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Salivary glands of the cat flea, *Ctenocephalides felis*: Dissection and microscopy guide

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A B S T R A C T

Fleas are morphologically unique ectoparasites that are hardly mistaken for any other insect. Most flea species that feed on humans and their companion animals, including the cat flea (*Ctenocephalides felis*), have medical and veterinary importance. Besides facilitating blood acquisition, salivary biomolecules can modulate pathogen transmission. Thus, dissection of salivary glands is essential for comprehensive studies on disease vectors like the cat flea. Herein, we present the pictorial dissection protocol assisting future research targeting individual flea organs, for revealing their roles in vector competence and physiology. We provide a comprehensive guide, allowing researchers, even with limited practical experience, to successfully perform microdissection for collecting cat flea salivary glands. Furthermore, the protocol does not require expensive, sophisticated equipment and can be accomplished with routinely available tools. We illustrated expected results with morphological changes of salivary glands upon blood feeding as well as fluorescently stained these organs.

Introduction

Fleas are small, bilaterally compressed, flightless insects that belong to the order Siphonaptera. Adults with an obligate hematophagous lifestyle are predominantly ectoparasites of mammals (~94 % of known species) and occasionally of birds (the remaining 6 %). Fleas adapted saltatorial hind legs in addition to piercing-sucking mouthparts for blood acquisition (Whiting et al., 2008; Durden and Hinkle 2018). They are heavily chitinized and bear prominent sclerotized combs (ctenidia), and on their head possess lateral noncompound eyes (Gage 2004) (Fig. 1A).

The cat flea, *Ctenocephalides felis* (family Pulicidae), is the most important pervasive flea pest occurring worldwide (Clark et al. 2018). It feeds primarily on domestic and feral cats (*Felis catus*) and dogs (*Canis lupus familiaris*), but also on humans, several livestock species [*e.g.*, horses (*Equus caballus*), goats (*Capra hircus*), and cattle (*Bos taurus*)], and wild mammals, such as opossums (*Didelphis* spp.) and small rodents (Linardi and Santos 2012; Rust 2017). Flea infestation is a significant problem and can develop into flea allergy dermatitis, *i.e.*, hypersensitization of hosts to flea bites caused by certain compounds found in the cat flea's saliva (Carlotti and Jacobs 2000; Contreras et al., 2018). Being a generalist blood-feeder, parasitizing a wide-range of potential hosts of zoonotic infections, the cat flea is capable of transmitting pathogenic microorganisms to humans, including *Rickettsia felis* and *Rickettsia typhi* (causing flea-borne spotted fever and murine typhus, respectively) (Caravedo Martinez et al., 2021), *Bartonella henselae* (agent of cat-scratch fever) (Sepúlveda-García et al., 2023), and potentially *Yersinia pestis* (causative bacterium of plague) (Bland and Hinnebusch 2016). Moreover, the cat flea is an intermediate host of the tapeworm *Dipylidium caninum*, prevalent in pet populations and occasionally in children (Elston and Do 2010).

During blood feeding, insect saliva is injected into the skin. It contains a mixture of salivary proteins interfering with the host's immune responses. In addition to facilitating blood acquisition, salivary constituents can facilitate pathogen transmission (Arcà and Ribeiro 2018). Therefore, studies targeting salivary glands and components of the saliva are paramount as they can enhance our comprehension of the complex vector-pathogen-host interaction. Microdissection of disease vectors, including the cat flea, is required when studying salivary gland-derived molecules involved in pathogen transmission (Danchenko et al., 2021). For example, recently we demonstrated the necessity to dissect and collect flea salivary glands to determine the dissemination of

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R. felis to flea tissues essential for transmission (Laukaitis et al., 2022). Moreover, a bulk dissection (preparation of tissues from many individual fleas) was performed in our laboratory for nucleic acid and protein extraction (Ribeiro et al., 2012; Danchenko et al., 2021; Lu et al., 2023).

