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OVIDUCT, EGG, AND EGG SAC STRUCTURES IN THE SIBERIAN SALAMANDER, *Salamandrella keyserlingii* (CAUDATA, HYNOBIIDAE): A HISTOLOGICAL AND HISTOCHEMICAL STUDY

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Unraveling morphological and chemical features of the eggs' jelly layers and other clutch elements, formed by the oviduct of a female, is necessary for the understanding of reproductive adaptation in amphibians. Our study is the first microstructural and histochemical descriptions about the complexity of oviduct, ovisac, jelly layer of the ovum, and egg sac in hynobiid salamanders. We examined female *Salamandrella keyserlingii* in preovulatory (gravid) and ovulatory (spent) conditions using anatomical, histological, and histochemical methods. Each ovum was covered with two jelly layers. All ova from one oviduct fell into one egg sac. Inner space of the oviduct, in which ova were passed through, was filled with jelly-like substances designated as "intermediate fluids." We named the membrane-like outer layer of the egg sac as "theca." Jelly layers surrounding the ovum were formed by the oviducal tubular glands of the *pars recta* and *pars convoluta* of the oviduct, consisting of acidic and neutral glycoproteins. In the ovisac (i.e., homologous uterus), there were three glandular regions and one aglandular dilatable ovisac. Of the ovisac, glands A, B, and C secreted acidic and neutral glycoproteins for the intermediate fluids, neutral glycoproteins for the theca, and mucus-like materials with a neutral glycoprotein nature, respectively. We suggest that these mucus-like materials are essential to attaching the adhesive tips of the egg sacs to some substrates during oviposition.

Keywords: amphibians; salamanders; anatomy; female reproductive system; clutch; egg jelly; reproduction.

INTRODUCTION

Different reproductive modes of salamanders are associated with a variation of egg and clutch structures (Salthe, 1963; Altig and McDiarmid, 2007). This variety is possible due to the diversity of oviduct structure and functions (Wake and Dickie, 1998; Greven, 2003). In case of deposition of eggs, the structure and composition of egg jelly and clutch elements depend on the glandular subdivisions of the oviduct. Additionally, glandular and aglandular regions are combined in the oviduct. These structural features are different among taxa.

Members of the family Hynobiidae have some reproductive traits, distinguished from other salamander families (Poyarkov, 2010; Hasumi, 2015). A unique type of a full clutch, consisting of a pair of egg sacs that contain all eggs, is recognized in these traits (Salthe, 1963; Altig and McDiarmid, 2007). Several traits of egg sacs (e.g., type, coloration, nature of the sac surface) are different among hynobiids and thus are used for species identification (Matsui and Matsui, 1980; Nishikawa et al., 2008; Poyarkov, 2010). However, after oviposition, qualitative parameters of egg sacs change under influence of the absorption of water (Hasumi et al., 1994). Like other amphibians, the clutch formation in hynobiids occurs by the oviducts (Makino, 1934; Hasumi, 1996a; Wake and Dickie, 1998; Greven, 2003). In hynobiids, the oviduct maturation is a seasonal process. In female *Hynobius nigrescens*, mass of the oviduct plus ovisac fluctuates seasonally (Hasumi, 1996b). In *Salamandrella keyserlingii*, the same changes are described via quantitative analyses of the diameter of *pars convoluta* and width of the ovisac (Yartsev and Kuranova, 2015) and qualitative descrip-

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tions about anatomical and histological states of oviducts and ovisacs (Bulakhova and Berman, 2015). In these hynobiids, oviducts and ovisacs are hypertrophied before "spawning" (i.e., simultaneous extrusion of sperm and oviposition) during spring. Mass, diameter, and width of these oviducts and ovisacs decrease after spawning. Gamete maturation induces the initiation of increase in these structures during the second half of the long-term nonbreeding season toward the breeding season throughout the year. During the aquatic phase of the breeding season, ova are coated with jelly in the oviduct after ovulation. All jelly-coated ova accumulate inside the ovisacs after passing through the posterior part of the oviduct, i.e., *pars convoluta* (Makino, 1934; Hasumi, 1996a). The distal part of the ovisac, named the dilatable ovisac (Hasumi, 1996a), can storage a large number of eggs as the result of forming an egg sac.

Despite the existence of these data, mechanisms of oviductal jelly-secretion and egg sac formation have not yet been determined in hynobiids (for reviews, see Joly and Boisseau, 1995; Wake and Dickie, 1998; Greven, 2003; Altig and McDiarmid, 2007). The aim of our study was to examine histological and histochemical structures of the oviduct, ovisac, jelly layer of the ovum, and egg sac in *S. keyserlingii* for the understanding of a specific role of these structures and a process of egg sac formation in hynobiid salamanders.

MATERIAL AND METHODS

We examined 10 adult females of *S. keyserlingii* that were captured during the aquatic phase of the breeding season of April – May 2006, 2009, and 2013 in the vicinity of Tomsk (Southeast of western Siberia, Russia: 56°26′ N 85°00′ E; 150 m elevation), were fixed and preserved in 10% formalin, and were stocked in the Collection of the Department of Vertebrate Zoology and Ecology of National Research Tomsk State University. We measured snout-vent length (SVL, distance from the tip of the snout to the posterior angle of the vent) of these preserved specimens to the nearest 0.1 mm with vernier calipers.

