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Morphology of accessory ovaries in adult males of *Perla marginata* (Plecoptera: Perlidae)

ELŻBIETA ROŚCISZEWSKA[1] and TOMÁŠ SOLDÁN[2]

[1]Institute of Zoology, Jagiellonian University, Ingardena 6, PL-30060 Kraków, Poland; e-mail: roscis@jetta.if.uj.edu.pl
[2]Institute of Entomology, Academy of Sciences of the Czech Republic, Branišovská 31, CZ-37005 České Budějovice, Czech Republic; e-mail: soldan@entu.cas.cz

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Abstract. Bisexual gonads in the stoneflies *Perla burmeisteriana*, *P. pallida* and *Dinocras cephalotes* are reported for the first time. Gross morphology and ultrastructure of the accessory ovaries of mature larvae and adult males of *Perla marginata* are described in detail. There are 36–58 male ovarioles situated distal to the paired testes and opening into fused termini of the lateral ducts in abdominal segments II and III. These correspond in structure to the ovarioles of adult females but are significantly smaller (maximum size of proximal oocyte 9.0 × 45 μm) and each usually contains 10–14 linearly arranged previtellogenic oocytes. Oogenesis ceases at the end of previtellogenesis or at the onset of vitellogenesis. The ooplasm contains either regularly dispersed or irregularly accumulated particles in different regions of the cell with accumulations occurring near mitochondria and Golgi complexes. Based on results of metachromatic staining, these are thought to represent either lipid droplets (most) or yolk globules. The oolemma rarely develops short microvili and few pycnotic vesicles. Development of the follicular epithelium (influencing vitellogenesis and secretory activity during choriogenesis) is abnormal. Follicular cell growth is not synchronized with that of the oocytes, and the follicular cells of the terminal (distal) oocytes show neither patency nor secretory activity. The mechanism controlling degeneration of male ovarioles and the evolutionary significance of hermaphroditic gonads in the Plecoptera are discussed.

INTRODUCTION

Functional hermaphroditism – the production of mature eggs and fully developed, functional spermatozoa by the same individual has been reported in many parasitic and free living animals (e.g. Brusca & Brusca, 1990; Dorit et al., 1991; Heller, 1993; Graham et al., 1993). It undoubtedly represents an adaptation to a sessile way of life, parasitism, or to the considerably restricted mating opportunities associated with extremely low vagility or population density and is mostly accompanied by reduced or absent sexual dimorphism (Heller, 1993). Considering the exceptionally high species diversity of insects, hermaphroditism seems unusually rare within this group (Schroeder, 1928). Of the 32 extant insect orders, obligate hermaphrodites have been reported only in the Plecoptera (Schoenemund, 1912; Junker, 1923), Isoptera (Geyer, 1951), Blattodea (Heymons, 1890; Brooks & Kurtti, 1972) and Coccoidea (Cholodkowsky, 1902; Huges-Schrader, 1927, 1930, 1963; Johnston, 1912; Royer, 1973). In addition, incidental gynandromorphs with bisexual but mostly non-functional gonads have been found in the Lepidoptera, Coleoptera, Hymenoptera and Diptera (see Wasmann, 1890; Schultz, 1897; Wenke, 1906 or Schroeder, 1928). It should be stressed that true and functional hermaphroditism, exhibiting exchange of genetic material between hermaphrodites and resulting in fertile offspring, seems to be restricted to representatives of the Sternorrhyncha: Coccoidea such as *Icerya purchasi* (Hughes-Schrader, 1925, 1930; Bronns, 1939; Royer, 1973), *Icerya bimaculata* (Hughes-Schrader, 1963), and *Icerya zeteki* (Hughes-Schrader & Monaham, 1966).

However, non functional or accessory hermaphroditism occurs more frequently. Although it has been investigated in detail only in three insect species, the termite *Neotermes zuluensis* (Geyer, 1951; see also Richards & Davies, 1977 for further references), the cockroach *Blattella germanica* (Brooks & Kurtti, 1972), and the stonefly *Perla marginata* (Schoenemund, 1912; Junker, 1923; Matsuda, 1976; Richards & Davies, 1977; see also Zwick, 1980 for further references). However, no data concerning the ultrastructural arrangement of a hermaphroditic gonad has been published. The objective of this paper is thus to describe the arrangement of a hermaphroditic internal reproductive system and the ultrastructure of rudimentary ovarioles in males of *P. marginata* with emphasis on the degeneration of early oocytes.

