THE EFFECTS OF HYPERSONALINE UPON THE EGGS AND PROLARVAE
OF THE GULF OF CALIFORNIA GRUNION, LEURESTHES
SARDINA (JENKINS AND EVERMANN 1888)

by

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STATEMENT BY AUTHOR

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ABSTRACT

The Gulf of California grunion, *Leuresthes sardina* (Jenkins and Evermann 1888), eggs were artificially fertilized and incubated in nine salinities (35 o/oo to 85 o/oo) to determine the effect of hypersalinity on development and hatching success. Both fertilization and hatching success decreased significantly with increases in salinity -- mean percentages of eggs hatched from laboratory experiments ranged from 61.1% at 35 o/oo to 10.0% at 60 o/oo; none hatched in salinities from 66 o/oo to 85 o/oo. Field experiments corroborated the results of the laboratory experiments.

Eggs incubated in the laboratory (18.5°C) completed development in nine days, whereas eggs in beach sand (20.4 to 33.8°C) developed in seven days. The optimum moisture content for developing embryos in the field was 3.0 to 4.5%.

Heavy infestations of parasitic dipteran larvae and adults (*Fucellia rejecta*) were found in grunion pods spawned near bird resting sites, with mortalities ranging from 10 to 90%.

Metabolic rate increased with increasing salinity. Embryos at 60 o/oo had heart beats of 203/minute compared to 140/minute for the control group. Although Gulf grunion embryos were able to acclimate to hypersaline conditions (to 60 o/oo), the viability of hatched prolarvae was impaired at salinities of 45 o/oo and greater, due to excessive yolk absorption prior to hatching.
INTRODUCTION

Organisms living in the upper intertidal zone are exposed to great fluctuations and extremes in temperature, salinity, solar radiation, wave action, and currents. Kinne (1971) states that temperature and salinity are the two most influential physical properties affecting marine biota. These variables produce a profound effect on the vertical distribution of organisms. Animals near the high tide mark tend to be more resistant to temperature and salinity fluctuations than those found near the low tide mark (Kinne, 1963; Copeland, 1967; Vernberg and Vernberg, 1972).

In recent years, the arid coastal beaches and esteros of the northern Gulf of California are becoming hypersaline habitats due to the decreased flow of the Colorado River (Thomson, Mead, and Schreiber, 1969). These beaches are the principal spawning grounds of the Gulf grunion _Leuresthes sardina_ (Jenkins and Evermann 1888). Grunion juveniles and adults thrive in an environment having extreme temperatures (8-35°C = viable range) and salinities (37 to 60 o/oo). From January to May, the Gulf grunion swim onto the beach on descending high spring tides to spawn in the semi-fluid sand. As each female digs into the sand, several males wrap themselves around the erect female, aiding extrusion of eggs which is accompanied by the males' simultaneous release of milt, and achieving fertilization of the eggs (Fig. 1). Then, all participants return to the sea on the ebb of the succeeding wave (Thomson and Muench,
Fig. 1 Erect female Gulf of California grunion, *Leuresthes sardina* (Jenkins and Evermann 1888), surrounded by several males during spawning activities on April 24, 1970, at Punta Machorro, El Golfo de Santa Clara, Sonora, Mexico.
in press). The eggs incubate in the beach from seven to nine days, depending upon the temperatures. The eggs are then washed from the beach and stimulated to hatch by the turbulence of the waves of the next spring tide series.

Over the past ten years, the United States and Mexico have been discussing the feasibility of constructing a nuclear desalination plant in the vicinity of El Golfo de Santa Clara, Sonora, Mexico. The facility would produce an effluent of approximately $945.6 \times 10^6$ gal/day at a temperature of 39.2°C and a salinity of 48.5 to 70 o/oo (Thomson et al., 1969). This heated hypersaline effluent could drastically affect the reproductive cycles of many inshore fishes, including the grunion. During early embryonic development, grunion eggs and prolarvae must tolerate widely fluctuating environmental conditions; a further increase in these fluctuations could exceed their tolerance limits.

The purpose of this investigation is to determine the effects of increasing salinities on developing grunion eggs. The criteria that will be used for determining salinity effects are fertilization success, rate of embryonic development, cardiac response, abnormalities, and hatching success.
MATERIALS AND METHODS

This investigation was conducted under both controlled laboratory conditions and under natural field conditions in the vicinity of El Golfo de Santa Clara, Sonora, Mexico. Laboratory experiments spanned three years (1969-1971). Field experiments were conducted during the spring of 1970 at Punta Machorro, 3 miles southeast of El Golfo de Santa Clara.

Laboratory Experiments

Grunion eggs for the laboratory experiments were collected from eight spawning runs: four in 1969, three in 1970, and one in 1971. Adult grunion were captured with a 50 x 4 ft bag seine having 1/4 in mesh. The sexual products of both males and females were stripped simultaneously by applying slight pressure to the posterior ventral side of the abdomen near the anal fin, as described by Strawn and Hubbs (1956).

