



Egg temperature and initial brood patch area determine hatching asynchrony in Magellanic penguin *Spheniscus magellanicus*

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In birds, the adaptive significance of hatching asynchrony has been under debate for many years and the parental effects on hatching asynchrony have been largely assumed but not often tested. Some authors suggest that hatching asynchrony depends on the incubation onset and many factors have been shown to influence hatching asynchrony in different species. Our objective was to analyze the exact timing of the onset of incubation and if this affects hatching asynchrony; and, in addition, which other factors (brood patch development, incubation position, adult body condition, intra-clutch egg dimorphism, laying date and year) affect hatching asynchrony in Magellanic penguins *Spheniscus magellanicus*. We first estimated the eggshell temperature at which embryo development starts, with a non-destructive and novel method. We then recorded individual egg temperatures in 61 nests during incubation, and related them, and other breeding parameters, to hatching asynchrony. We also observed incubation positions in 307 nests. We found a significant positive relationship between hatching asynchrony and the temperature that the first-laid egg experienced during egg laying and between hatching asynchrony and the initial brood patch area. We also found a negative relationship between hatching asynchrony and the difference in temperature between second and first-laid eggs within a clutch, measured after the egg-laying period was finished. We ruled out position of the eggs during incubation, adult body condition, egg volume, laying date, and study year as factors influencing hatching asynchrony. The egg temperature during laying and the difference in temperature between eggs of a clutch are determinants of hatching asynchrony in Magellanic penguins.

In birds, most species hatch their clutches of eggs asynchronously (Clark and Wilson 1981, Stoleson and Beissinger 1995), with an appreciable time lag between the hatching of the first egg and the last egg of the clutch. Birds cannot physiologically lay all the eggs of a clutch on the same day so, typically, they lay eggs with an interval of 1–2 d. The laying period lasts a few days and many birds start incubating within that period, leading to hatching asynchrony (Clark and Wilson 1981, Magrath 1990). Hatching asynchrony creates a developmental hierarchy between siblings that sometimes entails differential mortality between nestlings within nests (O'Connor 1978, Mock and Schwagmeyer 1990). In some species, asynchronous hatching produces fewer but higher quality chicks than synchronous clutches (Amundsen and Slagsvold 1991).

The adaptive significance of hatching asynchrony has been under debate for many years (Stoleson and Beissinger 1995). On one hand, it might be advantageous to start incubation during egg laying and not wait until laying is finished, so hatching asynchrony could be a by-product of an early onset of incubation. For example, the 'egg viability hypothesis' proposes that early incubation could contribute to maintaining the egg viability, selecting for mechanisms or behaviors that initiate incubation before egg laying is finished (Arnold et al. 1987). Similarly, the 'egg protection hypothesis' (Oring

1982, Bollinger et al. 1990), states that early incubation protects the eggs from dangers other than unfavourable environmental conditions (e.g. nest predators) by shortening the total time of exposure of the eggs/nestlings during a very vulnerable stage. Alternatively, hatching asynchrony could also be adaptive if size asymmetry between siblings is advantageous because it maximizes nestling survival or minimizes the costs of reproduction to the parents (see Table 1 in Stoleson and Beissinger 1995 for a detailed description of the hypotheses proposed regarding these trade-offs).

While in different species many factors have been shown to influence hatching asynchrony, no single factor has been found to explain hatching asynchrony across species. Given the many explanations for hatching asynchrony in the literature, there must be a variety of factors that regulate asynchrony. For example, hatching asynchrony can be influenced by female's body size (Bortolotti and Wiebe 1993), egg size variation between eggs of a clutch (St Clair 1996, Massaro et al. 2004), egg composition (Brown 1988), egg position during incubation (St Clair 1996), pore density of the shell (Massaro and Davis 2005, but see Boersma and Rebstock 2009), laying date (De León et al. 2001, Cresswell and McCleery 2003), and length of the foraging

trips of the adults during incubation when both parents incubate (Boersma 1990).

Most authors agree that hatching asynchrony depends on the exact time when incubation begins during the laying sequence; the earlier the incubation starts, the greater the asynchrony (Nilsson 1993, Vedder 2012). Nonetheless, only a few studies have actually tested whether the onset of incubation correlates with the degree of hatching asynchrony (Haftorn 1981, Wiebe et al. 1998, Wang and Beissinger 2009, Johnson et al. 2013), and it has been proposed that the adults incubation behavior during egg laying is the key factor affecting hatching asynchrony (Ricklefs and Smeraski 1983, Ardia et al. 2009, Rebstock and Boersma 2011).

In order to understand the adaptive significance of hatching asynchrony, we first need to study the parental effects that affect and cause hatching asynchrony. Hence, our objective in this paper is to analyze the factors that influence asynchrony and whether the incubation onset and the patterns of egg temperature during incubation influence hatching asynchrony in Magellanic penguins *Spheniscus magellanicus*. We define hatching asynchrony as the number of days that elapse between hatching of the first and last egg within clutches. Here, we study the relationship between hatching asynchrony and egg volume, egg position in the nest during incubation, brood patch development, laying date, year and the parents body condition. We use year in this study as an indicator of the environmental conditions experienced by penguins during a breeding season (Boersma and Rebstock 2009), 'bad years' result in low breeding success and 'good years' in high breeding success. To understand when incubation begins, we first estimated the eggshell temperature at which the embryo development starts, with a non-destructive and novel method in a set of nests. The temperature experienced by the embryo and at which its development starts is known as physiological zero, which is assumed to occur at temperatures $\geq 24^{\circ}\text{C}$ (Webb 1987). It has been estimated in other penguins in the genus *Pygoscelis* to be 26°C (Weinrich and Baker 1978). In this work, we did not estimate the embryo temperature, but the temperature of the eggshell when development starts. In another set of nests in the field, we measured the eggshell temperature at the surface of each egg of the clutch from the day it was laid and until a few days before hatching, and recorded in those nests the hatching asynchrony of the clutch. By taking these measurements we were able to investigate if and how variables related to egg temperature during the whole incubation span, and especially during the egg-laying period – when the onset of incubation is supposed to happen, affected hatching asynchrony. To our knowledge, this is the first study to investigate individual egg temperature, and not total nest temperature, in the context of understanding hatching asynchrony. Moreover, although it has already been established that the incubation behavior of adults influences hatching asynchrony in Magellanic penguins (Rebstock and Boersma 2011), our study analyzed and rejected other important factors previously proposed to influence asynchrony. We describe in detail how egg temperature, which is controlled by the parents, is important to the generation of asynchrony.

