

RESEARCH ARTICLE

Modulation of heart rate response to acute stressors throughout the breeding season in the king penguin *Aptenodytes patagonicus*

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ABSTRACT

'Fight-or-flight' stress responses allow animals to cope adaptively to sudden threats by mobilizing energy resources and priming the body for action. Because such responses can be costly and redirect behavior and energy from reproduction to survival, they are likely to be shaped by specific life-history stages, depending on the available energy resources and the commitment to reproduction. Here, we consider how heart rate (HR) responses to acute stressors are affected by the advancing breeding season in a colonial seabird, the king penguin (*Aptenodytes patagonicus*). We subjected 77 birds (44 males, 33 females) at various stages of incubation and chick-rearing to three experimental stressors (metal sound, distant approach and capture) known to vary both in their intensity and associated risk, and monitored their HR responses. Our results show that HR increase in response to acute stressors was progressively attenuated with the stage of breeding from incubation to chick-rearing. Stress responses did not vary according to nutritional status or seasonal timing (whether breeding was initiated early or late in the season), but were markedly lower during chick-rearing than during incubation. This pattern was obvious for all three stressors. We discuss how 'fight-or-flight' responses may be modulated by considering the energy commitment to breeding, nutritional status and reproductive value of the brood in breeding seabirds.

KEY WORDS: Acute stress, Energy cost, Fasting, Heart rate, Penguin, Reproductive value, Risk assessment, Seabird

INTRODUCTION

Animals facing environmental disturbances respond by mounting a series of physiological and behavioral modifications known as the stress response (Romero, 2004). Those adaptive changes are intended to redirect energy resources towards increasing fitness, in a so-called 'emergency life-history state' (Wingfield et al., 1998; Boonstra et al., 2001). Because stress responses can be costly in terms of energy, health or missed breeding opportunities (e.g. McEwen and Wingfield, 2003), they are likely shaped to increase lifetime fitness according to the life-history characteristics of considered organisms and the risk associated with specific disturbances (Nephew et al., 2003; Boonstra, 2013). For instance, physiological responses to stress may depend on the energy reserves of the animal (Cyr et al., 2008) or mechanistically underlie parental decisions (Lendvai et al., 2007; Bókony et al., 2009; Goutte et al.,

2011), considering a trade-off between the cost of missing a breeding opportunity versus the expected benefits of surviving to breed in the future (Williams, 1966).

In response to acute disturbances (e.g. predation events, sudden storms), an early and short-lived phase of the stress response involves a sympathetic discharge from the nervous system, increasing heart rate, muscle tone, mobilizing energy substrates (e.g. neoglucogenesis) and priming the body to action (Wingfield, 2003). This acute 'fight-or-flight' response occurs within seconds, and is controlled by central sympathetic command neurons (Jansen et al., 1995). Heart rate (HR) has been shown to increase with increased sympathetic input (Cyr et al., 2009), and can be used to investigate the fine tuning of 'fight-or-flight' responses to acute stressors of various nature. For instance, we recently found that the HR response of colonial king penguins (*Aptenodytes patagonicus*) to acute experimental stressors increases with stressor intensity (Viblanc et al., 2012). Similarly, several studies have documented stimuli-dependent modulations of HR responses to stress in other species (Nephew et al., 2003; Tarlow and Blumstein, 2007; Wascher et al., 2011), including other penguins (Giese, 1998; Holmes et al., 2005; Ellenberg et al., 2006, 2013). To our knowledge, however, whether acute stress responses are modulated according to life-history stages in interaction with stressor intensity is unknown. This study thus examined whether HR stress responses in king penguins were modulated by changes in energy and reproductive status throughout the breeding season, depending on stressor type and associated risk.

King penguins provide an interesting model to answer such questions. Their energy commitment to reproduction is especially high, because parents alternate between long-term fasting shifts on land to care for their single egg or chick and foraging trips at sea (Groscolas and Robin, 2001). Fasting shifts shorten with advancing reproduction (Weimerskirch et al., 1992) as efforts to provision the chick increase. The higher workload experienced while rearing young chicks is likely reflected in the higher glucocorticoid levels of the parents at that time (Viblanc et al., 2014a; Bonier et al., 2009). Chicks only fledge 14–16 months later, and birds that lose a chick can not replace it in the same season (Weimerskirch et al., 1992). Thus, the value of reproduction in a given season is expected to increase with advancing breeding shift (Winkler, 1987; Côté, 2000) and acute 'fight-or-flight' responses may be shaped accordingly. For instance, given that stress responses typically redirect behaviour and energy from reproduction to survival, HR responses associated with 'flight' initiation could be attenuated during later breeding stages to prevent chick desertion by the parents (Redondo and Carranza, 1989; Albrecht and Klvana, 2004). Alternatively, HR stress responses could increase with increasing investment in relation to chick defence, as penguin parents are more defensive of their breeding territory during chick-rearing (Côté, 2000) and exhibit heightened glucocorticoid levels at this time (Viblanc et al., 2014a). In addition, penguin pairs that breed successfully in a given

