HISTOCHEMICAL AND BIOCHEMICAL CHARACTERIZATION OF OVIDUCTAL SECRETORY GLYCOPROTEINS AND THE EFFECT OF PROGESTERONE ON GLYCOPROTEIN MODIFICATION IN *Rana tigerina*

INTRODUCTION

At present, several economic animals in Thailand such as chicken and shrimp have been attached with many epidemic problems leading to an export banning. Rural rice field frog (*Rana tigerina*), as its preferable taste and much lower epidemic problem, should thus be considered to replace those sources of meat and promoted to be an important economic animals for the routine practice in the future. The import value of frog meat to many targeted countries, e.g., Hongkong, Japan, Malaysia, Singapore, Germany and France and has been increased annually. While the demand of frog meat in the domestic and international markets become higher, the supply of frogs especially those caught from the natural habitats, is severely declined. Disruption or destruction of ecological environment suitable for frog breeding renders more severity to the declination of frog's population. The higher supply of these economic animals can only be achieved by introducing a good practical system to the farmers, along with applicable biotechnology.

The increasing demand of *R. tigerina* has made it a challenging animal to be raised in culture for the development of breeding and mass production. The total gross production of the frog is still far inferior from the market demand. One possible reason would be the lack of the fundamental knowledge about frog's reproduction and its endocrinological control. To the best of our knowledge, there are very few reports on *R. tigerina's* reproduction available. Weerachatyanukul *et al.* (1993), Prachaney (1996) and Nakiam (1994) have reported the morphological studies of the ovary, oviduct and testis during breeding and non-breeding period in this species. In this study, we aimed to extend the more insightful knowledge in oviductal secretion and its influence by sex steroid hormone. Oviduct of *R. tigerina* is composed of two major

parts; pars recta (PR) and pars convoluta (PC). Secretions from these two parts are jelly-like substances which are enriched in carbohydrate compositions and serve many crucial functions during fertilization. Alonso-Bedate (1976) revealed that progesterone was the most effective steroid hormone that stimulates the jelly release of *Rana ridibunda* oviducts. Despite the importance of these jelly secretions, the characterization of their sugar moieties is scarcely available. This prompted us to study properties of carbohydrates in the oviductal secretion in *R. tigerina* during breeding, non-breeding and progesterone-priming periods, using lectin histochemistry as a tool. The obtained information will lead us to understand the application of progesterone in controlling the process of oviductal secretions, mimicking the natural process, which can be utilized to improve frogs' reproduction in the aspect of their breeding.

OBJECTIVES

1. To characterize the sugar moieties of glycoprotein substances in all portions of the oviductal secretions using various lectin staining.

2. To identify the cells types that are involved in jelly secretion as well as their distribution in each part of oviduct.

3. To determine the progesterone effect on the patterns of glycoprotein secretion in the jelly composition of oviduct.

LITERATURE REVIEW

1. Taxonomy and biology of *Rana tigerina*

A rural rice field frog, *R. tigerina*, occupies a taxonomic position within:

Phylum Chordata

Class Amphibia

Order Anura

Superfamily Ranoidea

Family Ranidae

Genus Rana

(Taylor, 1962; Webb *et al*., 1981)

Rana tigerina, a native frog, is one of the most abundant species in Thailand. These frogs are found in ponds and rice fields throughout the country. The appearance of the head of this species is triangular, having a greater side to side diameter than longitudinal one. Skin of the front head is smooth and appears olive-brown. The female frog is bigger than male. Adult mature female weighs about 250 to 300 g whereas male weighs approximately 150 to 200 g. In contrast to the disparity in the weight, the length between snout to vent in both female and male is relatively similar (11-13 cm). The width of abdomen in female during non-breeding period is ~ 6-8 cm while it is ~ 5 cm in male (Taylor, 1962). However, the abdominal width in female can be increased drastically when its ovaries reach maturation and fill with millions of mature oocytes. Male frog can be distinguished from female by three sexual characteristics; 1) the large pink thumb pads which change thickness and color intensity during breeding season, 2) more slender body than that of female (Nakiem, 1994), and 3) a low guttural croaking sound made by the two lateral enlarged air vocal sacs located under the side of the head (Taylor, 1962).

2. Gross anatomy and morphology of the oviduct

Oviduct is the major female reproductive organ that provides transparent gelatinous-like substances overlaying the ovulated eggs. These substances from all

parts of oviducts, collectively called “jelly coats” are deposited in several layers during the passage of eggs down in the oviducts. In young female frogs, oviducts are quite straight, thin-walled, and small calibre (Ecker, 1889). In mature females, oviducts become convoluted tubules that lie over both sides of kidneys and sometimes part of the ovaries. The ducts are attached to the body wall by dorsal mesenteries. Oviducts appear differently among amphibian species; straight in caecilians, slightly convoluted in salamanders and greatly convoluted in anurans (Duellman, 1985). The anterior ends of the oviducts (ostia tubes, ostium) are semilunar slits directed inwards and open dorsally into the pleuro-peritoneal cavity (Christensen, 1931; Ecker, 1889; Warren and Walker, 1967). Behind the ostium of each oviduct, the slender duct maintains its even size for a short distance and then become convoluted until it reaches the base where it expands into the uterus (Underhill, 1969). During the breeding season, alternative of oviductal structure is apparent in both the size and convolution which is believed to be influenced by intrinsic hormonal level (Ecker, 1889).

Oviducts of amphibian are divided into various regions based on different criteria. Based on the gross and/or histological appearance, oviduct of *R. pipiens* can be divided into three parts: anterior straight portion, pars recta (PR); a middle convoluted part, pars convoluta (PC); and the thin-walled posterior portion, pars uterus (PU) (Christensen, 1931). Using the same criteria, oviduct of *Xenopus laevis* can be divided into seven portions: the uppermost, thin-walled, ostial portion. Next to the ostial portion is the PR portion which can be further subdivided into PR1 and PR2. Subsequence to the PR is the main body of the oviduct, the PC, which can be further subdivided into four portions, PC1, PC2, PC3 and PC4, according to their color differences and sizes. The color of PC1 is grayish and that of PCs 2-4 is milky white. The size of these PC portions is apparently larger in PC1 and PC3, while they are smaller in PC2 and PC4 (Yoshizaki, 1985). Similar criteria have been applied to *Rana tigerina* (Prachaney, 1996), with a minor difference in PR portion. Briefly, the oviduct of *Rana tigerina* can be divided into 5 parts based on external and internal characteristics: the anterior straight portion called pars recta (PR) and the highly coiled portion named pars convoluta (PC) which is further subdivided into PC1, PC2, PC3 and PC4. The proximal part of PC1 is small and gradually increases in size in the

middle region and becomes smaller in the distal part as it enters PC2. PC2 is generally smaller than PC3. The size of PC4 gradually decreases again throughout this portion, and at the distal end of PC4, the oviductal wall is thinner and expanded to become the ovisac. The oviduct of *Bufo melanostictus* can be classified into four distinct regions based on their gross morphology. The proximal end of the oviduct is called infundibulum, which is funnel-shaped. The infundibulum continues into a convoluted tube which progressively enlarges towards the posterior portion. Because of its large size, it is thus called "magnum" and can be divided into the upper, middle and lower magnum, respectively. The lower magnum continues into a short, straight, narrow tube, so named isthmus, and the distal-most ovisac (Low *et al.*, 1967). The authors also demonstrated the function of each part of the oviduct in contributing the jelly layers on the egg strings in this species. Eggs taken from the coelom and the proximal infundibulum possess no jelly coat, whereas eggs taken from distal infundibulum and upper magnum are covered with a single layer of jelly. The second and third layers of jelly are acquired as the eggs passed through the middle and the lower magnum, respectively. No jelly is added to eggs in the ovisac. In *R. pipiens*, the oviduct can be divided into six jelly-secreting regions, anterior region 1 to posterior region 6, based on the differential staining of the jelly gland. Its size becomes wider from the anterior to posterior end. These regions correspond to the layers of jelly deposited to the eggs as they pass the oviduct (Shiver and James, 1970).

3. <u>Histological studies of the oviducts</u>

In *R. pipiens*, ostium at the superior straight portion of the paired oviduct is consisted of a folded mucosa, lined with a single layer of columnar epithelial cells. The epithelial layer is mainly composed of ciliated cells. This region lacks tubular glands and is enclosed by serosa. Tubular glands and longitudinal folds can be found in the PC region. The height and branching of the folds diminish as the oviduct progresses towards the posterior part of the PC region. Tubular glands initially appear in the early PC portion and increase in depth and basal diameter in the more distal portions. The ciliated and mucous producing cells are lining the lumen of PC portions forming a ridge-pattern and running longitudinally throughout the PC region (Lee,

1967). Lying in between these ridges are the openings of the tubular glands. Two types of cells, small ciliated cells and large vesicular cells, have been found in the anterior portions of the oviducts. Also in the anterior one-third of the oviducts, the epithelia form the well-recognized ridges which extend longitudinally. These ridges are gradually lower posterior and become smooth at the distal-most portion. Interestingly, the epithelial cells lining the ridges become larger in size and the vesicular cells are more prominent from the anterior towards posterior, corresponding to the decrease in diameter of the oviducts. Cilia extending from epithelial cells are more numerous in the anterior two-third of oviduct than in the posterior region (Shiver and James, 1970).

In *R. tigerina*, Prachaney (1996) reported that the oviduct is composed of three layers; mucosa, muscular layer and serosa (a thin layer of peritoneum). Pars recta (PR) is characterized by the appearance of long and branchy mucosal folds. The mucosa of distal PR is more extensively folded than those in the proximal part. These folds are lined with a simple columnar epithelium which is composed of two cell types, ciliated cells and non-ciliated cells. Two major characteristics of PC's mucosa are longitudinal ridges of epithelia and the abundancy of mucosal glands. The mucosal ridges are lined with the simple columnar epithelia and contain two types of cells, ciliated and non-ciliated cells, similar to those of PR. PC1 and PC2 are relatively similar in histological structures both in ridge-like mucosal folds and glands. The mucosal glands of PC3 are different from those in PC1 and PC2 as they are consisted of various heterogeneous secretory products. Unlike the other mucosal glands in the proximal PC portions or in other species, mucosal glands of PC4 possess a unique feature in which they contain two types of mucosal glands; the upper serous-like and the lower large-mucosal glands. The two mucosal glands stained differently with toluidine blue; the upper one reveals stronger metachromatic reaction than the lower one, suggesting a different carbohydrate property in these two glands.

4. The property of jelly

The jelly, secreted from the oviducts to enclose ovulated eggs, possesses many important functions during fertilization. These jelly coats help maintaining the ionic balance during sperm penetration and also protect the developing embryo from mechanical injuries, from fungi and other infections after fertilization. Moreover, they also conserve the heat produced by metabolism of the embryo during development (Warren and Walker, 1967; Rugh, 1951). The jelly is a complex organic secretion made up chiefly of carbohydrates and to a minor extent of proteins. The carbohydrate moieties are highly variable within the jelly with the majority being both acid and neutral mucopolysaccharides (Hamphries and Haghes, 1959; Shivers and James, 1970). Considering the great variable chemistry of this jelly secreted by the oviduct, it is interesting to know that these various constituents of jelly are secreted from which part(s) and which cell types of the oviduct. Histochemical observation on amphibian oviductal glands and epithelium has revealed the jelly's contents and its properties. In *B. melanostictus*, periodate-reactive and diastase resistant mucosubstance can be found along the entire length of the epithelium from the infundibulum to lower magnum (Low *et al*., 1967) confirming the enrichment of carbohydrates in the cells producing and secreting jelly. In *R. cyanophlyctis*, components in the jelly glands and luminal epithelium exhibit neutral polysaccharide and protein containing tyrosine residues, sulhydryl and disulfide groups according to positive reactions with periodic acid-Schiff stain (PAS), Millon's reaction, and alkaline tetrazolium reaction, respectively. Moreover, based on the positive staining with alcian blue jelly glands are proven to produce acid mucopolysaccharides which are detectable in non-ciliated cells (Suvarnalatha *et al*., 1975). Histochemical staining of the oviducts of *R.tigerina* has revealed metachromasia reaction with toluidine blue and positive staining with alcian blue in non-ciliated cells and mucosal glands of PC1, PC3 and upper mucosal gland in PC4, suggesting that the oviductal epithelium of this species also synthesize and secrete acid mucopolysaccharide substances (Prachaney, 1996).

