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RESEARCH ARTICLE

The oxidative debt of fasting: evidence for short- to medium-term costs of advanced fasting in adult king penguins

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ABSTRACT

In response to prolonged periods of fasting, animals have evolved metabolic adaptations helping to mobilize body reserves and/or reduce metabolic rate to ensure a longer usage of reserves. However, those metabolic changes can be associated with higher exposure to oxidative stress, raising the question of how species that naturally fast during their life cycle avoid an accumulation of oxidative damage over time. King penguins repeatedly cope with fasting periods of up to several weeks. Here, we investigated how adult male penguins deal with oxidative stress after an experimentally induced moderate fasting period (PII) or an advanced fasting period (PIII). After fasting in captivity, birds were released to forage at sea. We measured plasmatic oxidative stress on the same individuals at the start and end of the fasting period and when they returned from foraging at sea. We found an increase in activity of the antioxidant enzyme superoxide dismutase along with fasting. However, PIII individuals showed higher oxidative damage at the end of the fast compared with PII individuals. When they returned from re-feeding at sea, all birds had recovered their initial body mass and exhibited low levels of oxidative damage. Notably, levels of oxidative damage after the foraging trip were correlated to the rate of mass gain at sea in PIII individuals but not in PII individuals. Altogether, our results suggest that fasting induces a transitory exposure to oxidative stress and that effort to recover in body mass after an advanced fasting period may be a neglected carryover cost of fasting.

KEY WORDS: Re-feeding signal, Oxidative stress, Hormesis, Foraging effort

INTRODUCTION

The ability of animals to face prolonged periods of food shortage is under strong natural selection (Geiser and Stawski, 2011; Lindstedt and Boyce, 1985; McCue, 2012; Millar and Hickling, 1990; Staples, 2016). Body reserves (i.e. glycogen, lipids and proteins) play a key role in promoting survival under conditions of low energy intake or complete fasting (e.g. Cherel et al., 1994b; Phillips and Hamer, 1999; Secor and Carey, 2016). However, as storage energy is limited, vertebrates have evolved biochemical and physiological mechanisms allowing them to preserve body reserves while fasting, and to trigger re-feeding when energy reserves are critically depleted (Groscolas and Robin, 2001; Groscolas et al., 2008; McCue, 2010; Spée et al., 2010; for a review, see Secor and Carey, 2016). The management of body reserves is tightly linked to changes in metabolic rates: reducing metabolism and physical activity helps extend the period during which energy stores sustain metabolism, while increased metabolism and physical activity promote food searching (Cherel et al., 1994b; Groscolas and Robin, 2001; Nordøy et al., 1990; Rey et al., 2008). Although such metabolic changes provide immediate lifesaving responses, medium- to long-term costs associated with metabolic changes during fasting remain little investigated in wild species that typically cope with repeated and sometimes prolonged periods of food shortage (Vázquez-Medina et al., 2010). Because mitochondria are cornerstone organelles implicated in metabolic responses to fasting (Monternier et al., 2014), but also the first site of production of damaging reactive oxygen species (ROS) (Andreyev et al., 2005), direct oxidative costs to prolonged fasting may be expected (e.g. Chausse et al., 2015; Geiger et al., 2012; Sorensen et al., 2006; Wasselin et al., 2014).

In this study, we test for links between prolonged fasting and oxidative stress in a long-lived seabird, the king penguin (*Aptenodytes patagonicus* Miller 1778), for which fasting is a natural and important part of the life cycle. King penguins breed on land but forage for marine resources, mostly myctophid fish species (Bost et al., 1997), at the oceanic polar front several hundreds of kilometres away from their breeding site (Charrassin and Bost, 2001). Breeding partners must therefore alternate periods of prolonged fasting on land (caring for a single egg or chick) and foraging trips at sea (Olsson, 1996; Weimerskirch et al., 1992). While on land, adults rely entirely on energy reserves during fasting periods of up to 3–5 weeks (Groscolas and Robin, 2001). The longest fasting bout during reproduction is undertaken by the male and covers the month-long period of courtship and the first incubation shift, i.e. the male is the first to incubate the egg while the female replenishes her energy reserves at sea (Stonehouse, 1960; Weimerskirch et al., 1992). The trade-off between current reproduction and survival is therefore particularly important in this species (because breeding and foraging grounds are separated by long distances), and the efficient management of stored energy is crucial to breeding success (adults generally abandon reproduction if stores are critically depleted; Gauthier-Clerc et al., 2001; Groscolas et al., 2008; Olsson, 1997; Robin et al., 2001).

Long-term fasting in penguins (and in animals in general) is characterized by metabolic transitions that can be divided into three distinct phases (Cherel et al., 1994b; Groscolas, 1990; Groscolas and Robin, 2001; for a review see Secor and Carey, 2016). First, individual metabolism relies mostly on carbohydrates as the main energy substrate, and body mass loss per day rapidly drops during fasting phase I (hereafter referred to PI). At the same time as glycogen stores are depleted, lipids become the principal energy

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Received 27 June 2016; Accepted 8 August 2016

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List of abbreviations

GC	glucocorticoid
LMM	linear mixed model
OXY	total plasma antioxidant capacity
PI	fasting phase I
PII	fasting phase II
PIII	fasting phase III
ROM	reactive oxygen metabolite
ROS	reactive oxygen species
SOD	superoxide dismutase

resource. During this period, energy expenditure decreases to a minimum, and individuals enter a long energy-sparing period, the so-called fasting phase II (PII) (Cherel et al., 1994b; Nordøy et al., 1990). If fasting is prolonged further, for instance when the breeding partner's return from sea is delayed, individual body mass will decrease even further and challenge an individual's investment in reproduction (Gauthier-Clerc et al., 2001; Groscolas and Robin, 2001; Groscolas et al., 2008). This final fasting phase (phase III, hereafter PIII) is accompanied by drastic metabolic changes, i.e. the close exhaustion of body fat reserves giving way to muscular proteolysis as a last extreme energy resource (Belkhou et al., 1991; Cherel et al., 1988a; Goodman et al., 1981; Le Maho et al., 1981; Robin et al., 1988). This critical state heralds a physiological limit beyond which adult survival may be compromised (Robin et al., 1998). During PIII, energy expenditure increases again (Le Maho et al., 1981; Cherel et al., 1994b) along with glucocorticoid (GC) levels and non-reproductive behaviour (Kitaysky et al., 1999; Robin et al., 2001; Groscolas et al., 2008). This gradual reallocation of energy towards soma preservation rather than current reproduction results from a complex network of metabolic and (neuro-) hormonal changes called the 're-feeding signal' (Bertile et al., 2009; Groscolas and Robin, 2001; Minokoshi et al., 2004; Groscolas et al., 2008; Spée et al., 2010), and is expected to lead to the restoration of adult body condition at the expense of reproductive success.

