporation as described in Bernard et al. (1999), an aqueous and an organic extract were obtained and resuspended in ethanol:water (1:1, v:v) and hexane:isopropanol (3:2, v:v), respectively.

Glycerol

A volume of aqueous extract equivalent to 300 µl of plasma was used to determine glycerol concentration. It was dried under nitrogen and resuspended in hydrazine buffer. Glycerol concentration was measured enzymatically using an Uvikon spectrophotometer at 340 nm. Total tritium activity was counted on another sample of aqueous extract equivalent to 150 µl of plasma using Ecoscint A scintillation fluid (National Diagnostics, Hesse Hull, England) and a Wallac 1409 counter (Wallac, Turku, Finland). At this step of analysis, tritium activity in the aqueous extract was found to be incorporated not only into glycerol but also into other compounds, mostly glucose. Glycerol was purified using thin layer chromatography and the percentage activity determined according to Bernard et al. (1999). The percentage of tritium activity in glycerol was found to be similar in phase II (28.9 ± 3.7%) and phase III (32.3 ± 4.5%). The specific activity

of glycerol was calculated as total tritium activity times percentage of activity in glycerol divided by glycerol concentration.

Fatty acids

Total NEFA concentration was measured on 10 µl of plasma with an analytical test-kit (NEFA C, Wako Chemicals, Osaka, Japan). The palmitate concentration was obtained by multiplying NEFA concentration by the fractional contribution of palmitate to total NEFA, determined by gas-liquid chromatography. Briefly, plasma lipids were extracted according to Dole and Meinertz (1960) and separated by thin layer chromatography (silica gel plate 60, Merck, Darmstadt, Germany) using hexane:diethyl ether:acetic acid (70:30:1, v:v:v) as the developing solvent. The NEFA fraction was isolated and converted to methyl esters using 14 % boron trifluoride in methanol. Fatty acid methyl esters were separated and quantified by gas-liquid chromatography using a Chrompack CP 9001 gas chromatograph equipped with an AT-WAX capillary column [0.25 mm (i.d.) × 30 m, 0.25 µm thickness, Alltech, Templeuve, France] and a flame ionization detector. Helium was used as the carrier gas and the oven temperature was maintained at 200 °C. Fatty acid peaks were identified by comparing their retention times with authentic standards (Nu-Chek Prep, Elysian, MN, USA) and quantified with an integrator (model SP 4290, Spectra-Physics, Les Ulis, France).

Because phospholipids are not completely extracted by the method of Dole and Meinertz (1960), the total ^{14}C activity and its distribution in plasma lipids (TAG, diacylglycerols, NEFA and phospholipids) was determined on the organic extract obtained from extraction, according to Folch et al. (1957). Lipids were separated by thin layer chromatography, as described above. Each fraction was scraped into separate scintillation vials, resuspended in ethanol:water (1:1, v:v) and counted in Ecoscint A scintillation fluid. Because no ^{14}C is incorporated into fatty acids other than palmitate, palmitate activity was calculated by multiplying total ^{14}C activity found in the organic extract by the percentage activity found in the NEFA fraction. Palmitate activity divided by palmitate concentration yielded palmitate specific activity.

Other plasma metabolites and hormones

Plasma glucose and β -hydroxybutyrate levels were determined on deproteinized plasma by enzymatic methods (Test-Combination, Boehringer-Mannheim GmbH, Germany). Uric acid and TAG levels were estimated by enzymatic colorimetric methods using commercial kits (UA plus for uric acid and Peridochrom triglycerides GPO-PAP for TAG; Boehringer-Mannheim GmbH, Germany). Plasma glucagon and insulin levels were determined by radioimmunoassay. Glucagon was estimated using a commercial kit (Linco, St Charles, MS, USA); the intra- and inter-assay coefficients of variation were 6 % and 7 %, respectively. Insulin was estimated using the insulin-CT kit from CIS bio international (Gif-sur-Yvette, France). The intra- and inter-assay coefficients of

variation were 5 % and 6 %, respectively. Plasma obtained after reaching an isotopic steady state was used in all the measurements.