5. Seasonal variation in the oviduct

Reproductive patterns of most animals, particularly cold-blooded vertebrates are correlated with the environmental fluctuation. Slight annual variation in the environment, such as rainfall, photoperiod and temperature may interfere the breeding cycles. Annual variation in the precipitation seems to be the main factor inducing periodicity of breeding activity. In equatorial habitats with a constantly warm and humid climate, amphibians may reproduce throughout the year, for example, the frogs, *Rana erythrses* in Borneo (Inger and Greenbery, 1956) and the toads, *B. melanostictus* in Singapore (Berry, 1964).

In Thailand, breeding period of *R. tigerina* has been reported to be during April to September based on the histology of testes and numbers of staging oocytes in the ovaries (Nakiem, 1994; Sretarugsa *et al.*, 2001). Moreover, the high concentrations of sperms during spermiation observed during April to September have supported the timing of breeding period in this species (Vichatrong, 1996). The annual variation in the weight of the oviducts has been found to be secondary to the ovarian cycle both in urodeles (Adam, 1940) and anurans (Jørgensen and Vijayakumar, 1970). Corresponding to this observation, the oviducts of *R.tigerina* have shown to decrease in size and weight during September to February which is the non-breeding season (Prachaney, 1996).

6. The hormonal control of the amphibian oviduct

The oviducts of annually reproducing urodeles and anurans undergo a cycle which is secondary to the ovarian cycle. Thus, their weight falls abruptly during spawning and increases slowly during subsequent months. It has been reported that ovariectomy in the amphibians has resulted in growth retardation and atrophy of the well-developed oviducts, while exogenously administered estrogens has led to oviduct regeneration (Lee, 1967). The annual variation in oviductal weight in mature amphibians reflects the amount of secretory granules present in the glands. These glands are depleted during spawning since they are discharged into the lumen as the

jelly substances to coat the passing eggs (Lee, 1967). The depletion, thus, depends on the number of eggs which pass through the oviduct (Jørgensen and Vijayakumar, 1970). The female toad lays each year a large number of eggs which are enveloped with jelly; the outermost transparent layers around the ovulated eggs, as the eggs pass down the oviducts. Several observations have indicated that jelly release in the toad is provoked by an ovarian hormone, and analysis of the cause of jelly release seems to give insight into the endocrine activity of anuran ovary around the point of ovulation. In the toad, *Bufo bufo*, jelly secretion is initiated by an ovarian hormone which is released in response to pituitary stimulation, and this hormone is probably the same progestin which is responsible for ovulation and maturation (Thornton and Evennett, 1969). The mode of action of gonadotropic hormones in causing jelly release has indicated that although hCG does not cause jelly release in ovariectomized toads, the castrated animals 12 hours after hCG injection release some jelly from the oviducts, while those operated on 18 hours after injection give the normal response. Thus, the oviductal stimulus appears to arise from pre-ovulatory rather than post-ovulatory follicles. This finding has been confirmed by the demonstration of a peak of progestin activity 8-9 hours after hCG injection. Progestin levels in the plasma are undetectable earlier than 4 hours following hCG and later than 20 hours. Ovulation occurs after 24 hours (Thornton, 1972).

Although the oviduct seems to be controlled by both estrogens and progestins, the comparative functional aspect between amphibians and other well-studied species, such as birds, cannot be stated with certainty at this time since little biochemical work on the control of amphibian oviduct growth has been performed. In the avian oviducts, estrogen injection results in an increase in a general protein synthesis, while progesterone acts to selectively stimulate the synthesis of avidin, a specific egg white protein. So far, it has not yet been ascertained, in the amphibian species, whether progesterone, present perhaps in very low amount throughout the oviductal growth period, has a similar promoting effect on jelly protein synthesis within the gland cells. Possibly only when this subliminal dose is greatly exceeded, i.e., 12 hours prior to oviposition, does the actual release of stored jelly occur (Redshaw, 1972).

In *Bufo arenarum*, the injection of hCG stimulates release of jelly into the oviductal lumen and that the action of hCG is dependent on the presence of the ovaries and a factor in the extracts of toad ovaries which caused release of jelly when injected into the oviducts of testing animals (Thornton, 1972). The substance in ovarian extracts that exerts the function to induce jelly release is functionally proven by experimental evidence to be progesterone as reported by Fernández *et al*. (1997), demonstrating that, progesterone which is present of 17β-estradiol appears to be responsible for triggering the release of the secretory granules into PR lumen of this species. In adult females of *Rana esculenta*, plasma concentration of progesterone is highest before ovulation and drops dramatically to reach a minimal level when ovulation occurs. Preceding the postovulatory phase, the hormonal level is again increased. This is consistent with the role assigned to progesterone, both inducing the mature division of the oocytes and regulating the jelly release by oviductal glands (Vitaioli *et al*., 1990). This is similar to *Rana dybowskii*, progesterone induces oocyte maturation by acting initially at the oocyte surface where it triggers generation of membrane-mediated second messengers during oocytes maturation in amphibians (Bandyopadhyay, 1998). As a result of these studies, the possibility that an ovarian hormone produced by the post-ovulatory follicle is responsible for stimulating jelly release during the normal breeding cycle has been discussed (Smith, 1955). There is some indirect evidence indicating the nature of the ovarian secretion, i.e., progesterone, administered either locally or systemically, provokes jelly release in toads while estrogens have much less provocative effect than progesterone (Lodge and Smith, 1960; Thornton and Evennett, 1969).

Progesterone is synthesized and secreted by full-grown oocyte during the long period of vitellogenesis in amphibian (Redshaw and Nicholls, 1971; Thibier-Fouchet *et al*., 1976; Sretarugsa and Wallace, 1997). In *X. laevis* (Mullner *et al*., 1978; Fortune, 1983; Sretarugsa and Wallace, 1997), the stage VI follicles (1.2-1.3 mm diameter) are primarily responsible for the synthesis and secretion of progesterone. Similar studies have distinguished estrogenic and progestogenic ovarian follicles in *Rana nigromaculata* (Kwon *et al*., 1991; 1993).

Progesterone is normally produced as a response to circulating gonadotropin and is important for reproduction success. Progesterone resumes meiosis of the full-grown oocyte (Masui and Clarke, 1979) and induces the oviductal secretions that coat the ovulated eggs rendering them fertilizable as they pass down the oviduct (Thornton and Evennett, 1969; Katagiri, 1987).

7. Lectins and its histochemical applications

The current definition (Nomenclature Committee of the IUB and IUB-IUPAC Joint Commission on Biochemical Nomenclature, 1981) emphasizes the ability of lectins to agglutinate cells and precipitate glycoconjugates. Lectins are defined as "proteins of non-immunoglobulin nature capable of specific recognition and reversible binding to carbohydrate moieties of complex carbohydrates without altering covalent structure of the recognized glycosyl ligands" (Kocourek and Horejsi, 1983). It should be noted that by its emphasis on complex carbohydrates, this definition excludes some toxins and chemotactic proteins that bind only to simple sugars (Etzler, 1992).

The word "lectin" was originally applied to proteins extracted from plants and had the property of agglutinating mammalian erythrocytes (Boyd, 1970). Such proteins are also called phytohaemagglutinins. One lectin molecule can bind to two or more glycoprotein molecules on the outside surfaces of cells and thereby join the cells together. Comparable proteins are now known from fungal and animal sources, and any carbohydrate-binding protein of non-immune origin that agglutinates cells or precipitates polysaccharides or glycoconjugates is considered to be lectin (Liener *et al.*, 1986).

Lectins are classified into five groups according to their affinities for different sugars (Appendix Table 1). Binding is principally to the terminal sugar of a polysaccharide or oligosaccharide and can be competitively inhibited by adding the free sugars or appropriate glycosides to a solution of the lectin (Kiernan, 1999). The binding of a lectin molecule to a carbohydrate does not involve the formation of

covalent bonds. It is similar in nature to the attachment of an antigen to its specific antibody.

Lectins are proteins with large molecular weight (MW 20,000-300,000), which make it possible to be visualize markers. Lectins are oftenly modified their free amino groups by certain labels without interfering the carbohydrate-binding properties. The most frequently used labels are fluorochromes, biotins, histochemically demonstrable enzymes (horseradish peroxidase, alkaline phosphatase), ferritin (an electron-dense protein) and [^{3}H]-acetyl groups (for subsequent autoradiography). Bound lectins are sometimes detected by immunohistochemical methods, using anti-lectin antisera (Kiernan, 1999). Histochemical methods involving the use of lectins share many common technical methods with immunohistochemical techniques. Lectin histochemistry is useful for the demonstration of cells that are not easily distinguished by ordinary dye-based staining. For example, lectin affinities allow the recognition of microglial cells in nervous tissue (Streit, 1990; Hauke and Korr, 1993; Glenn *et al*., 1993; Acarin *et al*., 1994), nerve fibers in the peripheral nervous system (Spicer *et al*., 1996), muscle fiber types in paraffin sections (Bardosi *et al*., 1989) and capillary blood vessels in various organs (Tyler and Burns, 1991; Qu *et al*., 1997). With these useful properties of lectins, we thus utilize a number of lectin based staining methods as comparison tools to study the carbohydrate composition of oviductal secretory products during breeding, non-breeding and hormonal administration of *R. tigerina*.

MATERIALS AND METHODS

1. Experimental animals and collection of oviducts

The native rice field female frogs of Thailand, *Rana tigerina*, with the age more than 12 month-old were maintained in cement tanks at a farm in Ayutthaya Province. Water in the tanks was kept at ambient temperature and partly replaced daily. The animals were fed with pellet feed once a day. Oviducts were collected from 24 sexually mature female frogs during breeding period (April - September) and non-breeding period (October - February). To determine the effect of progesterone on the level of glycoprotein synthesis and release in oviductal epithelial cells, the frogs were intramusculary injected with oil soluble progesterone at 1.6 and 3.2 µg/g body weight or with 60 µl nut oil (for control). The oviducts were collected post-hormonal administration following the method described below.

2. Lectins

Six types of biotinylated lectins were used: BSL-I, ConA, LCA, RCA-I, UEA-I, and WGA (Vector Laboratories, Inc.) and their specificity to sugar termini are shown in Appendix Table 1. The biotinylated lectins were reconstituted by adding 1 ml distilled water to the final concentrations of 2 mg/ml (UEA-I, BSL-I) and 5 mg/ml (ConA, LCA, RCA-I, and WGA).

3. Lectin histochemistry with oviductal sections

3.1 Tissue preparation

The frogs were anesthesized by hypothermia under ice until no movement is detected and decapitated. The anterior abdominal walls were opened, and the oviducts were carefully removed from the covering peritoneum. They were divided into five portions based on the criteria described by Prachaney (1996). All portions of oviducts were flushed gently with Frog Ringer's solution (96 mM NaCl, 2 mM KCl, 4 mM

$MgCl_2$, 0.6 mM $CaCl_2$, 5 mM HEPES, pH 7.6) followed by perfusion with Bouin's solution to fix epithelial cells. The specimens were further fixed in Bouin's solution for additional 12 h, and then washed in several changes of 70% ethanol to remove the residual fixative. The tissues were dehydrated in a graded series of 70% to 100% ethanol (30 min for each step). Methylbenzoate was used as an intermediate clearing solution for at least 24 h, before clearing for 1 h with two changes of benzene. Thereafter, the tissues were infiltrated in the mixture of benzene and paraffin at the ratio of 1:1 (v/v) and pure paraffin for 1 h and 3 h, respectively. The tissue blocks were cut at five micron-thick and processed for hematoxylin and eosin (H&E) staining or lectin histochemistry.

3.2 Lectin histochemistry

Five micron-thick paraffin sections were deparaffinized with xylene 3 times, 5 min each, and rehydrated through a decreasing series of ethanol: 100%, 95%, 80% and 70% ethanol for 5 min each. The endogenous peroxidase activity in the specimen was blocked with 70% ethanol containing 1% hydrogen peroxide (H_2O_2) for 10 min. Then, the sections were immersed in 70% ethanol containing 1% saturated lithium carbonate ($LiCO_3$) for 5 min to eliminate residual picric acid. To block residual aldehyde, sections were immersed in the 300 mM glycine in water for 5 min. Non-specific binding sites were blocked with 4% bovine serum albumin (BSA) in Tris-buffered saline (100 mM Tris-HCl, 0.9% NaCl) for 30 min. The sections were then exposed to the individual biotinylated lectin (1:100 in TBS) for 90 min at room temperature, then washed 3 times for 5 min each in TBS containing 0.1% Tween 20 (TBS-T). Subsequently, the sections were incubated with horseradish peroxidase (HRP) conjugated streptavidin (1:250 in TBS) for 1 h and washed repeatedly in TBS-T. Binding of lectins to carbohydrate moieties were detected by NovaRed substrate kit (Vector Laboratories, Inc.) for 5-15 min until red color on the sections was notified. The sections were washed with distilled water for 5 min to stop the reaction and the nuclei was counterstained with hematoxylin for 1 min. Sections were then dehydrated in a graded series of 70% - 100% ethanol for 5 min each, cleared in 3 changes of

xylene for 5 min each and mounted by permount. The binding of lectins in the cells were observed under a Nikon E600 light microscope and the images were captured by Nikon DXM 1200 digital camera.

