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First observations of ovarian regeneration in an amphipod, *Ampelisca eschrichtii Krøyer, 1842*

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Background. Females with signs of regeneration of previously atrophied ovaries were found in populations of the gammaridean amphipod *A. eschrichtii* on the northeastern shelf of Sakhalin Island (Russia), a phenomenon unknown in any other amphipod species. Most *A. eschrichtii* have a two-year life cycle and reproduce in winter and spring at the age of two years. However, the rare surviving females of the third year are able to regenerate the ovaries and again participate in reproduction. The precursors of these individuals - a small number the second-year females with an asynchronous (summer) breeding period and ovaries atrophied due to seasonal starvation - apparently possess sources of cells for the restoration of the germinal and somatic components necessary for ovarian regeneration. **Methods.** Histological preparations of the second year females with ovarian atrophy and normal ovaries, of the third year female with ovarian regeneration, as well as testes of immature and sexually mature males were examined to determine the sources of cells of the germinal and somatic lines necessary for ovarian regeneration.**Results.** A new germinal zone is formed in the ovaries of the third year females from germ cells preserved in the atrophied ovaries and eosinophilic cells of the starving second year females. Eosinophilic cells form the mesodermal component of the germinal zone. A large number of these cells appear in second year females that have atrophied ovaries. These cells settle and multiply on the intestinal wall of the third year female, and then migrate to the regenerating ovaries.**Conclusions.** Germ cells of second year females are not lost and permit subsequent ovarian regeneration. Eosinophilic cells involved in ovarian regeneration are of mesodermal origin. Morphological signs of eosinophilic cells are characteristic of quiescence cells, which under certain conditions are able to activate regeneration.

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ABSTRACT

Background. Females with signs of regeneration of previously atrophied ovaries were found in populations of the gammaridean amphipod *A. eschrichtii* on the northeastern shelf of Sakhalin Island (Russia), a phenomenon unknown in any other amphipod species. Most *A. eschrichtii* have a two-year life cycle and reproduce in winter and spring at the age of two years. However, the rare surviving females of the third year are able to regenerate the ovaries and again participate in reproduction. The precursors of these individuals - a small number the second-year females with an asynchronous (summer) breeding period and ovaries atrophied due to seasonal starvation - apparently possess sources of cells for the restoration of the germinal and somatic components necessary for ovarian regeneration.

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Conclusions. Germ cells of second year females are not lost and permit subsequent ovarian regeneration. Eosinophilic cells involved in ovarian regeneration are of mesodermal origin. Morphological signs of eosinophilic cells are characteristic of quiescence cells, which under certain conditions are able to activate regeneration.

Keywords. Amphipoda, *Ampeliscidae*, *histology, Okhotsk Sea*, germ cells, eosinophilic cells, mesoderm cells.

Does this mean they reproduce for the first time 2 years after fertilization of the egg?

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INTRODUCTION

We report ovarian regeneration in the gammaridean amphipod, *Ampelisca eschrichtii*, a previously unknown reproductive adaptation in amphipods. Ovarian regeneration permits some individuals of *A. eschrichtii* of the northeastern Sakhalin Island Shelf to reproduce in the third year of life. *Ampelisca eschrichtii* are gonochoristic. All amphipods extrude their ova into an external brood pouch where they are fertilized and brooded until hatch. Nearly all *A. eschrichtii* have a two year life cycle (Demchenko et al., 2016; Durkina et al., 2018). *A. eschrichtii* food sources in the Offshore western gray whale feeding area on the northeastern Sakhalin Island Shelf are most abundant in winter and spring and reduced in summer and autumn (Demchenko et al., 2016). Mating and embryo production occurs in winter and spring. Second year *A. eschrichtii* females produce ova in summer and autumn from lipid reserves accumulated in the previous winter and spring. A few females however, survive to a third year. These later females use embryos (produced from first generation of vitellogenic oocytes) and their eggs as trophic sources in summer through fall (Durkina et al., 2018).

The *A. eschrichtii* ovaries, as in all studied amphipods (Charniaux-Cotton & Payen, 1985), extend dorsolaterally to the digestive tract from the second to the seventh thoracic segment. Vitellogenic oocytes form over the length of the ovaries and are the dominant element of the ovaries of mature summer and autumn females. Previtellogenic oocytes are located at the base of vitellogenic oocytes. The previtellogenic and vitellogenic oocytes are covered with follicular epithelium (derived from mesoderm cells) and form, respectively, primary and secondary follicles. Proliferation of oogonia occurs only among mesodermal cells in a germinal zone (Charniaux-Cotton & Payen, 1985) is a rapid process and thus, difficult to observe (Subramoniam, 2016). The oogonia leave the germinal zone after a series of mitotic divisions and then enter a meiosis prophase to become previtellogenic oocytes. The germinal zone and follicular epithelium are preserved in the ovaries throughout the life of amphipods (Charniaux-Cotton & Payen, 1985), but we (Durkina et al., 2018) did not find a germinal zone in mature *A. eschrichtii* ovaries.

The regenerating ovaries of third year *A. eschrichtii* females consist of three parts. The anterior part of ovaries remain an empty tube of connective tissue - a sign of previous ovarian atrophy. The middle part consists of a new germinal zone. The posterior part is filled with newly formed primary follicles (Durkina et al., 2018). Formation of the germinal zone and primary

follicles indicates that the females have sources of germline cells and mesodermal cells.

