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Defective neurulation in frog embryos exposed *to* dilute sea water

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Embryos of *Microhyla ornata* were exposed to dilute sea water from early gastrula stage onwards for a period of 48 h. The process of neurulation was studied in control and treated embryos by using optical and scanning electron microscopy. In the treated embryos, the neural folds formed normally in the initial period of exposure and subsequently approached each other. However, they failed to fuse mid-dorsally in the cephalic region. In the posterior half of the embryos, the neural folds fused to form the neural tube. Irrespective of this, the treated embryos continued differentiation of the brain, as was evident from the development of the eyes. Failure of ectodermal cells to cover the neural cells may be related to the dramatic surface modifications induced due to high concentration of cations like Na^+.

EFFECTS of saline medium on amphibian embryos have been widely studied for various reasons. For example, Ely[1] has studied effects of dilute sea water on embryos and tadpoles of a toad, *Bufo marinus*, and has described the tolerance levels. To find out if acidity of breeding ponds is a limiting factor determining distribution of amphibians in New Jersey, Gosner and Black[2] have investigated effects of altered pH and salinity on the embryonic development of several species of frogs. With a view to understanding the ecological relationship of amphibians to brackish water, Ruibal[3] has studied effects of salinity on embryos of *Rana pipiens*. Salthe[4], who was interested in finding out the mechanism of increase in the volume of perivitelline space during early amphibian development, has also reported effects of low pH, various cations and anions on the embryos of *R. pipiens*.

In recent years, Beebee[5] has studied salt tolerance of the embryos of natterjack toad (*Bufo calamita*) because breeding ponds of these toads are subjected to salt spray and tidal inundation in some coastal areas of Britain. Desiccation of breeding ponds due to irregular rainfall and possibility of tidal inundation of ponds in coastal areas prompted Padhye and Ghate[6] to investigate salt tolerance of the embryos of *Microhyla ornata*.

While studying the ecology of brackish water population of *R. pipiens* from California, Ruibal[3] has made an interesting observation regarding neurulation of the embryos exposed to near-lethal concentration of sea water – it has been mentioned that at salinities above 5‰ the surviving embryos displayed anteriorly open neural groove. Similar effects have latter been photographically documented along with brief histology of defective neural tube[6].

In this paper we provide additional evidence in the form of histological and scanning electron microscopic (SEM) analyses of defective neurulation in *M. ornata* embryos exposed to dilute sea water. The interesting facts emerging out of this work are: (1) the mechanism of neural tube closure may be different along the anteroposterior axis of the neural tube, (2) exposure to saline medium leads to collapsing of elevating neural folds as well as detachment of nonneural and neural ec-

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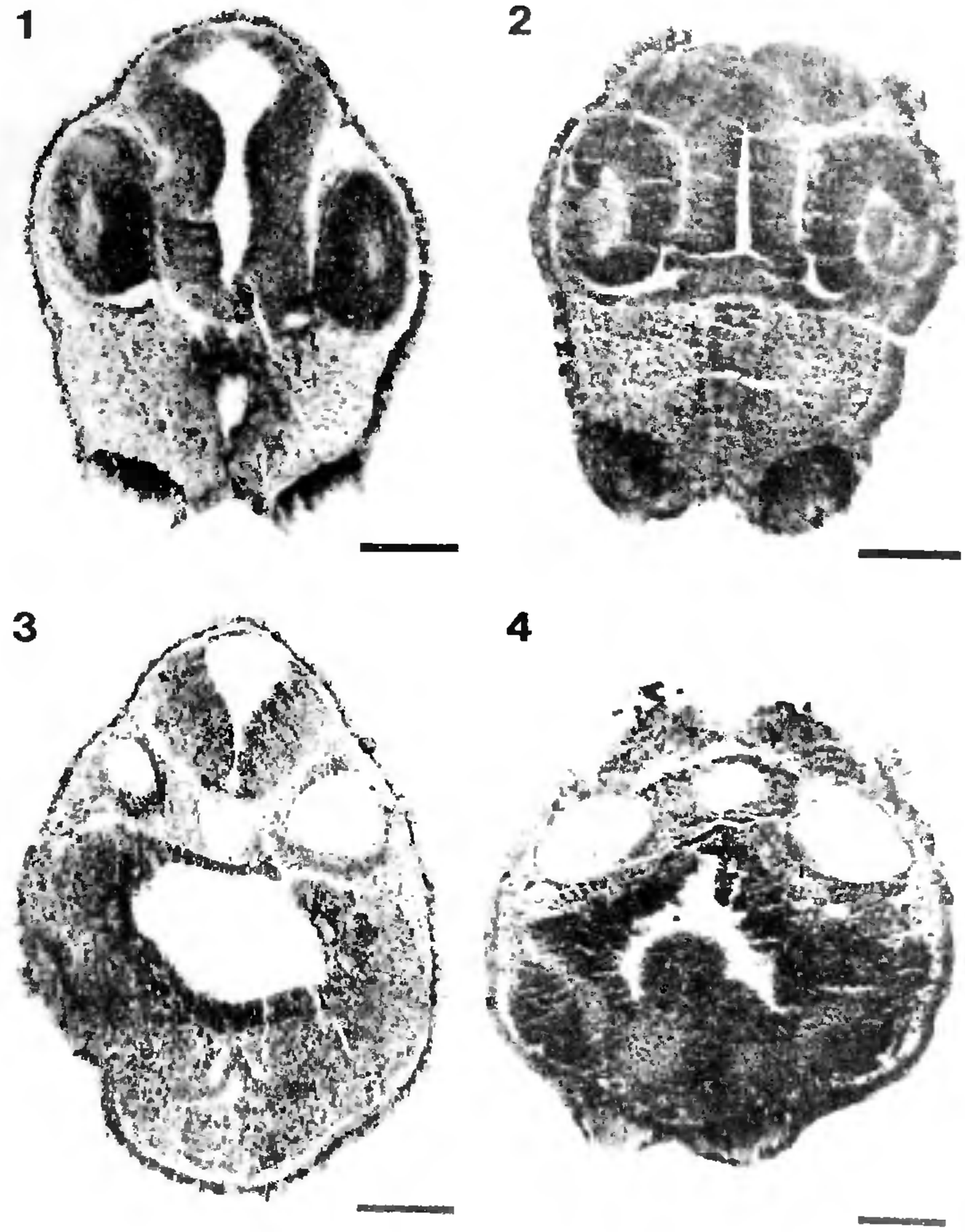


