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THE TOLERANCE OF DEVELOPING CIRRIPEDE EMBRYOS TO SALINITY AND TEMPERATURE[1]

BY

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Intertidal barnacles are exposed to wide variations in temperature, and their external surface to desiccation at low tide. During these periods of exposure to the air, water is expelled from the mantle space and the valves closed except for a small opening which allows diffusion of air into the mantle space and gills (MONTEROSSO 1927, CRISP & SOUTHWARD 1961). In *Chthamalus*, often exposed to conditions of prolonged insolation and extreme desiccation without harm, the valves may close completely or be opened only intermittently (MONTEROSSO 1927). The mantle space needs to lose very little water by evaporation to cause the egg masses to be exposed to salinities in excess of those normally occurring in the sea. Nevertheless the eggs of *Chthamalus stellatus* were able to develop even when the adults containing them had been kept out of water for periods of a week or even longer (PATEL & CRISP 1960).

Some species, such as *Elminius modestus*, *Balanus improvisus* and *B. eburneus* live in estuaries where, as adults, they experience periodically very low salinities; some can even survive in virtually fresh water (HOLMES & PRIOR 1938). *Balanus amphitrite* is apparently able to live and sometimes to breed in abnormally high and low salinities (NILSSON CANTELL 1948, SHATOURY 1958).

It is not clear, however, to what extent the eggs are in fact exposed to extreme conditions, since the adult might temporarily protect the mantle cavity by closing the valves or by reducing pumping activities when external conditions were unsuitable. It is therefore of interest to know how tolerant the egg masses can be to prolonged variations in salinity.

Egg masses can be removed from the parent and their development observed *in vitro*, both in pedunculate (BATHAM 1946) and in sessile forms (CRISP 1959; BARNES & BARNES 1959; PATEL & CRISP 1960). Removal from the parent in itself has no influence on development (PATEL & CRISP 1960).

Three species were investigated by rearing egg masses in water of abnormally high and low salinity – *Balanus eburneus* Gould, *Balanus amphitrite amphitrite* Darwin[1] and *Chelonobia patula* Ranzani.

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Methods

Specimens of *Balanus eburneus* and *B. amphitrite* were obtained from wooden piles opposite Duke University Marine Station; in October it was found better to collect specimens of *B. amphitrite* from Shark Shoal jetty in order to obtain sufficient of the younger egg masses, as individuals growing in the more enclosed waters had almost ceased to breed. Specimens of *Chelonobia patula* were obtained from the carapace of the blue crab, *Callinectes sapidus*, collected outside the Marine Station.

The egg masses were removed as soon as possible on bringing specimens into the laboratory, and each pair from a single individual, if found to be at the required stage of development, was further subdivided and the pieces placed individually each in a glass tube 1 inch in diameter and 4 inches in height. The tubes were each partially filled with water of the required salinity, stoppered, and placed at the appropriate temperature in a thermostatically controlled cabinet at 30°C, 25°C, 20°C or 15°C. Each egg mass was examined microscopically at intervals of between 6 and 10 hours and the water changed daily for a fresh supply at the same temperature in order to reduce the possibility of fungal infection. The majority of experiments, especially the more critical ones, were made in the cabinet at 25°C which was provided with an oscillating platform which caused eggs masses in the tubes to be constantly moved about but not damaged. Preliminary experiments were first carried out to investigate whether this rocking technique had any significant effect on development. These experiments showed that rocking the tubes promoted more uniform embryonic development in large pieces of egg mass, particularly in the later stages when the embryos were acquiring brown pigment. It had otherwise no significant effect. In the other cabinets the tubes were assembled in beakers, and were gently shaken manually once or twice between each set of observations. By this means, and by using small portions of egg masses, reasonably uniform development of the embryos in each piece of egg mass was achieved. Egg masses attacked by fungi were ignored, but these were only a small proportion of the whole. Evaporated

[1] J. P. HARDING, in a personal communication informs us that the variety previously known as *B. amphitrite denticulata* Broch, is identical with a specimen in the British Museum labelled by Darwin *B. amphitrite*. In Harding's view this variety, which now occurs as an immigrant form in Britain, should be referred to as *B. amphitrite amphitrite*.

