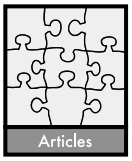

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Intra-clutch pattern of albumen $\delta^{13}C$ and $\delta^{15}N$ in yellow-legged gulls *Larus michahellis*: female dietary shift or resource allocation strategy?

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Energy or nutritional constraints associated to female dietary shifts during the clutch production period may play a role in generating intra-clutch egg size variation in yellow-legged gulls *Larus michahellis*. To explore this possibility, we determined albumen $\delta^{13}C$ and $\delta^{15}N$ values in three-egg clutches (modal clutch size) from three different breeding episodes: Ebro Delta 2004 and 2006, and Columbretes Islands 2004. Rather than a shift in females' diet, consistent intra-clutch patterns of variation in egg size and albumen isotopic values (particularly in the case of albumen $\delta^{13}C$, which values held constant throughout the laying sequence) pointed to an intrinsic mechanism as the most feasible cause for the relatively smaller size of third/last-laid eggs. However, diet ''quality'' for breeding females seemed to affect intra-clutch egg size variation. In particular, a deficit of specific nutrients for egg formation associated to refuse scraps exploitation (as suggested by depleted albumen isotopic values) likely resulted in the more apparent intra-clutch egg size profile for the Ebro Delta 2004. In the absence of dietary shifts, the observation of consistently higher $\delta^{15}N$ values for third-albumens suggested a greater contribution of endogenous resources to their synthesis, as conversion of stored reserves into egg proteins results in greater isotopic fractionation, thereby yielding enriched isotopic signatures (particularly for $\delta^{15}N$ that shows greater isotopic fractionation with respect to that commonly assumed for $\delta^{13}C$). We point to reabsorbed material derived from the hormonally-mediated regression of the female reproductive system (which is likely the intrinsic mechanisms resulting in the intra-clutch pattern of egg size variation: the hormonal hypothesis) as the most feasible endogenous source of nutrients for the synthesis of last-laid eggs, as optimize reproductive investment and maximize female fitness.

Historically, avian life-history research strongly focused on the chick rearing phase, whereas earlier stages of the breeding cycle, such as egg formation, were largely neglected (Navarro and González-Solís 2007, but see Monaghan et al. 1998). This was the result of considering that the peak in demand for breeding occurs when parents are delivering food to their dependent young. However, it now becomes increasingly clear that egg formation is as demanding as chick rearing (reviewed by Nager 2006), thereby being potentially traded against competing components of animals' fitness such as self-maintenance. On the other hand, the amount and quality of resources allocated to eggs profoundly influence the growth and survival of the progeny (Mousseau and Fox 1998), thus strongly affecting maternal fitness. Indeed, several studies have shown a positive relationship between egg size and offspring fitness (Christians 2002, Krist 2011). Understanding resource acquisition and allocation to egg formation is therefore crucial for our full comprehension of avian life-history strategies.

Currently, we have some understanding of the major physiological and hormonal mechanisms underlying egg formation (e.g., Sockman et al. 2000, 2006 Williams et al. 2001), but little is known about how this process in wild birds might be energetically or nutritionally constrained. Previous studies directed to address this issue have mainly focused on the relationship between energy/nutrient availability for breeding females and clutch/egg size (Nager 2006). However, the effect of such constraints on determining the intra-clutch pattern of egg size variation where egg size typically declines with laying sequence in many birds (Slagsvold et al. 1984, Leblanc 1987, Reid 1987), has received minor attention (but see Hargitai et al. 2005).

The intra-clutch egg size pattern of variation in most avian species has been related to the common hormonal control regulating the onset of incubation (which usually takes place before clutch completion) and the regression of the female's reproductive system (leading to a decline in the size of those eggs formed currently with such regression; the hormonal hypothesis, e.g., Leblanc 1987, Sockman et al. 2000). However proximate energy/nutritional constraints on the laying female have been also put forward as possible explanations for such pattern of egg

size variations (see Hargitai et al. 2005). In particular, depletion of stored reserves of laying females (Houston et al. 1983, Pierotti and Bellrose 1986), increasing restrictions in females' foraging options associated to the early onset of the incubating behaviour (Bollinger et al. 1990, Bollinger 1994), or daily changes in food resources exploited by laying females (Perrins 1970) may also play a fundamental role in generating intra-clutch egg-size variation. Tracing nutrient acquisition and allocation to particular eggs within the laying sequence may improve our comprehension of potential factors constraining egg production. Fortunately, this is now feasible through application of naturally occurring isotopic tracers, such as the stable isotopes of carbon ($^{13}C/^{12}C$, expressed as $\delta^{13}C$) and nitrogen ($^{15}N/^{14}N$, expressed as $\delta^{15}N$; reviewed by Hobson 2006).