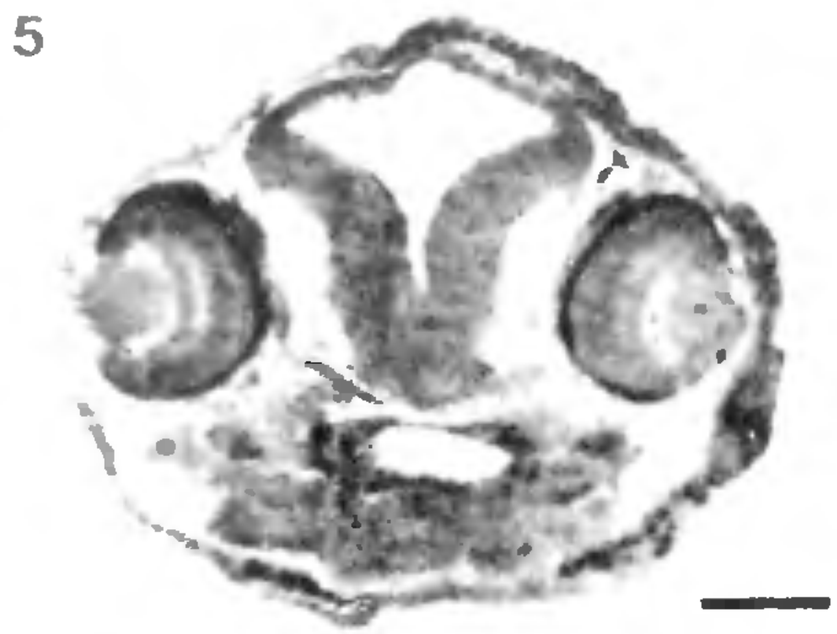
Figures 1–4. 1, T. S. control embryo at 24 h (post gastrulation). Note developing eyes and suckers. Neural tube is completely closed and covered over by ectoderm. Scale bar = 100 µm. 2, T. S. experimental embryo (30% sea water) of the same age as above. Note open neural tube, nonneural ectoderm detaching from the neural cells. Neural folds have, however, approached midline and partially fused (see also Figure 7 for magnified view). Scale bar = 100 µm. 3, T. S. control embryo (as in Figure 1) through otic capsule region. Again the neural tube is completely closed and has typical morphology. Notochord and otic capsules are well developed. Scale bar = 100 µm. 4, T. S. experimental embryo of the same age as above. Note highly abnormal neural tube. The ectoderm is widely separated and neural cells are actually falling away. Notochord and otic vesicles are normal. Suckers are also seen on the ventral side. Scale bar = 100 µm.