and pasteurised sea water was used to provide the medium of highest salinity, and the same batch of sea water, suitably diluted with distilled water was used for all the lower salinities. Salinities of 85, 70, 60, 50, 40, 35, 30, 25, 20, 15, 10 and 5‰ were used in most of the experiments. The highest salinity was measured by titration against silver nitrate solution, and the lower salinities were checked by means of a sensitive hydrometer using the data on sea water density in relation to salinity and temperature supplied by the U.S. Department of Commerce, Coast and Geodetic Survey, Serial Publ. 298.

In two species, namely in *Balanus amphitrite* and *Chelonobia patula*, preliminary experiments showed that the development of embryos corresponded very closely whether the salinity change was made suddenly or through a graded series of sea waters. The eggs which were brought gradually to extreme salinities developed no faster than would have been anticipated from the fact that a longer time elapsed before they reached the salinity level at which development was retarded or stopped. Sudden changes in salinity of themselves did not therefore appear to be harmful. No attempt was thereafter made in the majority of experiments to give the embryos a gradual change of salinity. Towards the end of development some egg masses, especially those of *Chelonobia patula*, appeared to contain embryos ready to hatch, but they required hatching substance to induce vigorous hatching movements. In order to assess the development of such egg masses, a small quantity of an extract containing hatching substance was added. The hatching extract was made as described by CRISP (1956), but from crushed specimens of *B. amphitrite*.

Representative samples of larvae hatched at different salinities were measured as stage 1 and stage 2 nauplii using a micrometer eyepiece and their tolerance to different salinities investigated. The stages of embryonic development quoted refer to the series described by CRISP (1954) for embryos of *Balanus porcatus* (=*B. balanus* (L)).

Tolerance of eggs of *Chelonobia patula* to salinity variations

The influence of salinity was first investigated by subdivision into twelve parts of a large pair of egg masses at a fairly early stage of development (Stage 5) taken from a single individual of *C. patula*. Under these conditions variation between individual barnacles was eliminated. The egg masses were incubated at 25°C for 90 hours, and examined as follows. Hatching extract was first added and after twenty minutes a few ml. of formalin. The hatched nauplii were counted and the unhatched eggs, which nearly all remained in the egg mass were placed under a coverglass and counted, the number which were abnormal or undeveloped being noted separately. Curves (b) and (c) in Fig. 1 illustrate respectively the percentage which were apparently developing normally and the small

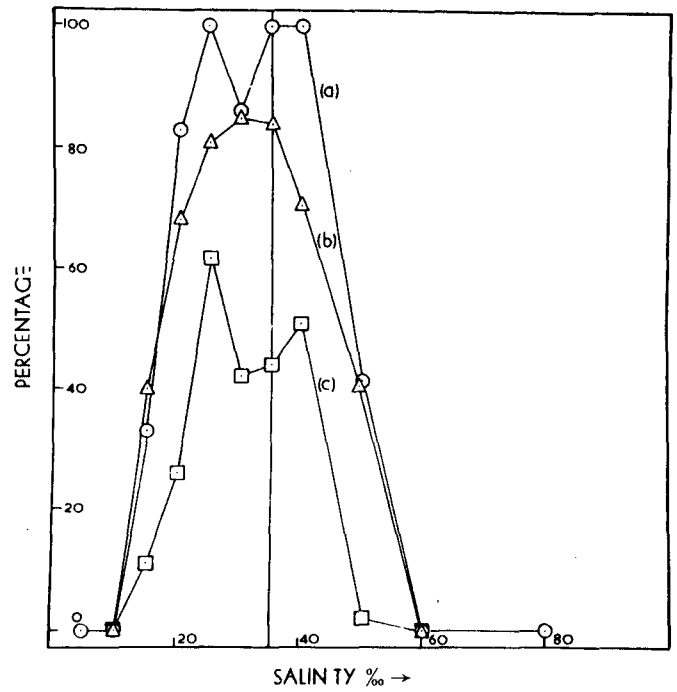

Fig. 1. Tolerance of stage 5 *Chelonobia* embryos at 25°C measured by different criteria. (a) Percentage of tubes with successfully hatched nauplii; (b) percentage of embryos which had hatched or which, though unhatched, appeared to be developing normally; (c) percentage of embryos which had hatched after the egg masses had been treated with hatching extract.

er proportion which had reached the hatching stage at the time the experiment ended. It can be seen that between 25‰ and 40‰ some 50% of the eggs were hatched, and some 75–80% were developing normally. Outside this salinity range the percentage of eggs hatching decreased sharply, as also did the percentage hatching normally.

