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Effects of tributyltin(IV) chloride on fertilization of *Styela plicata* (Ascidiacea: Tunicata): II. Scanning and transmission electron microscopy studies

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The morphological aspects of *Styela plicata* fertilization after treatment with tributyltin(IV) chloride are described by means of scanning and transmission electron microscopy investigations. Alterations have been shown both on female and male gametes; spermatozoa, all the egg envelopes and the mitochondria of the egg cortical cytoplasm are modified in relation to incubation time. As a consequence, the damage to gametes blocks sperm–egg interaction and fertilization does not occur.

KEYWORDS: tributyltin(IV) chloride; fertilization; ascidians

INTRODUCTION

Numerous environmental problems are being brought about by toxic chemicals in industrial areas. The biocide agent tributyltin (TBT) is used mainly in marine antifouling paints, in wood preservation, disruption of circulating industrial cooling waters, and slime control in paper mills. Recently, trisubstituted organotin salts have been widely detected at higher levels in fish and waters of harbours and inland seas.[1] Moreover, assessments of acute toxicity are supplemented by studies designed to assess the effects of chronic exposure on reproduction, by altering embryonic development, metamorphosis.[2,3] 'Imposex' in neogastropods[4] and the disappearance of some species in areas where there are substantial levels of TBT contamination, both in water and sediments, have been demonstrated.[5] Studies of metabolic alterations in the *Ciona intestinalis* female gonad cells incubated in 10^{-5} or 10^{-7} M TBT solutions have revealed the presence of variations in some compounds and enzymatic activity. Ultrastructural observation in *Ascidia malaca* gametes has shown that the eggs and the spermatozoa are highly affected and that fertilization does not occur: the spermatozoa arrest in the vitelline coat.[6] There is, at present, no well-established study that permits the appraisal of contaminant effects applicable to any species. The aim of this work is to extend analyses of effects of TBT to another species of ascidians in order to compare variation in the incidence of impact of contaminant exposure among different species, by means of scanning and transmission electron microscopy observations. The gametes of Tunicates and of Ascidians, in particular, although fundamentally similar in their architectural plane, show a different morphology in the various species so far studied; this could be related to different spawning and sperm–egg biology in the various species.[7] Fertilization in ascidians is a multistep process where egg envelopes, mainly follicle cells and the vitelline coat, are involved.[8,9]

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MATERIALS AND METHODS

Chemicals

Tributyltin(IV) chloride (TBTCl) was a kind gift from Witco GmbH (Bergkamen, Germany). Concentrated stock solutions were obtained by dissolving stoichiometric amounts of the compound in 0.07% dimethylsulfoxide (DMSO) containing

Millipore-filtered sea water (MFSW). The total tin content was checked using a Perkin Elmer model 3100 atomic absorption spectrometer (equipped with a Perkin Elmer model 100 flow injection analysis system for atomic spectroscopy) according to standard procedures. The solvent DMSO, used because of the low solubility of the compound in non-coordinating solvents, was a Merck (Darmstadt, Germany) reagent. Working solutions (pH 7.25–8.5) were obtained by further dilution of the stock solutions in MFSW. Freshly prepared 10^{-5} and 10^{-7} M TBTCl solutions were used.

Biological material

Adult specimens of *Styela plicata*, an ascidian self-fertile species, were collected in the Gulf of Palermo and its surroundings. Female and male gametes were removed from the gonads and gonoducts of 30 dissected animals. The eggs still retain their germinal vesicles when they are liberated; in this way, at least 40 min are required for germinal vesicle breakdown and first meiotic division to occur, after which the eggs become fertilized.[10] Before insemination, dry sperm was diluted in MFSW at pH 7.8, and about 22 °C to a final concentration of approximately 0.1% v/v. After insemination the eggs were washed twice in the same medium. Some of the living eggs was examined using a Leitz Diaplan light microscope (LM); some were fixed for electron microscope observation.

In particular, observations have been carried out on eggs fertilized in TBTCl solutions in order to follow the event of fertilization, for 2, 3 and 5 h.

All experiments were repeated in triplicate ($n = 3$). Control fertilized eggs, both in MFSW and MFSW + 0.07% DMSO, were observed to develop up to the swimming larval stage.