Methods

Study area and species

We conducted this study in Isla Quiroga, Puerto Deseado, Santa Cruz, Argentina ($47^{\circ}45'\text{S}$, $65^{\circ}53'\text{W}$). In this island there is a colony of 1500 reproductive pairs of Magellanic penguins. This species lays two eggs 3–4 d apart during the first weeks of October. Both parents alternate incubation tasks, taking each one an initial incubation bout of 15 d and then shorter shifts until hatching; females usually take the first incubation bout (Boersma et al. 1990). Egg temperatures are usually lower than 25°C during the laying period, and four days after the first egg is laid, when the clutch is complete with the laying of the second egg, the temperature reaches 26.3°C (Barrionuevo and Frere 2012). From that point onwards, egg temperature progressively increases up to the 17th day when it becomes stable at 34.2°C (Rebstock and Boersma 2011, Barrionuevo and Frere 2012). Previous studies in this species at our colony show that brood patch area increases its size as incubation proceeds, reaching its maximum and fixed size on the 10th day from the laying of the first egg. We found that this pattern of brood patch development explains the pattern of variation in egg temperature during incubation (MB unpubl.). Brood patch temperature reaches its maximum temperature and stays constant from the moment egg laying has finished and onwards (Barrionuevo and Frere unpubl.). In other northerly-located study-colonies, after 40 d of incubation, both eggs hatch with an asynchrony of 2 d (range: $-1-4$ d, Boersma et al. 1990).

General field methods

During the breeding seasons 2010–2013, we chose a random sample of 200 nests each year and checked nests daily from the beginning of October, before laying started. Of these 200 nests, we used for each experiment a different sample size. These nests were marked with flagging tape (Tapebrothers, Longwood, FL) with a unique nest number. We omitted those nests that were used in the previous years of our study, given that the same couple usually returns to the same nest every breeding season (Boersma et al. 1990). In doing this, we avoided taking repeated measurements of the same pair of breeders. Furthermore, all adults were identified with permanent metal tags with a unique number (2×10 mm; National Band and Tag Company, Newport, KY) attached to the foot webbing.

Egg incubation positions

Most penguin species normally hold their two eggs one in front of the other during incubation. In *Eudyptes* penguins the rear position is beneficial for the incubation of the eggs and directly affects hatching asynchrony (Derksen 1977, Burger and Williams 1979, St Clair 1996, Massaro and Davis 2004), so we wanted to investigate whether the same was true for Magellanic penguins. During the years 2011 and 2012, we checked 133 and 174 nests – respectively – every 4 d, starting after both eggs were laid, to determine which egg was in the rear position. On the laying day of

each egg, we marked the eggs with their order number using a waterproof marker in order to recognize them in future visits, and we measured length and width (± 0.1 mm; Vernier calipers, Mitutoyo, IL, USA) to estimate egg volume, using different formulas for first or second-laid eggs (Boersma and Rebstock 2010). We determined hatching asynchrony of 30 and 37 nests of these checked nests, in 2011 and 2012, respectively.

Egg-temperature of early embryo development

Understanding the mechanisms that affect hatching asynchrony requires knowing when the embryos start their development – it is important to know when the first-laid egg starts the process of incubation and development, and if the first-laid egg is incubated before the second. Therefore we needed to measure the physiological zero temperature in Magellanic penguins in our colony to analyze how hatching asynchrony is established. We did not, however, measure the strict physiological zero in this work because that would require destructive sampling and the end of embryo development, as we would have had to measure the temperature experienced by the embryo on the embryo itself (Weinrich and Baker 1978). Instead, we measured the temperature of the eggshell at which the embryo development started in the lab, and then we used the same method to measure the temperature on eggs at nests in which we studied the hatching asynchrony in the field, allowing us to establish when those eggs started incubation.

We based our calculation of the minimum temperature at which development starts on the fact that the yolk starts to swell around the 2nd day of effective incubation, because the yolk takes up water from the albumen (Romanoff 1960, see also Enemar and Arheimer 1980, 1989, where the usage of this method and the different stages of yolk swelling are described). We considered the swelling evidence of early development, and registered the temperature at which it occurred.

During 2011, on the laying day of the eggs, we removed 30 first-laid eggs from their nests, previously marked with the nest number, and left plaster eggs instead. We divided the eggs into three treatments in which the eggs in each group were exposed to a set incubation temperature for four days following removal from the nest: 24°C ($n = 10$ eggs), 25°C ($n = 10$ eggs) and 26°C ($n = 10$ eggs). In this way we simulated the laying period of 4 d. The eggs were placed in a Simen Premium incubator in the lab under controlled conditions. We measured the temperature of the eggs in the incubator by fixing a thermochron temperature data logger (iButton DS1921G#F50, $\pm 0.5^\circ\text{C}$, size: 17.35 \times 5.89 mm, Maxim Integrated, San José CA, US) to the shell surface between both egg poles with medical adhesive tape to one of the eggs in each treatment. We registered the egg temperature every 5 min. To maintain the humidity of the incubator, we filled a container at a constant level with tap water and we also rotated the eggs once a day, manually. To measure the yolk swelling, we took a picture of the eggs in an Ovoscope, especially designed to trans-illuminate penguin eggs (for more information see Barrionuevo and Frere 2014), on the laying day of each egg, and 4 d later after being exposed to the different tem-

peratures. Then, with the computer program Image Pro-Plus 4.5 we measured total egg area and yolk area for day 0 and day 4. For each egg we estimated the proportion of the total egg area occupied by the yolk (yolk area/total egg area), and used the difference of the proportion between day 0 and day 4 as a swelling index. All these eggs were returned to the original nests and hatched successfully, if they were not predated during incubation. On these nests we did not perform any further manipulation or observation other than checking if the eggs normally hatched. This technique allowed us to establish the temperature at which the embryo development started without destroying the eggs.