MATERIAL AND METHODS

Males and male larvae of *Perla pallida* Guérin-Méneville, *P. marginata* (Panzer), *P. burmeisteriana* Claassen and *Dinocras cephalotes* (Curtis) (family Perlidae) were collected in the Bieszczady mountains (southeast of Poland) and, except for *P. pallida*, in the Šumava mountains (South Bohemia) during the summers of 1987, 1990, 1991, and 1995. Adults were collected by sweeping riparian vegetation, larvae by the usual hydrobiological sampling techniques (see e.g. Hynes, 1972). Twenty-four specimens of *P. marginata*, 15 specimens of *P. burmeisteriana*, 6 specimens of *P. pallida*, and 16 specimens of *D. cephalotes* were dissected. Larvae more than 1–2 years-old and of maximal body length and some younger larvae of *P. pallida* were also investigated.

Dissected male internal reproductive systems of *P. marginata* were fixed in 2.5% glutaraldehyde in 0.5 M phosphate buffer for

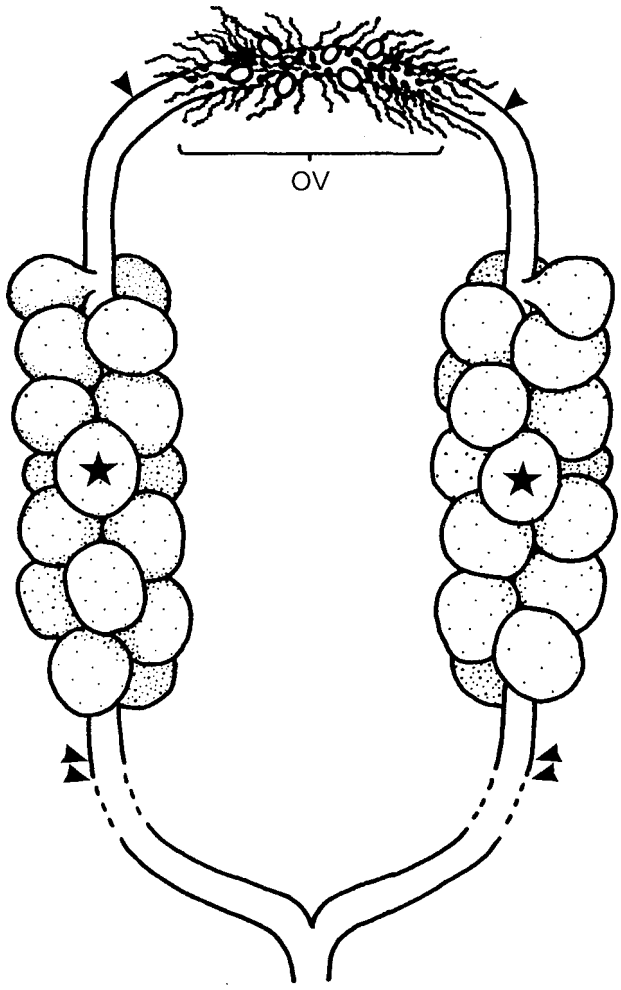


Fig 1. Sketch of bisexual gonad of adult male of *Perla marginata*. OV – paired ovary; testicular vesicles marked with asterisks. Note fused proximal parts of the lateral oviducts (arrowheads) and distal parts of the spermatic ducts (double arrowheads) forming a ring.

24 h, rinsed three times in phosphate buffer containing 5.8% sucrose and postfixed for 1.5 h with 1% osmium tetroxide in 0.1 M phosphate buffer. Throughout fixation a pH of 7.4 was maintained. Specimens were rinsed three times in water, dehydrated in a graded series of alcohol and acetone and embedded in Epon 812. Blocks were cut using a Tesla BS 490 A ultramicrotome. Ultrathin sections contrasted with uranyl acetate and lead citrate were examined under a Tesla BS 500 transmission electron microscope (TEM). Semithin sections (1.5 μm) stained with 1% methylene blue in 1% borax or by means of the PAS method (Litwin, 1985) were examined under a Peraval Interphaco Zeiss light microscope. For details and nomenclature of structures see Rościszewska (1989, 1995).

