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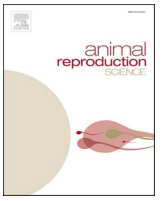

Morphological evidence for the physiological nature of follicular atresia in veiled chameleons (*Chamaeleo calyptratus)*

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ABSTRACT

Follicular atresia (FA) has been assumed to serve different functions in reptiles, e.g. helping to develop hierarchies, limiting clutch size, and regression of ovarian structures. Reproductive output is dependent on a balance between ovulations and FA. Excessive rates of FA may not only be detrimental for the survival of a population, but have also been associated with pathological conditions. In order to gain insights into the physiological and potentially pathological processes of FA, we performed a decriptive study on the morphological features of the ovaries in sexually mature female veiled chameleons (*Chamaeleo calyptratus*, VC). Of 60 clinically healthy female VC with continuous ovarian cycling and at least one confirmed cycle with FA over at least 1.5 years, 30 were selected for macroscopic evaluation of ovarian appearance and 7 were subjected to histology and immunohistology. While FA of previtellogenic follicles happened at a low rate, expected for a species with two germinal beds per ovary and polyautochronic reproductive pattern, atresia in the late vitellogenic stage affected entire generations of follicles, consequential to ovulatory failure. Histologically, no pathological processes were identified in any of the animals. Rather, three stages of FA (early, middle, late) were defined and vitellogenic follicles showed two distinct morphological types of FA: yolky and cystic. Yolky FA was found in 21/30 (70%) animals, while cystic FA co-occurred in 9/30 (30%) of the animals.

1. Introduction

Follicular atresia (FA) is a common phenomenon during the oogenesis of vertebrate species and includes a complex process involving cell death (apoptosis), the resorption of cellular material by the cells themselves (autophagy) and by other cells (heterophagy) (Saidapur, 1978; Thomé et al., 2009; Morais et al., 2012; Corriero et al., 2021). Follicular atresia may occur at any stage of follicular development, but serves different purposes depending on the timing. While FA of developing follicles on the preovulatory ovary serves to develop follicular hierarchies, limit clutch or litter size, and determine species-specific ovulation patterns, FA of mature follicles is instrumental in the resorption of ovarian structures, e.g. during refractory breeding periods (Crews and Licht, 1974; Jones et al., 1978; Guraya, 1989; Etches and Petitte, 1990).

Species-specific reproductive output is dependent on a delicate balance between ovulations and follicular atresia, influenced by extrinsic factors, such as environmental and social factors, resources and food intake, season, and intrinsic factors, such as gonadotropin hormones, corticosteroids, growth hormone and insulin-like factor (Saidapur, 1978; Corriero et al., 2021). Excessive rates of FA

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may not only be detrimental for the survival of a population (e.g. captive breeding programs) but have also been associated with pathological conditions, such as fatal yolk coelomitis (Stacy et al., 2008). In clinical cases, the accumulation of atretic follicles is commonly called pre-ovulatory follicular stasis (POFS) (Scheelings, 2008; Sykes, 2010; Miles, 2019; Cermakova, 2023) and is usually addressed with ovaryhysterectomy (Naguib, 2018; Lock, 2000; Sykes, 2010); however, etiology and pathogenesis have never been fully elucidated.

There is a vast amount of descriptive literature on FA in non-mammalian ovi- and ovoviviparous vertebrate species. Despite various classification schemes, the general morphological aspects are similar, consisting of stages during which the zona radiata undergoes fragmentation to allow hypertrophied granulosa cells to invade the oocyte and phagocytose the yolk, resulting in gradual reduction of oocyte material and a remnant pigment scar with lipofuscin and melanin content. All the while, the thecal cell layer shows blood vessel proliferation and may or may not be involved in the invasion and phagocytosis of the oocyte (Saidapur, 1978). In fish, a four-stage scheme of FA is widely accepted, such as initial, intermediate, advanced, and final atresia and alpha (α), beta (β), gamma (γ) and delta (δ) stages (Miranda et al., 1999; Hunter and Macewicz, 1985). In birds, classification is very heterogenous and has led to the description of various types of atresia (Gupta and Maiti, 1986; Gupta et al., 1988; Madekurozwa and Kimaro, 2006; Islam et al., 2010; and references mentioned therein) but atretic follicles were often generally divided in two types: non-bursting and bursting, the first affecting mostly small primordial follicles and the latter larger, vitellogenic follicles (Gupta et al., 1988; Islam et al., 2010). In reptiles, descriptions of follicular atresia refer to generally one type of atresia which is staged based on the progression of the follicular degeneration, such as early, middle and late stages (Betz, 1963; Goldberg, 1973; Gupta and Duda, 1982; Calderón et al., 2004; da Silva et al., 2018). Depending on the reptile species, FA may occur mainly in previtellogenic (Moodley and Van Wyk, 2007) or in both previtellogenic and vitellogenic follicles (Guraya, 1965). Although the fate of the yolk upon follicular atresia remains unclear, a stromal ovarian structure called the chordolacunar system was described in avian, chelonian and crocodilian species, consisting of irregular, empty stromal spaces lined with flattened epithelial cells, most likely involved in the extrafollicular phagocytosis of yolk (Nili and Kelly, 1996; Callebaut et al., 1997; Uribe and Guillette, 2000; Islam et al., 2010).

The veiled chameleon (*Chamaeleo calyptratus,* VC), a prolific oviparous squamate reptile species with a polyautochronous ovulation pattern, i.e. ovulating numerous follicles from each ovary at once, has been established as model animal for reproductive research in oviparous reptiles (Kummrow, 2009; Pimm et al., 2015; Cigler et al., 2023). A large proportion of the female VC in the research populations mentioned above underwent long phases of their sexual maturity without reproductive output (egg production), despite repeated cyclical investment in follicular development (Kummrow et al., 2010; Cigler et al., 2023). While the animals were not obviously clinically affected, the continuous process of cyclical maturation of large batches of follicles without reproductive output intuitively appeared to present an energetic malinvestment without evident adaptive value. This study describes the morphological features of FA in VC to gain insight into the physiological processes and further our understanding of the biological functions of FA in this species.

2. Material and methods

2.1. Animals

This study is based on 60 female VC from two different study populations (Toronto, n = 38: Kummrow et al., 2010; Zurich, n = 22: Cigler et al., 2023). All animals were captive born, purchased at the age of 2 months, and housed in individual terrariums up to the age of at least 1.5 years. A daily 12-hour photoperiod of full spectrum lighting with a UV index of 1.5–2.5 was provided. Ambient temperature of 24–28 °C was maintained throughout the day, with basking spots reaching 35 °C, and a nighttime drop to 18–22 °C. Environmental conditions were kept constant throughout the year to avoid seasonal quiescence. Terrariums were misted twice daily with an automated rain system, maintaining humidity between 55–80%. Animals were fed gut-loaded and calcium carbonate dusted house crickets (*Acheta domesticus*) and lettuce three times a week.