In king penguins, birds reaching fasting PIII are able to subsequently rebuild their energy reserves. However, PIII individuals may – but do not systematically – recover body mass as fast as PII individuals (Robin et al., 2001). Notably, for birds breeding late in the season, PIII birds take longer to restore their body mass than PII birds, whereas this is not the case early in the breeding season (Robin et al., 2001). Such differences in recovering dynamics within a breeding season for early and late PIII birds suggest that costs of fasting up to PIII and/or of re-feeding are likely to exist, and may perhaps only be compensated for (or worth paying) under specific environmental (or life-history) circumstances. For instance, breeding birds may not be willing to increase their foraging effort (at a potential cost) to catch up for lost body mass late in the season because of their extremely low likelihood of breeding success (Weimerskirch et al., 1992).

Because of its implications for life-history traits (Costantini, 2008, 2014; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009; Selman et al., 2012; Stier et al., 2012), one important cost to assess for penguins at an advanced stage of fasting (i.e. PIII) is the production of ROS and the balance between ROS and antioxidant defences. Indeed, oxidative stress, i.e. an imbalance between ROS and counteracting defences, in PIII individuals may be associated (1) in the short-term with the transition from PII to PIII and associated increase in metabolic rate (Cherel et al., 1994b; Le Maho et al., 1981); and (2) in the medium-term with the intense foraging effort likely required by PIII birds to recoup their initial body condition (Andreyev et al., 2005; Hulbert et al., 2007; Isaksson et al., 2011; Speijer et al., 2014). Indeed, in addition to high metabolic rates that may result in the by-production of large quantities of ROS (Beckman and Ames, 1998; Stier et al., 2014a,b; but see Speakman and Selman, 2011), king penguins forage in apnoea during repetitive diving bouts (>1000 foraging dives per trip; Bost et al., 2007) carried out at high ambient pressure (>100 m diving depth; Kooyman et al., 1992). This temporarily exposes their tissues to critically low levels of oxygen (hypoxemia), before the transient re-perfusion of oxygen-rich blood when reaching the surface again – a situation known as ischemia-reperfusion (Meir and Ponganis, 2009), which may cause massive bursts of oxidative stress (Chouchani et al., 2016). Whereas deep-diving animals have typically evolved efficient antioxidant defences to deal with such a situation (Vázquez-Medina et al., 2011, 2012; Zenteno-Savin et al., 2010), penguins at an advanced stage of energy depletion may have compromised defence mechanisms, making it harder to cope with oxidative stress.

We experimentally tested whether pre-reproductive king penguins forced to reach PIII (advanced fast) experienced short- to medium-term oxidative costs compared with individuals leaving the colony in a higher body condition (PII, medium fast). We specifically focused on male king penguins as those naturally experience the longest fast of the breeding cycle under natural conditions (Stonehouse, 1960; Weimerskirch et al., 1992). Thus, in a species repeatedly exposed to long-term periods of energy depletion and intense foraging effort, we questioned: (1) whether PIII was associated with the onset of oxidative stress (short-term cost), (2) whether PIII individuals coped with increased oxidative stress while foraging at sea and (3) whether, when returning from sea, oxidative homeostasis was re-established.

MATERIALS AND METHODS

General methods

This study was performed in the breeding colony of 'La Baie du Marin' (approximately 20,000 breeding pairs), Possession Island, Crozet Archipelago (46°26′S, 51°52′E), over two field sessions: December 2011–February 2012 and December 2013–February 2014. Over the 2011–2012 and 2013–2014 summers, we identified 23 males based on their structural size and song during courtship (Stonehouse, 1960). They were caught and housed in open wooden pens of 3×4 m within 10 m of the breeding colony, and thus subjected to natural climatic conditions and colony sounds. Sixteen birds (five birds in 2011–2012 and 11 birds in 2013–2014) were kept captive and released during fasting PII (mean fast duration±s.e.=20.78±1.66 days), and seven birds (three birds in 2011–2012 and four birds in 2013–2014) were kept captive until they entered fasting PIII (mean fast duration±s.e.=28.7±1.88 days). The fasting status of birds was determined by changes in mass-specific daily body mass loss (see below). Birds were left undisturbed except for mass measurements and blood sampling. We ensured that all birds departed to sea upon release, usually within a couple of hours after release.

Ethical statement

All experiments described in this study were approved by the Ethics Committee of the French Polar Institute. Authorizations to enter the colony were obtained from Terres Australes et Antarctiques Françaises. The experiments comply with the current laws of France.

Body mass measurements

Birds were left undisturbed except for weighing once a day (±1 g) for the first 8 days of the fast, and every second day until the end of

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PII. Weighing occurred every morning between 09:00 and 09:30 h. We determined transitions between fasting phases (PI–PII–PIII) based on changes in the rate of mass-specific daily body mass loss (dm/mdt, where m is mass and t is time), which decreases from PI to PII, is low and stable in PII, and increases in PIII (Cherel et al., 1988a,b). King penguins typically enter PIII at a critical mass threshold of approximately 9.3 kg (Cherel et al., 1994a; Gauthier-Clerc et al., 2001; Viblanc et al., 2012). We used both the critical mass threshold and changes in dm/mdt to determine the entry to PIII in the field. Those results were later validated by determining plasma uric acid concentrations (see Fig. S1), an index of protein catabolism in birds (Robin et al., 1988). During fasting in PIII, birds were weighed daily until their release. Birds in the PII or PIII groups had similar body mass when first captured and put in the enclosure (see Results). A final mass measurement was taken when birds returned from their foraging trip.

