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Starting with a handicap: phenotypic differences between early- and late-born king penguin chicks and their survival correlates

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Summary

1. The exceptionally long (*c.* 11 months) growth period of king penguin chicks (*Aptenodytes patagonicus*) is interrupted by the Austral winter. As a consequence, penguin chicks born late in the breeding season have little time to build-up their energy reserves before the drastic energy bottleneck they experience during winter and face greater risks of mortality than early-born chicks.

2. Whereas it is well known that breeding adults alternate between early- and late-breeding attempts, little is known on the phenotype of early- and late-chicks, and on the potential existence of specific adaptive phenotypic responses in late-born individuals.

3. We investigated phenotypic differences between early- and late-chicks and tested their survival correlates both before the winter and at fledgling. Chicks were sampled 10 days after hatching to measure body mass, plasma corticosterone levels, oxidative stress parameters and telomere length.

4. Late-chicks were heavier than early-chicks at day 10. Late-chicks also had higher corticosterone and oxidative stress levels, shorter telomere lengths and suffered from higher mortality rates than early-chicks. For both early- and late-chicks, high body mass close to hatching was a strong predictor of survival up to, and over, the winter period.

5. In late but not early-chicks, high corticosterone levels and long telomeres were significant predictors of survival up to winter and fledging, respectively.

6. Our study provides evidence that late- and early-king penguin chicks showed marked phenotypic differences 10 days after hatching. We provide an integrative discussion on whether these differences may be adaptive or not, and to what extent they may be driven by active maternal effects, indirectly induced by environmental effects, or stem from individual differences in parental quality.

Key-words: corticosterone, early-life conditions, growth, individual quality, oxidative stress, phenotypic plasticity, reproductive timing, telomere

Introduction

In seasonal environments, breeding timing is key to the reproductive success of most animals. Food resources often decline as the breeding season advances, which conflicts with the good nutritional conditions required by offspring to reach a body condition that promotes their survival over the growth period and after independence (Roff 1980). As a consequence, the offspring of late-breeders typically suffer from higher mortality rates than those

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of early breeders (Perrins 1970; Daan *et al.* 1988; Verhulst & Tinbergen 1991; Olsson 1996), and sometimes appear to differ in phenotype (Brinkhof 1997). However, sources of variation in offspring phenotype may be highly diverse, and their consequences on offspring survival may differ greatly depending on environmental context (Monaghan 2008). The processes accounting for differences in early-life phenotypes of early- vs. late-born individuals, and the extent to which those differences reflect constraints or adaptations to a seasonal environment remain to be adequately defined.

Conventionally, it is assumed that an individual's phenotype is determined both by the genes it inherits and by the environment in which those genes are expressed (Nylin & Gotthard 1998). Among environmentally driven modulations of gene expression, early-environment and/or maternal effects may be important factors affecting offspring phenotypes (Wolf & Wade 2009). Further, by influencing offspring phenotypes and their possible resilience to environmental change, perturbations during early development (*e.g.* food shortage, social stress, maternal hormones) are likely to drive the survival and fecundity prospects of entire cohorts of juveniles (Lindström 1999; Forchhammer *et al.* 2001; Metcalfe & Monaghan 2001; Saraux *et al.* 2011). To adaptively increase reproductive success and enhance individual fitness, the modulation of offspring phenotypes should follow environmental cues, such as changes in food availability (Muller & Groothuis 2013). For example, one possible strategy for late-born offspring to compensate for the short remaining favourable season is to exhibit higher-than-normal growth rates (Abrams *et al.* 1996). Such specific growth responses are enabled by developmental plasticity (Bize, Metcalfe & Roulin 2006) and may be adaptive if they improve individual fitness (Arendt 1997).

Adaptive phenotypic changes, however, should be discriminated from developmental constraints (Dmitriew 2011). Indeed, poor environmental conditions during early development (*e.g.* as early as the hatchling stage or shortly after) may also preclude the development and maturation of essential (but costly) protections or reserves and may lead to the production of damaged or pathological phenotypes (Dmitriew 2011). For instance, by enhancing oxidative stress, poor developmental conditions have been suggested to accelerate telomere loss, a tell-tale sign of accelerated ageing in young organisms (Tarry-Adkins *et al.* 2008, 2013). Telomeres are noncoding nucleoproteins structures located at the end of eukaryotic chromosomes that protect the integrity of DNA. Yet, when organisms are in a state of oxidative stress, the unbalance between pro-oxidants (mainly generated by the mitochondria during normal energy processing) and antioxidant defences may lead to oxidative damage on various biomolecules, including DNA (Halliwell & Gutteridge 2007). This process contributes to increasing telomere erosion rates, ultimately leading to cell death or replicative senescence (Monaghan & Haussmann 2006). Poor nutritional conditions during early growth may also increase circulating levels of stress hormones such as corticosterone or cortisol (Honarmand, Goymann & Naguib 2010). Although elevated concentrations of stress hormones help organisms mobilize resources over short-term periods, they are also known to have deleterious effects over extended periods (Kitaysky *et al.* 2003; Wingfield, Williams & Visser 2008). In addition, it has recently been shown that early exposure to corticosterone may induce oxidative stress and telomere erosion (Haussmann *et al.* 2012). Thus, the putative links between growth conditions, stress hormones, oxidative stress and telomere erosion may contribute to explain differences in mortality rates between early- and late-born offspring.

Another, nonmutually exclusive, hypothesis for explaining differences in the phenotypes of early- and late-born offspring, is that those phenotypes may be related to adult quality, and not the mere consequence of timing constraints. For instance, early breeders are often more experienced or higher quality parents (Forslund & Pärt 1995), and the higher survival prospects of early-born offspring may be partly due to intrinsically higher parental phenotypic quality (Daunt *et al.* 1999). Phenotypic heterogeneity between early- and late-born offspring, and within each offspring group, may then reflect both intrinsic variability in the experience/quality of parents (individual quality hypothesis), and extrinsic deterioration in the environment as the breeding season progresses (timing of breeding hypothesis). Thus, by scrutinizing phenotype differences between offspring produced early and late in the breeding season, and investigating potential links with survival, it should become possible to disentangle adaptive from nonadaptive phenotypic responses to a stressful late environment (Kitaysky *et al.* 2003).