4. Sodium dodesyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining

4.1 Preparation of epithelial cytoplamic proteins and secretions

To obtain secretions from five different oviductal portions: pars recta (PR), pars convoluta (PC) 1-4, portions of excised oviducts were flushed with 1 ml Frog Ringer's solution. The flow-through oviductal secretion was reloaded into syringe and repeatedly flushed into the same oviductal portion. Protease inhibitors; phenylmethanesulfonyl fluoride (PMSF) and ethylenediaminetetraacetic acid (EDTA) at the final concentration of 1 mM were added into these oviductal secretions. Samples were stored at -20°C until use.

For epithelial cells preparation, five portions of the oviducts were removed 12 h after ovulation and they were opened longitudinally. The adluminal surface of epithelial cells was washed thoroughly and then gently scraped in Frog Ringer's solution to totally eliminate oviductal secretion that might be associated with cilia of epithelial cells. The secretion-freed oviductal tissues were then homogenized in the Frog Ringer's solution containing protease inhibitors. The homogenate was centrifuged at 3,000 g for 30 min and recentrifuged at 12,000 g for 10 min to eliminate cellular debris. The supernatant was stored at -20°C until use.

4.2 SDS-PAGE and silver staining

Glycoproteins of oviductal secretions and epithelial cells taken from breeding, non-breeding period and progesterone administered animals were resolved by 12.5% SDS-PAGE with 8% stacking gel. Samples were solubilized with SDS-PAGE loading buffer and denatured by heating at 95^{o}C in a boiling-water bath for

5 min. Approximately 2-5 µg of total proteins of each sample and prestained protein marker (Amersham Pharmacia Biotech) were loaded into the wells of duplicated gels and electrophoresed under a reducing condition at 20 mA under a constant voltage mode. The duplicated gels were subjected to either silver staining or lectin-streptavidin blotting.

For silver staining, the gel was placed in a fixative (25% ethanol, 7% acetic acid) for 1 h with gentle shaking. Thereafter, it was submersed into 10% ethanol twice for 7 min each and washed twice with distilled water, 10 min each. The gel was incubated in dithiothreitol (DTT) solution and shaken slowly for 30 min and washed briefly with distilled water. Developing solution (3% sodium carbonate containing 0.018% formaldehyde) was added to the gel, shaken briefly, discarded and the fresh developing solution was then replaced to visualize the appropriate intensity of the protein bands. Thereafter, the developer was removed and the gel was rinsed briefly with distilled water and Destain II to stop the reaction, and kept wet in distilled water or dried in the duplicated layers of cellophane.

5. <u>Western blotting and lectin staining</u>

Separated proteins in the gel were electrotransferred to a 0.45 µm nitrocellulose membrane (Amersham Pharmacia Biotech) at 100 volts for 55 min under a constant voltage mode condition. The membrane was submersed at least 1 h at room temperature in blocking solution containing 4% BSA in TBS and 0.05% Tween 20 to block the non-specific binding sites. The proteins were incubated with 1:500 dilution of the biotinylated lectin in TBS-T for 90 min, and then washed with TBS-T 3 times, 5 min each. The membrane was exposed to HRP conjugated streptavidin (1:5,000 dilutions in TBS-T) for 1 h followed by washing with TBS-T 3 times, 5 min each. To detect the binding of other lectins, the bound lectin-strepavidin complexes were stripped from the membrane and the proteins were re-incubated with the other biotinylated lectins and the subsequent incubations were followed under the same conditions described above. Lectin reactivity was visualized by an enhanced chemiluminescence method using ECL kit (Amersham Pharmacia Biotech) for 2 min

followed by exposure to the BioMax film specific for chemiluminescent visualization (Amersham Pharmacia Biotech).

RESULTS

1. Immunohistochemistry of various lectins in oviductal tissues

The presence and distribution of lectins in the oviductal epithelial cells and their secretory products of breeding, non-breeding and progesterone treated animals were investigated using lectin histochemistry. Six types of lectins including BSL-I, ConA, LCA, RCA-I, UEA-I and WGA were used in this study. The results in Figure1 revealed that in the PR region, a highly intense reactivity (as the red enzymatic product) was apparent in the non-ciliated cells situated at the upper epithelial folds (NCC) when the lectins LCA and RCA-I were applied (panels C and D). In addition, a moderate staining intensity was observed with ConA and WGA lectins (panels B and F). The staining of BSL-I and UEA-I in NCC, however, showed much less staining intensity compared to other type of lectins (panels A and E). The moderate staining of all lectins were uniformly distributed throughout the cytoplasm of the NCC situated at the basal part of epithelial folds. In the ciliated cells (CC), all lectins gave negative staining (panels A-F).

During non-breeding period, overall diameter of the oviducts and the size of epithelial cells became smaller. Positive staining of all lectins in both upper and lower parts of oviductal epithelium decreased drastically, giving only a faint staining level, with the exception of UEA-I in which a moderate staining level was still recognizable in NCC located at the basal part of the epithelial folds (Figure 2, panel E). Staining intensity of the given lectins in the oviductal epithelium of the progesterone treated animals was even more pronounced than those of seasonal breeders. This included the staining of BSL-I and UEA-I in NCC at the basal part of epithelial folds. Moreover, a highly intense staining of BSL-I was clearly observed in the NCC at the upper part of epithelial fold (Figure 3, panel A), while UEA-I gave negative staining in the same cell type (Figure 3, panel E). Other lectins including RCA-I and WGA showed a comparable staining intensity as found in the seasonal breeders in NCC along the entire epithelial folds (Figure 3, panels D, F). Notably, the staining intensity of LCA was greatly declined in the progesterone treated animals compared to the staining level

detected in the seasonal breeders. This, in turn, suggested the selective enhancing effect of progesterone on the glycosylation pattern of the oviductal proteins.

In the PC1 region of breeding animals, the faintly moderately and highly positive reactivity was observed in the NCC when the lectins ConA, RCA-I and WGA, respectively, were applied (Figure 4, panels B, D and F). The staining of other three lectins, BSL-I, LCA and UEA-I in both cell types of epithelial ridge showed minimal staining intensity (Figure 4, panels A, C and E). The results also revealed that the entire mucosal glands were specifically stained with only RCA-I and UEA-I with mild to moderate staining intensity, accordingly (Figure 4, panels D and E). Other lectins showed only background staining intensity. Positive reactivity at the apical cilia of CC was likely to be the aggregation of accumulative secretory products which may be part of secretion from the PR oviduct. During non-breeding, the structure of cells in epithelial folds and glandular tissues were poorly recognized. In the epithelial ridges, only RCA-I and WGA showed an intense reactivity in NCC (Figure 5, panels D and F). In the mucosal glands, lectins ConA, LCA and UEA-I gave mild to moderate staining reactivity (Figure 5, panels B, C and E). A background staining level of all lectins was observed in the CC cells situated in epithelial ridge. In the progesterone treated animals, the lectins RCA-I and UEA-I showed a highly intense reactivity in NCC and CC (Figure 6, panels D and E). In the CC, lectins ConA and LCA gave a mild staining intensity (Figure 6, panels B and C), while, in the NCC, lectins BSL-I and WGA gave a mild staining intensity (Figure 6, panels A and F). The only UEA-I showed an intense reactivity in the mucosal glands (Figure 6, panel E).

In the PC2 region of breeding animals, a moderately intense reactivity was noticed in the NCC in the epithelial ridges when lectins BSL-I, RCA-I and WGA (Figure 7, panels A, D and F) were applied. On the other hand, lectins ConA, LCA and UEA-I gave a mild to moderate staining intensity specific to the CC in epithelial ridges (Figure 7, panels B, C and E). Notably, only LCA positively stained both NCC and CC (Figure 7, panel D). The contents of the mucosal glands below the epithelial ridges were uniformly and moderately reactive with WGA (Figure 7, panel F) and faintly reactive with BSL-I and RCA-I (Figure 7, panels A and D). During

non-breeding period, a marked decrease in all lectin staining was observed in both epithelial ridges and the mucosal glands. However, lectins UEA-I and WGA remained to have an intense reactivity with CC and NCC, respectively, in the epithelial ridges (Figure 8, panels E and F). In addition, these two lectins also gave faint reactivity in the contents of the mucosal glands. In the progesterone treated animals, similar staining results for all lectins were comparable to those of the seasonal breeders (Figure 9). The only exception was applied to UEA-I which showed an enhanced staining intensity in the NCC (Figure 9, panel E). Interestingly, the staining in the contents of mucosal glands, particularly with RCA-I and WGA apparently decreased compared to that found in the seasonal breeders (Figure 9, panels D and F).

In the PC3 region of breeding animals, the NCC in the epithelial ridges exhibited a highly intense staining level in the entire cytoplasm when lectins RCA-I and WGA were applied (Figure 10, panels D and F). In contrast to NCC, the CC in the epithelial ridges were faintly stained with BSL-I and UEA-I and moderately stained with ConA (Figure 10, panels A, E and B). BSL-I gave the positive staining in both NCC and CC (Figure 10, panel A). In the mucosal glands, WGA was the only lectin that gave a positive reactivity in their contents (Figure 10, panel F). Notably, the staining pattern appeared to be varied from one region to another. During non-breeding period, the overall declination of all lectin staining as shown for PC2 was also achieved in this oviductal region (Figure 11). NCC remained faintly reactive with BSL-I, RCA-I and moderately reactive with WGA (Figure 11, panels A and D), while, CC were positively staining with only ConA (Figure 11, panel B). The positive reactivity in the mucosal glands was detected only when lectin WGA was used (Figure 11, panel F). In the progesterone treated animals, the staining intensity of most lectins were generally enhanced as opposite to that shown for the non-seasonal breeders. Staining intensity of the lectins in the NCC appeared to be extensively enhanced (from no staining to become moderate staining) when lectins BSL-I, ConA and UEA-I were applied (Figure 12, panels A, B and E). The staining intensity with NCC cells was also increased with lectins LCA and RCA-I (Figure 12, panels C and D). Surprisingly, the staining of the NCC and mucosal glands with WGA was opposite to the highly intense reactivity detected in the seasonal breeders (Figure 12, panel F).

Morphological appearance of PC4 differed greatly from other PC regions in which its mucosal glands were subsequently divided into two portions: upper and lower mucosal glands. In the breeding animals, the NCC in the epithelial ridges showed a moderately intense reactivity when lectins BSL-I and WGA were applied and highly intense reactivity when lectin RCA-I was used (Figure 13, panels A, D and F). The CC in the epithelial ridges was faintly stained with lectins ConA and UEA-I (Figure 13, panels B and E). The minimal positive reactivity in both NCC and CC was seen following the staining of LCA (Figure 13, panel C). Content of the upper mucosal glands was specifically reactive with RCA-I and UEA-I, while the other lectins gave negative staining results (Figure 13, panels D and E). Content of the lower mucosal glands was also highly specific to WGA staining, but not with the other lectins (Figure 13, panel F). During non-breeding period, all lectins staining in epithelial ridges was markedly diminished, namely, no positive reactivity was detected in both epithelial cell types. The only mild reactivity could only be found in the content of the upper mucosal glands with lectins BSL-I and UEA-I (Figure 14, panels A and E). In the progesterone treated animals, a similar enhancing effect of the hormone, particularly on the NCC was clearly observed with lectins BSL-I, LCA, RCA-I and UEA-I (Figure 15, panels A, C, D and E). Some enhancement of lectin staining in the CC could be found when lectins ConA, LCA, RCA-I and UEA-I were used (Figure 15, panels B, C, D and E). The effect of hormone on the upper and lower mucosal glands was much less pronounced than the epithelial ridges. Comparing to breeding period the slight differences in the lectin staining that could be detected were the staining of ConA in the UMG and the heterogeneous staining of UEA-I in LMG (Figure 15, panels B and E). As similar in PC3, WGA gave negative staining in all sub-portions of PC4 (Figure 15, panel F).

Figure 1 Lectin histochemistry of the PR region during the breeding period. The PR region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). Note the intense reactivity at the non-ciliated (NCC) in the basal part of epithelial folds marked by solid arrows. CC = ciliated cell, NCC = non-ciliated cell.

