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Life-history evolution at the molecular level: adaptive amino acid composition of avian vitellogenins

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Avian genomes typically encode three distinct vitellogenin (VTG) egg yolk proteins (VTG1, VTG2 and VTG3), which arose by gene duplication prior to the most recent common ancestor of birds. Analysis of VTG sequences from 34 avian species in a phylogenetic framework supported the hypothesis that VTG amino acid composition has co-evolved with embryo incubation time. Embryo incubation time was positively correlated with the proportions of dietary essential amino acids (EAAs) in VTG1 and VTG2, and with the proportion of sulfur-containing amino acids in VTG3. These patterns were seen even when only semi-altricial and/or altricial species were considered, suggesting that the duration of embryo incubation is a major selective factor on the amino acid composition of VTGs, rather than developmental mode alone. The results are consistent with the hypothesis that the level of EAAs provided to the egg represents an adaptation to the loss of amino acids through breakdown over the course of incubation and imply that life-history phenotypes and VTG amino acid composition have co-evolved throughout the evolutionary history of birds.

1. Introduction

Because the approximately 10 000 species of birds all lay eggs yet differ markedly in such aspects of reproductive life history as clutch size and developmental pattern, birds have provided important model organisms for theoretical and empirical studies of life-history evolution [1–7]. Avian developmental patterns occupy a continuum from precocial (in which young are able to move and feed themselves upon hatching) to altricial (in which young are initially helpless) [8]. Precocial species generally have longer embryo incubation times than altricial species; but even among altricial and semi-altricial species, there is substantial variation in the length of the incubation period [9]. The selective factors favouring longer embryo incubation times in altricial and semi-altricial species have been the subject of much debate [9–12]; but whatever the selective factors at work, avian developmental patterns represent a trade-off between provisioning the young through energy and nutrients provided to the developing embryo through yolk and provisioning the young through feeding after hatching [13].

Avian eggs contain fat and water in excess of the embryo's needs, but not excess protein [14]. The egg yolk proteins known as vitellogenins (VTGs) are the primary source of amino acids incorporated into structural and non-structural proteins translated by the embryo [15,16]. VTGs are synthesized in the maternal liver in response to oestrogen and selectively taken up by developing oocytes, where the mature polypeptide is cleaved into three polypeptides: lipovitellin, the highly phosphorylated phosvitin, and a smaller peptide, which is variously named depending on the VTG [17]. In producing VTGs for inclusion in egg yolk, the breeding female must make use of essential amino acids (EAAs) which neither she nor the embryo can synthesize and which therefore must originate either in the female's dietary intake or in mobilization from sources such as maternal protein [18–21].

Because VTGs and other egg proteins are the only source for EAAs used in all proteins synthesized by the embryo prior to hatching and because precocial

Figure 1. Phylogenetic tree shows the relationships of the 34 avian species from which the VTG1, VTG2 and VTG3 sequences were analysed. The tree is based on genome-scale data and replotted from [26], with additional species [27–30]. Embryo incubation times (in days post hatch) are mapped (colour-coded) along the branches of the tree by parsimony reconstruction [31]. (Online version in colour.)

species hatch at a more fully developed state than altricial species, natural selection is predicted to favour more extensive provision of EAAs in VTGs of precocial species. Moreover, the incorporation of both absorbed and recycled amino acids in proteins is an inevitably inefficient process, with an estimated efficiency of about 70–80% in growing domestic chickens [22]. The inefficiency of amino acid recycling results from the fact that breakdown of amino acids occurs continuously in animals, regardless of their nutritional state, because the enzymes involved in breakdown of amino acids are constitutively expressed [23]. Given that the pool of EAAs available to the embryo is fixed, the inefficiency of amino acid incorporation will have a cumulative effect over the course of the incubation period. As a consequence, if two species hatch at the same level of development but differ in the duration of embryo incubation, the embryo will have a greater total requirement for EAAs during incubation in the species with a longer embryo incubation time. Therefore, even among altricial species, selection is predicted to favour an increased provisioning of EAAs in VTGs of species with longer incubation periods.

Taking advantage of the recent availability of multiple avian genome sequences [24], I test these predictions by examining the evolution of amino acid composition of VTGs across the phylogeny of birds. I test for an effect of embryo incubation time independent of developmental mode by analysing subsets of the data consisting of species with semi-altricial and/or altricial development. I focus both on the 10 amino acids generally considered dietary essentials (EAAs) and, in addition, on the two sulfur-containing amino acids, methionine (M) and

cysteine (C). M is considered a dietary essential and can be used in the synthesis of C, yet if C is present in sufficient quantities it is said to have a ‘sparing’ effect on the dietary requirement for M [25].

