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Delayed start of full incubation is related to a delayed brood patch development in Magellanic Penguins *Spheniscus magellanicus*

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Capsule: Most bird species start full incubation before the clutch is complete, others long after laying the last egg, gradually increasing the incubation temperature as incubation progresses, as is the case in Magellanic Penguins *Spheniscus magellanicus*.

Aims: In this study, we analysed the possible mechanisms underlying this delayed start of full incubation in Magellanic Penguins. We postulated two non-mutually exclusive, possible mechanisms: delayed development of the brood patch and/or variation in the adults' incubation postures.

Methods: We conducted our work at Isla Quiroga, Argentina during 2011–13. We measured the development of the brood patch area, its temperature, the egg temperature and observed the postures of adult penguins.

Results: We found no association between the adults' postures and the delayed start of full incubation, nor between brood patch temperature and the increase in egg temperature as incubation proceeded (egg age). The intercept of the curve between egg temperature and egg age was positively related to brood patch area, while the slope of the curve was negatively related.

Conclusion: Our results support the hypothesis that the shape of the curve of the increase in egg temperature as incubation proceeds is a consequence of the increase in the extension of the brood patch area.

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Incubation has been identified as an energy-demanding phase of reproduction (Nilsson 1994, Monaghan & Nager 1997, Reid *et al.* 2000), especially in birds with long breeding cycles (Hanssen *et al.* 2005). For birds that lay more than one egg, the laying period lasts several days, as birds are unable to lay all the eggs of a clutch on the same day because of physiological constraints. In some species it has been shown that incubation starts during the laying period (Wang & Beissinger 2011). Several hypotheses have been proposed to explain why an early onset of incubation would be evolutionarily favourable ('Nest Predation Hypothesis': Clark & Wilson 1981, 'Hurry-up Hypothesis': Slagsvold 1986, 'Egg-viability Hypothesis': Veiga 1992).

Although it has been shown that it would be beneficial for adults to start incubation during egg laying, in some penguins it appears to be normal to reach full incubation (defined in Wang & Beissinger 2011; stable and high incubation temperatures) well into the incubation period (Yellow-eyed Penguin *Megadyptes antipodes*,

Massaro *et al.* 2006; Magellanic Penguin *Spheniscus magellanicus*, Rebstock & Boersma 2011, Barrionuevo & Frere 2012; Gentoo *Pygoscelis papua* and Jackass *Spheniscus demersus* Penguins, Burger & Williams 1979). This delayed onset has been linked to: (a) a mechanical constraint, such as delayed development of the brood patch (St Clair 1992, Massaro *et al.* 2006) and/or (b) a change in the incubation postures as incubation proceeds (St Clair 1992). The brood patch is a de-feathered area through which the parents heat their eggs and achieve a successful incubation (Lea & Klandorf 2002). In many passerines the de-feathering process occurs before breeding or during nest building and copulation (Jones 1971, Zann & Rossetto 1991), but in some penguin species there is a two-week delay in brood patch development after egg laying is finished (St Clair 1992). On the other hand, in some penguin species the posture of the adults changes as incubation progresses, going from standing next to the eggs to lying on the eggs (St Clair 1992, Williams 1995). This variation in postures as incubation progresses is

consistent with lower egg temperatures before clutch completion in the Fiordland Crested Penguin (*Eudyptes pachyrhynchus*) (St Clair 1992).

The objective of this study is to discuss these two mechanisms for the delayed start of full incubation found in the Magellanic Penguin. We propose that the delayed start could be a consequence of: (a) a delayed brood patch development and/or (b) the change in adults' incubation postures as incubation progresses. This work contributes to the general knowledge of incubation patterns and temperatures, and their variation, and helps to understand how incubation temperatures are established in penguins, especially in Magellanic Penguins.

Methods

Study site

This study was conducted at Isla Quiroga (47°45'S, 65°53'W), Santa Cruz, Argentina, which holds 1500 breeding pairs of Magellanic Penguins, with most nests located beneath shrubs (E. Frere, unpubl. data). The island is flat, located at only 80 m off the coast, and measures 600 by 98 m at the widest points. We have direct access to the nests.

Study species

Magellanic Penguins lay two eggs, four days apart, beginning in early October. Both parents incubate their eggs, each one taking incubation bouts of approximately 15 days, after which the bouts become progressively shorter until the eggs hatch, approximately 40 days after laying. Usually females take the first incubation bout (Boersma *et al.* 1990). The adults' postures during incubation have not been previously described in this species, but from our own previous observations we know they spend most of the time in a static position, not changing their postures very frequently (M. Barrionuevo, pers. obs.). Egg temperatures are usually low (<25°C) immediately after laying the first egg and, four days after this, when the clutch is complete with the laying of the second egg, the mean temperature reaches 26.3°C (Barrionuevo & Frere 2012). From that point onwards, egg temperature progressively increases up to the 17th day when it becomes stable at 34.2°C (Rebstock & Boersma 2011, Barrionuevo & Frere 2012).