All animals showed continuous and regular ovarian cycling with 2–4 consecutive follicular cycles monitored by fecal steroid hormone patterns (Kummrow et al., 2010) or imaging diagnostics (Cigler et al., 2023) of which at least 1 cycle showed ovulatory failure with follicular atresia. All were clinically healthy throughout the entire study and were either euthanized or castrated at the end of the respective study at the age of 1.5–2 years.

Ovaries of 30 females (n = 19 from Toronto study, n = 11 from Zurich study) from the above mentioned animals were available for further examination (ovaries of the other 30 animals had been subjected to other studies or not been collected). The Toronto study was performed under Animals for Research Act of Ontario, the recommendations of the Canadian Council for Animal Care, and approved by University of Guelph Animal Care Committee and the Toronto Zoo Animal Care and Research Committee (Kummrow et al., 2010), and the Zurich study was performed under the Swiss Animal Experimentation Ordinance and followed the issued Animal Experiment permit Nr. ZH223/19 (Cigler et al., 2023). For counting germinal beds and description of early stages of follicles, ovaries of three surplus juvenile, premature VC were provided by a registered laboratory reptile breeding facility in Switzerland.

2.2. Macroscopic appearance of ovaries

Ovaries of 30 animals from two separate study groups were evaluated either by photodocumentation (n = 19, Toronto study group) or grossly upon postmortem examination or following castration (n = 11, Zurich study group) and categorized by the presence and appearance of atretic follicles, based on previously published criteria (Cigler et al., 2023). As described by Cigler et al., 2023, the relative age of the batches of atretic follicles can be determined by size and shape, color, and degree of vascularization. With increasing

age, yolky atretic follicles turn from orange and vascularized to bright yellow with decreasing to no external vascularization, eventually lose turgidity and become irregular in shape and smaller. Towards later stages of atresia, some AF become more orange in color, without external vascularization. Shortly before complete resorption, atretic follicles present as a small, dark orange to red, round-to-oval structure, with very little to no yolky content. In this study, the youngest batch of atretic follicles was considered 1st generation, followed by 2nd and 3rd generation, representing follicular batches of one or two previous cycles (Fig. 1A). In animals that ovulated in the latest cycle (n = 3), the remaining AF were assigned to the 2nd and 3rd generation. Differentiation into yolky and cystic atresia was based on the distribution and appearance of the follicular content: yolky atretic follicles presented a homogenous yellow filling (consistent with yolk), whereas cystic atretic follicles were characterized mainly by a segregation of clear fluid and a focal accumulation of more firm, yellow material (consistent with yolk) attached to the follicular wall (Fig. 1B). Note that in this descriptive context, the term "cystic" does not refer to a pathologic condition but solely to the fact that the follicle is filled with fluid. All macroscopic evaluations were confirmed using the developmental data collected for each individual animal within their respective study groups.

2.3. Ovarian anatomy and germinal beds in premature VC

The paraffin embedded ovaries of two premature VC were submitted to a series of sections until the whole organ was processed. The 4–5 μm sections (50 per animal) were then stained with hematoxylin and eosin (HE) and the total number of germinal beds (GB) per ovary was counted. Histologically, GB were identified the presence of oocytes and/or oogonia, embedded in ovarian stroma underneath the germinal epithelium.

2.4. Histology, special stains and immunohistochemistry

Seven animals (n = 6, Zurich study; 1 premature female) with representative macroscopic stages of follicular development (Cigler et al., 2023), in particular yolky and cystic FA, were chosen to further describe the different stages of follicular development and FA histologically. For histological examination, tissues were fixed in 4% formalin solution and routinely embedded in paraffin. For description of follicular atresia, paraffin blocks were sectioned at 4–5 μm, and stained with HE. The terminology for describing the histomorphological picture of the ovarian architecture and the atretic follicles was based on studies on non-vitellogenic follicles in other reptile species (Betz, 1963; Guraya, 1965; Moodley and Van Wyk, 2007; da Silva et al., 2018).

Immunohistochemistry was performed using a Dako autostainer (Dako, Glostrup, Denmark) and the horseradish peroxidase method, to detect Iba1 (monocytes/macrophages) (Shannon et al., 2021; Dervas, and Dervas et al., 2020, 2023), factor VIII–related

Fig. 1. Ovaries of female Veiled chameleons (*Chamaeleo calyptratus*). A. Three generations of atretic follicles, numbered according to increasing age: 1st (1), 2nd (2), 3rd (3, encircled) generation B. Vitellogenic follicles show two types of follicular atresia: yolky (Y) and cystic (C), amongst large, vascularized, late vitellogenig follicles (LVF).

antigen (Factor VIII) (endothelial cells) and α–smooth muscle actin (α-SMA) (smooth muscle cells). Briefly, after deparaffinization, antigen retrieval for Iba1 and Factor VIII was performed by incubation of the slides with citrate buffer (pH 6) at 98 °C for 10 min. Endogenous peroxidase was blocked by incubation with peroxidase blocking solution (Dako) for 10 min. Slides were incubated with the primary antibodies [rabbit pAB Iba1 (1:350, Wako, Osaka, Japan) overnight at 4 °C, rabbit pAb Factor VIIIra (1:100, A0082, Agilent, Santa Clara, US) for 40 min at room temperature and mouse mAb α-SMA (1:400, M0851, Agilent, Santa Clara, US) for 1 h at room temperature]. Afterwards, matching secondary antibodies (EnVision+ HRP Rabbit (Dako) for anti-Iba1 and anti-Factor VIIIra and Dako REAL (Dako) for α-SMA) were applied according to the manufacturer's protocol. Sections were washed with phosphate buffered saline between each incubation step (pH 8). Finally, sections were counterstained with hematoxylin for 20 s and mounted. Sections from spleen (Iba1), small intestine (α-SMA) and the heart (Factor VIII) of an adult veiled chameleon (*Chamaeleo calyptratus*) served as positive controls for testing for antibody specificity. Slides incubated with nonimmune serum from the species in which the primary antibodies were raised instead of the primary antibodies served as negative controls.

3. Results

3.1. Macroscopic appearance of ovaries and frequency of atresia types

In the majority of animals (28/30, 93.3%) a 1st generation of yolky AF was seen. Only two animals (2/30, 6.8%) from the examined photodocumentation had ovulated in the latest cycle and thus contained no 1st generation AF. In many VC, two generations of atretic follicles could be identified (18/30, 60.0%), while in considerably fewer (9/30, 30.0%) a third generation, often a remnant, was still present (Fig. 1A). 21/30 (70.0%) animals showed only yolky atresia throughout all generations. 9/30 (30.0%) animals had both yolky and cystic generations of AF (Fig. 1B), while no animal had only cystic AF. Furthermore, cystic AF was only seen in 2nd generation follicles, i.e. follicles older than ca. 4 months. Incidence of cystic AF was 21.1% (4/19 animals) and 45.5% (5/11 animals) in the two study groups, respectively.