Egg volume, egg temperature, adults body condition and hatching asynchrony

During the breeding seasons 2010–2013, we checked 61 nests daily. On the laying day of each egg, we marked the eggs with their laying-order number (E1 = first laid egg, E2 = second laid egg), estimated egg volume (Boersma and Rebstock 2010), and added a thermochron temperature data logger (iButton) to the shell surface between both egg poles with medical adhesive tape; this data logger registered egg temperature every 15 min. We removed the iButton 34 d after the laying day to prevent hatching failures (for more details see Barrionuevo and Frere 2012). Although the iButton was attached to one side of the egg only, and the temperature recorded might have fluctuated with the rotation of the egg, in a previous study we showed that the temperature on opposite sides of the egg did not vary significantly (in the study we attached two iButtons to the same egg on opposite sides and recorded the temperature with both iButtons, Barrionuevo and Frere 2012). Moreover, the average temperature recorded was the same all around the egg, although it was higher when the iButton was in contact with the patch and lower when in contact with the ground. Many other methods that are used to measure the egg temperature record the temperature of the embryo with more precision than the iButtons (Webb 1987, Weathers and Sullivan 1989, Hepp 2004, Niizuma et al. 2005), but are not useful for our objectives because they interfere with the size of the clutch and/or do not allow the development of the embryo by using fake or unviable eggs, so we would not have been able to record egg temperature in both eggs at each nest.

From the egg temperature data, we extracted the following variables to analyze their influence on hatching asynchrony: $T_{E2} - T_{E1}$ = difference in the average egg temperature of second (E2) and first (E1) laid eggs from the end of the laying period until the 34th day of incubation. T_{E1} = average egg temperature of first-laid egg during the laying period. $p \geq F0^\circ\text{C}$ = proportion of time that the temperature of first eggs exceeded the temperature estimated in the item: egg-temperature of early embryo development.

On the laying day of the second egg and when the males returned from the sea to take their incubation bout, we estimated female and male body condition, respectively. We measured length and depth of bill with a caliper (± 0.1 mm), and length of foot and flipper with a ruler (± 1 mm), and we weighed them using a spring balance (± 25 g; Pesola AG, Baar, Switzerland) (Yorio et al. 2001). We did a PCA (principal component analysis) on all the size measurements and

we regressed the adults weight on the principal component from the PCA. We used the residuals of this regression as the estimates of body condition.

In order to establish hatching asynchrony – number of days elapsed between hatching of the first and second egg within clutches – we started checking the nests daily 35 d after the laying of the first egg. We registered the date of hatching of each egg at the nests we sampled.

Brood patch

The brood patch may affect the temperature that the eggs receive, because the temperature of the patch determines the heat that the eggs receive, and the size of the patch determines how many eggs can be effectively incubated (Lea and Klandorf 2002). The initial patch temperature and area only affect the first egg, as this is the only egg at the nest during the three to four days following its laying. Then, when the second egg is laid, the patch area and temperature affect both eggs and, at this point, they would have no effect on the hatching asynchrony, as both eggs are experiencing the same patch conditions.

In 19 nests of the 61 previously mentioned, we measured the brood patch of the adults during 2012 and 2013 ($n = 9$ nests and $n = 10$ nests, respectively). On the laying day of the first egg, we measured the adults' patch area and temperature. We measured its width with calipers (± 0.1 mm), stretching the patch and measuring it at the widest point, and we measured its length with a ruler (± 1 mm). We estimated patch area as: $(\text{length} \times \text{width})/2$ (St Clair 1992). We also registered patch temperature with an electronic thermometer ($\pm 0.1^\circ\text{C}$; Pro-check, CABA, Argentina), placing the thermometer in direct contact to the skin of the bottom part of the patch, wrapping the thermometer with the same skin of the patch to impede contact with the air. To register the temperature we waited until the electronic thermometer stabilized. We only measured the female's patch, as usually females take the first incubation bout (Boersma et al. 1990).

Incubation onset experiment

We evaluated the effect of the temperature during egg laying on hatching asynchrony through a manipulative experiment in which we deprived eggs of the parents' heat. We also analyzed how different laying intervals affect the incubation span. We defined the incubation span as the days elapsed between laying and hatching, although no strict incubation during egg laying might occur. During 2010–2012, as part of another experiment that would not be used in this work in which we manipulated the incubation onset of eggs, we stored first-laid eggs in a plastic container at ambient temperature, from the day they were laid up to 3 or 4 d, mimicking the laying period found in Magellanic penguins. We covered the plastic container with a white cardboard, which prevented the eggs from being exposed to the sun, but did not alter their exposure to the ambient temperature. When the second egg was laid, we returned the eggs to the nest; at this point of the laying-incubation period the egg temperature in eggs under natural conditions is above 26°C (Barrionuevo and Frere 2012), so we assume the eggs

returned to their nests started their development immediately. We compared the incubation span of these stored first-laid eggs with the incubation span of first-laid eggs of control nests in which the eggs were not removed from their nests and the incubation temperature during the laying period was not the ambient temperature but the temperature that parents transferred to the eggs. We obtained the hourly ambient temperature from the National Weather Center (Puerto Deseado Station – 2.5 km from our study site in Isla Quiroga) and we averaged the temperature for each day.

Data analysis

To evaluate if there exists a preferential incubation position between eggs of a clutch and if it is related to hatching asynchrony we ran a generalized linear model with Poisson distribution and log link function, for data collected on 2011–2012. Hatching asynchrony was the response variable and the predictor variables were: the proportion of visits in which the first laid egg was found in the anterior position, the ratio between egg volume of second and first laid eggs of a clutch, and the interactions between both variables.

