Morphology of the avian yolk sac

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Abstract
The avian yolk sac is a multifunctional extraembryonic organ that serves not only as a site of nutrient (yolk) absorption, but also for early hemopoiesis, and formation of blood vessels. Although the yolk sac membrane being specialized to function as an extraembryonic absorptive organ, it is neither morphologically nor functionally part of the embryonic gut. Yolk absorption is by the phagocytic activity of the extraembryonic endoderm. I used cryohistology and resin embedding histology of complete developmental series of Japanese quail to document the development of the avian yolk sac and changes of the microscopic anatomy throughout development. This material is complemented by complete series of MRT-scans of live ostrich embryos from beginning of incubation through hatching. Considerable changes of size and shape of the yolk mass are documented and discussed as resulting from water flux from albumen to yolk associated with the biochemical activation of yolk sac proteins. During embryogenesis, the yolk sac endoderm forms villi that increase the absorptive surface and reach into the yolk ball. The histology of the absorptive epithelium is specialized for phagocytic absorption of yolk. During early developmental stages, the extraembryonic endoderm is single layered, but it eventually becomes several layers thick during later stages. The extraembryonic mesoderm forms an extensive layer of hematopoietic tissue; deep in this tissue lie the yolk sac vessels. During late stages of development, the erythropoietic tissue disappears, blood vessels are obliterated, and the yolk sac epithelium becomes apoptotic. Results are discussed in the light of the evolutionary history and phylogeny of the amniote egg.

1 | INTRODUCTION
The avian egg is an almost paradigmatic model of amniote eggs. However, diverging evolutionary pathways have led to different functional, developmental, and morphological features of the eggs in the diverse groups of amniotes (e.g. Packard & Packard, 1980; Elinson, Stewart, Bonneau, & Blackburn, 2015; Starck, Stewart & Blackburn, this issue of JMMOR). However, knowledge of the amniote egg is scattered through the published literature, incomplete for morphology and clades providing the necessary phylogenetic coverage, and rarely comprehensive and integrative in an evolutionary context. While the eggshell and the chorioallantois have been covered to some degree by morphological studies, the analysis of the morphology of the yolk mass and the cellular yolk sac has been neglected. Therefore, this paper focuses on the morphology and histology of the avian yolk sac so that comparisons with other amniote eggs may be made and placed in a phylogenetic and evolutionary framework.

The avian egg is a cleidoic egg; that is, a protective layer of eggshell membranes, and a calcified, but semi-conductive eggshell (only water vapor and respiratory gases diffuse across shell membranes and shell) enclose proportionally large volumes of yolk and albumen. Yolk, albumen, and eggshell are the main constituents of the egg. The ovary...
Among bird species, the yolk content of eggs ranges between 15% and 70% of the egg fresh weight, depending on egg size, length of incubation period, phylogeny, and mode of post-hatching development (Carey, Rahn, & Parisi, 1980; Ricklefs, 1977; Sotherland & Rahn, 1987). The yolk content of eggs of altricial birds is ca. 15%, in most precocial birds it ranges around 35%. In species with extended incubation periods, like megapodes (Megapodiidae), the yolk content may reach up to 50% of the egg fresh weight; in the Kiwi (Apteryx australis) the yolk content of the egg may reach 70% of fresh weight.

At laying, the yolk is a sphere, centered in the middle of the egg. A thin, noncellular vitelline membrane surrounds it. The yolk contains almost all energy and other components necessary for the development of the bird, from first cleavage to hatching; only respiratory gases and water vapor pass through the eggshell. About 50% of the yolk is water, 16% is protein, 32% is fat, and about 2% is ash (Romanoff & Romanoff 1949; Mehner 1983). The yolk also contains vitamins (A, B-complex, C, E), minerals (Ca, Cu, Fe, K, Mg, Mn, Na, P, Sr, Zn; Hopcroft, Cowieson, Muir, & Groves, 2019), hormones (e.g. Merrill, Chiavacci, Paitz, & Benson, 2019), enzymes, antibodies, and carotenoids. Proteomic analysis revealed 119 different proteins from (unincubated) chicken egg yolk (Mann & Mann, 2008). More recent datasets list a total of 260 distinct proteins for egg yolk (and 148 for egg white, respectively). Proteomics and interactomics point to yolk proteins being functional in cell development and proliferation, cell–cell interaction and hematopoiesis, egg white proteins were mainly related to cell migration (D’Alessandro, Righetti, Fasoli, & Zolla, 2010).

All components contained in the yolk need to be absorbed, processed, and transferred to the developing embryo. The cellular yolk sac that serves these functions develops during early stages of embryogenesis. It continuously grows and adjusts to the increasing (energy) requirements of the developing embryo. Being functionally similar to the intestine, but distinct in histology, morphological origin, and time of functioning, the cellular yolk sac essentially functions as a non-gut absorptive organ. The mesodermal component of the cellular yolk sac also is the site of primary hematopoiesis and immune cells (review in Sheng, 2010).