Repair of damaged organs and tissues occurs due to stem cells (Stoltz et al., 2015; Mahla, 2016) which are undifferentiated, can self-renew and can also produce differentiated offspring. Germline stem cells (GSCs) are the source of invertebrate and vertebrate gametes (Lin, 1998; Dansereau & Lasko, 2008; Dunlop et al., 2014; Grieve et al., 2015; Truman et al., 2017). Each GSC division produces a daughter GSC, and a differentiated daughter cell (Lin, 1998). Stem cells remain in microenvironments or niches – adjacent to specialized somatic cells whose signals regulate stem cell function (Spradling et al., 2001). In addition to cellular niches, stem cells also occur on basement membranes, and function in response to extracellular matrix signals (Xie & Li, 2007). Gilboa and Lehmann (2004) observed that GSCs in *Drosophila* can be derived from primordial germ cells (PGCs), which populate the anlagen of the gonad during embryonic development. PGCs origins in amphipods were resolved in *Orchestia cavimana* (Wolff & Scholtz, 2002) and *Parhyale hawaiensis* (Extavour, 2005). PGCs come from a single cell, which is the smallest at the 8th cell stage of embryo development (g-blastomere) (Gerberding et al., 2002). The fate progenitor of PGCs is determined by the localization of germline determinants it contains (Extavour, 2005). Removal of the g-blastomere in *P. hawaiensis* embryos impedes the formation of PGCs. Adults obtained from these embryos however, are fertile and produce offspring (Modrell, 2007). Modrell's (2007) results ~~indicate~~ an empty GSC niche remains in the somatic tissues of g-removed juvenile *P. hawaiensis* ovaries. A signal from an empty GSC niche in the amphipods appears to recruit surrounding somatic gonad cells to become GSCs (Kaczmarczyk, 2014). The germline replacing cells in g-remote *P. hawaiensis* were of mesodermal origin (Winchell et al., 2017).

The mesoderm, the middle germ layer produced during embryonic development (Kimelman & Griffin, 1998), is the source of mature crustacean somatic tissues including the gonads, muscles, connective tissue, the vascular system and parts of the excretory organs (Saxena, 2005). The mesodermal component of 18 mm male *A. eschrichtii* testes are readily apparent and resemble wide cords (Durkina et al., 2018). The undifferentiated genital apparatus of amphipods is composed of two thin strands of mesodermal cells in the postembryonic period (Charniaux-Cotton & Payen, 1985). The transformation of thin mesodermal strands into wide cords during the growth of male amphipods can result from mesodermal cell multiplication in the testes themselves, or from migration of mesodermal cells into the testes from the outside tissues.

We used information on mesodermal cord formation in male *A. eschrichtii* to infer how the mesodermal component of the germinal zone is formed during ovarian regeneration in the third year females.

We examined the germinal zone in immature female *A. eschrichtii* ovaries to determine whether this structure is preserved during ovarian atrophy in starving females. We compared the mesodermal fomation in immature male testes and third year female ovaries and mesodermal cell responses to ovarian atrophy in second year females. We also searched for where amphipod mesodermal cells involved in the regeneration of the ovaries are normally located.

MATERIALS & METHODS

We previously recognized ovarian regeneration in two 32 mm third year *A. eschrichtii* females and ovarian atrophy preceding regeneration in two second year *A. eschrichtii* females (24 and 27 mm length) carrying embryos in summer in 2013 samples (Site B61_13), but not in 2015 samples (Durkina et al., 2018, sec. S1 Table). We give herein a more detailed analyses of these previous observations. We describe the ovarian regeneration on the basis of histological preparations of one of the above third year females. Ovarian regeneration was nearly complete in the second female of the third year of life. The degree of ovarian atrophy differed in the two females observed. We found accumulation of eosinophilic cells in the anterior part of body in the 24 mm female (with more pronounced atrophy of the ovaries) with signs of migratory activity that we did not find in the 27 mm female (with less pronounced ovarian atrophy). We used histology to examine ovarian atrophy of the 24 mm female, immature (13.5 mm) and sexually mature (18 mm) males and a sexually mature (24 mm) female with normal ovaries. Our cross comparisons permitted us to track ovarian regeneration and the role eosinophilic cells in the process. We previously reported our histological preparation methods (Durkina et al., 2018). We measured germ and eosinophilic cell positions and estimated cell volumes V (Data S1) from photographs of our histological sections using Videotest (http://www.videotest.ru; VideoTesT Ltd., St. Petersburg, Russia) and the relation:

$$V=4/3*\pi*R*r^2 \qquad (1),$$

where: R is the radius of the long axis and r is the radius of the short axis of the cell.