To analyze which variables affect hatching asynchrony we ran two generalized linear models with Poisson distribution and log link function. In both the response variable was the hatching asynchrony. In one model a) ($n = 61$ nests) the predictor factor was the year (entered as a four level factor: 2010, 2011, 2012 and 2013), and the continuous variables were: the difference between the egg volume of the second minus the first-laid egg (from now on: intra-clutch egg dimorphism), female and male body condition, laying date of the first-laid egg (standardized as the deviation of the median laying date of each year), and the variables previously defined: $T_{E2} - T_{E1}$ (difference in the average egg temperature of E2 and E1 from the end of the laying period until the 34th day of incubation), T_{E1} (average egg temperature of E1 during the laying period), and $p \geq F0^\circ\text{C}$ (proportion of time that the temperature of E1 exceeded 26°C during egg laying).

For the other model b) ($n = 19$ nests) we used hatching asynchrony as the response variable and the predictor variables were: T_i and A_i (initial patch temperature and area, respectively – measured when the first egg was laid).

There was no need to correct any model for overdispersion (Zuur et al. 2009) and in all models we used a backwards selection procedure removing the terms one by one according to a decreasing p value and comparing the models with and without the eliminated variable with an ANOVA test using the goodness-of-fit chi-squared test (χ^2 parameter). We used the χ^2 parameter because as the data followed a Poisson distribution, the chi-squared test is the most appropriate (Crawley 2007). The output of the ANOVA shows the residual deviance of each model (the one with and the other without the variable analyzed), the deviance (the difference between these residual deviances) and the p-value. We reported the deviance and the p-value.

For the models analyzed we used the package 'stats' from the R software ver. 2.12.1 (R Development Core Team) and the code 'glm'. We report values as mean \pm SE and considered differences to be significant at $p < 0.05$. All graphs were plotted using Sigma Plot 10.0 (Systat Software).

Results

Egg incubation positions

During 2011 the first-laid egg was found to be in the anterior position in 0.51 ± 0.02 proportion of the total visits ($n = 133$ nests), while in 2012 this proportion was 0.50 ± 0.01 ($n = 174$ nests). In 2011 the ratio second-laid egg volume/first-laid egg volume was 1.028, and in 2012 it was 1.025, meaning that the second-laid eggs were larger than the first (paired t -test first vs second laid egg; 2011: $t_{132} = -3.42$, $p = 0.0008$; 2012: $t_{173} = -4.23$, $p < 0.0001$).

There was no significant relationship between hatching asynchrony and the proportion of time that first eggs spent in the anterior position (Deviance = 0.39, DF = 65, $p = 0.53$), nor did we find a significant relationship between hatching asynchrony and the ratio of second-laid egg volume and first-laid egg volume (Deviance = 0.0028, DF = 64, $p = 0.99$), and the interaction between both variables (Deviance = 0.96, DF = 63, $p = 0.33$).

Egg-temperature of early embryo development

In the treatments at 24°C and 25°C, the yolk area/total egg area relationship between the day_0 and day_4 of incubation did not increase significantly (24°C: \bar{x} yolk area/total egg area $_{day0} = 0.752$, SD = 0.059, \bar{x} yolk area/total egg area $_{day4} = 0.785$, SD = 0.047, paired t -test yolk area/total egg area $_{day0}$ vs yolk area/total egg area $_{day4}$: $t = -1.93$, $p = 0.09$, $n = 10$ eggs; 25°C: \bar{x} yolk area/total egg area $_{day0} = 0.808$, SD = 0.019, \bar{x} yolk area/total egg area $_{day4} = 0.811$, SD = 0.047, paired t -test yolk area/total egg area $_{day0}$ vs yolk area/total egg area $_{day4}$: $t = -0.15$, $p = 0.88$, $n = 10$ eggs). But in the experiment at 26°C we found an increased in the yolk area within those 4 d (26°C: \bar{x} yolk area/total egg area $_{day0} = 0.763$, SD = 0.025, \bar{x} yolk area/total egg area $_{day4} = 0.798$, SD = 0.034, paired t -test yolk area/total egg area $_{day0}$ vs yolk area/total egg area $_{day4}$: $t = -3.74$, $p = 0.005$, $n = 10$ eggs). So, we assume that the egg-shell temperature at which the swelling of the yolk occurs is somewhere between 25°C and 26°C, we will be referring to this temperature as 26°C.

Variables affecting hatching asynchrony

Throughout the study, hatching asynchrony averaged 1.80 ± 0.13 d ranging from 0 to 4 d, and the laying interval was on average 3.82 ± 0.06 d ranging from 3 to 4 d. During egg laying, the temperature of the first-laid egg was above or equal to 26°C for $35 \pm 2\%$ of the time (Fig. 1). The average egg temperature of first-laid egg during laying (day 0) was 22.50 ± 0.36 °C, much lower than 26°C (Fig. 1). There was one egg that spent 4.5% of the time above 26°C and another that spent 91% of the time above 26°C. These eggs reached on average 15.17°C and 30.66°C during laying and had an asynchrony of 1 and 3 d, respectively. Only 4 eggs (from 122 eggs analyzed) had average temperatures during egg laying above 26°C and the asynchrony of these nests ranged from 2 to 4 d.

There was a significant and positive relationship between the temperature of first-laid eggs during egg laying and hatching asynchrony, and a significant and negative relationship between the egg temperature difference between both eggs of the clutch (recorded from the end of the laying period

Table 1. Generalized linear model with Poisson distribution and logit link function of Magellanic penguins' *Spheniscus magellanicus* hatching asynchrony against fixed variables. The final significant model and all the non-significant variables analyzed are shown ($n = 61$ nests).