Blood sampling

To control for possible effects of season, time of day and stress (Dawson et al., 2001; Romero and Romero, 2002), we standardized captures. Blood sampling was performed before weighing the birds at the same period of the year and at the same time of day (every day at 09:00 h ±30 min). The bird's head was covered with a hood to minimize stress and agitation, and blood samples (1 ml) were taken from the brachial vein using a G22-1½ needle fitted to a 2.5 ml heparinized syringe. All blood samples were obtained within 3 min of handling. After centrifugation (3000 ***g*** for 10 min), plasma was kept frozen at –20°C and moved to a −80°C ultra-cold freezer at the end of the day until assayed.

Laboratory measures of oxidative stress, uric acid and protein content

Total plasma antioxidant capacity

Total plasma antioxidant capacity (OXY) was measured using the OXY adsorbent test (5 µl of 1:100 diluted plasma) (Diacron International, Grosseto, Italy) in accordance with methods reported in previous studies (e.g. Costantini and Dell'Omo, 2006; Stier et al., 2013). The OXY adsorbent test quantifies the ability of the plasma to buffer massive oxidation through hydroperoxide acid. All sample measurements were duplicates. Intra-individual variation was 3.35% and inter-plate variation based on a standard sample repeated over plates was 4.25%.

Antioxidant enzymatic activity

The enzymatic activity of superoxide dismutase (SOD) in plasma was measured using a commercial SOD activity kit following the manufacturer's protocol (25 µl of 1:6 diluted plasma) (Enzo Life Sciences, Villeurbanne, France). All sample measurements were duplicates. Intra-individual variation based on duplicates was 3.11% and inter-plate variation based on a standard sample repeated over plates was 6.92%.

Reactive oxygen metabolites (ROMs)

Plasma ROM levels were measured using the d-ROMs test (8 µl of plasma) (Diacron International), in accordance with methods reported for previous studies (e.g. Costantini and Dell'Omo, 2006; Stier et al., 2013). The d-ROMs test measures hydroperoxides, which are the main compounds contributing to the oxidant ability of the plasma (Costantini, 2016) and are expressed as mg H_2O_2 equivalent dl^{-1}. All sample measurements were duplicates. Intra-individual variation was 5.29% and inter-plate variation based on a standard sample repeated over all plates was 5.39%.

Uric acid measurements

OXY measurements may be influenced by the concentration of uric acid and proteins in plasma (Costantini, 2011). Hence, the plasma concentration of uric acid (mg dl^{-1}) was determined using an enzymatic method (10 µl of 1:25 diluted plasma) (Uric Acid Assay, Randox Laboratories, Roissy, France). All sample measurements were duplicates. Intra-individual variation was 3.23% and inter-plate variation based on a standard sample repeated over all plates was 3.55%.

Total protein content

We also measured plasma total protein concentration (g l^{-1}) using a colorimetric assay (10 µl of 1:10 diluted plasma) (Bradford Reagent B6916, Sigma-Aldrich). All runs were duplicates. Intra-individual variation was 3.12%.

As plasma protein and uric acid levels did not significantly explain variation in plasma OXY levels [linear mixed model (LMM) with individual ID as a random factor; F=2.41, P=0.124 and F=−0.01, P=0.938, respectively], we did not control for these variables in our statistical analyses.

Statistical analyses

All analyses were run in the statistical computing software R (v.3.1.1; R Foundation for Statistical Computing, Vienna, Austria). To investigate effects of our fasting experiment on bird variation in body mass and exposure to oxidative stress, we divided our statistical analyses into three parts.

First, for each individual, we characterized the exact day of transition between each phase (PI, PII and PIII) using segmented regression models with the R package 'segmented' (Muggeo, 2008). We searched for break points in uric acid levels in relation to the number of days of fasting (see Fig. S1). From a starting value for the linear predictor (fasting days) describing the response (uric acid concentration), an iterative algorithm was used to fit a new linear regression model at each iteration, identifying marked changes in slope coefficients as breakpoints (for details on the procedure, see Muggeo, 2008). Break-point estimates confirmed the transition stages obtained from segmented relationships with mass-specific daily body mass loss (dm/mdt).

Second, we ran LMMs to compare variation in body mass, plasma oxidative damage (ROMs) and antioxidant capacity (OXY and SOD) for birds released in PII or PIII at the three following periods: (1) beginning of the fast, (2) end of the fast and (3) when birds returned from their post-fast foraging trip at sea. Body mass, oxidative and antioxidant markers were entered as dependent variables in separate LMMs. A six-level (PII-beginning, PIII-beginning, PII-end, PIII-end, PII-return and PIII-return) fixed factor was considered in the models. Individual ID and year of sampling were considered as random effects to control for intra-individual and yearly variation in response variables. Tukey HSD *post hoc* comparisons ('glht' function from the 'multcomp' R package; Bretz et al., 2010) were used to compare responses of PII and PIII individuals. Considering a set of statistical inferences simultaneously and to limit multiple testing issues (Type I errors), only biological relevant comparisons were investigated: (1) PII versus PIII separately at each period and (2) within each bird group (PII or PIII), the differences between values at the beginning of the fast, end of the fast, and return from the post-fast foraging trip. Effect sizes (Hedges' unbiased d) for differences between PII and PIII groups, as well as 95% CI were calculated following Nakagawa and Cuthill (2007) and are provided in the figures.

Third, we used LMMs to investigate whether PII and PIII individuals paid a different oxidative cost in terms of the foraging

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effort required to rebuild energetic resources at sea (defined as birds' daily mass gain during their foraging trip in g day^{-1}). We specified ROMs, SOD and OXY levels measured at birds' return from their foraging trip as the dependent variables under scrutiny and tested for an interaction between fasting phase and foraging effort (daily body mass gain). Individual ID and year were treated as random effects in the models. All results are reported as means±s.e. For each model, the numbers of observations (n) and of individuals (N) are given.