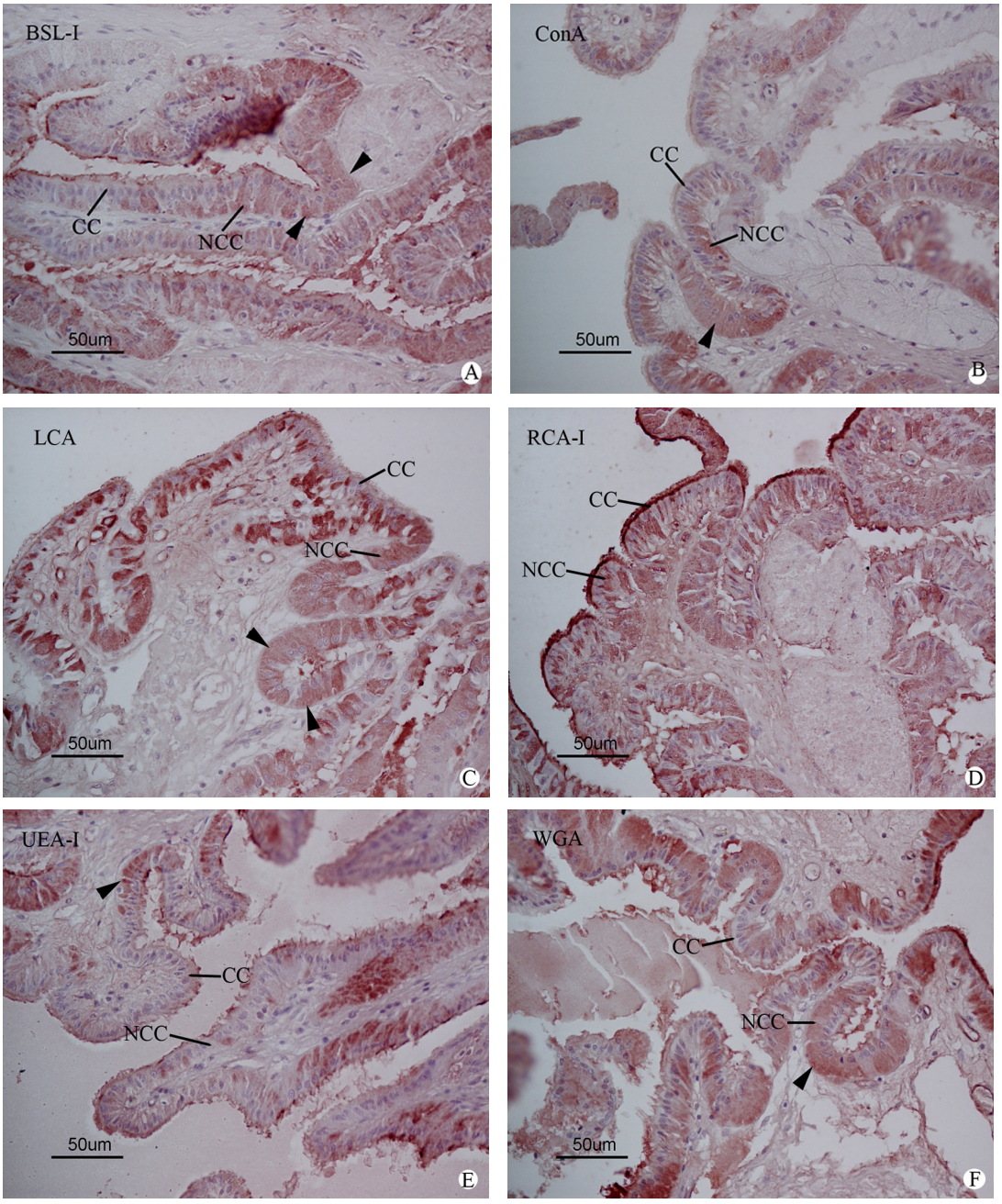

BSL-I
CC
NCC
50um
A
ConA
CC
NCC
50um
B
LCA
CC
NCC
50um
C
RCA-I
CC
NCC
50um
D
UEA-I
CC
NCC
50um
E
WGA
CC
NCC
50um
F

Figure 2 Lectin histochemistry of the PR region during the non-breeding period. The PR region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). Positive staining of all lectins in both upper and lower parts of oviductal epithelium decreased, giving only a faint staining level. CC = ciliated cell, NCC = non-ciliated cell.

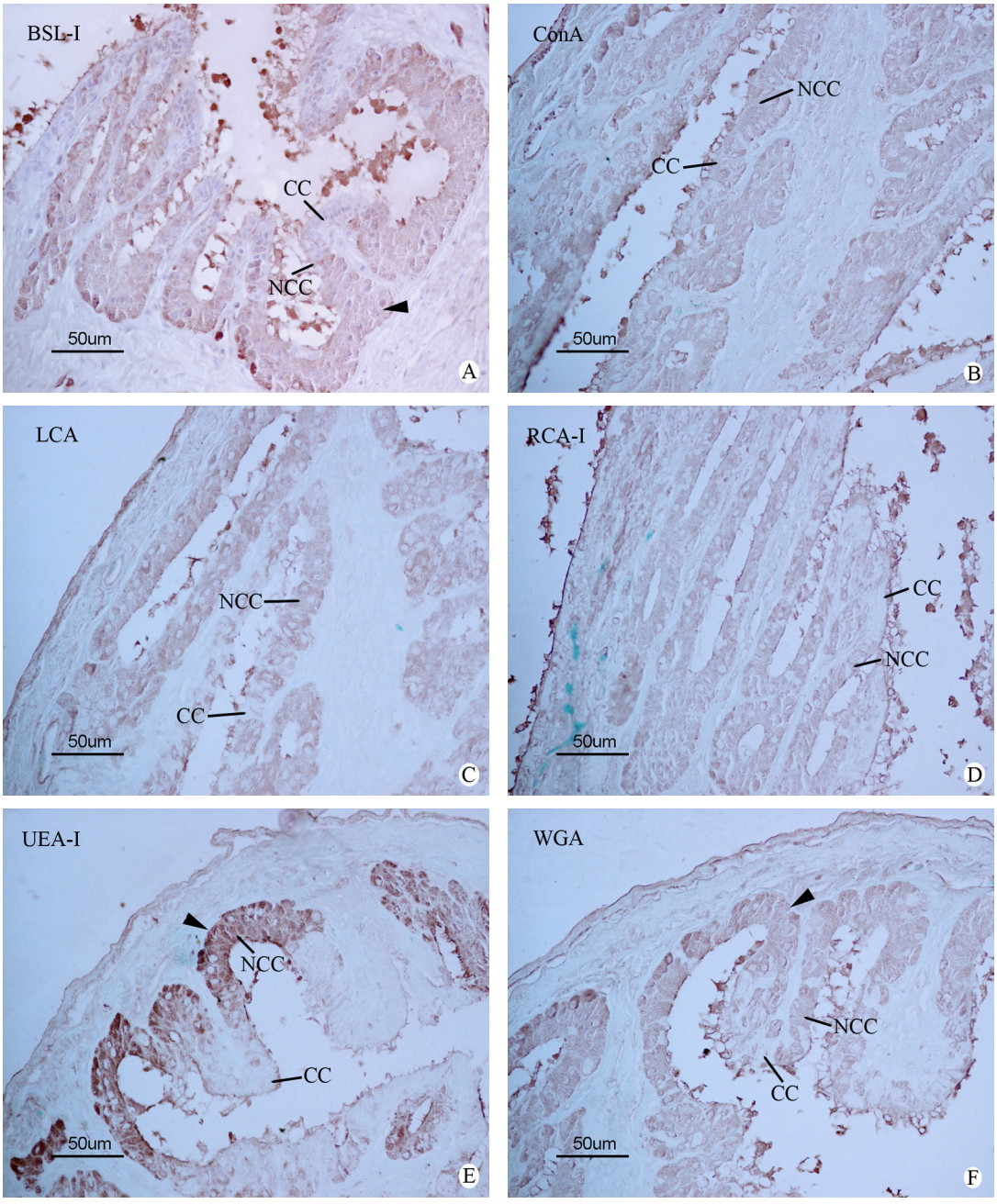
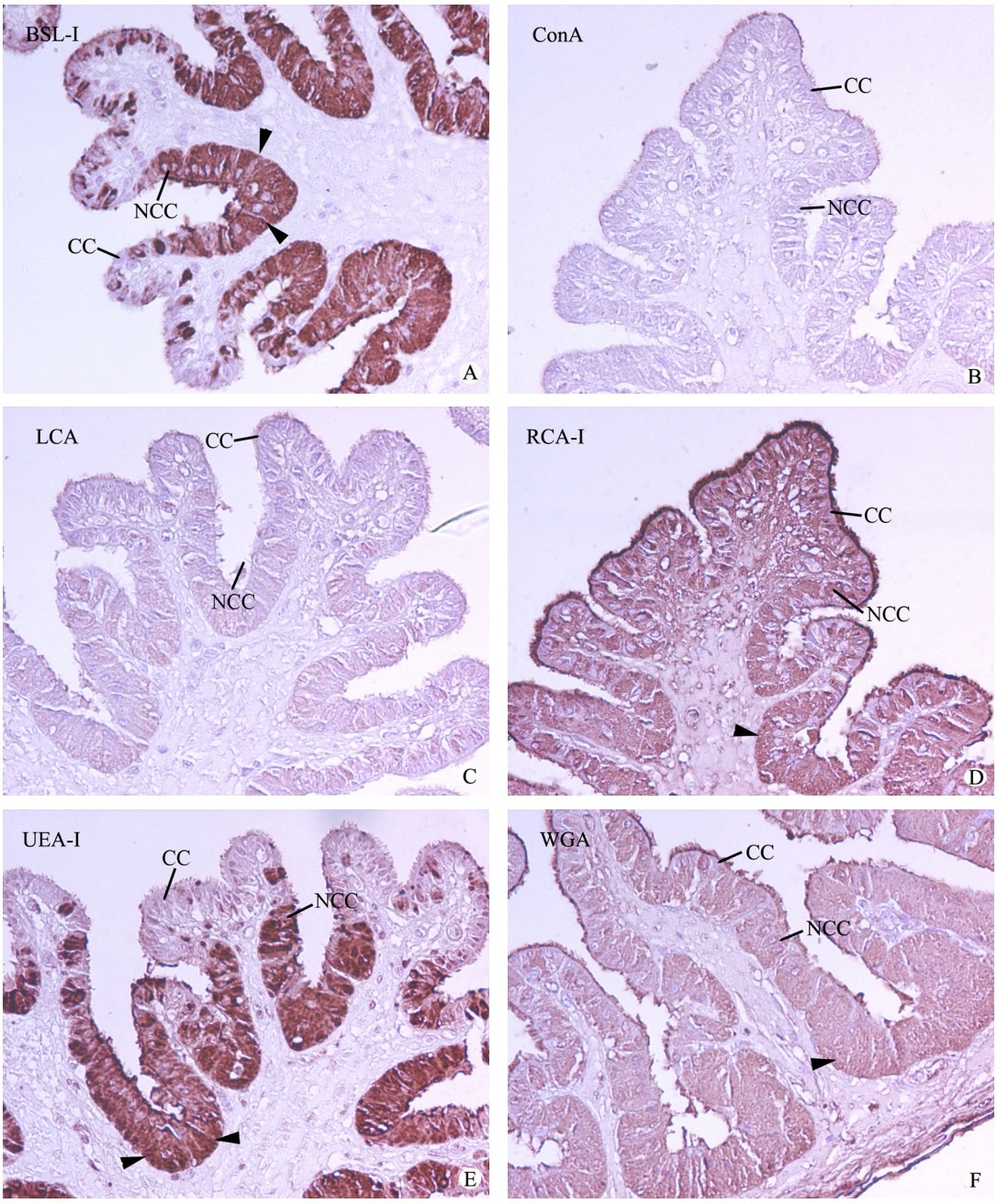

BSL-I
CC
NCC
50um
A
ConA
NCC
CC
50um
B
LCA
NCC
CC
50um
C
RCA-I
CC
NCC
50um
D
UEA-I
NCC
CC
50um
E
WGA
NCC
CC
50um
F

Figure 3 Lectin histochemistry of the PR region during the hormonal administration period.

The PR region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). The level of reactivity was more pronounced than those of seasonal breeders. CC = ciliated cell, NCC = non-ciliated cell.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NCC
CC
A
ConA
CC
NCC
B
LCA
CC
NCC
C
RCA-I
CC
NCC
D
UEA-I
CC
NCC
E
WGA
CC
NCC
F

Figure 4 Lectin histochemistry of the PC1 region during the breeding period.
The PC1 region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). The faintly moderately and highly positive reactivity was observed in the NCC when the lectins ConA, RCA-I and WGA, respectively, were applied.
CC = ciliated cell, MG = mucosal gland, NCC = non-ciliated cell.