Changes of size and shape of the yolk ball during the initial period of incubation have received little attention. However, they are not only universal among birds, but also indicative of functional and compositional processes concerning the yolk that occur even before the yolk sac membrane starts growing as an extraembryonic organ. Much later, before hatching, when the embryo prepares for independent living outside the egg, the cellular yolk sac regresses and yolk residues are incorporated into the body cavity of the young bird. Basic research on the avian yolk sac has been reported/summarized by Lillie (1908), Patten (1929), Raginosa (1961), Mossmann (1987), and Bellairs and Osmond (2005). Lambson (1970) has studied the ultrastructure of the endodermal cells of the yolk sac, for chicken, and Yoshizaki et al. (2004) that of Japanese quail. They provided convincing ultrastructural evidence for phagocytotic yolk uptake by the extraembryonic endoderm. Mobbs and McMillan (1979, 1981) studied the early period of chick embryogenesis showing that the hypoblast endoderm cells actively phagocytose yolk. Ectodermal cells were not involved in yolk phagocytosis. Physiological and biochemical evidence support phagocytotic yolk uptake by endodermal cells of the yolk sac (e.g. Sheng & Foley, 2012).

Many papers focus on specific detailed questions (e.g. protein composition: Mann & Mann, 2008; hemopoiesis: Sheng, 2010), or noninvasive imaging (Dupe, Morrison, Welten, Baggott, & Tickle, 2011), but few efforts have been made at bringing morphological data together to allow reconstructing the evolutionary history of the amniote egg. However, the changing microscopic anatomy of the extraembryonic tissues throughout development will be essential information for any comparative analysis of the evolution of the amniote egg. Therefore, this paper aims at providing knowledge of the types of tissues and cells of the avian yolk sac (and their possible functioning), and how they change during development. To reach that goal, this report documents and reviews the changing histology of the avian yolk sac from the earliest embryonic stages through hatching and compares these data with that of other sauropsids in an evolutionary framework. Results of this study will be included in a phylogenetic analysis at the end of this issue of the Journal of Morphology (Starck et al. this issue of JMOR).

2 | MATERIALS AND METHODS

Fertilized eggs of Japanese quail (Coturnix japonica Temminck and Schlegel, 1849) were obtained from the animal livestock facility at the University of Halle. Eggs were incubated in an automated incubator at 38°C and 78% humidity, turned automatically during intervals of 11 hr with a 1 hr break, and were allowed to cool for 1 hr per day. Eggs were collected every day and either preserved by (a) freezing, by placing them in a commercial freezer (at ~25°C) in natural position, or (b) by tissue samples of yolk sac dissected and fixed in 4% paraformaldehyde in 0.1 mol L⁻¹ phosphate buffer. Staging of embryos was according to Starck (1989). Frozen eggs were peeled, mounted on stubs and sectioned (whole egg) using a cryostat. Sections were collected on permakove slides, dried, and stained with Sudan black and basic fuchsin (Table 1). Paraformaldehyde preserved tissue samples of the yolk sac membrane were dehydrated through graded series of ethanol, embedded in hydroxyethyl methacrylate (Historesin; Leica Microsystems, Wetzlar, Germany), and thin sectioned at 2 µm section thickness using an AO Spencer No. 820 rotary microtome. Sections were mounted on slides.
and stained with Rüdeberg solution (0.1% methylene blue, 0.1% thionin and 0.1 mol L$^{-1}$ Na$_2$HPO$_4$ in distilled water; Rüdeberg, 1967; Table 2). Microphotographs were taken using either an Olympus dot slide scanner microscope or a Zeiss Axiophot equipped with a Plan-Apochromat (63×1.4 oil DC, ∞/0.17) and an Axiocam ERc 5 s. Microphotographs taken at the Axiophot were processed (removing background shade) using Image J software (Rasband, 1997–2018), and stitched using Microsoft Image Composite Editor, version 2.0.3.0 (64 bit) 2015. I used Adobe Photoshop CS2 Version 9.0 for cropping images, removing background noise, contrast enhancement where necessary, mounting images on plates, and for labeling.

Fertilized ostrich eggs (*Struthio camelus* Linnaeus, 1758) were purchased from a local farmer near Tübingen, Germany. Egg were incubated in an artificial incubator (same settings as given above), and submitted to magnetic resonance imaging once a week. After hatching, chicks were returned to the farmer. Magnetic resonance imaging was performed using a Vision Siemens Megatron 1.5 T, 64F tomograph at the Department of Neuroradiology, Section for Experimental Nuclear Resonance of the Central Nervous System, University of Tübingen. Images were taken as T1- or T2-weighted images (details given in figures captions).

Data were collected between 1996 at University of Tübingen and 1998 at University of Jena. Despite the long time since material collection and slide preparation, results of this study have not been published, except for two conference abstracts (Starck, 1999, 2019).