Due to their particular reproductive cycle, king penguins (*Aptenodytes patagonicus*, Fig. 1) provide a unique opportunity to test whether chicks raised in markedly contrasted breeding environments differ in their early-life phenotypes and survival rates. King penguins are pelagic marine birds with a complex 'annual' cycle of *c.* 13 months (Weimerskirch, Stahl & Jouventin 1992; Heezik *et al.* 1994). Chick development (from hatching to fledging) occurs over *c.* 11 months and includes an energy-constraining winter period (April–September) during which chicks are seldom fed (Weimerskirch, Stahl & Jouventin 1992) and generally lose substantial body mass (Cherel, Stahl & Le Maho 1987; Descamps *et al.* 2002; see Fig. 2 for an example of body mass growth curve). Following chick fledging, parents are compelled to forage in order to restore their lost energy reserves and moult, which can take up to two additional months before they are able to breed again (Weimerskirch, Stahl & Jouventin 1992). It follows that successful breeders in 1 year may only attempt reproduction as late-breeders in the next. As late-born chicks tend to accumulate smaller reserves than early-chicks before their winter fast (Heezik *et al.* 1994), king penguin parents that reproduce late have drastically reduced chances of breeding

success (Cherel, Stahl & Le Maho 1987); with even zero success some years (Olsson 1996). To date, however, no study has investigated phenotypic heterogeneity within and between chicks born early or late in the breeding season, to address whether such heterogeneity (if it exists) could reflect adaptive phenotypic responses to environmental deterioration during the season.

Here, we compared the phenotypes and associated survival probabilities of early and late-hatched chicks in colonial king penguins. Specifically, we (i) investigated phenotypic differences between early- and late-chicks in terms of body mass, stress hormones (corticosterone), antioxidant defences, oxidative stress/damage markers and telomere length; and (ii) investigated whether specific phenotypes (early vs. late) were associated with higher survival probabilities both before and after the critical winter period.

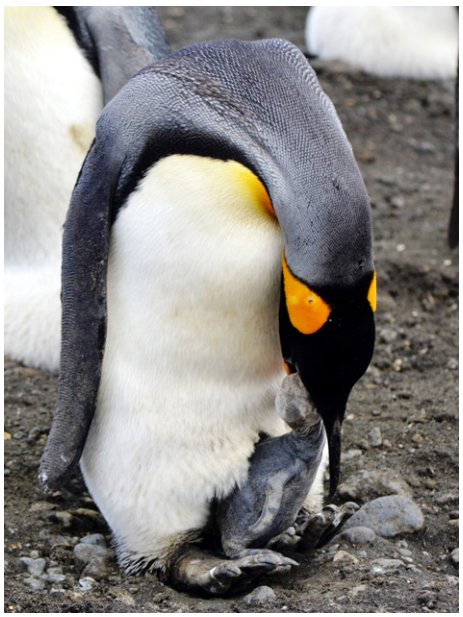

Fig. 1. Adult king penguin (*Aptenodytes patagonicus*) feeding its chick (Copyright permitted by G. Lemonnier).

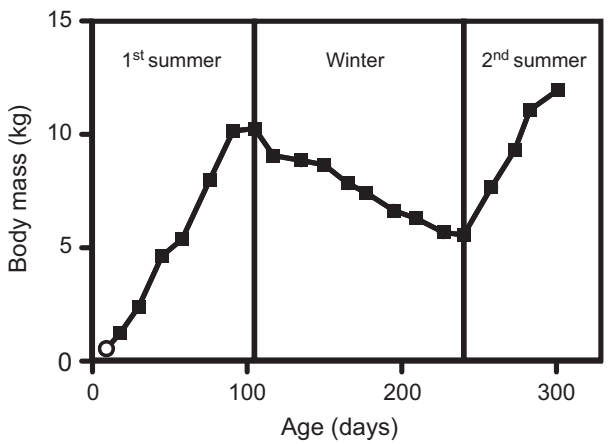

Fig. 2. Body mass growth trajectory of one king penguin chick (individual n°PE19, 2010) over its entire growth period (from 28 January 2010 to 16 November 2010). Growth periods (first summer, winter and second summer) were standardized for each individual according to the time point at which individual body mass (i) stopped to increase (end of the first summer), and (ii) started to increase again after the winter break (beginning of the second summer). See text for details. The open point indicates the very first body mass measurement taken 10 days after hatching, which also correspond to the time at which physiological markers were determined.

Materials and methods

GENERAL PROCEDURE

This study took place on Possession Island in the Crozet Archipelago (46°25′S; 51°52′E). Data were collected during the 2009 and 2010 breeding seasons in the king penguin colony of 'La Grande Manchotière' (*c.* 24 000 breeding pairs). To precisely determine hatching date (at ± 1 day) and start monitoring chicks shortly after, pairs of breeding adults were marked during courtship using a nonpermanent animal dye (Porcimark, Kruuse, Langeskov, Denmark). They were subsequently monitored daily throughout incubation. Breeding pairs were selected in the same area of the colony. We monitored pairs of both early (mean hatching date ± SE = 20 January ± 0·9 days) and late (25 February ± 0·7 days) breeders.

CHICK MONITORING

In total, 39 early-hatched chicks (19 in 2009 and 20 in 2010) and 45 late-hatched chicks (16 in 2009 and 29 in 2010) were monitored (hereafter referred to as early- or late-chicks). Chicks were followed from hatching to the beginning of their final (prefledging) moult the subsequent year. Within 3 days of hatching, chicks were individually identified using colour-coded fish tags (Floy Tag and MFG, Inc. Seattle, WA, USA) attached subcutaneously to their upper-back.