	Variables	z	DF	p
Final model	T_{E1} ^a	2.64	1,56	0.005
	$T_{E2} - T_{E1}$ ^b	-2.78	1,56	0.008
		Deviance	DF	p
Non-significant	Intra-clutch egg dimorphism ^c	0.205	1,54	0.651
	Male body condition	0.002	1,52	0.968
	Year	0.649	3,51	0.885
	$p \geq F0^\circ C$ ^d	0.152	1,55	0.696
	Female body condition	0.075	1,53	0.785
	Laying date	-0.001	1,48	0.981

^aAverage egg temperature of first-laid egg (E1) during the laying period.

^bDifference in average egg temperature of second (E2) minus first (E1) eggs from the end of the laying period until the 34th day of incubation.

^cDifference between the egg volume of the second minus the first-laid egg.

^dProportion of time that temperature of first egg is $\geq 26^\circ C$ during the laying period.

until the 34th day of incubation) and hatching asynchrony (Table 1). These significant variables explained 26.89% of the variation in hatching asynchrony.

We found that when the clutch hatched synchronously (in this group we include clutches hatching synchronously and with one day asynchrony - 0/1) the average temperature of the first egg during laying was lower ($\bar{x}_{egg\ temperature} = 20.82 \pm 0.51^\circ C$) than when the asynchrony was 2, 3 or 4 d ($\bar{x}_{egg\ temperature} = 23.52 \pm 0.42^\circ C$) (Tukey HSD test 0/1 vs 2/3/4: $p = 0.0004$, DF = 56; Fig. 2a). Breaking down the laying period, the temperature of the first egg was lower at those nests with synchronous hatching (0/1) compared with the nests with asynchronous hatching (2/3/4), on all days, except for days 3 and 4 of the interval of 4 d, (0/1: $\bar{x}_{day0} = 20.82 \pm 0.52^\circ C$, $\bar{x}_{day1} = 18.05 \pm 0.92^\circ C$, $\bar{x}_{day2} = 18.13 \pm 0.70^\circ C$, $\bar{x}_{day3} = 22.06 \pm 0.87^\circ C$, $\bar{x}_{day4} = 24.48 \pm 0.91^\circ C$; 2/3/4: $\bar{x}_{day0} = 23.52 \pm 0.42^\circ C$, $\bar{x}_{day1} = 21.12 \pm 0.68^\circ C$, $\bar{x}_{day2} = 21.61 \pm 0.59^\circ C$, $\bar{x}_{day3} = 24.34 \pm 0.51^\circ C$, $\bar{x}_{day4} = 26.21 \pm 0.65^\circ C$) (two-sample t -test 0/1 vs 2/3/4: $p_{day0} = 0.0002$, $p_{day1} = 0.0085$, $p_{day2} = 0.0004$, $p_{day3} = 0.0183$, $p_{day4} = 0.1233$, the Bonferroni adjusted level of significance is $p = 0.1$; Fig. 3).

If we evaluate the rate of increase in egg temperature of the first laid egg on the day the second egg is laid (measured as the slope of the line of the regression of egg temperature against hour of the day), we found that those first-laid eggs that were kept at lower temperatures during egg laying (lower T_{E1}) had a faster warming on the laying day of the second egg than those eggs that were kept at higher temperatures (warming rate of $E1 = 15.4162 - 0.5276 \times T_{E1}$, $R = -0.29$, $p = 0.02$; Fig. 4). All eggs reached the temperature of the second-laid egg on the day following the laying of the second egg.

We also found that, when the eggs hatched more synchronously the temperature difference between the second and first-laid eggs during incubation was higher than when the eggs hatched more asynchronously (i.e. the second egg had higher temperature than the first, Table 1, Fig. 2b). The hatching asynchrony progressively decreased as second eggs reached higher temperatures than first eggs. We found

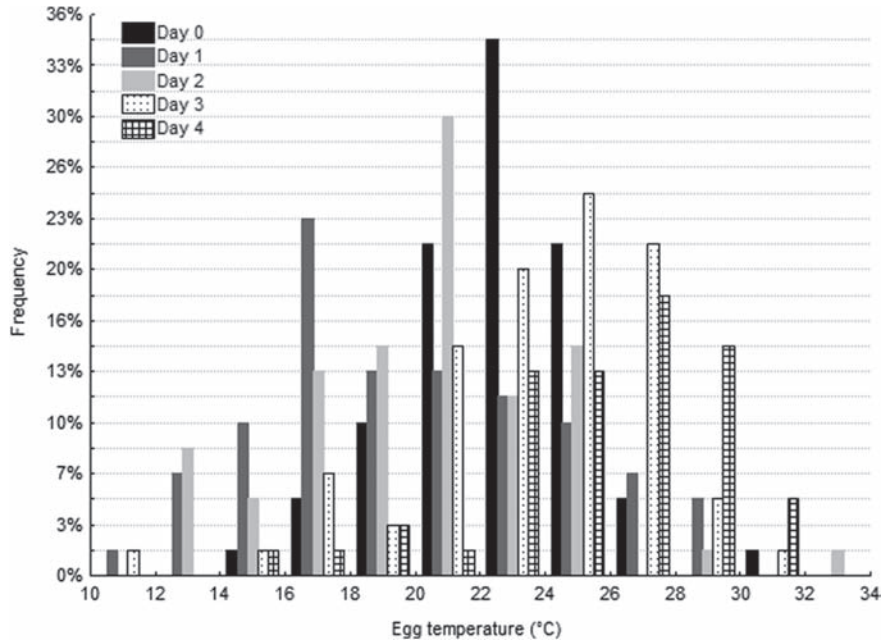


Figure 1. Frequency histogram of the temperature of first-laid eggs during the laying period. Each day of the laying period from the laying of the first egg (day 0) until the laying of the second egg (day 4) is represented here by a different pattern. We recorded every 15 min the temperature of 61 eggs.