RESULTS

Bird fasting duration and variation in body mass

PIII birds fasted significantly longer (28.7±1.9 days) than PII birds (20.8±1.8 days; LMM: F=30.01, P<0.001, n=23, N=23). In both groups, body mass showed significant differences according to metabolic status (LMM: F=67.68, P<0.001, n=69, N=23; Fig. 1). At the start of the fast, PII and PIII birds did not differ significantly in body mass (13.52±0.23 versus 13.26±0.32 kg for PII and PIII birds, respectively; Tukey's HSD: z=0.67, P=0.979), but as expected, at the end of the fast PIII birds had a significantly lower body mass than PII birds (10.29±0.23 versus 9.06±0.32 kg for PII and PIII birds, respectively; Tukey's HSD: z=3.25, P=0.010). When returning from their foraging trip at sea, both PII and PIII birds had restored their body reserves. Body mass was no longer different between the two groups and was not different to that at the beginning of the fast (Tukey's HSD: $-0.68<z<0.54$, $0.978<P<0.996$).

Re-feeding period at sea in relation to fasting

The mean time individuals spent at sea rebuilding their energy stores following fasting was similar for PII and PIII individuals (LMM: F=0.51, P=0.48, n=23, N=23; Fig. 2A). Birds released in PIII gained significantly more mass than PII birds during the time they spent foraging at sea (LMM: F=7.38, P=0.013, n=23, N=23; Fig. 2B). Daily mass gain was 176.3±36.0 g day^{-1} for PII birds and 239.2±43.9 g day^{-1} for PIII birds, though the difference was not significantly different (LMM: F=1.98, P=0.17, n=23, N=23; Fig. 2C).

Variation of oxidative stress measurements in relation to fasting

Plasmatic variation in antioxidant SOD activity and pro-oxidant markers (ROMs) were significantly different between PII and PIII groups (LMM: SOD, F=6.08, P<0.001, n=69, N=23, ROMs, F=3.01, P=0.017, n=69, N=23). *Post hoc* tests showed that SOD levels significantly increased between the beginning and end of the fast in both PII and PIII individuals (Fig. 3). In PIII individuals, however, SOD significantly decreased between the end of fast and the subsequent return from the foraging trip. In contrast, the decrease in PII birds was not significant (Fig. 3). There was nonetheless no difference in SOD levels between the start of the fast and the return from sea in both PII and PIII individuals (Fig. 3). ROM levels significantly increased between the start and end of the fast in PIII individuals only (Fig. 4). After foraging, ROM levels were similar to those observed at the beginning of the fast in both PII and PIII individuals (see Fig. 4). OXY levels did not differ throughout the fasting period (LMM: F=1.7, P=0.15, n=69, N=23; Fig. 5).

Re-feeding effort and oxidative stress

Re-feeding effort (calculated as the mass gain per day during foraging) may come at the cost of maintaining oxidative balance, especially in individuals enduring long fasting periods. Accordingly, re-feeding effort was significantly positively related with ROM levels measured at the return from sea in PIII individuals only (LMM: group×mass gain per day, F=5.02, P=0.038, n=23, N=23; Table 1, Fig. 6). No relationship was found between re-feeding effort and OXY (F=0.88, P=0.36, n=23, N=23) or SOD levels (F=0.73, P=0.40, n=23, N=23).

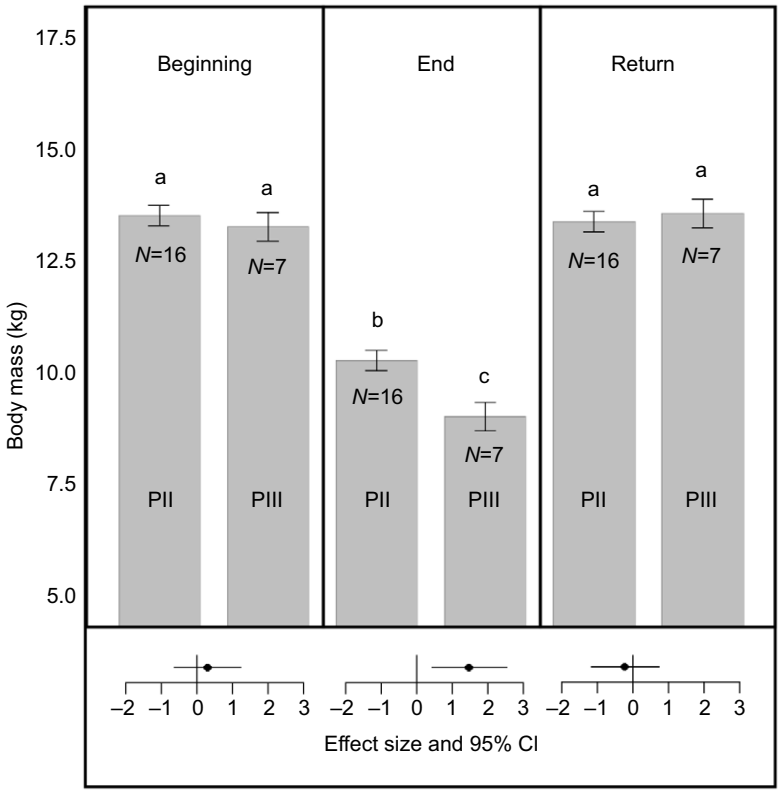

Fig. 1. Body mass at different stages of fasting (beginning, end and when returning from a post-fast foraging trip at sea) in king penguins (*Aptenodytes patagonicus*) having undergone an experimental fasting period up to fasting phase II (PII) or fasting phase III (PIII). Marginal means±s.e. from the model are presented (n=69, N=23). Values not sharing a common letter are statistically different at P<0.05 (Tukey HSD test). Effect sizes (Hedges' unbiased d) for differences between PII and PIII groups, as well as 95% CI are provided below each panel.