Although a handful of publications showed immunofluorescent and transmission electron microscopy of flea salivary glands (Macaluso et al., 2008; Thepparit et al., 2013; Brown et al., 2016; Laukaitis et al., 2022), descriptions of the preparation and dissection process (Andersen et al., 2007; Bland et al., 2015) are very limited in the literature. A detailed outline of the dissection procedure is valuable for identifying the actual physical appearance of different tissues and provides a useful complement to drawn illustrations of flea internal anatomy (Durden and Traub 2002; Service 2012; Mehlhorn 2012; Brown 2019; Dougas et al., 2021). Therefore, the purpose of this article is to provide a comprehensive instruction guide for cat flea dissection, with an emphasis on salivary glands morphology and cell architecture. The pictorial descriptions presented in this Method Report constitute helpful guidelines for all investigators interested in *C. felis* dissection, salivary gland microscopy, and sialome research.

Material and methods

Source of fleas

The colony of cat fleas (originally obtained from Elward II Laboratory, Soquel, CA, United States) was maintained under standardized insectary conditions at 24 ± 2 °C and 75 ± 5 % relative humidity, on a 16:8 h light:dark schedule. Newly emerged adult fleas were fed defibrinated bovine blood (HemoStat Laboratories, Dixon, CA, United States) in an artificial feeding system (Wade and Georgi 1988) as previously described (Reif et al., 2011).

Dissection station and specimen preparation

After continuous blood-feeding for 24 h to 14 days, fleas were collected and sequentially surface washed with 10 % bleach (sodium hypochlorite) for 2 min, 70 % ethanol for 2 min, and 3 × sterile distilled water for 2 min to remove surface debris, dried flea feces, host blood, and microorganisms. Next, fleas were immobilized on ice (approximately 30 min). Aliquots of 15 μL of 0.01 M sterile phosphate-buffered saline (PBS) pH 7.2 were placed in the wells of a glass depression slide under a stereomicroscope. Immobilized individual fleas were then laid down on their side into the drop of PBS using fine-tipped forceps. For flea dissection, a sharp needle (27 G) attached to a 1 mL slip-tip syringe was held in each hand. A very fine needle (30 G) attached to a syringe and a dissection probe (microneedles/*minuten nadeln* mounted to a wooden applicator stick) were necessary for tissue manipulation (Fig. 1B). The materials and tools used for flea dissection are listed in Table S1. The manufacturer/supplier with a corresponding identifier is provided for each item. All essential steps of a flea dissection are thoroughly described in the following Section 2.3-2.4, and File S3.

Note: Fine-tipped forceps can be cleaned by rinsing in 10 % bleach or 70 % ethanol and dried with Kimwipes disposable wipers between different sample groups. Surface cleansing and recycling of needles