Calculations and statistics

Fat mass (FM) was calculated from BM (both in kg) as: $\text{FM} = 0.552 \times \text{BM} - 4.260$ ($r^2 = 0.74$, $N = 81$, $P < 0.0001$). This equation was determined in a preliminary study for king penguins with BM 8.5–14.7 kg (M.-A. Thil and R. Groscolas, unpublished data).

Glycerol and palmitate R_a values were calculated using the steady state equation of Steele (1959): $R_a = \text{tracer infusion rate (d.p.m. min}^{-1}) / \text{specific activity (d.p.m. mmol}^{-1})$, and are expressed per unit of body mass or fat mass. R_a NEFA was determined by dividing R_a palmitate by the fractional contribution of palmitate to total NEFA (on average 26 % and 14 % in phase II and III, respectively; see Results).

TAG:FA cycling occurs both primarily (i.e. where fatty acids are re-esterified in adipose tissue without entering the circulation) and secondarily (i.e. where fatty acids arrive at their site of re-esterification through the circulation) (Klein et al., 1989; Wolfe et al., 1990). In the present study, only the absolute and relative rates of primary cycling could be assessed. Upon hydrolysis, each TAG molecule yields three NEFA and one glycerol molecules so that $3 \times R_a$ glycerol represents total fatty acid release by lipolysis. Glycerol cannot be metabolized directly because of the absence of glycerokinase in adipocytes (Wolfe et al., 1990). Therefore, primary TAG:FA cycling was calculated according to Wolfe et al. (1990) as:

$$\text{TAG:FA cycling } (\mu\text{mol kg}^{-1} \text{ min}^{-1}) = (3 \times R_a \text{ glycerol}) - R_a \text{ NEFA},$$

$$\text{TAG:FA cycling } (\%) = 100 \times (\text{TAG:FA cycling}) / (3 \times R_a \text{ glycerol}).$$

It has been shown that substantial and significant rates of primary cycling can be obtained only if the ratio R_a NEFA: R_a glycerol is well below 3 (Wolfe, 1992).

Changes in glycerol and palmitate specific activities were assessed by two-way analysis of variance (ANOVA) or Kruskal–Wallis ANOVA on ranks (when populations were not normal or homoscedastic). Relationships between plasma concentration and R_a glycerol and R_a NEFA were assessed by linear regression analysis. In all other cases, statistical differences were estimated using the Student's *t*-test or the Mann–Whitney Rank Sum test. Percentages were transformed to the arcsine of their square root before statistical analysis. Values are means \pm S.E.M. The criterion of significance was $P < 0.05$.

Results

Metabolic and hormonal status

Physiological data presented in Table 1 were used to characterize phases II and III of fasting. Compared to phase II,