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year can only attempt to breed late in the subsequent season; however, it is extremely rare that they succeed (Weimerskirch et al., 1992; Stier et al., 2014). Thus, ‘fight-or-flight’ responses are also expected to change over the season given the higher likelihood of success of early-breeding birds. Documentation of the modulation of HR responses to acute stressors with advancing breeding season would help to further our understanding on whether ‘fight-or-flight’ responses can be adaptively shaped by specific life-history stages and energy constraints in wild animals.

RESULTS

Effects of advancing breeding shift, sex and stressor type on heart rate

Regardless of sex and stressor type, the HR excess (number of heart beats produced above initial resting rate) in breeding penguins upon acute stress decreased with advancing breeding shift (Table 1, Fig. 1). Indeed, controlling for colony area and stressor order, the best model selected by AICc did not retain any significant interaction between sex, stressor type and advancing breeding shift (see supplementary material Table S1). Similarly, regardless of sex and stressor type, the HR response to acute stress was lower during chick-brooding than during incubation (Table 2, Fig. 2; see supplementary material Table S2). Both the model with advancing breeding shift and the model with breeding stage (incubation versus chick-rearing) explained a similarly high portion of the variance in HR excess ($R^2_{\text{mar}}=0.83$ and 0.84 ; $R^2_{\text{cond}}=0.88$ and 0.88 , respectively). For captures, the distances at which birds detected the approaching experimenter (based on the onset of HR increase) was not affected by advancing breeding shift or breeding stage (incubator versus brooder), and was not different between the sexes. Indeed, although advancing breeding shift was retained in the final model, the effect was not significant (LMM; $t=-1.58$, $P=0.12$, $n=83$, $N=60$; see supplementary material Table S3). For breeding stage, only the intercept was retained in the final model (see supplementary material Table S4).

Effects of fasting on heart rate

We found no significant difference between the HR excess measured in the same eight birds stressed both at the beginning and at the end of their incubation shift (Wilcoxon paired signed-rank

Table 1. Mixed-model estimates for the effects of stressor type and advancing breeding shift on breeding king penguin heart rate excess caused by acute stress

Term	Estimate±s.e.	d.f.	<i>t</i>	Prob.> <i>t</i>
Intercept	5.85±0.38	201.35	15.46	<0.0001*
Breeding shift no.	-0.29±0.08	147.13	-3.82	0.0002*
Stressor ‘capture’	6.45±0.25	181.73	25.61	<0.0001*
Stressor ‘sound’	-2.32±0.25	191.09	-9.29	<0.0001*
Stressor order	0.04±0.07	220.38	0.56	0.58
Colony area ‘area B’	-1.18±0.47	104.97	-2.51	0.01*

The model presented is the best model retained by AICc (see supplementary material Table S1). The starting model included all two-way interactions between independent variables. Colony area and stressor order were entered as a covariates in the model to control for area-related differences in stress responses (see Viblanc et al., 2012) and potential habituation or sensitization effects. Bird identity was specified as a random factor. Factors are reported in reference to the level ‘10 m approach’ for stressor type, and ‘area A’ for colony area. The marginal and conditional R^2 for the model were 0.83 and 0.88, respectively. * $P<0.05$. The model was run for a total of $n=246$ observations on $N=77$ breeding birds (44 males, 33 females).

tests for 10 m approaches and captures; $V=11$ and 29 , $P=0.69$ and 0.15 , $N=7$ and 8 , respectively). Similarly, detection distances based on HR increase during approach for capture did not differ significantly between the beginning and the end of the incubation shift ($V=21$, $P=0.74$, $N=8$).