3.2. Histologic ovarian architecture and germinal beds

The ovaries presented two histologically distinct regions: the cortex and the medulla. The cortex contained numerous ovarian follicles and two germinal beds (GB), which were embedded in the ovarian stroma. The ovarian stroma consisted of loose connective tissue that was rich in small caliber vessels and formed a meshwork of thin interconnecting trabecles. The GB presented as two elongated regions at the surface of the ovary in which oogonia and oocytes (at different developmental stages) were aligned underneath the flat monolayered germinal epithelium. Oogonia were round to ovoid with a central round nucleus and a peripheral rim of pale basophilic homogeneous ooplasm. The nuclei showed loose chromatin and an evident nucleolus. The primary oocytes had large round centrally located nuclei and the cytoplasm was more abundant, fibrillar and eosinophilic than that of the oogonia. At early stages of development, primary oocytes were often surrounded by a monolayer of spindeloid to elongated germinal stromal cells. At later stages of development, oocytes started protruding deeper into the ovarian stroma and were surrounded by a more cell rich, multilayered row of germinal stromal cells. The medulla of the ovaries consisted of dense connective tissue that was interspersed with

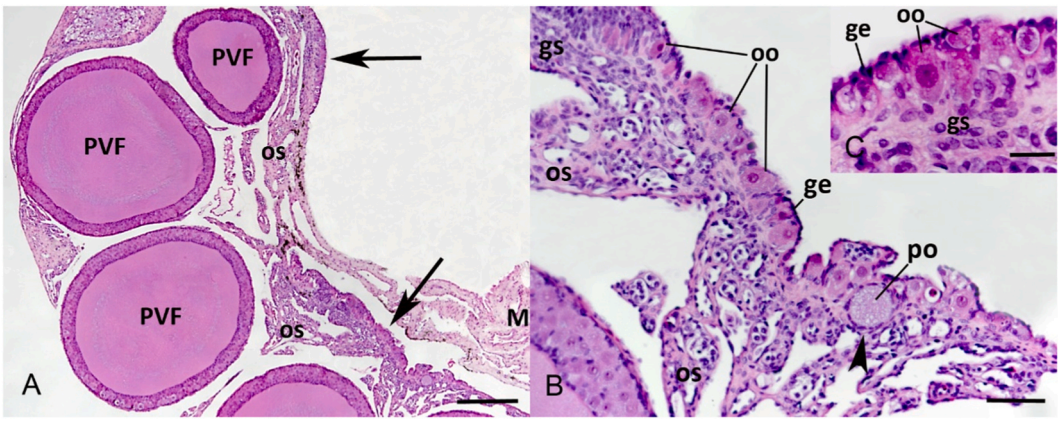

Fig. 2. A. Ovarian architecture. The ovaries in veiled chameleons (*Chamaeleo calyptratus*) are divided into the cortex and the medulla (M). The cortex contains two germinal beds (arrows) and numerous previtellogenic follicles (PVF) embedded in the ovarian stroma (os). The medulla consists of a dense collagen-rich connective tissue. HE stain, bar= 500 μm. B and C. Germinal bed. Elongated region at the surface of the ovary in which oogonia (oo) and oocytes at different developmental stages (f.e. primary oocytes, po) are embedded in the germinal stroma (gs), covered by a flat monolayered germinal epithelium (ge). At early stages of development, primary oocytes were often surrounded by a single layer of spindeloid to elongated germinal stromal cells (arrowhead). HE stain, bar= 100 μm. C. Oogonia are round to ovoid structures with a central round nucleus and a peripheral rim of pale basophilic homogeneous ooplasm. HE stain, bar= 25 μm. PVF: previtellogenic follicles, os: ovarian stroma, gs: germinal stroma, oo: oogonia, ge: germinal epithelium, po: primary oocyte, M: medulla.

medium caliber vessels (Fig. 2A-C).

3.3. Histological frequency of atresia types

Five of the 7 histologically evaluated animals revealed atresia of only vitellogenic follicles, while two showed rare atresia of previtellogenic follicles in addition to the predominating atresia of vitellogenic follicles. In vitellogenic follicles, yolky atresia was observed in all 7 animals, whereas 4 of the 7 animals contained both generations of yolky and cystic atresia (Table 1).

3.4. Atresia of previtellogenic follicles

Atresia of previtellogenic follicles was rarely observed (Table 1) and, although not clearly evident grossly, was recognized upon histological examination by distinct morphological features. Previtellogenic atretic follicles could be easily distinguished histologically from vitellogenic follicles by their smaller size (< 1 mm) and the presence of ooplasm in the follicle center (in contrast to vitellogenic follicles that contained yolk substance). Three stages of previtellogenic FA could be differentiated, early, middle and late stage atresia.

At an early stage, atretic follicles showed a mild distortion in the shape, as the follicle started losing its original spherical shape, due to an infolding of the follicular wall. The ooplasm was less homogenous and irregularly distributed, with multifocal areas of rarefication and vacuolization. A beginning mild irregular proliferation of the granulosa was visible. Granulosa cells were hyperplastic and showed occasional intracellular vacuolization and/or presence of bright eosinophilic material in the cytoplasm (interpreted as phagocytized ooplasm). The zona pellucida was discontinuous and folded inward toward the ooplasm. At this stage, occasional aggregates of amoeboid macrophages are seen, characterized by a small hyperchromatic, eccentric nucleus and abundant cytoplasm that contained a foamy to intensely eosinophilic material (also interpreted as phagocytosis of ooplasm) (Fig. 3A and B).

At middle stages of atresia, the follicle further diminished in size and the cells and the ooplasm was variably dispersed with amoeboid macrophages. At this stage the granulosa was more pleomorphic and markedly diffusely proliferated, invading the ooplasm. Granulosa cells showed marked intracellular and intercellular vacuoles and with some cells occasionally exhibiting mitotic figures. Occasionally, granulosa cells also showed intracytoplasmic intensely eosinophilic droplets (consistent with phagocytosed ooplasm). The theca interna also showed hypertrophy, characterized by numerous fibrocytes, fibroblasts, smooth muscle cells (SMA-α+) and small vessels, which lead to an increased overall thickness of the follicular wall. The zona pellucida was entirely lost at this stage (Fig. 3C-E).

Upon late stage atresia, the center of the follicle was entirely effaced by a high number of large cells with abundant foamy to vacuolated cytoplasm, that could be addressed as either granulosa cells or macrophages (Iba1+). The theca cell layer further hypertrophied and consisted of connective tissue, smooth muscle cells (SMA-α+), fibrocytes and fibroblasts. An increased number of small capillaries (Factor-VIII +) was also noted, extending from the theca into the center of the follicle (Fig. 3F -H).

3.5. Yolky atresia of vitellogenic follicles

Atresia of vitellogenic follicles could be morphologically divided in two forms, based on distinct gross and histological features, the yolky and the cystic type.

Grossly, yolky atretic follicles were yellow in colour and, compared to vitellogenic follicles, showed less prominent (hyperemic) thecal vessels and a beginning loss of turgidity (Fig. 1B). Histologically, this type of atresia could be divided into an early, middle and later stages.