RESULTS

The germinal zone in immature and atrophied ovaries

The germinal zone of immature 13-18 mm female ovaries is well defined. The mesodermal cells are round, with a thin rim of cytoplasm and a large nucleus, thin filaments of chromatin, they lack a nucleolus (Fig. 1A) and readily stain with eosin. Follicular epithelial cells (a derivative of mesoderm), unlike mesodermal cells, have nuclei that are intensely stained with hematoxylin. Germ cells are commonly in pairs, next to the mesodermal cells (Fig. 1B). Occasionally, oogonial mitoses is apparentin the germinal zone (Fig. 1B).
Paired germ cells (Fig. 1C) with a pair of oogonia, which form after mitosis, and rare previtellogenic oocytes, are apparent in the atrophied 24 mm female ovaries (Fig. 1D). Mesodermal cells lose their rounded shape in atrophied ovaries (Fig. 1D). The volumes of few germ cells in female with ovarian atrophy are the same as those of the smallest germ cells in immature female ovaries (Fig. 2).

Mesodermal component formation in immature male testes and a regenerating third year female ovaries

The mesodermal component of the immature testes and of the regenerating ovaries form in similar ways. The testes of 13.5 mm male (double arrow) are located in the 2nd and 3rd thoracic body segments and are adjacent to the intestinal wall which is represented (in investigated males and females) only by its middle layer – of the intestinal basal membrane (ibm) (Fig. 3A). The testes contain eosinophilic mesodermal cells, the nuclei of which almost fill the entire cell volume. The nuclei chromatin form a network structure, the nucleolus is absent. The mesodermal cells are part of the germinal zone of the regenerating ovaries (Fig. 3B) and have the same characteristics as the mesodermal cells of the immature testes. No mitoses was found among mesoderm cells in the immature testes or in the regenerating ovaries. Rare degrading previtellogenic oocytes that remained from the old ovary were found in the germinal zone of the regenerating ovaries (Fig. 3B).

The eosinophilic cells morphologically similar to mesodermal cells of the testes and the germinal zone of the ovaries are located on the intestinal basal membrane in the 13,5 mm male (Fig. 3A) and in the female with regenerating ovaries (Fig. 3B). The eosinophilic cells have a narrow rim of the cytoplasm, large nuclei without a nucleolus and filamentous chromatin, and differ in size between the male (Fig. 3C) and the female (Fig. 3D). These cells often have

lamellipodia (wide processes of the cytoplasm), which are a sign of cell motility. In contrast to 13.5 mm males, the 18 mm male testes are formed (Demchenko et al., 2016) and there are no eosinophilic cells on the intestinal wall (not shown). In females with regenerating ovaries, eosinophilic cells are abundant in the section of the intestinal basal membrane opposite the germinal zone (Fig.3B), but absent in the section of the intestinal basal membrane opposite the posterior restored part of the ovary with primary follicles (not shown). Reproduction of eosinophilic cells occurs in nests (cell groups) on the intestinal basal membrane (Fig. 3E).

The formation of the mesodermal component of the testes and the new germinal zone of the ovaries occurs as a result of the immigration of eosinophilic cells from the intestinal wall. In females, we found single migrating eosinophilic cells near the ovarian germinal zone (Fig. 3B), outside the intestinal wall (Fig. 3E), in the intestinal lumen (not shown) and among adipose tissue (Fig. 3F).

Rare mesodermal cells, which are related to the former germinal zone, occur in the narrow atrophied area that connects the anterior and middle parts of the ovaries. The cells of the old mesoderm, in contrast to the cells mesoderm of the new germinal zone, are flattened (Fig. S1A). Rare nests of 3-5 eosinophilic cells occur on the intestinal basal membrane opposite of the atrophied part of the ovary (Fig. S1B).

A massive appearance of eosinophilic cells in the female's body occurs even before the regeneration of the ovaries. The most extensive accumulations of these cells were in the anterior part of the body near the intestine and on its wall in one of the two second year females, which had the most pronounced degree of ovarian atrophy (Fig. 4A). The same cells, but in smaller numbers, are located in the anterior part of the body above the intestine in females with normal ovaries (Fig. 4B). The volumes of eosinophilic cells in females with normal and atrophied ovaries are similar, but they increase significantly during ovarian regeneration (Fig. 5). Unlike in females, male eosinophilic cells do not increase in volume during the formation of testis mesoderm (Fig. 5).

DISCUSSION

Regeneration of atrophied ovaries can occur in third year *A. eschrichtii*. Starving second year females can resorb vitellogenic oocytes for nutrition, which leads to atrophy of the ovaries (Durkina et al., 2018). Germ cells and mesodermal cells can survive, however, in the atrophied

ovaries. *A. eschrichtii* appear to have stem cells among the surviving germ cells, that can, as in other invertebrates and vertebrates (Lin, 1998; Dansereau & Lasko, 2008; Dunlop et al., 2014; Grieve et al., 2015; Truman et al., 2017) produce new germ line. Ovarian regeneration by *A. eschrichtii* thus occurs in 4 major stages (see Fig. S2; Box S1).