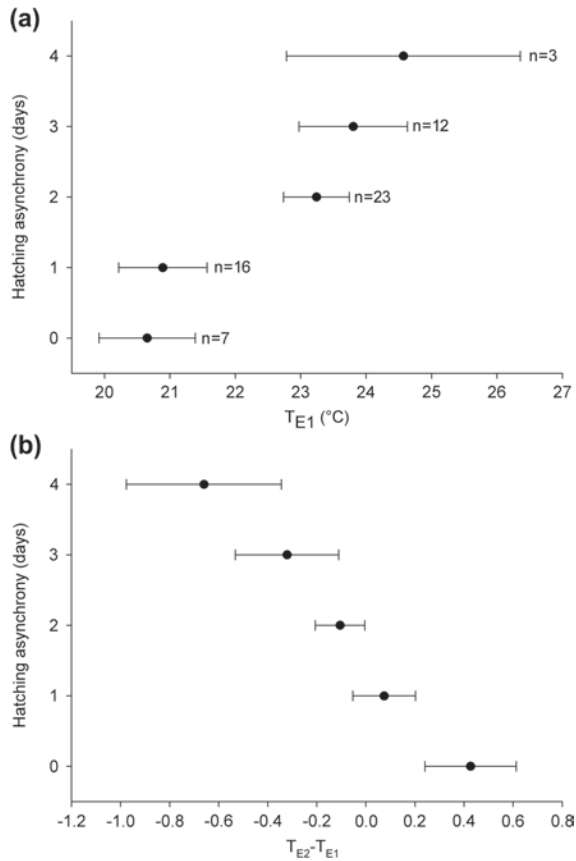


Figure 2. (a) Average egg temperature of first-laid egg during the laying period (T_{E1}) and (b) difference in average egg temperature of second (E2) and first (E1) eggs from the end of the laying period until the 34th day of incubation ($T_{E2} - T_{E1}$), with respect to hatching asynchrony. Negative values in the x-axis show higher temperatures for E1 and positive values higher temperatures for E2. Mean \pm SE and sample size of nests (n) are shown.

significant differences in egg temperature during incubation between hatching asynchronies: 0 vs 3 d ($p = 0.02$), and 4 d ($p = 0.04$) (Tukey HSD test, Fig. 2b).

All other variables analyzed in the model (i.e. proportion of time spent $\geq 26^\circ\text{C}$, intra-clutch egg dimorphism, laying date, year and adults body condition) did not affect hatching asynchrony (Table 1).

The mean initial brood patch area was $12.83 \pm 3.01 \text{ cm}^2$, and the mean initial brood patch temperature was $35.35 \pm 1.285^\circ\text{C}$ (means \pm SD). The egg temperature (T_{E1}) and the initial brood patch area were correlated ($R^2 = 0.32$, $F_{1,17} = 7.41$, $p = 0.015$), but we found no significant relation between the egg temperature and the brood patch temperature

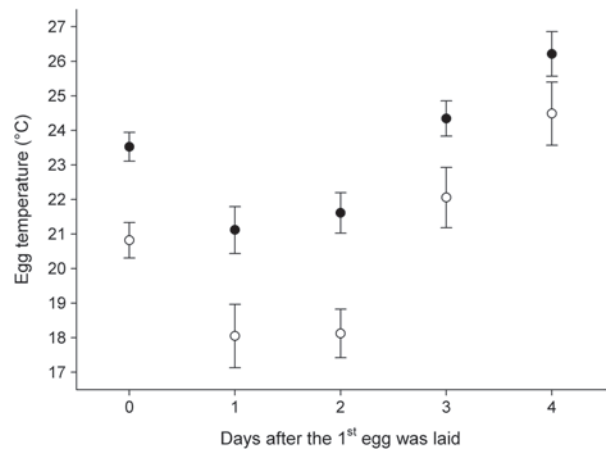


Figure 3. Open circles represent the egg temperature of first-laid eggs during the laying period, since the first egg was laid (day 0) and until the second egg was laid (day 4), where the asynchrony was of 1 d or less. The filled black circles show the temperature of the first-laid egg over time since laying, where hatching asynchrony was 2 d or more. Mean \pm SE are shown.

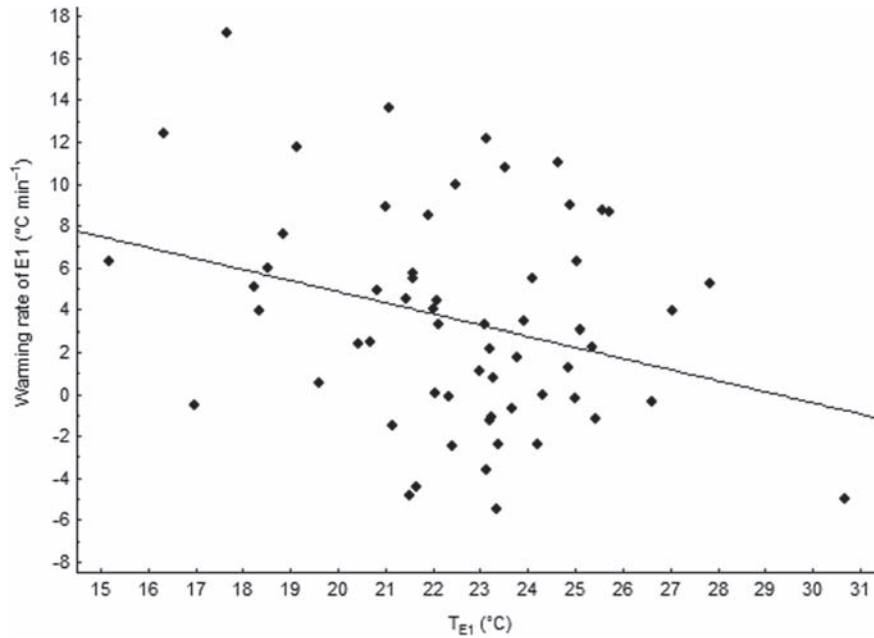


Figure 4. Relation between the warming rates of the first-laid egg on the last day of the laying period – when the second egg is laid – and the mean temperature of the first-laid egg during the laying period. The rate is calculated for each nest analyzed ($n = 61$) as the slope of the linear relation between the temperature and the hour of the day.