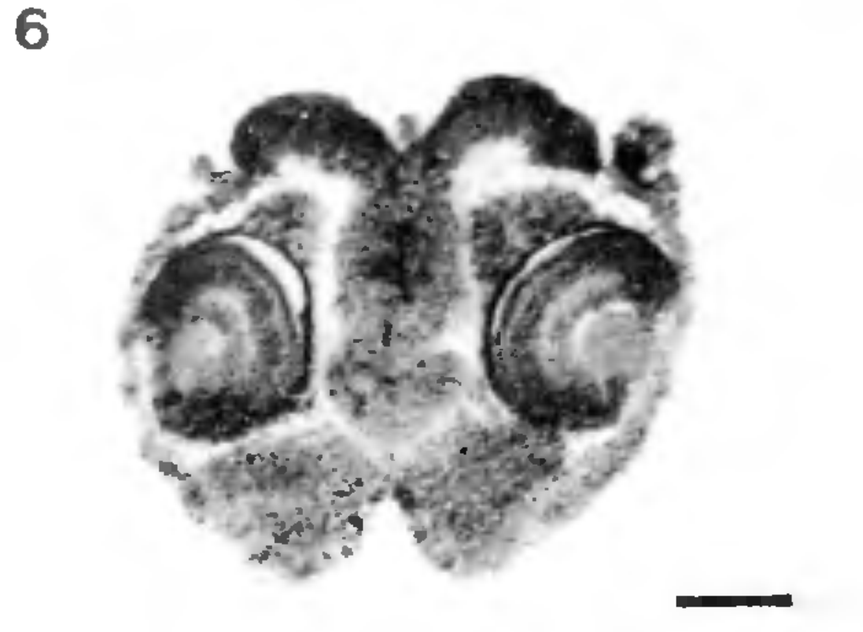
toderm from each other and (3) in spite of defective neurulation, development of eye and otic capsule proceeds normally at least for some time.

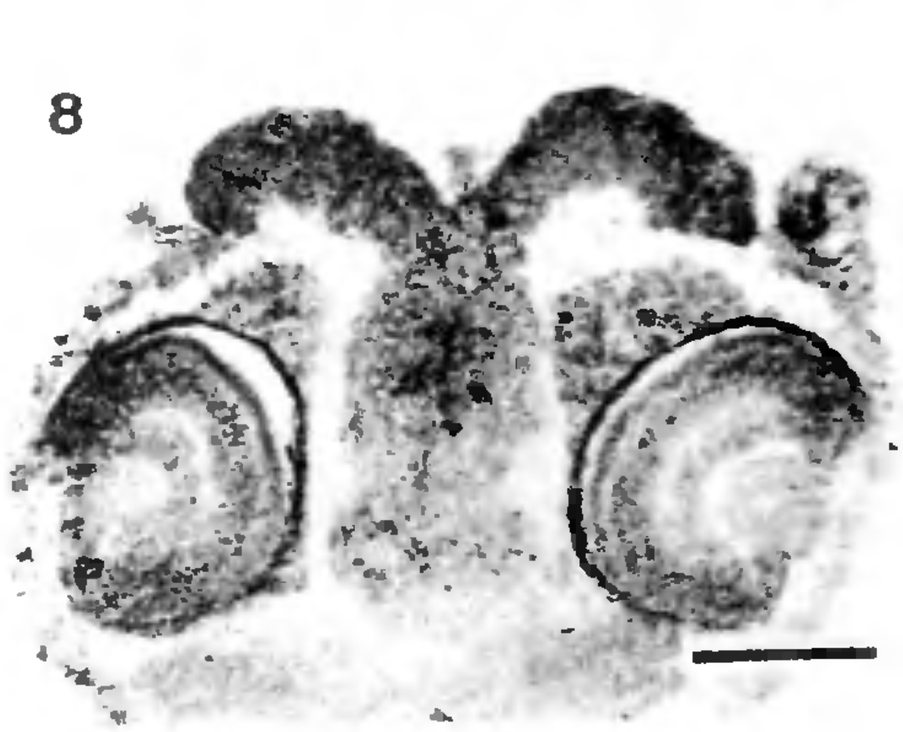
Embryos of the frog *M. ornata* were collected from local freshwater ponds, jelly was removed manually using forceps and the embryos in early gastrulation (stage 10 of Gosner[7]) were exposed to different dilutions of sea water according to the methods described earlier[6]. The sea water was collected from an apparently unpolluted site on the west coast and stored in a sealed polypropylene jar at about 10°C until use. It was diluted with distilled water so as to obtain 10%, 20% ... 70% sea water. The sea water used was collected from the west coast during heavy monsoon rains and it had salinity of 24 parts per thousand. The total sodium and potassium concentration measured by flame photometry was 7200 mg/l and 250 mg/l, respectively.

Since one of the prominent effects of sea water exposure on embryos was defective neurulation, we carried out histological examination as before[6]. In addition, SEM examination was carried out on whole embryos processed as described by Ghaskadbi[8].

Sea water at a concentration of 60% and more was found to be rapidly lethal to developing embryos of *M. ornata*. At 60% and 70% concentrations, sea water killed all the exposed embryos within a few hours of exposure. Sea water concentration of 40% and 50% was tolerated by most embryos up to about 48 h. In 30% sea water, the embryos survived up to 72 h while 10% and 20% sea water was tolerated beyond 96 h. The experi-







Figures 5–8. 5, T. S. control embryo at 48 h. Note well-developed typically shaped neural tube, eyes and gut. (To choose the appropriate matching section, we had to include this section which unfortunately has a knife mark.) Scale bar = 100 µm. 6, T. S. experimental embryo at the same age as above. The neural tissue is abnormally fused in the region between the eyes while it is uncovered by ectoderm dorsally. The ectoderm appears to be completely detached from neural tissue and neural folds are collapsing (see Figure 8, magnified view). Scale bar = 100 µm. 7 and 8, Slightly magnified views of the sections presented in Figures 2 and 6 respectively. Scale bars = 100 µm.

ments were terminated at 120 h because the purpose was to see effects on early development.