The sexual products of 10 males and 10 females were mixed in a 1-liter glass jar containing 200 ml of synthetic sea water (Instant Ocean) of the desired salinity. Nine different salinities were prepared. The control solution had a salinity of 35 o/oo, while the eight experimental solutions were prepared at salinities of 40, 45, 50, 55, 60, 66, 72, and 85 o/oo. A portable refractometer (American Optical, model No. 10419) was used to monitor the salinities of the prepared solutions, while a Beckman Zeromatic II pH meter was used to measure the pH of the solutions, which were maintained at a constant pH of 8.1.
After the gametes of both males and females were deposited in the 1-liter jars, they were gently but thoroughly agitated and then placed in a dark, cool (18°C) Coleman cooler for at least 30 minutes to obtain the maximum possible fertilization rate. When the 30 minutes had elapsed, the excess milt was carefully decanted from the eggs. This was done in the shade of a tent, with care not to expose the eggs to damaging solar ultraviolet radiation (McHugh, 1954). To reduce contamination, the eggs were then rinsed at least four times with clean synthetic sea water of the same salinity used during fertilization.

The cleansed eggs were placed in plastic incubation trays (28 cm wide x 35 cm long x 15 cm deep). Each tray was fitted with a snap-on plastic cover to minimize evaporation. A plastic aquarium subsurface filter was placed on the bottom of the tray to keep the eggs out of the water. The aquarium filter was 30 cm long x 25 cm wide x 2 cm high. The filter was covered with several layers of paper towels which were dampened with the prepared sea water solution. The paper towels stayed moist throughout the incubation period by capillary action. The eggs were arranged in three layers, each layer separated by several thicknesses of paper towels (see Fig. 2). The incubation trays were then stored in dark, cool (18°C) Coleman coolers for transportation back to the laboratory.

Upon arrival at the laboratory, the nine trays of eggs were immediately placed in an incubator (G.E., model 805), and maintained at a constant 18.5 ± 0.1°C, which corresponded approximately to the mean sea surface temperature during the spring grunion runs.
Fig. 2 Incubation tray containing three layers of developing grunion eggs.
Temperature, salinity, and pH of the water in each tray were regularly monitored from the instant of fertilization to hatching. Moisture content and salinity were kept constant by the addition of proper amounts of distilled water. Random samples of eggs were collected from each tray and were preserved in 10% formalin solution for observation at a later date.

Each tray of embryos was observed twice and sampled once daily to record their rate of development. Embryonic heart rates and total wet weights were also recorded. A random sample of eggs from each of the nine incubation trays was counted with an eight-key laboratory counter and categorized into one of fourteen developmental stages (see section on Embryology). The percentages of the quantity of eggs in each category were compared with the overall total number of eggs sampled at that time from each of the nine different salinities. Comparisons of the growth rates, and the types and numbers of abnormalities occurring at each developmental stage, in the different salinities were also recorded.

**Field Experiment**

Throughout the grunion spawning season, air, water, and beach sand temperatures were monitored along with sea water salinity and moisture content of the beach. After each spawning run, egg pods were removed from the sand and preserved with a 10% formalin solution. The temperature, depth, and dimensions of each pod were also recorded.

A field experiment (April 24, 1970) was devised to check the effects of natural environmental conditions upon the artificially fertilized grunion eggs and to compare this information with that obtained
from the three laboratory experiments. The sexual products from both adult females and males were collected and artificially fertilized in the desired salinities, as described previously. Instead of placing the fertilized eggs in incubation trays, they were buried in the sandy beach at Punta Machorro during early evening (Fig. 3). Small trenches (30 cm long x 10 cm wide x 5-8 cm deep) were excavated in the beach within the boundaries of the grunion run of that day. Care was taken to excavate the trenches in areas void of any naturally fertilized pods. The depth of the trenches corresponded to the mean maximum depth (8 cm) and the mean minimum depth (5 cm) of natural egg pods. Artificially fertilized eggs were poured in thin layers in each trench and covered with a thin layer of damp sand removed from that same level. Several layers were poured into the trench and covered with sand until a 3-cm thickness of eggs and sand was achieved. Then, all the eggs were covered with sand containing the proper moisture content. Temperatures of the sand at the pod level and at the surface were recorded. A wooden stake marked each trench. Eggs fertilized in seven test salinities (35, 40, 45, 50, 55, 60, and 66 o/oo) were incubated in seven separate trenches. Figure 4 illustrates the location of the experimental trenches in relationship to the natural pods and the high water marks on the beach.