As most other gull species (Laridae), the yellow-legged gull *Larus michahellis* typically shows a modal clutch size of three eggs, with the last-laid egg being significantly smaller than the first two (Reid 1987). Given its great feeding plasticity and opportunistic foraging behaviour, this species is particularly likely to shift its diet so as to adapt its daily dietary intake to the most abundant/available local food sources (Ramos et al. 2009, Ramos et al. 2011). As this species mainly rely on local nutrient sources for egg formation (Ramírez et al. 2010), energy or nutritional constraints associated to dietary shifts incurred by breeding females during the clutch production period may play a role in generating intra-clutch egg size variation. We explored this possibility through a bivariate isotopic approach. In particular, we determined albumen $\delta^{13}C$ and $\delta^{15}N$ values in three-egg clutches (modal size of the clutches) of yellow-legged gulls for three different breeding episodes: Ebro Delta 2004 and 2006, and Columbretes Islands 2004. Albumens were selected as suitable material for the present study since they are synthesized during non-overlapping short time periods (Ruiz et al. 2000), and are therefore potentially affected by different dietary inputs. We predicted that any dietary shift occurred during the clutch production period should be reflected in variations of the intra-clutch pattern of albumen isotopic signatures.

Methods

Study are and sampling strategy

In order to evaluate the effect of resource use on the intra-clutch patterns of egg size and albumen isotopic signatures, this study was conducted during the 2004 breeding season at two gulleries with marked differences in their feeding habits: the Ebro Delta and the Columbretes Is., both located on the Spanish Mediterranean coast. The Ebro Delta gullery is located on the Peninsula de la Banya (40° 40′N, 0° 45′E), a protected area of salt marshes near human settlements, whereas the Columbretes Is. (39° 54′N, 0° 41′E) are a small volcanic archipelago which lies 55 km off the coast. One of the area's most important fishing fleets operates near to both colonies (between 39° 50′N and 41° 00′N, and 0° 35′E and 1° 30′E), and fishery discards from this activity are exploited by individuals of both colonies (Arcos et al. 2001). However, whereas the gull population on the Columbretes Is. feeds almost exclusively on this marine resource, the diet of Ebro Delta gulls also includes a lesser proportion of terrestrial resources, mainly meat waste coming from refuse dumps (Ramos et al. 2009, Ramos et al. 2011). Inter-annual variations in the intra-clutch profiles of gulleries were also evaluated by sampling eggs at the Ebro Delta in 2006. The great diversity of potential dietary resources in this locality makes this gullery particularly prone to inter-annual dietary shifts (Ramos et al. 2009, Ramos et al. 2011).

During peak egg laying of different breeding episodes, nests were tagged when the first egg was laid and then inspected daily until clutch completion in order to ascertain final clutch size and laying order. Fresh, newly-laid eggs (<24 h after laying) were sampled and replaced by dummy eggs, marked and measured (maximum length and width) with a digital calliper to the nearest mm, placed under refrigeration and transported to the laboratory pending preparation for stable isotope determination.

Laboratory procedures

Eggs were boiled to allow separation of yolks and albumens. Albumens were lyophilized to constant mass and ground to powder. Subsamples of powdered material were then weighed to the nearest μg and placed into tin capsules for carbon and nitrogen isotope ratio determination. Isotopic analyses were carried out at the Serveis Científico-Tècnics of the University of Barcelona (Spain) by means of a Thermo-Finnigan Flash 1112 (CE Elantech, Lakewood, NJ, USA) elemental analyser coupled to a Delta-C isotope ratio mass spectrometer via a CONFLOIII interface (Thermo Finnigan MAT, Bremen, Germany), with IAEA standards being applied every 12 samples to calibrate the system. Stable isotope ratios were expressed in the standard δ notation relative to Vienna Pee Dee Belemnite ($\delta^{13}C$) and atmospheric N_2 ($\delta^{15}N$). Replicate assays of standards indicated analytical measurement errors of $\pm 0.1‰$ and $\pm 0.2‰$ for $\delta^{13}C$ and $\delta^{15}N$, respectively.