Early stages of atresia were characterized by a beginning loss of turgidity of the follicle, indicated by a mild invagination of the follicular wall towards central yolk mass. The yolk nucleus substance in the follicle center at this stage mostly consisted of dense yolk granules and rare accumulations of eosinophilic pale amorphous fluid. The granulosa was characterized by cells that were cuboidal to polygonal in shape (hypertrophy) and that were arranged in multiple irregular layers along the follicular wall (hyperplasia). Granulosa cells were polymorphic, with distinct round to oval nuclei and an, often prominent, nucleolus. The cytoplasm showed frequent micro- and macrovesicular vacuolation and contained dark basophilic droplets (yolk droplets, indicating phagocytosis). A moderate number

Table 1
Stages and types of atresia in seven veiled chameleons (*Chamaeleo calyptratus),* based on histological examination. PVF: previtellogenic follicles, VF: vitellogenic follicles.

Animal Nr	Atresia						
	PVF			VF			
				Yolky			Cystic
	Early	Middle	Late	Early	Middle	Late	
1					x		x
2						x	x
3				x			x
4						x	
5	x	x		x			x
6					x	x	
7			x	x			

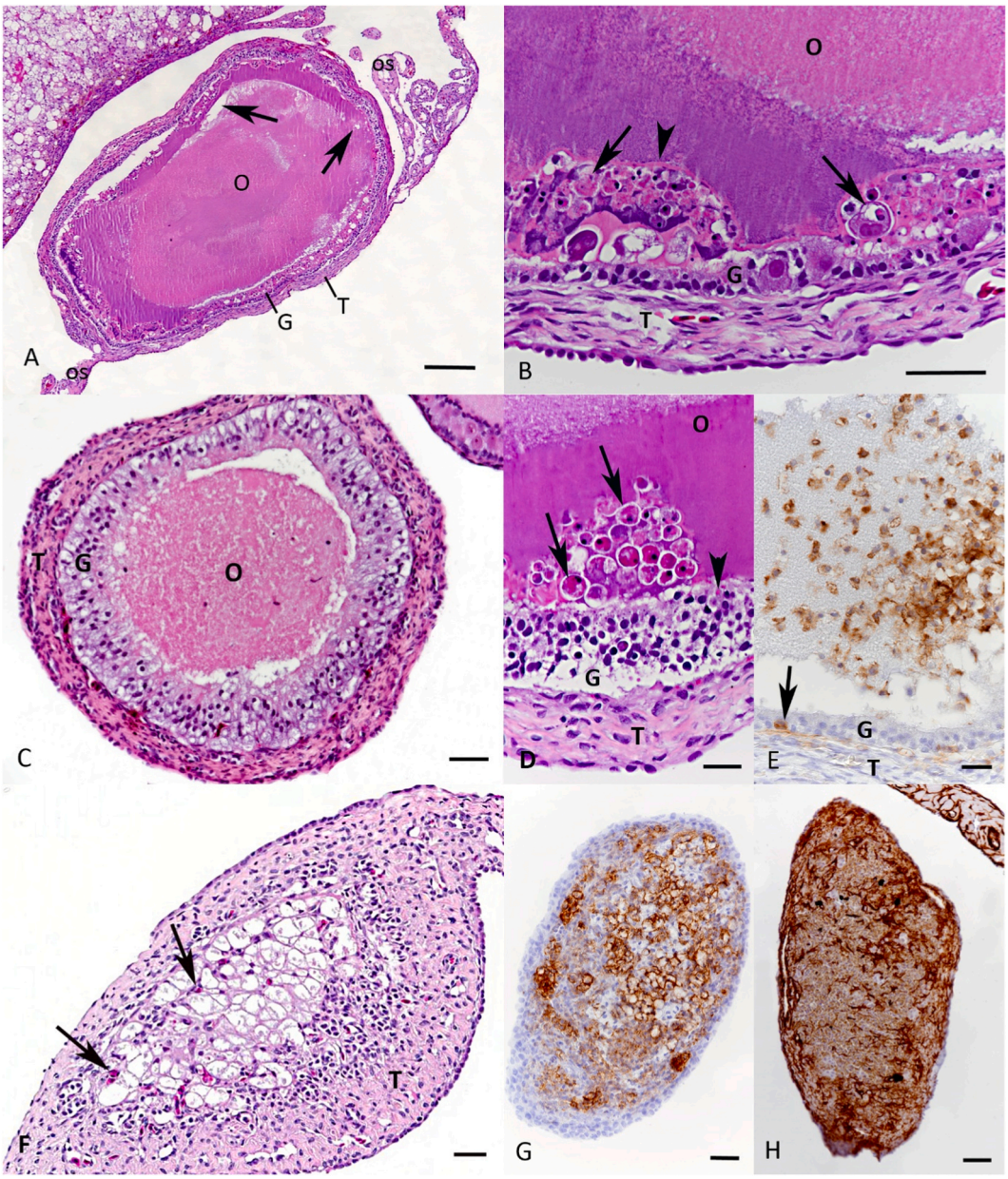

Fig. 3. Morphological features of atresia of previtellogenic follicles (PVF) in veiled chameleons (*Chamaeleo calyptratus*). A, B. Early stage atresia. A. The follicle shows a beginning shrinkage and irregularity (infolding) of the follicular wall. The ooplasm has lost its homogenous appearance due to multifocal areas of rarefication and vacuolization (arrows). HE stain, bar= 250 μm. B. The zona pellucida multifocally infolds towards the ooplasm (arrowhead) and the granulosa cells show beginning hypertrophy. A moderate number of amoebic macrophages lie underneath the zona pellucida (arrows) and occasionally show intracytoplasmic eosinophilic droplets (indicating phagocytosis of ooplasm). HE stain, bar= 50 μm. C-E. Middle stage atresia. C. The follicle shows progressed shrinkage and proliferation of both the granulosa and theca, the former gradually invading the ooplasm. HE stain, bar= 250 μm. D. The granulosa is diffusely hyperplastic and more pleomorphic, the granulosa cells display intracellular vacuolization and occasional intracytoplasmic accumulations of eosinophilic droplets, indicating phagocytosis of ooplasm (arrowhead). A high number of amoebic macrophages are also noted (arrows). HE stain, bar= 50 μm. E. Amoebic macrophages (Iba1 +) invade the ooplasm and multifocally the granulosa (arrow), Iba1, bar= 20 μm. F-H. Late stage atresia. F. The follicle shows a complete loss of the ooplasm, the center is replaced by a high number of large, foamy cells and small vessels extending from the hypertrophic theca layer (arrows), HE stain, bar= 250 μm. G. A large proportion of the foamy cells in the follicle center are of histiocytic origin (macrophages, Iba1 +). Iba1, bar= 20 μm. H. The theca consists of a high number of smooth muscle cells (SMA-α +) that invade the follicle center, SMA-α, bar= 20 μm. O: ooplasm, G: granulosa cell layer, T: theca cell layer, os: ovarian stroma.