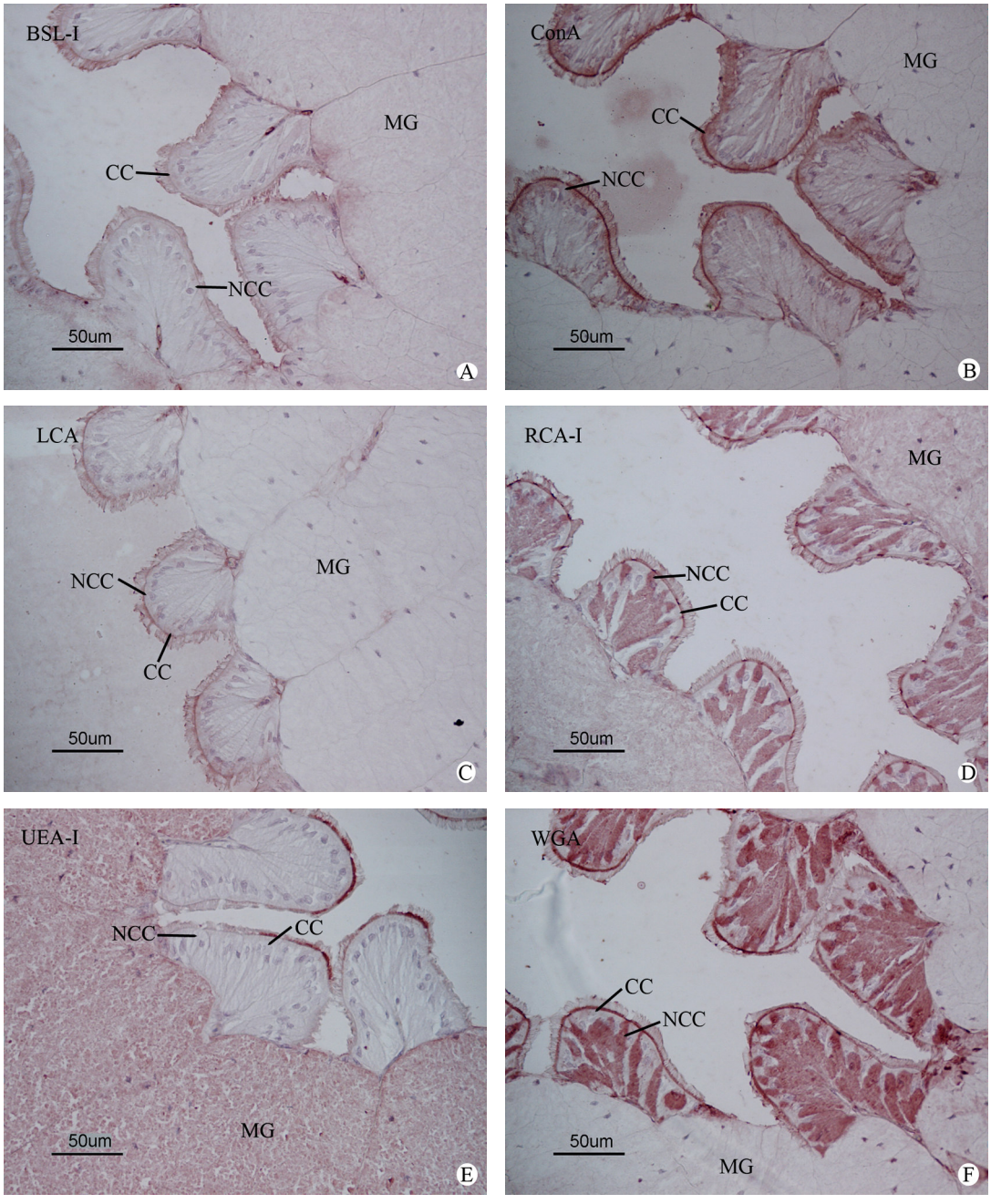
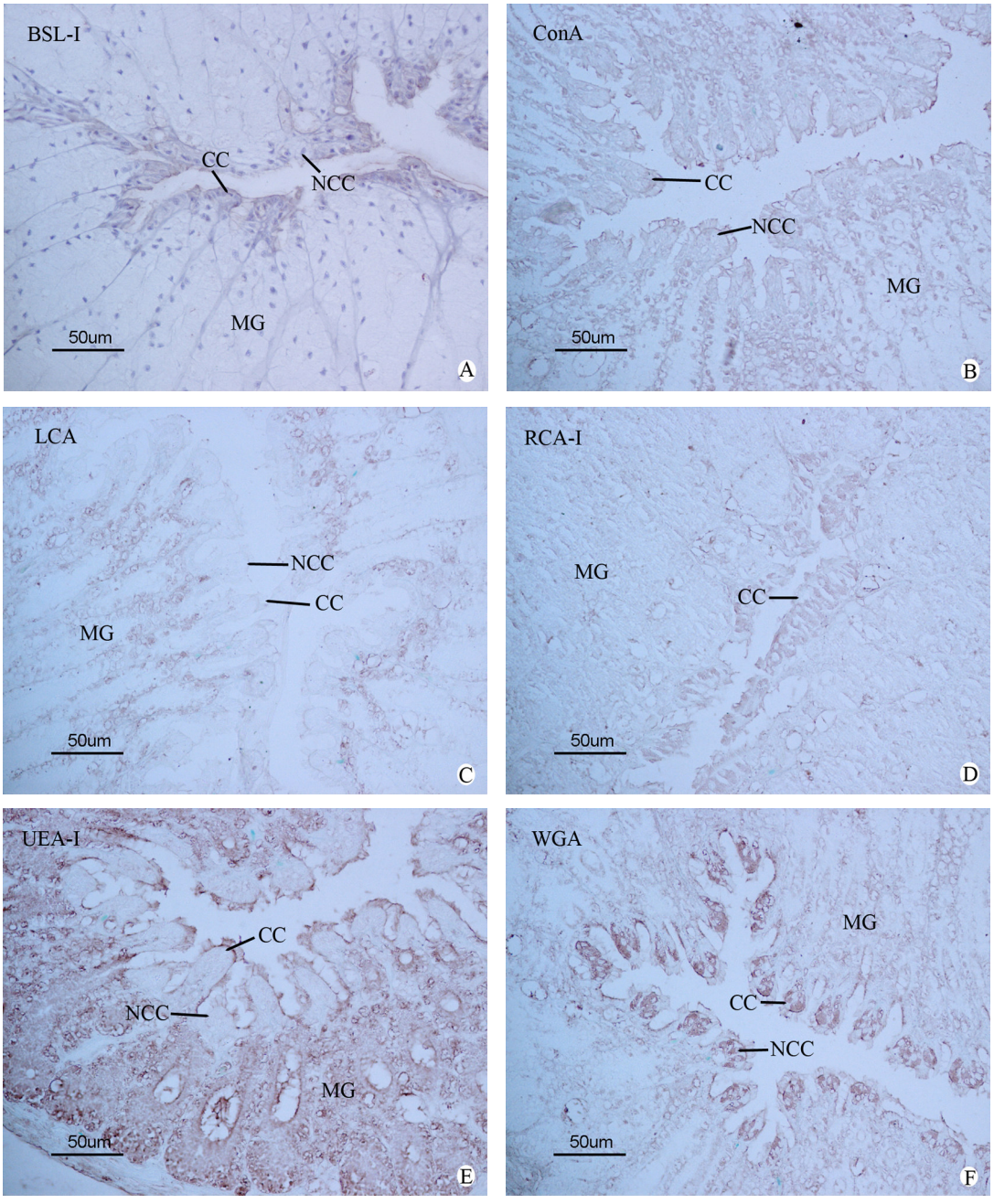

BSL-I
MG
CC
NCC
50um
A
ConA
MG
CC
NCC
50um
B
LCA
NCC
MG
CC
50um
C
RCA-I
MG
NCC
CC
50um
D
UEA-I
NCC
CC
50um
MG
E
WGA
CC
NCC
50um
MG
F

Figure 5 **Lectin histochemistry of the PC1 region during the non-breeding period.**

The PC1 region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). The structure of cells in epithelial folds and glandular tissues were poorly recognized. CC = ciliated cell, MG = mucosal gland, NCC = non-ciliated cell.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ConA
CC
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LCA
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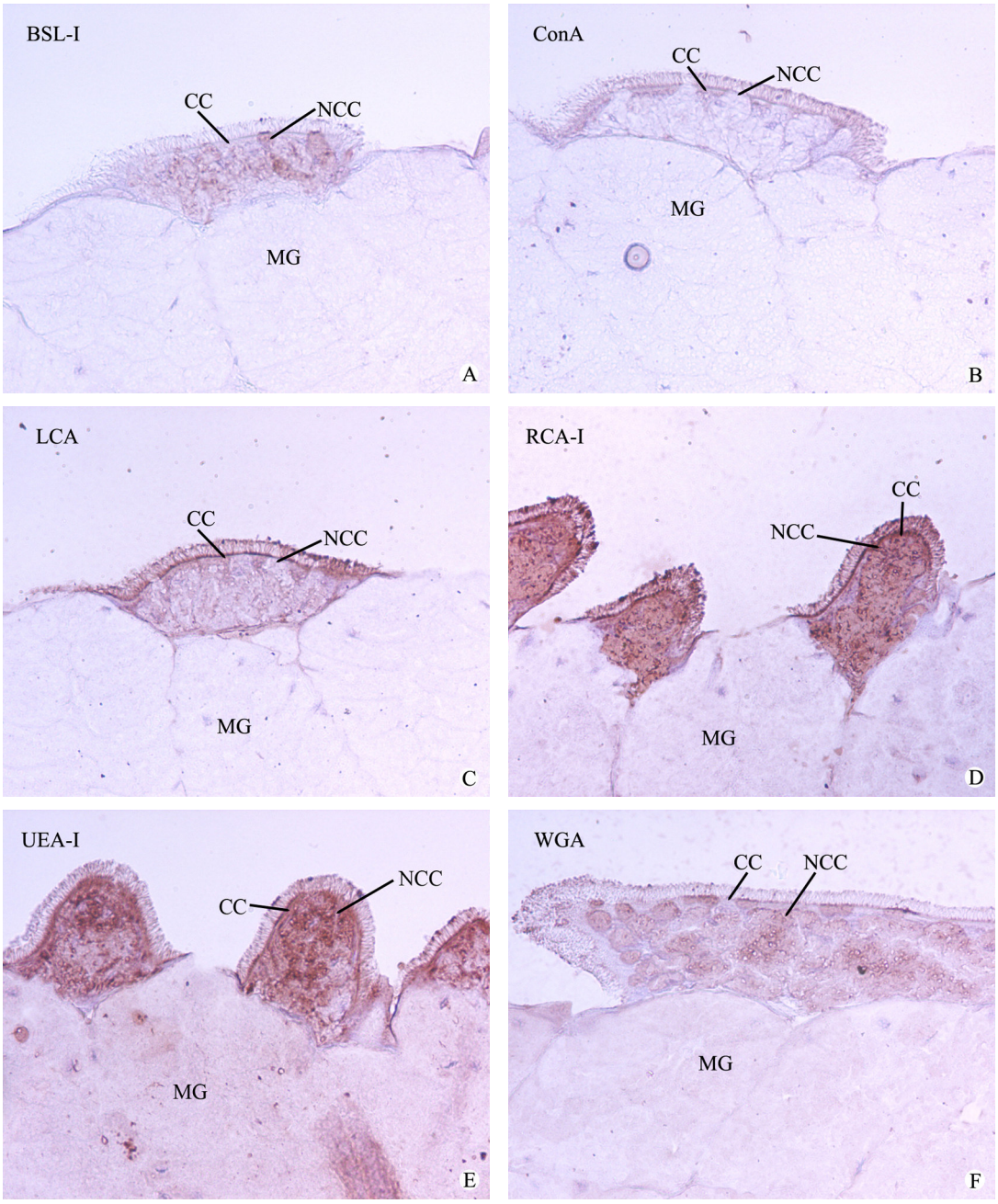
CC
MG
50um
C
RCA-I
MG
CC
50um
D
UEA-I
CC
NCC
MG
50um
E
WGA
MG
CC
NCC
50um
F

Figure 6 Lectin histochemistry of the PC1 region during the hormonal administration period.