We first described a gross appearance of ovaries and oviducts in female genital tracts. For histological and histochemical examinations, we used four females (SVL = = 52.3, 53.5, 55.6, and 57.4 mm): two were gravid (= preovulatory condition), one was ready for oviposition (= ovulatory condition), and one was after oviposition (= spent condition). We excised small parts of the *pars recta* and *pars convoluta* (lengths about 5 – 8 mm) of the oviduct and whole ovisac from the left side in all females. We also removed a part of one egg sac from the cloaca of an ovulatory female. We dehydrated specimens in ethanol, cleared in butanol, and embedded in paraffin. We made transverse sections at 5 – 7 μm with a rotary microtome. In addition, we prepared sagittal sections of the right ovisac of one graved female in order to verify the ovisac microstructure. We affixed sections to albumenized or SuperFrost slides. We stained slides with modified azan, Mayer's hematoxylin-eosin (AM and HE, respectively; for general histology), alcian blue at pH 2.5 (AB, for acidic mucous carbohydrates detection), periodic acid-Schiff's (PAS, for neutral carbohydrates), and Coomassie's Blue methods (CB, for proteins). All histological procedures followed Exbrayat (2013a, 2013b), but in some cases we replacednuclear fast red by Groat's hematoxylin. We observed stained sections with an Axio Lab A1 compound microscope (Carl Zeiss Microscopy, Germany). We marked positive histochemical reactions as "+" and negative as "–". Digital micrographs were obtained with an AxioCamERc 5s camera and ZEN 2012 software (Carl Zeiss Microscopy, Germany).

We used terminology by Altig and McDiarmid (2007) for designating of egg and clutch structures and by Greven (2003) for description of oviductal anatomy and histology. Followed by Hasumi (1996b), we named the distal elastic part of the ovisac as "dilatable ovisac." In addition, we designated the other part of the ovisac as "glandular ovisac." We termed the external membrane-like structure of the egg sac "theca" and inner jelly of the one "intermediate fluid."

RESULTS

Gross anatomy of the mature oviduct

Mean SVL of females was 61.3 mm (S.E. = 2.0, *n* = = 10, range = 52.3 – 69.8). The oviducts (= Müllerian ducts) stretched through the dorsolateral surfaceofthe body cavity. Each oviduct had a separate mesentery attached to the dorsal wall of the body cavity. From anterior to posterior direction, the mature oviducts were divided into the *pars rectum* and the *pars convolutum*. The *pars recta* began with the ostium tubuae, located near the heart sac region. The *pars convoluta* was the longest part of oviduct with a coiled form. The posterior part of this division was the ovisac, which was connected with the cloacal cavity. This part was wider than other part of the *pars convoluta*. In the gravid females, the wall of the anterior part of ovisac had the same coloration as other parts of the oviduct. Its middle part was lighter and denser, but the dilatable ovisac was rugate and transparent.

Gravid females in a preovulatory condition had greatly swollen oviducts and mature ovaries, the main

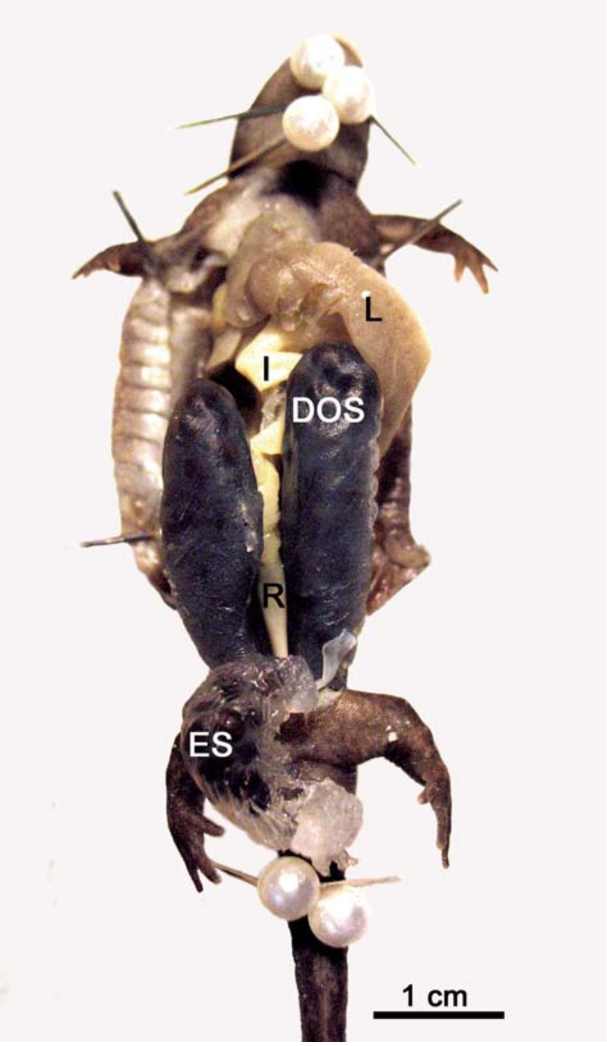

Fig. 1. Ventral view showing two dilatable ovisacs (each including an egg sac), one of which was partially extruded from the cloacal opening, in a female *Salamandrella keyserlingii* that was ready for oviposition. SVL = 55.6 mm; formalin fixed specimen. Abbreviation: L, liver; I, intestine; R, rectum; DOS, dilatable ovisac; ES, egg sac.