RESULTS

Occurrence of accessory ovaries and gross morphology

Accessory ovaries were found in the males of all four species investigated. They occurred in older larvae and adult males from both Polish and Czech populations, in 100% of the specimens investigated. In most specimens, accessory ovaries were located in abdominal segments II and III, rarely extending into segments I or IV.

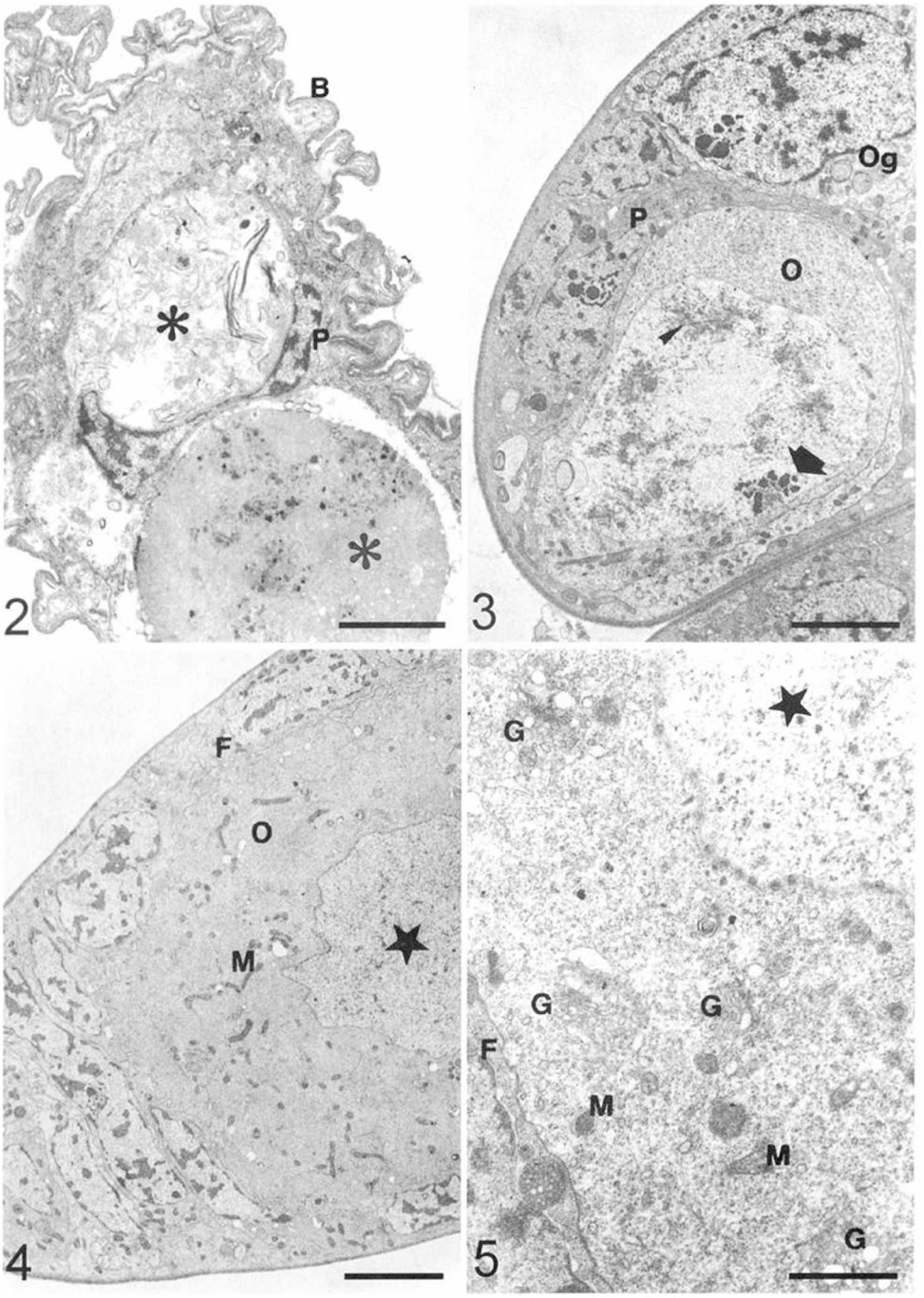
An ovarian mass (ov) forms the anterior end of the male internal reproductive system (Fig. 1). Although it seems to be unpaired, this structure undoubtedly represents a paired organ connecting the anterior extremities of the two testes or, more precisely, their exit ducts. In the species investigated, the gonoducts form a ring (the lateral oviducts fuse anteriorly and vasa deferentia proximally, see Fig. 1). The accessory ovary contains numerous ovarioles opening individually into the exit ducts. The number of ovarioles ranges from 35–58, with an average of 48 in *Perla* species and 39 in *Dinocras cephalotes*. Each ovariole is enveloped by an acellular, simple and at many places folded tunica propria (basal lamina) (Fig. 2, B), and consists of two regions: an apical germarium (Figs 2, 8, 9, Ge) and a proximal vitellarium (Figs 4–9). Germaria of individual ovarioles have degenerated at the life cycle stages investigated (Fig. 2). Prefollicular cells are apparent containing numerous electron-dense bodies in their cytoplasm (Fig. 2, P). There is no clear separation between germarium and vitellarium and, in the transition zone, the primary oocytes enter the growth phase (Fig. 3). Synaptonemal complexes were observed with the oocyte nuclei (Fig. 3). A distinct nucleolus can also be seen in the oocyte nucleus (Fig. 3, arrow). The oocyte cytoplasm (Fig. 3, O) is rich in ribosomes but poor in other organelles. Both oogonia (Fig. 3, Og) and oocytes are of an irregular shape, with most oocytes being suboval or oboval (Fig. 3, O).