### 3 | RESULTS

I present results for ostrich and Japanese quail together. This is under the assumption that the morphological features reported here are universal for modern birds (Neornithes; e.g., Braun, Cracraft, & Houde, 2019; Livezey & Zusi, 2001, 2007; Prum et al., 2015).

### 3.1 | Shape and size of the yolk ball

At egg laying, the yolk has the form of a sphere, centered in the middle of the egg (Figure 1a). A thin vitelline membrane (not documented) covers the yolk ball superficially. At an equatorial position, two spiral bands, that is, chalazae, extend from the vitelline membrane reaching into albumen. They ultimately connect with the inner eggshell membrane at the blunt pole and the pointed pole of the egg. The yolk is deposited in layers. It has a core of liquid yolk (high water content), that is, nucleus of Pander (Figure 1a), which continues into the latebra

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**TABLE 1** *Coturnix japonica* eggs used for cryostat sections$^a$

| Specimen # | Age (hours) | Age (days) |
|------------|-------------|------------|
| 401        | 111 hr 15 min | 4 days 15 hr 15 min |
| 400        | 140 hr 50 min | 5 days 20 hr 50 min |
| 336        | 162 hr 46 min | 6 days 18 hr 46 min |
| 333        | 208 hr 5 min | 8 days 16 hr 5 min |
| 329        | 208 hr 5 min | 8 days 16 hr 5 min |
| 330        | 208 hr 5 min | 8 days 16 hr 5 min |
| 328        | 208 hr 5 min | 8 days 16 hr 5 min |
| 331        | 208 hr 5 min | 8 days 16 hr 5 min |
| 349        | 237 hr 45 min | 9 days 21 hr 45 min |
| 339        | 262 hr | 10 days 22 hr 00 min |
| 402        | 278 hr 27 min | 11 days 14 hr 27 min |
| 335        | 309 hr 33 min | 12 days 21 hr 33 min |
| 332        | 309 hr 33 min | 12 days 21 hr 33 min |

$^a$All material is deposited at the Bavarian state collection of Zoology, Munich and is accessible upon request.

**TABLE 2** *Coturnix japonica*, specimen numbers of histological semi-thin sections

| Specimen # | Embryonic stage |
|------------|-----------------|
| 281, 282, 284 | 14 |
| 280 | 15 |
| 283 | 17 |
| 287 | 19 |
| 286, 288, 289, 294 | 20 |
| 291, 292, 293 | 21 |
| 290 | 22 |
| 296, 298 | 26 |
| 295, 297 | 27 |
| 96, 97, 122, 124, 130 | 27/28 |
| 125, 126, 127, 185 | 28 |
| 183, 184 | 28/29 |
| 120 | 29 |
| 111, 176 | 30/31 |
| 112, 113, 118, 156, 161, 163 | 31 |
| 117, 157 | 31/32 |
| 27 | 32 |
| 181, 87 | 32/33 |
| 152 | 33 |
| 22 | 33/34 |
| 149 | 34 |
| 26, 150 | 34/35 |
| 60, 69, 75, 76, 103 | 35 |
| 100, 102 | 35/36 |
| 98 | 36 |
| 74 | 36/37 |
| 55, 56, 57, 73, 107, 146, 147, 148 | 37/38 |
| 33, 39, 106, 108 | 38 |
| 139 | 38/39 |
| 43, 39 | 39 |
| 136 | 40/41 |
| I, II, III Hatchling |

$^a$All material is deposited at Department of Biology II, LMU Munich and is accessible upon request.
(Figure 2a), that is, a stalk of liquid yolk that reaches to the surface of the yolk ball, right below the blastodisc.

With beginning incubation, the yolk ball moves into an eccentric position under the eggshell (Figure 2a) with the blastodisc being closest to the eggshell. Connected to the chalazae, the yolk ball always turns so that the blastodisc is up, that is, at the highest position within the egg, and closest to the eggshell. Also, the air cell develops as an air filled space between inner and outer shell membrane at the blunt pole of the egg (Figure 2a, b). The primary function of the air cell is to compensate for volume changes due to water loss during incubation;
thus, throughout incubation, it continuously changes its shape. The size of the air cell increases continuously during development until the end of incubation.