The PC1 region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). CC = ciliated cell, MG = mucosal gland, NCC = non-ciliated cell.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NCC
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A
ConA
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NCC
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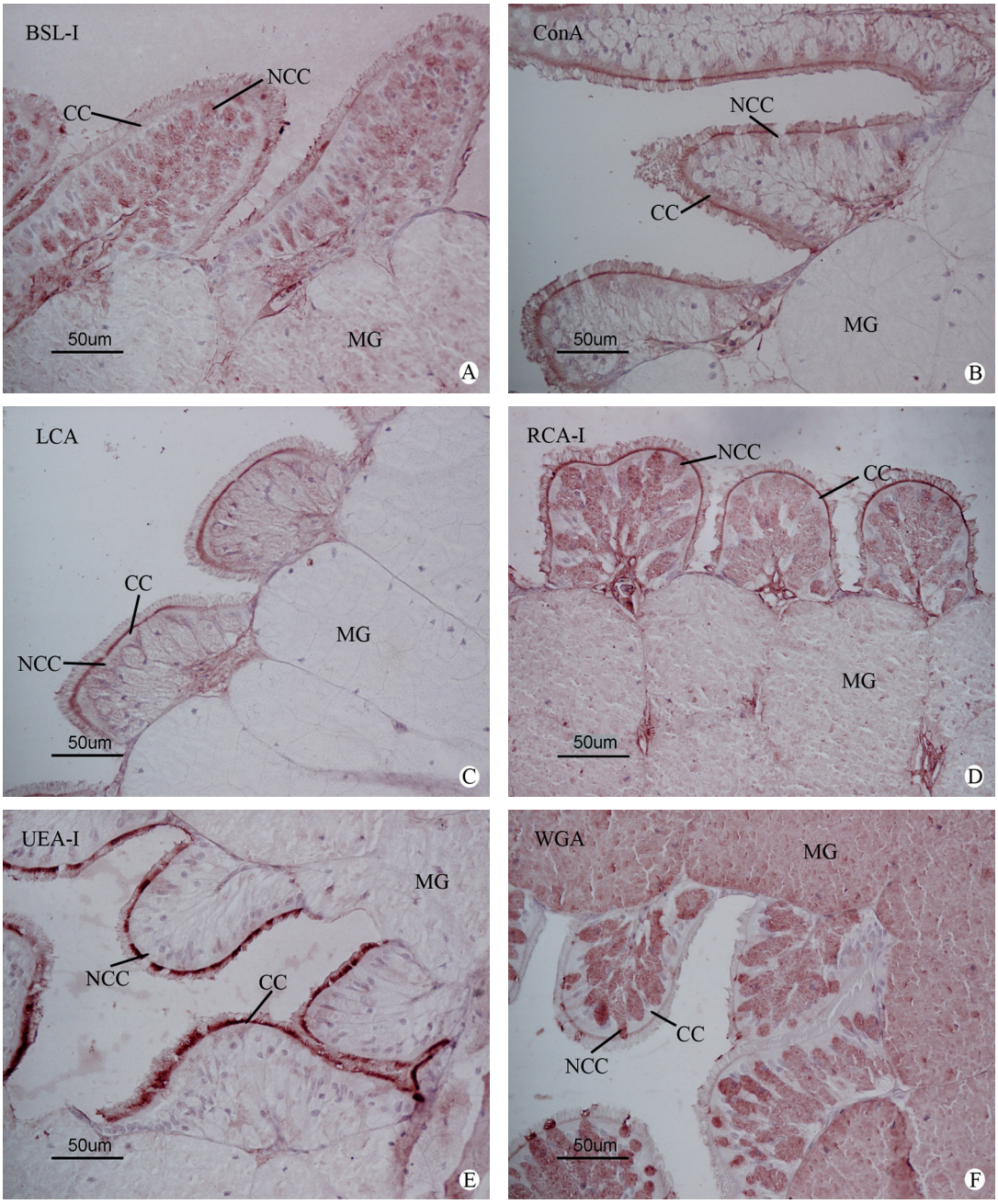
B
LCA
CC
NCC
MG
C
RCA-I
CC
NCC
MG
D
UEA-I
NCC
CC
MG
E
WGA
CC
NCC
MG
F

Figure 7 Lectin histochemistry of the PC2 region during the breeding period.
The PC2 region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). CC = ciliated cell, MG = mucosal gland, NCC = non-ciliated cell.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50um
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ConA
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MG
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LCA
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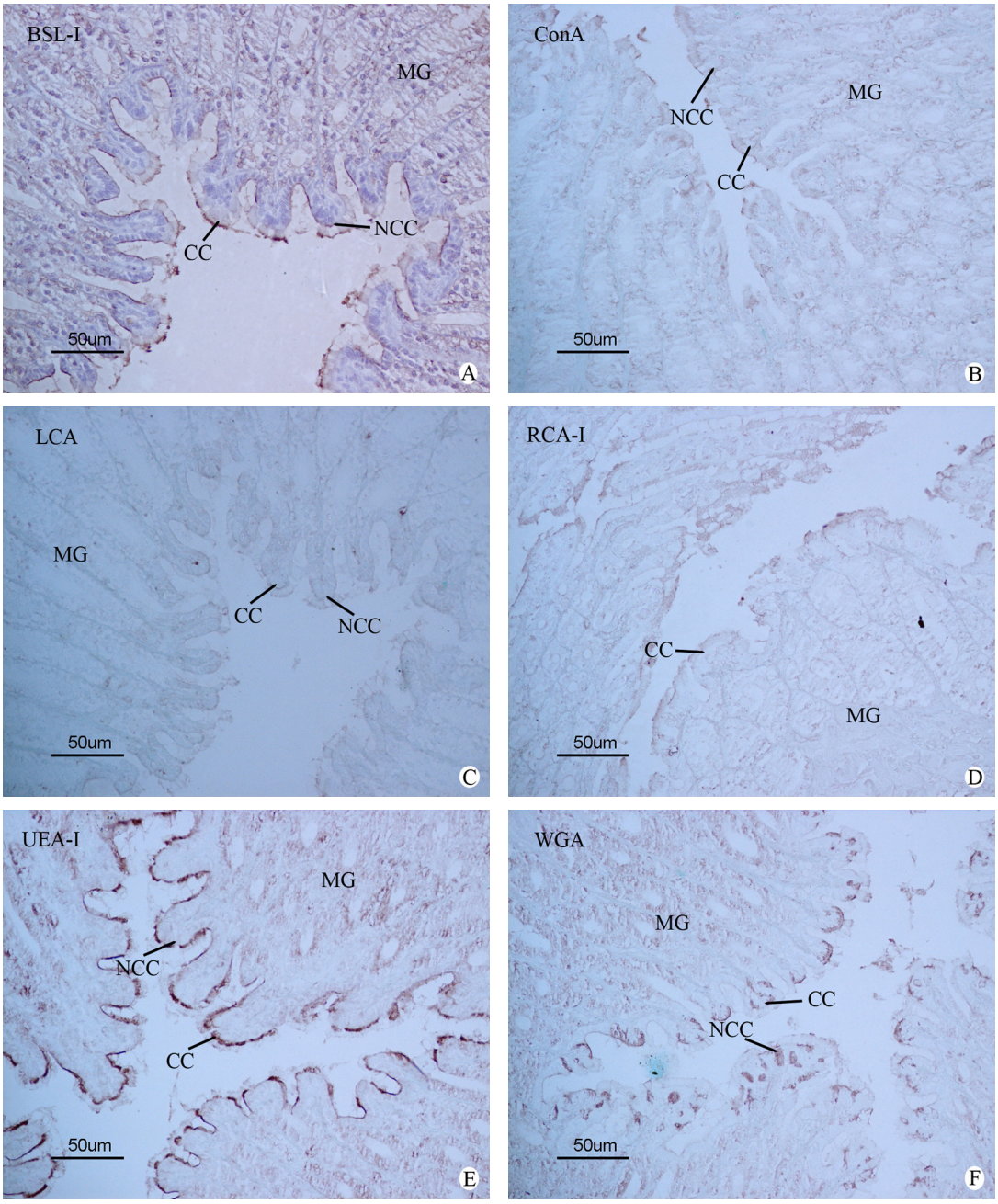
RCA-I
NCC
CC
MG
50um
D
UEA-I
MG
NCC
CC
50um
E
WGA
MG
CC
NCC
50um
F

Figure 8 **Lectin histochemistry of the PC2 region during the non-breeding period.**

The PC2 region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). CC = ciliated cell, MG = mucosal gland, NCC = non-ciliated cell.


BSL-I
MG
NCC
CC
50um
A
ConA
NCC
MG
CC
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B
LCA
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CC
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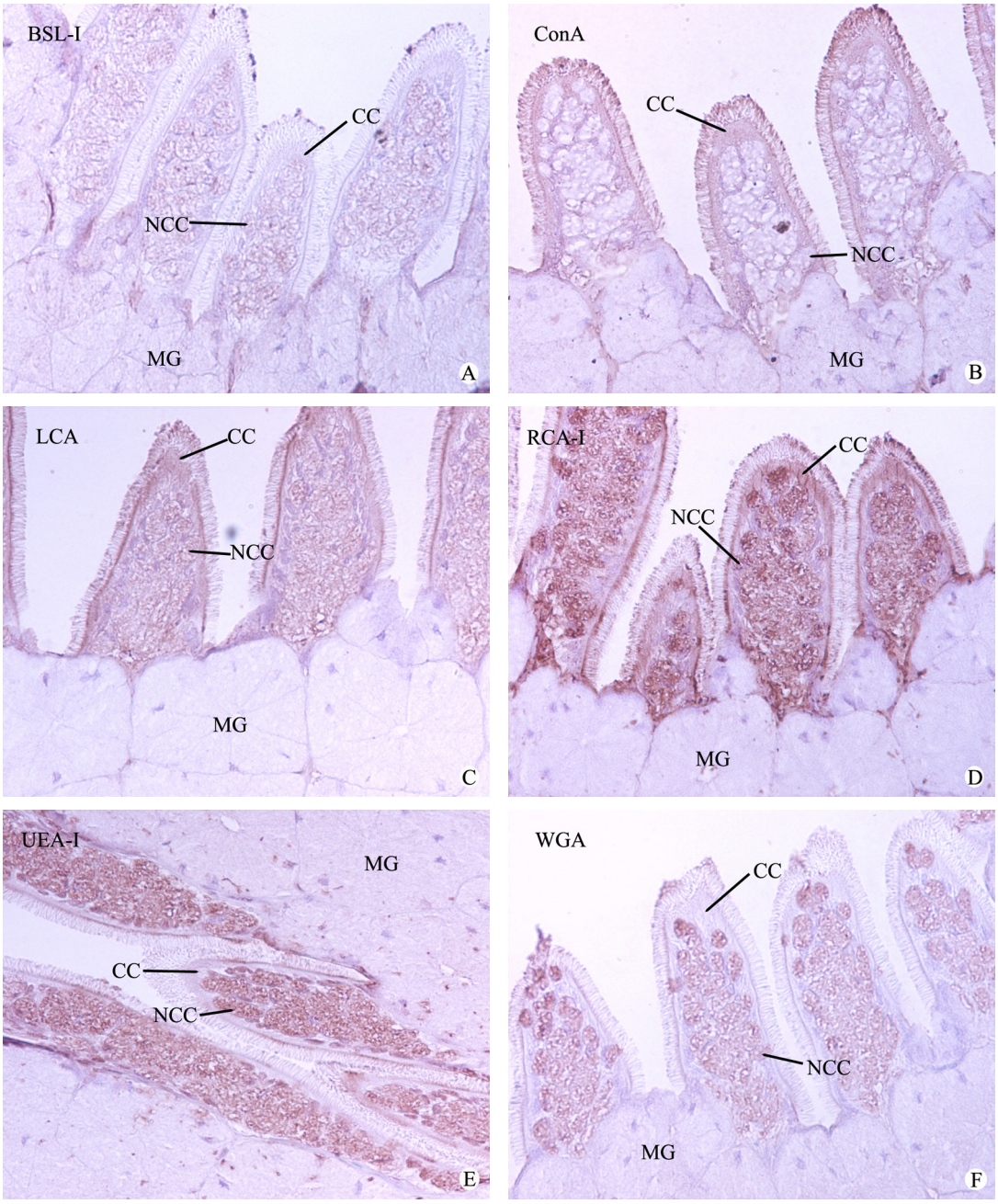
50um
C
RCA-I
CC
MG
50um
D
UEA-I
MG
NCC
CC
50um
E
WGA
MG
CC
NCC
50um
F

Figure 9 Lectin histochemistry of the PC2 region during the hormonal administration period.