($R^2 = 0.21$, $F_{1,17} = 0.71$, $p = 0.41$). In the model in which we analyzed the effect of brood patch development on asynchronous hatching we found a significant and positive relationship between patch area and hatching asynchrony (Table 2). When the asynchrony was of 3 and 4 d the initial patch area was larger ($\bar{x}_{\text{patch area}} = 15.36 \pm 0.78 \text{ cm}^2$) than when the hatching asynchrony was of 0, 1 and 2 d ($\bar{x}_{\text{patch area}} = 11.66 \pm 0.76 \text{ cm}^2$) (Fig. 5). The initial patch area explained 27.58% of the variation in hatching asynchrony. Initial patch temperatures did not affect asynchrony (Table 2).

Incubation onset experiment

We compared the incubation span of first-laid eggs stored during 3 or 4 d at ambient temperature (i.e. eggs that did not receive any parental heat transfer during the laying period) with the incubation span of control eggs left at the nest untouched, that may have received some heat transfer by the adults, and that also had a laying period (LP) of 3 or 4 d. The ambient temperature during the days in which the eggs were stored was on average $11.51 \pm 0.17^\circ\text{C}$

Table 2. Generalized linear model with Poisson distribution and logit link function of Magellanic penguins' *Spheniscus magellanicus* hatching asynchrony against fixed variables. In this model we analyzed the effect of brood patch development. The final significant model and all the non-significant variables analyzed are shown ($n = 19$ nests).

	Variables	z	DF	p
Final model	A_i^a	2.02	1,18	0.04
Non-significant	T_i^b	-0.64	1,17	0.52

^aInitial brood patch area – measured when first egg was laid.

^bInitial brood patch temperature.

(maximum temperature = 23.5°C , minimum temperature during daylight = 1.4°C) and the eggs stored for 3 and 4 d were exposed to the same ambient temperature because both intervals were performed simultaneously. We found that the eggs incubated by the adults hatched earlier than the eggs stored at ambient temperature (t -tests incubation span of control vs stored eggs: LP = 3, $t_{65} = 8.85$, $p < 0.0001$; LP = 4, $t_{166} = 12.09$, $p < 0.0001$; Table 3). Eggs stored for 3 d hatched earlier than eggs stored for 4 d (t -test incubation span stored eggs for 3 vs 4 d: $t_{110} = 5.15$, $p < 0.0001$; Table 3), we found this same pattern in control nests (t -test LP = 3 vs LP = 4: $t_{120} = 4.01$, $p < 0.0001$; Table 3). Despite these significant differences in the incubation span between eggs with 3 and 4 d of laying period, there was no significant differ-

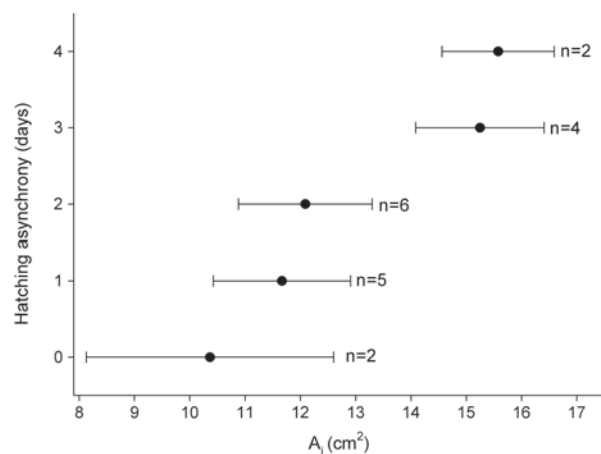


Figure 5. Initial patch area, measured when the first egg was laid (A_i), with respect to the hatching asynchrony. Mean \pm SE and sample size of nests (n) are shown.

Table 3. Incubation span (days elapsed since laying and until hatching) of first-laid eggs of Magellanic penguins *Spheniscus magellanicus* for those eggs in which their clutch was laid within 3 or 4 d (laying period) and that were normally incubated by their parents (control eggs) or were stored at ambient temperature during 3 or 4 d and then returned to the nests. The mean (\bar{x}) \pm SD and sample size (n) for each treatment is shown.

Laying period	Control eggs	Stored eggs
3	$\bar{x} = 39.95 \pm 1.13$ n = 19	$\bar{x} = 42.31 \pm 0.93$ n = 49
4	$\bar{x} = 41.13 \pm 1.86$ n = 104	$\bar{x} = 43.22 \pm 0.92$ n = 64

ence in the average egg temperature during the laying period in the control nests (\bar{x} egg temperature_{LP=3} = 22.77°C, SD = 3.96, \bar{x} egg temperature_{LP=4} = 21.99°C, SD = 4.28, t-tests: $t_{286} = -1.33$, $p = 0.18$).

Discussion

Although it was already known that adult behavior during incubation determines hatching asynchrony of nestlings in Magellanic penguins (Rebstock and Boersma 2011), in this study we explored the factors influencing hatching asynchrony in greater depth and we were able to identify how asynchrony is established by the egg-temperature patterns during incubation. We were able to discard many factors that could possibly influence asynchrony (incubation position, adult body condition, intra-clutch egg dimorphism, laying date and year). But we found that the higher the egg temperature of the first egg during laying and the larger the brood patch on the laying day of the first egg, the greater the hatching asynchrony. In contrast, we also found that the larger the difference in egg temperature between second- and first-laid eggs, the smaller the hatching asynchrony.

The eggshell temperature at which we found a swelling of the yolk (as an index of the onset of embryo development) for Magellanic penguins was the same as the physiological zero found in other penguin species (26°C) (Weinrich and Baker 1978), which is higher than in other avian species (Webb 1987). The novelty of our finding is in the method, which allowed us to look deeper into the study of egg temperatures and their effects on hatching asynchrony without destroying the eggs. It is interesting to point out, however, that the temperature of 24°C caused a greater swelling of the yolk when compared with the temperature of 25°C (although both were not significant). Also, we found that in asynchronous nests the average temperature during laying for first eggs was between 23°C and 26°C, while synchronous eggs had temperatures below 23°C. This indicates that some development of the embryo may be occurring at 23°C, possibly at the level of a cell division that does not result in changing the yolk appearance. We were not able to capture this development with our experiment, but we believe that if we had dissected eggs, we would have found no signs of embryonic development, since Weinrich and Baker (1978) in their work with penguins, and dissecting eggs, got the same onset temperature of embryonic development as we did. Increasing the sample size, controlling the treatments by the yolk size and enlarging the range of temperatures

analyzed would help narrow down this methodology, which would be very helpful in future studies because of the ethical implications of this non-destructive method.