At 10 days, chicks were captured for blood sampling and morphometric measurements. To avoid disturbing breeders during these procedures, chicks were carried to a quiet laboratory facility on the outskirts of the colony (<5-min walking distance). Blood was sampled from the chick's marginal flipper-vein generally within 10 min of capture. Body mass was measured using a platform balance (Kern IT60K2LIP, ±4 g). Chicks were returned to the parent's brood pouch as soon as measurements were completed. During chick handling, the parent was provided with a dummy egg, and it's head was covered with a hood to keep it calm. One observer remained close to the adult during that time. This procedure never resulted in chick abandonment or breeding failure. When collected, blood samples were stored on ice for <10 min before being centrifuged for 5 min at 2000 G. Plasma and red blood cells were separated and immediately transferred to a −20 °C freezer. Samples were then transferred to a −80 °C freezer for long-term storage by the end of the day.

Subsequently, chicks were captured for similar morphometric measurements every 15 days over their yearly growth period (from January until their departure at sea 1 year later). Measurements were taken as explained previously, and during procedures, the chick's head was always covered with a hood to reduce handling stress.

GROWTH ANALYSIS

During the winter period, food resources are drastically reduced and parental food-provisioning naturally varies between chicks (Cherel & Le Maho 1985). Therefore, we standardized chick-growth

trajectories by defining the 'winter period' as the period when chicks face their winter fast and lose substantial body mass (±2 weeks, Fig. 2). Growth periods were accurately determined for each individual chick according to its body mass dynamics over the pre- to postwinter periods. The first summer growth period was defined as the period of time between hatching and the date at which chick body mass gain ceased. The winter phase corresponded to the time duration during which body mass was stabilized or decreased. Finally, the second summer of growth was the time duration from the date at which body mass started to increase over two successive body mass measures (*i.e.* 30 days) and the time from which the first signs of moult were detected (Corbel *et al.* 2009). Each period was defined by analysing chick-growth curves individually, and chick survival was monitored until fledging (*i.e.* departure at sea).

OXIDATIVE STRESS

Plasma concentration of reactive oxygen metabolites (ROMs) in king penguin chicks was measured using the D-ROM test (5 μL of plasma; Diacron International, Grosseto, Italy) following the manufacturer protocol, as previously described in penguins by Beaulieu *et al.* (2011). The D-ROM test is based on the Fenton reaction and measures primarily hydroperoxides, the results being expressed as mg of H_2O_2 equivalent dL^{-1}. Thus, D-ROM indicates potential exposure to oxidative stress. Intra-individual variation based on duplicates was low (CV = 4·67 ± 0·57%) as well as interplate variation based on a standard sample repeated over plates (CV = 5·59%). In addition, we measured an indicator of oxidative damage on DNA. We quantified the plasmatic concentration of 8-hydroxy-2′-deoxyguanosine (8-OHdG) using a competitive immunoassay (Assay Designs DNA damage ELISA Kit; Enzo Life Sciences, Farmingdale, NY, USA). 8-OHdG is one of the predominant forms of free radical–induced oxidative lesion on DNA and has been widely used as a marker of oxidative stress (Halliwell & Gutteridge 2007). For this assay, 8-OHdG levels were determined only for 58 of 84 chicks due to plasma quantity limitations. Intra-assay variation based on seven duplicates (due to limited available plasma) was low 4·16 ± 1·24%.

Antioxidant defences in chick plasma were evaluated using the Oxiselect™ Total Antioxidant Capacity (TAC) assay kit (Cell Biolabs, Inc., San Diego, CA, USA) following manufacturer instructions. The TAC assay measures the antioxidant power of biomolecules from the plasma via a single-electron transfer mechanism, and results are expressed as mM UAE (Uric Acid Equivalent). This assay measures nonenzymatic antioxidants only, which are mostly derived from the diet (for a discussion on the limitations of the TAC assay, see Sies 2007). Intra-individual variation based on duplicates was low (CV = 1·81 ± 0·27%), as was interplate variation based on a standard sample repeated over the plates (CV = 2·37%).

TELOMERES MEASUREMENTS AND SEX DETERMINATION

Chick telomere length was determined following DNA extraction from blood cells (Nucleospin® Blood QuickPure; Macherey-Nagel, Düren, Germany), using quantitative real-time amplification (qPCR) previously adapted for birds (Criscuolo *et al.* 2009) and successfully used in king penguins (Geiger *et al.* 2012). Primer sequences for telomere amplification were similar to those used by Criscuolo *et al.* (2009). For a single control gene, defined as a gene nonvariable in copy numbers within our population (hereafter non-VCN; Smith, Turbill & Penn 2011), we used the *Aptenodytes patagonicus* zinc finger protein, with primer sequences defined by Primer 3 software (Howard Hughes Medical Institute, MD, USA) as: (Royal1: 5′-TACATGTGCCATGGTTTTGC-3′; Royal2: 5′-AAGTGCTGCTCCCAAAGAAG-3′). Primer concentrations in the final mix were 200 nM for telomere length determination and 300 nM for the control gene. Telomere and control gene PCR conditions were: 2 min at 95 °C followed by 40 cycles of 15 s at 95 °C, 30 s at 56 °C, 30 s at 72 °C and 60 s at 95 °C. We used 2·5 ng DNA per reaction and the BRYT Green® fluorescent probe (GoTaq®qPCR Master Mix; Promega, Charbonniere, France). DNA samples were analysed on different plates (or runs), telomere and non-VCN amplification of each sample being carried out on the same plate. Each plate was also composed of a mix of 2009–2010 and early- and late-breeder samples to avoid any undesired plate effect to interfere with the final calculations. Mean amplification efficiency of the qPCR runs were comprised between 100·9 and 103·1 for telomere, and between 100·6 and 102·9 for the non-VCN gene. Intraplate mean coefficients of variation for C_t values were 1·35 ± 0·06% for the telomere assay, and 0·79 ± 0·04% for non-VCN assay. Interplate coefficients of variation based on repeated samples were low: 1·56% for the telomere assay and 1·35% for non-VCN assay (C_t values again). Final calculation of telomere length (T/non-VCN ratio) was carried out by (Pfaffl 2001) using the telomere- and non-VCN-specific efficiencies of each plate.