Within a few days after incubation, the yolk sac increases considerably in size (Figure 3a), and changes its shape, now expanding through the egg, forming several horizontal layers of yolk (Figures 1b, c, e and 2b–d). For the quail, three layers were recognized: judged by the staining intensity, Y1 is dense yolk, similar to the original yolk ball, Y2 appears diluted, and Y3 is a highly diluted, watery yolk right under the embryo. These layers are transitory and disappear as the embryo grows and demands more space in the egg. For later stages, it is not possible to describe the shape of the yolk ball precisely, because it is largely liquefied, and its shape becomes irregular and adjusted to the space remaining in the egg (Figures 1g and 2e). In the MRT-images of ostrich, only two layers of yolk were recognized, but differences in water content could be documented in a semi-quantified manner and compared with the yolk ball right after egg laying (Figure 3b–d). Magnetic resonance spectroscopy allows measuring differences in chemical shift as a semi-quantitative measure of water and lipid components of the yolk. When comparing the chemical shift spectra of an ostrich egg at the beginning of incubation (Figure 3b), they show two peaks indicative of water and lipid contents. After 3 weeks of incubation, two layers of yolk can be distinguished (Y1, Y2 in Figure 2c) that clearly differ in their chemical shift profile (Figure 3c and d). Changes in chemical shift indicate a higher water content in the measured yolk volumes as compared to the original yolk sphere at the beginning of incubation (Figure 3b). Although magnetic resonance spectra were taken only from one egg at two moments during incubation, they clearly coincide with the volume increase of the yolk. It suggests that water flux from the albumen to the yolk causes the measured volume increase and "dilutes" the yolk.

Depending on the overall length of the incubation period, the stratification of the yolk disappears later during incubation. However, the shape of the yolk remains that of a cushion with the embryo resting on it (Figures 1e and 2c and d). In the quail, the stratification has almost disappeared at the ninth day of incubation (residues being faintly recognizable). In the ostrich, the stratification disappeared after 5 weeks of the much longer incubation period. At the end of the incubation, the residual yolk sac is completely incorporated into the body cavity (Figure 2e and f).

### 3.2 Yolk sac membrane

#### 3.2.1 Growth of the yolk sac membrane

Growth of the cellular yolk sac begins with the formation of the area opaca and continues until the cellular yolk sac surrounds the entire yolk sphere. In the Japanese quail, the yolk mass is completely overgrown by cellular yolk sac after about 7–8 days of incubation. In the sample of eggs studied here, the yolk sac was still open at the lower pole at days 6 (Figure 1c) and 7 of incubation, but an 8-day-old embryo (Figure 1e) had a fully closed yolk sac membrane. The closure of the yolk sac (yolk sac umbilicus) has not been studied here. The yolk sac umbilicus is morphologically complex since it includes elements of the chorioallantois and a supposed splitting of the extraembryonic mesoderm (see discussion). No isolated yolk mass remains, all yolk is included in the cellular yolk sac.

During the growth period, the cellular yolk sac develops folds that reach into the superficial layers of the yolk mass (Figure 1d, f, h). When the cellular yolk sac has fully overgrown the yolk mass, the development of the cellular yolk sac continues by extending the folds deeper into the yolk. These folds, however, never penetrate the entire yolk sphere; they always remain as a superficial layer of folds. In older quail embryos (e.g. 12–13 days of incubation; Figure 1g and h), they reach about 3–4 mm deep into the yolk mass, the more central parts of the yolk mass remain noncellularized.

As the embryo grows during development, it sinks deeper and deeper into the yolk, which, in the beginning, appears like a cushion, but later fills available space between the embryo and the eggshell. There is no predictable pattern of how the embryo sinks into the yolk sac, and the distribution of the yolk in the egg during the latter part of the incubation appears random. During the last period of incubation, between aeration of the lungs and actual hatching, the residual yolk sac is incorporated into the body cavity (Figure 1g and 2e, f). It remains connected with the small intestine through the yolk stalk (not documented).

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**FIGURE 1** *Coturnix japonica*, developmental changes of the yolk and cellular yolk sac (all cryostat sections, peeled, stained with Sudan black and basic fuchsine). (A) Unincubated egg. Nucleus of Pander in the center of the yolk mass. The Latebra not shown; Blastodisc is up. (B) Egg after 5 days, 20 h, 50 min incubation; histological slide with two consecutive sections in a mid-sagittal position. The yolk mass extends through the entire egg and is stratified. (C) Egg after 6 days of incubation; yolk is stratified and residues of the nucleus of Pander are visible, embryo (slightly cross section through abdominal region and head) residing on yolk. White arrow heads indicate the margin of the growing cellular yolk sac; the space between the white arrow heads is not yet covered by cellular yolk sac. (D) Detail of (C), showing a thin cellular yolk sac and short yolk sac folds reaching into the yolk (black arrows). (E) Egg after 8 days 16 h incubation. The embryo lies on the yolk like on a cushion. Black arrow heads point to yolk sac folds. (F) Detail of (E); folds of the cellular yolk sac extend deeper into the yolk; there is, however, only the superficial layer of yolk in contact with the folds of the yolk sac membrane. Arrows point to folds of the yolk sac. (G) Egg after 12 days, 21 h, 33 min incubation. The embryo fills almost the entire egg, residues of yolk and albumen. (H) Detail of (G) showing folds of the endodermal cellular yolk sac reaching into the superficial layer of yolk. ab, albumen; ce, cerebellum; ey, eye; f, feathers; Fe, Femur; H, heart; Li, liver; lt, latebra; Lu, lung; mSt, muscular stomach; Si, small intestine; Y, yolk; Y1-3 layers of yolk that were observed a few days after incubation. Arrows point to folds of the yolk sac.
3.2.2 | Histology and cytological differentiation of the yolk sac membrane