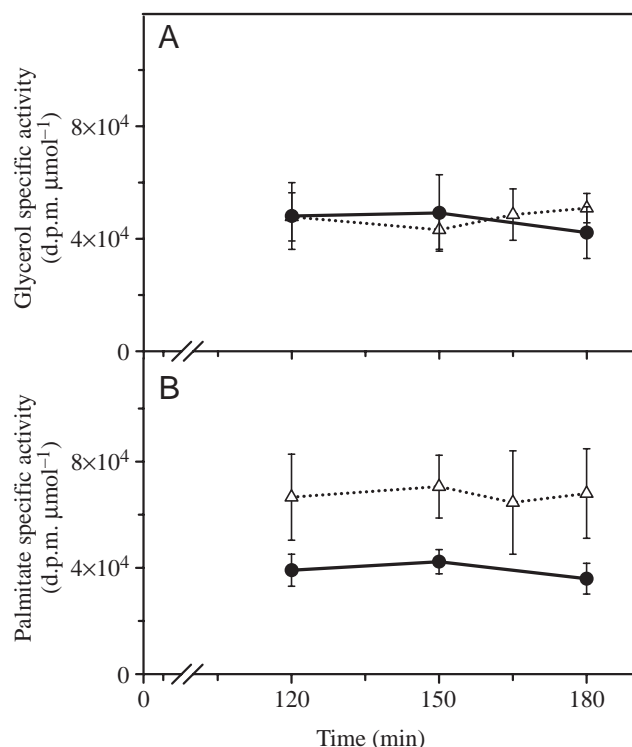


Fig. 1. Glycerol (A) and palmitate (B) specific activities during the final 60 min of 2-[³H]glycerol and 1-[¹⁴C]palmitate infusion. Closed circles, solid line, phase II; open triangles, dotted line, phase III. Values are means \pm S.E.M. for 8 (phase II) and 9 (phase III) birds.

birds in phase III had a 25 % lower BM, nearly fourfold lower FM (7 % *versus* 20 % adiposity) and 2.4-fold and 3.5-fold higher plasma uric acid and glucagon levels, respectively ($P < 0.001$). Plasma glucose concentration was also slightly lower in phase III ($P < 0.05$). Plasma β -hydroxybutyrate, TAG and insulin levels were not significantly different between the two fasting phases.

Isotopic steady state

In both fasting phases, the isotopic steady state existed during the last 60 min of infusion, as indicated by the non-significant changes in specific activities of glycerol and palmitate ($P > 0.90$; Fig. 1A,B). Glycerol specific activity was similar in phases II and III ($P = 0.84$) and averaged $4.62 \pm 0.60 \times 10^4$ disintegrations $\text{min}^{-1} \text{mol}^{-1}$. Palmitate specific activity was significantly higher in phase III ($6.74 \pm 1.16 \times 10^4$ disintegrations $\text{min}^{-1} \text{mol}^{-1}$) than in phase II ($4.09 \pm 0.45 \times 10^4$ disintegrations $\text{min}^{-1} \text{mol}^{-1}$) ($P < 0.05$).

Concentration and rate of appearance of lipolytic metabolites

Concentration and R_a glycerol, R_a palmitate and R_a NEFA are shown in Table 2. Glycerol and NEFA concentrations were slightly but not significantly higher in phase III than in phase II, the palmitate level in phase III being about two-thirds that of phase II ($P < 0.01$). The percentage contribution of palmitate to total NEFA did not change throughout the

Table 2. Mean plasma concentrations and rates of appearance of glycerol, palmitate and total non-esterified fatty acids during phases II and III of fasting in king penguins

	Phase II (N=8)	Phase III (N=9)
Glycerol (mmol l^{-1})	0.05 ± 0.01	0.08 ± 0.01
Palmitate (mmol l^{-1})	0.16 ± 0.01	$0.10 \pm 0.02^{**}$
NEFA (mmol l^{-1})	0.61 ± 0.03	0.74 ± 0.08
R_a glycerol ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	5.72 ± 0.79	5.89 ± 0.72
R_a glycerol ($\mu\text{mol kg FM}^{-1} \text{min}^{-1}$)	30.00 ± 4.62	$85.06 \pm 11.61^{***}$
R_a palmitate ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	2.71 ± 0.32	2.18 ± 0.30
R_a palmitate ($\mu\text{mol kg FM}^{-1} \text{min}^{-1}$)	13.97 ± 1.68	$31.08 \pm 3.79^{***}$
R_a NEFA ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	10.49 ± 0.39	$15.63 \pm 1.92^*$
R_a NEFA ($\mu\text{mol kg FM}^{-1} \text{min}^{-1}$)	53.99 ± 6.80	$223.78 \pm 26.82^{***}$
TAG:FA cycling ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	8.06 ± 1.59	$2.18 \pm 1.02^{**}$
TAG:FA cycling ($\mu\text{mol kg FM}^{-1} \text{min}^{-1}$)	43.15 ± 8.98	31.46 ± 15.25
Percentage of re-esterification (%)	40.7 ± 7.0	$11.7 \pm 4.0^{**}$

Calculated rates of TAG:FA cycling and percentage of re-esterification are also presented.