Sex determination was also performed on DNA extracted from blood cells following a method adapted from Griffiths *et al.* (2002).

CORTICOSTERONE ASSAYS

Circulating corticosterone levels were determined at 10 days from blood samples taken within 10 min of capture. Thus, the levels presented here are likely not basal values. Plasma corticosterone (CORT) levels were determined by immunoassay according to guidelines provided by the manufacturer (Corticosterone EIA Kit; Enzo Life Sciences). For this assay, corticosterone (CORT) levels were determined only for 71 of 84 chicks due to plasma quantity limitations. Intra-assay variation based on five duplicates (due to limited available plasma) was low 5·92 ± 0·87%. The detection limit provided by the manufacturer is 26·99 pg mL^{-1}.

DATA ANALYSIS AND STATISTICS

Data were analysed in two steps. First, we analysed changes in chicks' body mass from 10 days posthatching to fledging. Second, we investigated posthatching phenotypic differences between early- and late-chicks and their relationship with survival.

To investigate whether growth period durations and growth trajectories over the 1-year-growth period of king penguin chicks differed between early- and late-chicks, we used a linear mixed model (LMM) with chick identity specified as a random factor and the different growth phases (*i.e.* 10 days, end of the first summer, end of the winter and end of the second summer) as a repeated factor. Year, sex, chick group and all interactions were initially included as fixed factors. To anticipate the fact that the duration of growth phases were likely to differ between early- and late-chicks (late-chicks necessarily having a shorter first summer), the duration of each growth periods was initially used as a covariate in the model. However, as it turned out to be nonsignificant, it was not included in the final model. Nonsignificant terms were dropped (starting with interactions) in a stepwise procedure to obtain the model with the best Akaike Information Criterion (AIC) value. Multiple *post hoc* comparisons were made using Bonferroni correction.

At 10 days posthatching, we tested for differences among blood parameters [corticosterone, telomere length, oxidative stress (D-ROM), DNA damage and plasmatic antioxidant capacity] between chick groups (early vs. late) using ANOVA with year, sex

and chick groups as fixed factors, as well as all interactions. Determinants of chick survival between groups (early vs. late) were evaluated using a generalized linear modelling procedure (GzLM) with a logistic binary distribution of the dependent variables. Pre-winter and long-term survival were analysed by running two different models where the binary dependent variable was either (i) chick survival/death (0/1) before the winter period; or (ii) chick survival/death (0/1) at the time of departure at sea (survival at fledging). Year, sex and chick groups (early vs. late-born chicks), as well as starting values of blood parameters (corticosterone, telomere length, D-ROM, DNA damage and plasmatic antioxidant levels at 10 days) were used, respectively, as fixed factors and covariates in the analysis. All interactions between starting values of blood parameters and chick groups were tested to determine whether our physiological markers recorded after hatching predicted differently the survival rate of chicks in each group. Again, nonsignificant terms (starting with interactions) were sequentially dropped to produce minimum adequate models with lowest AIC value. All analyses were run on SPSS v. 18.0) IBM SPSS Statistics, Armonk, NY, USA). Tests were two-tailed, and P values < 0·05 were considered significant. Means are given ± SE.

Results

CHICK-GROWTH DURATION AND BODY MASS CHANGES

Although the duration of growth phases did not vary significantly between sex and years (Table 1a), the interaction *growth phase* × *chick group* was significant (Table 1a). A LMM analysis split by phases indicated that late-chicks experienced a significantly shorter winter period (87·50 ± 6·66 days) than early-chicks (126·05 ± 2·89) (Winter, $F = 26{\cdot}90$, $P < 0{\cdot}001$), other comparisons being nonsignificant: day 10, 10·31 ± 0·67 vs. 10·65 ± 0·67 days; first summer, 93·35 ± 2·30 vs. 87·23 ± 4·98 days; second summer, 84·33 ± 3·12 vs. 89·17 ± 7·68 days).

Over the entire growth period (Figs 2 and 3, Table 1b), chicks' body mass first increased from 10 days posthatching (583·5 ± 104·3 g) to the end of the first summer period (7988·4 ± 195·6 g), then subsequently decreased during the winter fast to reach an average of 5094·5 ± 228·8 g by the end of the first growth period. Finally, chicks resumed their growth to reach an average of 12473·4 ± 253·2 g at the end of the second summer. Chicks' body mass was neither influenced by year or group factors (Table 1b) but was significantly influenced by sex, males being on average heavier than females over the entire growth period (6761·5 ± 127·7 g vs. 6308·4 ± 124·2 g). The interaction *Group* × *Stage* was significant, indicating that early- and late-chicks experienced different growth trajectories (Table 1b, Fig. 3). Thus, we ran separate LMMs (with chick group, sex and year as fixed factors) to compare the body mass of early- and late-chicks at each growth phase. Although late-chicks were significantly heavier than early-chicks at 10 days posthatching ($F = 5{\cdot}84$, $P = 0{\cdot}018$), the reverse was observed by the end of the first summer ($F = 12{\cdot}82$, $P = 0{\cdot}001$, Fig. 3). This difference remained significant, even when only late-chicks that survived until winter were kept in the analysis (statistics not shown). During the winter period and subsequent summer, body mass did not differ significantly between the two groups of chicks (Fig. 3, all $P > 0{\cdot}38$).