Angelo and Van Gilst (2009) revealed experimentally that the nematode *Caenorhabditis elegans* germline stem cells (GSCs) do not die when they starve, that surviving GSCs regenerate a new germ line when food is resumed, and that fasting extends the reproductive longevity of nematodes. Three year female *A. eschrichtii* may also be products of starvation. Flattened mesodermal cells remaining in ovaries of ~~the third year~~ *A. eschrichtii* may be too rare to form new germinal zone and a new follicular epithelium. Eosinophilic cells that restore the somatic component of the ovaries have a high nuclear-cytoplasmic ratio, nuclei that lack a nucleolus and condensed chromosomes. Small numbers of eosinophilic cells occur the anterior sections of female *A. eschrichtii* with normal ovaries. Ovarian atrophy induced by starvation ~~appears to~~ may stimulate the proliferation of these cells, and their massive appearance in the anterior part of the body (on the intestinal wall and next to it). Eosinophic cells may migrate posteriorly along the intestine and settle on its wall for ovarian regeneration. We observed the proliferations of eosinophilic cells on the intestinal wall of female in the process of ovarian regeneration. The densest accumulations of these cells were on the intestinal wall next to the forming ovary germinal zone. The volumes of eosinophilic cells are greater in females with regenerating ovaries than in females with normal and atrophied ovaries. The cause of this phenomenon is unknown.

The mesodermal component of new germinal zone in regenerating ovaries and immature male testes is produced from eosinophilic cells that migrate from the intestinal wall. The lack of proliferating mesodermal cells in immature male testes and regenerating ovaries also ~~indicates~~ suggests that the ~~reproduction~~ of these cells occurs outside the gonads. Eosinophilic cells are of mesodermal origin. ~~They are not belong to the ectoderm (epidermis and nerve tissue) or to the endoderm (epithelium of the intestine and hepatopancreas).~~ Eosinophilic cells ~~are characterized~~ by migration activity, which is inherent mesodermal cells (Nakatsuji et al., 1982). High nuclear-cytoplasmic ratios, condensed chromosomes, and absence of the nucleolus are characteristic of *A. eschrichtii* eosinophilic cells and are also characteristic of quiescence cells. Quiescence cells, which include stem cells, do not undergo genome replication, have altered cellular metabolism and are resistant to various stressors (Valcourt et al., 2012; Rumman et al., 2015). Quiescence

cells do not divide but can re-enter the cell cycle and resume proliferation if they receive necessary microenvironmental signals (Mohammad et al., 2019). Reactivation of quiescence cells into proliferation is crucial for tissue repair and regeneration (Yao, 2014).

CONCLUSIONS

These results open to question whether summer starvation can extend female *A. eschrichii* life spans to a third year. Females that form the first generation of vitellogenic eggs in summer and autumn and hatch in winter have a sufficient reserve of nutrients to survive in the summer. A small number of females that breed in the summer have spent their energy reserves for their production of a first brood of eggs and, accordingly, the first batch of embryos. They can use the second generation of vitellogenic oocytes for nutrition, which leads to ovarian atrophy during extreme summer starvation. Those few females surviving until food is again abundant may have extended lives and restored ovaries. Regeneration of ovaries is by repopulation from cells of the former germ line and eosinophilic cells of mesodermal origin.

We (Durkina et al., 2018) also examined a third year female with completely regenerated ovaries and another third year female with large vitellogenic oocytes that were likely to be used in the upcoming reproductive season. Ovarian regeneration and extended lives of amphipods may be common. *Ampelisca macrocephala* also have a similar a two-year life cycle but nevertheless, 3-year-old females that breed the second time have been observed (Kanneworff, 1965). *Orchestia gammarellus* females typically live 12 to 15 months, but can survive and reproduce at approximately 36 months (Persson, 1999). Most females of the giant predatory amphipod *Eusirus perdentatus* brood only once during their approximately 6 year life span, however, rare individuals (probably reaching 8 years of age), apparently, carry a second brood (Arntz et al., 1992).

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Author Contributions

VBD performed histological slide analysis, data interpretation and MS preparation. VBD and JWC wrote the article. The NLD assisted with the study design and preparation of the final MS. All authors read and approved the final version of manuscript.

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Competing interests

The authors declare there are no competing interests.

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345 **Abbreviations**
346
347 dpvo, degrading previtellogenic oocyte;
348 ec, eosinophilic cell;
349 fc, follicular cell;
350 gc, germ cell;
351 gz, germinal zone;
352 ibm, intestine basal membrane (= middle layer of intestine wall);
353 mc, mesodermal cell;
354 mec, migrating eosinophilic cell
355 obm, ovary basal membrane;
356 og, oogonia;
357 om, oogonial mitosis;
358 pvo, previtellogenic oocyte;
359 t, testis;
360 vo, vitellogenic oocyte.

Figure 1

Components of the germinal zone of *A. eschrichtii* ovaries

? in these images the stain appears fc > mc

(A) mesodermal cells (mc), in contrast to follicular cells (fc), are intensely stained with eosin in the ovary of an immature 13 mm female. The ovary contains previtellogenic (pvo) and vitellogenic (vo) oocytes. (B) a germ cells pair (gc) (one out of focus), mesodermal cells (mc) and oogonial mitosis (om) in ovary of an immature 18 mm female. (C) germ cells (gc) and previtellogenic oocyte (pvo) and (D) mesodermal cell (mc), oogonia (og) and previtellogenic oocyte (pvo) on the basal membrane of atrophied ovary of a second year 24 mm female. All scales in µm.