Field methods

During early October of the breeding seasons 2011–13, we randomly selected a total of 184 nests and checked

them daily to establish laying day of the eggs. We marked these nests with flagging tape (Tapebrothers, Longwood, FL) with a unique nest number. Each year we chose different nests to avoid taking repeated measurements of the same pair of breeders, as Magellanic Penguins reuse the same nest year after year. The first time we captured adults, we attached a unique and permanent metal tag (2 × 10 mm; National Band and Tag Company, Newport, KY) to their foot webbing for identification during future visits.

Brood patch development

We measured individual egg temperature throughout the incubation period in a subset of the previously randomly chosen nests during the breeding seasons of 2012 ($n = 12$ nests) and 2013 ($n = 12$ nests). On the laying day of each egg, we removed the eggs from the nests using a stick with a tin on the end; the tin was properly covered to prevent egg damage and the egg fitted perfectly in it. We fixed one thermochron temperature data logger (iButton DS1921G#F50, $\pm 0.5^\circ\text{C}$, Maxim, CA, USA) on each egg to the shell surface between the egg poles with a small strip of medical adhesive tape, and returned the eggs to the nests. We programmed iButtons to log the temperature every 15 minutes, until the egg was approximately 34 days old (we have previously validated this methodology, for more details see Barrionuevo & Frere 2012). In this way, we registered the individual egg temperature of both eggs in each nest. We also measured length and width of the eggs with a Vernier calliper (± 0.1 mm, Mitutoyo, IL, USA) and estimated egg volume using the equations proposed for each egg (first and second laid eggs) in Boersma & Rebstock (2010).

In these same nests in which we measured egg temperature, we captured adults to measure brood patch development on the laying day of the second egg (first-laid egg age = 4 days) and then every 6 days until the first-laid egg was 34 days old. Egg age concerns the days elapsed between the day the egg was laid and the day the measurement was taken. Normally, there was only one adult at the nest during the visits, but if both parents were present, we captured the adult that was sitting on or lying on the eggs. By not capturing both adults, we reduced nest disturbance. We captured adults at their nests by using a 1 m long iron hook around the tarsus and pulling them towards the captor until they could be caught by hand. Adults returned immediately to their nests once released, so the egg temperature was not significantly affected. We measured brood patch temperature using an electronic probe thermometer ($\pm 0.1^\circ\text{C}$, Pro-check, CABA, Argentina). We placed the thermometer in direct

contact with the brood patch, wrapping it with the skin, until the temperature stabilized (for more details see Barrionuevo & Frere 2015). We measured the length of the brood patch of the adults with a ruler (± 1 mm), and its width with a Vernier calliper (± 0.1 mm), stretching the brood patch area and measuring it at the widest point (for more details see Barrionuevo & Frere 2015). We calculated the brood patch area as half the product of length and width (St Clair 1992). First, we measured the brood patch temperature to avoid handling time affecting the brood patch temperature. The latency, that is the time between capture and the measurement of the brood patch temperature, was less than a minute and the ambient temperature is not so low as to affect the brood patch temperature (15°C , data given by the National Weather Center, Puerto Deseado Station, 2.5 km from the study site). During the first visit, we identified the sex of the adult by measuring the bill depth with a Vernier calliper (± 0.1 mm) because males have deeper bills than females (Gandini *et al.* 1992), and recorded this measure together with the unique number on the metal tag. In later visits, to identify the sex, we used the foot webbing metal tags.

Incubation posture

During the breeding seasons of 2011 and 2012, we registered adult incubation posture at 160 nests throughout the incubation period; these were not the same nests where we measured the brood patch development of adults. On the laying day of each egg, and then every 4 days until hatching, we visited the nests to check the incubation posture of adults from 5 m away to avoid influencing the posture. The visits were mostly during mornings unless the weather conditions were unfavourable. We defined the postures as: lying, sitting or standing. When the adult was in a horizontal position and no egg was visible with the naked eye it was registered as a lying posture. When the adult was standing on the nest, partially hunched, and eggs were partially exposed we registered this as the sitting posture. Finally, when the adult was standing on the nest and eggs were fully exposed it was registered as the standing posture. Although our data are point samples of incubation posture, we think they are suitable for assessing whether postures change along with incubation given the behaviour previously observed in Magellanic Penguins during this nesting period (see study species section). Furthermore, although we did not take many observations per nest (10), many nests (160) were sampled for adult incubation postures.