The PC2 region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). CC = ciliated cell, MG = mucosal gland, NCC = non-ciliated cell.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A
ConA
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LCA
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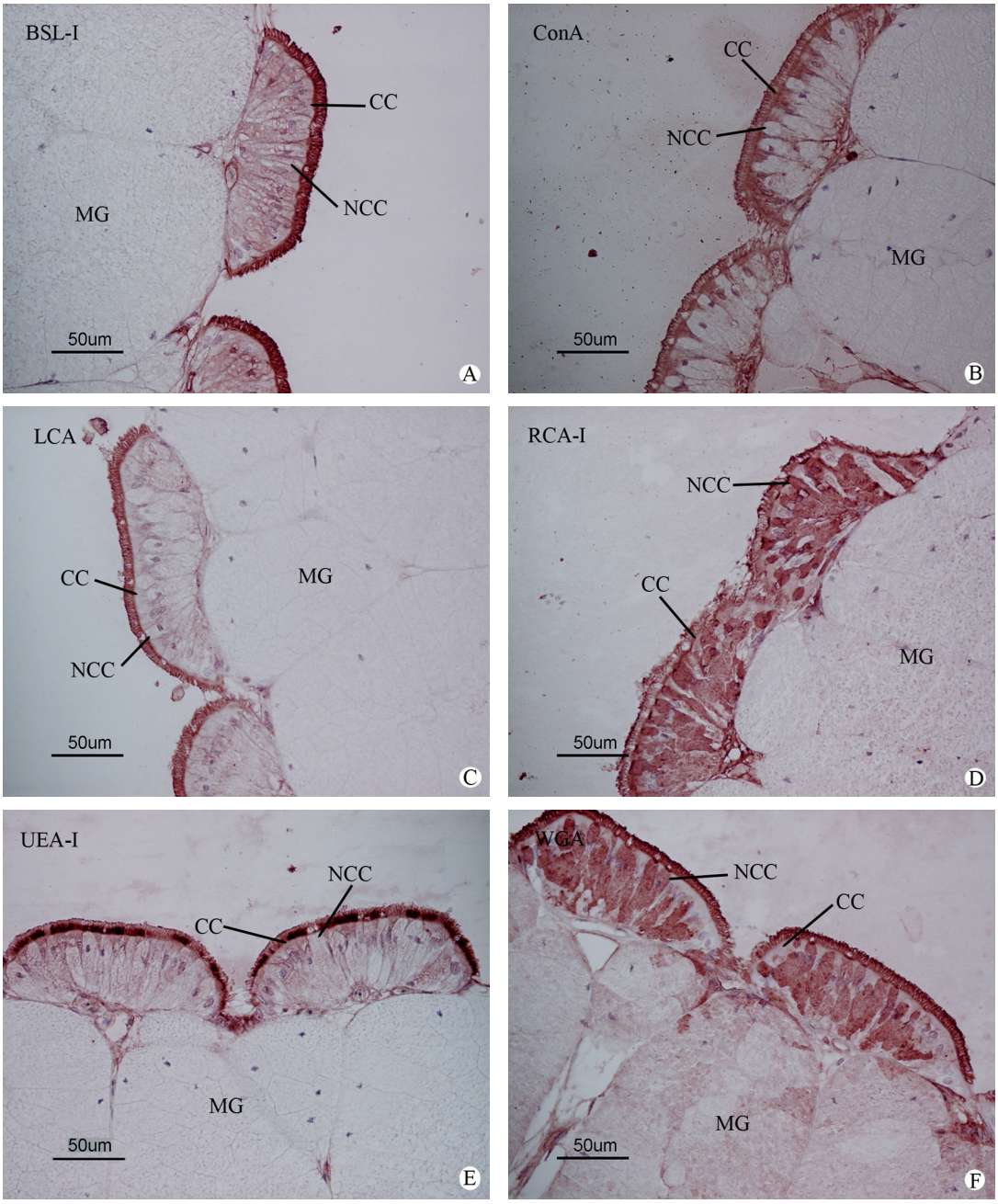
NCC
MG
C
RCA-I
CC
NCC
MG
D
UEA-I
MG
CC
NCC
E
WGA
CC
NCC
MG
F

Figure 10 Lectin histochemistry of the PC3 region during the breeding period. The PC3 region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). CC = ciliated cell, MG = mucosal gland, NCC = non-ciliated cell.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CC
NCC
MG
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ConA
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MG
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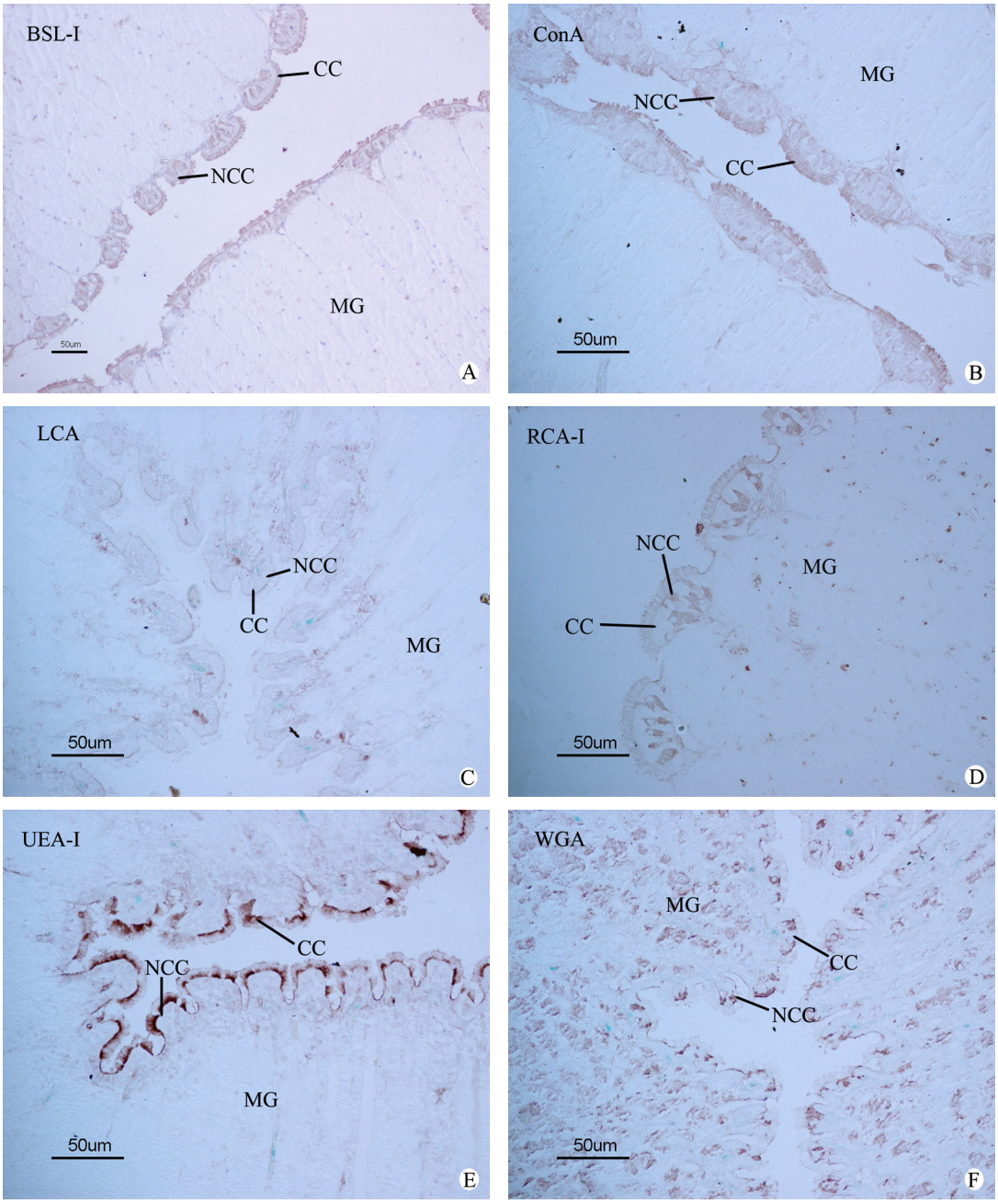
C
RCA-I
NCC
CC
MG
50um
D
UEA-I
NCC
CC
MG
50um
E
WGA
NCC
CC
MG
50um
F

Figure 11 **Lectin histochemistry of the PC3 region during the non-breeding period.**

The PC3 region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). CC = ciliated cell, MG = mucosal gland, NCC = non-ciliated cell.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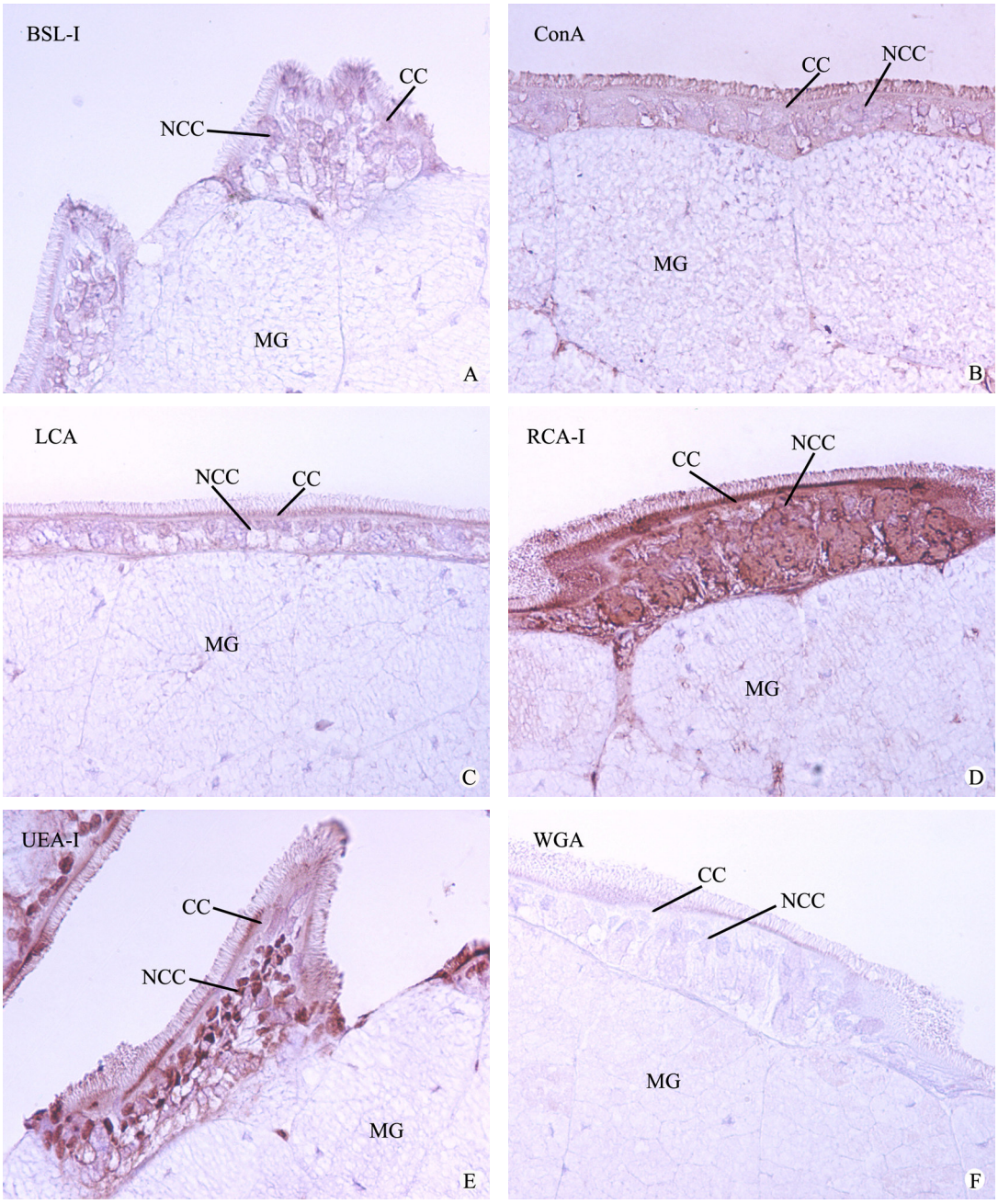
RCA-I
NCC
MG
CC
50um
D
UEA-I
CC
NCC
MG
50um
E
WGA
MG
CC
NCC
50um
F

Figure 12 **Lectin histochemistry of the PC3 region during the hormonal administration period.**