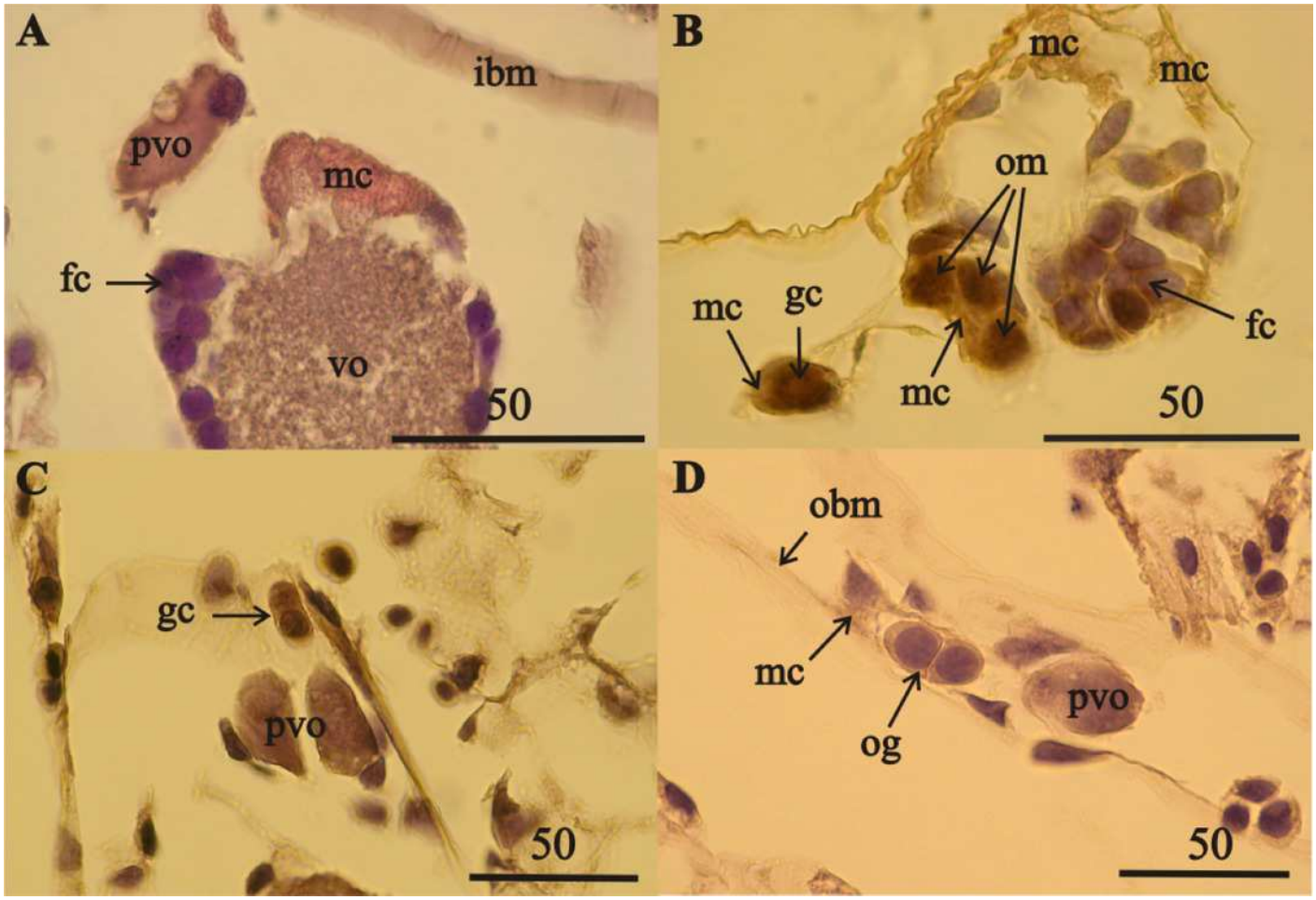


Figure 2

Volumes of germ cells (μm^3) in *A. eschrichtii* females

(Fimm_13) immature 13 mm female. (Fimm_18) immature 18 mm female. (Fatr_24) 24 mm female with ovarian atrophy.