Table 1. Results of linear mixed model analyses describing the variability in (a) growth phase durations and (b) body mass changes occurring over the 1-year-growth period of king penguin (*Aptenodytes patagonicus*) chicks. Growth phase duration and body mass were analysed in relation to year, date of hatching in the breeding season ('*Chick group*' factor; early and late-chicks), chick sex, and Growth phase ('*Growth phase*' factor; hatching, first summer, winter and second summer). The significant interaction '*Chick group* × *Growth Phase*' indicates that early and late-chicks differed in their growth phase duration and body mass growth trajectories. Chick identity was used as random factor and random factor estimates are indicated in italics. Estimates of fixed effects were calculated using 2010 (*Year*), late chicks (*Chick group*), males (*Chick Sex*), fledging (*Growth phase*) and late-chicks during the winter (interaction for growth phase duration) or at 10 days (interaction for body mass) as reference values. The model is based on 83 individuals and 180 body mass values

Variables	Estimates	SE	d.f.	*F*	*P*
(a) Growth period duration					
Random factor estimates					
Identity 135·45 ± 14·65					
Year	1·41	0·41	1, 77·5	0·06	0·805
Chick group	−7·96	8·93	1, 79·6	26·46	<0·001
Chick sex	0·05	0·39	1, 77·4	0·04	0·844
Growth phase	−81·5	8·30	3, 51·0	1408·13	<0·001
Chick group × Growth phase	49·82	12·0	3, 51·0	20·94	<0·001
(b) Body mass					
Random factor estimates					
Identity 1·78 ± 5·77					
Year	−258·0	147·0	1, 72·2	3·08	0·083
Chick group	−1138·9	509·1	1, 80·9	1·91	0·171
Chick sex	−453·1	143·2	1, 70·7	10·01	0·002
Growth phase	−12444·9	493·6	3, 154·3	865·15	<0·001
Chick group × Growth phase	2954·0	636·2	3, 154·2	8·68	<0·001

STARTING VALUES OF PHYSIOLOGICAL MARKERS (DAY 10)

Values for the blood parameters measured 10 days after hatching for early- and late-chicks are presented in Table 2. Plasmatic concentrations of corticosterone were significantly higher in late than in early-chicks. Corticosterone levels were also significantly higher in 2009 than in 2010 (10·59 ± 0·85 vs. 7·55 ± 0·68 ng mL^{-1}) but did not differ significantly between the sexes (see Table 2). Late-chicks presented higher levels of oxidative stress (D-ROM) and DNA damage than early-chicks at 10 days (see Table 2). These levels were neither significantly influenced by year or sex (Table 2) but were positively correlated with each other ($r = 0{\cdot}48$, $P < 0{\cdot}001$). In contrast, the antioxidant capacity of the chick's plasma did not appear to significantly differ between early- and late-chicks (see Table 2). Similarly, TAC was neither affected by year or sex (Table 2). Telomeres length measured 10 days after hatching did not differ between the sexes (see Table 2).

However, telomeres were longer in 2009 than in 2010 (1·43 ± 0·11 T/S ratio vs. 1·14 ± 0·08 T/S ratio, Table 2), and they were significantly shorter in late- than early-chicks (see Table 2).

PHENOTYPE AT HATCHING AND CHICK SURVIVAL

There was no significant year effect on chick survival rate ($\phi^2 = 2{\cdot}26$, $P = 0{\cdot}133$). Interestingly, we found that chick survival rates recorded before winter or before fledging differed between early and late-chick groups (Fig. 4, Table 3). While early-chick survival to the beginning of winter was high (85%), only few late-chicks (14%) made it that far (Fig. 4). Similarly, whereas survival rate over the entire growth period reached 65% in early-born chicks, only 10% of the late-born chicks made it to fledging (Fig. 4). This shows that early/late difference in survival rates principally occurred during the first summer, during which 86% of late chicks died.

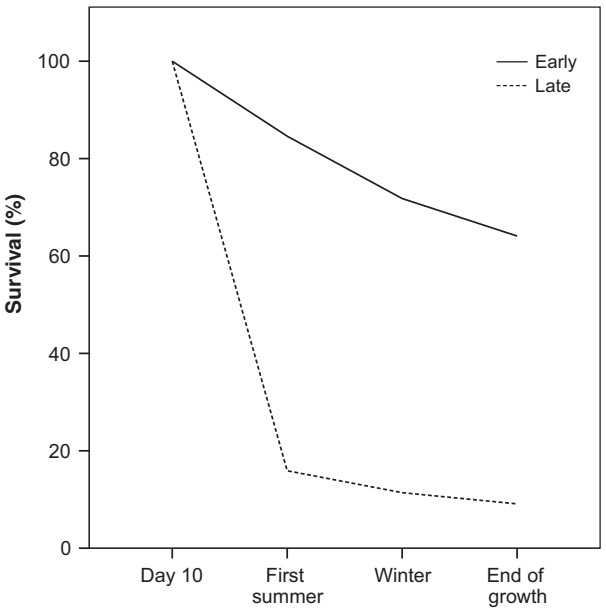

Fig. 4. Survival curves of king penguin chicks over their 1-year-growth period in relation to their hatching period (early or late in the season). Curves are based on the survival of 39 early and 44 late-born chicks followed during two successive breeding seasons (2009 and 2010). See text for methodological and statistical details.

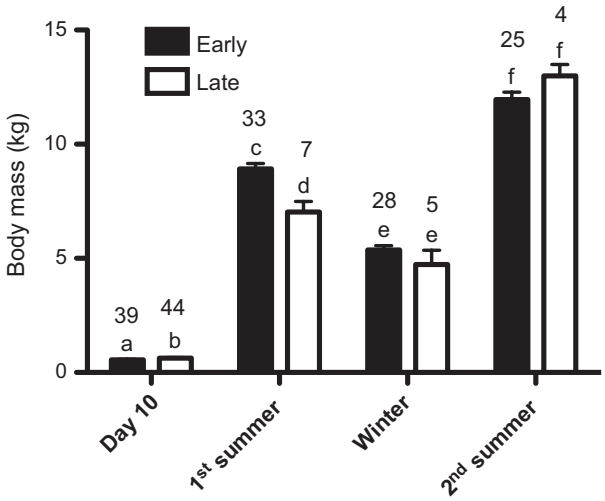

Fig. 3. Differences in mean body mass (±SE) of early and late-born king penguin chicks over the growth period. Different letters indicate significant differences between groups within the same period. See text for statistics and differences among growth periods. Sample sizes (early/late) are indicated for each period.

