FULL PAPER

Wildlife Science

Testicular regulation of seasonal change in apocrine glands in the back skin of the brown bear (*Ursus arctos*)

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ABSTRACT. Brown bears communicate with other individuals using marking behavior. Bipedal back rubbing has been identified as a common marking posture. Oily substances are secreted via enlarged sebaceous glands in the back skin of male bears during the breeding season. However, whether apocrine gland secretions are associated with seasonal changes remains unknown. The present study aimed to identify histological and histochemical changes in the secretory status and the glycocomposition of the apocrine glands in the back skin of male bears in response to changes in seasons and/or reproductive status. The apocrine glands of intact males during the breeding season were significantly larger and more active than those of castrated males during the breeding season and those of intact males during the non-breeding season. Lectin histochemical analyses revealed a more intense reaction to *Vicia villosa* agglutinin (VVA) in the cytoplasm, mainly Golgi zones of apocrine cells during the breeding season among castrated, compared with intact males. Positive staining for VVA was quite intense and weak in intact males during the non-breeding and breeding seasons, respectively. Ultrastructural analysis revealed VVA positivity in the Golgi zone, especially around secretory granules in apocrine cells. Changes in lectin binding might reflect a change in the secretory system in the apocrine cells. The present histological and histochemical findings of changes in the secretory status and glycocomposition of the apocrine glands according to the season and reproductive status suggest that these glands are important for chemical communication.

KEY WORD: glycocomposition, olfactory communication, skin gland, tree-rubbing, *Ursus arctos*

Chemical communication is crucial for the transmission of information in many species [11], and the feces, urine and secretory substances from skin glands were used as the chemical signals. In the chemical communication, apocrine and sebaceous glands are utilized, the volatilities of two glands are different, and it is hypothesized that the secretions from these glands play a different role in the chemical communication [11].

Chemical communication probably can be effective communication in brown bears (*Ursus arctos*) that are solitary and live in vast home ranges [3, 22], because chemical signals, unlike visual and auditory signals, can convey information even in the absence of the producer. In addition, the vomeronasal organ that detects species-specific chemicals is well-developed in the bears [25]. In general, the location of mammalian scent glands is often linked to behavioral patterns of scent marking [17, 19, 23]. Brown bears demonstrate complicated marking behaviors, such as rubbing, biting, clawing and urinating, and bipedal back rubbing is a common

Table 1. Number of animals and seasonal examinations

Sexual status	ID[a]	Age (y)	Histology		Lectin histochemistry					TEM	Facility[b]
			June	October	February	April	June	August	October	June	
Intact	I	18			✓	✓	✓	✓	✓		A
	II	8			✓		✓	✓	✓		A
	III	8			✓		✓	✓	✓		A
	IV	22	✓	✓		✓	✓		✓		B
	V	22	✓	✓		✓	✓		✓		B
	VI	23	✓	✓		✓	✓		✓		B
	VII	19	✓	✓							B
	VIII	19	✓	✓							B
	IX	24	✓	✓							B
	Total		6	6	3	4	6	3	6		
Castrated	X	14	✓				✓				A
	XI	7	✓				✓			✓	B
	XII	15	✓				✓			✓	B
	Total		3				3			2	

a) Samples from Bear I, II, III, IV, V, VI, IX, X, XI and XII were included in a previous study [26]. b) A, Noboribetsu Bear Park; B, Sahoro Bear Mountain.

marking posture [2, 6, 18]. During the breeding season (May to July: [7, 27]), enlarged sebaceous glands and oily substances with a sweet aroma are observed in the back skin of male bears [26]. Apocrine glands that seem to secrete pheromones [1, 9, 19] were also observed in the back skin of male brown bears [20, 26]. However, whether apocrine gland secretions are associated with seasonal changes remains unknown.

Carbohydrates are crucial substances that are involved in secretion by many exocrine glands [21]. Lectin histochemistry was used for the evaluation of glycoconjugates, because lectins bind to specific carbohydrates. Glycoconjugates in the scent glands of many species have been analyzed using lectin histochemistry [14, 23, 31]. Seasonal changes in carbohydrates in the apocrine glands of the forehead skin of the male impala revealed by lectin histochemistry seem regulated by androgens during the breeding season [30]. The present study aimed to determine histological and lectin histochemical changes in the secretory status and glycocomposition in the apocrine glands in the back skin of the brown bear in response to seasons and reproductive status.