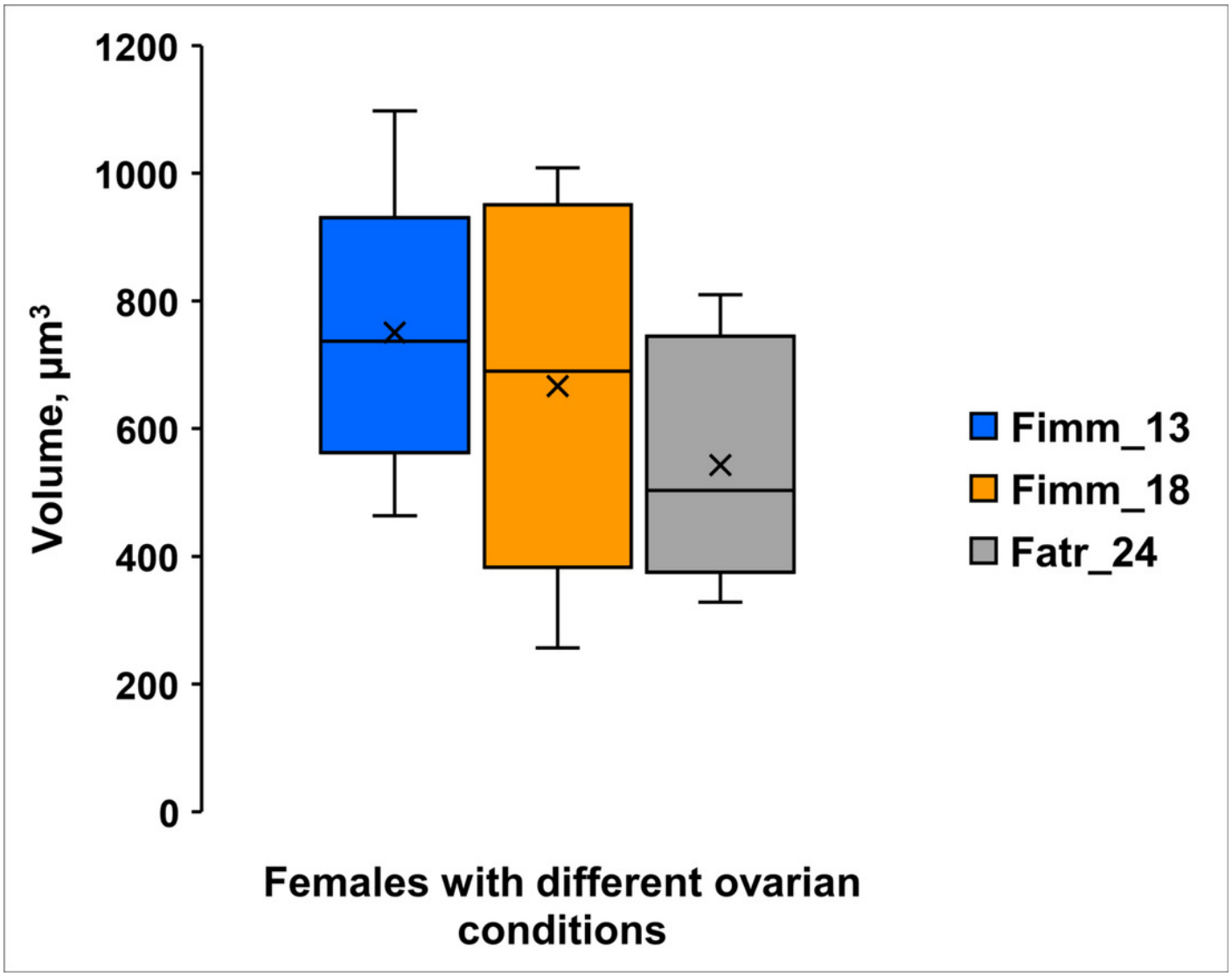


Figure 3

Mesodermal component formation in *A. eschrichtii* gonads

(A) immature 13.5 mm male testis (t) containing mesodermal cells (mc) contacts the intestinal wall, which is presented by its middle layer – of basal membrane (ibm), occupied by eosinophilic cells (ec). The cells of outer layer of the intestinal wall are extremely rare, and the ectoderm cells – its inner layer – are exfoliated. (B) the accumulation of eosinophilic cells (ec) on the intestinal basal membrane (ibm) is located opposite the germinal zone (gz) of the regenerating ovary, migrating eosiniphilic cells (mec) near the germinal zone and disintegrating previtellogenic oocyte (dpvo) (upper left). (C) eosinophilic cells (ec) on the intestinal basal membrane (ibm) of an immature 13.5 mm male. (D) eosinophilic cells (ec) on the intestinal basal membrane (ibm) of a female with regenerating ovary. (E) nest of eosinophilic cells on the intestinal basal membrane (ibm) of a female with regenerating ovary contains small cells formed after mitosis (open head arrows), a disintegrating previtellogenic oocyte (dpvo) (upper right) in the germinal zone, and migrating eosinophilic cells (mec) (solid line arrows). (F) migrating eosinophilic cells (mec) (arrows) in adipose tissue of a female with regenerating ovary. All scales in μm.