A stake was placed on the lower edge of the grunion spawning area to measure the amount of sand being transported to or from the beach during the incubation period. The stake was calibrated in 0.5 cm increments.
Fig. 3 Gulf grunion spawning within the boundaries of the experimental area at Punta Machorro where artificially fertilized eggs were buried and incubated for seven days.
Fig. 4 Stakes marking experimental egg pods at Punta Machorro. Stakes in lower left and upper left corner mark the high tide of the day of the run. The photograph was taken five days after the run.
Throughout the field experiment, naturally fertilized grunion egg pods were dug from the beach, their depth and width measured, and their temperatures recorded. Moisture content samples were collected and analyzed using wet sand weight versus dry sand weight to determine percent of moisture. Each day (during morning, afternoon, and evening hours) the temperatures of the air, water, and sand were monitored in the artificial pods or trenches. The artificial pods were sampled only during the evening to eliminate the possible harmful effects of ultraviolet radiation. After a random sample of artificially fertilized eggs were procured from the trench, the trench was carefully covered with sand of the same moisture content as that of the level of the eggs. This procedure was performed nightly for each salinity within the study site. Also, at approximately the same time each night, natural pods were excavated from the beach at approximately the same level as that of the test site and compared morphologically to the artificially fertilized eggs. Each random sample of eggs was observed with a dissecting microscope to record the developmental stage, possible abnormalities, and embryonic heart rates before preservation in 10% formalin.
RESULTS

The data represent an extensive survey and collation of individual observations of more than 135,000 grunion eggs and embryos during the entire project.

Embryology

The embryology of the Gulf grunion, *Leuresthes sardina*, has not been studied, although David (1939) described the development of the congeneric California grunion, *L. tenuis* (Ayres). Developmental stages for *L. sardina* were based on both *L. tenuis* stages and teleostean embryology according to Oppenheimer (1937), Bolin (1936), Joseph, Massmann, and Norcross (1964), Miller (1952), Lasker and Tenaza (1968), Budd (1940), Clark (1938), McMynn and Hoar (1953), Lasker (1965), Berrill (1971), Mansueti and Hardy (1967), and Costello et al. (1957). Fourteen stages of *L. sardina* development were recognized. The name of each stage, its abbreviation, approximate hours of development (based upon incubation at 18.5°C), and a brief description are as follows:

Stage 1. Unfertilized egg (U.F.; 0 hr). The color of the eggs varied from a pale yellow for immature eggs to a bright reddish orange for mature, ripe eggs. The eggs were 1.1 to 1.4 mm in diameter and weighed from 1.2 to 2.2 mg, with a mean weight of 1.8 ± 0.27 mg (100 eggs). The egg's outer wall, the chorion, was very flexible and complete except for a tiny dimple-like aperture, the micropyle.
The vitelline membrane lining the inner surface of the chorion formed the outer perimeter of the perivitelline space which is formed by the bulk of the egg, the yolk, which is enclosed by the plasma membrane. There were numerous (50-85) oil globules within the yolk and each globule had a diameter less than 100 μ. This complex of oil globules at the top of the yolk sac constituted a mass that was approximately 0.1 of the total volume of the yolk sac.

Stage 2. Four to eight cells (4-8; 4 to 8 hr). This stage occurred 4 to 8 hr after fertilization. The micropyle was closed and the vitelline membrane became the fertilization membrane. The egg became turgid upon completion of the water hardening process. The blastomere, a small spherical mass of cells, was located in the upper portion of the egg surrounded by the oil globules.

Stage 3. Early blastula (E.B.; 20 to 28 hr). In the early blastula stage, meroblastic cleavage occurred, thus spreading out the mass of blastomeres into the blastodisc, a semi-flat disc atop the yolk sac in the zygote. This usually occurred after 25 hours of development in 70 to 80% of the embryos observed. Oil globules could still be seen in the upper portion of the yolk sac.

Stage 4. Middle blastula (M.B.; 23 to 40 hr). The middle blastula stage represented both an enlarging and flattening process
of the blastodisc, which now encompassed nearly half of
the yolk sac. This usually occurred after 30 hr of
development, but was often observed after 23 hr.

Stage 5. Late blastula (L.B.; 26 to 50 hr). At this stage the
flattened blastula had nearly completely engulfed the yolk
sac, leaving approximately one-third of the total surface
area of the zygote exposed.

Stage 6. Early gastrula (E.G.; 40 to 60 hr). The cells of the
blastula became increasingly more dense at a central loca-
tion, producing the germ ring and henceforth the embryonic
shield.

Stage 7. Late gastrula (L.G.; 60 to 75 hr). This stage represented
the completed formation of the neural keel and the con-
tinual narrowing of the embryonic shield.

Stage 8. First somite (F.S.; 70 to 90 hr). Formation of the rudiments
of the central nervous system and the presence of
the first somite represented this developmental stage.

Stage 9. Extra-embryonic coelom formation (E.E.C.; 60 to 102 hr).
At this stage the numbers of somites had increased, and the
auditory placodes had begun to form adjacent to the forma-
tion of the forebrain. The optic vesicles were faintly
visible and the yolk sac epithelium produced the extra-
embryonic coelom. The posterior portion of the tail was
separated from the yolk sac. Sixty-five to seventy-five
percent of the embryos observed at this stage were recorded near the 85-hr mark of embryological development.

Stage 10. **Lens of eye formation (L.E.F.; 76 to 126 hr).** In this stage the auditory placodes were well-formed and easily discernible. The ectoderm thickened to form the lens of the eye, which appeared to be light brown in color. The olfactory pit was also developed and this anatomical structure usually appeared after approximately 110 hr.