Starting values of body mass and blood parameters measured at the age of 10 days were tested as predictors for survival of individuals both before winter and at the end of the growth period (respectively Table 3a,b, Fig. 5). While D-ROM, DNA damage, plasmatic antioxidant defences and telomere lengths were not related to survival rate of chicks until winter, body mass was a strong predictor of survival, in both chick groups (Table 3a, Fig. 5a). The significant interaction *Chick group* × *Corticosterone* indicates that a higher corticosterone plasma concentration in late-chicks was significantly related to higher survival rates before winter, while no relationship was apparent in early-chicks

Table 2. Blood parameters of early- and late-king penguin chicks [corticosterone, oxidative stress (D-ROM), DNA damage, total antioxidant capacity (TAC) and relative telomeres length] measured 10 days after hatching

Blood variables at 10 days posthatching	Early-born chicks	Late-born chicks	Group	Sex	Year
Corticosterone (ng mL^{-1})	7·91 ± 0·63	9·59 ± 0·88	$F = 4{\cdot}34$	$F = 0{\cdot}01$	$F = 7{\cdot}72$
			***P* = 0·041**	$P = 0{\cdot}931$	***P* = 0·007**
Oxidative stress (D-ROM) (mg H_2O_2 dL^{-1})	1·60 ± 0·13	2·09 ± 0·14	$F = 6{\cdot}51$	$F = 0{\cdot}54$	$F = 0{\cdot}55$
			***P* = 0·013**	$P = 0{\cdot}464$	$P = 0{\cdot}463$
DNA damage (8-hydroxy-2′-deoxyguanosine) (ng mL^{-1})	57·9 ± 6·6	97·4 ± 9·0	$F = 12{\cdot}45$	$F = 2{\cdot}60$	$F = 0{\cdot}46$
			***P* = 0·001**	$P = 0{\cdot}113$	$P = 0{\cdot}500$
TAC (mM UAE)	0·98 ± 0·05	1·07 ± 0·06	$F = 1{\cdot}63$	$F = 2{\cdot}04$	$F = 0{\cdot}34$
			$P = 0{\cdot}206$	$P = 0{\cdot}157$	$P = 0{\cdot}562$
Relative telomere length (T/S ratio)	1·44 ± 0·10	1·11 ± 0·09	$F = 17{\cdot}90$	$F = 0{\cdot}01$	$F = 4{\cdot}70$
			***P* < 0·001**	$P = 0{\cdot}924$	***P* = 0·033**

Mean ± SE are reported both for early and late-chicks, and results of statistical models (ANOVAS) reporting the effects of group (early vs. late), sex and year (2009 vs. 2010) are indicated, with significant terms appearing in bold.

Table 3. Separated analyses of generalized linear models following a binary distribution of survival rates (1 = death) of king penguin (*Aptenodytes patagonicus*) chicks until the winter period (a) or until chick departure at sea (b). Chick groups include early and late-chicks. The presented model was obtained after backwards deletion of least significant terms and presented the lowest Akaike Information Criterion (see text for details on model selection)

Variables	Wald ϕ^2	ddl	*P*
(a) Survival rate until winter (n = 63)			
Chick group	5·55	1	0·001
Mass	10·53	1	0·008
Corticosterone	7·02	1	0·721
Chick group × Corticosterone	4·07	1	0·044
(b) Survival rate until departure (n = 66)			
Chick group	9·77	1	0·002
Mass	7·50	1	0·006
Telomere length	0·99	1	0·319
Chick group × Telomere length	4·09	1	0·043

(Table 3a, Fig. 5c). Concerning chick survival to fledging (Table 3b), body mass of 10-day-old chicks was also a strong predictor of survival in both groups (Fig. 5b). Interestingly, the interaction *Chick group* × *Telomere length* pointed out that late-chicks with longer telomeres at 10 days were more likely to survive until their final moult and departure at sea (Table 3b, Fig. 5d).

Discussion

This study reveals marked differences in the phenotypes of king penguin chicks born either early or late during the breeding season. Phenotypic differences were apparent from the early stages of life (as close as 10 days after hatching) and were tightly related to offspring survival prospects. Importantly, correlates between chick phenotype and survival appeared context-dependent. Although late-chicks were heavier at 10 days, they presented higher plasma levels of corticosterone and oxidative stress, as well as shorter blood cell telomeres. Late-chicks suffered from a higher mortality than early-chicks, mostly during the first weeks of their life. Interestingly, in late – but not early – chicks, plasma corticosterone levels significantly predicted short-term (prewinter) survival, and telomere length at 10 days significantly predicted survival to fledging. Such strong phenotypic differences between early- and late-born king penguin chicks shortly after hatching may reflect adaptive or pathological responses, that could either be driven by active maternal effects, induced indirectly by environmental effects, or stem from differences in parental qualities.

CHICK BODY MASS AND SURVIVAL

As frequently reported, our results show that offspring body mass strongly affected offspring survival, both in early- and late-chicks. The positive effect of body mass on chick survival was detectable as early as 10 days after hatching. Surprisingly, however, late-chicks were heavier than early ones at day 10, which contrasts with results from other species that typically exhibit a decline in the body condition of offspring born late in the season (Brinkhof 1997). Interestingly, and consistently with our previous findings (Geiger *et al*. 2012), we found that small (rather than late) chicks were those that grew faster during