part of which contained many mature follicles with the surface of dark-brown coloration (their diameter was over 2 mm). A female during ovulation also had greatly swollen oviducts and ruptured ovaries with several remaining follicles and many immature small- and white-follicles. In this female, three ova (= mature oocytes) during migration toward the two openings of the oviducts were found in the space near the liver, one of the ovulated ova entered the right of the *pars recta* and two entered the left, three ova were in the *pars convoluta* of the left oviduct, and the other ripe ova were accumulated inside the two dilatable ovisacs (Fig. 1). Due to this accumulation, each dilatable ovisac increased in size. At this time, adhesive tips of the egg sacs were extruded from the cloacal opening. Size of the left ovary was 21.8 × 6.2 mm (length × width), and that of the right was 20.5 × 7.2 mm. Spent females after oviposition had ruptured ovaries composed of only numerous immature small- and white-follicles (up to 0.5 mm) and attenuated oviducts.

Microstructure and histochemistry of the mature oviduct

From the outside to the inside, the wall of the oviduct included the following layers: the squamous pleuroperitoneal epithelium, smooth muscle layers, vascularized loose connective tissue, and oviducal epithelium. In the *pars recta*, *pars convoluta*, and ovisac, the epithelium formed the tubular oviducal glands between which the connective tissue was stretched. Among the oviducal epithelium, three types of cells were detected: secretory cells of the tubular glands and cells, located around the opening of the tubular glands. Epithelial cells of this group were secretory or ciliated cells. The muscle layer was more pronounced in the ovisac.

Pars recta. In this part of the oviduct, there were only ciliated cells around the opening of the tubular glands (Fig. 2). These cells had a goblet-like shape. The ovoid, rob-shaped or triangular apical nucleus of these cells occupied much of the cell volume. The tubular glands formed the main part of the oviducal wall. In gravid females in a preovulatory condition, these glands produced CB+, PAS+, and AB- secretory granules (Fig. 2A and 2B). In a female during ovulation, these granules became AB+ in the process of granule evacuation from those glands (Fig. 2C). In a spent female after oviposition, tubular glands were empty (Fig. 2D). Their secretory cells were small. They had the vacuolated cytoplasm with AB+ staining reticular structures, residual secretory granules and large nuclei with irregular chromatin distribution.

Pars convoluta. Peripheral cells of the epithelial layer consisted of both ciliated and secretory cells (Fig. 3). These ciliated cells resembled the cells in the *pars recta*. The secretory cells had cubic, prismatic or pyramidal shapes. Their irregular nuclei were basal. The cytoplasm was filled with mucus-like materials, which stained PAS+, AB+, and CB+ (Fig. 3A and B, Table 1). These cells were developed in all the studied group of females (Fig. 3). The tubular glands were well developed in this part of the oviduct. Preovulatory females had greatly hypertrophied glands (Fig. 3A and B). They formed the largest part of the oviducal wall, and the connective tissue stretched as thin layer between them. The secretory cells of tubular glands were very large. Irregular nuclei of these cells were located at the basis. The