Data analysis

To determine which variables affected egg temperature as incubation proceeded, measured as egg age, we performed a non-linear mixed model (NLMM), which allowed us to check for differences in the parameters of the curves within the variability of the variables analysed. The curve that describes daily mean egg temperature as a function of egg age (counted since the first egg was laid) has a logarithmic shape:

$$\text{Egg temperature} = b + m \times \ln(\text{egg age})$$

The parameters of the curve are: the intercept b , initial egg temperature and the slope m , rate of increase in egg temperature. This curve has been shown to be the one that best describes the daily mean egg temperatures as incubation proceeds in Magellanic Penguins (Rebstock & Boersma 2011).

The brood patch was measured on days 4, 10, 16, 22, 28 and 34 since the first egg was laid. We averaged the egg temperature recorded on the days previous to each brood patch measurement for each egg, intervals: 0–3, 4–9, 10–15, 16–21, 22–27 and 28–33 days, counted since the first egg was laid. During the interval 0–3 days we only had temperatures of first-laid eggs, in later intervals we averaged the temperature of both laid eggs. In the model, we linked each brood patch measurement with the corresponding average egg temperature interval (response variable). In the fixed part of the model, each parameter (m and b) of the curve has its own linear model in which we added the same predictor variables (Pinheiro *et al.* 2015). The predictor variables for the parameters were: brood patch area, brood patch temperature and the sum of the egg volumes present in the clutch for each interval (for interval 0–3 we only had egg volume of first egg and in later intervals we summed both egg volumes). All variables were added as continuous variables. To account for the lack of independency of the measurements at the same nest, we added nest as a random factor. We included random effects for both parameters in the model, m and b , allowing for the parameter estimates to vary across nests or individuals.

We tested the random factors comparing the full model with and without the random factors with a Likelihood Ratio test, before testing the fixed factors (Zuur *et al.* 2009). We used a backwards selection procedure, removing the terms one by one in a decreasing order of complexity and according to a decreasing P value (Crawley 2007). In the NLMM we eliminated each variable from the linear models of each parameter one at a time, that is, a variable could be eliminated from the linear model of parameter m , but

not from the model of parameter b . The statistics (L -ratio) and the P value reported for each variable are the results of the comparisons of the models with and without each variable, through a Likelihood Ratio Test performed with the function 'anova'. In some cases, we also report the output of the fixed effects. We used the R Software v. 2.12.1 (R Development Core Team 2010) for running the models and the packages 'nlme' and 'lattice' for the NLMM. All the other statistical analyses were performed with Statistica v. 7 (Statsoft Inc. 2004). We report means \pm se. We considered differences to be significant when $P < 0.05$. All graphs were plotted using Sigma Plot v. 10.0 (Systat Software Inc. 2006).

Results

Brood patch development

Brood patch area was related to the slope and intercept of the curve between daily mean egg temperature and egg age (Table 1). In contrast, brood patch temperature and egg volume were not related to any of the parameters of the curve between egg temperature and egg age (Table 1). The larger the brood patch, the higher the initial egg temperature ($t_{1116} = 2.94$, $P = 0.004$). Nonetheless, the larger the brood patch, the slower the rate of increase in egg temperature ($t_{1116} = -2.43$, $P = 0.02$).

Both the egg temperature and the brood patch area increased with egg age (One-way ANOVA; dependent variable: average egg temperature on the days previous to each brood patch measurement, categorical factor: first-laid egg age; $F_{(5,255)} = 251.8$, $P < 0.0001$. One-way ANOVA; dependent variable: brood patch area, categorical factor: first-laid egg age; $F_{(5,255)} = 7.88$, $P < 0.0001$; Figure 1). After the tenth day since the first egg was laid there were no differences in brood patch area as the incubation progressed (Tukey honest significant

Table 1. NLMMs for Magellanic Penguins' egg temperatures as a function of first-laid egg age. Volume of the clutch, brood patch temperature and brood patch area were analysed, and nest was added as random factor. b and m are parameters of a logarithmic function where, b is the intercept and m is the slope ($n = 24$ nests). Brood patches measured at days 4, 10, 22, 28 and 34 since the first egg was laid were linked with averaged egg temperature on the days prior to each brood patch measurement (temperature intervals: 0–3, 4–9, 10–15, 16–21, 22–27 and 28–33 days). Significant results are shown in bold.

Variable	Parameter	L -ratio	P
Egg volume	b	0.25	0.62
	m	0.01	0.93
Brood patch area	b	8.56	0.003
	m	5.96	0.02
Brood patch temperature	b	0.001	0.97
	m	0.38	0.54

difference (HSD) Test: significant differences only between day 4 and 10, 16, 22, 28 and 34: all $P < 0.0001$; Figure 1), while egg temperature was still increasing until the last part of the incubation (Tukey HSD Test: significant differences between first-laid egg age: all $P < 0.008$, except between days 28 and 34, where $P = 0.20$; Figure 1).