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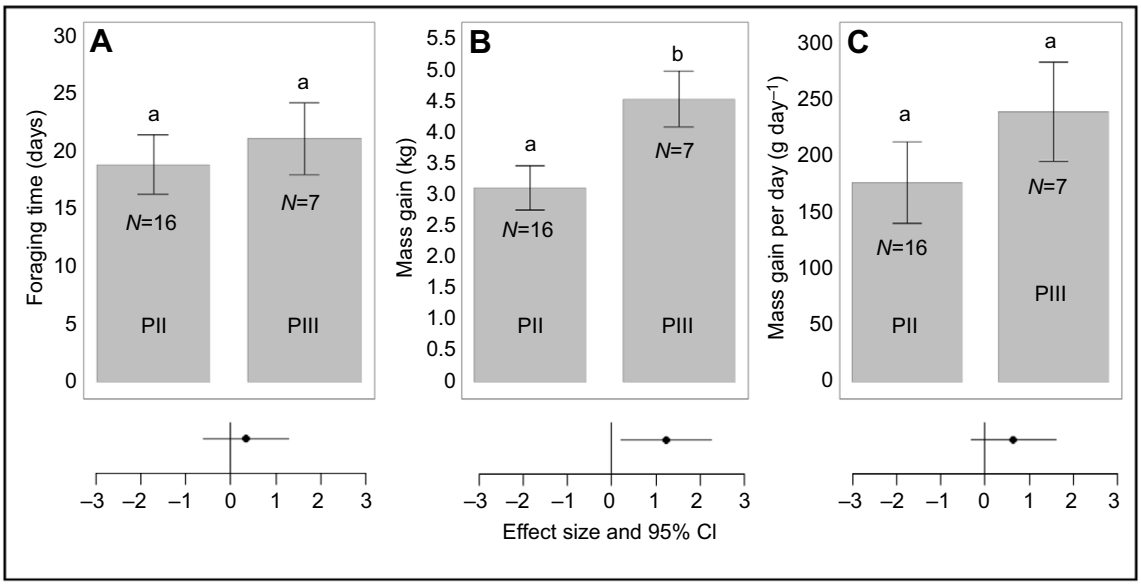

Fig. 2. Comparison of at sea post-fast foraging durations in king penguins (*Aptenodytes patagonicus*) having undergone an experimental fasting period up to fasting phase II (PII) or fasting phase III (PIII). (A) Mean time spent (days) at sea and (B) mean mass gain (kg) of foraging king penguins (*Aptenodytes patagonicus*) during their re-feeding trip following release from an experimental fasting period up to PII or PIII. (C) Foraging effort in term of body mass gain per day (g day^{-1}) by birds during their foraging trip whether they were released in PII or PIII (n=23, N=23). Marginal means±s.e. estimated by LMM models are presented. Values not sharing a common letter are statistically different at P<0.05 (LMMs with year as random factor). Effect sizes (Hedges' unbiased d) for differences between PII and PIII groups as well as 95% CI are provided below each panel.

DISCUSSION

In this study, we tested for a potential oxidative cost of long-term fasting in king penguins, for which fasting is a natural and important part of its life cycle (Groscolas, 1990; Stonehouse, 1960). Our results suggest that birds fasting up to PIII (advanced fast) paid an additional cost of recovering from that fast compared with birds fasting up to PII (medium fast), i.e. a debt paid in terms of oxidative imbalance to restore body reserves. Our results complement previous findings in captive ducks and rats (Geiger et al., 2012; Wasselin et al., 2014) describing an oxidative cost of entering PIII.

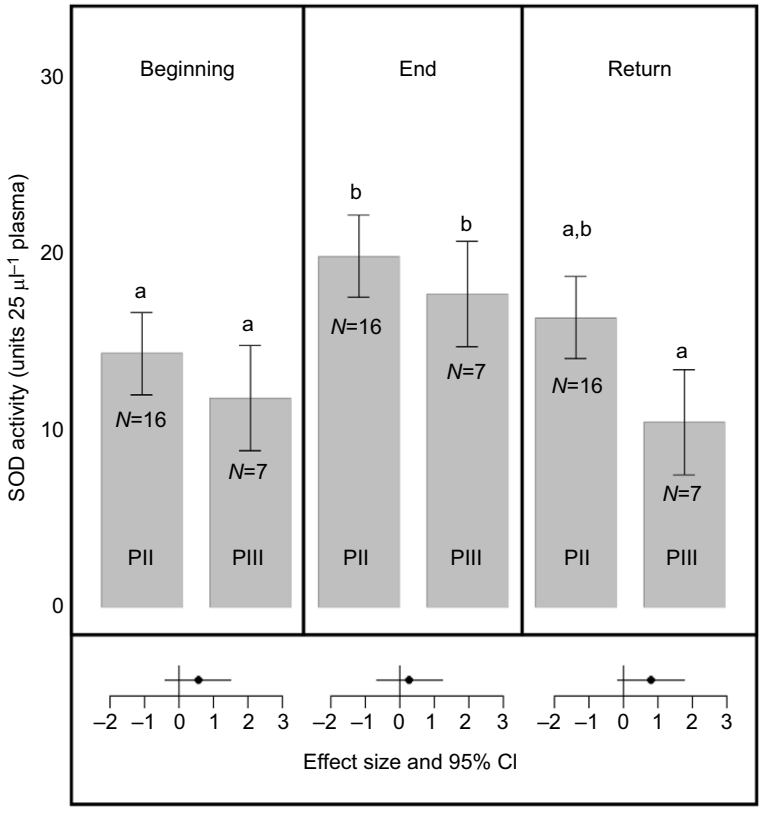

Fig. 3. Plasmatic superoxide dismutase (SOD) activity at different stages of fasting (beginning, end and when returning from a post-fast foraging trip at sea) in king penguins (*Aptenodytes patagonicus*) having undergone an experimental fasting period up to PII or PIII. Marginal means±s.e. from the model are presented (n=69, N=23). Values not sharing a common letter are statistically different at P<0.05 (Tukey's HSD test). Effect sizes (Hedges' unbiased d) for differences between PII and PIII groups as well as 95% CI are provided below each panel.