MATERIALS AND METHODS

Experimental design

In order to investigate the testis-regulated seasonal change in the apocrine glands, we classified the seasons as being non-breeding (February, August and October), transitional (April) and breeding (June). The period with high plasma testosterone concentration before the breeding season was regarded as the transitional season (April) [26]. We compared the size and the activity of apocrine glands between intact males (n=6) during the breeding and non-breeding seasons, and between intact (n=6) and castrated (n=3) males during the breeding season to determine secretory status in response to seasons and to reproductive status.

We initially screened 21 lectins in intact males during the breeding season (n=1) and non-breeding season (n=1), and in a castrated male during the breeding season (n=1) to identify seasonal and testis-dependent differences in glycoconjugates in the apocrine glands. We selected *Vicia villosa* agglutinin (VVA), lectin binding to *N*-acetyl-galactosamine (GalNAc) [24], as the most appropriate lectin, because the intensity of VVA in the apocrine glands of the intact male during the breeding season was dramatically different from those of the intact male during the non-breeding season and the castrated male during the breeding season. We then histochemically compared VVA reactions between intact (n=6) and castrated (n=3) males during the breeding season. We also investigated seasonal effects in the glycocomposition of intact males (n=6) once every two months using VVA. We then investigated ultrastructures that were positive for VVA in apocrine cells using transmission electron microscopy (TEM). Table 1 shows details of the samples included in each experiment.

Animals

Total of 12 captive Hokkaido male brown bears (*U. a. yesoensis*) from the Noboribetsu Bear Park, Hokkaido, Japan (42°N, 141°E; three intact and one castrated males), and Sahoro Bear Mountain, Hokkaido, Japan (43°N, 142°E; six intact and two castrated) were used in the present study. Some samples from bears were included in a previous study [26], as described in Table 1. The living conditions and maintenance of the bears at each facility were as described previously [26]. Back rubbing was evident during the experimental periods, and this marking behavior was observed more often during the breeding season. This study was approved by the Animal Experiment Committee at Obihiro University of Agriculture and Veterinary Medicine, Japan (no. 28-218), and proceeded according to Institutional Regulations on the Management and Operation of Animal Experiments.

Anesthesia

The bears were anesthetized by the intramuscular administration of 2.5–3.5 mg/kg (body weight) of a 1:1 mixture of zolazepam HCl and tiletamine HCl (Zoletil; Virbac, Carros, France) with either 0.03 mg/kg medetomidine HCl (Domitor; Orion Corporation Animal Health, Turku, Finland) or 1 mg/kg xylazine HCl (Selactar; Bayer Healthcare, Leverkusen, Germany) using blow darts. After sample collection, anesthesia was reversed by the intramuscular administration of 0.03 mg/kg atipamezole HCl (Antisedan; Orion Corp. Animal Health).

Tissue collection

Skin samples were obtained from the center of the back between scapulae as follows. The fur of skin samples in each bear was shaved, the skin was repeatedly washed with 20 mg/m*l* povidone iodine (Isodine; Meiji Seika, Tokyo, Japan) and 50% isopropanol (Yakuhan, Kitahiroshima, Japan). Skin biopsies were then collected using an 8-mm BP-L80K Biopsy Punch (Kai Ind. Ltd., Seki, Japan) at a depth of 25 mm. The wounds were sutured with Coated Vicryl® Plus Antibacterial Suture (Ethicon, Somerville, NJ, U.S.A.). The bears were then subcutaneously administered with 0.2 mg/kg of meloxicam (Metacam; Boehringer Ingelheim, Germany) and 5 mg/kg of Enrofloxacin (Baytril; Bayer, Leverkusen, Germany) for analgesia and antimicrobial activity, respectively. Veterinarians conducted all of the above procedures.

Size and activity of apocrine glands

Columnar skin biopsy specimens were fixed in 10% formalin, embedded in paraffin and cut into 4-μm thick sections parallel to the long axis. The area of apocrine glands was measured within 6×10-mm^2 rectangles on 10 sections of skin tissues (cut at 200-μm intervals) using ImageJ software (National Institutes of Health). The summarized value was taken as the estimated index of the size of the glands as described previously [26]. Activity was evaluated as the ratio of terminal portions containing apical projections to the total terminal portions of the apocrine glands as described [12].