Effects of breeding timing on heart rate

For incubating males at shift 1, HR excess was not significantly different in the 10 males that bred early versus the 12 males that bred late in the season (birds with high versus low breeding success potential). Indeed, breeding timing was not retained as an important factor influencing HR excess in the final model (see supplementary material Table S5). Similarly, there was no significant difference between early and late breeding males in terms of detection distances during capture ($t=1.56$, $P=0.13$, $n=31$, $N=22$).

DISCUSSION

We show that the ‘fight-or-flight’ cardiovascular stress response to disturbance may be modulated by breeding advancement in

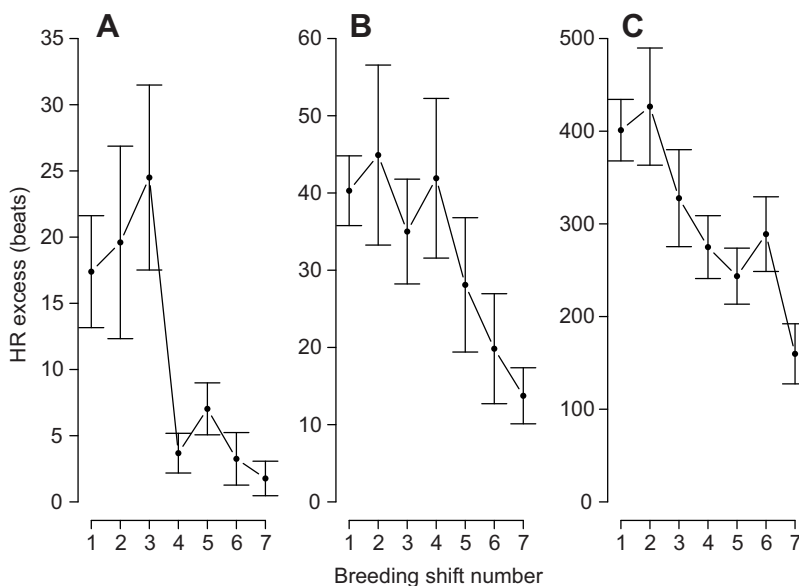


Fig. 1. Changes in heart rate excess caused by three different acute stressors with advancing breeding shift in breeding king penguins. Number of heart beats produced above initial resting HR in response to (A) sound stress, (B) 10 m approach and (C) capture as a function of the breeding shift number.

Table 2. Mixed-model estimates for the effects of stressor type and breeding stage on breeding king penguin heart rate excess caused by acute stress

Term	Estimate±s.e.	DFDen	t Ratio	Prob.> t
Intercept	5.42±0.31	221.14	17.51	<0.0001*
Breeding stage 'incubation'	-1.12±0.28	146.11	-4.03	<0.0001*
Stressor 'capture'	6.33±0.25	180.45	25.57	<0.0001*
Stressor 'sound'	-2.33±0.25	190.64	-9.47	<0.0001*
Stressor order	0.04±0.07	218.61	0.52	0.61
Colony area 'area A'	-1.51±0.42	97.18	-3.60	0.0005*

The model presented is the best model retained by AICc (see supplementary material Table S2). The starting model included all two-way interactions between independent variables. Colony area and stressor order were entered as a covariates in the model to control for area related differences in stress responses (see Viblanc et al., 2012) and potential habituation or sensitization effects. Bird identity was specified as a random factor. Factors are reported in reference to the level 'chick-brooding' for breeding stage, '10 m approach' for stressor type and 'area A' for colony area. The marginal and conditional R^2 for the model were 0.84 and 0.88, respectively. * $P<0.05$. The model was run for a total of $n=246$ observations on $N=77$ breeding birds (44 males, 33 females).

seabirds. The HR excess caused by acute stressors in penguins decreased with (1) advancing breeding shift, and (2) with the transition from incubation to brooding. The models including those variables explained substantial and similar amounts of variation in the HR response to stressors. Consistent with previous results, HR excess was also affected by the specific nature (intensity/risk) of acute disturbances (Viblanc et al., 2012) (see similar findings in other bird species; Nephew and Romero, 2003; Nephew et al., 2003; Wascher et al., 2011, including penguins; Giese, 1998; Holmes et al., 2005; Ellenberg et al., 2006, 2013), but the decrease in HR excess with advancement of breeding was not stressor dependent.