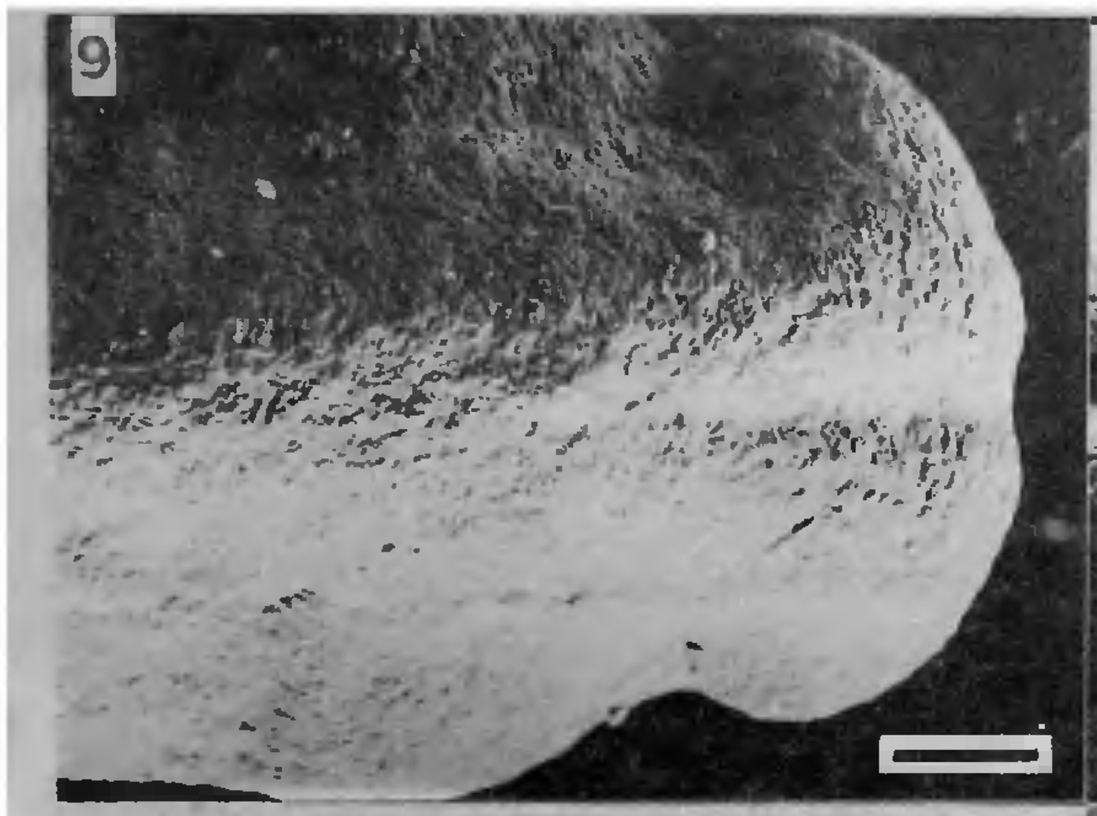
Prominent effects of sea water on embryonic development were observed between 30 and 50% concentration. The effects observed were: failure of the enlargement of perivitelline space, curling of the body axis, reduced percentage of hatching, slight retardation of development and reduced pigmentation. The severity of these effects increased with the increasing percentage of sea water in the medium.