We were able to rule out the position of the eggs during incubation as a source of variation in hatching asynchrony because, first, we did not find differences in the incubation position of first and second-laid eggs, and second, we found no effect of the positions on hatching asynchrony. Data on which position is thermally beneficial in penguins are contradictory, but most studies found differences in incubation positions between eggs (Derksen 1977, Burger and Williams 1979, St Clair 1992, 1996, Massaro and Davis 2004). These studies are based on *Eudyptes* penguins, which have an egg size dimorphism for second eggs that is 1.2–1.7 times the egg volume of the first egg (Lamey 1990). Probably, this high dimorphism is responsible for the difference in incubation position between eggs found in *Eudyptes*. Magellanic penguins, on the other hand, have only a 2% egg volume dimorphism (Boersma and Rebstock 2010, Barrionuevo and Frere 2014), and this could be the reason why we did not find differences in the incubation position and, therefore, no effect on hatching asynchrony.

In our study we found that hatching asynchrony in Magellanic penguins is not influenced by the environmental and parental factors analyzed: the variable that would indicate possible differences in the food abundance from one season to the other (year), the body condition of the parents, the laying date, and the intra-clutch egg size dimorphism were not related to hatching asynchrony. We need to call attention to two of these factors. First, during the study years the breeding success in the colony Isla Quiroga and nearby colonies was high (2010 = 1.16 fledglings/nests, 2011 = 1.14 fledglings/nests, 2012 = 0.64 fledglings/nests, 2013 = 0.89 fledglings/nests, Barrionuevo and Frere unpubl.). Therefore, none of our study years was too ‘bad’, as has been shown to be common in Magellanic penguins in other colonies (Boersma et al. 1990), to see if variation in food abundance could affect hatching asynchrony. Second, the lack of significance of the intra-clutch dimorphism could lie in the small size difference between eggs. Nonetheless, De León and coauthors (2001) observed in other penguin species that, while the mean egg volume difference between both eggs of the clutch was not significant, hatching asynchrony increased with within-clutch egg size asymmetry.

We found that the key to understanding how hatching asynchrony is established depends on the temperature at which the first-laid egg is kept during egg laying: the higher the temperature, the greater the hatching asynchrony. A 3°C difference in temperature of first-laid eggs can affect the asynchrony period from 0/1 to 2/3/4 d. Those eggs kept at temperatures below 23°C do not start to develop until the second egg is laid, resulting in synchronous nests, while those eggs kept above 23°C may experience some embryonic development and develop at a rate proportional to the temperature received during egg laying, resulting in asynchronous nests.

Another issue that supports the idea that the temperature at which the first egg is kept during the laying period can have an effect on the development and may influence hatching asynchrony is that the rewarming period of the first egg is very quick and, thus, cannot be responsible of the hatching

asynchrony found. Gwynn (1953) and Lack (1968) speculated that, as the first egg is not effectively incubated in *Eudyptes* penguins (similar to what we found with our yolk-swelling experiment), a rewarming period of those eggs when incubation starts, would cause the first egg to hatch later than expected. We have already found that, the next day following the laying of the second egg, all the first-laid eggs reached a temperature similar to the temperature of these second-laid eggs (Barrionuevo and Frere 2012) – so the rewarming period in our study is short, and we found that eggs exposed to low temperatures during egg laying had higher warming rates than those exposed to high temperatures.

The difference in incubation span between eggs kept in the nests and those experimental ones kept at ambient temperature shows that, during the laying period, adults clearly transfer some heat to the eggs that start development. This difference translates to approximately 2 d, which is the mean hatching asynchrony found in Magellanic penguins. Also, the shorter incubation span found in eggs with a laying period of 3 d with respect to those with 4 d, might be because in the shorter laying period the eggs start to be incubated earlier than in the longer period (the second egg is laid earlier and effective incubation begins sooner). This is consistent with the difference in incubation span found of almost one day.

In our colony, we found initial brood patch area to be positively related to egg temperature during egg laying and, therefore, also related to hatching asynchrony. We had already found that egg temperature is determined by patch development (Barrionuevo and Frere unpubl.), so this result was one we expected. Nonetheless, the initial patch temperature was not related to egg temperature or to hatching asynchrony, maybe because the temperature did not vary between nests so drastically as the area did.

We also found that the difference in egg temperature between eggs of the same clutch significantly affected the hatching asynchrony: when second-laid eggs received more heat than first eggs the asynchrony period was shorter. We were not expecting to find this significance because we did not find differences in the incubation positions (this work) or in the egg temperatures (Barrionuevo and Frere 2012) between both eggs. But it seems that no matter how small the egg temperature difference between the eggs was, it was enough to generate a difference in hatching asynchrony.

Although it had been previously proposed that adult incubation behavior in Magellanic penguins controls hatching asynchrony (Rebstock and Boersma 2011), we have shown through this study that the temperature that the eggs receive is the main factor determining asynchrony. This temperature is controlled by the development of the brood patch, and not by the incubation postures (Barrionuevo and Frere unpubl.). So, the brood patch, which is known to be influenced by hormones (Jones 1971, Lea and Klandorf 2002), is what controls hatching asynchrony in Magellanic penguins. Furthermore, the egg-temperature difference within a clutch is key to hatching asynchrony. To conclude, we did not find any factor other than egg temperature that may control hatching asynchrony. All variables related to food abundance or parents' body condition were dismissed as factors influencing asynchrony. Hatching asynchrony seems to be determined by a constraint of the brood patch development

during the laying period that influences egg temperature of first-laid eggs.

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