Stress responses may vary according to the energy demands of various reproductive stages. Chick-rearing is typically a period of high energy commitment in penguins (Gales and Green, 1990; Green et al., 2009) and down-regulating stress responses could allow substantial energy savings during this period. For instance, hormonal stress responses of grey-faced petrels (*Pterodroma macroptera gouldi*) to acute challenges are higher during incubation than chick-rearing (Adams et al., 2005). Down-regulated stress responses (including HR) also occur during energetically demanding periods, such as molt (e.g. in European starlings *Sturnus vulgaris*; Cyr et al., 2008).

Interestingly, the adrenocortical response of magellanic penguins (*Spheniscus magellanicus*) to handling stress increased over a shorter breeding timeframe (i.e. during incubation), although this was probably related to long-term fasting and decreased body condition during this period (Hood et al., 1998) (see below). In king penguins, parents increase the frequency of foraging trips during chick-rearing (Weimerskirch et al., 1992) to meet the energy requirements of their growing offspring. As foraging comes at a substantial metabolic cost during chick brooding (Kooyman et al., 1992; Charrassin et al., 1998), down-regulating stress responses during this period could allow substantial energy savings for adults.

In contrast to results in magellanic penguins (Hood et al., 1998), our data on males stressed both at the beginning and end of an incubation (fasting) shift suggest that no specific changes in HR excess occur in response to the variation in energy status over a shorter timeframe. However, it is likely that in our study, males at the beginning and at the end of their incubation shift were at a similar fasting stage (phase II) and energy status, during which body fat mainly fuels metabolism (Groscolas and Robin, 2001). Indeed, males typically depart the colony to re-feed before entering a more critical stage of fasting (phase III) where protein catabolism occurs (Groscolas and Robin, 2001), and only a very small fraction (~3%) of birds are found to reach this critical stage in natural conditions (Gauthier-Clerc et al., 2001). It would be interesting to consider whether HR stress responses are down-regulated in those extreme cases by maintaining birds in captivity beyond the onset of phase III. Previous studies indeed suggest that stress responses can be down-regulated in situations of energy deficits (Kitaysky et al., 2005) or limited food availability (Kitaysky et al., 1999) in seabirds (but see Hood et al., 1998). However, those studies considered the responsiveness of the HPA axis, and whether the cardiovascular (sympathetic) response to stress may also be under the control centres responsible for monitoring energy balance in fasted birds remains to be determined (see Mager et al., 2006 for some evidence in rats).

In European starlings, when comparing breeding and non-breeding conditions, Dickens et al. (2006) reported similar differences in the HR response of males to an acute stressor (intruding bird). Males kept under a long-day photoperiod (breeding conditions) showed significantly higher HR responses to the intruder than males under short-day photoperiod (non-breeding

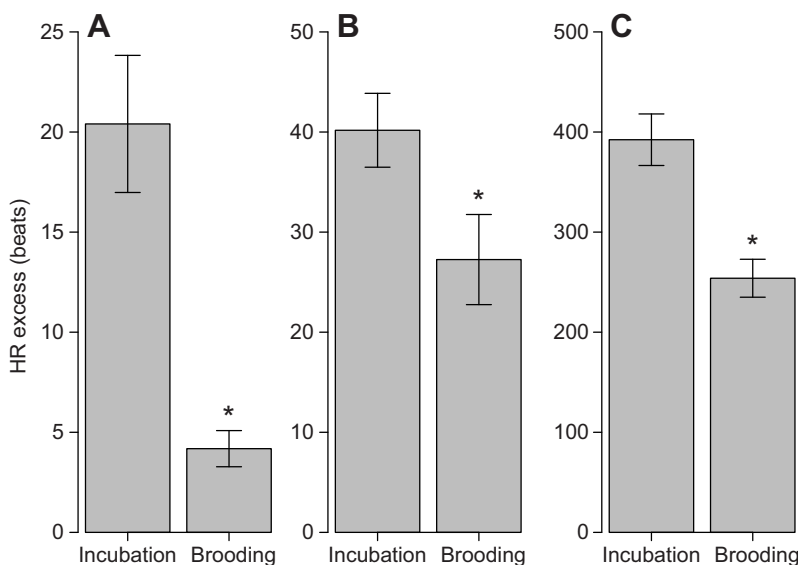


Fig. 2. Heart rate excess caused by three different acute stressors in breeding king penguins either incubating an egg or brooding a young chick. Number of heart beats produced above initial resting HR in response to (A) sound stress, (B) 10 m approach and (C) capture in birds incubating an egg or brooding a chick. Values are means±s.e.m. * $P<0.05$.