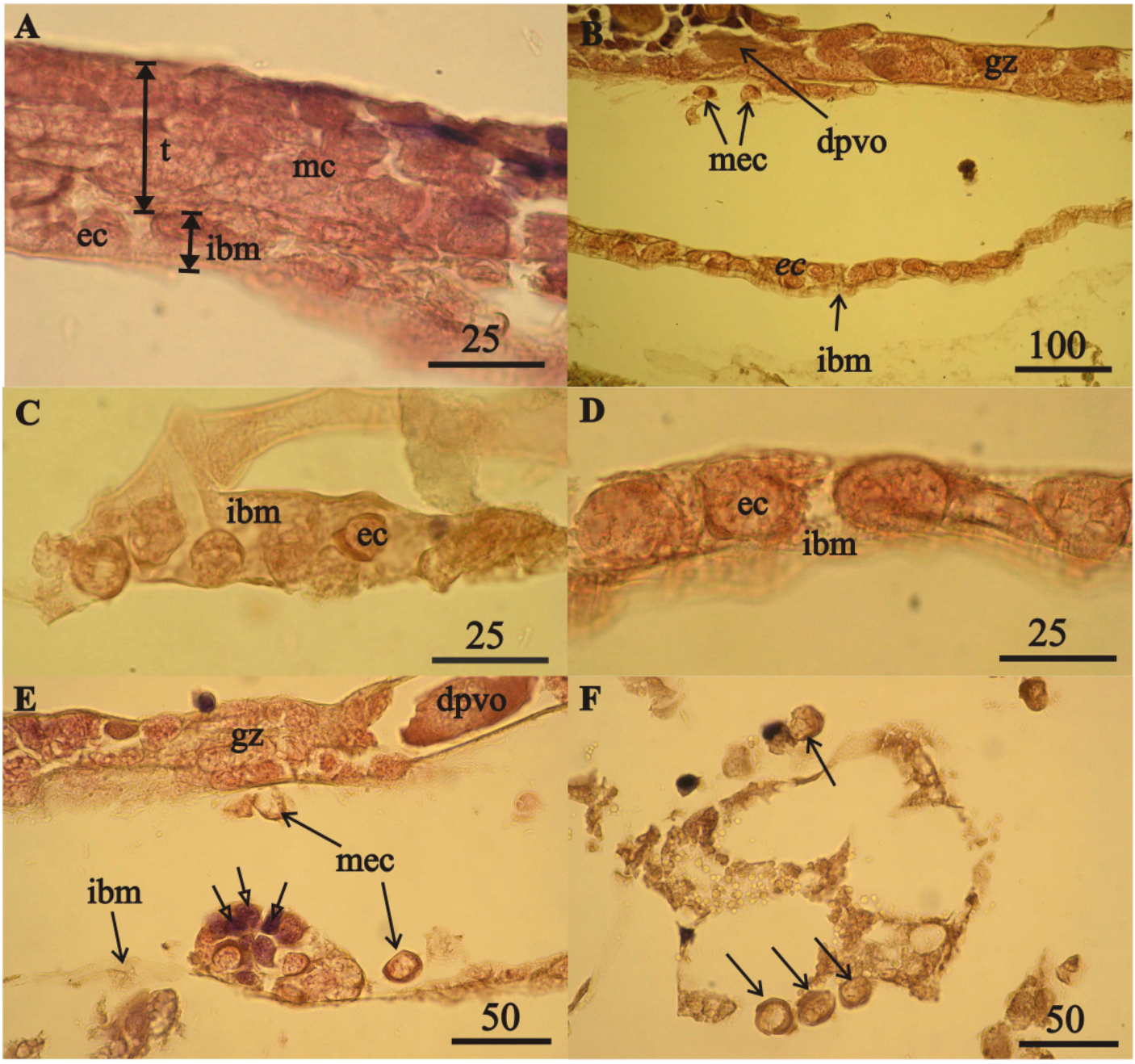

A
t
mc
ec
ibm
25
B
gz
mec
dpvo
ec
ibm
100
C
ibm
ec
25
D
ec
ibm
25
E
dpvo
gz
mec
ibm
50
F
50

Figure 4

Anterior body of *A. eschrichtii* females of the second year of life with different ovarian conditions .

(A) eosinophilic cells (ec) are accumulated in masses next to and on the intestinal basal membrane (ibm) of the female with an atrophied ovaries. (B) eosinophilic cells (ec) are in sparse numbers in a female with normal ovaries. All scales in µm.