Statistical analyses

Egg volume (hereafter egg size) was estimated as $KV \times \text{length} \times \text{width}^2$, where $KV = 0.485$ (Ruiz et al. 1998). Generalized linear mixed models (GLMM) with normal link functions were used to fit egg size, albumen $\delta^{13}C$ and albumen $\delta^{15}N$. A set of competing models was built by considering laying order (first-, second- and third/last-laid egg) and breeding episode (Columbretes Is. 2004, Ebro Delta 2004 and Ebro Delta 2006) as fixed effects; an individual random effect (i.e., nest effect) was always included in the models so as to account for the dependence among eggs laid by the same female. Overall variability among nests is the result of intrinsic biological variability among females plus the variability induced by particular conditions prevailing in a breeding episode. Thus, we considered models including both common and different random effects (i.e., interaction between random nest effect and breeding episode). Residual variance (i.e., variability not explained by the effects included in fitted models) may be homogeneous or not, and it is potentially heterogeneous among eggs or breeding episodes. Models

accounting for such heterogeneities were also considered. Model selection was based on the Akaike information criterion corrected for small sample sizes (AIC_C, Johnson and Omland 2004) and the corresponding AIC_C weights. Posterior pairwise comparisons between groups were made using Hochberg's approach (Hochberg 1988) so as to maintain the overall type I error at 0.05. Statistical analyses were performed using SAS 9.1 (SAS Institute Inc., Cary, NC, USA) and SPSS 15.0 (SPSS Inc., Chicago, USA) software.

Results

The model selected to fit egg size (Table 1) showed this variable to be related to all considered factors. Total variance, i.e., the sum of random nest and residual variances, was clearly higher in Columbretes Is. 2004. In addition, the random nest component accounted for more than half the total variability in egg size, regardless of the breeding episode considered (Table 2). In relation to fixed effects, last-laid eggs were consistently smaller than the first two, although the intra-clutch patterns of egg size variation were slightly different among the three breeding episodes (as revealed by the interaction between laying order and breeding episode). In particular, clutches from Ebro Delta 2004 showed the most apparent intra-clutch profile, with significant differences between the three eggs, whereas egg sizes for the first two eggs were not significantly different in the Columbretes Is. 2004 and Ebro Delta 2006 samples (Table 3, Fig. 1A).

With respect to albumen $\delta^{13}C$, the best-supported model (Table 1) included a random nest effect, which accounted for about half the observed variability. Residual heterogeneity among breeding episodes was only weakly supported. In terms of fixed effects, breeding episode had a marked effect and all the pairwise comparisons showed significant differences (Table 3). Estimated parameters showed the lowest $\delta^{13}C$ values in the Ebro Delta 2004 sample ($-20.3‰$), followed by Columbretes Is. 2004 ($-19.3‰$) and Ebro Delta 2006 ($-18.9‰$) (Table 3, Fig. 1B).

In relation to albumen $\delta^{15}N$ values, the best-supported model (Table 1) showed an interaction between the random nest factor and breeding episode, i.e. random nest effect was different among sites and years. Moreover, the model indicated that residual variance was greater in the Ebro Delta 2004. As regards fixed effects, the results supported main effects of laying order and breeding episode (Table 2). The detected laying order effect was such that the value of the $\delta^{15}N$ signature for the last-laid egg (11.18‰) was significantly higher than those for the first- (10.8‰) and second-laid eggs (10.9‰). Moreover, overall $\delta^{15}N$ values for Ebro Delta 2004 (10.5‰) were consistently lower than those for Columbretes Is. 2004 (11.2‰) or Ebro Delta 2006 (11.2‰, Table 3, Fig. 1C).

Discussion

Egg size variation is highly consistent within individuals and shows a high repeatability and heritability (reviewed by Christians 2002). Several female characteristics, including body size, age or experience have slight positive influences on egg size in most species, as do some other intrinsic characteristics of females such as that ones ascribed to the physiological system specific of egg production (e.g., oviduct mass, Christians 2002). Accordingly, our data showed that more than half of the egg size variability was related to the random nest factor, which may account for the above mentioned female intrinsic traits as well as for the influence of corresponding mates (e.g., through behaviours like courtship feeding).