2. Methods

(a) Statistical methods

Analysis of amino acid composition involved sequences from 34 avian species (figure 1) for which complete VTG1, VTG2 and VTG3 sequences were available (electronic supplementary material, table S1 and figure S1). All sequences analysed included domains homologous to the serine-rich phosvitin domain and the von Willebrand factor type D domain of chicken VTG2, as annotated in Swiss-Prot accession P02845 (electronic supplementary material, figure S1). Published phylogenetic studies have suggested that the avian VTG genes arose by duplications that occurred prior to the most recent common ancestor of birds [32,33]. Phylogeny-based statistical analyses assumed species relationships following the phylogenetic hypothesis illustrated in figure 1, based largely on the genomic phylogeny of Jarvis *et al.* [26], with relationships of passerine families derived from other sources [27–30]. Data on body mass and embryo incubation time are summarized in the electronic supplementary material, table S1. Mean body mass values for each species were obtained from Dunning [34]. When mean values for males and females were given separately, the mean of those two means was used. For one species in the sample (*Tauraco erythrolophus*), data were not available in Dunning [34]. For the latter species, I used the mid-point of the body mass range provided by del Hoyo *et al.* [35]. Data

on the duration of embryo incubation were obtained from del Hoyo *et al.* [35] and other literature [36,37]. When a range of incubation times was given, the midpoint was used. Because no information was available for *Manacus vitellinus*, the value for the closely related *Manacus manacus* was used. Based on data from del Hoyo *et al.* [35], the developmental mode of each species was placed in standard categories [38] across the precocial–altricial spectrum (electronic supplementary material, table S2). In statistical analyses, body mass and incubation times were natural-log-transformed to improve linearity.

VTGs showed high frequency of S (serine), mostly located in poly-S repeats of highly variable length, most (but not all) of which were in found in the region homologous to the chicken phosvitin domain (electronic supplementary material, figure S1). Because S showed the highest frequency of all amino acids in every VTG sequence analysed, frequencies of S were compared among VTG1, VTG2 and VTG3; and percentages of other amino acids were expressed as percentages of non-S residues. All reported residue percentages were based on the mature protein, excluding the signal peptide. The following 10 amino acid residues were classified as dietary EAAs: F (phenylalanine), H (histidine), I (isoleucine), K (lysine), L (leucine), M (methionine), R (arginine), T (threonine), V (valine) and W (tryptophan). All of these except H are considered indispensable amino acids for birds, because the animal cannot synthesize them; H is also included, because synthesis occurs at a low level, insufficient for dietary needs [16]. Additional analyses involved the two sulfur-containing amino acids, M and C (cysteine).

In analyses of amino acid composition in a phylogenetic framework, the percentage of essential amino acids (%EAA) and the percentage of M and C (%M + C) in the mature protein sequence were correlated with log body mass and log incubation time using phylogenetically independent contrasts (PIC) estimated by the PDAP method [39] in MESQUITE v. 2.75 [31], assuming the phylogeny in figure 1 and arbitrary branch lengths. For each variable used in PIC, the correlation between the absolute values of contrasts and their standard errors was used to test for adequacy of standardization [39]; no significant correlations were found. Partial correlations among PIC were estimated without fitting an intercept [40]. Based on pruned phylogenetic trees, PIC were estimated separately for (i) altricial and semi-altricial species ($n = 24$); and (ii) altricial species only ($n = 20$).

I also conducted statistical analyses not taking into account phylogenetic relationships (designated below as ‘conventional statistical analyses’). These analyses used randomization tests (which do not require the assumption of independence). In each randomization test, 10 000 pseudo-datasets were created by sampling with replacement from the data. The relevant test statistic was computed on each of the pseudo-datasets; and the test statistic computed on the actual data was compared with the distribution of values obtained from the pseudo-datasets. The test statistics used in randomization tests were the correlation coefficient and the paired *t*-statistic (for comparisons between different VTG subfamilies). All reported *p*-values are two-tailed. Means are reported $\pm$ s.e.m.