Values are means \pm S.E.M.

Asterisks indicate a significant difference between fasting phases (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

R_a , rate of appearance; NEFA, non-esterified fatty acids; FM, fat mass.

infusion experiment. It was lower in phase III (14 ± 2 %) than in phase II (26 ± 3 %; $P < 0.001$). When expressed relative to BM, R_a glycerol and R_a palmitate were similar in phases II and III ($P > 0.20$) and averaged 5.85 ± 0.51 and $2.43 \pm 0.22 \mu\text{mol kg}^{-1} \text{min}^{-1}$, respectively. Only R_a NEFA was 1.5-fold higher in phase III than in phase II ($P < 0.05$). However, when expressed per unit of FM, R_a glycerol, R_a palmitate and R_a NEFA were 2.8-, 2.2- and 4.1-fold, respectively, higher in phase III than in phase II ($P < 0.001$).

As shown in Fig. 2A, there was a significant direct relationship between plasma concentration of glycerol (x) and R_a glycerol (y) in phases II and III. At any given plasma glycerol level, R_a glycerol was higher in phase III than in phase II when expressed per unit FM ($P < 0.05$). When R_a glycerol was expressed relative to BM, the regression equations were not different between the two fasting situations ($P > 0.05$; not shown). Fig. 2B illustrates that NEFA levels in plasma and R_a NEFA expressed per unit FM were directly related in phase III. This relationship was not significant in phase II but, at any given plasma NEFA level, R_a NEFA was lower than in phase III.

TAG:FA cycling

The rate of primary TAG:FA cycling expressed relative to BM and the percentage of re-esterification were about 3.5-fold lower in phase III than in phase II ($P < 0.01$, Table 2). The rate of TAG:FA cycling expressed in relation to FM was not different in the two fasting situations ($P = 0.53$).

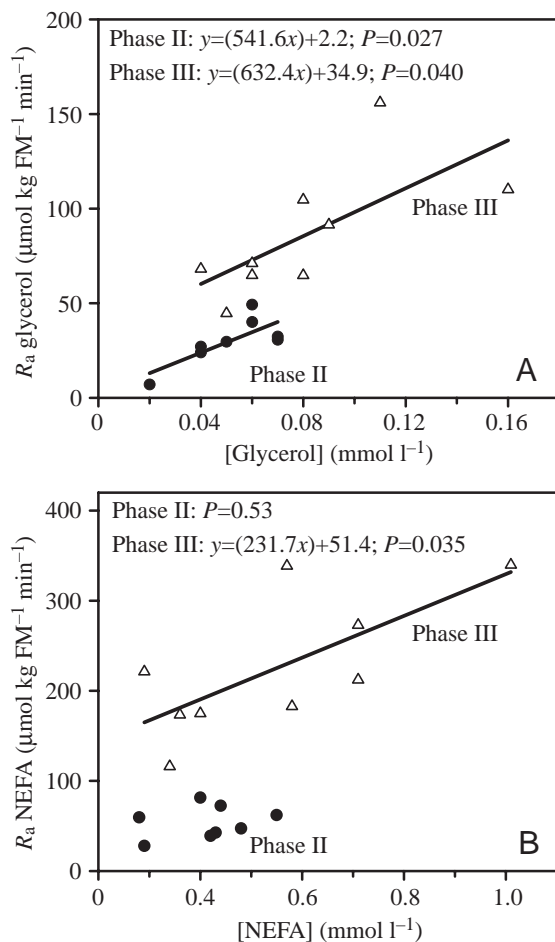


Fig. 2. Regressions of rates of appearance (R_a) of glycerol (A) and NEFA (B), expressed per unit of fat mass (FM) on plasma concentration. Each symbol represents an individual observation. Closed circles, phase II; open triangles, phase III. Differences in regression slopes for the two fasting phases were tested with an analysis of covariance (ANCOVA), using the glycerol or NEFA concentration \times phase of fasting interaction as factor. Differences in intercept were tested for with an ANCOVA, with the fasting phase as factor.