Previtellogenesis and early vitellogenesis

Ten to 14 linearly arranged oocytes can be distinguished in each vitellarium, with most of them in the previtellogenic stage (Figs 4, 8, 9, O). Each oocyte nucleus contains a prominent, well stained nucleolus (Figs 8, 9, arrows) and its nuclear envelope is densely perforated by pores (Figs 4, 5). The ooplasm is rich in ribosomes and contains elements of rough endoplasmic reticulum, numerous mitochondria (Figs 4 and 5, M) and Golgi complexes (Fig 5, G).

The terminal oocytes are the largest in each ovariole, but differ greatly in size between ovarioles. The maximum size of oocyte observed was 0.09 mm × 0.045 mm, with its large nucleus still containing the prominent nucleolus of differently sized electron-dense particles (Fig. 6, D). Numerous tiny particles can be distinguished in the karyoplasm, most frequently near the nuclear envelope. An accumulation of "nuage" material can be seen in perinuclear regions of the oocyte (Fig. 6, n). The ooplasm contains inclusions which can either be regularly dispersed throughout the ooplasm or accumulated irregularly in different regions of the cell (Figs 6, 7).

Based on metachromatic staining with methylene blue (Litwin, 1985), these inclusions seem to be either lipid droplets (most) or yolk globules. When investigated by TEM, aggregations of such entities can be seen near mitochondria, RER (rough endoplasmatic reticulum) elements and Golgi complexes (Fig. 7, G, M).