3. Results

(a) Amino acid composition

S was the most abundant amino acid in VTGs, mainly in the form of long poly-S repeats in the phosvitin domain (electronic supplementary material, figure S1). Mean %S was significantly higher in VTG1 ($15.02 \pm 0.13\%$) than in VTG3 ($10.03 \pm 0.06\%$; $p < 0.001$; randomization test; figure 2a). Likewise, mean %S was significantly higher in VTG2 ($13.44 \pm 0.24\%$) than in VTG3 ($p < 0.001$; randomization test;

Figure 2. Plots of amino acid composition variables in VTG1 (open circles) and VTG2 (filled circles) versus VTG3: (a) %S; (b) % essential amino acids; (c) %M + C. In each case, the line is a 45° line.

figure 2a). Of the amino acids other than S, the 10 EAAs constituted a significantly greater mean percentage of residues in VTG1 ($54.04 \pm 0.11\%$) than in VTG3 ($52.23 \pm 0.09\%$; $p < 0.001$; randomization test; figure 2b). Likewise, the mean %EAA was significantly higher in VTG2 ($53.62 \pm 0.10\%$) than in VTG3 ($p < 0.001$; randomization test; figure 2b). By contrast, mean %M + C was significantly greater in VTG3 ($4.46 \pm 0.07\%$) than in VTG1 ($2.57 \pm 0.09\%$) or VTG2 ($2.65 \pm 0.04\%$; $p < 0.001$ in each case; randomization tests; figure 2c).

The highest %EAA among VTG1 sequences in the dataset was that of *Tauraco erythrolophus*, with 874 EAA out of 1578 non-S residues (55.4%). The lowest %EAA among VTG1 sequences in the dataset was that of *Leptosomus discolor*, with 828 EAA of 1590 non-S residues (52.1%). Although the difference between the highest and lowest percentages was just 3.3%, this corresponded to a difference of 46 EAA residues per VTG1 molecule. Similarly, the highest %EAA among VTG2 sequences was that of *Caprimulgus carolinensis*, with 857 EAA out of 1575 non-S residues (54.4%), whereas the lowest %EAA among VTG2 sequences was that of *Zonotrichia albicollis*, with 817 EAA of 1569 non-S residues (52.0%). In this case, the difference between the highest and

Figure 3. (a) %EAA versus log incubation time for VTG1 (filled circles and solid regression line; $r = 0.469$; $p < 0.001$; randomization test) and VTG2 (open circles and dashed regression line; $r = 0.482$; $p < 0.001$; randomization test). (b) %M + C in VTG3 versus log incubation time with linear regression line ($r = 0.492$; $p < 0.004$; randomization test).

lowest percentages was only 2.4%, but this corresponded to a difference of 40 EAA per VTG2 molecule. Thus, given the large number of amino acid residues in VTG1 and VTG2, modest percentage differences nonetheless amount to substantial differences in residue numbers per molecule.

The lowest %M + C in VTG3 was 4.6% in *Corvus brachyrhynchos*, and the highest was 5.7% in *Haliaeetus leucocephalus*. The former value corresponded to 71 of 1532 non-S residues, whereas the latter value corresponded to 85 of 1498 non-S residues. Thus, there was a difference of 14 M + C per VTG3 molecule between these two species.

(b) Correlates of embryo incubation time

Mapping of embryo incubation time over the phylogeny suggested that relatively long incubation times represent the ancestral condition for modern birds, as seen in the basal groups Palaeognathae (including the ostrich, *Struthio camelus*) and Galloanserae (including the mallard duck, *Anas platyrhynchos*; figure 1). The results implied that exceptionally short incubation times have evolved independently multiple times in the Neognathae, most notably the cuckoos (Cuculiformes), woodpeckers (Piciformes) and passerines (Passeriformes; figure 1). Longer incubation times have evolved in certain lineages, especially the penguins (Sphenisciformes).

In conventional statistical analyses, %EAA was significantly positively correlated with log incubation time in the case of VTG1 ($r = 0.469$; $p < 0.006$; randomization test; figure 3a) and VTG2 ($r = 0.482$; $p < 0.001$; randomization test; figure 3a) but not in the case of VTG3 ($r = -0.225$; n.s.; randomization test). On the other hand, %M + C was not significantly correlated with log incubation time in the case of VTG1 ($r = 0.254$; n.s.; randomization test) or VTG2 ($r = 0.257$; n.s.; randomization

test), but %M + C was significantly correlated with log incubation time in the case of VTG3 ($r = 0.493$; $p < 0.004$; randomization test; figure 3b).

PIC showed a similar pattern to that seen in conventional analyses (table 1). In VTG1 and VTG2, PIC in %EAA were positively correlated with PIC in log incubation time, whereas in VTG3, PIC in %M + C were positively correlated with PIC in log incubation time (table 1). There was also a relatively weak but significant positive correlation between PIC in %M + C in VTG1 and those in log incubation time (table 1).