Discussion

Methodological considerations

This study was based on the measurement of the rate of appearance of glycerol (R_a glycerol) and of non-esterified fatty acids (R_a NEFA) during phase II and phase III of fasting in king penguins. In addition to data on fasting duration and BM (see Materials and methods), measurements of plasma concentration of metabolites support the view that the birds were actually in these fasting states. In phase II birds, plasma uric acid and β -hydroxybutyrate levels were low and high, respectively, and similar to those observed in penned incubating (Robin et al., 2001) and non-incubating king penguins (Cherel et al., 1988b) during phase II. These metabolite concentrations indicate a low protein catabolism and a high fatty acid oxidation, which is the signature of phase II of fasting in penguins and other birds (Cherel et al., 1988a;

Robin et al., 1988, 1998). In phase III individuals, plasma uric acid level was increased by a factor of 2.5, which is indicative of the high rate of protein degradation that characterizes phase III of fasting (Cherel et al., 1988b; Robin et al., 1998). This plasma uric acid level was slightly higher than in penned incubating birds at egg abandonment (approximately 0.36 mmol l^{-1}) (Robin et al., 2001), suggesting that in our study the phase III penguins had reached a state of critical energy depletion similar to that of breeders abandoning their egg.

Here we report the first simultaneous *in vivo* measurements of glycerol and NEFA kinetics in birds. How valid are the lipolytic fluxes estimated by the tracer method we used in fasting penguins? In phase II penguins, R_a glycerol and R_a NEFA averaged 5.7 and $10.5 \mu\text{mol kg}^{-1} \text{ min}^{-1}$, respectively. Given the molecular weight of glycerol (92 g mol^{-1}) and NEFA (on average 280 g mol^{-1} in king penguins; R. Groscolas, unpublished data), these rates are equivalent to a daily release of 9.1 and 50.8 g of glycerol and NEFA, respectively. Assuming all glycerol and NEFA released into the circulation are oxidized (no secondary recycling), these numbers approximate daily loss. The daily loss of glycerol and NEFA can also be estimated from daily BM loss, considering that (i) in the king penguin, lipids (TAG) steadily contribute to 47 % of BM loss during phase II (Cherel et al., 1994) and (ii) the glycerol and fatty acid moieties, respectively, contribute 10 % and 90 % of TAG. To avoid repeated manipulations, daily BM loss was not measured in this study. However, based on the 140 g day^{-1} BM loss observed in king penguins under similar conditions (BM = 12 kg, birds caught at pairing and penned for 1 week; R. Groscolas, unpublished data), a daily loss of approximately 7 g of glycerol and 59 g of fatty acids could be estimated. The reasonable agreement between the two estimates supports the conclusion that realistic lipolytic fluxes are obtained by continuous infusion of 2- ^3H glycerol and 1- ^{14}C palmitate in phase II fasting king penguins. Estimates of the daily loss of glycerol and fatty acids from BM loss cannot be made during phase III because the lipid contribution to daily BM loss progressively decreases during this phase and is not known for animals of 9 kg BM.