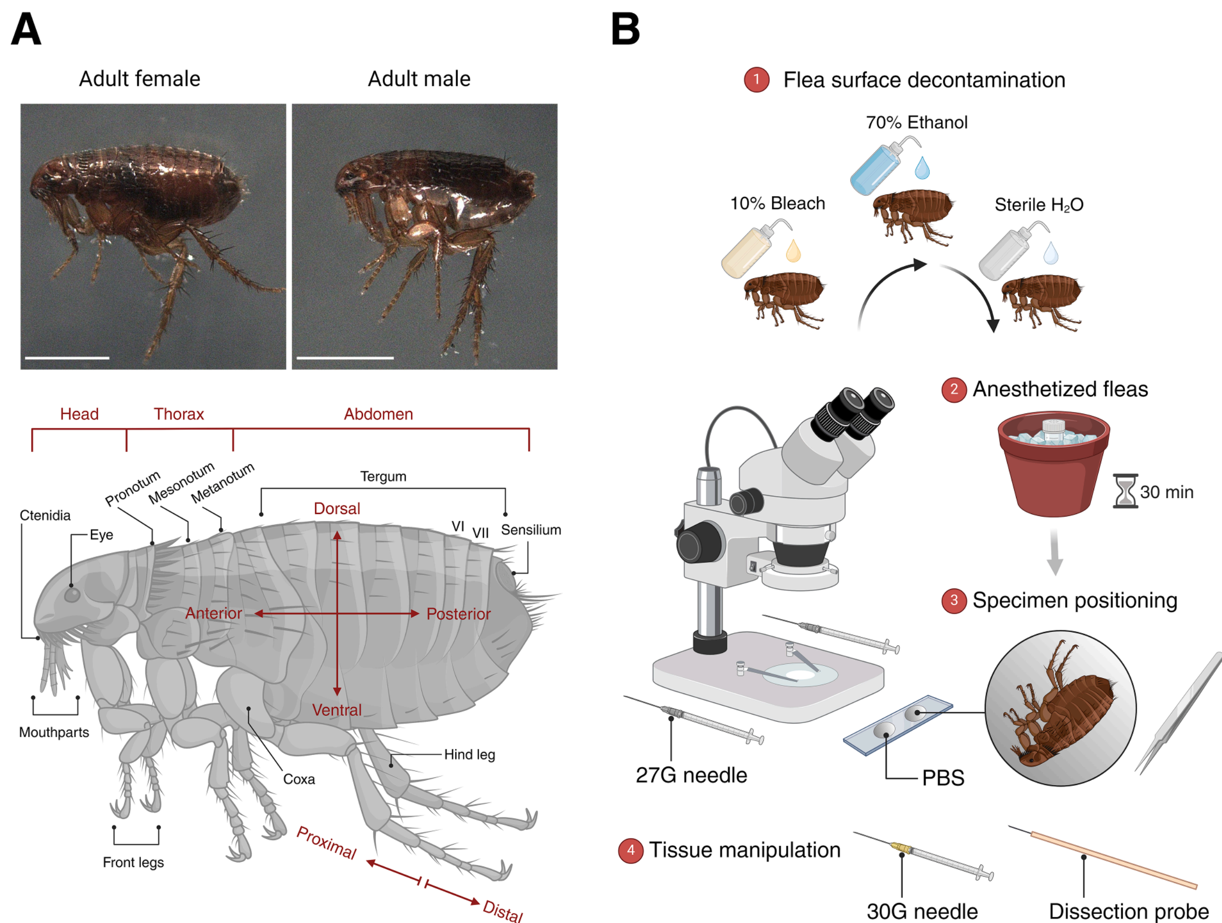

Fig. 1. Overview of sample preparation. **(A)** Lateral view of adult female and male cat flea (*C. felis*) and their anatomical diagram with key morphological features. Photographs were taken with a Moticam 1080 camera attached to a Motic SMZ-143 stereomicroscope. Scale bar represents 1 mm; **(B)** Dissection station and specimen preparation. The protocol details are given in Materials and Methods, Section 2.2. Abbreviation: PBS, phosphate-buffered saline, pH 7.2. Created with BioRender.com.

should be avoided for safety reasons and hazard of contamination. If needles are compromised during the dissection process, we recommend replacing them with a new item.

Instruction for salivary glands dissection

Using the needle held in one hand, the flea should be gripped by the head capsule and securely pressed against the slide. Next, a small incision is made between the mesonotum and metanotum with the needle held in the other hand, and the exoskeleton is gradually separated into two halves (Fig. 2A-B). To remove the salivary glands from the body cavity, the posterior end of the abdomen is pulled away gently and very slowly from the head (Fig. 2C). The paired salivary glands, still attached with thin ducts, should appear as translucent ovoid-shaped organs (Fig. 2D). Lastly, clip the salivary ducts with a needle to detach the glands for tissue collection. In some instances, careful manipulation with a 30 G needle is necessary to separate the salivary glands from fat body cells or any connective tissue that remains attached to them.

Note: The critical part is pulling the abdomen away from the head. If the salivary ducts are severed during the process, it is possible to find the broken glands amongst the surrounding organs; however, that may be of limited success and require dexterity with dissection tools.

Dissection of the alimentary canal and female reproductive organs

Once the exoskeleton is dissected into two parts, the alimentary canal is freed to be isolated. If the oesophagus is broken (Fig. 2D), the digestive tract may be withdrawn from the abdomen as follows: the needle held in one hand is pressed firmly to the coxa and the midgut and hindgut are released from the abdomen by gently pulling the apex of the proventriculus with the needle held in the other hand (Fig. 2E-G). If the oesophagus is not severed during the procedure described in Section 2.3, the proventriculus, midgut, and hindgut should remain attached to the head (Fig. 2H).

After the alimentary canal is removed from the body cavity, the reproductive organs can be recovered intact. Firstly, the needle held in one hand is inserted inside the anterior end of the flea body and firmly pressed against the slide (Fig. 2I). Next, the chitin exoskeleton is nicked between the VI and VII tergum segments, and the posterior end is opened using the needle held in the other hand (Fig. 2J). While the sensillum is carefully pulled away from the rest of the abdomen, the ovarioles are dislodged and spread out on the slide (Fig. 2K-L). Lastly, the ovaries are dissected by slitting the tergum with the needle. The ovarioles can be debrided from the fat body using a dissection probe if necessary.