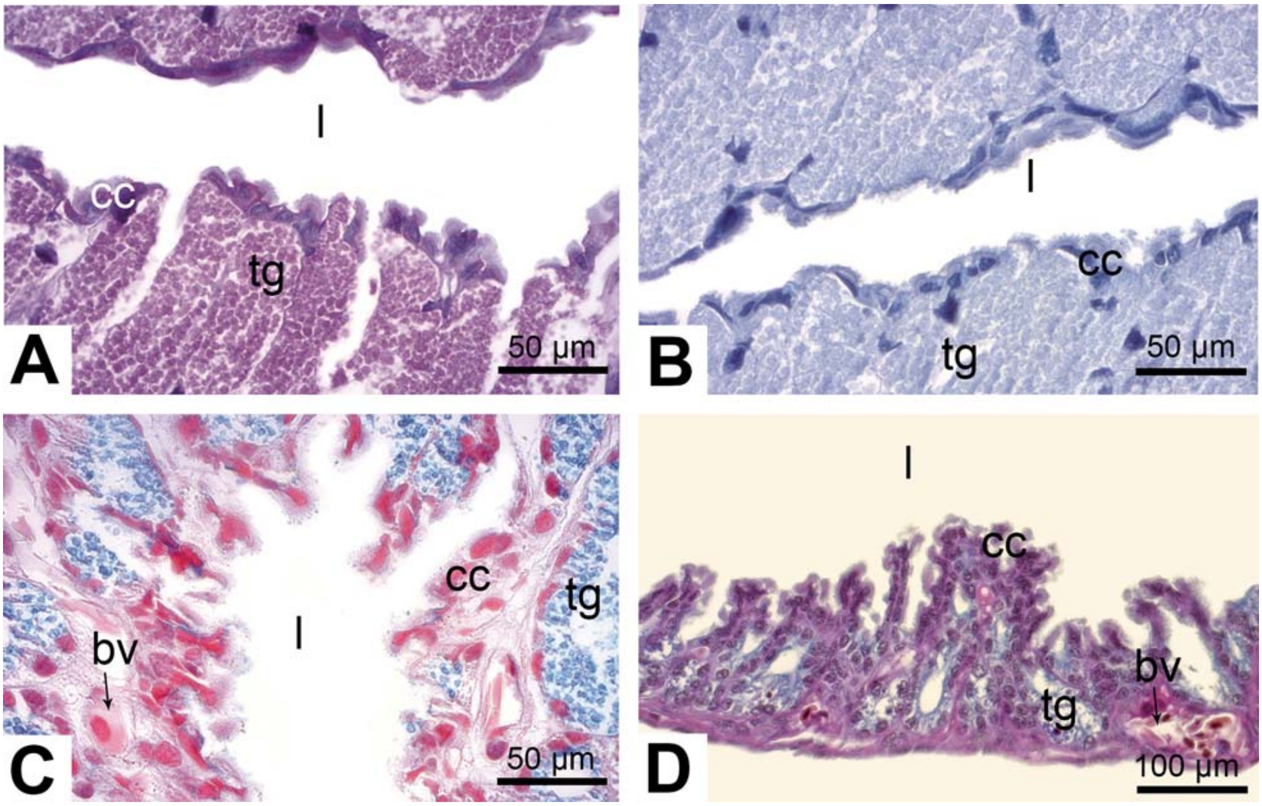

Fig. 2. Histological structure and histochemistry of the oviduct of *Salamandrella keyserlingii*: the *pars recta*. Transverse sections stained with PAS in A, AB in B and C, and AB+PAS in D. A, Apical parts of oviducal secretory folds in a gravid female. Secretory granules of tubular glands stained as PAS+; B, the same part of oviducal wall in a gravid female. Secretory granules of tubular glands stained as AB–; C, apical parts of secretory folds in a female during ovulation. Secretions of tubular glands stained as AB+; D, the oviducal wall in a female after oviposition. All tubular glands were empty. The AB+ reaction present in reticular elements of the cytoplasm of glandular cells. Abbreviation: bv, blood vessel; cc, ciliated cells; l, lumen of the oviduct; tg, tubular glands.

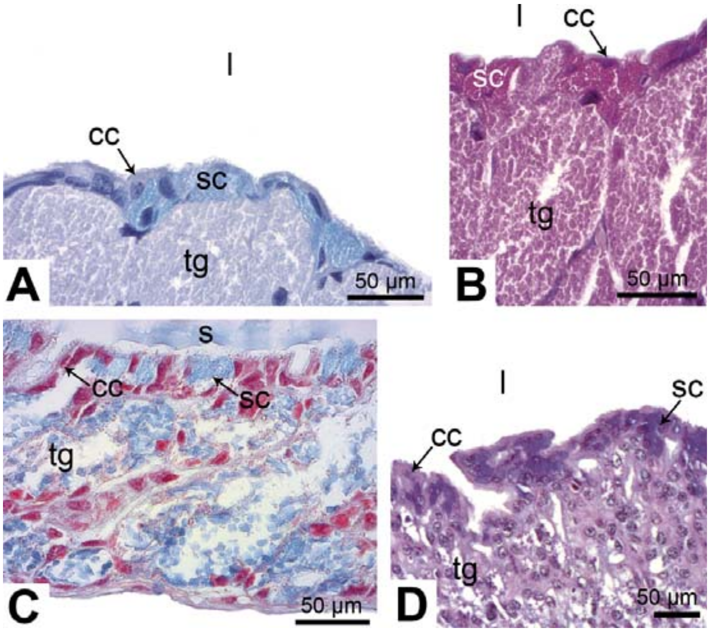

Fig. 3. Histological structure and histochemistry of the oviduct of *Salamandrella keyserlingii*: the *pars convoluta*. Transverse sections stained with AB in A and C, PAS in B, and AB+PAS in D. A, apical parts of oviducal secretory folds in a gravid female. Secretory granules of tubular glands stained as AB–, while secretory cells of the peripheral epithelium contained AB+ mucus; B, the same part of oviducal wall in a gravid female. Secretory granules of tubular glands and mucus of the peripheral secretory cells stained as PAS+; C, the oviducal wall in an ovulatory female. Both, secretory materials of tubular glands and mucus of the peripheral secretory cells stained as AB+; D, apical parts of secretory folds in a female after oviposition. Residual granules stained as PAS+ and AB–. Secretory cells of peripheral epithelium had PAS+ and AB+ staining. Abbreviation: sc, secretory cells of peripheral epithelium; other abbreviations as in Fig. 2.