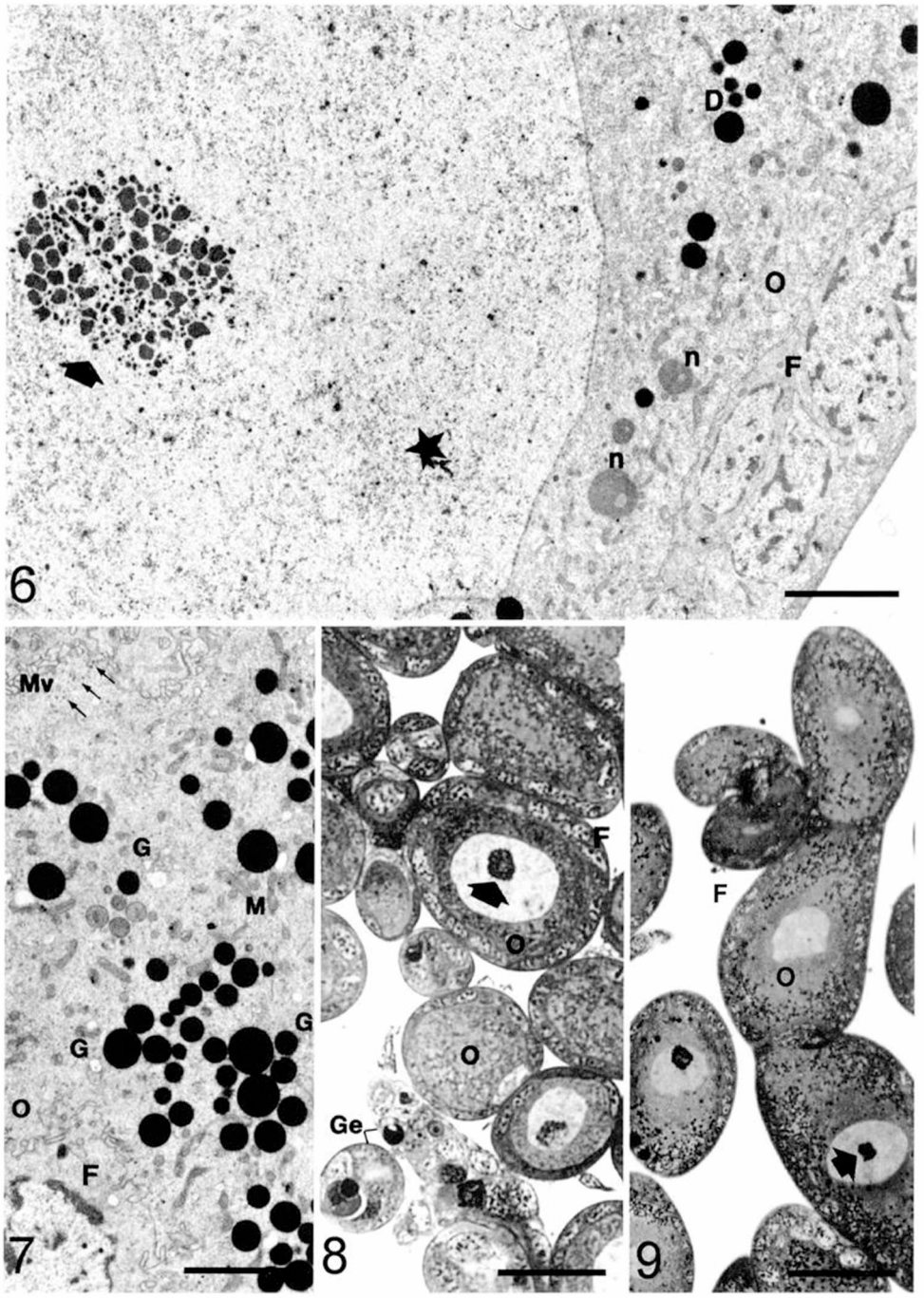


Figs 2–5. *Perla marginata*, male rudimentary ovaries. 2 – TEM, longitudinal section through fragment of apical part of degenerating germarium (bar = 2.5 μm); B – basal lamina = tunica propria; P – prefollicular cell; degenerated germ cells marked with asterisks. 3 – TEM, longitudinal section through fragment of transient zone between germarium and vitellarium (bar = 3.0 μm); arrow – nucleolus; Og – oogonium; O – oocyte; P – prefollicular cell. Note the irregular shape of the oocyte; synaptonemal complex marked with an arrowhead. 4 – TEM, longitudinal section through fragment of previtellogenic ovarian follicle (bar = 2.5 μm); F – follicular cell; M – mitochondrium; O – oocyte; oocyte nucleus marked with an asterisk. 5 – TEM, section through fragment of previtellogenic oocyte (bar = 1.0 μm); F – follicular cell, M – mitochondrium. Note numerous Golgi complexes (G) in ooplasm and pores perforating nuclear envelope; oocyte nucleus marked with an asterisk.

The oolemma occasionally develops short microvilli (Fig. 7, MV) and a few pinocytotic vesicles (Fig. 7, arrows). Oogenesis ceases completely in early vitellogenesis (stage II of Rościszewska, 1995), with developing oocytes rarely reaching the beginning of stage III (mid vitellogenesis) and never reaching stage IV (late vitellogenesis).