Transmission electron microscopy (TEM)

The samples were pre-fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer in sea water (pH 7.2) containing 4% sucrose for 30 min at room temperature and post-fixed in 1% osmium tetroxide in the same buffer for 1 h at 4 °C. They were then dehydrated in an ethanol–propylene oxide series and embedded in Dow epoxy resins.[11] Sections were stained with uranyl acetate and lead citrate[12] and examined with a Philips EM 410.

Scanning electron microscopy (SEM)

Specimens were fixed as described for TEM. After dehydration they were critical-point dried, sputter-coated with gold and observed with a Cambridge Stereoscan 200 scanning electron microscope.

RESULTS

Controls

S. plicata eggs are surrounded by complex ovular envelopes, i.e. follicle cells, vitelline coat and test cells (Figs 1–8). The follicle cells under a LM (Figs 1 and 2) appear columnar in shape and highly vacuolated; as observed by SEM they appear as a layer of spaced, cylindrical box-like cells that are arranged hexagonally (Figs 6 and 8). At their apical end lies a large vacuole (Fig. 2) containing a sponge-like granule (Fig. 8), which probably exerts an attracting role during fertilization.[13] Several mitochondria are detected in the cortical ooplasm; although many of them occur singly or in clusters, others surround the cortically placed lipid droplets (Fig. 3).

The vitelline coat is a three-layered matrix embedding the test cells in its innermost layer (Figs 1–3 and 7); its outermost layer shows a fine surface ornamentation. The mature *S. plicata* spermatozoon (Figs 4 and 5) shows only two parts: the head and the tail. The head consists of a slightly curved tapering nucleus with a roundish tip, partially flanked by a single mitochondrion; the tail has an axoneme with the usual $9 + 2$ tubular pattern.[14,15]

Eggs and spermatozoa incubated in 10^{-5} M TBTCl solution for 2–5 h

Incubation in TBTCl (10^{-5} M) solution for from 2 to 5 h causes several degrees of damage to the various components of eggs and spermatozoa (Figs 9–20). The vitelline coat appears to be the structure most affected, and after 2 h of incubation it appears thicker, probably due to high hydratation (Figs 9 and 10). Almost all the follicle cells are detached from the outermost layer of the vitelline coat; the rare follicle cells that are present appear very elongated (Fig. 10), but, under TEM observation, they do not show other signs of modification at this stage. The outermost layer of the vitelline coat, naked after the loss of the follicle cells, shows various surface modifications: fine ornamentations marking the boundaries of the fallen follicle cells, small protrusions, holes and crater-like cavities (Figs 13 and 14; arrows); the glycocalix of the outer layer of the vitelline coat is also well developed (Fig. 12; arrowheads). In the egg, cortical cytoplasm mitochondria associated with lipids show some degree of damage (Fig. 11); test cells and spermatozoa appear quite normal, like the controls, but the eggs do not cleave.

After 3 h of incubation in 10^{-5} M TBTCl solution (Figs 15–18), spermatozoa are not detected and the rare remaining follicle cells are lengthened (Fig. 15). The vitelline coat shows various degrees of damage until the breakage; in this case its inner layer, the test cells and the highly microvillated egg plasma membrane are evidenced (Figs 17 and 18).

After 5 h of incubation (Figs 19 and 20), the vitelline coat appears much more damaged and frequently broken; also, test cells show some degenerative modifications, i.e. myelinic figures (Fig. 20). The very rare spermatozoa detected at this stage show a strongly modified head; at the periphery of the egg, cytoplasm mitochondria surrounding lipid droplets are also highly modified (Fig. 19). The eggs are uncleaved.