Lipolysis during prolonged fasting: phase II versus phase III

Lipolytic fluxes have rarely been measured during prolonged fasting in mammals, and never during natural fasting or during phase III. The turnover of two fatty acids has been measured in naturally fasting pups of elephant seal, but no data on the R_a glycerol and R_a NEFA were obtained (Castellini et al., 1987). Table 3 summarizes data obtained in the only two mammal species, humans and dogs, studied after fasting durations (1–4 weeks) comparable to that of the king penguin during phase II. R_a glycerol in penguins is within the range reported for humans, whereas R_a NEFA is at the lower end of the range reported in dogs. More abundant data is available for short-term fasting laboratory animals and humans. In chickens fasted for 2 days, Emmanuel et al. (1983) reported R_a glycerol ($6.6 \mu\text{mol kg}^{-1} \text{ min}^{-1}$) similar to that measured in phase II penguins. In resting non-obese humans

Table 3. Rate of appearance (R_a) of glycerol and NEFA in humans, dogs and king penguins during phase II of fasting

Species	Fast duration (days)	R_a glycerol ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	R_a NEFA ($\mu\text{mol kg}^{-1} \text{min}^{-1}$)	References
Human (lean)	6–7	4.3	–	Bortz et al., 1972
Human (obese)	21–35	2.7–5.7	–	Bortz et al., 1972
Dog	4–5	–	31.3	Armstrong et al., 1961
Dog	7–11	–	14.1	Steele et al., 1968
King penguin	approx. 8	5.7	10.5	This study

fasting for 60–96 h, R_a glycerol ranges from 3.8 to $6.4 \mu\text{mol kg}^{-1} \text{min}^{-1}$ (Klein et al., 1986; Wolfe et al., 1987; Jensen et al., 2001), and was $5.9 \mu\text{mol kg}^{-1} \text{min}^{-1}$ in dogs fasted for 18 h (Nurjhan et al., 1988). R_a NEFA values of 16.5 and $10.0 \mu\text{mol kg}^{-1} \text{min}^{-1}$ have been reported in normal and obese humans, respectively, fasted for 84 h, respectively (Wolfe et al., 1987). Thus, the lipolytic fluxes measured in the present study of king penguins during phase II (R_a glycerol = $5.7 \mu\text{mol kg}^{-1} \text{min}^{-1}$, R_a NEFA = $10.5 \mu\text{mol kg}^{-1} \text{min}^{-1}$) are within the range reported for fasting animals of comparable body size. The value of 41 % obtained for the percentage of primary TAG:FA cycling in penguins during phase II is within the range of 20–49 % reported for short-term fasting humans (Wolfe et al., 1990; Campbell et al., 1994) and laboratory mammals (Commerford et al., 2000; Kalderon et al., 2000; McClelland et al., 2001). However, it must be mentioned that a percentage of recycling that was lower than 13 % or even insignificant has been reported by others (Coppack et al., 1994). It therefore seems that primary recycling is comparatively high in phase II fasting king penguins.

In this study, the whole body lipolytic rate (R_a glycerol in relation to BM; Klein et al., 1986) remained unchanged during phase III in comparison to phase II, despite a fourfold lower FM. It has been shown previously that in penguins, entrance into phase III corresponds to a decrease in the contribution of lipid to energy production, compensated by an increase in the contribution of protein (Robin et al., 1988; Groscolas, 1990). On the other hand, the resting metabolic rate expressed per kg BM is not affected by entrance into phase III (Dewasmes et al., 1980; Cherel et al., 1988a). In our experimental setup, animals in phase III did not show a higher level of physical activity than animals in phase II. Thus it is likely that the metabolic rate per kg BM was similar in the two fasting situations, which suggests that in long-term fasting penguins, the whole body lipolytic rate is related to energy needs rather than to FM. A similar suggestion has been made for short-term fasting humans on the basis of the determination of the whole body lipolytic rate at different levels of adiposity (Klein et al., 1986). Lipolysis (R_a glycerol per FM unit; Klein et al., 1986) and NEFA availability for oxidation (R_a NEFA per BM unit; Klein et al., 1986) were respectively 2.8-fold and approx. 50 % higher in phase III than in phase II, despite high depletion of fat stores in phase III. This observation indicates that, in long-term fasting penguins, NEFA availability is not directly proportional to the size of the TAG stores, i.e. that fat mass is not the only

regulator of fatty acid availability. A similar conclusion was drawn from the comparison of R_a NEFA in obese and lean overnight-fasted humans (Lillioja et al., 1986). Here we show that the same applies to prolonged fasted animals with very small fat stores.