In conventional analyses, log incubation time was positively correlated with log body mass ($r = 0.827$; $p < 0.001$; randomization test). Likewise, PIC in log incubation time were positively correlated with PIC in log body mass ($r = 0.668$; $p < 0.001$). Therefore, partial correlation was used to test whether the observed relationships between PIC in amino acid composition and those in log incubation time were independent of the relationship between incubation time and body mass (table 1). Controlling for log body mass, the first-order partial correlations between PIC in %EAA and PIC in log incubation time were significantly positive in the case of VTG1 and VTG2 (table 1). Likewise, the first-order partial correlation between PIC in %M + C and those in log incubation time, controlling for log body mass, was significant in the case of VTG3 (table 1).

(c) Mode of development

In order to control for effects of developmental mode on incubation time, PIC were estimated for reduced datasets (table 1). When altricial and semi-altricial species ($n = 24$) were included, patterns of correlation between PIC in %EAA and those in log incubation time were essentially the same as those for the complete dataset (table 1). Again, PIC in log incubation time were significantly positively correlated with those in %EAA in VTG1 and VTG2 (table 1). Similar significant relationships were seen in partial correlations controlling for PIC in log body mass (table 1). Likewise, a positive correlation between PIC in log incubation time and those in %M + C was observed in the case of VTG3; and a similar significant relationship was seen in the partial correlation controlling for PIC in log body mass (table 1).

When the same analyses were applied to a dataset consisting of altricial species only ($n = 20$), the relationships were generally similar (table 1). Both univariate and partial correlations between PIC in log incubation time and those in %EAA in VTG1 and VTG2 remained significant (table 1). In this reduced dataset, the univariate correlation between PIC in log incubation time and PIC in %M + C of VTG3 was not significant, but there was a significant partial correlation between PIC in log incubation time and PIC in %M + C of VTG3 controlling for PIC in log body mass (table 1).

4. Discussion

Phylogeny-based statistical analyses supported the hypothesis that the EAA composition of avian VTG1 and VTG2 is positively correlated with embryo incubation time and that this effect was statistically independent of body mass. VTG3 differed from the other two avian VTGs in having lower proportions of S and of EAA but higher proportions of the sulfur-containing amino acids M and C. In the case of VTG3,

Table 1. Correlation coefficients (with p -value^a) between phylogenetically independent contrasts in log incubation time and those in %EAA and %M + C in avian VTG1, VTG2 and VTG3.

		VTG1	VTG2	VTG3
%EAA	all species ($n = 34$)			
	univariate correlation	0.539 (0.001)	0.437 (0.011)	−0.059 (n.s.)
	partial correlation ^b	0.463 (0.007)	0.471 (0.006)	0.143 (n.s.)
	altricial + semi-altricial species ($n = 24$)			
	univariate correlation	0.624 (0.001)	0.530 (0.009)	−0.081 (n.s.)
	partial correlation ^b	0.565 (0.005)	0.569 (0.006)	0.157 (n.s.)
	altricial species only ($n = 20$)			
	univariate correlation	0.608 (0.006)	0.488 (0.034)	−0.228 (n.s.)
	partial correlation ^b	0.521 (0.022)	0.557 (0.015)	0.120 (n.s.)
%M + C	all species ($n = 34$)			
	univariate correlation	0.346 (0.049)	0.238 (n.s.)	0.446 (0.009)
	partial correlation ^b	0.261 (n.s.)	0.251 (n.s.)	0.475 (0.005)
	altricial + semi-altricial species ($n = 24$)			
	univariate correlation	0.278 (n.s.)	0.132 (n.s.)	0.505 (0.014)
	partial correlation ^b	0.279 (n.s.)	0.182 (n.s.)	0.524 (0.010)
	altricial species only ($n = 20$)			
	univariate correlation	0.238 (n.s.)	0.114 (n.s.)	0.401 (n.s.)
	partial correlation ^b	0.235 (n.s.)	0.213 (n.s.)	0.498 (0.030)

^a p -values in parentheses. Significant ($p < 0.05$) correlations in *italics*.

^bFirst-order partial correlation controlling for log body mass.

there was a positive correlation between decreased %M + C and shorter incubation time, which was again independent of body mass, suggesting that avian VTG3 may play a specialized role as a source of sulfur-containing amino acids. These effects were seen even when only altricial and semi-altricial species were considered and when only altricial species were considered, supporting the hypothesis that selection on amino acid composition of VTGs is a function of incubation time, independent of the mode of development.