conditions). Such changes could be linked to changes in hormone titres (testosterone) mediating territoriality during the breeding season (Dickens et al., 2006). In king penguins, changes in hormones occur between breeding stages (Mauget et al., 1994; Viblanc et al., 2014a). For instance, chick-rearing birds exhibit higher baseline glucocorticoid (corticosterone) levels than incubators (Viblanc et al., 2014a). However, the relationship between cardiovascular and endocrine responses to stress is complex. Both pathways may be modulated independently in response to acute stressors (e.g. Nephew et al., 2003), but may also be coupled. For instance, in adrenalectomized rats, corticosterone implants of increasing concentration appear to upregulate HR responses to novelty in open-field tests (van den Buuse et al., 2002). In this study, however, adrenalectomy likely affected the adrenomedullary regulation of blood pressure and HR (van den Buuse et al., 2002), making the results hard to interpret. In king penguins, future studies should consider to what extent circulating hormone levels play a permissive or suppressive effect on HR. In addition to corticosterone (Viblanc et al., 2014a), testosterone and prolactin are likely modulators of the ‘fight-or-flight’ response in relation to parental care, and their interplay with HR and glucocorticoid responses to stress remain to be thoroughly examined (Angelier and Chastel, 2009; Angelier et al., 2013). In fact, Fig. 1 suggests that the incubation–brooding transition is only a threshold for the sound stressor, the slow degradation of the HR response for 10 m approaches and captures being consistent with changes in hormone titres.

Another possibility to explain changes in stress responses with advancing reproduction may involve breeding investment. Parental commitment may be higher and stress responses modulated with advancing breeding because of the higher probability of offspring reaching sexual maturity (Winkler, 1987). Because king penguins are more defensive of their breeding territory during chick-brooding than incubation (Côté, 2000), acute HR responses to stressors could be expected to increase according to a ‘fight’ strategy during later stages of breeding. Yet, we observed a decrease in HR responses over incubation and brooding. Alternatively, a decrease in acute HR responses would be advantageous if it prevented breeding parents from deserting the brood (‘flight’ strategy) when faced with an acute stressor at an advanced stage of reproduction. In this sense, stress responses could mechanistically underlie parental decisions about offspring investment (Lendvai et al., 2007; Bókony et al., 2009; Goutte et al., 2011). Interestingly, we found no substantial difference between detection distances (based on HR) to approaching experimenters in relation to breeding advancement or seasonal timing, suggesting that king penguins may be similarly sensitive to the presence of an intruder throughout breeding but stress responses nonetheless downregulated with advancing breeding shifts. In addition, if the relative reproductive value of the brood were to explain changes in HR responses to stress, we would expect penguins breeding at different periods of the reproductive season (associated with strong differences in reproductive success) to exhibit different HR responses. The relative reproductive value of the offspring should be much greater in early breeders (Weimerskirch et al., 1992), and stress responses attenuated compared with late breeders. Of course, this would hold true if some extrinsic (environmental; e.g. photoperiod) or intrinsic (physiological) cue was used to link breeding success and seasonal timing. Potential mechanisms for king penguins might for instance include changes in environmental resources (Gauthier-Clerc et al., 2002) and adult body condition (Dobson et al., 2008) early and late in the season. Yet, we did not find a difference in HR

stress responses between early and late breeders. Given our relatively small sample size (10 early breeders and 12 late breeders), differences may not have been apparent. More likely is the fact that our sample selection was not fully representative of early and late breeders. Indeed, because of time constraints with fieldwork, we only compared the stress response of early and late males during the first incubation shift. It seems that at this very early stage of breeding, the reproductive value of the egg is perceived as similar (and likely minimum) by early and late breeders. Given that the HR stress response is primarily attenuated during chick-brooding, it would have been more relevant to compare early versus late breeders at this stage.

Finally, a few considerations should be discussed. First, to avoid colony disturbance, we only sampled birds on the periphery. How might this affect the stress profile of penguins? Côté (2000) suggested that peripheral territories were of lesser quality than central ones because of higher predation pressure, which may influence stress profiles. Actually, we found that baseline stress hormone levels were higher in central territories, likely due to higher social density and aggressiveness (Viblanc et al., 2014a; Côté, 2000). Thus, it appears clear that a proper comparison of incubating versus brooding birds should be done in the same colony location (as present) or that breeding territory location should be accounted for, to control for potential differences in stress responses between locations. Second, could the observed attenuation in stress responses be a mere consequence of habituation (Cyr and Romero, 2009)? This appears unlikely: the order in which stressors were applied had no significant effect on HR responses. In addition, the same males that were successively stressed at the beginning and the end of their incubation shift some 9 days apart did not differ significantly in their HR response, suggesting that no habituation occurred.