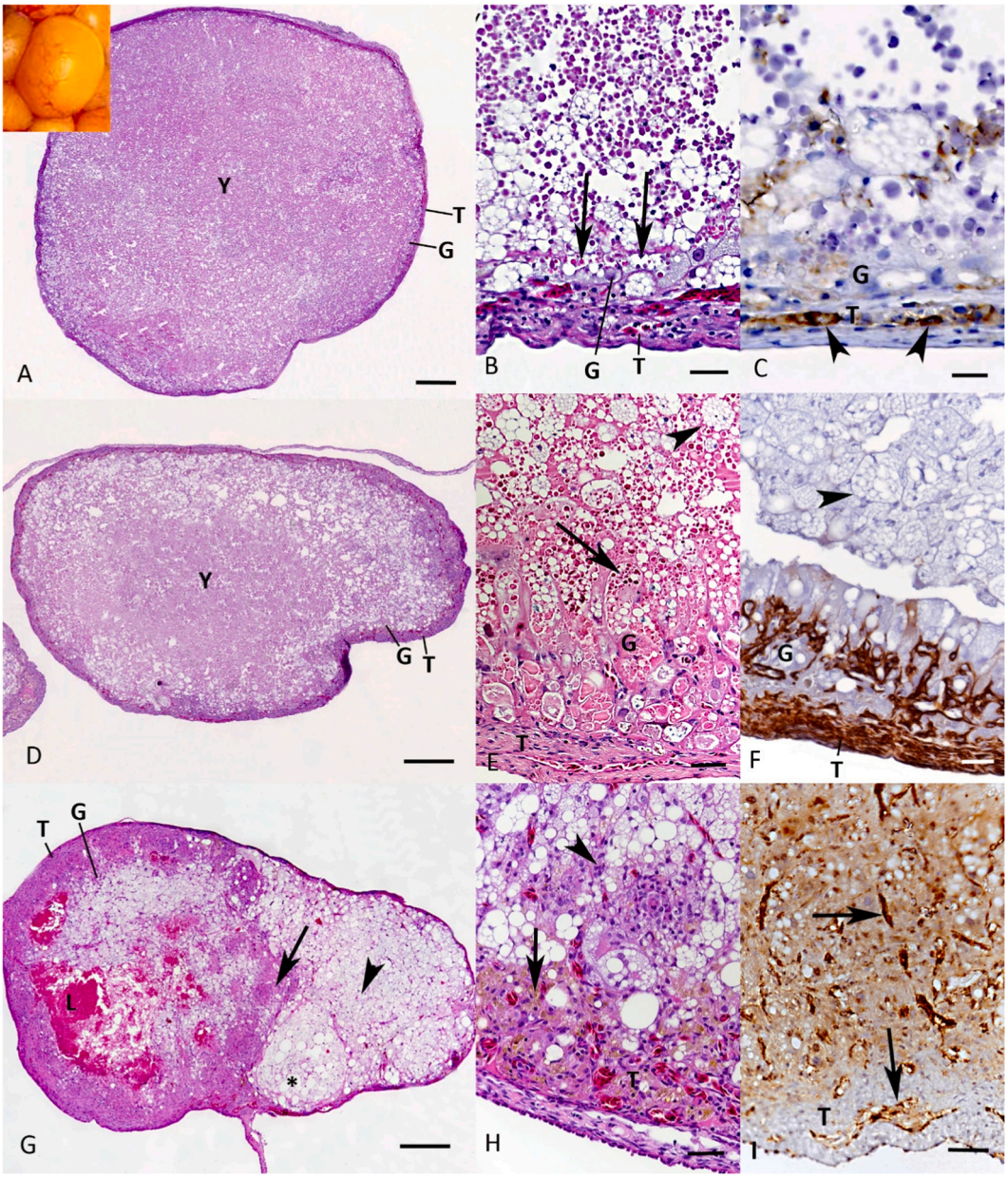

Fig. 4. Morphological features of yolky atresia of vitellogenic follicles (VF) in veiled chameleons (*Chamaeleo calyptratus*). A-C. Early stage atresia. A. The follicle shows a mild invagination of the follicular wall towards central yolk mass. The central yolk mass still consists of dense yolk granules. HE stain, bar= 250 μm. Inset: The yolky atretic follicle grossly shows a loss of turgidity. B. The granulosa cells are cuboidal to polygonal in shape (hypertrophy) and show frequent micro- and macrovesicular vacuolation and intracytoplasmic dark basophilic droplets (yolk droplets, arrows). The vessels of the theca are hyperemic. HE stain, bar= 25 μm. C. Macrophages (Iba1 +) are found either intravascularly in theca vessels (monocytes, arrowheads) or invading the granulosa and yolk substance (arrow), Iba1, bar= 25 μm. D-F. Middle stage atresia. D. The central yolk mass shows a decrease in the number of yolk granules and is increasingly replaced by a high number of optically clear variably sized vacuoles (lipid droplets). HE stain, bar= 250 μm. E. The granulosa is severely hyperplastic and pleomorphic (marked anisocytosis and anisokarryosis) and invades the central yolk mass. A high number of granulosa cells show accumulations of intracytoplasmatic basophilic droplets (indicating yolk phagocytosis, arrowhead). Abundant foamy macrophages invade the central yolk mass. HE stain, bar= 25 μm. F. The theca consists of a high number of spindeloid, elongated smooth-muscle-like cells (SMA-α +), that protrude into the granulosa. SMA-α, bar= 20 μm. G-I. Late stage atresia. G. The follicle is shrunken and shows an irregular outline. The yolk substance is entirely replaced by foamy enlarged cells (granulosa cells and macrophages, arrowhead), lipid droplets (asterisk) and amorphous acellular material (lutein mass, L). Note the thickened theca invading the follicle and forming small concentric "whorls" (arrow). HE stain, bar= 25 μm. H. A high number of macrophages with gold-brown intracytoplasmic granular material and small capillaries (arrowhead) are visible at the transition of the theca to the granulosa (arrow). I. Many small capillaries (Factor VIII+) extend from the theca and invade the granulosa. Factor VIII, bar= 25 μm. Y: yolk substance, G: granulosa cell layer, T: theca cell layer, L: lutein mass.

of foamy or vacuolated, enlarged macrophages (Iba1+) were also found interspersed in the granulosa and invading the central yolk mass. The zona pellucida was mostly absent or, if present, partially disintegrated. The vessels of the theca were hyperemic and often filled with round cells of histiocytic origin (monocytes, Iba1+) (Fig. 4A-C).