The yolk sac membrane develops from the area opaca of the blastoderm. At its early stages, it is a bilaminar omphalopleure consisting of a thin, superficial layer of extraembryonic ectoderm, and a deep layer of relatively large cells filled with yolk droplets (Figure 4a), which is derived from the hypoblast. (i.e. "area opaca endoderm" as described in Bellairs & Osmond, 2005). The bilaminar omphalopleure is a transitory structure and represents the growing margin (precisely, the leading
edge is ectoderm only, the endoderm follows a few cells later [Yoshizaki et al., 2004; Figure 2d]) of the cellular yolk sac membrane that ultimately embraces the entire yolk ball. Mesodermal cells from the embryonic lateral plates soon immigrate between the extraembryonic ectoderm and endoderm, thus establishing a thin layer of extraembryonic mesoderm resulting in the trilaminar omphalopleure. The first blood islands and blood vessels develop in the extraembryonic mesoderm (Figure 4b, c).

Histologically, the extraembryonic ectoderm of the bilaminar omphalopleure is a single layered, epithelium. Cells do not contain yolk vesicles. The extraembryonic endoderm of the bilaminar omphalopleure is a single-layered epithelium consisting of large prismatic cells with numerous vesicles of different sizes, some filled with intensively staining particles. While the extraembryonic ectoderm maintains flattened, squamous cells throughout development, the cytological appearance of the extraembryonic endoderm quickly changes, notably becoming a multilayered epithelium. The cells facing the yolk mass directly are always large and prismatic. Some cells contain large vesicles that do not stain (or stain only very pale using Ruedeborg solution), others contain large vesicles with numerous intensively staining droplets of variable size. At this stage (stage 15, ca. 2 days incubation), the extraembryonic mesoderm is a thin layer of mesenchymal cells between ectoderm and endoderm. Some mesoderm cells are visibly enlarged, probably representing early hemopoietic stages (Figure 4c). From stage 14 (ca. 2 days incubation) on, rudiments of developing blood vessels are found in the extraembryonic mesoderm layer; their endothelial lining is still incomplete (Figure 4c, d).

At 4 days (stage 20), blood vessels of various size have formed in the extraembryonic mesoderm, now bulging into the yolk (Figure 5a). An endothelium of flattened cells covers the lumen of the large blood vessels, but in the midsized and small vessels, it appears undifferentiated and incomplete. A thin layer of mesenchyme and a thick layer of hemopoietic tissue surround the blood vessels. Almost all cells of the hemopoietic tissue contain mitotic figures, that is, undergoing cell division. Some, nucleated blood cells have entered the blood vessels. Large vacuoles characterize the cells of the endoderm; their content does not stain with methylene blue and thionine (Figure 5a). A few hours later (4–5 days, stage 22; Figure 5b), the blood vessels reach deeper into the yolk, now forming folds with the blood vessel running in the apical part of the fold. The hemopoietic tissue also reaches into the folds and partially surrounds the blood vessels. The extraembryonic endoderm is a thick, stratified layer of vacuolated cells as described above.

During the following developmental stages until day 14 (stage 38), the folds containing blood vessels at their apical ends continue to grow deeper into the yolk, and the hemopoietic tissue surrounding the blood vessels forms an increasingly thick layer of proliferating cells while the lumen of the actual blood vessel is relatively small (Figure 6b–d). Notably, the blood vessels are surrounded not only by their endothelium, but also by a thick layer of hemopoietic tissue, and the multilayered extraembryonic endoderm. The apical border of the endoderm cells contains vesicles that stain like yolk vesicles. Most certainly, they represent phagocytosed yolk vesicles. The extraembryonic ectoderm does not contribute to the formation of the yolk sac folds.

During the last few days of incubation, before hatching (days 17–18; stages 38–42), the cellular integrity of the cellular yolk sac deteriorates. The cellular layers of the yolk sac membrane become necrotic, many cells containing extremely large vesicles. Blood vessels in the folds have collapsed and are nonfunctional; at least from a microscopic anatomical point of view; only the superficial blood vessels on the cellular yolk sac remain functional (Figure 6e and f). The hemopoietic tissue surrounding extraembryonic blood vessels has disappeared. Shortly before hatching, roughly around the initiation of pulmonary ventilation, the residues of the yolk sac are incorporated into the body cavity of the hatching bird. Judged by the cytology of the cellular yolk sac, it is not functional anymore as an absorptive organ. The incorporated yolk residues may constitute a considerable portion of the hatching body weight (Figure 2e,f).