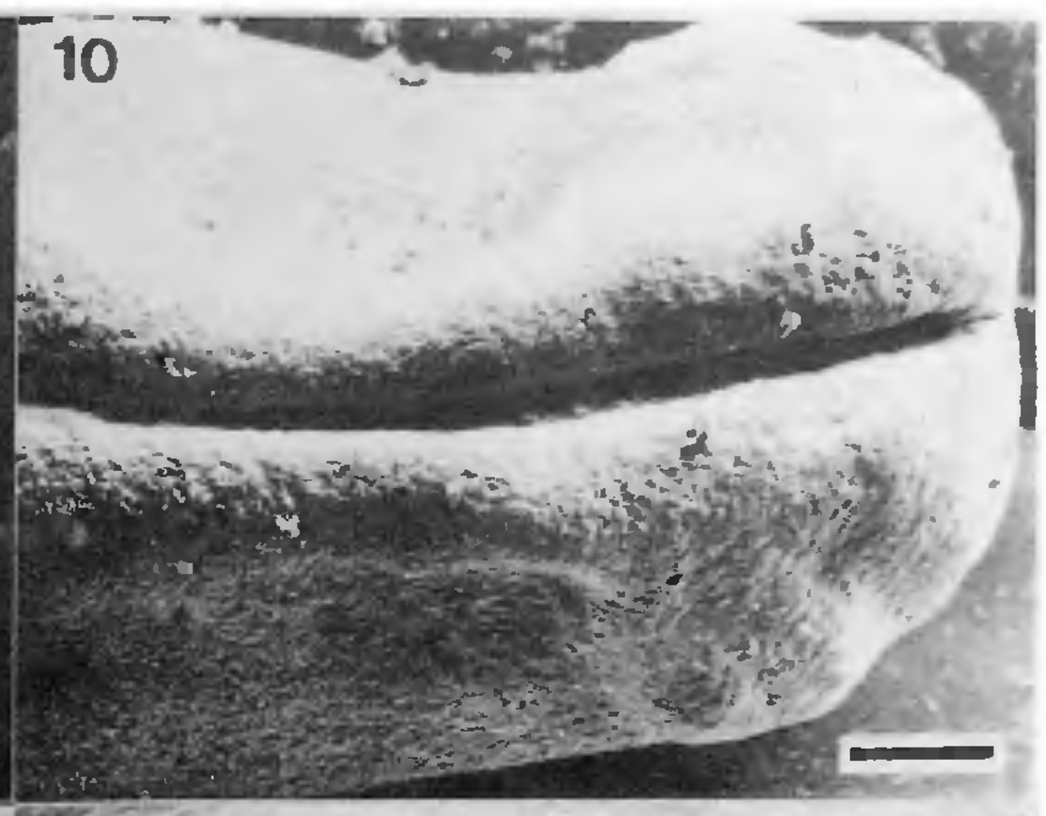
With respect to neural tube development, all embryos exposed to 30–40% sea water showed open neural tube in the head region. In 30% sea water, the embryos were otherwise well-developed and showed normal movement within the vitelline membrane. Most of these abnormal embryos survived for at least 72 h or more. In 40% sea water, however, the embryos were more stunted and, although they also showed open neural tube, most were dead by 48 h. All histological and SEM work was therefore carried out on embryos that survived exposure to 30% sea water.

In 50% sea water, embryonic development was arrested within a few hours of exposure, at about neurula or tail bud stage. Here again the neurulation was not normal. Beyond 50% the embryonic development was arrested as soon as the embryos were exposed, so the event of neurulation could not be observed.

In control embryos, development was quite rapid and normal in all the embryos. There was no mortality or signs of abnormal development in any. By the end of 48 h most control embryos hatched as tadpoles-with-suckers and attached themselves to the wall of the container. In the next 24 hours, eyes and pigmentation developed and the tadpoles were actively swimming. Different regions of the body could be easily observed in these tadpoles (head, trunk, tail).

Comparison of the transverse sections of control and experimental embryos revealed that, in the embryos exposed to 30% sea water, the neural tube was open dorsally in the head region as well as in the anterior part of the trunk region. In the tail region, however, the neural tube had closed. A section passing through the head re-