The PC3 region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). CC = ciliated cell, MG = mucosal gland, NCC = non-ciliated cell.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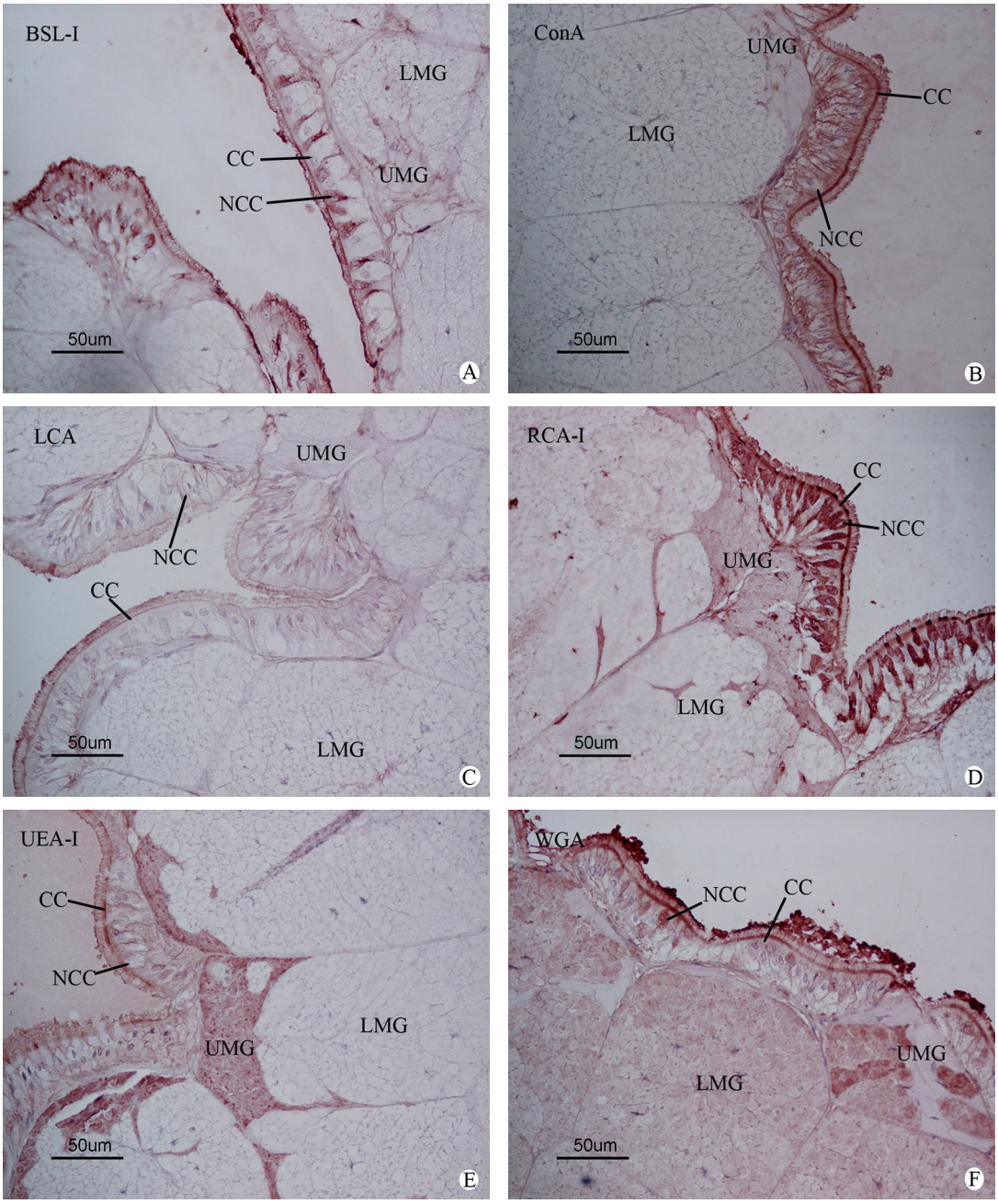
NCC
MG
D
UEA-I
CC
NCC
MG
E
WGA
CC
NCC
MG
F

Figure 13 Lectin histochemistry of the PC4 region during the breeding period. The PC4 region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). CC = ciliated cell, LMG = lower mucosal gland, NCC = non-ciliated cell, UMG = upper mucosal gland.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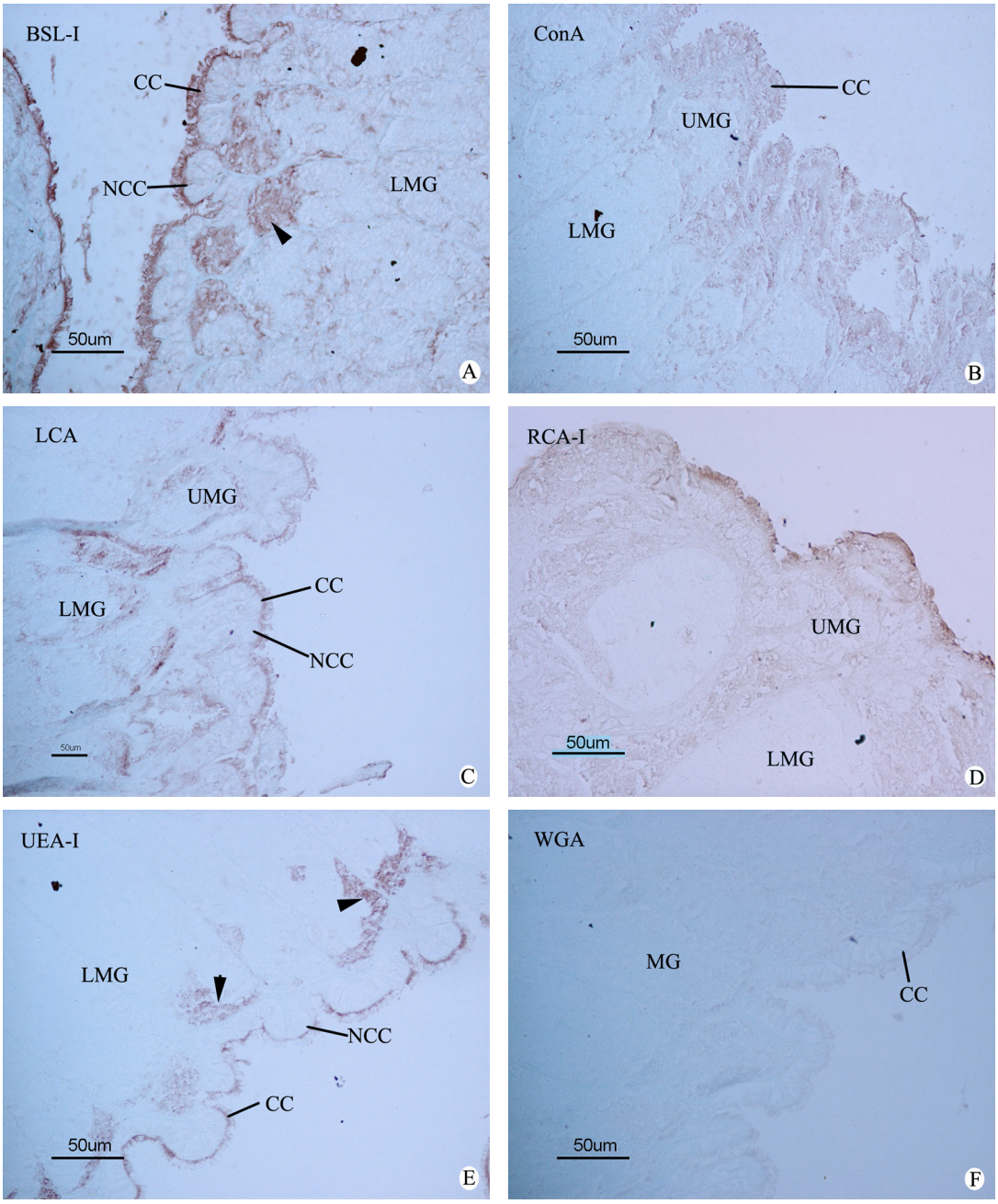
CC
50um
LMG
C
RCA-I
CC
NCC
UMG
LMG
50um
D
UEA-I
CC
NCC
LMG
UMG
50um
E
WGA
NCC
CC
UMG
LMG
50um
F

Figure 14 **Lectin histochemistry of the PC4 region during the non-breeding period.**

The PC4 region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). CC = ciliated cell, LMG = lower mucosal gland, NCC = non-ciliated cell, UMG = upper mucosal gland.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ConA
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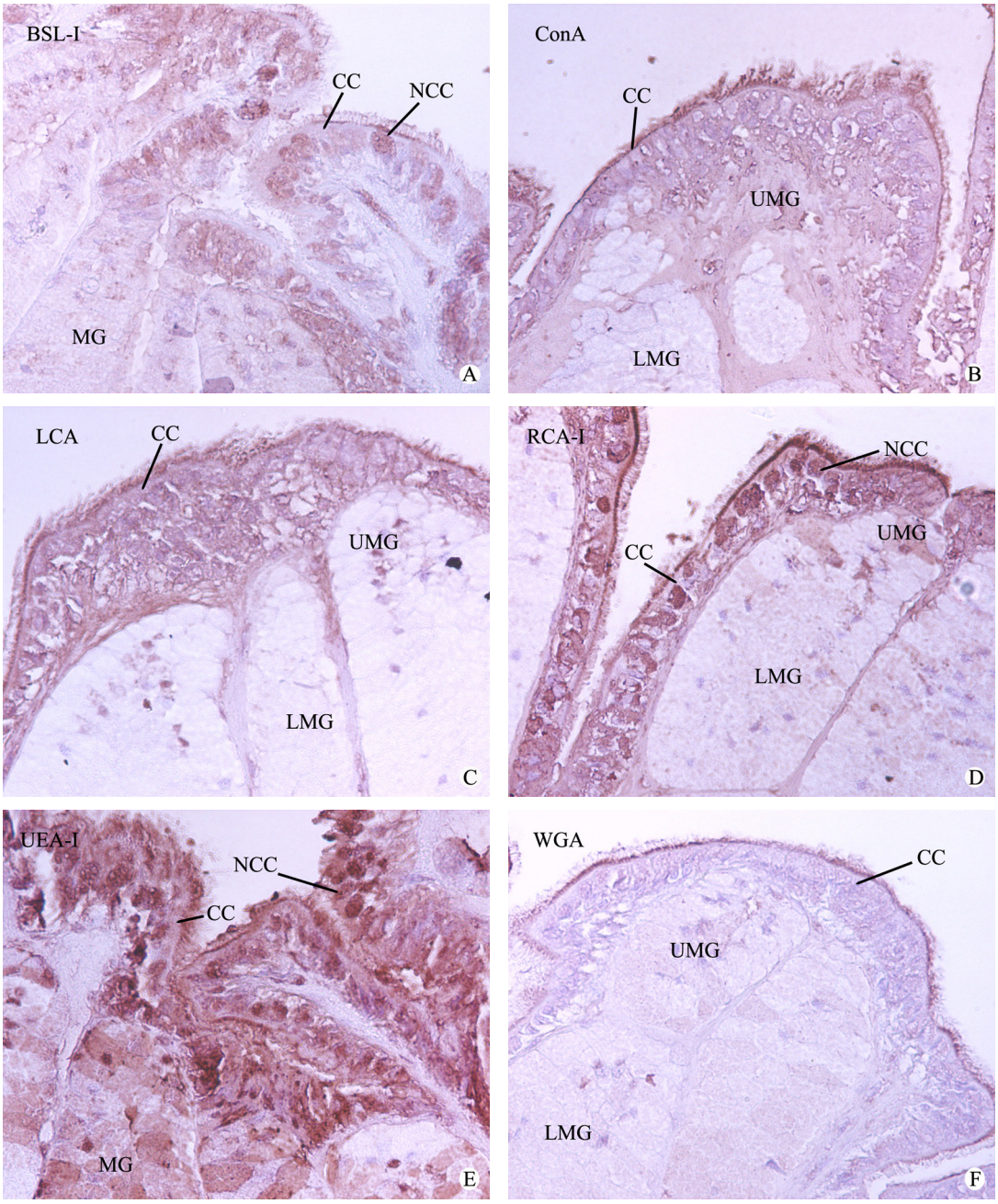
NCC
50um
C
RCA-I
UMG
50um
LMG
D
UEA-I
LMG
NCC
CC
50um
E
WGA
MG
CC
50um
F

Figure 15 **Lectin histochemistry of the PC4 region during the hormonal administration period.**

The PR region of oviductal tissues were subjected to various types of lectin staining including BSL-I (panel A), ConA (panel B), LCA (panel C), RCA-I (panel D), UEA-I (panel E) and WGA (panel F). CC = ciliated cell, LMG = lower mucosal gland, NCC = non-ciliated cell, UMG = upper mucosal gland.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LCA
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UMG
LMG
C
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NCC
CC
UMG
LMG
D
UEA-I
NCC
CC
MG
E
WGA
CC
UMG
LMG
F