Regardless of the variability detected in the overall egg size among individuals (as suggested by the random nest factor), and despite observed inter-colony and inter-annual differences in gulls' diets (as revealed by detected differences in the overall isotopic composition of their albumens), the intra-clutch egg size pattern stood for all the breeding episodes being the last-laid egg consistently smaller than the first two. In our view, this consistency points to an intrinsic mechanism (likely the hormonal-mediated regression of female reproductive system) as the most feasible cause for the establishment of such profile. However, slight differences in the intra-clutch patterns of egg size variation were detected among breeding episodes, with clutches from the Ebro Delta 2004 showing the greatest egg size differences according to laying sequence. In turn, this breeding episode showed the most depleted albumen isotopic values, suggesting a greater reliance of breeding females on refuse scraps (whose isotopic values are lower than those of marine resources; Boutton 1991, Hebert et al. 1999, Ramos et al. 2009). Due to the poorer ''quality'' of this food resource with respect to the common marine ones (Bertellotti et al. 2001), a deficit of specific nutrients for egg formation associated with garbage exploitation might contribute to the more apparent intra-clutch pattern of egg size variation observed for this breeding episode (see Bolton et al. 1992). In addition, the greater influence of female intrinsic traits when explaining albumen $\delta^{15}N$ variability (i.e., the greater random nest effect) suggested that the lack of specific nutrients for egg formation might be partially compensated for by a greater contribution of females' endogenous resources (Bolton et al. 1992, Nager 2006).

In addition to the observed isotopic differences among breeding episodes, the most salient feature of our results was the consistent intra-clutch isotopic profile based on the relatively enriched $\delta^{15}N$ values for last-laid eggs. According to our a priori expectations, observed intra-clutch pattern of $\delta^{15}N$ values could be the result of a dietary shift incurred by females during the clutch production period. Owing to its opportunistic foraging behaviour, this species may shift its diet from common marine preys towards more available and predictable food resources, such as refuse scraps, according to its foraging options and linkage to nests (see Ramos et al. 2011). The early onset of the incubation behaviour (which is effective after the second egg is laid, Parsons 1972) and the consequent restrictions in female' foraging options may cause, therefore, a shift in females' diet towards this poorer quality food resource before clutch completion, thereby constraining third-laid egg production. However, a greater reliance of laying females on refuse scraps after the onset of incubation should result in depleted $\delta^{15}N$ values for third albumens, which clearly contrast with

Table 1. Models evaluated to fit egg size (volume, cm^3) and albumen isotopic values ($\delta^{13}C$ and $\delta^{15}N$, ‰), and their corresponding Akaike's information criterion corrected for small sample sizes (AIC_C), Akaike increments (ΔAIC_C) and weights ($AIC_{C\ W}$). Fixed factors considered were breeding episode (BE), laying order (LO) and its interaction. Female effect (nest) and its interaction with BE were also evaluated, as well as common or heterogeneous residual terms among BE or LO. Total number of parameters estimated in each model is denoted by k. Asterisks denote selected model for each variable.