At middle stages, the central yolk mass showed a decrease in the number of yolk granules and increased accumulations of eosinophilic pale amorphous fluid (formation of lutein mass), admixed with a high number of optically clear variably sized vacuoles

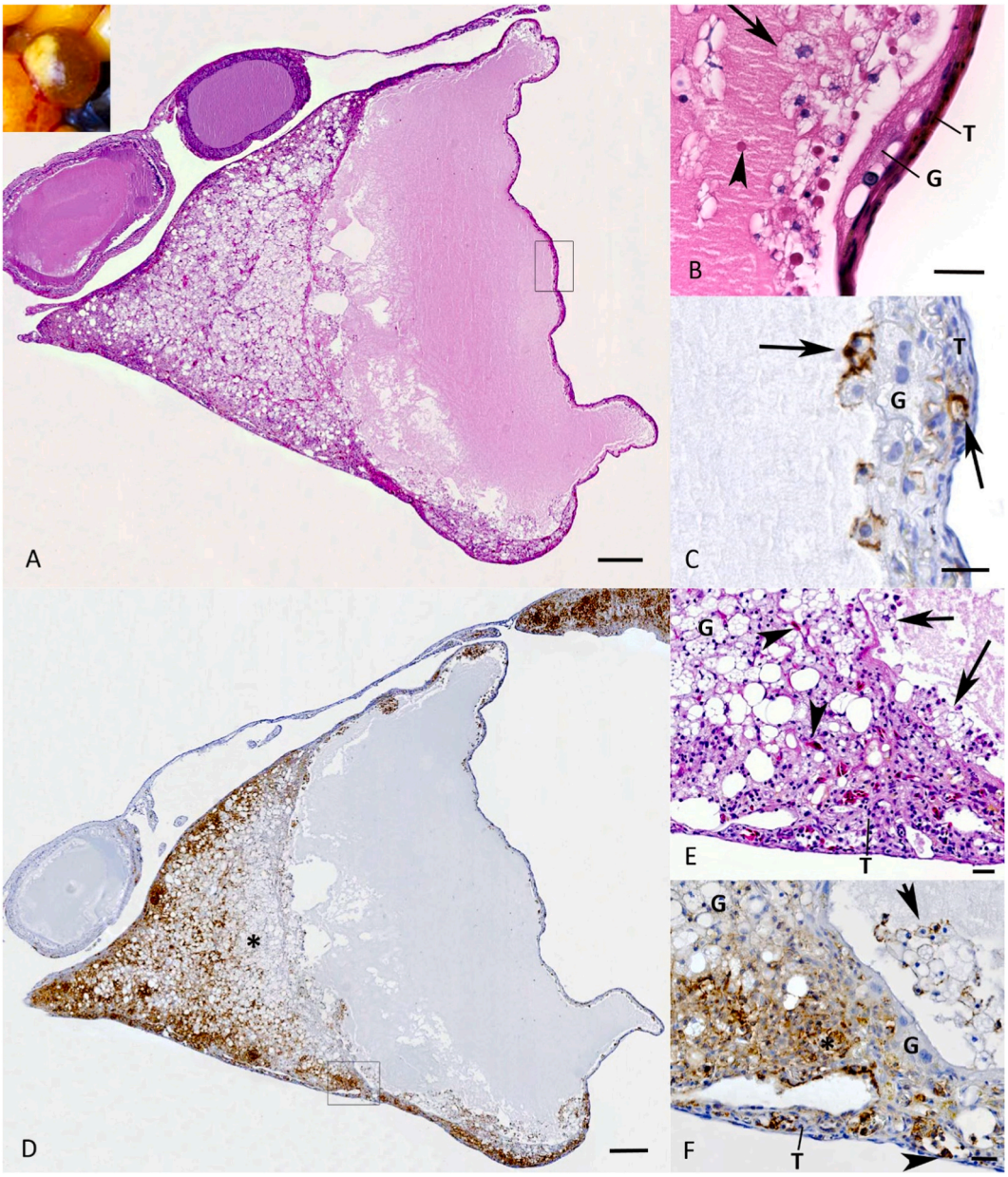

Fig. 5. Morphological features of cystic atresia (CA) of vitellogenic follicles (VF) in veiled chameleons (*Chamaeleo calyptratus*). A. The lumen of the cystic follicles is filled with a high amount of pale eosinophilic homogenous material. The follicular wall is mostly thin and collapsed (processing artefact), HE stain, bar= 250 μm. B. The thin-walled aspects of the cystic atretic follicle consist of a monolayered, occasionally vacuolated flat granulosa cell layer and a thin theca. A mild to moderate number of foamy macrophages (arrow) and few yolk droplets (arrowhead) are found in the central cytoplasm, HE stain, bar= 50 μm. C. Iba1 + cells are attached to the granulosa (macrophages, arrow) and/or in the lumen of thecal vessels (monocytes, arrowhead), Iba1, bar= 50 μm. D. The follicular wall is focally thickened by hyperplastic granulosa cells admixed with a high number of macrophages (asterisk), Iba1, bar= 250 μm. E. The hyperplastic granulosa consists of severely vacuolated, enlarged cells and is infiltrated by small capillaries extending from the theca (arrowheads). In the adjacent central cytoplasm free macrophages are seen (arrows), HE stain, bar= 50 μm. F. Many Iba1 + cells are seen inside (monocytes, arrowhead) and close to thecal vessels (asterisk), as well as in the central cytoplasm (arrow), Iba1, bar= 50 μm. G: granulosa cell layer, T: theca cell layer.

(lipid droplets). Both the granulosa and the theca showed progressing thickness and disorganization. Multifocally, the irregular hyperplastic granulosa invaded the central yolk mass, accompanied by numerous foamy macrophages (Iba1+). Granulosa cells showed increased pleomorphism (marked anisocytosis, anisokarryosis and strong variation in shape) and a higher degree of vacuolization. Also at this stage, the granulosa cells and the macrophages showed evidence of intracytoplasmic basophilic droplets (indicating yolk phagocytosis). The theca was thickened and consisted of a high number of spindeloid, elongated smooth muscle like-cells (SMA-α+) and fibroblasts, that multifocally started invading the granulosa (Fig. 4D-F).

Late stage atretic follicles showed prominent shrinkage and became increasingly irregular in shape. The central cytoplasm was partially to fully occluded by granulosa cells and/or foamy macrophages interspersed with some islets of strongly eosinophilic homogenous material (remnants of the lutein mass) and variably sized round optically empty spaces (lipid droplets). A high number of macrophages (Iba1+) with abundant gold-brown intracytoplasmic material were found in the theca and at the transition to the granulosa. The theca was severely thickened and consistent of dense connective tissue (mostly SMA-α+ cells) and small caliber vessels (Factor-VIII+), that increasingly invaded and protruded into the follicle center. Theca cells were frequently vacuolated and occasionally formed small “whorls” (Fig. 4G-I).

3.6. Cystic atresia of vitellogenic follicles

Grossly, cystic follicles showed a thin, translucent wall and contained a clear accumulation of watery to viscous, brown to orange fluid. In most of the cystic follicles a variably sized aggregate of white to bright yellow, firm material was noted, that was focally protruding from the follicular wall to the follicular lumen (Fig. 1B).