The increase in lipolysis observed in phase III could have been subject to lipolytic hormone control. In birds, glucagon has a strong lipolytic effect (Hazelwood, 1984) and its intravenous injection markedly increases the concentration of circulating plasma NEFA in the emperor penguin (Groscolas and Bézard, 1977). Here we found that the plasma level of glucagon was 3.5-fold higher in phase III than in phase II, in accordance with the previous observation that in fasting non-incubating king penguins, glucagon increases progressively during phase II and more sharply at entrance into phase III (Cherel et al., 1988b). This increase in glucagonemia might have stimulated lipolysis. Unlike in mammals, insulin has no antilipolytic effect in birds (Hazelwood, 1984), including the emperor penguin (Groscolas and Bézard, 1977). Moreover, in the present study the plasma concentration of this hormone was not different in birds fasting in phase II and in phase III. It is therefore unlikely that insulin intervened in the phase III increase of lipolysis.

The triacylglycerol:fatty acid substrate cycle and NEFA availability

Compared to phase II, total fatty acid release *via* TAG hydrolysis ($3 \times R_a$ glycerol) relative to total BM remained nearly unchanged in phase III, whereas the percentage of fatty acids that were primarily re-esterified decreased from 41 % to 12 %. Thus, the decrease in primary TAG:FA cycling ($-5.9 \mu\text{mol kg}^{-1} \text{min}^{-1}$) accounts entirely for the increase in NEFA availability ($+5.1 \mu\text{mol kg}^{-1} \text{min}^{-1}$). Regulation of the primary TAG:FA cycle is poorly understood (McClelland et al., 2001). Potential factors include hormones, substrate concentrations, adipose tissue blood flow and interactions among them (Wolfe et al., 1990; McClelland et al., 2001). It is unlikely that the reduced percentage of primary TAG:FA cycling in phase III was due to a direct inhibition of re-esterification within the adipocyte. Indeed, the absolute primary TAG:FA cycling in relation to FM was not significantly lower in phase III than in phase II. The availability of glucose, which is a precursor of glycerol 3-phosphate (Wolfe et al., 1990; McClelland et al., 2001), was probably not a limiting factor of primary re-esterification. Glucose concentration was only slightly lower in phase III

than in phase II, and in penguins the glucose turnover rate did not change between phases II and III (Groscolas and Rodriguez, 1981). Blood flow per unit mass of adipose tissue is known to increase as fat cell size decreases (Di Girolamo et al., 1971), and in the emperor penguin the average fat cell size at entrance into phase III is fivefold smaller than at the onset of the fast (Groscolas, 1990). Here, an increase in adipose tissue blood flow in the leaner birds is supported by the finding that the regression lines between glycerol and NEFA concentration and their respective R_a per kg FM are shifted upwards during phase III (Fig. 2). Indeed, since the R_a of metabolites has been shown to be strongly dependent on their convective transport through the circulation, i.e. concentration \times blood flow (Weber et al., 1987), a higher R_a at a given concentration indicates a higher blood flow. Thus, simultaneously with improving glucose supply to adipocytes, an increased adipose tissue blood flow in phase III could have caused the reduced percentage of primary TAG:FA cycling by providing adequate albumin binding sites to carry away fatty acids released by lipolysis (Leibel and Edens, 1990; Wolfe et al., 1990).

Our method allowed us to measure only the primary TAG:FA cycling, so we do not know the fraction of R_a NEFA that is re-esterified secondarily after NEFA transit through the circulation. Such an estimation would have required determining fatty acid oxidation (from the measurement of energy expenditure, RQ and N_2 excretion), which was not feasible under our field conditions. NEFA availability, i.e. NEFA available either for oxidation or secondary cycling after their release into the circulation, was increased in phase III. Because energy expenditure does not increase during phase III whereas the contribution of fatty acids to energy production decreases (see above), it is very likely that the rate of fatty oxidation was lower in phase III than in phase II. Consequently, it is conceivable that a larger part of the circulating NEFA was re-esterified back to TAG through secondary TAG:FA cycling in phase III than in phase II. A higher secondary cycling could counterbalance the decrease in primary cycling and prevent the remaining fatty acid stores from being oxidized, perhaps because they are needed for a more vital role than energy production.