A different method was adopted to allow for variability between egg masses from different individuals. Six pairs of egg masses were used, each being subdivided, and each pair being kept isolated at a given salinity. After sufficient time had elapsed to allow normal development to be completed each tube was examined to ascertain if hatching had occurred. Generally there was either a cloud of nauplii present, which was regarded as a success, or there were none; but in the critical salinities between 10‰ and 20‰ and between 50‰ and 60‰ occasional tubes had only a few nauplii swimming, less than half the total egg masses having hatched. We adopted the convention that in the trials in which egg masses gave a poor hatch, the result should be scored as half a success.

It will be seen that when the fraction of egg masses that hatched successfull was expressed as a percentage (Curve (a) fig. 1) the result was very similar i form to curve (b) representing the potential hatching of embryos from a sing egg mass. Indeed the salinity at which curves (a) and (b) cut the 50% level wer almost identical. The results illustrated by curve (a), which represent the propor tion of egg masses which hatched successfully at each salinity, were clear more representative than those of curves (b) and (c) which were derived fro a single egg mass. Ideally all the embryos in each of many egg masses shoul be counted at each salinity and the variability between and within egg mass analysed. The labour attached to such a task was quite impracticable; we therefor replicated egg masses and classified them as successfully hatched, partiall hatched, or not hatched.

Results with fairly small numbers of *Chelonobia* revealed that embryos whic at the outset of the experiment were in a late stage of development and wer therefore immersed in abnormal salinities for a shorter period, were mor tolerant than early embryos. Classifying the egg masses as early (stages 1–9) o late (stages 10–12) therefore improved the precision of the method by increasin the slope of the line connecting percentage success with salinity, and als revealed the degree to which the age of the embryos influenced their tolerance In *Chelonobia patula* the salinity extremes compatible with successful develop ment of the whole egg mass were found to be from approximately 25 to 40 ‰ for early stages and from 15‰ to 50‰ for late stages, indicating a fairly wid tolerance. A more accurate measurement was afforded by reading off th salinity at which half the egg masses develop successfully, since the relatio between percentage success and salinity changes most sharply in this region For *C. patula*, using the results illustrated in fig. 1, the range of tolerance s defined lay between 17‰ and 48‰, written for convenience as 32.5‰ ± 15.5 ‰

Influence of temperature on salinity tolerance

Since tolerance of temperature and salinity may not be additive but may sho interaction, as in larvae of *Sesarma cinereum* (COSTLOW *et al.* 1960), the relatio between successful hatching and both temperature and salinity was investigated Another species, *B. eburneus*, was used, choosing egg masses of as nearly th same age as available material allowed; these ranged from stage 5 to stage 9, th majority were at stage 6–7. The results are shown in the form of surviva percentages on a salinity-temperature grid (fig. 2). The contours are relativel simple and have been drawn in by hand. Interaction was small and scarcel significant. There was a slight tendency for the area of survival to enlarge a the higher temperatures, due possibly to the shorter time the embryos wer exposed to unfavourable conditions. The experiments covered only the rang