Lectin histochemistry

Specimens were processed using the avidin–biotin complex (ABC) method with 21 biotinylated lectins (Table S1) of the lectin screening kits I-III (Vector Laboratories, Burlingame, CA, U.S.A.) as described previously [8]. Briefly, deparaffinized sections were incubated with 0.3% H_2O_2 in methanol for 30 min, followed with 0.1 M phosphate buffered saline (PBS) containing 2.5% bovine serum albumin for 30 min. Then slides were reacted with biotinylated lectins in PBS at 4°C overnight. The VVA concentration was 4 μg/m*l*, and Table S1 shows those of the other lectins. The samples were incubated with the ABC reagent PK-6100 (Vector Laboratories) at room temperature for 30 min, and then visualized using 0.02% 3,3′-diaminobenzidine tetrahydrochloride and 0.006% H_2O_2 in Tris-HCl buffer. The negative controls were performed using PBS instead of lectins. They were counterstained with hematoxylin. The intensity of reactions to lectins was evaluated on a scale of 0 (no staining), 1 (faintly staining), 2 (moderate staining) and 3 (intense staining).

Transmission electron microscopy

Formalin-fixed skin biopsies were cut into slices 50–100-μm thick and processed for VVA histochemistry as described above. After lectin staining, the skin samples were fixed in 0.1 M phosphate buffer (PB; pH 7.4) containing 3% glutaraldehyde for 60 min, washed with PB, post-fixed with 1% OsO_4 in PB for 60 min, dehydrated and embedded in LR white resin. Semi-thin sections (1-μm thick) were stained with toluidine blue for observation by light microscopy. Ultrathin sections (80-nm thickness) were examined using an HT7700 TEM (Hitachi, Tokyo, Japan) without uranyl acetate and lead citrate staining.

Statistical analysis

In the paired group (breeding and non-breeding seasons), the significant differences in the sizes and activities of apocrine glands were determined by testing for normality with the Wilk-Shapiro test, and using the paired *t*-test or the Wilcoxon signed rank test. In the unpaired group (intact and castrated males), the significant differences in the sizes and activities were determined by the unpaired *t*-test (groups have normal distribution by testing Wilk-Shapiro test, and the groups were equal variance by using *F*-test) or Wilcoxon rank sum test (groups do not have normal distribution by testing Wilk-Shapiro test). *P*-value of <0.05 indicated significant difference. All data were statistically analyzed using R software (R Development Core Team, 2015).
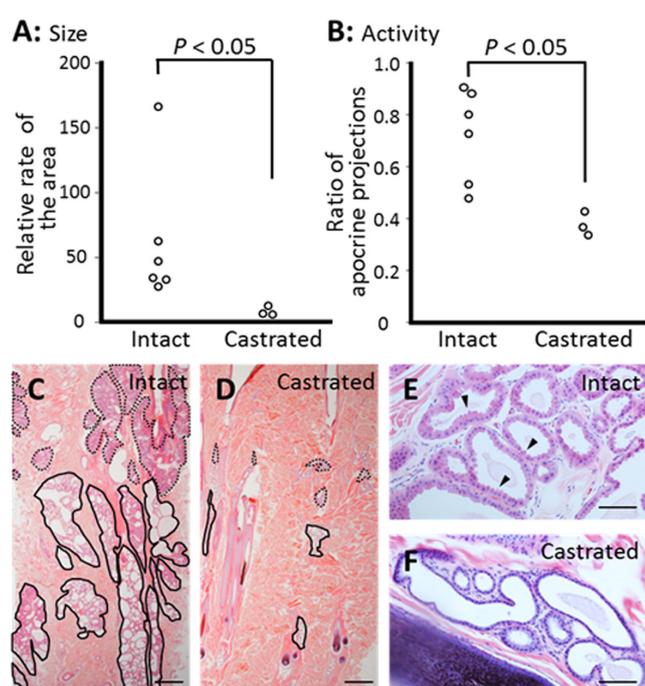
RESULTS