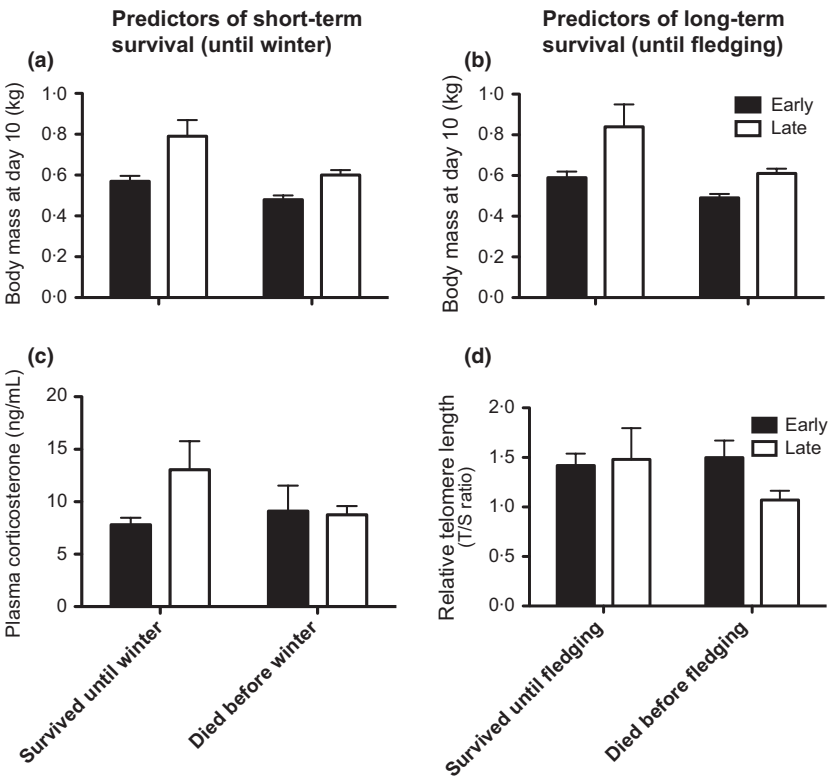

Fig. 5. Phenotypic predictors (mean ± SE) at day 10 of short (until winter, a/c) and long-term (until fledging, b/d) survival of king penguin chicks. (a) Body mass at day 10 of chicks that survived vs. died before winter. (b) Body mass at day 10 of chicks that survived vs. died before fledging. (c) Plasma corticosterone concentration at day 10 of chicks that survived vs. died before winter. (d) Blood cell telomere length at day 10 of chicks that survived vs. died before fledging. See Table 3 for statistics.

the second summer, independently of their hatching date (data not shown). Remarkably, late-chicks also faced a shorter winter period (corresponding to the time during which parents rarely fed their chicks (Weimerskirch, Stahl & Jouventin 1992), supporting the idea that parental investment was high for late chicks that survived up to (and past) winter. Survival rate of young penguins during their first years at sea is up to 0·75, independently of whether they were born early or late in the season (Saraux *et al.* 2011). Hence, because of the strong (and widely reported) positive links between body mass and survival, it is likely that this phenotypic response is adaptive. However, the proximate factors causing late-chicks to be heavier than early-chicks close to hatching, and the potential costs of this phenomenon, remain open questions to which several hypotheses can be formulated.

ENVIRONMENTAL EFFECTS ON CHICK PHENOTYPE

Late-chicks may have benefited from transient favourable nutritional conditions just after hatching. Indeed, in the Southern ocean, productivity is high until the end of the summer (March) and the hatching date of late chicks may coincide with prime nutritional conditions. Accordingly, previous findings have shown that late-breeding adult king penguin return from their foraging trip close to hatching with higher food energy content stored in their stomach than early breeders (Gauthier-Clerc *et al.* 2002). Nevertheless, this greater energy content was also associated with longer foraging trips, thus resulting in much lower energetic output per day foraging at sea. In turn, this suggests greater foraging difficulties in late than early breeders (Gauthier-Clerc *et al.* 2002).

POTENTIAL MATERNAL EFFECTS ON CHICK PHENOTYPE AND SURVIVAL

Besides altering foraging behaviour at sea (with direct effects on chick body mass) females may also indirectly modulate their offspring's growth trajectories by transferring hormones or nutrients to the egg. Maternal effects and the transfer of environmental cues to the embryo and are key parameters guiding an embryo's development (Mousseau & Fox 1998; Wingfield, Williams & Visser 2008). Such maternal effects might help to adjust the young's phenotype to current environmental conditions, with for instance a recent study in red squirrel reporting that a high conspecific density triggers maternal hormones that increase the post-natal growth of offspring (Dantzer *et al.* 2013). In birds, the transfer of maternal corticosterone into the egg yolk during laying (Hayward & Wingfield 2004) is for instance known to affect embryo's development and to shape chick phenotype even long after hatching (Groothuis *et al.* 2005), potentially through higher corticosterone plasma levels or stress responses at the chick stage (Spencer, Evans & Monaghan 2009; Haussmann *et al.* 2012).

Accordingly, we found higher corticosterone levels at day 10 after hatching in late- than early-born chicks. Although the hypothalamic-pituitary adrenal-axis activity and corticosterone responses to acute stressors are not known for chicks this young in king penguins, Corbel, Geiger & Groscolas (2010) have previously reported baseline levels for king penguin chicks at the onset of their fledging moult to be 7·2 ± 0·5 ng mL^{-1}. Those values are remarkably close to the values observed for early chicks in this study, suggesting that our corticosterone measures may not be too far from basal even though it generally took us over 5 min to sample the chicks. Because corticosterone can provide short-term benefits but may incur long-term costs (Hayward & Wingfield 2004; Bonier *et al.* 2009; Haussmann *et al.* 2012), cautious is needed when interpreting the adaptive value, or not, of such hormonal differences. High levels of corticosterone in late-chicks could merely reflect environmental stressful conditions experienced by mothers (*i.e.* exogenous transfer of corticosterone via the egg yolk) and/or by chick themselves (*i.e.* endogenous production of corticosterone). Early modulation of chick corticosterone plasma levels could for instance be due to differences in the colonial environment in which they were raised. Differences in colony density for instance might affect stress levels in adults king penguins (Viblanc 2011) and trigger differential corticosterone deposition in the egg of early vs. late-breeders, with higher concentrations in the latter. Still, the fact that late-chicks were in better condition and that higher levels of corticosterone early in life were associated with higher survival rate during the first weeks or so of growth pleads towards an adaptive value rather than an environmentally driven cost of higher corticosterone levels for late-chicks (for another example of positive impact of corticosterone on survival, see Cote *et al.* (2006)). Positive effects of elevated corticosterone levels in growing birds may for instance be linked to increased begging and aggressive behaviour, allowing chicks to solicit more food from their parents (Wingfield *et al.* 1998; Kitaysky, Wingfield & Piatt 2001; Kitaysky *et al.* 2003). High corticosterone levels of late-chicks might thus account for the greater foraging effort of late king penguin parents (Gauthier-Clerc *et al.* 2002), and in turn the higher body mass of late-chicks close after hatching. Experiments are required to examine the impact of high corticosterone levels on king penguin chick behaviours, and whether those changes affect parental foraging strategies. In addition, accurate measures of basal and stress-induced levels of corticosterone in chicks, of egg hormonal content or embryo ageing parameters, are needed to fully appreciate the origin and nature of the phenotypic heterogeneity observed among early and late king penguin chicks.