Fixed terms	Variance terms			Egg size (cm^3)			$\delta^{15}N$ (‰)			$\delta^{13}C$ (‰)		
	Nest	Residual	k	AIC_C	ΔAIC_C	$AIC_{C\ W}$	AIC_C	ΔAIC_C	$AIC_{C\ W}$	AIC_C	ΔAIC_C	$AIC_{C\ W}$
Constant	Common	Common	3	1156.7	132.1	$< 10^{-7}$	336.1	53.7	$< 10^{-7}$	362.3	46.3	$< 10^{-7}$
LO	Common	Common	5	1074.8	50.2	$< 10^{-7}$	319.9	37.5	$< 10^{-7}$	368.4	52.4	$< 10^{-7}$
BE	Common	Common	5	1149.5	124.9	$< 10^{-7}$	313.8	31.4	$< 10^{-7}$	316.0	0.0	0.3336*
LO, BE	Common	Common	7	1068.2	43.6	$< 10^{-7}$	297.2	14.8	0.0003	322.0	6.0	0.0166
LO,BE, LOxBE	Common	Common	11	1039.9	15.3	0.0004	299.0	16.6	0.0001	326.1	10.1	0.0021
constant	Common	By LO	5	1130.4	105.8	$< 10^{-7}$	334.4	52.0	$< 10^{-7}$	366.2	50.2	$< 10^{-7}$
LO	Common	By LO	7	1072.6	48.0	$< 10^{-7}$	319.9	37.5	$< 10^{-7}$	372.2	56.2	$< 10^{-7}$
BE	Common	By LO	7	1123.2	98.6	$< 10^{-7}$	310.9	28.5	$< 10^{-6}$	318.3	2.3	0.1056
LO, BE	Common	By LO	9	1065.8	41.2	$< 10^{-7}$	296.6	14.2	0.0004	324.1	8.1	0.0058
LO,BE, LOxBE	Common	By LO	13	1040.0	15.4	0.0003	298.6	16.2	0.0002	328.1	12.1	0.0008
constant	Common	By BE	5	1158.8	134.2	$< 10^{-7}$	320.5	38.1	$< 10^{-7}$	363.3	47.3	$< 10^{-7}$
LO	Common	By BE	7	1059.9	35.3	$< 10^{-7}$	308.8	26.4	$< 10^{-6}$	369.1	53.1	$< 10^{-7}$
BE	Common	By BE	4	1151.7	127.1	$< 10^{-7}$	296.0	13.6	0.0006	316.7	0.7	0.2351
LO, BE	Common	By BE	9	1053.2	28.6	$< 10^{-6}$	284.2	1.8	0.2104	322.3	6.3	0.0143
LO,BE, LOxBE	Common	By BE	13	1024.6	0.0	0.7496*	296.6	14.2	0.0004	326.3	10.3	0.0019
constant	By BE	Common	5	1159.1	134.5	$< 10^{-7}$	319.8	37.4	$< 10^{-7}$	358.8	42.8	$< 10^{-7}$
LO	By BE	Common	7	1077.6	53.0	$< 10^{-7}$	303.0	20.6	$< 10^{-4}$	364.8	48.8	$< 10^{-7}$
BE	By BE	Common	7	1152.0	127.4	$< 10^{-7}$	304.4	22.0	$< 10^{-4}$	317.4	1.4	0.1657
LO, BE	By BE	Common	9	1071.1	46.5	$< 10^{-7}$	287.2	4.8	0.0469	323.4	7.4	0.0082
LO,BE, LoxBE	By BE	Common	13	1043.1	18.5	0.0001	289.1	6.7	0.0182	327.4	11.4	0.0011
constant	By BE	By LO	7	1133.9	109.3	$< 10^{-7}$	319.9	37.5	$< 10^{-7}$	360.1	44.1	$< 10^{-7}$
LO	By BE	By LO	9	1075.7	51.1	$< 10^{-7}$	302.9	20.5	$< 10^{-4}$	365.8	49.8	$< 10^{-7}$
BE	By BE	By LO	9	1126.8	102.2	$< 10^{-7}$	303.8	21.4	$< 10^{-4}$	320.5	4.5	0.0352
LO, BE	By BE	By LO	11	1069.0	44.4	$< 10^{-7}$	287.8	5.4	0.0348	326.3	10.3	0.0019
LO,BE, LOxBE	By BE	By LO	15	1043.4	18.8	0.0001	289.7	7.3	0.0134	330.2	14.2	0.0003
constant	by BE	By BE	7	1161.9	137.3	$< 10^{-7}$	310.9	28.5	$< 10^{-6}$	360.2	44.2	$< 10^{-7}$
LO	by BE	By BE	9	1061.4	36.8	$< 10^{-7}$	298.1	15.7	0.0002	365.9	49.9	$< 10^{-7}$
BE	by BE	By BE	9	1154.9	130.3	$< 10^{-7}$	295.6	13.2	0.0007	319.2	3.2	0.0673
LO, BE	by BE	By BE	11	1055.1	30.5	$< 10^{-6}$	282.4	0.0	0.5175*	324.9	8.9	0.0039
LO,BE, LOxBE	by BE	By BE	15	1026.8	2.2	0.2495	284.8	2.4	0.1559	328.9	12.9	0.0005

Table 2. Parameter estimates for the GLMM models selected to fit egg size (volume, cm^3) and albumen isotopic signatures ($\delta^{15}N$ and $\delta^{13}C$, ‰). For fixed effects reference categories were breeding episode of Ebro Delta 2006 and 3rd-laid egg.