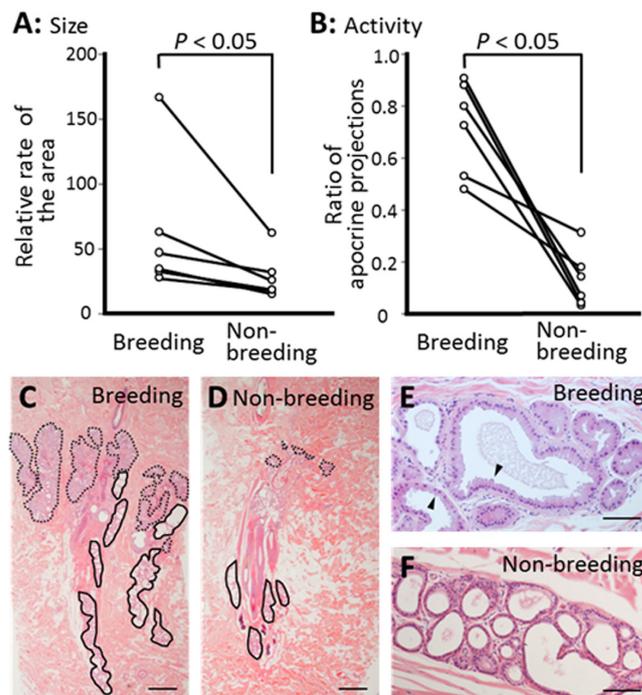*Changes in size and activity of apocrine glands*

Enlarged apocrine glands were located more deeply than enlarged sebaceous glands in the back skin of intact male during the breeding season, and both types of glands were situated next to hair follicles (Figs. 1 and 2). The apocrine glands of intact males were significantly larger and more active than those of castrated males during the breeding season (*P*<0.05, Wilcoxon rank sum test and *P*<0.05, unpaired *t*-test, respectively; Fig. 1). Apocrine glands of all six intact males were significantly larger and more active during the breeding, than the non-breeding season (*P*<0.05, Wilcoxon signed rank test and *P*<0.05, paired *t*-test, respectively; Fig. 2).

Screening test

Screening for 21 lectins revealed Soybean agglutinin, *Bandeiraea simplicifolia* lectin-I, VVA, Jacalin and Peanut agglutinin react

Fig. 1. Size and activity of apocrine glands in the back skin of intact and castrated male brown bears during the breeding season. Size (A) and activity (B) of apocrine glands. Histological structure of apocrine glands of intact (C) and castrated (D) males. Solid and dashed lines, apocrine and sebaceous glands, respectively. Apocrine glands with apical projections (arrowheads) in intact males (E), and without apical projections in castrated males (F). Scale bars: 1 mm (C, D), 100 μm (E, F).

Fig. 2. Seasonal changes in size and activity of apocrine glands in the back skin of intact males. Size (A) and activity (B) of apocrine glands. Histological structure of apocrine glands of intact males during the breeding (C) and non-breeding (D) seasons. Solid and dashed lines indicate apocrine and sebaceous glands, respectively. Apocrine glands with apical projections (arrowheads) during the breeding season (E), and without apical projections during the non-breeding season (F). Scale bars: 1 mm (C, D), 100 μm (E, F).

to apocrine glands in the intact male during the non-breeding season and in the castrated male during the breeding season, but not in the intact male during the breeding season (Table S2). In particularly, VVA strongly bound to the apocrine glands of the intact male during the non-breeding season, and the castrated male during the breeding season.

Comparison of VVA staining between intact and castrated males

Staining for VVA was positive in cytoplasm, mainly Golgi zone of the apocrine cells of castrated males (Fig. 3c). The intensity of the VVA reaction during the breeding season was much higher in castrated, than in intact males (Fig. 3).

Seasonal changes of VVA staining in the apocrine glands of intact males

Figure 4 shows that staining for VVA was intensely positive in cytoplasm, mainly Golgi zone of the apocrine cells of intact males during the non-breeding season (February, August and October), moderate during the transitional period (April) and very weak during the breeding season (June).

*Ultrastructural analysis of apocrine glands using VVA histochemistry*

Staining for VVA was positive around the secretory granules of apocrine glands, and relevant to the positive staining in the Golgi zone determined by light microscopy (Fig. 5).