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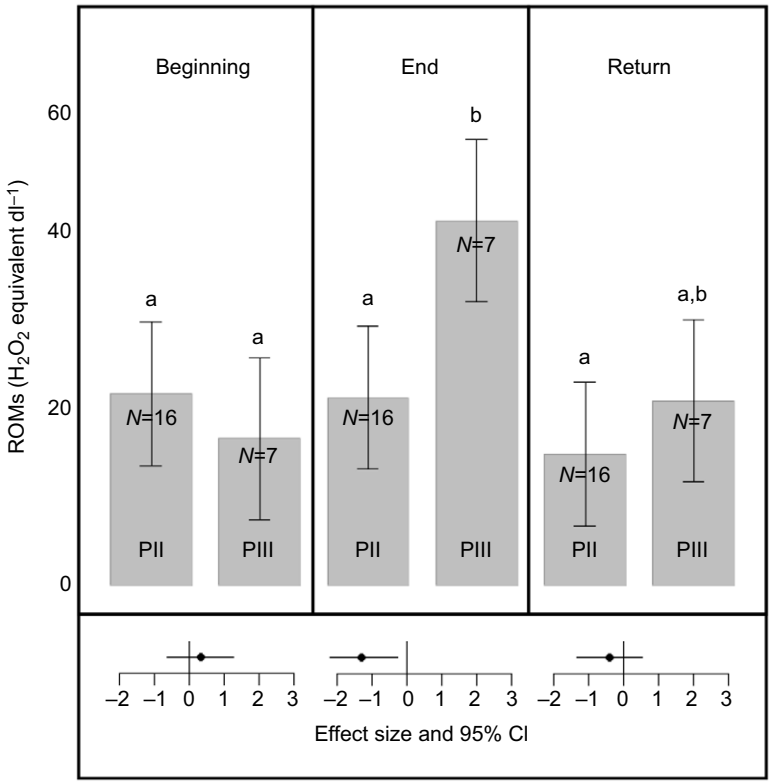

Fig. 4. Total oxidative plasmatic damage [reactive oxygen metabolites (ROMs)] at different stages of fasting (beginning, end and when returning from a post-fast foraging trip at sea) in king penguins (*Aptenodytes patagonicus*) having undergone an experimental fasting period up to PII or PIII. Marginal means±s.e. from the model are presented (n=69, N=23). Values not sharing a common letter are statistically different at P<0.05 (Tukey's HSD test). Effect sizes (Hedges' unbiased d) for differences between PII and PIII groups as well as 95% CI are provided below each panel.

Birds that fast up to PIII utilize more energy reserves than birds that stop fasting in PII, PIII being characterized by the onset of protein (muscle) catabolism once fat stores are close to exhaustion (Cherel et al., 1994b; Groscolas and Robin, 2001). Our study shows that all individuals (PII and PIII birds) had fully recovered their body mass when returning from their foraging trip at sea (Fig. 1), both PII and PIII individuals spending a similar amount of time at sea. These results suggest that PIII individuals either exhibited greater foraging effort (e.g. in terms of prospection of the water column while diving) or were more efficient at processing and/or assimilating

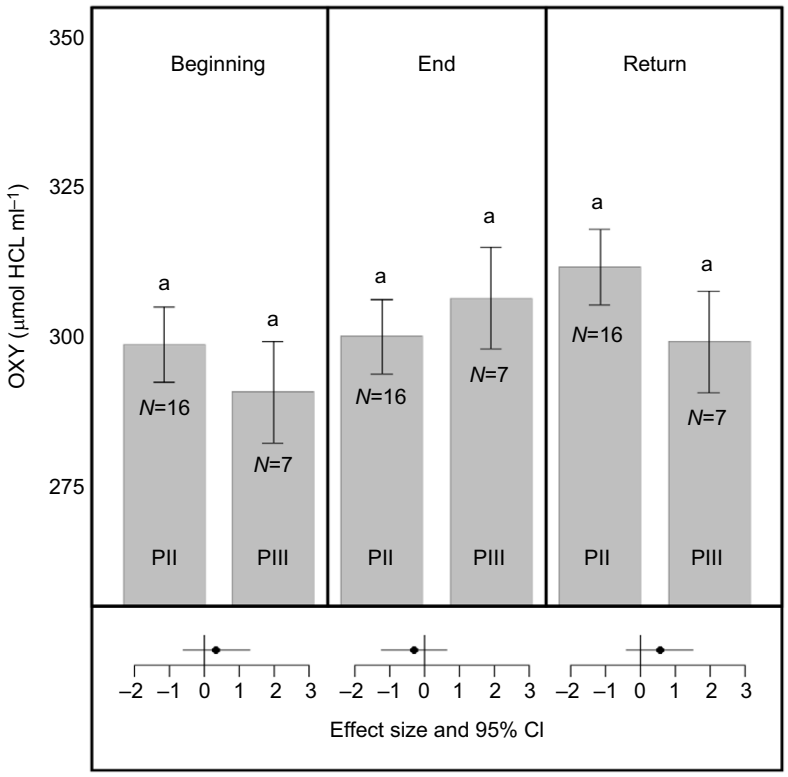

Fig. 5. Total antioxidant plasmatic defences (OXY) at different stages of fasting (beginning, end and when returning from a post-fast foraging trip at sea) in king penguins (*Aptenodytes patagonicus*) having undergone an experimental fasting period up to PII or PIII. Marginal means±s.e. from the model are presented (n=69, N=23). Values not sharing a common letter are statistically different at P<0.05 (Tukey's HSD test). Effect sizes (Hedges' unbiased d) for differences between PII and PIII groups as well as 95% CI are provided below each panel.

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Table 1. Linear mixed model estimates for the effects of foraging effort (daily body mass gain; g day^{-1}) in king penguins (*Aptenodytes patagonicus*) on total oxidative damage levels in plasma measured at the end of a post-fast foraging trip at sea (*n*=23, *N*=23)

Factor	Estimate	s.e.	d.f.	*F*	*P*
Fasting group (PII)	12.188	8.626	18.1	2.00	0.175
Daily mass gain	0.082	0.032	18.2	5.65	0.029
Fasting group (PII)×Daily mass gain	–0.078	0.035	18.1	5.02	0.038

Birds had either undergone a previous fasting period up to fasting phase II (PII) or fasting phase III (PIII). Estimates for the fasting group and interaction are considered against the reference level PIII. Year was specified as a random factor.

caught food resources. Apparently, fasting up to PIII was achieved at an oxidative cost, as we observed higher oxidative damage in PIII birds both at the end of the fast (higher plasma levels of ROMs) and after the re-feeding trip (decreased enzymatic antioxidant defences, SOD). In addition, foraging effort was positively related to ROM levels when returning from the foraging trip in PIII but not PII birds.