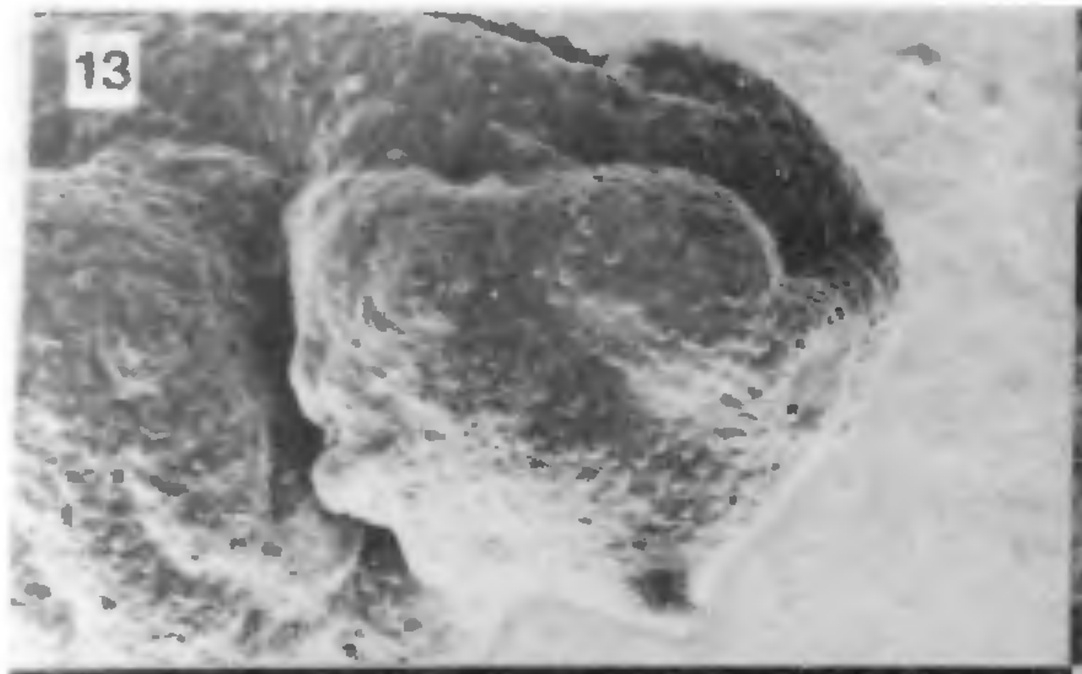


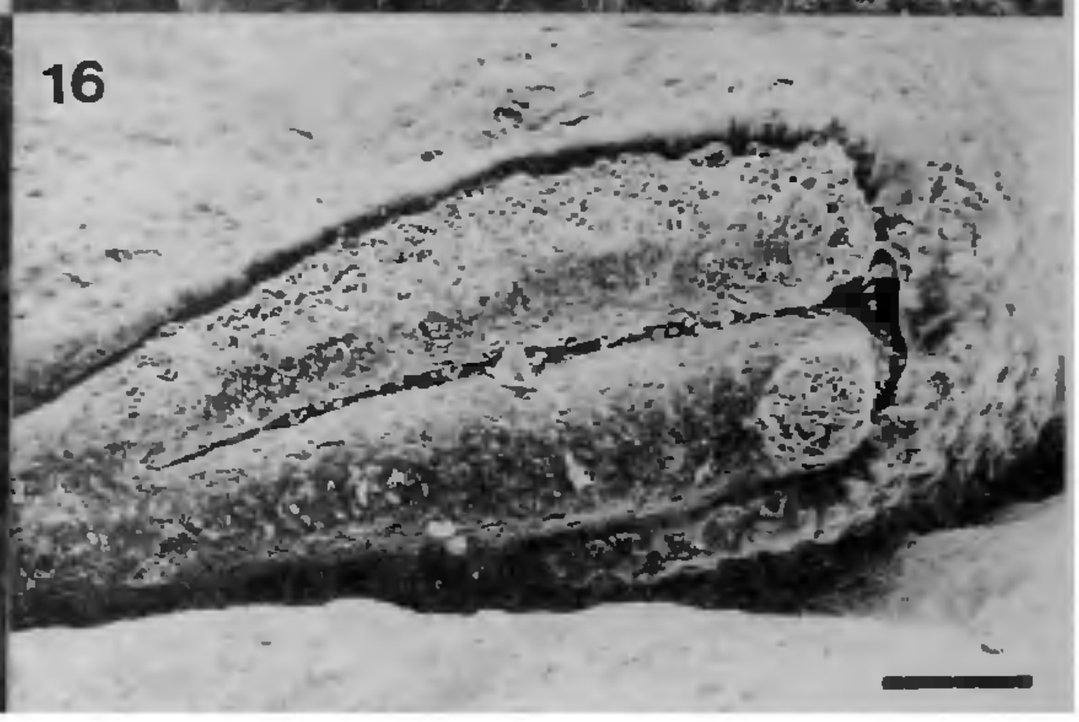
Figures 9–12. **9**, Dorsal view control neurula. Note complete closure of the neural folds. Scale bar = 100 µm. **10**, Dorsal view of the corresponding experimental embryo. The neural folds have approached towards the midline and there is delay in fusion. At this stage there is no evidence of detachment of nonneural and neural ectoderm. Scale bar = 100 µm. **11**, Later view of 24 h (post gastrulation) control embryo. Note head, trunk and tail as distinct regions of the embryo. Suckers and external gills are also visible. Scale bar = 500 µm. **12**, Lateral view of the corresponding experimental embryo. The neural tissue in the anterior two third part is not covered by the ectoderm and the tail is curved. Scale bar = 500 µm.

gion of the control embryo showed well-differentiated neural tube; the eyes and suckers were becoming evident at 24 h post gastrulation (Figure 1). A comparable section of the experimental embryo showed dorsally open neural tube with collapsing neural folds. Nonneural ectoderm had not closed over and neural cells were exposed (Figure 2). Sections passing through the otic-capsule region also indicated that neurulation was badly affected in the experimental embryos (Figures 3 and 4). Development of otic (ear) capsules and notochord was apparently unaffected.

At 48 h the control embryo showed well developed neural tube, eyes and gut region (Figure 5). In the case of experimental embryos, however, the neural tube was wide open and highly abnormal. The nonneural ectoderm had failed to close mid dorsally and the neural tissue formed a fused mass in between the developing eyes (Figure 6). Figures 7 and 8 are magnified views of the embryos shown in the Figures 2 and 6. These two figures (Figures 7 and 8) clearly point out the distorted development of the neural tube. Eye development was found to be nearly normal, however.