Follicular cells

Each oocyte in the vitellarium of the ovarioles found in the accessory ovaries is enveloped by a follicular epithelium, the development of which is similar to that observed in females (for details see Rościszewska, 1995). Further differentiation of follicular cells ceases when they reach



Figs 6–9. *Perla marginata*, male rudimentary ovaries. 6 – TEM, section through early vitellogenic oocyte (bar = 3.75 μm). Note large oocyte nucleus (asterisk) containing a prominent nucleolus (arrow), "nuage" material (n) in the perinuclear ooplasm and electron-dense spheres (D) of storage material. F – follicular cell; O – oocyte. 7 – TEM, section through early vitellogenic follicle (bar = 2.5 μm); O – oocyte. Note electron-dense ingredients accumulated in ooplasm close to Golgi complexes (G) and mitochondria (M) and irregularly infolded oolemma forming short microvilli (MV). There are only a few pinocytotic vesicles (arrows). 8 – light microscope, a semithin, methylene blue stained cross section through male accessory ovaries showing degenerating germaria (Ge) and oocytes (O) of different ovarioles, (bar = 37.5 μm). Note abnormally developed cells of follicular epithelium (F) and large oocyte nuclei containing prominent nucleoli (arrow). 9 – light microscope, a semithin, methylene blue stained longitudinal section through male accessory ovaries showing oocytes (O) of different ovarioles (bar = 37.5 μm). Note abnormally developed cells of follicular epithelium (F) and large oocyte nuclei containing prominent nucleoli (arrow).

the stage of elongate or columnar shape corresponding to early vitellogenesis (III) in female oocytes. The contact zone between oocyte and follicular cells is not extensive but is complex, with some cells starting to develop projections. However, a lack of proper synchronization of cells during development of the follicular epithelium was observed in most ovarian follicles (Fig. 8). Furthermore, the follicular cells of terminal oocytes undergoing vitellogenesis showed neither patency nor secretory activity.

DISCUSSION AND CONCLUSIONS

Dioecism (separation of sexes) is a common, genetically determined feature in insects and several mechanisms of sex determination have been reported (Schroeder, 1928; Laugé, 1985; Dorit et. al., 1991; Büning, 1994). In this respect, the most thoroughly investigated diploid insect is *Drosophila melanogaster*. The principal factor determining sex seems to be the ratio between sex chromosomes and autosomes (for details and further references see e.g. Dorit et al., 1991; Kaulenas, 1992; Bownes, 1992; Parkhurst & Meneely, 1994; Steimann-Zwicky, 1994). As already stressed, hermaphrodites occur rarely in insects and are considered an anomaly within this group (cf. Schultz, 1897). Our knowledge of their sex determination is fragmentary. In this respect some data are available on *Icerya purchasi* (Coccoidea). Royer (1973) reported that hermaphroditism in these insects is connected with the segregation of the germ cells into two large groups: (i) large diploid cells and (ii) small haploid ones. Large diploid cells will eventually develop into the female part of the bisexual gonad whereas smaller cells will develop into the male part. The fertilized eggs develop into diploid hermaphroditic individuals. Eggs are left unfertilized only occasionally and from such eggs haploid males develop. There are no true females in *Icerya purchasi* and the hermaphrodites do not copulate with other hermaphrodites. The self-fertilization seems to occur in this species (Hughes-Schrader, 1927, 1963; Royer, 1973).

Nonfunctional (accessory) hermaphroditism in stoneflies was discovered in *Perla marginata* by Schönemund (1912) and was studied for the first time by Junker (1923) in detail. Here we report on the presence of similarly organized, bisexual gonads in males of the related species *Perla burmeisteriana*, *P. pallida* and *Dinocras cephalotes*. Contrary to true hermaphrodites, both the males and females are diploid in their autosomes but differ in number of heterochromosomes. It is possible that the double number of heterochromosomes in females (in comparison to males) represents a factor influencing the normal development of the ovary. In contrast, the smaller number of male heterochromosomes can block further stages of oocyte development in the male ovary.