Whereas PII has previously been characterized by a decrease in energy expenditure along with a slow decrease in plasmatic antioxidant defences and oxidative damage (e.g. in ducks; Geiger et al., 2012), reaching the critical stage of PIII enhances oxidative respiration, allowing an individual to mobilize its last resources necessary to undertake foraging activities (Goodman et al., 1981; Groscolas and Robin, 2001). Increases in metabolic rate with the onset of PIII have indeed been observed in penguins (Cherel et al., 1994b; Groscolas and Robin, 2001; Groscolas et al., 2000) and rats (Koubi et al., 1991), and the transition from PII to PIII appears to be accompanied by an increase in oxidative damage (in ducks: Geiger et al., 2012; in rats: Wasselin et al., 2014).

Several mechanisms may be suggested to explain the onset of oxidative stress in fasting PIII. First, the energetic reserves mobilized during fasting might also include exogenous antioxidants (Mårtensson, 1986) (antioxidant compounds not produced by the organism but acquired from the diet, such as carotenoids, vitamin E, etc.). However, this seems unlikely in king penguins because OXY in our study did not appear to vary with fasting duration. Second, shifting from a lipid to a protein oxidative pathway has been suggested to increase amino-acid input into the Krebs cycle, leading to the generation of large amounts of NADH,

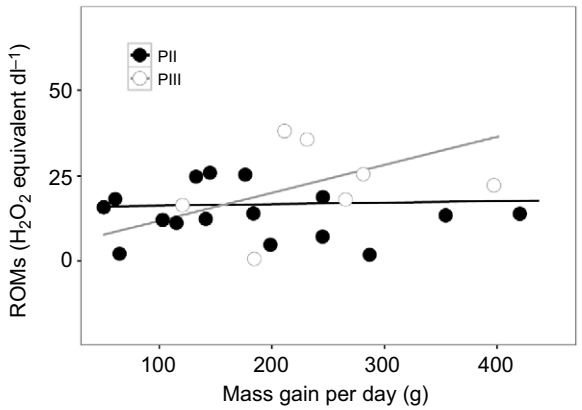

Fig. 6. Interactive effect of the effort invested during the re-feeding trip (body mass gain per day) on the total plasmatic oxidative damage of king penguins returning from their foraging trip depending on whether they were released in PII or PIII (*n*=23, *N*=23).

thereby enhancing oxidative respiration (Wasselin et al., 2014). Third, plasmatic concentrations of GC hormones (corticosterone in birds) known to increase rapidly at the onset of PIII, promote gluconeogenesis and enhance energy resource mobilization (Cherel et al., 1988a; Robin et al., 1998). The corticosterone increase (and a concurrent decrease in prolactin levels) has been associated with a 're-feeding signal' in various bird species, including king and other penguins, resulting in a decrease of current reproduction to the benefit of self-maintenance (Angelier and Chastel, 2009; Criscuolo et al., 2002; Groscolas and Robin, 2001; Groscolas et al., 2008; Spée et al., 2011). GC-related beneficial effects on adult survival are then likely to be counter-balanced by detrimental impacts on the oxidative balance when exposure to high levels of GCs is sustained over time (Costantini et al., 2011; Lin et al., 2004). Together, these processes likely add up to explain the oxidative status reached at advanced fasting stages (Lin et al., 2004; Morales et al., 2004; Sorensen et al., 2006; Wasselin et al., 2014).

One alternative explanation for the oxidative rise in phase III could be that it is adaptive. Indeed, ROS and/or reactive nitrogen species (especially H_2O_2 and NO) are involved in many transduction signalling pathways as secondary messengers (tyrosine kinase membrane receptors, MAP kinases, nuclear factor κB or Ras; Kamata and Hirata, 1999; Allen and Tresini, 2000), raising the question of whether oxidative stress in itself during PIII could play a role in the re-feeding signal (Geiger et al., 2012) by modulating cellular hormesis (Costantini, 2014; Ristow and Zarse, 2010). This hypothesis supports the idea that oxidative stress may act as an important mediator of life-history trade-offs (Costantini, 2008, 2014; Metcalfe and Alonso-Alvarez, 2010; Monaghan et al., 2009; Selman et al., 2012). Experimentally increasing oxidative stress level (e.g. using a pro-oxidant such as paraquat; Isaksson and Andersson, 2008) or decreasing antioxidant activity (e.g. using buthionine sulfoximine; Koch and Hill, 2016) in birds experiencing a moderate fast (PII) may allow testing of whether high oxidative stress leads to energy reallocations between breeding and foraging.

The oxidative cost of reaching fasting PIII might appear surprising given the life history and breeding cycle of long-term fasters such as king penguins (Cherel et al., 1988a,b). These birds indeed fast repeatedly throughout the life cycle (Cherel et al., 1987, 1988a,b). As seems to be the case, adaptation to fasting might actually prevent the occurrence of oxidative stress during advanced fasting. Penguins seem to be able to maintain high antioxidant defences (e.g. OXY levels) regardless of their energy depletion status (actually, SOD activity increases with advancing fasting), and those defences appear to shield the organism from oxidative stress during PII. In contrast, in humans, rats and mice, antioxidant defences decrease during fasting, which may trigger a pro-oxidative cascade, increasing mitochondrial oxidant generation (Ceriello and Motz, 2004; Sorensen et al., 2006; Souza Rocha et al., 2008; Szkudelski et al., 2004). However, once in PIII, the increase in antioxidant defences is no longer sufficient to counteract the damages caused by ROS or reactive nitrogen species, leading to short-term oxidative stress. Thus, the adaptation of those long-term fasters to oxidative stress relies on the fact that PIII birds seem to be able to recover rapidly from short-term oxidative stress. In fact, they recover to similar ROM levels as PII individuals over the same duration of foraging at sea. In addition, the oxidative cost of long-term fasting does not seem to affect body mass recovery (PIII and PII). Both PII and PIII birds appear to recover to similar body mass, and PIII birds appear to be more efficient at assimilating energy resources (body mass was recovered within a similar amount of time in PII and PIII birds without increasing re-feeding effort), as has

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been shown for rats (Robin et al., 2008). The oxidative cost of reaching PIII also likely did not affect future reproduction, as PIII penguins were observed returning to the colony to court and breed (Q.S., personal observations; Robin et al., 2001). Nonetheless, because oxidative stress may result in either deleterious effects (Bize et al., 2008) or adaptive (hormetic) responses (Costantini, 2014; Ristow and Zarse, 2010; Yun and Finkel, 2014), cumulative long-term effects of chronic exposure to oxidative stress are harder to predict. Notably, oxidative stress is known to be an important predictor of health and biological ageing through cumulative detrimental effects on DNA telomere length (Richter and Von Zglinicki, 2007; Von Zglinicki, 2000, 2002), and telomere length has recently been shown to be a good proxy of individual quality (breeding performance and immunity) in king penguins (Le Vaillant et al., 2015). Thus, it would be interesting to further consider whether birds repeatedly entering advanced fasting stages pay a long-term cost in terms of telomere attrition rates. This could be achieved using long-term monitored individuals followed through multiple breeding cycles (e.g. Le Vaillant et al., 2015).