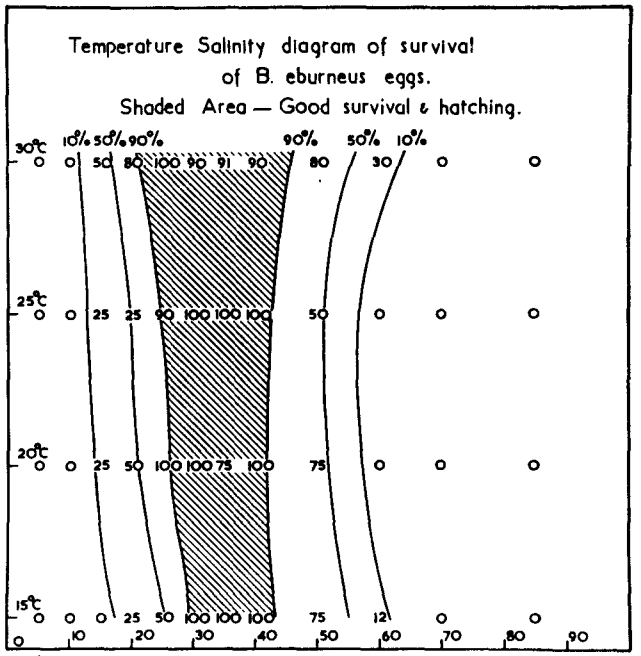

Fig. 2. Temperature salinity diagram of survival of *Balanus eburneus* embryos between 15°C and 30 C. The lines represent the probable position of contours of equal survival, and are based on the observed sample values of survival shown on the diagram.

of temperature normally experienced by the animal in the breeding season; more extreme temperatures might have shown more pronounced interaction. Similar observations on *C. patula* at 25°C and 30°C, and on *B. amphitrite* at 15°, 25 and 30°C indicated little influence of temperature on salinity tolerance in these species.

Influence of salinity on rates of development

In all three species the intervals of time between successive stages showed no relation to salinity within the range of 25‰ to 40‰; the egg masses almost always hatched normally and successfully. The observed intervals between embryonic stages were used to construct a linear relationship between stage of development and time, using the method described by PATEL & CRISP (1960). The intervals between stages were arranged on the ordinate so as to correspond with the proportion of the total time of development that each stage occupied in other species. The rates of development for *C. patula* and for *B. eburneus* at different temperatures have not previously been recorded and are illustrated in

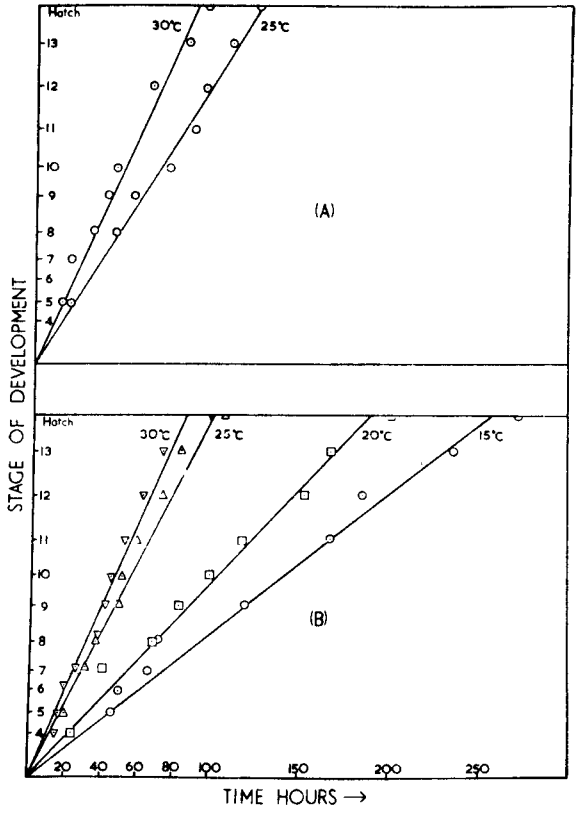

Fig. 3. Rates of development at various temperatures of the embryos of *Chelonobia patula* (A) and *Balanus eburneus* (B). The stages of development shown on the ordinate refer to those described in an earlier paper (CRISP 1954).

fig. 3, while the rates of development found for *B. amphitrite amphitrite* agreed well with those previously published for the same variety from British waters (PATEL & CRISP 1960). These linear relations conveniently allow intervals between stages to be converted to time intervals.