Note: After the dissection is completed, it is strongly recommended that the harvested flea tissues are rinsed with PBS (*i.e.*, placing them to a new drop of PBS in the second well of the depression slide) before transferring biological material into a collection tube, in order to remove residual hematocytes, debris of exoskeleton, and undesired off-target tissues. Depending on the aim of the study, organs can be collected in RNA*later* solution (for gene expression studies) or transferred directly to an extraction buffer, such as TRIzol Reagent (for nucleic acid isolation) or I-PER Reagent (for protein extraction). Alternatively, the dissected tissues may be stored in PBS at –80 °C.

Fluorescent staining of salivary glands

Fluorescent staining of the cat flea salivary glands was performed according to a previously published protocol (Suwanbongkot et al., 2019) with modifications. The procedure described here includes cell membrane permeabilization for having option to study intracellular

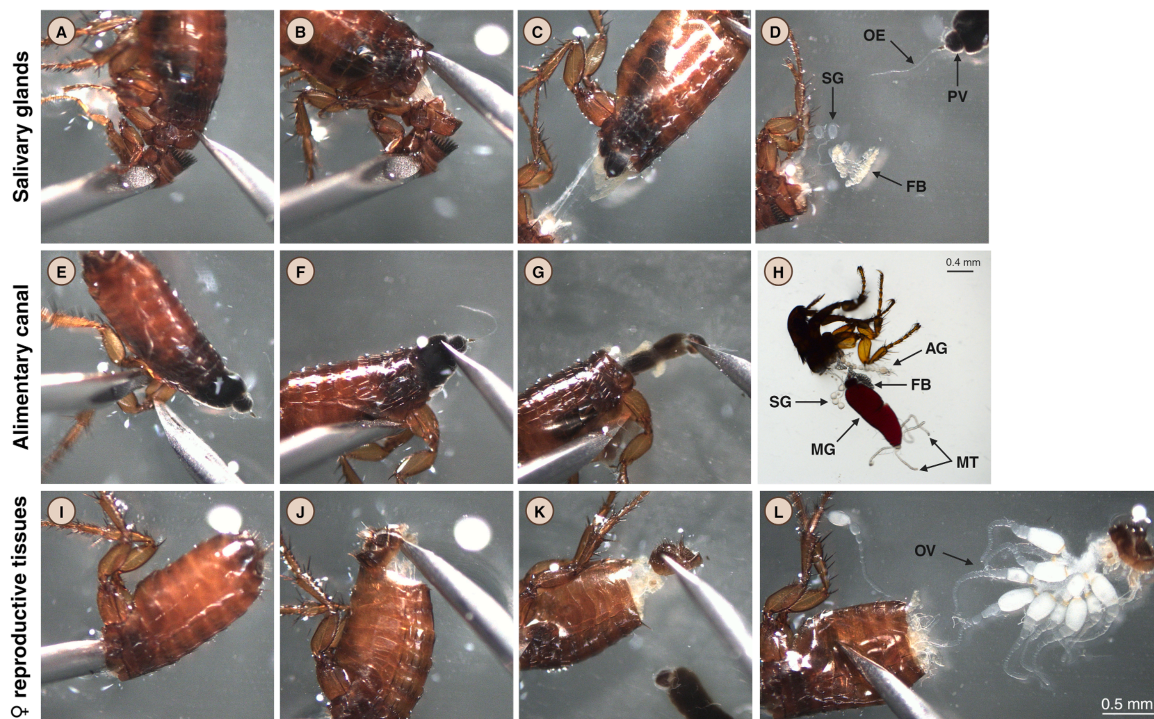

Fig. 2. Step-by-step guide of flea dissection. The depicted specimen is an adult female collected 7-days post-feeding, undergoing active oviposition. The pictorial dissection walkthrough of the salivary glands (panels **A-D**), the alimentary canal (panels **E-H**), and female reproductive organs (panels **I-L**) is extensively described in Materials and Methods, Section 2.3-2.4. If the operator is left-handed, all displayed hand motions should be reversed. For the fine microdissection of a cat flea, a 27 G needle attached to a thin syringe is recommended. The dissection procedure was recorded via Moticam 1080 camera attached to a Motic SMZ-143 stereomicroscope, using a reflective light source, apart from panel H, acquired with a transmissive light source. The terminology of the flea's internal anatomy is according to Rothschild et al. (1986). Abbreviations: AG, abdominal ganglia of the ventral nerve cord; FB, fat body; MG, midgut; MT, Malpighian tubule; OE, oesophagus; OV, ovarioles; PV, proventriculus; SG, salivary glands. Created with BioRender.com.