Histologically, the main features distinguishing yolky from cystic atresia were a noticeable difference in the content of the central cytoplasm and a marked overall variation in thickness of the granulosa cell layer. The lumen of the cystic follicles was mainly filled with a high amount of pale eosinophilic homogenous content. Multifocally, the material appeared more fibrillar or granular. Admixed with this material, yet to a noticeably lesser degree than in yolky follicles, a variable amount of dark basophilic droplets (yolk droplets) and free erythrocytes were seen. In larger cystic follicles, the center often appeared optically empty and the follicular wall was severely collapsed and wrinkled (most likely a processing artefact/rupture) (Fig. 5A).

The majority of the follicular wall was very thin, with the granulosa presenting as a flat to occasionally cuboidal monolayer of cells and the theca consisting of a thin rim of connective tissue (Fig. 4B). The granulosa cells showed with a variable amount of foamy to highly vacuolated cytoplasm and a variable number of intracytoplasmic yolk droplets (evidence of yolk phagocytosis). The nucleus was round to oval and, predominantly in the cells exhibiting evidence of phagocytosis, showed a hyperchromatic nucleus with densely clumped chromatin and a large nucleolus. In some areas the granulosa cell layer was histologically indistinguishable. Numerous foamy macrophages (Iba1+) were found irregularly attached to the granulosa cell layer or found lying freely in dense aggregates in the central cytoplasm (Fig. 5 B and C).

The grossly described focal aggregate of firm material protruding from the follicular wall presented histologically as a focally extensive area in which the granulosa was severely hyperplastic (up to 7 cell layers or more) and protruded into the central cytoplasm. The granulosa cells in this area were enlarged and polygonal, with highly vacuolated cytoplasm and also occasionally showed intracytoplasmic yolk droplets. Admixed with the hyperplastic granulosa cells were numerous macrophages (Iba1+), that also showed intracytoplasmic vacuolization or evidence of yolk phagocytosis. Iba1+ cells were also found at the transition of the theca to the granulosa in and around vessels (hence interpreted as macrophages and monocytes, respectively) (Fig. 5D-F).

3.7. No evidence of pathological findings

In all histologically examined VC (n = 7), FA represented a local process that was limited to the affected follicle. As mentioned above, with progression of atresia, an involvement of monocytes/macrophages (Iba-1+) was commonly observed. Invasion with those cells was in all cases solely noted during atresia (and absent in other follicular stages, such as vitellogenesis) and restricted to the follicular wall (granulosa and theca cell layer). Additionally, these macrophages frequently showed histological evidence of phagocytosis of yolk substance/ooplasm, supporting their primary phagocytic role in the process of yolk removal, as indicated in previous studies of reptiles and birds (Gupta and Duda, 1982; Gupta and Maiti, 1986; Moodley and Van Wyk, 2007).

In summary, all types and all stages of FA in the ovaries in this study showed no concurrent evidence of a pathological process (e.g. infiltration with inflammatory cells (heterophils etc.), oophoritis/coelomitis etc.).

4. Discussion

This study describes the macroscopic and histologic features of follicular atresia (FA) in veiled chameleons (VC). When compared to other non-vertebrate species with high incidences of follicular atresia, general morphological features and processes of FA are similar in the VC compared to other reptiles (Betz, 1963; Guraya, 1965; Goldberg, 1970; Varma, 1970; Saidapur, 1978; Moodley and Van Wyk, 2007; Morais et al., 2012; da Silva et al., 2018; Corriero et al., 2021). Elaborate categorization of various types of follicular atresia (lipoid versus non-lipoid, bursting versus in-situ, cystic, glandular, yolky, lipoglandular, liquefying, and involution versus invasion atresia), as described in previous publications in birds (reviewed in Gupta and Maiti, 1986), could not be applied to the VC of our study. In contrast, two types of morphologically distinct follicular atresia could be characterized in the VC: yolky and cystic atresia. Neither of the atresia types showed any association with pathogenicity; however, cystic atresia was only observed in older generations of follicles, i.e. in follicles at least 4 months into the process of resorption. The physiological mechanisms behind the less common, cystic form of

atresia could not be elucidated, but two non-exclusive hypotheses may be considered. Firstly, vascular constraint, due to stretching or compression secondary to dense conglomeration of several generations of AF and newly growing follicles, and/or reduced vascularization as part of the atresia process may lead to reduced activation of essential cellular components involved in the process of follicular atresia. This may not only reduce the stimulation of granulosa cells by hormones (e.g. FSH), which reach the follicle through the blood stream and passive diffusion though the theca layer (Goodman, 2009), but also impair the infiltration of blood-derived macrophages, the main phagocytic cell type involved in elimination of apoptotic cells during FA. Secondly, the effect of gravity and/or osmoregulation in larger AF may lead to differences in yolk density and therefore to the precipitation and separation of the yolky from the fluid content. The bi-directional close communication between oocytes (containing yolk protein) and granulosa cells is well-researched in mammals (e.g. mice), and it has been demonstrated that the former are capable of activating and inducing proliferation of granulosa cells (Su et al., 2009). Thus, the separation of fluid and yolky components in the center of cystic follicles in our study might be directly associated with the diminished hypertrophy and hyperplasia of the granulosa and, in turn, explain why the duration to complete resorption of cystic atretic follicles lasts longer than that of yolky atretic follicles (Cigler et al., 2023). It should be noted that pathological partial or full torsions of the ovarian pedicles produce a similar fluid-filled appearance of follicles on diagnostic imaging (Erokhina et al., 2021); however, these follicles are often filled with reddish, dark brown or murky green fluid (secondary to hemorrhage) and do not develop the clear precipitation observed in the cystic AF.

In birds, a peculiar form of “bursting atresia” has been described, affecting mostly large, vitellogenic follicles (Gupta and Maiti, 1986; Gupta et al., 1988; Islam et al., 2010). In this case, yolk leaks from a ruptured follicle into surrounding ovarian tissue or even extra-ovarian space and is phagocytosed by the endothelial lining of the stromal lacunae (Nili and Kelly, 1996). This chordolacunar system has also been described in crocodilian and chelonian species (Callebaut, 1988; Uribe and Guillette, 2000) but not in squamate species. The presence of bursting atresia needs to be considered controversial in reptiles with only two descriptions – in the lizard *Calotes versicolor* and the diamond-backed water snake *Natrix rhombifera* (Betz, 1963; Gouder et al., 1979). In this study, there was no evidence for a chordolacunar system or physiologic extra-follicular yolk resorption; therefore, any presence of ectopic yolk in VC would have to be considered pathologic, posing an imminent risk for inflammation in the coelomic cavity (Stacy et al., 2008; Gardner and Barrows, 2010).