Scanning electron microscopy further revealed that neurulation was affected early in the development. At a stage when control embryos had completed neurulation (Figure 9), the experimentals were lagging behind and the neural folds were still approaching the midline (Figure 10). In a few hours it was clear that the neural folds of the experimental embryos were abnormal and did not fuse in the mid dorsal line. After about 24 h from the commencement of the experiment, the control embryos had reached miniature tadpole stage with





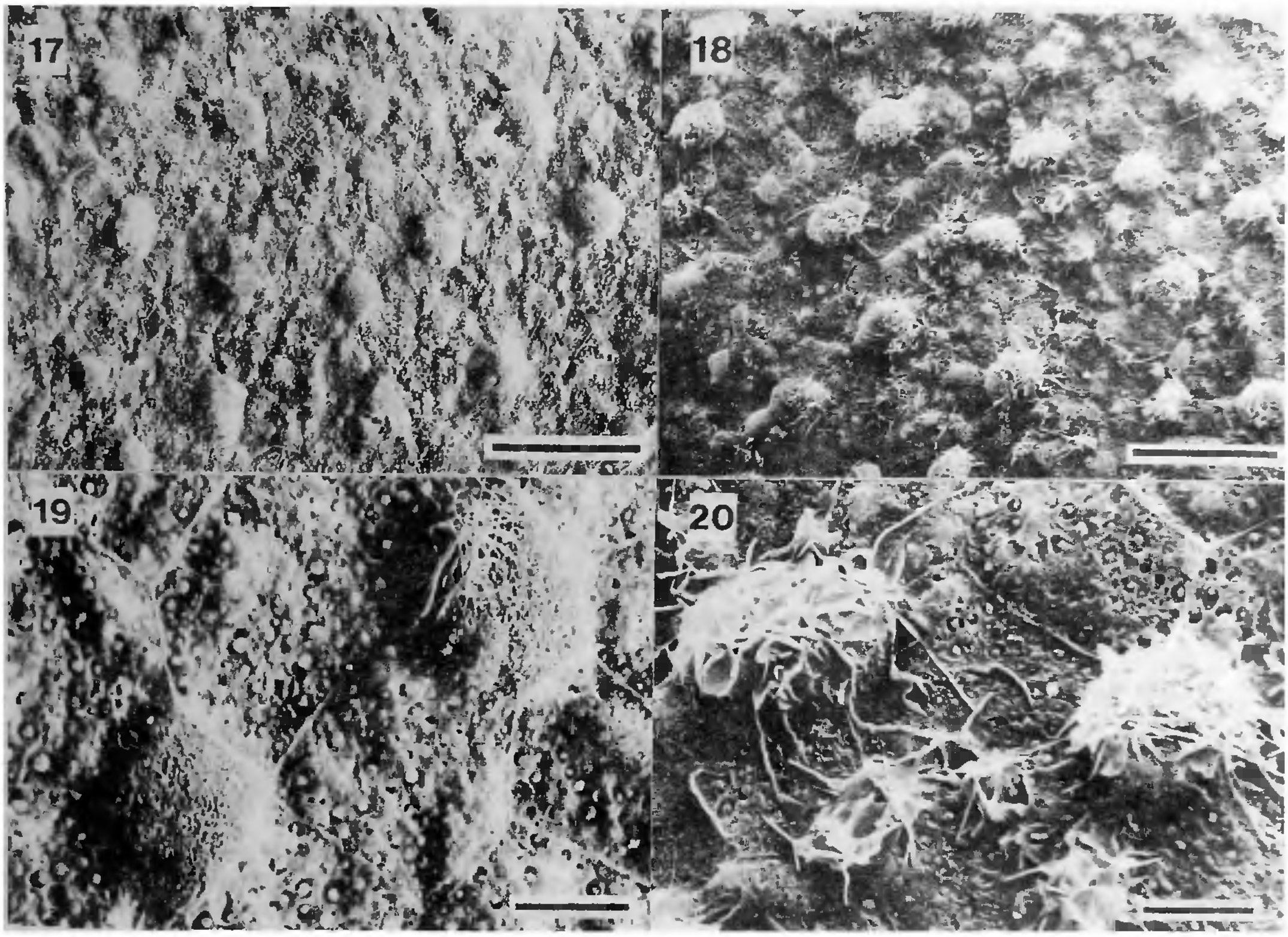
Figures 13–16. **13**, Magnified view of the head region of control embryo from Figure 11. Note head–trunk demarcation, external gills and suckers. **14**, Magnified view of the head region of the experimental embryo from Figure 12. The neural tissue appears completely detached from the ectoderm and the neural cells are exposed. Note abnormal head shape and absence of external gills. **15**, Dorsal view of the control embryo at 24 h. Note head–trunk demarcation. **16**, Dorsal view of the experimental embryo at 24 h. There is no clear-cut demarcation of head and trunk region. Neural tissue is not covered over by ectoderm and there is a wide gap between neural cells and ectoderm. Neural folds appear to have fused at least partially. Scale bar = 100 µm.

definite head, trunk and tail differentiation (Figure 11). The experimental embryos, however, showed considerably collapsed neural folds in the anterior two-third region of the neural tube. There was also overall incomplete differentiation of the head region while the tail was curved upwards (Figure 12). Slightly magnified views of these two figures are presented as Figures 13 and 14. Collapsed neural folds and protruding neural cells were clearly evident in the experimental embryos (Figure 14).

Dorsal view of the control and experimental embryos is presented in Figures 15 and 16, respectively. A considerable gap was observed between the neural and nonneural ectodermal cells of the experimental embryo. Nonneural ectoderm had apparently detached completely from the neural cells (Figure 16), a fact which was also evident from histological analysis (see Figures 6 and 8).