Taken together, those results suggest that the sympathetic-mediated response to stress is significantly down-regulated over the breeding season in king penguins. This modulation could be an active strategy to save energy during costly periods of the reproductive cycle or achieved to prevent desertion of the brood (perhaps via changes in titres of circulating hormones such as prolactin). Investigation of the underlying mechanisms (e.g. central or peripheral nervous control; Wingfield and Sapolsky, 2003) of stress attenuation in chick-brooding birds and the inter-relationships between the regulation of HPA and sympathetic responses to acute stress may prove particularly insightful.

MATERIALS AND METHODS

Animals

King penguins (*Aptenodytes patagonicus* Miller 1778) were studied on Possession Island, Crozet Archipelago (46°25'S, 51°45'E). Their breeding cycle is discussed at length elsewhere (Stonehouse, 1960; Weimerskirch et al., 1992; Descamps et al., 2002). Briefly, during the breeding season (November–March), parents alternate between periods caring for the egg or chick on land and periods foraging at sea. Males take charge of the first incubation shift, being relieved by their females 16–18 days later. Alternated incubation continues for a period of roughly 54 days, with the egg typically hatching during the fourth (female) shift. Parents then continue to alternate 6–12 day shifts ashore, brooding their chick for a period of approximately 31 days until the end of (male) shift 7. Subsequently, the chick is left unattended in the colony as both parents resume foraging trips feeding it until the end of the summer. Chick-provisioning is low during the winter and fledging only occurs during the subsequent season, i.e. some 11 months from hatching to fledging (Weimerskirch et al., 1992).

Over 2009–2011, a total of 77 birds (44 males and 33 females) equipped with HR loggers were stressed at shifts 1 to 7 of the breeding cycle. The data collected on brooding birds in 2011 (see Viblanc et al., 2012) was

supplemented with additional data collected on both incubating and brooding birds during the 2009–2010 breeding season. Three different stressors were applied to the birds (see below). Random breeding pairs were marked while courting at a distance of half a metre without capture using a non-permanent animal dye in the form of pressurized spray-paint (Porcimar[®] Kruuse, Langeskov, Denmark). They were later caught and flipper-banded for identification during field observations, either at the very onset of the first incubation shift (males) or at relief at the end of this shift (females). Marked birds were checked twice daily from a distance to determine egg laying and hatching dates, as well as the onset of each incubation and brooding shift. Birds stressed while incubating ($N=35$) were either males in shifts 1 ($N=22$) and 3 ($N=11$), or females in shift 2 ($N=10$). Birds stressed while brooding a non-thermally emancipated chick ($N=48$) were males in shifts 5 ($N=14$) and 7 ($N=9$), and females in shifts 4 ($N=14$) and 6 ($N=13$). Some birds were stressed at several time periods (different reproductive shifts) over the breeding season, which explains the above difference in sample size when considering all incubating birds together or incubating shifts separately. Thus, to account for potential effects of habituation or sensitization in HR responses, we included stress order as a covariate in subsequent analyses (see below).

The effect of seasonal timing (early versus late breeding) on HR responses was only investigated for males during incubation shift 1 using 10 m approaches and captures, because of logistical constraints. We compared the responses of 10 early (November–December) breeders with those of 12 late (February–March) breeders, sampled in the same colony area. In addition, we also considered whether fasting duration affected HR response. For this, we considered whether HR response varied in eight birds that were repeatedly captured 8.6±1.0 days (mean±s.e.m.) apart, between the beginning and the end of their incubation shift.

Bird handling, either when fitting HR loggers or during capture–immobilization protocols, was always performed within the colony on the birds' breeding territory and never resulted in egg or chick abandonment. Flipper bands were removed at the end of the study because of their known detrimental long-term effects on survival and reproduction (Saraux et al., 2011). Capture and tagging procedures were approved by the Ethical Committee of the Institut Polaire Français – Paul-Emile Victor. Authorization to enter the colony and manipulate birds was obtained from Terres Australes et Antarctiques Françaises. The experiments comply with the current laws of France.