TABLE 1. Histochemical Characteristics of the Structures of Oviducts, Ovisacs, Jelly Coats of the Ovum, and Egg Sacs in *Salamandrella keyserlingii*

	Category	Staining Reaction		
		PAS	AB	CB
Pars recta of the oviduct	Tubular glands	+	+/–*	+
Pars convoluta of the oviduct	Peripheral secretory cells of the epithelial layer	+	+/+*	+
	Tubular glands	+	+/–*	+
Ovisac	Tubular glands A	+	+	+
	Tubular glands B	+	–	+
	Tubular glands C	+	+/–*	+
Egg jelly	Inner layer (J_1)	+	+	+
	Outer layer (J_2)	+	+	+
Egg sac	Intermediate fluid	+	+	+
	Theca	+	–	+

* Gravid females in a preovulatory condition/a female during ovulation.

principal part of the cytoplasm contained numerous secretory granules that had been stained PAS+, AB-, and CB+ in gravid females (Fig. 3). In an ovulatory female, tubular glands actively excreted PAS+, AB+, and CB+ secretory products, which enveloped ova lying in the lumen of the oviduct (Fig. 3C). In a spent female after oviposition, a nature of tubular glands was not different between *pars convoluta* and *pars recta* (Fig. 3D, Table 1).

Ovisac. This part had a more complex structure in comparison with divisions of the oviduct. It was divided into two principal parts: the glandular ovisac and dilatable (aglandular) ovisac. Peripheral epithelial cells of these parts of the ovisac included only ciliated cells. In the anterior-posterior direction, the glandular ovisac contained three types of tubular glands, which were designated by “A, B, and C” (Fig. 4). Nuclei of glandular cells of all glands’ types had irregular shape and basal position.

The first region of the glandular ovisac (R_1) followed the *pars convoluta*. It had the tubular gland of the type A with large secretory granules, stained PAS+, AB+, and CB+ (Fig. 4A – C). Another type of glands was in the second region (R_2) of the glandular ovisac (Fig. 4D – F). In the R_2, the tubular glands of the type B had smaller secretory granules in comparison with those in the glands A. These granules were PAS+, CB+, and AB–. In gravid females, glands A and B of the glandular ovisac were filled with secretions, but they released the products in their lumens. In an ovulatory female, secretions were in the inner surface of the ovisac. The third region (R_3) of the glandular ovisac included tubular glands of the type C (Fig. 4G – I). Their glandular cells had mucous secretion and reticular structures in the cytoplasm. In females before ovulation, secretions of glands C were AB–, weak PAS+, and CB+. In the ovulatory female, reticular elements of the cytoplasm of glandular cells became PAS+, AB+, and CB+. In a female after oviposition, secretory cells of tubular glands had large nuclei, vacuolated cytoplasm with residual secretion material (Fig. 5B).

The dilatable ovisac was located posteriorly and had no glands (Fig. 5A). Here, there were clearly visible folds of the wall that were covered with ciliated cells.

Egg Jelly

In the *pars convoluta*, ripe ova (= mature oocytes) were coated with two jelly layers (Fig. 6A – C). Above the vitelline membrane, the first wide reticular-like layer occurred (J_1). It had AB+, PAS+, and CB+ stains, followed by the dense layer (J_2) with two different zones: one was AB–, PAS+, and CB+, and another was AB+, PAS+, and CB+.

There was an egg sac, including ova coated with jelly layers, in one dilatable ovisac where about half of a full clutch was formed. All ova in the ovisac possessed the same jelly-layer structure and histochemistry as those in the *pars convoluta* (Fig. 6D and E, Table 1). However, in these ova, zones of J_2 were indistinguishable and had obscure AB+ staining.

Egg sacs

The egg sac, shifted to the dilatable ovisac, was composed of two components. We designated the outer, dense, and membrane-like layer as “theca” for the first component (Fig. 7). The second component was the “intermediate fluid” with inner and jelly-like substances (Figs. 6D, E and 7). These two components wrapped the eggs transferred from one oviduct. The intermediate fluid was stained as PAS+, AB+, and CB+. The theca was