Lipolysis and the refeeding signal

The major aim of this study was to examine whether a reduction in NEFA availability through a decrease in the lipolytic rate is the first step of a metabolic and endocrinal cascade that leads to the stimulation of feeding behaviour in prolonged fasting penguins. Our results do not support this possibility. Indeed, entrance into phase III, which is known to trigger the refeeding signal, was not associated with a reduction but with an increase in R_a NEFA, the whole body lipolytic rate remaining unchanged. Thus, if entrance into phase III is due to the attainment of a critical fat mass, the lipolytic rate does not appear to be the link between reduced total body fat availability and increased protein catabolism. The observation that NEFA availability may positively affect

sparing of body protein (Hasselblatt et al., 1971) clearly does not apply in penguins during fasting phase III. It cannot be ruled out that other information arising from adipose tissue, including leptin secretion (Ahima et al., 1996), might inform the whole body or organs of fat store availability. On the other hand, alteration of metabolic pathways other than from adipose tissue can be suggested. Among them, a reduction of fatty acid oxidation in tissues such as the liver should be considered first. In prolonged fasting rats, entrance into phase III has been shown to be associated with a rapid decrease in the total hepatic activity of carnitine palmitoyltransferase and fatty acid oxidase, which are enzymes involved in mitochondrial and peroxisomal fatty acid oxidation, respectively (Andriamampandry et al., 1996). Such a decrease in hepatic fatty acid oxidation in the face of an increased R_a NEFA could explain the transitory (several days) increase in plasma NEFA concentration observed previously at entrance into phase III in young (Cherel and Le Maho, 1985) and adult (R. Groscolas, E. Mioskowski and J.-P. Robin, unpublished data) king penguins. Here, plasma NEFA concentration was slightly but not significantly higher in phase III than in phase II, perhaps because the experiments were done a little before or after reaching the peak in plasma NEFA concentration. A decrease in hepatic fatty acid oxidation in phase III fasted king penguins is also suggested by the slight decrease in the plasma concentration of β -hydroxybutyrate, a product of β -oxidation. Although non-significant, this decrease at least indicates that the progressive increase in plasma β -hydroxybutyrate concentration that is observed during phase II in penguins (Groscolas, 1986; Cherel et al., 1988b) has been reversed. Altogether, these observations support the view of a reduced fatty acid oxidation in penguins with the same energy status as those spontaneously departing to refeed at sea. A reduction of hepatic fatty acid oxidation has been shown to stimulate feeding behaviour in rats relying heavily on fat as energy substrate (Langhans and Scharrer, 1987). The same might apply in penguins, the reduction in fatty acid oxidation apparently being independent of fatty acid production by adipose tissue.

In conclusion, this study shows that at a stage of fasting (phase III) corresponding to spontaneous egg abandonment in fasting, incubating king penguins, lipolysis and NEFA availability are not depressed but are in fact increased. Thus, the phase III-associated refeeding signal that redirects behaviour from incubation towards the search for food does not appear to originate from a limited NEFA availability. The increased lipolysis seems related to a stimulated secretion of glucagon. On the other hand, the higher NEFA availability is attributable to a decrease in the primary TAG:FA cycling, suggesting that this cycling intervenes in the control of NEFA availability in prolonged fasting birds. The possibility that the metabolic and behavioural changes that accompany phase III are the result of a direct inhibition of fatty acid oxidation, and whether and how this inhibition is linked to the attainment of a critical depletion of fat stores, should be examined in further investigations.

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