Egg size (cm^3) Fixed parameters	Estimate ± SE	Variance parameters	Estimate	95% CI
Intercept	75.46 ± 1.61	Common nest effect	32.173	(21.90, 51.86)
Columbretes Is. 2004	2.08 ± 2.51	Columbretes Is. 2004 residual	32.088	(21.23, 54.10)
Ebro Delta 2004	−1.46 ± 2.11	Ebro Delta 2004 residual	7.851	(5.24, 13.07)
Ebro Delta 2006	Reference	Ebro Delta 2006 residual	12.018	(7.80, 20.89)
1st-egg	8.17 ± 1.19			
2nd-egg	5.71 ± 1.19			
3rd-egg	Reference			
1st-egg Columbretes Is. 2004	−3.50 ± 2.26			
1st-egg Ebro Delta 2004	4.37 ± 1.50			
2nd-egg Columbretes Is. 2004	−0.45 ± 2.26			
2nd-egg Ebro Delta 2004	3.37 ± 1.50			
$\delta^{15}N$ (‰) Fixed parameters	Estimate ± SE	Variance parameters	Estimate	95% CI
Intercept	11.43 ± 0.10	Columbretes Is. 2004 nest effect	0.008	(<0.01, 0.10)
Columbretes Is. 2004	−0.03 ± 0.11	Ebro Delta 2004 nest effect	0.204	(0.10, 0.70)
Ebro Delta 2004	−0.70 ± 0.15	Ebro Delta 2006 nest effect	0.088	(0.04, 0.47)
Ebro Delta 2006	Reference	Columbretes Is. 2004 residual	0.136	(0.09, 0.25)
1st-egg	−0.37 ± 0.08	Ebro Delta 2004 residual	0.348	(0.24, 0.55)
2nd-egg	−0.28 ± 0.08	Ebro Delta 2006 residual	0.151	(0.10, 0.27)
3rd-egg	Reference			
$\delta^{13}C$ (‰) Fixed parameters	Estimate ± SE	Variance parameters	Estimate	95% CI
Intercept	−18.90 ± 0.13	Common nest effect	0.224	(0.14, 0.41)
Columbretes Is. 2004	−0.38 ± 0.19	Common residual	0.225	(0.17, 0.30)
Ebro Delta 2004	−1.45 ± 0.17			
Ebro Delta 2006	Reference			

the observed intra-clutch isotopic pattern. In addition, the consistency of the intra-clutch pattern in $\delta^{15}N$ values among breeding episodes would imply similar dietary shifts for two years and at two different breeding sites, which makes such dietary shift an unlikely explanation for the observed isotopic trend (particularly at the Columbretes Is.,

Table 3. Mean least squares estimation, standard errors and 95% CI for egg size (volume, cm^3) and albumen isotopic signatures ($\delta^{15}N$ and $\delta^{13}C$, ‰). In agreement with selected models, values for egg size are shown for each combination of laying order and breeding episode; albumen $\delta^{15}N$ for each factor separately; and albumen $\delta^{13}C$ for each breeding episode (model selection is shown in Table 1). Asterisks denote significant differences between eggs (1st-2nd, 2nd-3rd, 3rd-1st, respectively) and superindexes homogeneous breeding episodes (results from post-hoc contrasts with Hochberg correction; Hochberg 1988).