4 | DISCUSSION

4.1 | Morphological changes of the yolk ball

As incubation begins, the yolk ball moves its position within the egg, increases in size, and changes its shape. Here, I reported these
changes for Japanese quail and Ostrich, representative species of two phylogenetically distinct clades, that is, Palaeognathae versus Gallinales. Falen, Szeverenyi, Packard Jr, and Ruocco (1991) documented the same changes of the yolk ball at the beginning of incubation in chicken eggs, that is, size increase, change of form and position in the egg, but the paper focuses on the layered structure of the yolk, the nucleus of Pander and the Latebra. The authors only marginally discuss shape changes of the yolk ball. Duce et al. (2011) unintentionally documented the same changes of the yolk sac in their publication on MRI of Japanese quail eggs. Despite applying MRI in an unnatural position of the egg (pointed pole down), and using T2-weighted imaging, so that much of the stratification and changes in lipid content cannot be seen with the same clarity, their images show the same dynamic shape changes of the yolk mass shortly after beginning of incubation. Other sources (Bain et al., 2007; Bain, Fagan, Mullin, McNaught, & Condon, 2005; Chickscope, 1996; Herrmann, Taylor, Murray, Poptani, & Sée, 2018) present MRI of chicken eggs although none of the studies focused on the cellular yolk sac. All unintentionally documented the same kind of size and shape changes as described here. Witschi (1956) presented a series of semi-schematic drawings of sparrow embryos in the egg, at about mid-embryogenesis, documenting a layered structure of yolk sac and albumen with the embryo residing on the yolk (roughly equivalent to the developmental stage of quail as shown in Figure 1e). Shojaei, Tavakoli, Aghasizade, and Sayyah-Badkhhor (2014) documented the embryonic development of live ostrich using T2-weighted MRI. Despite an unnatural position of the eggs (pointed pole down), their results are fully comparable to those presented here. Their study included a comparison of fertilized eggs with unfertilized eggs over the same length of incubation (18 days). While in the fertilized eggs the yolk ball changed its shape, it did not in the unfertilized eggs, providing comparative evidence that indeed developmental processes at early embryogenesis trigger the changes of the yolk mass. The overall species sampling is small and the general attention given to morphological changes of the yolk mass in developing birds is admittedly limited. However, the repeated (often unintentional), and consistent report of the same changes of the yolk shape in quail, chicken, ostrich and one passeriform species supports the view that the observed changes document universal (avian) physiological processes making the yolk accessible to the developing embryo. As a side note, it might be mentioned that a number of reviews and textbooks ignore the fact that the yolk sac is a transitory structure with a dynamic and independent development during avian embryogenesis. Too often, its morphology is erroneously illustrated as a yolk ball maintained throughout the incubation period.

Volume changes of the yolk sac during incubation have received even less attention. Shape and volume changes relate to a major shift of water from the albumen to the yolk during the first days of incubation. Yoshizaki et al. (2004) reported a peak of yolk volume at day 4 of incubation in Japanese quail. This is consistent with Bhagat, Zade, and Charde (2012) who recorded (wet) weight changes of the yolk sac in Japanese quail during the entire incubation period. Their data show a clear peak of yolk weight at day 4 of incubation. I have not measured yolk volume or yolk wet weight in Japanese quail, but the microscopic anatomy presented here is straightforward showing a considerable increase of the yolk (qualitatively described). The data on ostrich eggs presented here document quantitatively the same changes of volume and shape. Additionally, magnetic resonance spectroscopy showed that the formation of the stratified yolk and the increase of the volume of the yolk mass (in ostrich) coincides with a considerable transport of water from the albumen into the yolk. Volume changes and measurement of water flux into the yolk, are only cross-developmental signs of physiological and biochemical transformations of the yolk. It would be interesting to see how these morphological changes of the yolk mass relate to reported changes of the chicken egg proteome from unincubated eggs (e.g., Mann & Mann, 2008) to observed changes after 12 days of incubation (Zhu, Qiu, Sun, Meng, & Zhou, 2019).

4.2 Development of the cellular yolk sac

The cellular yolk sac is an extraembryonic organ designed to absorb yolk from the yolk sphere. As such, it overgrows the yolk sphere and finally surrounds the yolk. The closure of the yolk sac at the abembryonic pole of the egg was not studied here, but was subject of a detailed description by Lillie (1908; p. 217, figures 123, 129) and was referenced in numerous later publications (although unclear if repeated or just referenced Patten, 1929; Witschi, 1956; Mossmann, 1987; Raginosa, 1961). Lillie (1908) reported that an albumen-sac were established outside of the yolk-sac, when the expanding allantois reached the lower pole of the egg, and yolk sac and allantois were united by the undivided portion of the mesoblast around the yolk-sac umbilicus. This connection was never severed, and consequently the residuals of the albumen sac were incorporated into the body cavity together with the yolk sac before hatching. However, these epithelial configurations have never been documented using histological methods, thus the microscopic anatomical arrangement of the epithelia around the yolk sac umbilicus remains to be investigated.