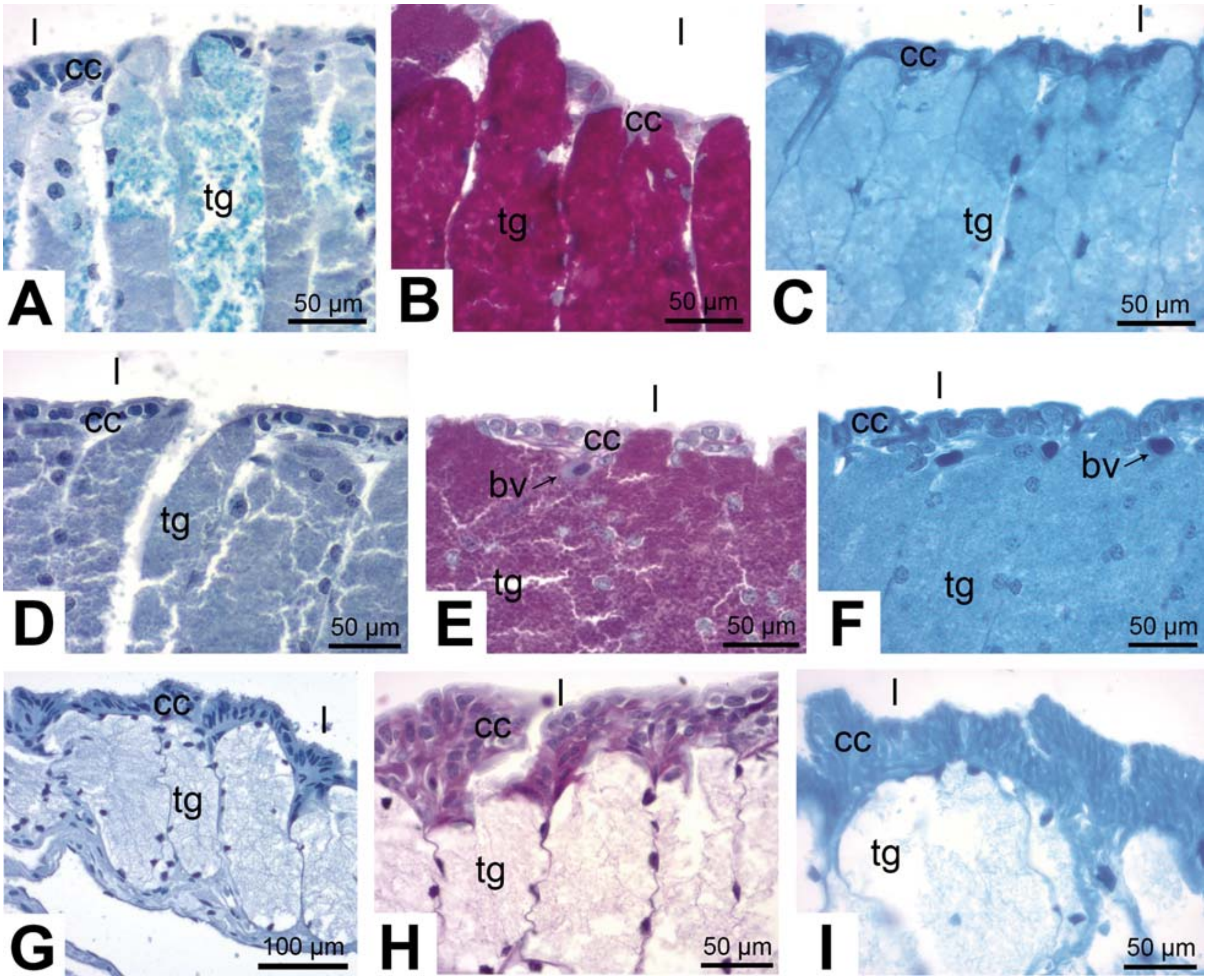

Fig. 4. Histological structure and histochemistry of the glandular ovisac in gravid females of *Salamandrella keyserlingii*. Sagittal sections stained with AB in A, D, and G; PAS in B, E, and H; CB in C, F, and I. A, The ciliated epithelium and tubular glands of the type A with AB+ secretory granules; B, the same region. Secretory granules stained as PAS+; C, the same region. Secretory granules stained as CB+; D, the ciliated epithelium and tubular glands of the type B with AB– secretory granules; E, the same region. Secretory granules stained as PAS+; F, the same region. Secretory granules stained as CB+; G, the ovisac wall with tubular glands of the type C, contained AB– secretory material; H, the same region. Secretory material stained as weak PAS+; I, the same region. Secretory material stained as CB+. Abbreviations as in Figs. 2 and 3.

characterized by different staining reactions: PAS+, AB–, and CB+ (Table 1).

DISCUSSION

The gross anatomy of the oviduct of *S. keyserlingii* (Yartsev and Kuranova, 2015; this study) is similar to the one of other hynobiids (Yamagiwa, 1924; Makino, 1934; Hasumi, 1996a, 1996b; Bulakhova and Berman, 2012). Gross anatomical observations of *H. retardatus* (Makino, 1934) and this histological study of *S. keyserlingii* indicate that parts of the oviduct have different functions. We found that the secretory unit of the oviduct of *S. keyselingii* was the tubular glands, which occurred in the *pars recta*, *pars convoluta*, and ovisac. Among the apical epithelial cells, the secretory cells were only in the *pars convoluta*, while the ciliated cells occurred in the entire oviduct. Glands of the *pars recta* and *pars convoluta* of the oviduct produced neutral and acidic glycoproteins forming jelly layers. In addition, Kusa and Ozu (1961) found fucose, mannose, and galactose in jelly layers of the eggs of *H. retardatus*, *H. nigrescens*, and *H. tokyoensis*.

The ovum of *S. keyserlingii* (= mature oocyte) is mesolecithal and telolecithal (Sytina et al., 1987; Yartsev