At salinities at or beyond the extremes of 20‰ and 50‰ development was retarded, even though sometimes some or even the majority of eggs eventually hatched. The retardation could be measured by comparing the stage reached by egg masses kept at the abnormal salinity with the stage reached by a part of the same egg mass maintained in water of 35‰ salinity. The former egg mass invariably developed more slowly, and its delay could be converted into hours by means of the relation between time and development illustrated in fig. 3 using the slope appropriate to the temperature of the experiment. The delay in hours was then converted to a percentage of the time of development of the

Table I. Relative delay in development at different salinities for three species of barnacle.

	Balanus amphitrite	Balanus eburneus	Chelonobia patula
Interval of stages of development at 35‰	Stage 7–Stage 13	Stage 4–Stage 12	Stage 5–Stage 12
Temperatures at which observations were made	15, 25 & 30°C	15, 20, 25 & 30°C	25 & 30°C
Mean delay as % of total time of development at salinity of			
10‰	Cytolysis	Cytolysis	Cytolysis
15‰	19%	25%	59%
20‰	5%	16%	33%
25‰	−1%	7%	8%
30‰	2%	−3%	5%
35‰	(0%	0%	0%)
40‰	6%	0%	10%
50‰	23%	7%	44%
60‰	50%	44%	91%
70‰	92%	–	No development
80‰	No development	No development	No development

egg mass which had been kept at normal salinity. Thus if the egg mass in abnormal sea water failed to develop at all the relative delay would be 100%. By this means it was possible to compare the relative delay at different temperatures and salinities. No relationship was evident between the relative delay and the temperature, but salinity had an overriding effect. Table I records the relative delays for egg masses of the three species averaged from all the results obtained. It can be seen that as the salinity was lowered beyond 20‰ or raised beyond 50‰ the normal rate of development was reduced, especially in *C. patula.*

Size of larvae

Larvae obtained from eggs which had developed at different salinities were compared with those which hatched at the salinity of normal sea water containing 35‰ of salt. The lengths and breadths of from three to eight individuals were determined in sea water of the same salinity as that in which the larvae had been reared, using a callibrated eyepiece micrometer. Stage 2 nauplius larvae were measured for all three species, and stage 1 nauplius larvae hatched directly from the egg were measured for *Balanus eburneus* at only two temperatures, namely at 15 and 20°C.

The detailed results will not be quoted, since successfully hatched individuals showed no significant deviations from the normal size whatever the salinity in which they had developed. A few of the larvae showed a slight deformity in that the caudal processes were not fully expanded at the moult between the stage I and the stage 2 nauplius, and in many larvae of *C. patula*, reared at a salinity of 15‰, these processes were not developed normally. Apart from this, none of the batches of larvae differed from the normal in mean size by as much as 5%, and few showed departures exceeding 3%. These small differences in mean size were quite unrelated to the salinity and were within the range of variation to be expected from the magnitude of the variability between individual eggs and larvae within a single egg mass.

Tolerance of larvae

The behaviour of hatched larvae in sea water of salinity ranging from 5 to 85‰ was investigated. At salinities which were outside the range of tolerance the rate of swimming was first reduced, then the larvae sank to the bottom and twitched, and finally showed a quivering of the limb muscles or remained quite motionless. Table II shows the tolerance of *Chelonobia patula* larvae developed at a salinity of 25‰ after remaining for various times in sea water of the salinity indicated in the first column. Relatively little change occurred after half an hour. The lower limit of tolerance of these larvae lay in the region of 15 to

Table II. Tolerance of larvae of Chelonobia patula to changed salinity.