4.3 Histological development of the yolk sac

Here, I documented the histological and cellular development of the yolk sac in detail throughout development. Some of the histology confirms earlier descriptions. In particular the consecutive developmental steps of bilaminar and trilaminar omphalopleure, the stratified organization of the yolk sac endoderm, and the phagocytotic uptake of yolk by those endoderm cells that are in immediate contact with the yolk. Lambson (1970) and Yoshizaki et al. (2004) already provided detailed ultrastructural evidence for phagocytotic yolk uptake in chick and quail embryos, respectively. Mobbs and McMillan (1979, 1981) used transmission electron microscopy, and injection and tracing experiments to document the endocytotic uptake of the yolk through endodermal cells of the early area vasculosa. More recently, Sheng and Foley (2012) provided biochemical evidence for phagocytotic yolk uptake while Bauer et al. (2013) studied differentiation processes that provide nutrient transport competence to yolk sac endodermal cells.
The formation of the folds of the yolk sac has been described here in detail. Although occasionally mentioned, their detailed microscopic anatomy has not, yet, been reported (to my knowledge). The important point here is that the folds reach only into the superficial layers of the yolk mass, and that they carry a blood vessel at their apical end. During the course of development, the folds reach deeper into the yolk, but never cellularize the entire yolk mass. The blood vessels are increasingly surrounded by hemopoietic tissue, so that at about mid-development a thick layer of hemopoietic tissue surrounds the blood vessels. Any nutrient transport from the yolk through the extraembryonic endoderm into the blood stream must pass through this layer of hemopoietic tissue. It is only towards the end of the incubation period that the hemopoietic tissue regresses from the yolk sac. At the end of incubation, that is, around aeration of the lungs, the blood vessels in the yolk sac folds cease functioning and the cells of the extraembryonic endoderm appear necrotic; this is about when the yolk sac is incorporated into the body cavity of the hatching bird. It is unclear and not studied how and if the cellular yolk sac is still functional and how the incorporated residual yolk is actually absorbed by the newly hatched birds.

Despite the clear cytological and histological distinction between extraembryonic yolk sac endoderm and the embryonic gut, the embryonic endoderm forming the intestinal mucosal epithelium and the extraembryonic endoderm are continuous. An open connection between the embryonic gut and the yolk sac has either implicitly been assumed or was postulated (van der Wagt 2020; Speake, Murray, & Noble, 1998). However, the only histological study of the stalk in hatching chicken connecting the yolk sac with the intestine is by Raginosa (1961, plate 10, in Russian). She presented drawings showing no open connection between the embryonic gut and the yolk sac; but, her drawings were not to the cellular level. Olah and Glick (1984) studied the yolk stalk of 2-week-old posthatching chicken using standard histology and showed that the *tunica muscularis* of the intestine forms a sphincter like thickening at the beginning of the yolk stalk. This might explain some of the contrasting reports about the connection between yolk sac and intestine. While the residues of the yolk mass are slowly absorbed during early posthatching life, the residues of the yolk stalk gain importance as extramedullary myelopoietic tissue (Meckel’s diverticulum) during later life of the bird (Olah, Glick, & Taylor, 1984). A modern documentation of the connection is missing.

How the residual yolk is absorbed remains largely unclear. From a physiological point of view, Romanoff (1944) showed that the residual

**FIGURE 3** Dynamic changes of the yolk sac volume and yolk composition in *Struthio camelus*. (a) Volume changes (measured from serial MRT-images, throughout the development) during development. After beginning of incubation the volume of the yolk mass increases until week 3, then it continuously declines until hatching at week 7. (b) Magnetic resonance spectroscopy of the yolk at beginning of incubation (see Figure 2a). Measurements are relative and indicate the relative portions of water and lipid in the yolk. The white square in the right hand MRT-images indicates site of measurement (measured volume 1 cm$^3$). (c) Magnetic resonance spectroscopy of the yolk after 2 weeks of incubation (see Figure 2c). Measurement of the lower layer of yolk (= Y1 in Figure 2c). (d) Same egg at same time as in (C), but measurement was taken in the upper layer of yolk (= Y2 in Figure 2c). Comparing the chemical shift spectra in b, c, and d shows a change from almost equal contribution of water and lipids to a high water content in the upper yolk layer.
yolk in freshly hatched birds is quickly absorbed when birds are fed ad libitum, but its absorption is delayed when birds are fasted. This indicates that yolk absorption is an active process that requires energy and, in contrast to prevailing paradigms, does not supplement periodically poor feeding conditions in newly hatched birds. These findings were orchestrated by Noy and Sklan (2001) who showed that feeding chicks stimulated the release of yolk through the yolk stalk.