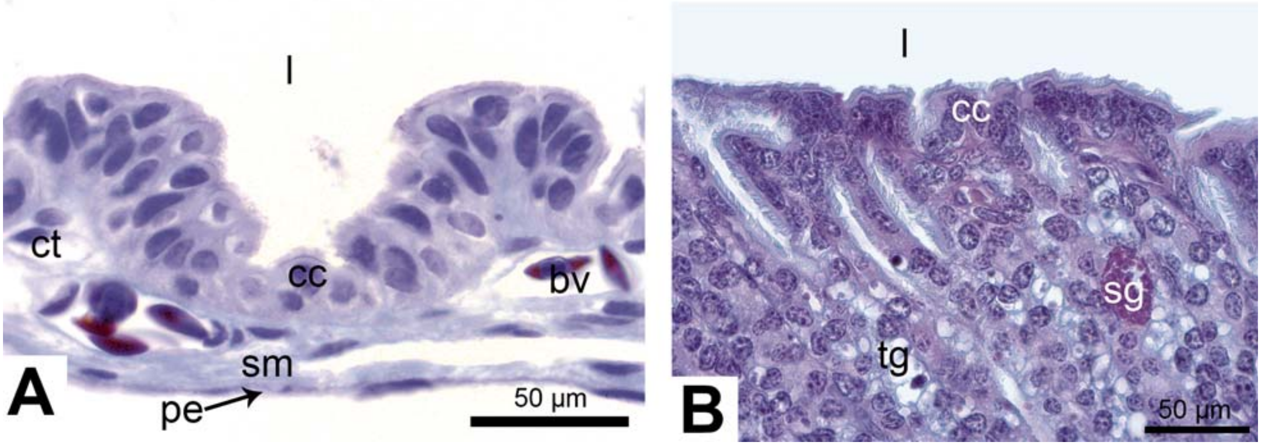

Fig. 5. Parts of the ovisac of *Salamandrella keyserlingii*. Sections stained with AM in A and AB+PAS in B. A, The dilatable ovisac of a gravid female. The peripheral epithelium includes only ciliated cells; B, the glandular ovisac of a female after oviposition. Secretory cells of tubular glands with reticular cytoplasm and residual secretory granules. Abbreviations: ct, connective tissue; sg, secretory granules of tubular glands; sm, smooth muscle layers; pe, squamous pleuroperitoneal epithelium; other abbreviations as in Figs. 2 and 3.

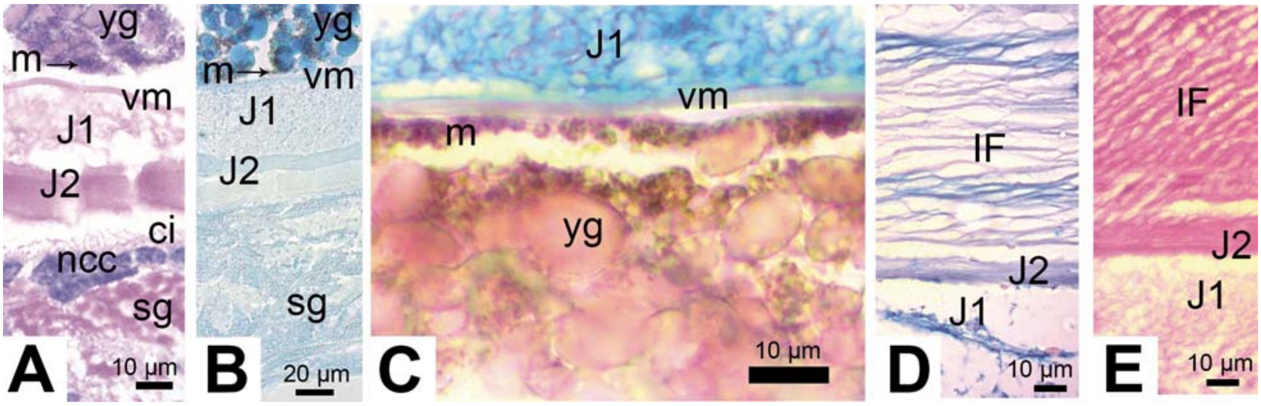

Fig. 6. Secretory materials around the ova (= mature oocytes) from different parts of the oviduct of *Salamandrella keyserlingii*. Transverse sections stained with PAS in A and E, CB in B and AB in C and D. A, Peripheral part of an ovum, two jelly layers, and inner part of the oviducal wall in the *pars convoluta* (from top to bottom). The vitelline membrane, jelly layers, and secretory granules of tubular glands stained as PAS+; B, the same region. The vitelline membrane, jelly layers, and secretory granules of tubular glands stained as CB+; C, inner jelly layer and peripheral part of an ovum in the *pars convoluta*. The vitelline membrane stained as AB–, while the inner jelly layer with strongly AB+ reaction; D, jelly layers and intermediate fluid of egg sac from the ovisac stained as AB+; E, the same envelopes stained as PAS+. Abbreviations: ci, cilia; ncc, nuclei of the ciliated cells; IF, intermediate fluid of an egg sac; J1, inner jelly layer; J2, external jelly layer; m, melanin granules; vm, vitelline membrane; yg, yolk granules; other abbreviations as in Figs. 2 and 3.