In many avian and mammalian species, follicular atresia affects primarily previtellogenic follicles, facilitating the development of a follicular hierarchy (McGee and Hsueh, 2000; Johnson, 2015) and therefore limiting number of eggs in a clutch or embryo. In reptiles, FA may happen at various stages of follicular development: FA of primarly late previtellogenic stages serves to regulate clutch size (Varma, 1970; Guillette et al., 1981; Etches and Petitte, 1990; Uribe et al., 1995; Lozano et al., 2014; Santos et al., 2015), whereas FA of in the late vitellogenic phase serves to eliminate late batches of mature follicles at the end of the reproduction season in preparation for the quiescent reproduction phase (Altland, 1951; Telford, 1969; Goldberg, 1973; Gupta and Duda, 1982). While in our study animals, infrequent FA of previtellogenic follicles indicated the process of follicular selection, FA of entire batches of late vitellogenic follicles was observed randomly throughout the entire year, leading to long-lasting massive accumulations of several generations of large atretic follicles independent of reproductive quiescence (Kummrow, 2009; Pimm et al., 2015; Cigler et al., 2023).

Follicular atresia of developing follicles serving follicular hierarchy appears to be primarily regulated intrinsically (Gupta and Duda, 1982), while FA of mature vitellogenic follicles upon failure to ovulate may be triggered by external stimuli, in the sense of an adaptive process to suboptimal conditions for oviposition and offspring rearing (Crews and Licht, 1974). One intrinsic factor concerns the different patterns of reproduction (monoallochronic, mono- and polyautochronic). In polychronic species, the absence of follicular hierarchy does not require follicular atresia during follicular development, and the recruited number of primordial follicles largely reflects the number of preovulatory follicles (Jones et al., 1982; Shanbhag and Prasad, 1993), which is in line with our findings in VC. It has also been proposed that species with multiple germinal beds (GB) show a tendency to higher rates of follicular atresia (Jones et al., 1982). In this study, serial sectioning of both juvenile VC revealed the presence of two GB in each ovary. This finding complements the list of previously examined reptile species, revealing significant interspecific variations with numbers of GB per ovary ranging from one to countless (Jones et al., 1982; Jones and Summers, 1984; Shanbhag and Prasad, 1993; Callebaut et al., 1997; Shanbhag et al., 1998; Amey and Whittier, 2000; Stewart and Florian, 2000; Sica et al., 2001; Calderón et al., 2004). Multiple GB per ovary represent multiple germinal lines and result in larger numbers of recruited follicles; therefore, the atresia rate is naturally higher in species with multiple GB per ovary, particularly in combination with monochronic reproduction patterns (Jones et al., 1982). The observed comparatively low rate of atresia in PVF in VC expectedly conforms to a species which does not show monochronic reproduction pattern nor contains a large number of germinal beds per ovary. For the significant rate of atresia of mature VF, however, the study results do not offer any explanation and external triggers may have to be considered. One explanation may lie in the aseasonal husbandry of the studied VC populations. For standardized experimental reproduction research, all three research populations were kept under standardized conditions with constant temperatures, humidity and nutritional resources throughout the entire year (Kummrow, 2009; Pimm et al., 2015; Cigler et al., 2023). In the wild, VC exhibit seasonal reproductive patterns, with breeding and oviposition occurring towards the end of the rainy season and hatching of offspring at onset of the next rainy and resource-rich spring season (Necas, 1999; Diaz et al., 2015). In many commonly kept pet reptiles, seasonal quiescence is often not adequately addressed by respective seasonal environmental conditions (Pees et al., 2014). The lack of environmental triggers for quiescent phases in reproduction may be one of the reasons for year-round and seemingly random FA of entire batches of vitellogenic follicles; however, this is likely coupled with other factors, either intrinsic (i.e. hormonal), and/or extrinsic (i.e. environmental, husbandry-, and resource-related, etc.) which may induce atresia of mature follicles in place of ovulation.

In contrast to other studies, we did not identify any pathological inflammatory processes associated with FA (Stacy et al., 2008; Rowland, 2016; Kophamel et al., 2018; Cermakova et al., 2023). In clinical context, the accumulation of follicles undergoing atresia is commonly called pre-ovulatory follicular stasis (POFS) (Scheelings, 2008; Sykes, 2010; Miles, 2019; Cermakova, 2023), necessitating

surgical intervention (Naguib, 2018; Lock, 2000; Sykes, 2010). Based on our data, we refute the perception of a static nature of follicular atresia but provide evidence for a continuous resorptive process, of which the completion is rarely appreciated due to the long duration. We are aware of the increased risk of pathologic consequences of accumulated atretic follicles, such as coelomitis secondary to bursting follicles (Stacy et al., 2008; Gardner and Barrows, 2010); however, we strongly caution against the direct causal association of the presence of accumulated atretic follicles with clinical disease. Without the clear description of individual diagnosis and elucidation of respective pathogeneses, we suggest that clinical illness is rather linked to secondary processes than to a primary effect of FA. Many of the descriptions of ovarian pathologies in reptiles lack histological evidence (Pimm, 2013; Vatansever et al., 2018; Gandar et al., 2020), and when present, we scrutinize the presence of a pathological inflammatory process, e.g. "granulomatous oophiritis" (Kophamel et al., 2018; Erokhina et al., 2021; Cermakova et al., 2023). Instead, the presence of macrophages in FA is interpreted as part of a physiological resorptive process, supported by the widely known high heterogeneity and adaptability of macrophages, which gives macrophages a crucial role in tissue-specific function and organ homeostasis (Gordon and Martinez-Pomares, 2017).

5. Conclusions

Veiled chameleons show a low rate of FA of previtellogenic follicles which is in line with follicular selection in a species with only two germinal beds per ovary and a polyautochronic reproductive pattern. Follicular atresia of VF shows two distinct morphological presentations: yolky and cystic. While yolky atresia is more common, cystic atresia most likely represents a delayed but still physiological follicular resorption process. Despite the accumulation of atretic follicles and the lack of association with seasonal reproductive quiescence, the absence of pathological processes and apparent clinical health supports the physiological and likely adaptive character of FA of late VF to external triggers. Due to lack of stromal lacunae, however, any extra-follicular presence of yolk would have to be considered pathological in VC.

Animal welfare/ethical statement

Animal management and procedures followed local legislation and ethics committees in accordance with the Swiss Animal Experimentation Ordinance and followed the issued Animal Experiment permit Nr. ZH223/19, the Animals for Research Act of Ontario, the recommendations of the Canadian Council for Animal Care, and approved by University of Guelph Animal Care Committee and the Toronto Zoo Animal Care and Research Committee.

CRediT authorship contribution statement

Eva Dervas: Investigation, Methodology, Visualization, Formal analysis, Roles/Writing -original draft, review & editing. **Pia Cigler:** Investigation, Formal analysis, Roles/Writing - review & editing. **Jean-Michel Hatt**: Roles/Writing - review & editing, Project administration, Funding acquisition.**Maya S. Kummrow:** Conceptualization, Investigation, Resources, Formal analysis, Roles/Writing -original draft, review & editing, Supervision, Project administration, Funding acquisition.All authors have made substantial contributions to all of the following: (1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data, (2) drafting the article or revising it critically for important intellectual content, (3) final approval of the version to be submitted.

Declaration of Competing Interest

The authors declare no conflicts of interest.

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