pathogens. Briefly, dissected and rinsed salivary glands were fixed with Bouin's solution (71 % saturated picric acid, 24 % formaldehyde, 5 % glacial acetic acid) or with 4 % paraformaldehyde in cytoskeleton-stabilizing PHEM buffer (Danchenko et al., 2019) for 15 min in an 8-well chamber slide. Next, samples were permeabilized with 0.1 % Triton X-100 in PBS for 10 min and blocked with 5 % milk solution in PBS overnight at 4 °C. To suppress unwanted autofluorescence in flea tissues, samples were then counterstained with 0.01 % Evans blue in PBS for 5 min or Vector TrueVIEW autofluorescence quenching kit. To visualize filamentous actin, salivary glands were probed with Alexa Fluor 647-tagged phalloidin (Invitrogen, reference #A30107, lot #2186396, United States). All incubation steps were completed in a humidified chamber. Lastly, tissue samples were mounted with Vectashield Medium containing DAPI (4′,6-diamidino-2-phenylindole). The permanent mounts were covered with round coverslips and ringed with clear nail polish. The preparations were observed using a Zeiss LSM 980 Airy Scan II confocal microscope or Nikon Eclipse 90i epifluorescent microscope. The materials used for fluorescent staining are listed in Table S2 with the corresponding vendor and item number information.

Results and discussion

The dissection method described here can be successfully performed on newly emerged adult females and males, as well as blood-fed cat fleas. The authors do not recommend the outlined protocol for fleas stored in 70 % ethanol/isopropanol and frozen specimens kept either at –20 or –80 °C (for > 10 min), because insect samples become fragile and shatter easily during dissection.

Morphology of the cat flea salivary glands

Fleas have two pairs of salivary glands (four in total), two on each side of the abdomen, attached to a pair of salivary ducts. The two single cuticle-lined salivary ducts unite to form one long common tube (Figs. 3-4). Representative photographs comparing salivary gland morphology of both sexes collected on different days post-feeding are shown on Fig. 3A. The maturation of cat flea gonads and oviposition results in changes of various internal organs, including the salivary glands, midgut epithelium, and fat body, which follow a developmental cycle. Specifically, in the female flea during oocyte maturation, the salivary gland cells greatly increase in size (Fig. 3A, top row; Fig. 4C), analogous to other members of the Pulicidae family: the rat flea *Xenopsylla cheopis* and the rabbit flea *Spilopsyllus cuniculi* (Rothschild et al., 1986).

Remarkably, among the numerous specimens studied, we spotted individuals with joined/fused and supernumerary salivary glands, while such abnormality was not sex-linked (Fig. 3B). Development and growth