Stage 11. **Mesencephalon formation (MES.; 115 to 140 hr).** The mesencephalon expanded to form the optic lobes. The lens of the eye became dark brown in color and more pronounced. The pericardium was established with the pulsating action of yellowish orange blood being circulated through the vessels of the yolk sac. The embryo was active, with periodic flexing and twisting of the entire torso within the chorion. These movements were most often observed after 126 hr of development.

Stage 12. **Pectoral fin formation (P.F.: 100 to 185 hr).** At this stage, which occurred after 150 hr of development, distinct concentration of cells in the location of the pectoral fin area was observed. The pericardium became well-developed with heart beats and the rhythmic pulsation of the blood. The eyes of the prolarvae were nearly black in color. The abdominal cavity contained green and orange pigments.
Stage 13. Melanophore formation (MEL.; 148 to 220 hr). The melanophores first appeared as small, round, light-brown spots just posterior to the pectoral fin on each side of the prolarvae. A pair of melanophores developed directly atop of the brain and lay in a centrally located straight line. On the head there were two rows of melanophores containing three or four each. On the body portion there were two rows of four or five each, and the number of melanophores found in the tail region varied from 12 to 21. This description of the locations and quantities on the prolarvae grunion were obtained after observing a random sample of 100 eggs.

The myomeres of the tail were well-developed and easily observed. The pectoral fins were pointed at their extremities and contained the individual ray elements. The pigment of the eyes was black and the lenses were well-developed. The jaw was developed but was incomplete. The gut appeared to be complete, including the formation of the anus. The dorsal, caudal, and ventral fins were developed and contained their fin rays. The yolk sac was greatly reduced and there existed only one large oil globule. The prolarvae were much more motile than in previous stages. Activities included violent movements of rotating and tail thrashing upon the slightest provocation from an external stimulus.
Stage 14. Hatching (HAT.; 210 to 230 hr). The appearance of the larvae was the same as found in the previously described melanophore formation stage. However, the prolarvae escaped from the egg case upon the slightest agitation with a stream of air bubbles from a laboratory pipette, and the freshly hatched grunion collided with each other in a chain reaction. The prolarvae weighed $1.08 \pm 0.16 \text{ mg}$ and their snout-peduncle length was $3.84 \pm 0.11 \text{ mm}$ (a mean of 100 larvae).

Laboratory Experimentation

Laboratory experiments performed in the spring of 1969 showed successful fertilization in 35, 40, 45, 50, 55, and 60 o/oo solutions at ambient air temperatures between 25.0 and 39.5°C. This preliminary experiment showed that with increasing salinity the fertilization and hatching rates decreased.

Hatch data obtained for Lab Experiment #1-3/11/70 (Fig. 5) confirmed the trend initially observed in 1969. As salinities increased, the hatching rates decreased with 60 o/oo having only a 17.5% hatch, whereas 77.0% of the embryos hatched in the control (35.0 o/oo). At 55.0 o/oo, 4.1% hatched, less than at 45.0 o/oo, but 9.8% greater than eggs incubated in 50.0 o/oo. The largest percentage of embryos with arrested development at an early stage were those in high salinities (66, 72, and 85 o/oo). At 85 o/oo the greatest advancement in development was to the 4-8 cell formation, while those incubated at 72 o/oo progressed to the middle blastula stage. Those incubated at 66 o/oo exhibited growth to
Fig. 5 Lab Experiment No. 1-3/11/70 exhibiting the hatching successes and failures of grunion embryos at nine different salinities on the ninth day of incubation.

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<tr>
<th>Abbreviation</th>
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<tr>
<td>U.F.</td>
<td>Unfertilized</td>
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<td>4-8</td>
<td>4-8 Cell Formation</td>
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<td>E.B.</td>
<td>Early Blastula</td>
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<tr>
<td>M.B.</td>
<td>Middle Blastula</td>
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<tr>
<td>L.B.</td>
<td>Late Blastula</td>
</tr>
<tr>
<td>E.G.</td>
<td>Early Gastrula</td>
</tr>
<tr>
<td>L.G.</td>
<td>Late Gastrula</td>
</tr>
<tr>
<td>F.S.</td>
<td>First Somite Formation</td>
</tr>
<tr>
<td>E.E.C.</td>
<td>Extra Embryonic Coelom Formation</td>
</tr>
<tr>
<td>L.E.F.</td>
<td>Lens of Eye Formation</td>
</tr>
<tr>
<td>MES.</td>
<td>Mesencephalon Formation</td>
</tr>
<tr>
<td>P.F.</td>
<td>Pectoral Fin Formation</td>
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<td>MEL.</td>
<td>Melanophore Formation</td>
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<td>HAT.</td>
<td>Hatching</td>
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Fig. 5 Lab Experiment No. 1-3/11/70 exhibiting the hatching successes and failures of grunion embryos at nine different salinities on the ninth day of incubation. — The dotted lines represent embryos with arrested development at a specific stage. Each of the 5,650 fish were placed in one of the 14 developmental stages.
melanophore formation but 95% of the embryos did not progress beyond the late blastula stage. The highest rate (18%) of unfertilized eggs occurred at 85 o/oo and decreased to less than 1% at 35 o/oo.