Egg size (cm^3)	1st-egg			2nd-egg			3rd-egg		
	Mean ± SE	95% CI		Mean ± SE	95% CI		Mean ± SE	95% CI	
Columbretes Is. 2004	82.21 ± 1.89	(78.46, 85.96)		82.80 ± 1.89	(79.06, 86.55)	*	77.54 ± 1.93	(73.72, 81.36)	
Ebro Delta 2004	86.54 ± 1.29	(83.98, 89.10)	*	83.08 ± 1.29	(80.52, 85.64)	*	74.00 ± 1.36	(71.29, 76.70)	*
Ebro Delta 2006	83.63 ± 1.61	(80.43, 86.82)		81.17 ± 1.61	(77.97, 84.37)	*	75.46 ± 1.61	(72.26, 78.65)	*
$\delta^{15}N$ (‰)	Mean ± SE	95% CI		Mean ± SE	95% CI		Mean ± SE	95% CI	
	10.81 ± 0.07	(10.67, 10.95)		10.91 ± 0.07	(10.77, 11.05)	*	11.18 ± 0.07	(11.04, 11.32)	*
Columbretes Is. 2004 [1]	11.18 ± 0.06	(11.06, 11.29)							
Ebro Delta 2004 [2]	10.51 ± 0.12	(10.28, 10.74)							
Ebro Delta 2006 [1]	11.21 ± 0.09	(11.03, 11.39)							
$\delta^{13}C$ (‰)	Mean ± SE	95% CI							
Columbretes Is. 2004 [1]	−19.28 ± 0.13	(−19.54, −19.02)							
Ebro Delta 2004 [2]	−20.35 ± 0.11	(−20.57, −20.12)							
Ebro Delta 2006 [3]	−18.90 ± 0.13	(−19.16, −18.63)							

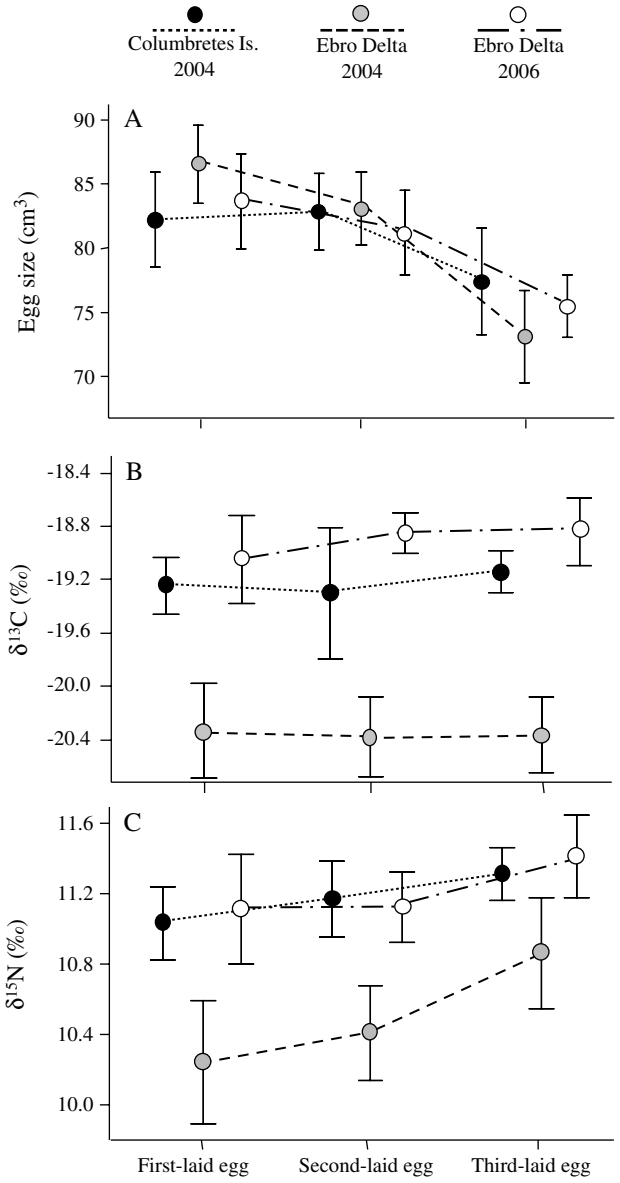

Figure 1. Intra-clutch pattern of variation for egg size (volume, cm^3) and albumen $\delta^{13}C$ and $\delta^{15}N$ values (A, B and C, respectively) in yellow-legged gull three-egg clutches (modal size of the clutches) for three different breeding episodes: Columbretes Is. 2004 (n = 18), and Ebro Delta 2004 (n = 24) and 2006 (n = 17). Values are mean and 95% CI.

the isolation of which restricts gulls' diet to marine resources, Ramos et al. 2011). Furthermore, the observation of relatively constant albumen $\delta^{13}C$ values throughout the laying sequence also argued against any dietary shift for laying females.