DISCUSSION

Sebaceous and apocrine glands are sources of chemical communication signals [11]. We previously showed that the volume of oily secretions and the size of sebaceous glands of the male brown bear are influenced by the season and by reproductive status [26]. The present study showed that seasons and reproductive status affect the secretory status of the apocrine glands as well as the glycocomposition that plays an important role in exocrine gland secretion [21]. Therefore, sebaceous and apocrine glands might both contribute to the transmission of reproductive information among bears. Sebaceous glands produce oily secretions that are long lasting and releasing their volatiles slowly, while apocrine glands produce watery volatile secretions that are generally involved in short-term signaling [5, 11]. Male brown bears might use mixtures of secretions from both apocrine and sebaceous
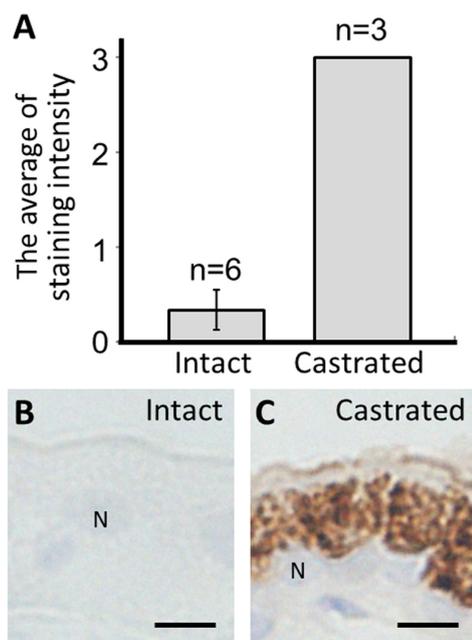
Fig. 3. Lectin histochemical VVA reaction in apocrine glands of the back skin of intact and castrated male brown bears during the breeding season. Staining intensity in intact and castrated males (A). The intensity of reactions to lectins was evaluated on a scale of 0 (no staining), 1 (faintly staining), 2 (moderate staining) and 3 (intense staining). Data are presented as means ± SEM. Representative staining features of apocrine cells of intact (B; score 0) and castrated (C; score 3) males. Abbreviations: N, nucleus. Scale bars: 5 μm.
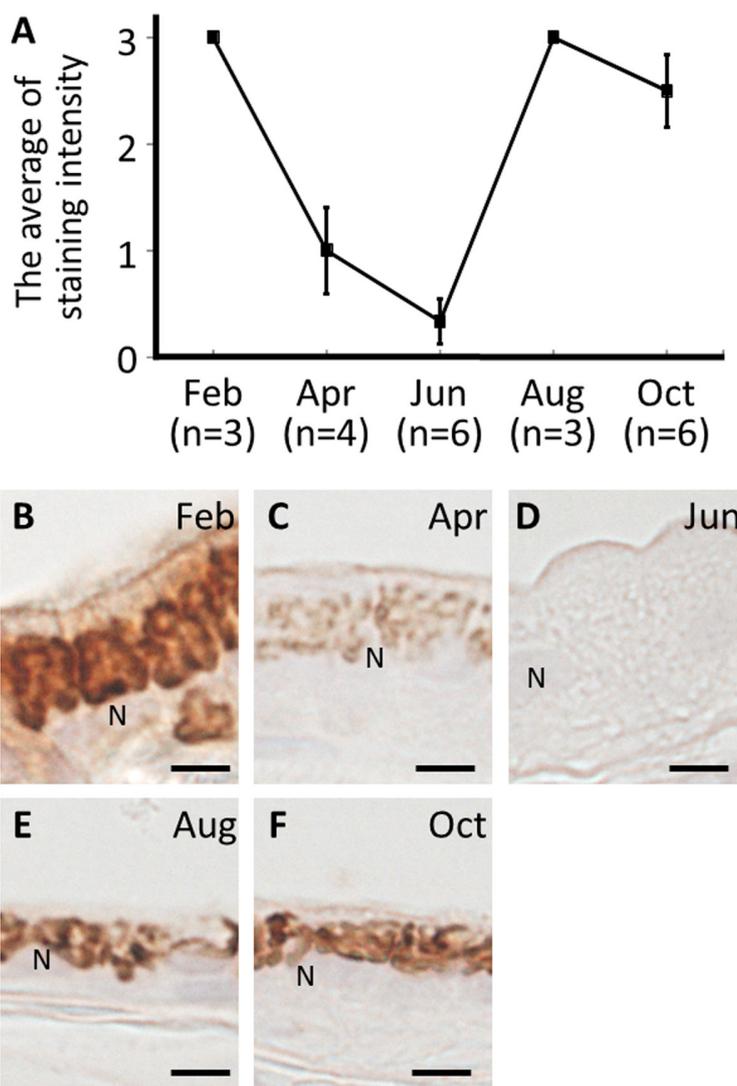
Fig. 4. Seasonal changes in VVA reaction by apocrine glands of intact males. Seasonal change of VVA staining intensity (A). The intensity of reactions to lectins was evaluated on a scale of 0 (no staining), 1 (faintly staining), 2 (moderate staining) and 3 (intense staining). Data are presented as means ± SEM. Representative staining features of apocrine glands in intact males during February (B; score 3), April (C; score 1), June (D; score 0), August (E; score 3), and October (F; score 3). Abbreviations: N, nucleus. Scale bars: 5 μm.