Similarly to our findings in king penguins, an increase in antioxidant defences to face increasing oxidative stress with advanced fasting has also been observed in seals (Vázquez-Medina et al., 2011). Interestingly, one common life-history feature of penguins and seals is the alternation of long deep-diving events with short surface events for breathing. These animals have to cope with prolonged apnoea, exposing tissues to high levels of hypoxemia due to high pressure when diving, followed by rapid tissue re-perfusion and transiently high oxygen concentration in tissues during brief surface episodes (Meir and Ponganis, 2009). This situation, known as ischemia-reperfusion, induces the mass activation of nitric oxide synthase (Huang et al., 1994; Iadecola et al., 1997) and xanthine oxidase (Granger, 1988), enzymes known to promote ROS. Coping with repeated exposure to high levels of ROS during repeated diving has been suggested to explain the higher levels and activity of antioxidant enzymes in the muscles and livers of diving birds (Zenteno-Savin et al., 2010), and to protect seals against oxidative damage during prolonged fasting (Zenteno-Savín et al., 2002). Land-based marine predators forage at sea but generally experience long-term fasts while breeding and/or moulting on land. It is likely that evolutionary pressures acting both on diving and fasting simultaneously selected for high antioxidant defence mechanisms in diving animals (Vázquez-Medina et al., 2012).

Nonetheless, our results suggest a limit to this adaptation. Indeed, although PII and PIII birds had similar ROM levels when they returned from foraging at sea, it appeared that an increase in foraging effort (mass gain per day) lead to an increase in oxidative stress in PIII birds but not in PII birds. Whereas those results suggest a cost for PIII birds to rapidly restore their energy reserves, our limited sample size warrants some caution. Increased foraging effort implies higher energy expenditure (Froget et al., 2004), which likely increases oxidative stress (Finaud et al., 2006). Thus, PIII birds recovering from an oxidative debt at the end of their fast are apparently not able to cope with the additional oxidative load imposed by high foraging effort. This might explain previous differences observed in the time taken by early- and late-breeding PIII birds to replenish their energy stores (Robin et al., 2001). Indeed, early in the season (as birds in our study), PIII birds appeared to recover from a foraging fast as rapidly as PII birds: the total duration of post-fast foraging trips are similar and they do not differ in terms of body mass upon return to the colony (Robin et al., 2001). In contrast, late-breeding PIII birds spend a significantly longer time at sea to recover their body mass. Given that late-breeding success is virtually zero (Olsson, 1996; Weimerskirch et al., 1992), late-breeding PIII birds may take longer to replenish energy reserves to avoid the oxidative costs of foraging.

To conclude, we highlight a short-term cost of prolonged (PIII) fasting in long-term fasting seabirds, which may play a role in the re-feeding signal promoting individual survival over current reproduction. This cost appears to be compensated for to a great extent (but not entirely) during the subsequent foraging trip at sea. The consequences of short-term costs may be carried over in the form of medium-term costs if birds increase their foraging effort at sea. Whether those short- to medium-term costs of fasting may accumulate over a longer time scale (individuals fast repeatedly during reproduction) to affect subsequent reproductions and adult fitness remains to be determined.

Acknowledgements
We are especially grateful to Dominic L. Cram and one anonymous reviewer for helpful comments on the paper.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Q.S. contributed to study design, data collection and analyses, and writing the manuscript. V.A.V. contributed to data analyses and writing the manuscript. H.S. contributed to data collection and laboratory analyses. A.S. and E.L. contributed to data collection. F.C. contributed to data analyses and writing the manuscript. P.B. contributed to study design, data analyses and writing the manuscript. J.-P.R. contributed to study design, data collection and writing the manuscript.

Funding
This research was funded by the Institut Polaire Français Paul Emile Victor (IPEV; Research Program 119) and the Centre National de la Recherche Scientifique (CNRS-INEE). Q.S. was funded by a doctoral fellowship from the Ministère de l'Éducation Nationale, de l'Enseignement Supérieur et de la Recherche. Field logistic support was provided by Terres Australes et Antarctiques Françaises.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.145250.supplemental

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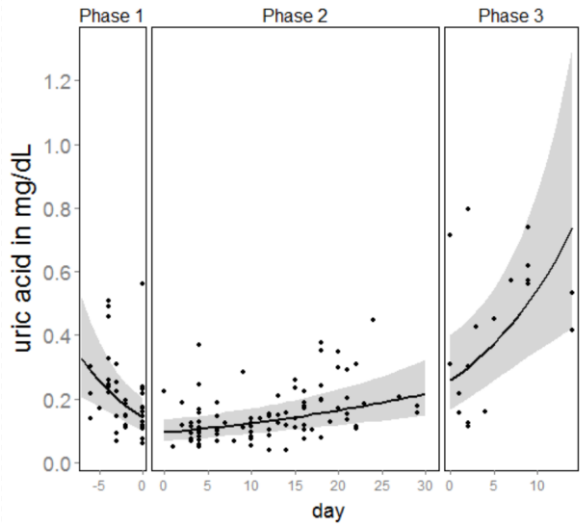

Fig. S1. Plasmatic Uric Acid dynamic in mg/dL during prolonged fasting. The exact day of transition between each phase (I; II; III) was determined using segmented regression models ('segmented' package in R; Muggeo, 2008) between the specific uric acid level and the time of fast.