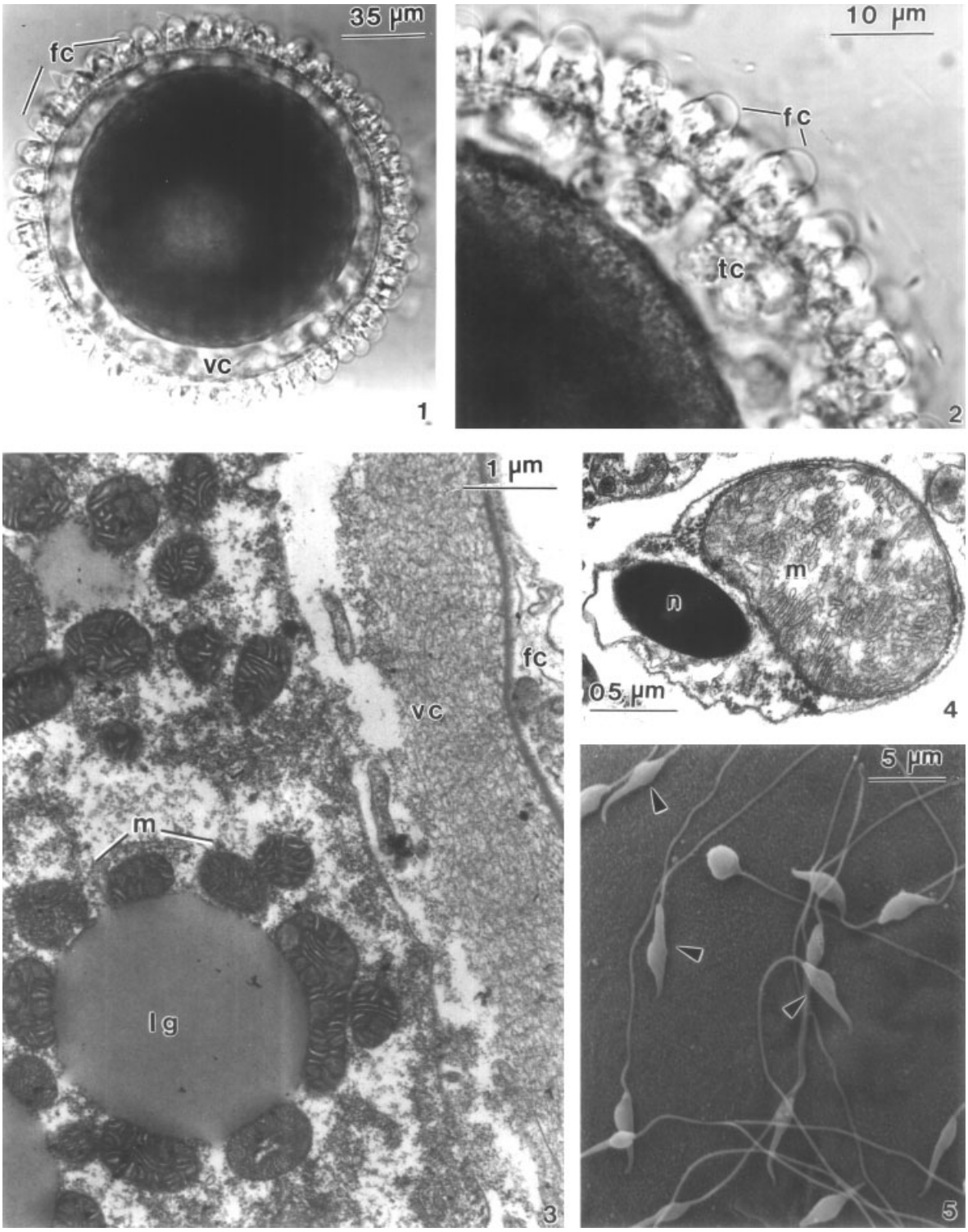

Figure 1. Phase contrast micrograph of a living *S. plicata* egg (control); fc = follicle cell, vc = vitelline coat.
Figure 2. As for Fig. 1; tc = test cell; fc = follicle cell.
Figure 3. Transmission electron micrograph of the envelopes and cortical cytoplasm of *S. plicata* egg (control); lg = lipid granule, m = mitochondrion.
Figure 4. TEM of a transverse section of an *S. plicata* spermatozoon (control); m = mitochondrion, n = nucleus.
Figure 5. Scanning electron micrograph of *S. plicata* spermatozoa (arrowheads) on the vitelline coat of the egg (control).