glands in back skin to mark and if so, such secretions might convey highly complex information.

The apocrine glands of intact males during the breeding season were significantly larger and more active than those of castrated males during the breeding season and those of intact males during the non-breeding season. These results indicated that apocrine gland secretions were activated during the breeding season under the influence of testicular sex hormones. The apocrine glands of forehead skin of the male impala become enlarged only during the breeding season [30], whereas those of metatarsal skin do not seasonally change. Preorbital apocrine glands in the male reindeer also vary according to the season, whereas apocrine glands in the caudal, interdigital and tarsal skin do not [10], and castration decreases the size of preorbital apocrine glands. In contrast, the size and activity of apocrine glands in the tarsal skin of white-tailed deer do not differ between sexes or among seasons [12]. Therefore, the regulatory mechanism of apocrine glands might depend on species and/or body region.

The lectin histochemistry screening test showed that reactions of some lectins, such as VVA, to apocrine glands were stronger in the intact male during the non-breeding season and in the castrated male during the breeding season compared with those in the intact male during the breeding season. In the previous study [30], staining intensity of lectin histochemistry does not always reflect to the activity of the apocrine secretory status such as the size, and it is consistent with our results.

The Golgi zone in apocrine cells was intensely positive for VVA during the non-breeding season in intact male brown bears,

and during the breeding season in castrated males. Furthermore, ultrastructural analysis revealed intensely positive VVA staining in the region around the secretory granules. *Helix pomatia* agglutinin, a lectin specifically binding to GalNAc like VVA [24], reacts intensely with the Golgi zone during the non-breeding season, but weakly during the breeding season in apocrine cells in the forehead of the male impala [30]. They postulated that changes in lectin binding in the Golgi zone reflect the mechanism of retaining secretory products in the cellular compartment [30]. VVA is the lectin that binds to a GalNAc [24]. In mammals, mucin-type *O*-linked glycan are starting with a GalNAc [29], and the *O*-linked glycosylation of protein plays a role in regulating polarized secretion [32]. In the apocrine glands, three secretion mechanisms; exocytosis, apocrine secretion and halocline secretion were known [4]. In intact males during the non-breeding season and castreated males during the breeding season, the apocrine secretion was inhibited, while the intensity of VVA was strong (the volume of GalNAc glycan may increase). On the other hand, in intact males during the breeding season, the apocrine secretion was activated, while the intensity of VVA was very weak (the volume of GalNAc glycan may decrease). Therefore, it is possible that the secretory system in intact males during the non-breeding season and in castrated males during the breeding season differs from that in intact males during the breeding season, and the exocytosis of apocrine glands might be activated in intact males during the non-breeding season.