Experimental stress protocols

We used the same experimental stress protocols as previously described by Viblanc et al. (2012). Penguins were submitted to (1) a distant (10 m) pedestrian approach; (2) a capture; or (3) a sound stress. We previously found that HR responses were lowest for sound stresses, intermediate for 10 m approaches and highest for captures (Viblanc et al., 2012). For 10 m approach and capture stresses, penguins were approached within their visual field by a walking observer starting from a distance of at least 30 m. The starting distance of ≥ 30 m was chosen as we found it to be greater than the detection distance of penguins to human observers in preliminary trials. During 10 m approaches, the observer stopped 10 m away from the bird whereupon he remained motionless for 1 min while dictating observations on the behaviour of the subject. He then retreated at a constant speed to his initial position, keeping the focal bird in sight and resuming behavioural observations. During captures, the observer walked directly to the focal bird and gently immobilized it for 3 min, covering its head with a hood to keep it calm. The hood was rapidly removed after the 3 min immobilization and the observer retreated at a constant speed to his original position, to continue observations for several minutes until the bird was resting again. During sound stresses, resting birds were discreetly approached from behind, until the observer was 15 m behind the focal individual. The experimenter then struck two hollow metal bars three times with a 1 s interval between strokes. Care was taken to keep out of sight of the animal (both during the approach, during the stress itself and when retreating) and the stress was only performed if the observer was certain that the focal individual was unaware of his presence.

After fitting HR loggers on breeding individuals (see below), we applied the three stressors randomly. Prior to the stress, a focal bird was observed to

ensure it was resting for at least 3 min before the stressor was applied, and not engaged in any form of activity (e.g. preening, territory defence) known to increase HR. All birds in this study were located in the periphery (i.e. 2–3 bird ranks from the edge) of the colony to avoid unnecessary disturbance of social congeners. Because we previously found that colony location (disturbed versus non-disturbed areas) had an effect on HR responses to acute stressors (Viblanc et al., 2012), we recorded the colony area in which the bird was to control for its effect on HR in further analyses. During the stress, bird behaviour (resting, vigilance, aggression) and the distances between the experimenter and the focal individual, were continuously dictated in real time to a digital audio recorder (VN5500[®] Olympus Europa, Hamburg, Germany). Behavioural observations continued several minutes after the end of the stress, until the bird reached a resting state again. Those observations allowed any effects of post-stress behaviour on HR (e.g. aggressive behaviour towards neighbours) to be taken into account. The time separating two successive stresses applied to a given individual was at least 6 h. When two birds were located in the same area of the colony, stresses applied to each of the birds were separated by at least 4 h.

Heart rate loggers

Acute HR responses to stressors were measured using external HR loggers (Polar[®] model RS800 and RS800CX, Polar Electro Oy, Kempele, Finland) specially adapted for use on king penguins (see Groscolas et al., 2010; Viblanc et al., 2012, 2014b). Loggers were made of two units: (1) a sensor-transmitter (30–40 g) composed of a HR processor which filtered out electrical background noise received from the electrodes (i.e. muscle activity) from heart activity; and (2) a receiver/logger (30 g). Electrodes were composed of two stainless-steel wires attached to gold-plated safety pins, which were inserted and secured in the subcutaneous fat layer (at a depth of approximately 5 mm, and over a length of 1 cm), 25 cm apart on the back of the bird. Iodine (Betadine[®]) and an alcohol based antiseptic solution were used to disinfect the electrodes before each use. The transmitter was attached in the middle of the back with Tesa[®] tape wrapped around several layers of feathers and the receiver was either secured to the flipper band or whenever possible fixed on a metal pole within a 5 m distance of the animal. Such a set up prevented the equipment from hindering the movements of the birds. This was confirmed by the fact that we never observed birds trying to remove electrodes or HR loggers, nor did we observe any adverse effects of the equipment on the birds' health or behaviour (>50 h of observations; Viera et al., 2011; Viblanc et al., 2011). The whole apparatus weighed less than 1% of the total body mass of the smallest bird. Further details on this method and how it accurately estimates HR of king penguins are provided by Groscolas et al. (2010).