Movement after:	Immediate	½ hr.	18 hrs.
Salinity ‰			
5	0	0	0
10	(+)	+	0
15	+	+	+ +
20	+ + +	+ + +	+ +
25	+ + +	+ + +	+ + + +
30	+ + + +	+ + + +	+ + + +
35	+ + + +	+ + + +	+ + + +
40	+ + + +	+ + + +	+ + +
50	+ + +	+ + +	+ +
60	(+)	(+)	(+)
85	0	0	0

( ) Motionless most of time, a few occasionally twitch
\+ Quivering or vibrating occasionally
\+ + Definite twitches of limb
\+ + + Sluggish swimming
\+ + + + Swimming well

20‰ a magnitude similar to that of the larvae of northern species studied by BARNES (1953). The upper limit lay at about 50‰. Larvae of *Chelonobia patula* were somewhat less tolerant than those of *B. eburneus* and *B. amphitrite*. The larvae of both the latter species continued swimming sluggishly for half an hour or more in a salinity of 5‰ and tolerated indefinitely a salinity from 10 up to 50‰. Below a salinity of 10‰ there was a change in behaviour, the majority swam to the unlit side of the dish. Motionless individuals recovered more frequently from sea water of abnormally high salinity than from low salinities.

A group of *Balanus amphitrite* larvae developed from eggs kept in sea water of salinity 15‰ was compared with another developed from eggs kept in sea water of salinity 50‰. The two groups showed an interesting difference in tolerance. At the lower limit of 5 to 10‰ the group reared at 15‰ swam slightly better at first, but eventually the two groups were indistinguishable. At the upper limit, however, they behaved quite differently; the group which was developed in high salinity sea water swam well in water of 60‰ salinity and swam sluggishly in water of 70‰. The other group which was developed in a medium or low salinity swam only for a short time in water of 50‰ and became quite motionless in this medium within two hours.

Salinity tolerance of different species

Since temperature had no noticeable effect on tolerance to salinity extremes the observations made on egg masses at different temperatures were pooled. This procedure increased the number of trials at the critical salinity level and so allowed more approximate measurements to be made of the salinity at which half the egg masses would hatch successfully.

The division of the results into those using initially early and initially late stage embryos reduced the number of observations in each set but greatly improved the precision. The six curves were all of similar form to curve (a) of fig. 1. The values for success were uniformly high in the middle salinities, a sharp fall occurred in percentage success at low salinity and a more gentle fall at the high salinity range. The intercepts of 50% success were used to read off the salinity ranges between which at least half the egg masses develop. The results are recorded in Table III.

The table establishes a similar wide tolerance for all three species and reveals an apparent slight increase in tolerance from *C. patula* and *B. eburneus*, to *B. amphitrite*. The numbers of trials are not however sufficient to establish this order with any high degree of certainty. The greater tolerance of late stage embryos is clearly illustrated in the table.

Table III.

Species	Salinity range for 50 % successful development	No. of egg masses used in critical range	
		10–20 ‰	40–50 ‰
Chelonobia patula			
Stages 1–9	32.5 ± 15.5 ‰	10	10
Stages 10–12	32.5 ± 17.5 ‰	6	6
Mean	32.5 ± 16.5 ‰	–	–
Balanus amphitrite			
Stage 7	33.0 ± 20 ‰	4	6
Stages 10–12	35.0 ± 23 ‰	7	9
Mean	34.0 ± 21.5 ‰	–	–
Balanus eburneus			
Stages 1–9	36 ± 16 ‰	12	18
Stages 10–12	32.5 ± 21.5 ‰	6	6
Mean	34.2 ± 18.7 ‰	–	–

Discussion

All three species inhabit estuarine areas. *Balanus amphitrite* occupies the more saline outer banks, and occurs at higher levels of the intertidal zone than *B. eburneus*. *Chelonobia patula* is to be found on crabs well up the estuaries as well as in fully saline conditions, but the crabs remain immersed for most of the time. *Balanus amphitrite* is therefore likely to be exposed to the most severe conditions of desiccation, and *C. patula* to the least. These facts are consistent with the order of increasing tolerance recorded in Tables I and III, and with the corresponding differences noted between the larvae. All these differences are however, quite small and they should not obscure the fundamental similarity in behaviour of all three species, both as embryos and as larvae. As embryos all three species continued to develop up to 50 ‰ beyond which point development became increasingly delayed, though the eggs did not cytolyse or appear markedly abnormal. At and below 10 ‰ however there was usually immediate damage to the tissues. Similarly the hatched larvae became motionless at high and low salinities of the same order, but recovered more readily after prolonged immersion in high salinity than in low.