Castration alters the cellular glycocomposition in accessory reproductive glands [13, 15, 16], suggesting that androgens can change carbohydrates in secretory cells [16]. Androgens appear to regulate seasonal changes in the glycocomposition of apocrine glands in the forehead of the male impala [30]. The present study showed that the intensity of VVA staining in the apocrine glands differed between castrated and intact males, and among seasons in intact males. The brown bear breeds seasonally and serum testosterone concentrations increase during the transitional and breeding seasons [26, 28]. Taken together, androgens might modulate changes in the size, activity and glycocomposition in the apocrine glands of male brown bears.

In conclusion, the present detailed histological and histochemical studies revealed that the secretory status and glycocomposition of the apocrine glands in the back skin of the brown bear change according to season and reproductive status. Therefore, these glands might be important for chemical communication among brown bears during the breeding season.
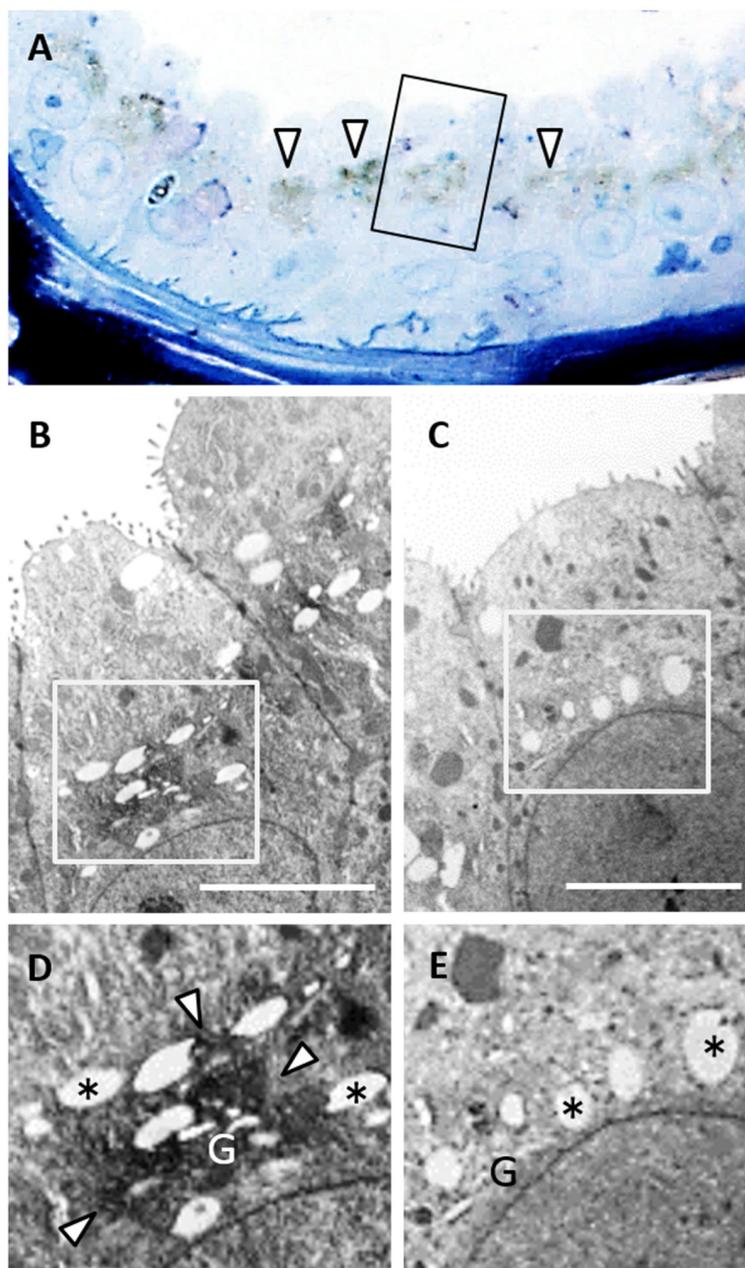
Fig. 5. Localization of VVA reaction in apocrine cells of castrated males during the breeding season. Semi-thin sections with VVA staining (A). Square (A) corresponds to panel (B). Arrowheads, positive VVA staining. Transmission electron microscopy (TEM) image of ultrathin section (B) adjacent to panel (A). TEM image without VVA staining from same individual (C). Square (B) and (C) correspond to panel (D) and (E), respectively. Asterisk, secretory granule. G, Golgi apparatus. Scale bar: 5 μm.

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