Hatching data for the Lab #2-4/9/70 grunion run (Fig. 6) represented a total sample of 41,458 eggs. The results from this experiment were similar to those obtained in the first experiment, but the overall percentage of hatching was diminished. It was felt that the lower percentages were due to the possible increased numbers of pale yellow, immature eggs stripped from the females. The eggs hatched after 9 days of incubation at 18.5°C. Once again, the largest percentage (42.5%) of hatched eggs was at 35 o/oo. The percentage decreased to 28.4% for the 40 o/oo eggs, then diminished further to 7.7% at 45 o/oo, with the smallest hatching percentage of 1.0% at 60 o/oo. The trend persisted, with 55 o/oo having nearly the hatching success of 40 o/oo with 29.0% hatched. Those eggs incubated at 66, 72, and 85 o/oo did not develop beyond the early gastrula stage. The maximum rate of unfertilized eggs appeared at 85 o/oo with greater than 20%, and the least amount appearing in the control.

The third laboratory experiment of 5/9/70 represented a total sample of 26,396 grunion eggs (Fig. 7). This experiment was identical to the previous two in every respect and the hatch data reflected the same trend. Hatching percentages decreased as salinities increased. Embryos incubated at 55 o/oo had a hatching success slightly less than those incubated and hatched at 45 o/oo. Again, the incubation time was nine days. In this experiment, the percentages were higher than the 4/9/70
Fig. 6 Lab Experiment No. 2-4/9/70 grunion run exhibiting the hatch data of prolarvae at nine different salinities on the ninth day of incubation. Each of the 8,500 larvae were staged in one of the 14 developmental stages. See Fig. 5 for legend.
EMBRYOS AT EACH STAGE (%)

DEVELOPMENTAL STAGES

Fig. 7 Lab Experiment No. 3-5/9/70 grunion run exhibiting the hatching data of embryos at nine different salinities on the ninth day of incubation. Each of the 7,200 larvae were staged in one of the 14 developmental stages. See Fig. 5 for legend.
run and the eggs were believed to be much more mature since they appeared blood-red in color. In this run, there were 64.9% hatched in the control, 56.2% hatched at 40 o/oo, and once again the embryos in the group 55 o/oo persisted with 44.0% hatching. Next trailed the 45 o/oo group with 38.8% hatching, 50 o/oo produced 36.5%, and the largest decrease in hatching occurred at 60 o/oo with 12.7%. All six salinities (35 o/oo to 60 o/oo) had embryos arrested at the melanophore formation stage on the day of hatching. The embryos fertilized and incubated at 66 o/oo and 72 o/oo exhibited increased mortality rates in the early blastula stage, while embryos in the 85 o/oo solution had 75.4% mortality at the 4-8 cell formation stage. The rate of fertilization was similar to the previous data with the highest rate occurring in the control and the least in the 85 o/oo salinity. In Lab #3, the maximum and minimum rates of unfertilized eggs were obtained, with 85 o/oo having 24.6% and the control with less than 0.1%.

In summary, a trend of increased salinities during the incubation period produced a significant reduction of hatched viable grunion planktonic larvae in all three laboratory experiments. Fertilization rates were the highest in the control group (35 o/oo) and the lowest in the 85 o/oo group. The percentage of deformed embryos increased with an increase in salinity.

**Field Experimental Results**

The purpose of the field experiment was to determine the effects of environmental conditions upon grunion eggs artificially fertilized and incubated at the same high salinities used in the laboratory.
On the days of Gulf grunion runs, after spawning activities had ceased, the beach temperatures (water saturated) recorded at the level of the egg pods ranged from 11.0 to 33.5°C (Fig. 8). Throughout the incubation period, the pod level temperatures reached levels of 20.4 to 33.8°C for those eggs located 2.5 cm below the surface, and 20.5 to 25.9°C for those located at a 10-cm depth (Fig. 9). Once again these temperatures lie within the temporal extremes recorded during the early hours of March 8 and the mid-afternoon of May 1, 1970. The beach temperatures were found to fluctuate greatly depending upon the time of day and the weather conditions during recording. It was observed that the moisture content of the beach helped to maintain a stabilized temperature regime within the vertical sand column. The moisture content of the beach was measured on 4/29/70, the fifth day of incubation, and it was observed that the eggs were developing normally in a moisture content between 3 and 5% (Fig. 10). Also, the temperature of the beach strata on this day showed that the greatest range of temperature existed nearest the surface, where the moisture content was the least. The temperatures in the drier sand near the surface fluctuated greatly due to warming by solar radiation and cooling due to evaporation. At depths below 10 cm, the moisture content gradually approached 60%, while the temperature remained constant. This trend was demonstrated in Fig. 8 during the days of grunion spawning in which the moisture content approached saturation levels and the vertical beach temperatures were nearly constant except near the surface where warming or cooling took place.
Fig. 8 Air and beach temperatures recorded on days of grunion runs after the spawning activities had ceased.
Fig. 9 Air and beach temperatures recorded during three incubation periods of Gulf grunion embryos.
Fig. 10  Moisture content of the beach strata in the experimental area recorded during the 4/29/70 field experiment. -- These moisture contents occurred during the 7-day incubation period.
The salinity of the beach was measured and was found to be 37 o/oo at the surface and approximately 40 o/oo at 50 cm on the day of the runs. Attempts were made to measure the salinities at various levels throughout the vertical beach strata but the techniques employed were futile.