SEM examination of the surface epithelial cells of the control and experimental embryos revealed some striking changes caused by sea water exposure. The surface of control embryo (trunk region) showed typical epithelial cells with surface projections and the usual ciliated cells arranged in a definite pattern (Figure 17). Corresponding region of the experimental embryo showed epithelial cells with abnormal surface projections. Ciliated cells were also abnormal (Figure 18). A more detailed view is presented in the Figures 19 and 20. Large 'ruffles' present on the cells from experimental embryo (Figure 20) were not at all observed in the controls.

The results presented herein clearly show that tidal inundation of the breeding pools will be detrimental to the embryos of *M. ornata*. Increased salinity of more than 30% concentration of sea water will either be fatal or it will cause abnormal development of the exposed embryos. Many aspects of salinity-caused abnormal development, such as prevention of swelling of perivitelline space, curved body axis, inhibition of hatching, etc. have been discussed earlier[6] and need not be reiterated here. The purpose of the present paper is to discuss abnormal neurulation and related phenomena.



Figures 17–20. 17, Ectodermal cells on the trunk region of the control embryo. The typical pitted appearance and surface projections are apparent. Also note pattern of ciliated cells. Cell boundaries are the distinct white raised lines (see magnified view Figure 19). Scale bar = 50 µm. **18,** Markedly changed ectodermal cells of an experimental embryo. Note reduced ciliated cells and abnormal surface projections on many cells (magnified view Figure 20). Scale bar = 50 µm. **19 and 20,** Magnified views of Figures 17 and 18 respectively. Scale bar = 10 µm.

Both processes, induction of neural tissue and morphogenesis of the neural tube, continue to attract the attention of research workers. A detailed review of recent advances in neural induction phenomena has recently been published[9] while the mechanism of neurulation has been reviewed several times during the past 20 years or so by different workers[10–12]. In addition, two excellent reviews of the experimental analysis of the shaping of the neural plate and tube are published by Jacobson[13,14]. In spite of this, the actual nature of neural induction phenomenon and the mechanical as well as physiological aspects of neurulation are not fully understood.

As far as neurulation is concerned, there is still controversy regarding the forces involved in the rolling of neural folds to form the actual neural tube – whether the forces are entirely intrinsic (to the neural tissue) or extrinsic factors (nonneural ectoderm, mesoderm) also help the process[12]. It is also becoming apparent now that the mechanisms involved may be different along the antero-posterior axis of the neural tube[12,14]. This may be the reason why only the anterior part of the nervous system was affected in our experiments. In fact, Burt[15] also reported that neurulation was more retarded in the head region than in the spinal cord when the *Amblystoma* embryos were exposed to saline water. While studying the uptake of Ca^{++} in developing frog embryo, Barth and Barth[16] also showed that cephalic regions incorporate more Ca^{++} than the spinal regions. In mouse, external Ca^{++} added in the medium was shown to augment the rate of neural tube closure in the hind brain region only and not in the other region, again pointing out that physiological mechanisms may be different at different places along the neuraxis[17].

An interesting aspect of our finding is that the nonneural ectoderm got detached from the neural ectoderm, somewhere in the latter stages of neurulation, in the em-

bryos exposed to sea water. Apparently, in spite of detached ectoderm, neurulation continued, albeit abnormally. *Microhyla* embryos exposed to NaCl also show defective neurulation[6], an observation in agreement with the present results. This is not surprising since the major cation in sea water is indeed sodium. It appears certain, therefore, that the concentration of cations/anions, especially that of sodium, in the water surrounding the embryos plays important role in morphogenesis. Exposure to cations like mercury and lead does not produce a similar effect in *Microhyla* embryos[18,19]. The collapse of neural folds reported here is, however, very similar to that reported in rat embryos cultured in Ca^{++}-deficient medium[20]. It may be worthwhile to investigate the effect of sea water/NaCl treatment on Ca^{++} balance in *Microhyla* embryos and its relation with neurulation.

On the whole, the SEM structure of the control *Microhyla* neurula is similar to that described by Tarin[21] and Löfberg[22] for other amphibians. The surface ectoderm is showing all the features described by these authors. In experimental embryos, there were considerable cell surface alterations as a result of exposure to drastic change in the salinity. But the mechanism involved is unknown at present. Keller[23] has, however, noted that in *Xenopus* embryos high salinity (0.385% salt concentration) causes the epithelium to weaken and even break down. He further noted abnormal ingression of surface cells, thickening of blastula and gastrula walls as well as exogastrulation. However, we did not observe exogastrulation in the present study. This may be because we did not remove the vitelline membrane.

In conclusion, it can be stated that *Microhyla ornata* embryos will not tolerate excessive tidal inundation and the resultant increase in the salinity of their ambient pond water. The embryos are very sensitive to more than about 0.2% NaCl or 20% sea water and that high concentration of salt will cause abnormal development.

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