OXIDATIVE STRESS, TELOMERE LENGTH AND SURVIVAL

The higher levels of oxidative stress and the shorter telomeres of late-chicks might have been induced by high

exposure to corticosterone (Haussmann *et al*. 2012) and/or by the alteration of early growth trajectories (Alonso-Alvarez *et al*. 2007; Tarry-Adkins *et al*. 2009). Indeed, an experimental manipulation of yolk corticosterone levels has been shown to lead to greater oxidative stress levels in chickens (*i.e*. D-ROM levels), but also to shorter telomeres (Haussmann *et al*. 2012). Interestingly, our results also point towards greater levels of D-ROM and shorter telomeres in late king penguins chicks (*i.e*. with higher corticosterone levels). Our two markers of oxidative stress/damage gave concordant results (see Sepp *et al*. 2012 for a discussion about the consistency of different oxidative stress markers), which strongly suggests that late-chicks were not able to generate an appropriate antioxidant response (both enzymatic and nonenzymatic). This point raises the question of a potential impairment of mitochondrial functioning in late-chicks, which might be one cause of their elevated oxidative stress levels (Halliwell & Gutteridge 2007). The early growth trajectory of late-chicks (the increased growth rate between hatching and day 10, leading to the higher body mass at day 10) may also account for part of the differences observed in terms of oxidative stress and telomere length between early and late-chicks. Indeed, the growth of late- chicks could be accelerated during their first 10 days of life as an attempt to catch up with early-chicks (see Benowitz Fredericks & Kitaysky 2005 for such an example in *Uria aalge* chicks). However, catch-up or accelerated growth has been associated with a variety of ecological and physiological costs (Metcalfe & Monaghan 2001), among which increased oxidative stress levels and accelerated telomeres erosion have been suggested in recent years (Alonso-Alvarez *et al*. 2007; Tarry-Adkins *et al*. 2009), including in this species (Geiger *et al*. 2012).

Oxidative stress levels may be associated with differences in individual survival probability or reproductive success (Bize *et al*. 2008; Stier *et al*. 2012), and thus, early differences in oxidative stress levels during offspring development may have substantial impacts on the future fitness prospects of late-born king penguins chicks. As such, oxidative stress may cause accelerated telomere erosion (in particular early in life) which has been negatively associated with long-term adult survival both in captivity and in the wild (Bize *et al*. 2009; Salomons *et al*. 2009; Heidinger *et al*. 2012). In the present study, telomere length also predicted survival up to fledging in late-chicks. However, given the limits of our oxidative stress measurements (Sies 2007), additional specific antioxidants (*i.e*. enzymatic) should be measured in the future to better understand how oxidative stress might be modulating survival in this species.

PARENTAL QUALITY AND BREEDING EXPERIENCE

Phenotypic variations between early- and late-chicks can also be associated with variability in breeder experience and parental (care) quality due to a mixed composition of experienced and inexperienced birds in the late-breeder population. In king penguins, young inexperienced adults typically moult late in the season (Stonehouse 1960), with the consequence of delaying their onset date of reproduction, which then coincides with that of experienced late-breeders (Stonehouse 1960; Weimerskirch, Stahl & Jouventin 1992). Such differences in parental experience during late-breeding might then account for the lower quality observed in late-chick phenotype. For instance, lower breeding skills might explain shorter telomere lengths due to suboptimal incubation and embryo developmental conditions (see Tarry-Adkins *et al*. 2009 for examples of links between early growth and telomere loss). In addition, the high rate of chick death during the first weeks of brooding could be attributed to reduced parental care ability in inexperienced adults (Descamps *et al*. 2005). Several studies considering age-related breeding performances have indeed supported the idea that first-time breeders may be constrained by their lower foraging/reproductive skills (Olsson 1997; Angelier *et al*. 2007; Lecomte *et al*. 2010), intrapair coordination or even lower ability to feed chicks with digested food (discussed in Daunt *et al*. 1999). Even more important is that brood care (*e.g*. feeding) ability in the very first period has been previously shown to be of tremendous importance for chick survival (Brinkhof & Cavé 1997). By contrast, experienced late-breeders may be able to buffer constraining environmental conditions (increased predation, social stress) and, as a consequence, may represent a large proportion of the couples that successfully raised their chicks. In line with this idea, manipulation of reproductive timing showed that old (experienced) shags (*Phalacrocorax aristotelis*) forced to breed in the same late environmental conditions than young (inexperienced) breeders maintain a higher reproductive success (Daunt *et al*. 1999). In addition, if early king penguins breeders and successful late-breeders are parents of higher quality, this may be reflected in adults having longer telomeres (Bauch, Becker & Verhulst 2013). Given that telomere length is a heritable trait (Horn *et al*. 2011; Voillemot *et al*. 2012), inheritance of longer telomeres may explain the early-/late-born chicks telomere difference at 10 days, as well as the link between telomere length and survival among late-born chicks. Longitudinal studies conducted over the first 3–5 years of a fledgling's life are anticipated to shed new results on the actual fitness differences arising between early- and late-born phenotypes. Finally, late breeding, even when unsuccessful, may nonetheless be of functional importance for young king penguin adults (4–8 years old), enabling them to acquire breeding experience and knowledge on the best breeding sites (Saraux *et al*. 2011).

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