In the field experiment of April 24, 1970 (Fig. 11), it was observed that the artificially inseminated grunion eggs developed more rapidly in fluctuating temperatures as opposed to those incubated at a constant 18.5°C. The total elapsed time of incubation was reduced from 9 days to 7 days. Since the previous lab experiments indicated that those eggs fertilized in 72 and 85 o/oo possessed 100% mortality rates at 18.5°C, it was decided to delete those from the field experiment where higher daily temperatures already existed. The 66 o/oo test salinity was retained since approximately 5% of the embryos reached the melanophore formation stage on the ninth day of incubation (Fig. 5).

Simultaneously, the hatching data from the artificially fertilized eggs paralleled the hatching data obtained from sampling 500 eggs from each of 25 randomly sampled naturally spawned grunion pods extracted from the beach. The total number of eggs surveyed in the grunion pods was 12,500 as compared to 29,665 artificially fertilized eggs in the experimental trenches.

The hatching results indicated that the trend as previously determined in the laboratory did exist under natural conditions but with some slight deviations. At 35 o/oo, maximum hatch of prolarvae was obtained with a 92.1% survival, but at 40 o/oo the live larvae hatched
Fig. 11  Hatching success and failures of grunion prolarvae incubated for seven days in the field (4/24/70 run).  -- Artificially fertilized eggs were incubated at seven different salinities.  Fucellia rejecta, a parasitic fly, destroyed all the eggs in the 50 o/oo trench.  See Fig. 5 for legend.
fell to 39.5%. In third place again, the 55 o/oo group persisted with a 30.6% hatch and 60 o/oo produced 5.9%, while 45 o/oo produced a low 4.4% hatch. The eggs fertilized in 66 o/oo produced embryos at various stages of development, including approximately 6% at the melanophore formation stage, but there were no embryos hatched on the seventh day of incubation. The salinities of 45 and 66 o/oo reflected the highest mortality rates at the early blastula and middle blastula stages. The 60 o/oo group had its highest mortality rate at the lens of eye formation through the melanophore formation stages.

In contrast to those eggs naturally fertilized, the hatch reached a maximum of 97.8% in pod 6 as compared to a minimum of 3.1% in pod 21. The mean of 25 naturally spawned pods was 81.5% hatched.

The fertilization rate for the artificially fertilized eggs reached nearly 100% for 35, 40, 45, and 55 o/oo, while those in the 60 and 66 o/oo group had 93.5 and 89.0% fertilized, respectively. Those eggs fertilized naturally by male grunion achieved nearly 99.0% success.

In the hatching results (Fig. 11), there were no embryos produced on the seventh day of incubation in the 50 o/oo trench due to the presence of parasitic dipteran larvae (Fucellia rejecta) which fed upon the grunion eggs. This peculiar phenomenon was first recorded in 1918 along the shores of southern California (Malloch, 1923) and now this same species of fly was observed devouring the young Gulf grunion embryos on the third day of incubation. By the fifth day, only egg cases remained. Upon the discovery of this phenomenon, other egg pods were excavated to see if this condition occurred elsewhere. It was found that in one m² more than
60% of the pods were infested with *Fucellia rejecta* larvae or adults. A total of 10 m\(^2\) was excavated at various levels and locations on Punta Machorro and the infestation ranged from approximately 10% to as high as 90% of the pods with a mean near 60%. It was also noted that the areas of highest infestation occurred close to resting sites of bird populations on Punta Machorro.

**Comparison of Laboratory vs. Field Results**

The purpose of this section is to compare the results of hypersaline conditions upon the success of fertilization (artificial and natural), abnormalities, heart rates, hatching success, and prolarvae viability.

The rate of unfertilized eggs (Fig. 12) increased from less than 1.0% at 35 o/oo to about 1.5% for 60 o/oo, with a gradual increase to 21.0% at 85 o/oo for the laboratory experiments. Those eggs that were found unfertilized in the field experiment were less than 1.0% in the 35 o/oo control group and nearly 8.8% in the 66 o/oo group. Approximately 1.2% of the eggs were unfertilized among the 12,500 eggs naturally fertilized. Thus, adequate fertilization rates in the different test salinities were achieved.

Another interesting correlation was observed concerning the various types and rates of abnormalities occurring at the different salinities (Fig. 13). There were four major abnormal conditions observed in all salinities, both in the laboratory and under natural field conditions. In nearly all cases the abnormalities occurred before the lens of eye formation in the embryo and only in rare cases were there abnormalities appearing after this stage. A few embryos exhibited abnormal
Fig. 12 The effect of salinity on the percent of unfertilized grunion eggs observed under both laboratory and field conditions.
Fig. 13 The effect of eight different salinities on the percent of abnormal embryos produced under laboratory and field conditions.
cleavage of the blastomere in the 4 to 8 cell formation stage. Instead of the cell dividing normally to form a compact sphere of cells, they produced a single strand numbering up to 8 cells. The most common abnormalities consisted of embryos being normally developed but either being totally blind or having one eye. Some embryos would have a cyclops appearance with a centrally located eye, while others would have the singular eye located on either side of the head. Other abnormal embryos exhibited well-developed bodies both externally and internally, except that their tails were deformed or bent severely. In many instances, the embryos had no tails but only a rudimentary stump just posterior to the anus. There were no live abnormal embryos hatched in any of the laboratory or field experiments.