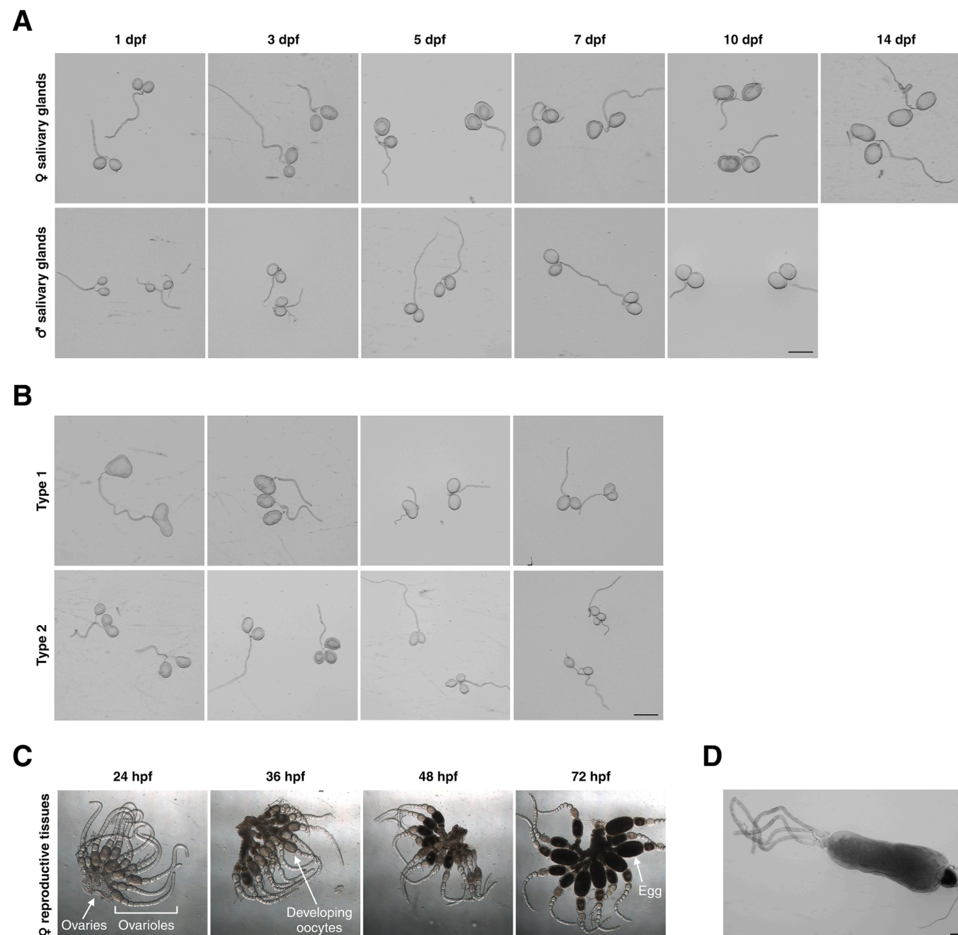

Fig. 3. Dissected cat flea salivary glands, reproductive tissues, and digestive tract observed with bright-field light microscopy. **(A)** Changing morphology of salivary glands through the course of blood-feeding. Differences were recorded between the sexes after 1-, 3-, 5-, 7-, 10-, and 14-days of continuous feeding (dpf). Scale bar represents 200 µm; **(B)** Variety of organ malformation and inter-individual variability of the flea's salivary gland morphology. Two types of tissue abnormality were observed: i) joined/fused glands, and ii) presence of an additional 5th gland. Scale bar represents 200 µm; **(C)** Maturation of female reproductive tissues and development of oocytes linked to blood acquisition, documented 24–72 h post-feeding (hpf). Scale bar represents 500 µm; **(D)** Digestive tract of the cat flea containing partially digested bloodmeal. Scale bar represents 200 µm. Micrographs displayed on panels A, B, and D were obtained using an Olympus DP72 camera attached to an MVX10 stereo dissecting microscope with a transmissive light source. Photographs on panel C were captured with a Moticam 1080 camera attached to a Motic SMZ-143 stereomicroscope using transmissive light. Created with BioRender.com.