Incubation posture

Researchers did not affect adults' postures given that none of them changed position during our observations (pers. obs.). Each nest was checked ten times throughout the incubation period. In 99% of the visits, penguins were lying, in 0.58% of the visits we found adults sitting and in 0.41% they were standing. The sitting and standing postures were registered only before the 15th day of the incubation period. However, as the penguins were almost exclusively lying down (99.01%), we could not conduct any further statistical analyses, and we conclude that adult behaviour did not change during the course of the incubation period.

Discussion

We found that brood patch area influenced the curve of egg temperature as incubation proceeded. The intercept of the curve was positively related to brood patch area, while the slope was negatively related to this variable. Larger brood patches resulted in higher initial egg temperatures, but slower rates of increase in egg temperatures. The asymptotic temperature, when full incubation is reached, is approximately the same for all eggs (Barrionuevo and Frere 2012); so if the brood patch area is larger at the beginning of incubation and

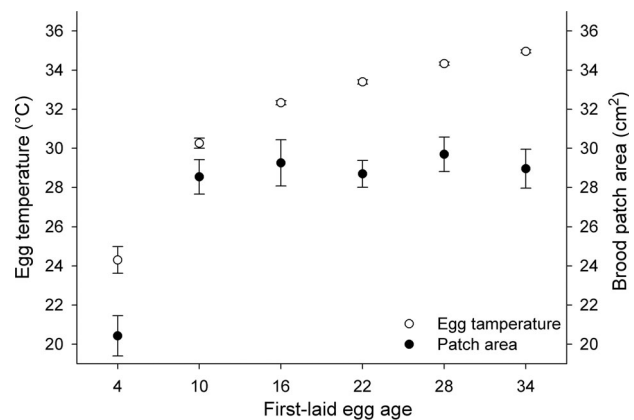


Figure 1. Mean \pm se egg temperature (white circles) on the days prior to each measurement of the brood patch and the corresponding brood patch areas (black circles) as incubation progressed, counted as days since the first egg was laid.

therefore the initial egg temperature is higher, there is no need to warm the eggs faster, and therefore the slope of the curve is lower if brood patch area is larger. On the other hand, in the last half of the incubation period there was still an increase in egg temperature, while brood patch area remained constant. Embryos in a stage of advanced development can have some level of internal heat production (Ar & Sidis 2002) that would explain this temperature increase late in the incubation period when the patch no longer increased in size.

Contrary to expectations, brood patch temperature did not affect egg temperatures. The brood patch temperature reaches its stable and maximum value once the laying period is finished, while the brood patch area reaches its peak later (Barrionuevo 2015), so at the beginning of incubation the brood patch temperature reaches its maximum while brood patch area is still small. Probably, the small area of the brood patch limits the temperature that the eggs receive, through a mechanical impediment, and although the brood patch temperature is high, if the brood patch area is still small the egg temperature will be low. Also, as incubation proceeded, the brood patch temperature, contrary to what happened with the brood patch area, did not vary significantly between individuals (Barrionuevo 2015).

We also did not find a relationship between egg volume and egg temperature. In Yellow-eyed Penguins (Massaro & Davis 2004) there is a positive association between egg volume and egg temperature, which is related to the position of the egg in the nest (penguins place one egg in front of the other anterior against posterior, parallel to their anterior-posterior axis). In Magellanic Penguins there is no consistently preferred position for larger eggs during incubation (Barrionuevo 2015), which may be related to the low intra-clutch dimorphism of Magellanic Penguins compared to *Eudyptes* penguins.

The incubation postures of adult Magellanic Penguins were constant during the entire incubation period: adults were lying most of the time (99%), while the egg temperature increased progressively. As we did not observe a change in incubation postures as incubation proceeded, we do not consider posture to be responsible for the delayed start of full incubation. In contrast, in the Fiordland Crested Penguin it has been shown that the incubation posture can be highly variable: 40% of the time penguins were standing (St Clair 1992), but the incubation temperature pattern is similar to that of Magellanic Penguins (Rebstock & Boersma 2011, Barrionuevo & Frere 2012). It is possible that this lack of variation in Magellanic Penguin incubation posture during the incubation

cycle is due to the presence of visual predators. In our study site there is an increasing population of Kelp Gulls *Larus dominicanus* that also breed on the island and which are the main predators of eggs and hatchlings (E. Frere, pers. comm.). Almost all penguins breed beneath bushes and some are poorly covered, so there might be an important nest predation pressure on defence behaviours: to lie down on the eggs may be a way to protect them and avoid nest predation.

We conclude that there is a relation between the delayed start of full incubation and the delayed development of the brood patch area, with both variables increasing together during the first half of the incubation period.

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