Penguins at the different incubating and brooding shifts were equipped (sometimes repeatedly) with HR loggers on their breeding territory within the colony, and then left to recover for at least 12 h (a night) before applying stressors. At capture, the head of birds was hooded to keep them calm, handling lasting between 5 and 10 min. Polar[®] monitors record HR by measuring the time lapse (in ms) between signals received from the transmitter. The lap was divided into 60,000 and the calculation was done every second. Monitors were set to store the data every 5 or 2 s, depending on logger model. The sampling rate was chosen to provide a memory autonomy of up to 99 h, and was appropriate considering the shortest durations of HR responses to applied stressors (see Viblanc et al., 2012). All loggers were removed from the birds within 1 day of application of the last stressor and a few days before their departure at sea, based on the estimated length of breeding shifts in king penguins (Weimerskirch et al., 1992).

Heart rate analyses

HR data were plotted and analysed using Polar Pro Trainer[®] v5.00.105 software. Audio recordings of each test were time-matched (by previous synchronization of the observer's digital watch with that of the HR logger) with the corresponding HR data. We defined HR_i (initial resting rate) as the HR at the moment preceding a rapid constant increase in HR. The duration of the HR response was characterized as the total time that HR increased above HR_i following the application of a stressor. Excess HR above resting values was then calculated as [(mean HR during stress–HR_i)×duration of HR elevation (in min)]. This corresponded to the number of heart

beats which were produced in excess of HR_i due to stress and presented an integrative measure of the HR stress response (the area under the curve). Behavioural observations were time-matched against HR profiles and occasionally used to identify and control for changes in HR independent of the stressors applied, viz. when HR increased because of aggression between neighbours when re-establishing territorial boundaries following colony disorganisation during capture. Additionally, for capture stresses for which the birds were approached continuously to contact, we used HR data and information from audio files to determine what distance the bird was from the experimenter when its HR started to increase (in other words, the distance at which the physiological response started or detection distance).

Statistics

Statistical analyses were performed using R v3.1.2. First, we used linear mixed models (LMMs) to investigate the effects of advancing breeding shift, penguin sex, stressor type (intensity) and all two-way interactions between those variables on HR excess (dependent variable). Breeding shift number, penguin sex, stressor type and interactions were specified as independent variables in the initial model. We then ran a model selection using the ‘dredge’ function from the ‘MuMIn’ package in R, and retained the model with the lowest Akaike’s information criterion corrected for small sample size (AICc) and the highest AIC weight as the best fit (Burnham and Anderson, 2002). Competing models were fitted by maximum likelihood during model selection. The final model was fitted using restricted maximum likelihood. Colony area and the order in which the stressor was applied were included as covariates in all models to account for colony site-specific differences in stress responses (Viblanco et al., 2012), and potential effects of habituation or sensitization on HR.

Second, we ran the same procedure as above, but specifying breeding status (incubation versus chick-brooding) rather than advancing breeding shift as an independent variable to consider its effect on heart rate excess. Specifically for captures, we used similar models to consider the effects of sex, advancing breeding shift (or breeding status) and their interaction, on detection distances while accounting for colony area and stressor order.

Third, we investigated the effects of breeding timing on HR excess in males during their first incubation shift by specifying seasonal timing (early vs late), stressor type (capture vs 10 m approach) and the interaction between those variables as independent variables in a LMM. We accounted for stressor order as a covariate in the model but not for colony area, because in this case, early- and late-breeding males were sampled in the same area of the colony.

Finally, to evaluate potential effects of fasting duration on HR responses, we used Wilcoxon paired signed-rank tests to compare the same eight birds that were stressed both at the beginning and the end of their incubation shift (we were only able to acquire HR responses for 10 m approaches on seven birds because of HR-logger malfunction).

Bird identity was specified as a random term in all LMMs to account for repeated measurements on the same individual (individuals used in different stress protocols). Model residuals were inspected for normality and where necessary, data were transformed using Box-Cox power transformations prior to analyses. Results are given as means±s.e.m. Significant effects are reported for $P < 0.05$. For LMMs, we report the marginal and conditional r -squares of the model (Nakagawa and Schielzeth, 2013). The marginal r -square (R^2_{mar}) represents the proportion of variance in HR excess explained by fixed effects, whereas the conditional r -square (R^2_{con}) represents the proportion of variance explained by fixed plus random effects. The number of observations used (n) and the number of birds concerned (N) for each model are also reported.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

V.A.V. and R.G. designed the study. V.A.V., B.G., M.K. and R.G. performed the field work. V.A.V. and A.D.S. did the analyses. V.A.V. wrote the paper. All authors contributed to its revision.

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Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.112003/-/DC1>

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