With an increase in salinity, the rate of abnormalities increased with the field experiment exhibiting a greater percentage at all salinities other than the control where it was less than 0.1%. At 40, 45, and 55 o/oo, a typical upward trend was established with 0.9, 1.5, and 2.2%, respectively. There were no abnormalities observed alive at 50 o/oo due to the insect infestation resulting in total destruction of the eggs. The sharpest increase in abnormalities occurred at 60 and 66 o/oo with 8.7 and 9.7%, respectively. In comparison with the laboratory incubated eggs and those eggs spawned naturally the rate of abnormalities ranged from less than 0.1 to 1.0%, respectively. Only in the 66 o/oo group of the laboratory experiments did abnormalities exceed 1.0% (1.6%).

On the sixth day of development, the embryos' heart rate was monitored (Fig. 14). In the laboratory experiments, the embryos
Fig. 14 The response of heart rate of increasing salinities in developing grunion. — Embryos (n = 50 per each salinity) under laboratory and field conditions and (n = 25) per each pod, were measured in the seven salinities. Measurements were recorded on the sixth day of embryonic development.
(n = 50/salinity) produced an increase in heart beat rate with an increase in salinity. Embryos in the control group had a rate of 140 beats/minute, 45 o/oo exhibited 158 beats/minute, and at 60 o/oo the embryos' hearts were pulsating at 189 beats/minute. Under field conditions, the embryos were apparently under stress due to a significant increase of heart rate at each higher salinity. The trend persisted with the control group having a mean of 147 beats/minute; at 45 o/oo the embryos' heart beat rate (158 beats/minute) was slightly less than the 40 o/oo group (162 beats/minute) but significantly more than the embryos in the control and those naturally spawned embryos at 37 o/oo (145 beats/minute). At 60 o/oo, a rate of 203 beats/minute was exhibited by the grunion embryos. Unfortunately, at 50 o/oo there were no live embryos to observe in the field experiment due to the complete infestation of the eggs by dipteran larvae.

Figure 15 illustrates the percent of embryos hatched live at each of the eight salinities tested. This illustration is a composite of all hatching data obtained on the first day of hatch. The mean of the three laboratory experiments encompassed the mean percentages of the hatched prolarvae after nine days. The field experiment and the mean of 25 naturally fertilized pods were incubated and hatched after seven days. The comparison reflected the previous established trend of increasing salinities decreasing the percentage of hatched prolarvae grunion. The trend was consistent except for those eggs fertilized and incubated in 55 o/oo. The percentage of laboratory hatched larvae ranged from 61.1% at 35 o/oo, 47.4% at 40 o/oo, 41.4% at 55 o/oo, 33.9% at 45 o/oo, 26.8%
Fig. 15 A comparison of hatching successes for artificially fertilized and naturally spawned grunion eggs, both in the laboratory and the field.
at 50 o/oo, 10.4% at 60 o/oo, and 0.0% at 66 o/oo. Eggs incubated in 72 and 85 o/oo produced no advanced embryos, and were excluded from this graph.

Under field conditions, the hatching percentages for the 35 o/oo group exceeded that of the laboratory experiments, but at other salinities the greatest decrease in the laboratory experiment occurred at 45 o/oo. There were no larvae hatched at 66 o/oo nor 50 o/oo where the dipteran infestation occurred. The mean of the 25 pods produced 81.5% live grunion larvae at 37 o/oo. At 37 o/oo, the hatch was significantly greater than the control at 35 o/oo in the laboratory but less than the control group in the field.

The next two graphs (Figs. 16 and 17) represent the maximum percentages of live prolarvae hatched from the beach. On the first day past the hatch date (Fig. 16), the probable hatch percentages were derived by combining those embryos remaining at the melanophore formation stage on the day of hatch with those that actually hatched. The trend from Fig. 15 was maintained with increases of hatch at all salinities including 66 o/oo. Embryos that developed in warmer fluctuating temperatures at salinities above 55 o/oo seemed to surpass those incubated at a constant 18.5°C. The percent of naturally spawned larvae approached the 90% hatch.