Most of our observations agree with those of Junker (1923) although he was not able to describe the then unknown synaptonemal complexes. Oogenesis begins as with females as described in detail by Rościszewska (1995). In oocyte nuclei, chromosomes were observed in leptotene and during the pairing of homologous chromosomes. Our observations support the presence of synaptonemal complexes in pachytene nuclei of *P. marginata*. Junker (1923) concluded that oocytes begin to degenerate from pachytene on, and thus that male ovaries never become fully functional. However, according to our observations, the process of oogenesis continues for much longer in male larva and the adult male ovaries, with previtellogenesis and early vitellogenesis taking place in most oocytes in the vitellarium. Moreover, ultrastructural data on male vitellogenic oocytes strongly suggests some limited yolk formation. Numerous Golgi complexes, rough endoplasm reticulum elements and mitochondria accumulate in the ooplasmic especially during previtellogenesis. Also, storage materials (fat droplets and yolk) appear to come into contact with the organelles appearing at the onset of vitellogenesis and this process does not differ from the "normal" oogenesis occurring in female larvae of the species studied (Rościszewska, 1997). Furthermore, it appears that slight pinocytotic activity takes place, though only a few microvilli and pycnotic pinocytotic vesicles were observed.

In addition, there are abnormalities in the development of the follicular epithelium which normally facilitates pinocytosis by forming intercellular spaces between adjacent cells (patency), allowing passage of vitellogenins to the oocyte surface (Kaulenas, 1992; Rościszewska, 1995). It is clear that a factor suppressing further development of the male ovary in *Perla* spp. must exist. It seems that such suppression begins in young larvae, since retardation in ovarian development, when compared with the development of testes and spermatogenesis, was observed in young larvae of *Perla pallida* (Rościszewska, in prep.).

What the agent blocking oocyte development might be has already been considered by Junker (1923), who suggested that the primary factor is the X : A ratio in male oogonia [identical to that in spermatogonia: 20 autosomes (AA) and 2 heterochromosomes (XX')]. However, 4 heterochromosomes called XXX'X' by Junker (1923) and 20 autosomes (AA) occur in oogonia of true females. This hypothesis seems to agree with results from studies of *Drosophila melanogaster* recently reviewed by Kaulenas (1992). Steinmann-Zwicky et al. (1989) analyzed the fate of XY germ cells in ovaries and XX germ cells in testes in this fly. Their results suggest that germ cells in the ovaries of *Drosophila* develop according to the X : A ratio rule. Thus, XX cells are supposed to go through oogenesis and XY cells go on to form spermatocytes. However, both XY and XX germ cells enter spermatogenesis in the testes. Consequently, some additional inductive signals from somatic cells in the testes might also play a role in differentiation. A somewhat similar mechanism might operate in the Plecoptera.

Rudimentary or accessory male ovarioles in *Perla* (Junker, 1923) and *Dinocras* are panoistic as in true female ovarioles. In the Plecoptera the first steps leading from panoism to meroism can be traced (Gottanka & Büning, 1990; Štys & Biliński, 1990; Endo & Matsuzaki, 1996). However, a connection between the emergence of meroism and of accessory ovaries in this order seems to be mere speculation since a similar situation has been described in termites (Richards & Davies, 1970) and the German cockroach (Brooks & Kurtti, 1972), insect orders that are characterized by panoistic ovarioles. As far as we know, bisexual gonads occur within the Plecoptera only in the family Perlidae (Zwick, 1980) which, moreover, represents one of the most advanced lineages within this order (Zwick, 1980). Presence of bisexual gonads seems to be reflected also in "hermaphroditic" behavioural patterns in members of this group. Contrary to those of other

families, males of *Perla* are frequently observed trying to mate with individuals of the same sex (unpublished observation).

To conclude, the evolutionary significance of bisexual gonads with a nonfunctional ovary in male Plecoptera remains unclear. Insects are generally bisexual and diploid and true hermaphroditism or haploidy seem to be highly derived within this group although true or rudimentary hermaphroditism occur in more primitive insect lineages (Hennig, 1981). Only parthenogenetic species are unisexual, being occasionally associated with male somatic haploidy (arrhenotoky). Since insect gonads mostly develop from diploid bisexual anlagen, the hermaphroditic gonads of the Plecoptera probably represent a simple atavism, when the suppression of female germ cells that usually takes place during embryogenesis or, at most, in first instars (Bownes, 1992; Parkhurst & Meneely, 1994; Steinmann-Zwicky, 1994) is postponed into the adult stage and is controlled by the action of somatic factors, such as absence of vitellogenins.

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