The differences between the highest and lowest observed values of %EAA and %M + C were modest. However, because of the length of VTG proteins, these amounted to substantial numbers of residues per VTG molecule. Moreover, given the abundance of these proteins in the yolk [41], even small differences in %EAA and %M + C per molecule are likely to lead to differences of many millions of amino acid residues available to the developing embryo.

Numerous hypotheses have been proposed to account for the evolution of differences in embryo incubation times among bird species, and it seems plausible that different selective factors are at work in different species [8–12]. Selective factors proposed to account for relatively long incubation times among altricial species include low levels of egg predation, as in cavity-nesting species [9]. Another class of hypotheses relates prolonged incubation to ecological factors that favour reduced incubation attentiveness by adults, including both foraging strategy and the potential for predation on the adult at the nest [8,10–12]. Whatever the selective factors, a longer incubation time without a change in the degree of development at hatching implies a slower process of embryonic development, with implications for nutrient utilization, particularly in the case of EAAs.

In any egg-laying species, all EAAs incorporated into the developing embryo's own proteins are ultimately derived from the EAAs provided to the egg (mainly via VTGs in the case of birds). Eukaryotic cells continually degrade proteins and recycle their constituent amino acids [42,43]; for example, the rate of protein degradation per day in chicken embryos has been estimated at 70–80% that of protein synthesis [44,45]. Thus, EAAs incorporated into embryonic proteins must come either directly from proteolysis of egg proteins such as VTGs or from recycling of EAAs from previously translated embryonic proteins. In either case, the amino acids themselves are subject to continual breakdown, because the enzymes involved in the breakdown of amino acids are constitutively expressed, regardless of the dietary needs of the organism [23]. The resulting inefficiency of amino acid use and recycling will have a cumulative effect over the course of incubation, resulting in a need for larger EAA stores in species with longer incubation times. The present results are consistent with the hypothesis that, in altricial and semi-altricial bird species with naturally long embryo incubation times, extra provisioning of the eggs with EAAs is an adaptation to counteract the potentially deleterious effects of inefficient amino acid incorporation. On the other hand, bird species with very short incubation times are likely to be less constrained with regard to nutritional provisioning of the eggs, consistent with the observed lower %EAA and %M + C in VTGs of species with short incubation times.

Prolonged absence of an incubating parent, as occurs in certain altricial and semi-altricial species with long incubation times [9,12], may be an additional factor decreasing the efficiency of amino acid utilization. When developing eggs of

passerines with naturally short incubation times were experimentally subjected to periodic cooling, as would occur in the absence of an incubating parent, longer periods of cooling led to decreased efficiency in the conversion of egg nutrients into embryonic tissue [46] and decreased innate immune function, presumably as a result of decreased synthesis of immune effector proteins [47]. The provisioning of eggs with larger EAA stores in certain species with long incubation times may thus serve to counteract the deleterious effects of intermittent parental absence, as well as the effects of inefficient amino acid use and recycling.

Testing hypotheses regarding selective factors not considered here will require data on additional avian lineages showing divergent reproductive and ecological adaptations. Because the present data included relatively few species with precocial young, future studies will be needed to test whether precocial species show relationships between embryo incubation time and amino acid composition of VTGs similar to those observed here in the case of altricial species.

The present results support the hypothesis that the amino acid composition of VTGs co-evolves with reproductive strategy in birds. Adaptive evolution of VTGs is not unique to birds; there is evidence that amino acid composition of VTGs in teleost fishes is likewise adapted to aspects of reproductive

biology [48–51]. For example, in barfin flounder, *Verasper moseri*, generation of free amino acids from one VTG paralogue regulates the buoyancy of spawned pelagic eggs [48]; and neo-functionalization of VTG duplicates may have played a key role in the adaptation of fishes to spawning in waters of different salinities [49]. Reconstruction of vertebrate VTG evolutionary history thus suggests that selection acting on life-history traits can have consequences at the level of protein sequence. Because of the direct role of VTGs in nutrition of the developing young, selection on these proteins as a result of life-history strategy is relatively direct. However, it is possible that selection arising from nutritional and reproductive adaptations might also occur in other proteins; for example, proteins that serve as amino acid stores in the adult that can be mobilized for reproduction. Such selection may constitute a previously unrecognized factor affecting the evolution of a variety of protein families.

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