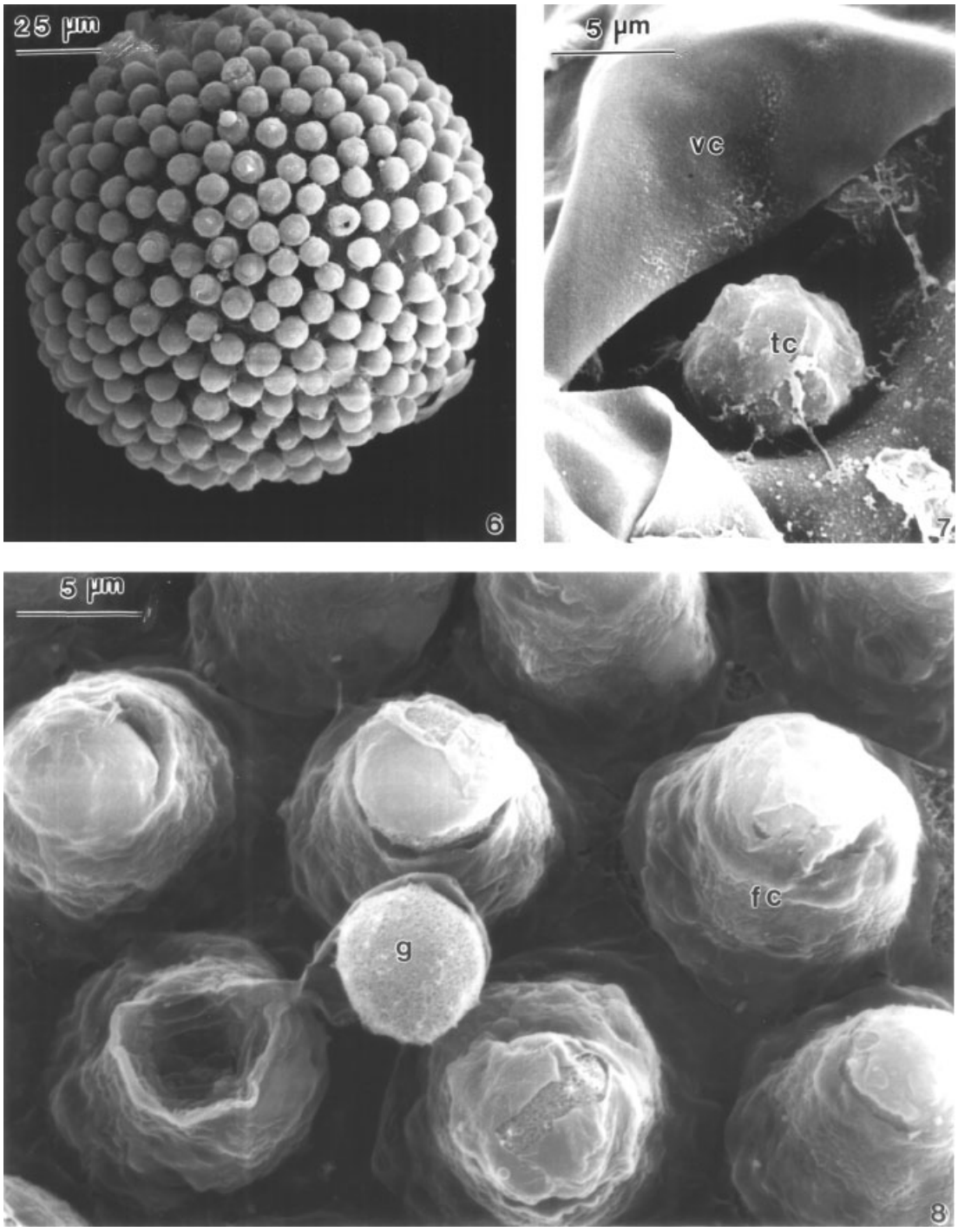

Figure 6. SEM of *S. plicata* egg (control): general view.
Figure 7. SEM of *S. plicata* egg control: particulars of the envelopes; vc = vitelline coat, tc = test cell.
Figure 8. As for Fig. 7; fc = follicle cell, g = granule of the fcs.