The egg membrane, or shell, must be freely permeable both to water and to substances of low molecular weight. Neither the eggs nor the hatched larvae showed any permanent change in size after being transferred to salinities of

osmotic pressure far removed from the normal, and early stage egg masses did not burst in the manner described by BARNES (1955) for the eggs of *B. balanoides* containing late embryos. The absence of any volume change indicates that the egg membrane must be either very strong and inelastic, or impermeable to water, or freely permeable to salt. The mature embryos can readily break the egg shell. According to BARNES (1955) this is achieved purely mechanically, but careful measurements carried out by one of us (D.J.C.) on the same species show that the eggs and embryos swell by about 30 % of their volume during this process. In either event, the egg cases are clearly neither very strong nor very inelastic. The egg cases are also known to be permeable to simple organic molecules, as witnessed by the rapid action of hatching extract (CRISP 1956) and of anaesthetics (CRISP & SPENCER 1959). It is inconceivable therefore that the egg membrane should be at the same time impermeable to water. It must therefore be freely permeable to salts. Consequently the embryos must be exposed in a very short time to the same salinity as that present outside the egg mass.

Since the eggs show a wide tolerance of salinity, even at cleavage, and long before organogenesis has occurred, it is unlikely that any mechanism is present to regulate the osmotic pressure of the embryonic tissues. Any regulation must be performed at the cellular level. Moreover the absence of permanent shrinkage or swelling of the embryonic tissues even at the most extreme salinities strongly supports the view that free diffusion of electrolytes occurs between them and the surrounding sea water. A wide range of cellular indifference seems far more likely than any regulatory mechanism to explain the salinity tolerance observed. The experiments on the larvae hatched from *B. amphitrite* embryos developed at different salinities showed that some degree of adaptation to salinity change might be possible during development and might help to account for the remarkably wide range of habitat successfully colonised by this species.

Summary

Egg masses of three warm water barnacles, *Balanus eburneus* Gould, *B. amphitrite amphitrite* Darwin and *Chelonobia patula* Ranzani were allowed to develop *in vitro* at a series of different temperatures and salinities. At salinities between 25 ‰ and 40 ‰ development took place at the normal rate. When exposed to salinities between 15 ‰ and 25 ‰ or between 40 ‰ and 60 ‰ only a proportion of the eggs hatched, and development was delayed. Salinity tolerance was not influenced appreciably by temperature within the range normally encountered by the animals.

The salinity tolerance of the first and second stage nauplii was similar to that of the embryos; however the actual limits were to some extent dependent

on the salinity to which the eggs had been exposed during development. The hatched nauplii were identical in size whatever the salinity at which they developed.

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THE SEXUAL BEHAVIOUR OF *HYMENOCHIRUS BOETTGERI*

BY

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In a previous paper (OLSSON and ÖSTERDAHL 1960) we have given a short repo of the breeding habits of the West African clawed toad *Hymenochirus boettge* (Pipidae). Since that time 15 new spawnings have been analyzed in which different males and 15 different females have taken part.

For breeding aquaria plastic containers measuring 55 × 35 × 30 cm we used, usually with the water level at 20 cm. The spawning act was induced l placing the container in a sunny position which usually caused the toads breed within a few days. The water temperature was kept at 20–25°C and t pH of the water was between 7 and 7.5.

The sexual behaviour of *Hymenochirus* can be described in three phas which we will call here "the territory behaviour of the male", "the clasping and "the oviposition".

The Territory behaviour of the Male

At nightfall the sexually activated male choses a territory at the bottom the aquarium in its darkest part. The size of the territory usually measur about 1–4 square decimeters. It could be noted that this territory was gradua freed from old leaves and other bottom deposits. This, however, was not t result of active cleaning efforts on the part of the male, but rather an effect his lively movements within the restricted area of his territory.

Sound production

An important part of the territory behaviour of the male toad is the prod tion of a sound. This sound is always produced whilst the toad is totally su merged and during this process it usually takes up a very characteristic positi with straight fore limbs, in its territory (Fig. 1 A and 2). The sound is no typical anuran croaking; we find that it more closely resembles the sou