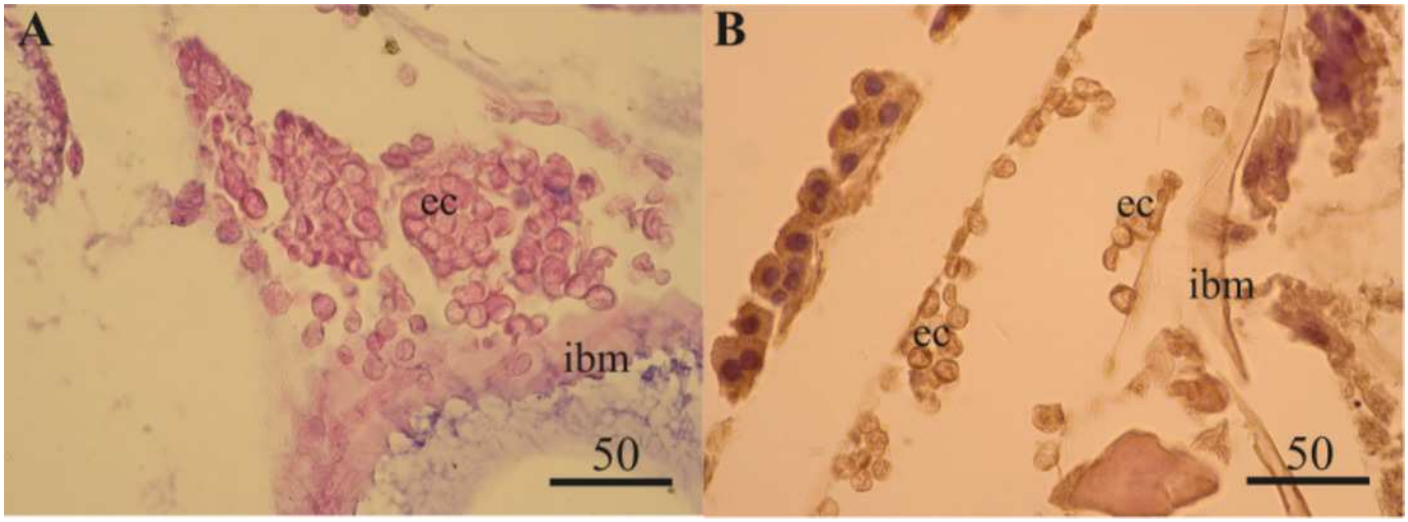
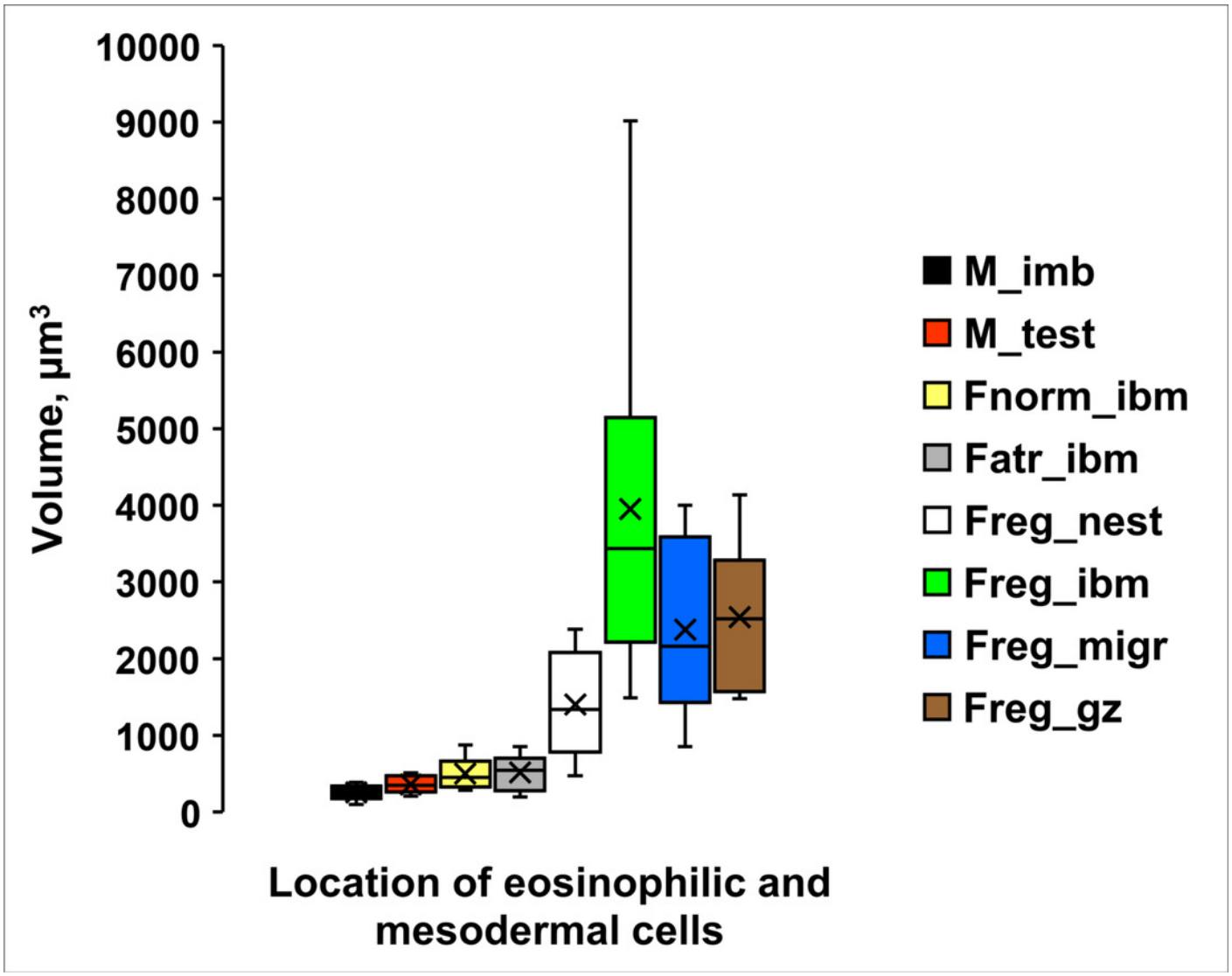


Figure 5

Volumes (μm^3) of eosinophilic cells (ECs) and mesodermal cell (MCs) in ~~the~~ *A. eschrichtii* of different reproductive status.

(M_ibm) – ECs on the intestinal basal membrane and (M_test) – MCs in the germinal zone of the testis in immature 13.5 mm male. (Fnorm_ibm) – ECs on the intestinal basal membrane in the anterior part of the body in a female with normal ovaries. (Fatr_imb) – ECs on the intestinal basal membrane in the anterior part of the body in a female with ovarian atrophy. (Freg_nest) – ECs in the "nests" on the intestinal basal membrane of the female with ovarian regeneration. (Freg_ibm) – ECs on the intestinal basal membrane opposite the germinal zone in the female with ovarian regeneration. (Freg_migr) – migrating ECs outside intestinal basal membrane in the female with ovarian regeneration. (Freg_gz) – MCs in germinal zone in the female with ovarian regeneration.


10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Volume, µm³
M_imb
M_test
Fnorm_ibm
Fatr_ibm
Freg_nest
Freg_ibm
Freg_migr
Freg_gz
Location of eosinophilic and mesodermal cells