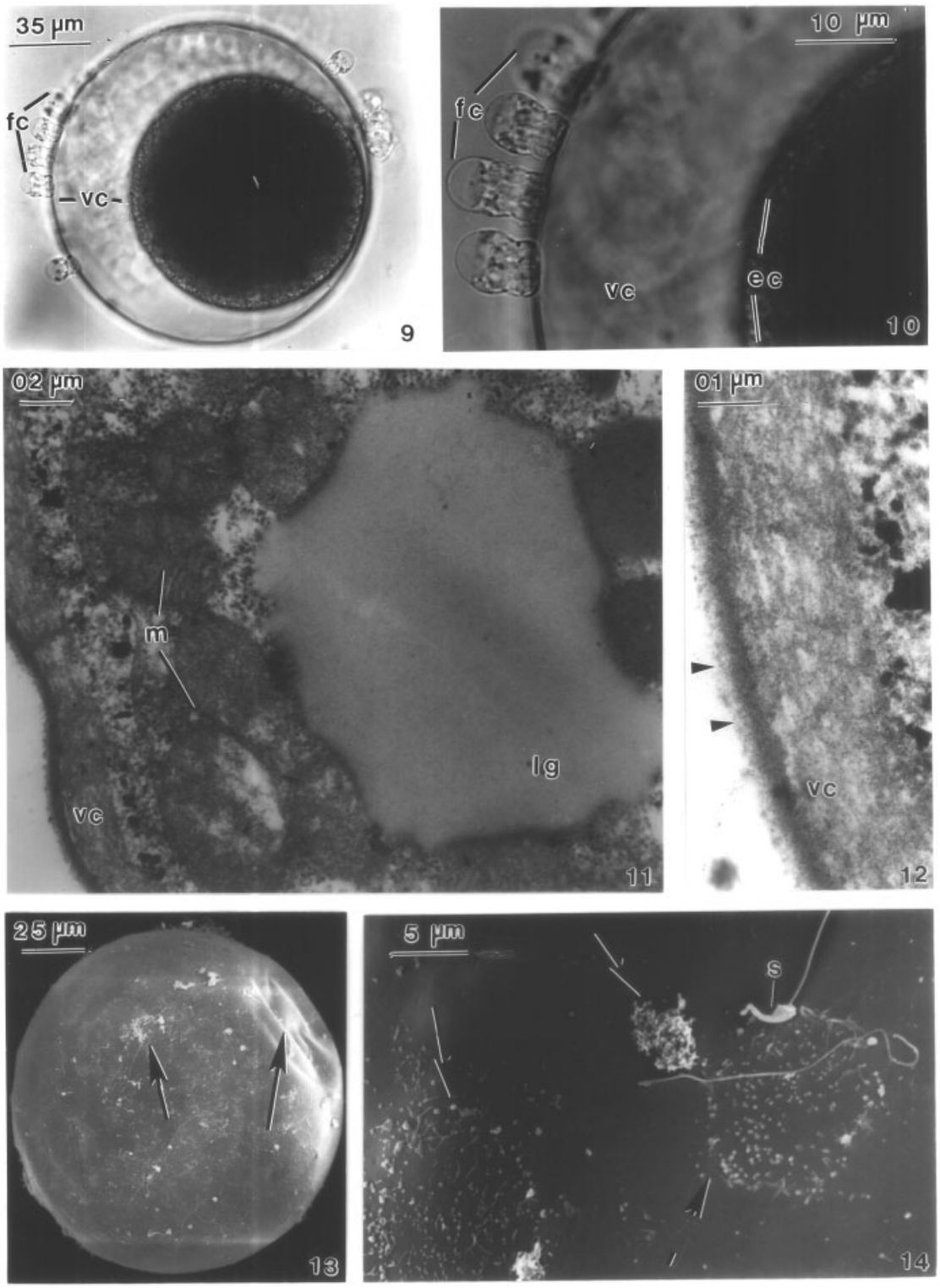

Figure 9. Phase contrast micrographs of *S. plicata eggs* incubated for 2 h in 10^{-5} M TBTCl solution; fc = follicle cell, vc = vitelline coat.
Figure 10. As for Fig. 9; ec = egg cortical cytoplasm, fc = follicle cell, vc = vitelline coat.
Figure 11. TEM of *S. plicata* eggs incubated for 2 h in 10^{-5} M TBTCl solution; lg = lipid granule, m = mitocondrion, vc = vitelline coat.
Figure 12. As for Fig. 11; vc = vitelline coat, arrowheads point to the glycocalix.
Figure 13. SEM of *S. plicata* eggs incubated for 2 h in 10^{-5} M TBTCl solution showing the surface modifications of the vitelline coat (arrows).
Figure 14. As for Fig. 13; s = spermatozoon.