et al., 2015). In females before ovulation, its mean diameter is about 1.5 mm (based on measurements in histological sections of ovaries; Yartsev et al., 2015). The ooplasm are rich yolk granules and contain numerous small melanin granules in the submembranous zone. Ovulated ovum has only the vitelline membrane of ovarian origin. In hynobiids, the number of jelly layers surrounding the egg or ovum is debatable. In *H. retardatus*, Makino (1934) described only two jelly layers: inner "elastic opaque capsule" and outer "not so thick." Using paraffin embedding and histochemical staining, Salthe (1963) found eight jelly layers in *H. lichenatus*. In embryological study of *S. keyserlingii*, Sytina et al. (1987) designated two jelly layers in eggs from egg sacs. In our study, an ovum in the *pars convoluta* had three "layers," but two external "layers" of ovisac ova were less distinguishable. Since, "a 'layer' ... is a morphologically discrete, easily recognizable region of jelly surrounding the ovum" (Altig and McDiarmid, 2007), we suggest that the egg of *S. keyserlingii* has only two jelly layers. Differences inside the external jelly layer are associated with the presence of two zones (i.e., areas that are less easily visualized than layers; for terminology, see Altig and McDiarmid, 2007). We assume that some of eight jelly layers detected in *H. lichenatus* (Salthe, 1963) should probably be considered as zones.

The egg sacs are formed by the ovisac, where we found three types of tubular glands. Based on histochemical comparisons, we assume that the glands A produce neutral and acidic glycoproteins of the intermediate fluid of the egg sacs, the glands B make the theca consisting of only neutral glycoproteins, and the glands C have secretions that are histochemically different from secretory materials for the elements of egg sacs (Table 1). We strongly suggest that a function of glands C is forming materials for the adhesive tips of the egg sacs, which are attached to substrates for egg sac deposition, such as plant leaves, twigs, and woody debris. Our results do not therefore support the Park and Sung's (2006) suggestion that materials extracted from the ventral glands of the female cloaca play a role in attaching egg sacs to some substrates in *Hynobius leechii*. This is reinforced by proteins' nature of secretions, small size, and extreme location of R_3 with these glands. For this reason, the ovisac has a more specific microstructure in comparison with the parts of the oviduct. These histological data for *S. keyserlingii*, corresponding to those of the experimental study of *H. nigrescens* (Hasumi, 1996a), indicate that the formation of egg sacs progresses with a complex process. In this species, egg sac making lasts for 50 h, whereas the passage of all eggs after ovulation into the ovisacs takes 32 h. Hasumi (1996a) also has stated that the dilatable ovisac is unique for hynobiids. In addition to this state, we can suppose that the whole ovisac as well is a structure unknown in other salamanders because it included three types of tubular glands. In salamanders from suborders Salamandroidea and Sirenoidea, the caudal portion of the *pars convoluta*, called "ovisac" (i.e., homologous uterus: see Hasumi, 1996a) has more simple structure, than the ovisac in hynobiids. The ovisac of sirenids is simple and has no tubular glands, but the ciliated cells occur in this part of the oviduct (Sever et al., 1996). In the suborder Salamandroidea, tubular glands are present in the uterine portion of some oviparous salamanders, but they are absent in other species (Greven, 2003). Viviparous salamanders do not have ciliated and secretory cells in the uterus (Greven, 2003). In conclusion, we affirm that both the egg sacs and the ovisacs are reproductive traits specific to hynobiid salamanders.

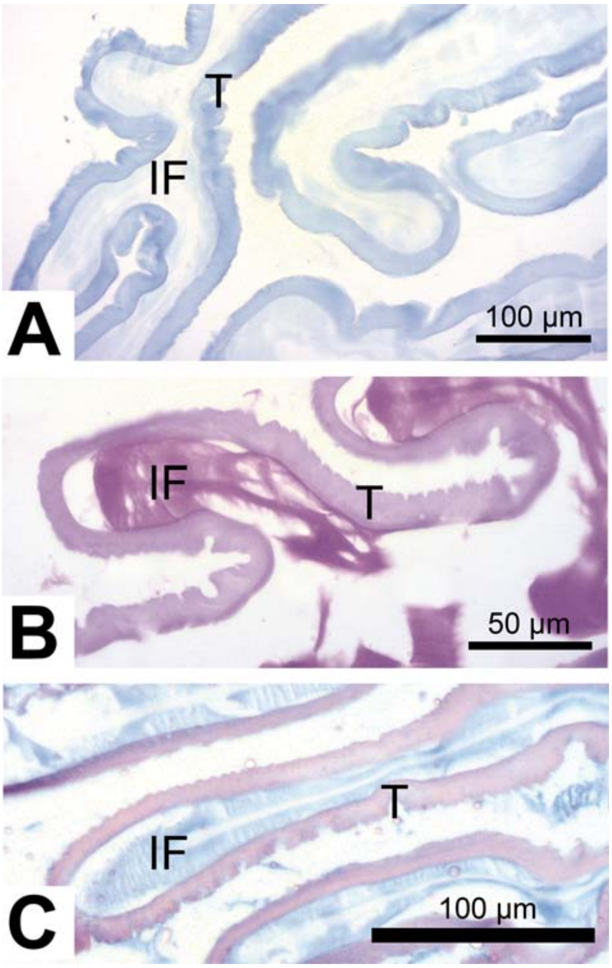

Fig. 7. Histochemical staining of the egg sac components of *Salamandrella keyserlingii*. Sections stained with CB in A, PAS in B, and AB in C. A, CB+ staining of the intermediate fluid (IF) and theca (T); B, both egg sac elements with PAS+ staining; C, different AB– reactions in the egg sac components: the intermediate fluid stainedas

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