4.4 Comparison of the avian yolk and cellular yolk sac with that of nonavian sauropsids

The nucleus of Pander and the Chalazae are typically avian features that have not been described for any nonavian sauropsid. Although it is generally problematic finding statements that structures are missing, Rathke (1866) and Reese (1908) explicitly state that there are no chalazae present in American alligator and other crocodilians. In birds, the
nucleus of Pander, the Latebra and the Chalazae together represent one complex character that serves to move the blastodisc up and close under the inner eggshell membrane. They are coupled to the typical avian behavior of egg turning. With the crocodilian archosaurs excluded, it is safe stating that this character complex is apomorphic for Neornithes.

Shape and volume changes of the sauropsid yolk after beginning incubation have rarely been described, but I assume that this has rather been neglected. Some papers document these changes for birds, I could not find any report on nonavian sauropsids. Shape and volume changes relate to a major shift of water from the albumen to the yolk during the first days of incubation. Packard and Packard (1980) discuss differences in water uptake comparing bird eggs with those of nonavian sauropsids. They highlight that birds depend on water stored in the egg, while nonavian sauropsids take up water from the environment (thus depend on environmental humidity). While this indicates a different system of water supply to the yolk mass, it does not necessarily imply that dilution of the yolk at the beginning of the incubation does not occur in nonavian sauropsids; it may just take different physiological pathways. Again, water uptake and activation of the yolk during early phases of development appear to be neglected topics in comparative and evolutionary developmental

**FIGURE 5** *Coturnix japonica*, cellular yolk sac. (a) Stage 20 (ca. 4 days), blood vessels have enlarged bulging into the yolk ball, their endothelium is now well developed. A layer of hemopoietic cells intermingles with extraembryonic mesodermal cells. (b) Stage 22 (ca. 4–5 days), the extraembryonic endoderm form folds of variable depth that reach into the yolk ball (yolk removed during preparation), blood vessels are located in an apical position of the folds. Cells of the extraembryonic endoderm contain large vacuoles. bv, blood vessel; eEn, extraembryonic endoderm; eEc, extraembryonic ectoderm; en, endothelium; ert, hemopoietic tissue
From a macroevolutionary point of view, utilizing internal water sources makes the avian egg independent of the substratum (as water resource), and allows birds to nest in trees or other remote places (probably at the parental cost of attending the eggs and incubating."

The microscopic anatomy, histology, and cellular details of the tetrapod yolk sac have been studied with little detail, and in few species that are systematically scattered. However, a series of recent studies showed that nonavian sauropsids (including crocodiles) follow different developmental pathways of cellularizing yolk, establish a different microscopic anatomy and absorb yolk differently (Elinson et al., 2015; Elinson & Stewart, 2014; Blackburn, Lestz, Barnes, Powers, & Langkilde, 2018; Blackburn et al., 2020; Blackburn, in press, this issue of JMOR). In all species studied so far, a bilaminar/trilaminar...
omphalopleure appears to overgrow the yolk mass, but the liquid yolk mass is invaded by proliferating endodermal cells, which phagocytose (and process?) the yolk material. These cells form clumps that progressively fill the entire yolk mass. Later, small blood vessels derived from the yolk sac vasculature invade the yolk sac cavity. Finally, the endodermal cells arrange in monolayers around these vessels (forming “spaghetti bands” as termed by Blackburn, this issue of the Journal of Morphology). This is substantially different from the avian pattern as described here, which progresses first as a bilaminar omphalopleure, then trilaminar omphalopleure, then the blood vessels move into folds of the extraembryonic endoderm, which ultimately becomes a stratified epithelium. The folds carrying the blood vessels reach only in the peripheral regions of the yolk while the center of the yolk mass remains uncellularized. The intensive development of hemopoietic tissue surrounding the blood vessels during most of the embryonic period, which regresses a few days before hatching, also is a feature that has not been described to non-avian sauropsids. There, blood islands are located on the external layers of the yolk sac, but appear not to form the circumvascular hemopoietic tissue. A detailed phylogenetic analysis is presented at the end of this issue if the Journal of Morphology.

5 | CONCLUSION

The avian yolk mass and the cellular yolk sac represent a number of clade specific features, that is, autapomorphic characters in a phylogenetic framework. These are nucleus of Pander, Latebra and Chalaza as a coupled character complex of the yolk mass. The cellular yolk sac of modern birds is characterized by a progressive development of endodermal/mesodermal folds that carry blood vessels and hemopoietic tissue surrounding the blood vessels. The folds reach only into the superficial layer of the yolk mass, the remainder of the yolk mass remains cell free. Uptake of yolk is by phagocytosis of endodermal cells. The cellular yolk sac deteriorates toward hatching, hemopoietic tissue disappears, blood vessels in folds obliterate and endodermal cells become necrotic. These morphological features and developmental processes are substantially different from non-avian sauropsids (including nonavian archosaurs) and highlight the unique organization of the avian yolk and cellular yolk sac.

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DATA AVAILABILITY STATEMENT

Histological slides are deposited at the Department of Biology II at Ludwig-Maximilians-University. They are available from the author upon request. CT-Image series will be made available at....

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