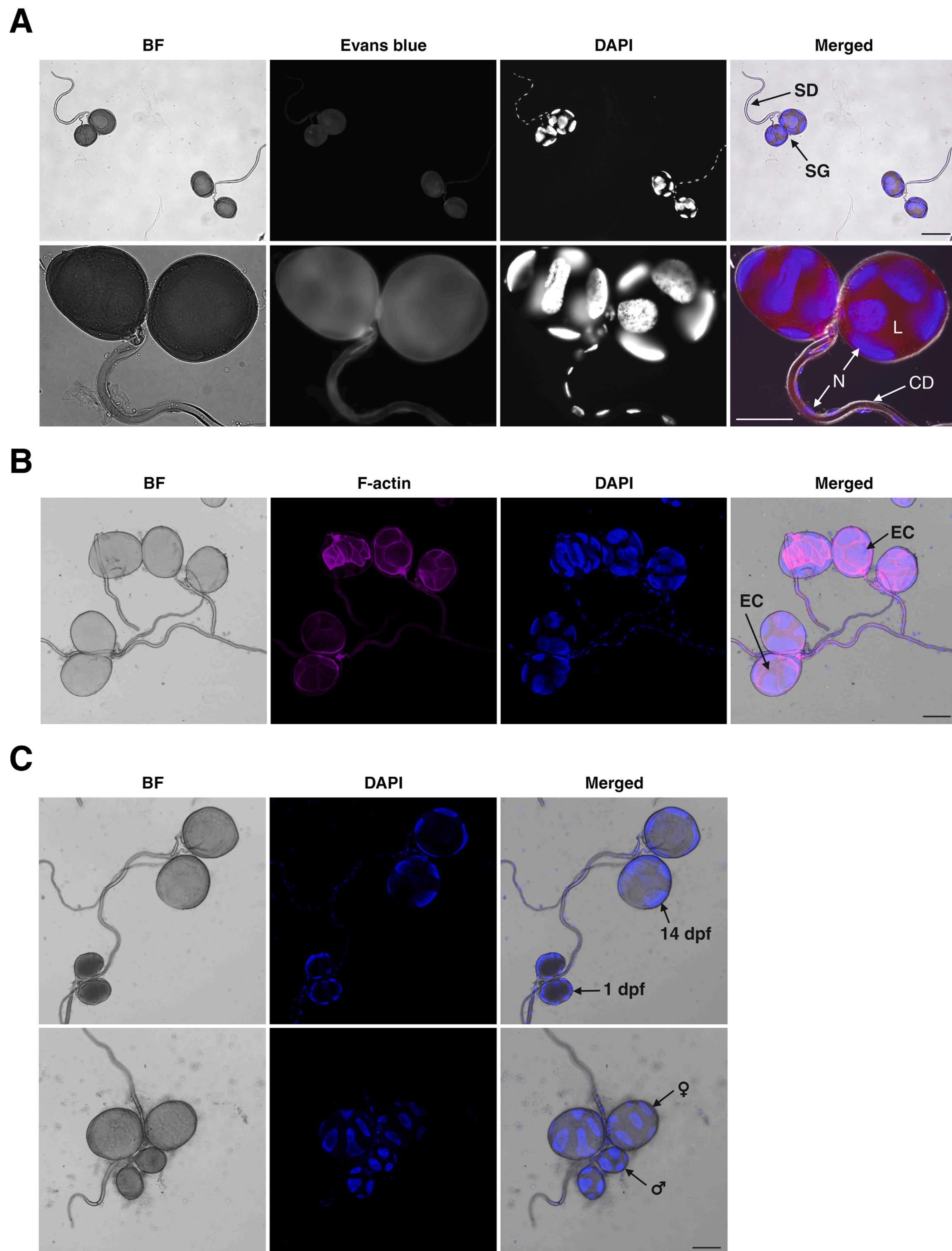

Fig. 4. Adult salivary gland cellular architecture of the cat flea (*C. felis*). **(A)** Fluorescent staining of female salivary glands dissected 3-days post-feeding. The tissues were fixed with Bouin's solution and permeabilized using 0.1 % Triton X-100 in PBS. Nuclei were labeled with DAPI (blue signal in merged images), and salivary glands were counterstained with Evans blue (red fluorescent signal in merged images). Scale bar represents 100 μm (upper row) and 50 μm (lower row); **(B)** Fluorescent staining of female salivary glands dissected 7-days post-feeding. The samples were fixed with 4 % paraformaldehyde in cytoskeleton-stabilizing PHEM buffer and permeabilized using 0.1 % Triton X-100 in PBS. Actin filaments were labeled with conjugated phalloidin (purple signal), and nuclei were stained with DAPI (blue signal). Salivary glands were treated with a Vector TrueVIEW autofluorescence quenching kit. Scale bar represents 100 μm; **(C)** Comparative images of female salivary glands 1- and 14-days post-feeding (dpf), and differences in salivary gland structure between sexes. The preparations were fixed with Bouin's solution and permeabilized with 0.1 % Triton X-100 in PBS. Gland and duct nuclei were visualized with DAPI (blue signal). Scale bar represents 100 μm. The terminology of the flea's internal anatomy is according to Rothschild et al. (1986). Abbreviations: BF, bright-field microscopy; CD, striated cuticle of duct; EC, epithelial (secretory) cell; L, lumen; N, nucleus; SD, salivary duct; SG, salivary glands. Micrographs on panel A were taken using a Nikon Eclipse 90i microscope, and images on panels B and C were captured using an LSM 980 Airy Scan II confocal microscope. Created with BioRender.com.