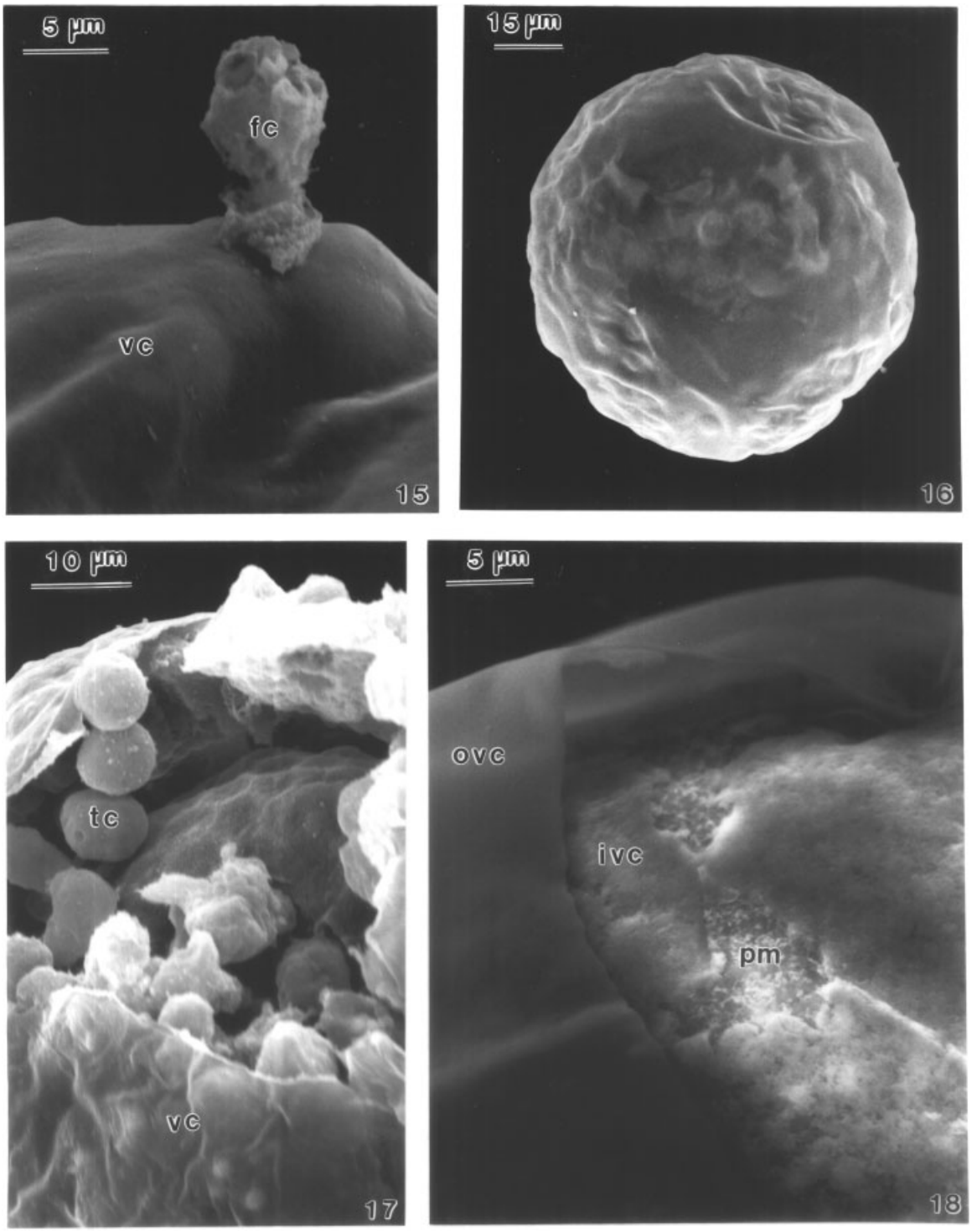

Figure 15. SEM of *S. plicata* eggs incubated for 3 h in 10^{-5} M TBTCl solution showing their modified envelopes; fc = follicle cell, vc = vitelline coat.
Figure 16. As for Fig. 15.
Figure 17. As for Fig. 15; tc = test cell, vc = vitelline coat.
Figure 18. As for Fig. 15; pm = egg plasma membrane, ovc = outer layer of vitelline coat, ivc = inner layer of vitelline coat.