Observed pattern in albumen $\delta^{15}N$ values could be attributed to a metabolic process affecting the mobilization of endogenous resources and leading to temporal shifts in the isotopic signatures of animal tissues (see Wiley et al. 2010). According to this idea, light isotopes (^{12}C or ^{14}N) are mobilized first from substrate reservoirs, thereby initially yielding isotopically lighter products. As substrate reservoirs diminish, heavy isotopes (^{13}C or ^{15}N) are transferred and the isotopic signature of the product increases in value. Thus, if endogenous protein stores were used up during clutch production, the relatively higher $\delta^{15}N$ values for third albumens could be the result of a nearly complete depletion of stored reserves and the consequent greater transfer of the heavy isotope (^{15}N) to last-laid eggs. However, this argument does not seem feasible since the contribution of endogenous resources for yellow-legged gull seems to be minor (Ramírez et al. 2010, Saino et al. 2010), although a partial contribution of this metabolic process to the intra-clutch pattern of albumen $\delta^{15}N$ cannot be completely ruled out.

In our view, the observed intra-clutch pattern in albumen $\delta^{15}N$ is more likely due to a greater contribution of endogenous resources to the synthesis of last-laid eggs. Conversion of stored reserves to egg proteins results in extra isotopic fractionation thereby yielding enriched isotopic signatures of eggs formed through a capital process (Gauthier et al. 2003, Hobson 2006). Accordingly to our isotopic results, the greater contribution of endogenous resources to the synthesis of last-laid eggs should result in enriched isotopic values particularly in the case $\delta^{15}N$, which shows greater isotopic fractionation (~3.1‰) with respect to that commonly assumed for $\delta^{13}C$ (~0.9‰; Hobson 1995, Hobson 2006). The idea that birds catabolise flight muscle for egg formation has a widespread support (e.g., Houston et al. 1995a). However, mobilization of material from the pectoral muscle may in itself be a relatively costly process and cause a negative effect on female foraging performance (Monaghan et al. 1998 and references therein, but see Houston et al. 1995a). In this context, re-utilizing body tissues that are no longer going to be used otherwise should be considered as a more beneficial reproductive strategy, since minimizing the costs of clutch production means maximizing individual fitness. In this regard, reabsorbed material derived from the hormonally-mediated regression of the female reproductive system could be considered a more feasible endogenous source of nutrients for the synthesis of last-laid eggs. To the best of our knowledge, Houston et al. (1995b) were the first in proposing the allocation of this reabsorbed material to egg synthesis in zebra finches *Taeniopygia guttata*. However, this was criticised by Williams and Ames (2004), who argued that the oviduct top-down regression in this species starts shortly after the passage of the last-laid egg. The latter was supported by the fact they found no decrease in absolute or relative mass of shell and albumen for the later-laid eggs. Contrastingly, the relative smaller size of the third-laid egg in yellow-legged gulls has been related to a lesser amount of albumen (an oviduct-dependent egg component) allocated to last-laid eggs (yolk size is rather constant throughout the laying sequence, Ruiz et al. 1998). Thus, the hormonally-mediated regression of female reproductive systems (including oviduct) in gulls might start shortly before the synthesis of last-laid albumens likely resulting in the typical intra-clutch pattern of egg size variation and allowing the inclusion of this reabsorbed material into third albumens.

Conclusions

Rather than a shift in females' diet, observed isotopic trends throughout the laying sequence suggested an intra-clutch pattern of resource allocation in yellow-legged gulls. In particular, the higher $\delta^{15}N$ values observed for third albumens might be attributed to a greater reliance on endogenous resources, likely derived from the regression of the female reproductive system, which follows the onset of incubation and probably results in the relatively smaller size of last-laid eggs. Observed intra-clutch patterns of variation in albumen isotopic values and egg size could be therefore ascribed to the common hormonal control regulating the onset of incubation and the regression of the female reproductive system. However, the "quality" of diet for breeding females may also play a role in generating the intra-clutch pattern of egg size variation. In particular, a deficit of specific nutrients for egg formation associated with dietary exploitation of refuse scraps may contribute to the more apparent intra-clutch pattern of egg size variation observed for the Ebro Delta 2004, and might be partially compensated for by a greater contribution of endogenous resources. Further isotopic studies including the isotopic composition of potential endogenous endpoints (such as flight muscle, ovary or oviduct biopsies) will provide more reliable insights into the actual role of stored reserves for clutch production. We encourage such isotopic research as it represents a feasible way in which information on resource allocation to egg production can be derived at a relatively low cost and effort.

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