of insects is controlled by hereditary and environmental factors (*e.g.*, nutrition and temperature). As a genetic factor, inbreeding may contribute to the emergence of morphological variation (Jong et al., 2017). In holometabolous insects, including the cat flea, tissue differentiation is connected to the growth and transcription factors that control metamorphosis within the pupa. Although organogenesis of model insect organisms is currently rather well understood (Nijhout et al., 2014; Texada et al., 2020; Nation 2022), the exact mechanisms that regulate the shape and size of different organs in the flea body remains unknown. Notably, it is unclear how phenotypic abnormalities recorded in our study affect the physiology and the secretory function of the salivary glands.

Architecture of cells constituting salivary glands

Using dyes with affinity to well-conserved organellar markers, we visualized the adult cat flea salivary glands by epifluorescent and confocal microscopy (Fig. 4). Employing an optimized protocol for fluorescent staining, we observed cellular characteristics of the glands. Cat fleas have reservoir-structure type glands, with a central lumen, surrounded by 8–10 secretory cells (Williams 1991; Ribeiro 1995). In agreement with previous reports, each gland consists of relatively large mono-nucleated cells, which create a unicellular epithelial layer around the bladder-like cavity. Applying Evans blue dye, we counterstained the permeabilized cells of glands, and using a higher magnification (*i.e.*, 400 ×) we captured the striated cuticle of the duct (Fig. 4A). To obtain a view of the salivary glands` cytoskeletal architecture, we utilized a conjugated phalloidin probe for selective labeling of filamentous actin (Fig. 2B). The striking differences in the salivary glands structure during the course of blood-feeding and between sexes are highlighted in Fig. 4C.

Microscopy of other cat flea organs

Fleas have panoistic type ovaries, characterized by direct development of oogonia to oocytes, lacking specialized nutritive cells and nutritive cord. Dissection of *C. felis* female fleas showed that this species has six ovarioles in each of the two ovaries (Osbrink and Rust 1984; Büning and Sohst 1988). Following the first available bloodmeal, oocyte development is initiated, and oviposition begins 48−72 h post-feeding (Fig. 3C). Endosymbionts, as well as pathogenic bacteria, possess the ability of infecting the flea reproductive organs (Azad et al., 1992; Driscoll et al., 2020), thus maintaining transovarial transmission to the next generation of insects. Consequently, ovarial tissues, harvested after dissection, are essential for pathogen detection and analysis of microbiome composition.

The alimentary canal of fleas consists of an epipharynx, an oesophagus, a proventriculus with numerous spines, a barrel-shaped midgut, a hindgut, and a small rectum. Besides, four Malphigian tubules are connected to the anterior portion of the hindgut (Service 2012) (Fig. 3D). Following bloodmeal ingestion, the digestive tract of fleas is the first organ affected by invading microorganisms and a gateway for their replication and dissemination (Dreher-Lesnick et al., 2010; Brown et al., 2021). Therefore, dissection of the alimentary canal is vital for medical entomology studies (Hinnebusch et al., 2017).

Conclusions

Traditional light microscopy remains the most frequent instrument for exploring changes in tissue morphology. Dissection of flea specimens on a glass slide with the aid of a stereomicroscope permits observing hidden anatomical structures. Cat fleas are petite insects (1.5–3.0 mm in length) with a sclerotized exoskeleton, making dissection challenging compared to other arthropods. This paper thoroughly describes the cat flea dissection technique and provides a detailed summary of salivary glands morphology and cellular architecture. We also outlined the preparation of permanent mounts of stained salivary glands. Importantly, this pictorial guide will be indispensable for advancing the study of flea salivary glands, and other internal organs, thus contributing to better understanding of vector-pathogen-host interactions.

CRediT authorship contribution statement

Monika Danchenko: Conceptualization, Methodology, Investigation, Visualization, Writing – original draft. **Kevin R. Macaluso:** Conceptualization, Methodology, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data in the current study are available in the article text and accompanying supplementary materials.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.cris.2024.100080.

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