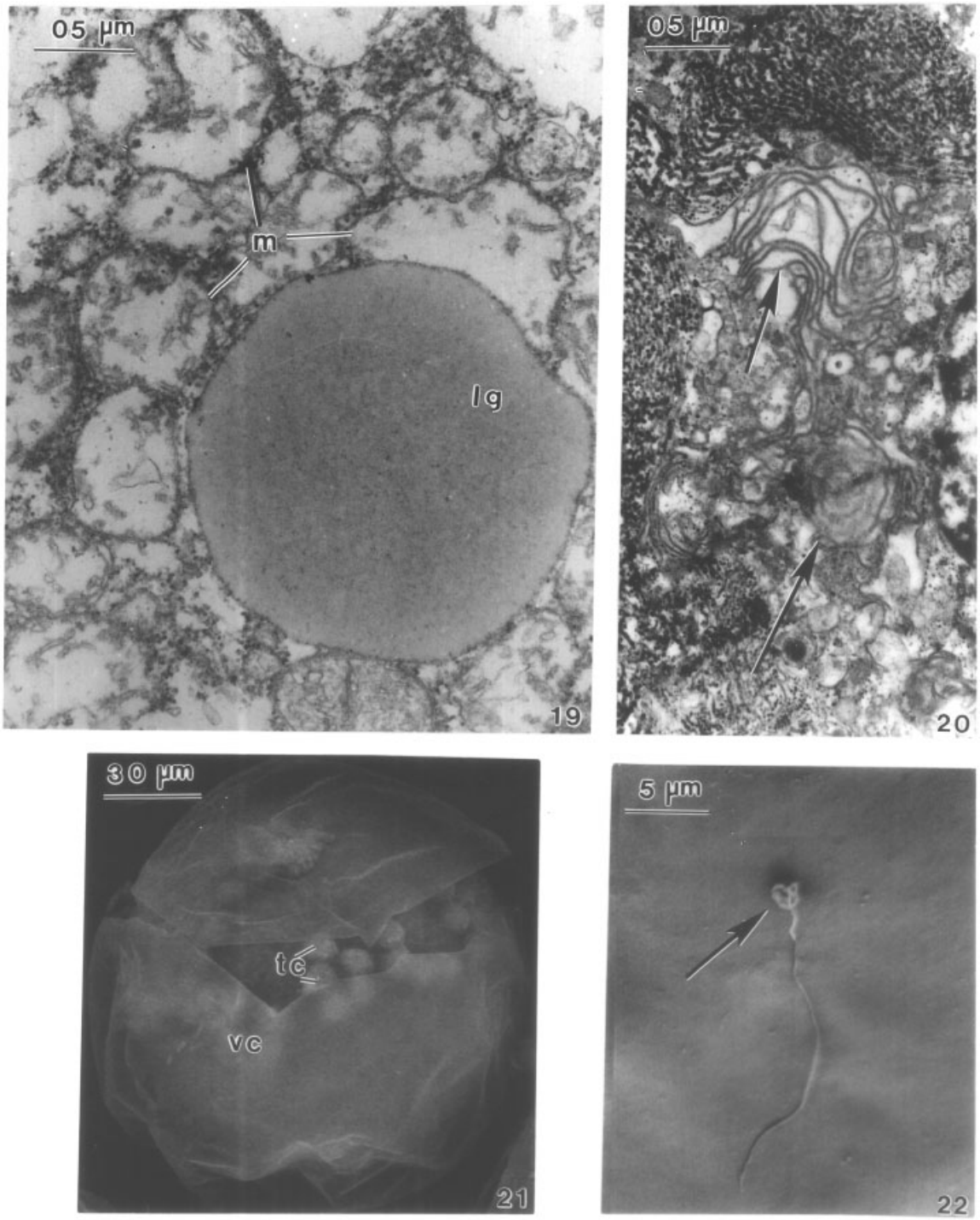

Figure 19. TEM of *S. plicata* eggs incubated for 5 h in 10^{-5} M TBTCl solution showing particulars of the damage to the egg cortical cytoplasm lg = lipid granule, m = mitochondrion.
Figure 20. As for Fig. 19, but for damage to the test cells (arrowed).
Figure 21. SEM of *S. plicata* eggs incubated for 5 h in 10^{-7} M TBTCl solution showing the modified vitelline coat (vc); tc = test cell.
Figure 22. As for Fig. 21, but showing a highly damaged spermatozoon (arrowed).

Eggs and spermatozoa incubated in 10^{-7} M TBTCl solution for 2 and 5 h

Incubation in 10^{-7} M TBTCl solution for 2 h shows that, under LM observation, many spermatozoa are motionless; some follicle cells detach and eggs are uncleaved. Under TEM observation, the rare follicle cells that are present appear very elongated. The eggs and spermatozoa do not show other signs of modification. After 5 h of incubation the results are the same as those of experiments in 10^{-5} M solution for 3 h: the few spermatozoa are damaged and the rare follicle cells are lengthened; the vitelline coat appears broken and egg plasma membrane is microvillated; the test cells seem to be like the controls. Also, mitochondria of the egg cortical cytoplasm begin to be damaged. In no case do the eggs cleave (Figs 21 and 22).

DISCUSSION

We have previously shown that TBTCl solution, either 10^{-5} or 10^{-7} M, induces anomalies in spermatozoa, unfertilized and fertilized eggs of *A. malaca*. In particular, the follicle cells detach from eggs and the test cells show anomalies in their nucleus and granules. Moreover, damaged spermatozoa are observed in the vitelline coat, but never in the egg cortical cytoplasm after incubation for 3 h in 10^{-5} and 10^{-7} M TBTCl. This signifies that fertilization does not occur.[6] In the present research, the ultrastructural aspects of fertilization in *S. plicata* are examined in order to compare the resistance of this species against the pollutant and to extend the study of the fertilization process using SEM. The most relevant alterations, in relation to incubation time, are observed in the follicle cells and the vitelline coat: a few follicle cells are present with a very elongated shape and the vitelline coat is highly damaged until breakage. The vitelline coat shows a different rearrangement: craters, blebs and holes appear as a new surface organization; also, test cells show signs of degeneration. The mitochondria of the egg cortical cytoplasm start to become damaged after 2 h of incubation in 10^{-5} M TBTCl solution. It is well known that triorganotins can disturb mitochondrial activity, binding to a component of the ATP synthase complex and inhibiting mitochondrial ATP synthesis, and thus disturbing the proton gradient.[16] It has been suggested that mitochondria serve as mediators of TBT effects and gene-regulatory signalling pathways.[17] No spermatozoa are seen on the egg surface or on the vitelline coat. After 5 h of treatment with 10^{-5} M TBTCl, a few spermatozoa, with very anomalous heads, have been detected. The absence of spermatozoa on the egg surface or on the vitelline coat could be explained by the absence of the follicle cells, which, in *S. plicata*, primarily play an attracting function.[18] In conclusion, the results reported above could give further early information on reproductive perturbation caused by TBTCl in ascidians resulting in altered functionality or even gametes death, leading to the species being unable to reproduce. As far as the ability of different species to contrast the effects of TBTCl, we can deduce that gametes of *S. plicata* seem to be more resistant than those of *A. malaca*. In fact, in *S. plicata*, a prolonged time of incubation and/or higher concentrations of TBT are necessary to detect the anomalies that prevent the fertilization process. However, in any case, fertilization does not occur for at least three reasons: the absence of follicle cells necessary for sperm–egg interaction[8] and the strong anomalies of the vitelline coat, which is considered to be the site of the species-specific binding,[9] and the lack of mobility and alterations of the spermatozoa.

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