The second day past the day of hatch (as in Fig. 17), the trend continued similarly to that seen in Fig. 16 with hatching percentages of the field experiment reaching 97.0% for the control, 84.0% for 55 o/oo, 57.5% for 60 o/oo, and 8.0% for 66 o/oo. A similar trend persisted with
Fig. 16 The maximum probable hatch for *L. sardina* embryos occurring one day beyond the hatch date for eight different salinities.
Fig. 17 The maximum probable hatch of grunion embryos occurring two days beyond the hatch date for eight different salinities.
those embryos incubated and hatched successfully in the laboratory with the control group having 82.0% hatch. As the salinity increased, the percent of hatch decreased except for those incubated at 55 o/oo (60.8%). Those larvae hatched from natural pods had the potential of producing 91.0%. The percentages for Fig. 17 were obtained by combining the hatching data from Fig. 15 with the pectoral fin formation and melanophore formation stages. It must be realized that the percentages of hatching from Figs. 16 and 17 represented the maximum possible hatch and in reality these figures may be diminished considerably due to other variables such as predation by insects and birds. Thus, the chances for embryos hatched at 66 o/oo to survive and mature to adulthood were nil.
DISCUSSION AND CONCLUSIONS

Fertilization Success

At spawning the gametes of marine fishes are often subjected to an abrupt salinity shock which might be expected to result in death, or at least considerably impairment of their ability to achieve fertilization. However, the gametes are remarkably tolerant to salinity change (Hoar and Randall, 1969; Kinne, 1971). Work by Holliday and Blaxter (1960), and Holliday (1965) showed that the gametes of the herring, Clupea harengus, and the plaice, Pleuronectes platessa, were tolerant to salinities as high as 60 o/oo. Lasker, Tenaza, and Chamberlain (1972) achieved very similar results for the sargo, Anisotremus davidsonii, the bairdiella, Bairdiella icistia, and the orangemouth corvina, Cynoscion xanthulus.

In the Gulf grunion, Leuresthes sardina, fertilization of the eggs was achieved in salinities between 35 and 72 o/oo with 99 and 92% success, respectively (Fig. 12). Fertilization success in all of the above species declined with an increase in salinity. At 85 o/oo, the fertilization success of L. sardina was questionable since Hubbs (1966) found parthenogenesis occurring in unfertilized California grunion (Leuresthes tenuis) eggs. They were able to produce blastodermal caps but did not gastrulate or form an early gastrula stage. It is suspected that parthenogenesis occurred in L. sardina eggs because development was arrested before gastrulation in all eggs fertilized and incubated in the 85 o/oo group (Figs. 5, 6, and 7).
Embryonic Development

There is ample evidence that the greatest degree of mortality occurs in fishes during the egg and larval stages of development (Sette, 1943; Ahlstrom, 1954). Different embryonic stages respond differently to changes in salinity. The blastula stage of plaice, *Pleuronectes platessa*, has little resistance to low salinities but after gastrulation, when the yolk surface is completely covered by a layer of cells, salinity tolerance is considerably greater (Holliday, 1965). McMynn and Hoar (1953) had observed similar results for developing *Clupea pallasii* eggs. This phenomenon is related to the fact that osmoregulation appears to be a property of overgrowing cells (Kinne, 1971).

In the four graphs (Figs. 5, 6, 7, and 11), the largest percentage of *L. sardina* embryos arrested during embryonic development occurred before gastrulation. As the salinities increased, the percentage of arrested embryos at the early blastula stage increased with 72 o/oo yielding the highest percentage.

Development Rate

The rate of embryonic development in *L. sardina* was found to be temperature dependent. Eggs incubated at a constant temperature (18.5°C) developed in nine days, while those incubated at higher fluctuating temperatures developed in seven days. Similar data for herring embryos by Holliday, Blaxter, and Lasker (1964) showed that as the temperature increased the metabolic rate increased. Lasker and Theilacker (1962) also recorded a similar phenomenon with a significant increase in metabolism of Pacific sardine eggs, thus decreasing developmental time.
Abnormalities

Effect of salinity on body proportions and external characteristics is an area of study in which it is difficult to separate the effects of a single environmental factor (Kinne, 1971). For this reason, most analyses include the combined effects of salinity, temperature, and oxygen content. Battle (1930) reported deformities of the caudal and cardiac regions of the rockling codfish, Enchelyopus cimbrius, in salinities up to 70 o/oo. Alderdice and Forrester (1968) studied the early development and hatching of the English sole, Parophrys vetulus, in various combinations of salinities and temperatures. They found that with an increase in temperature and salinity the numbers of abnormal embryos also increased, and none were produced under optimum incubating conditions (9°C and 25-27 o/oo).

The results obtained from the grunion embryos incubated at 18.5°C showed that with an increase in salinity the percent of deformities increased (Fig. 13). Magnification of this relationship was observed in the field (with increased temperatures) where 10% (at 66 o/oo) of the embryos were abnormal.

Cardiac Response

Cardiac activity (heart beats/minute) was used as an index to measure the metabolic activity of developing L. sardina embryos in relation to salinity. Remane and Schlieper (1971) used identical criteria to determine the effects of increased salinities on metabolic activities of a North Sea mussel, Mytilus edulis. They concluded that at a higher salinity the mussel appeared to be under additional stress due to an...
increased rate of heart beats. Spaargaren (1973) found similar results with two marine shrimps, Palaemon serratus and Crangon crangon. In hyper-normal salinities (at a constant 20°C), the heart rates of the shrimps increased significantly. He also noted that with rapid temperature changes the heart rate followed the increase or the decrease in temperature, thus showing that the cardiac rate was also temperature-dependent.

Oxygen concentrations also influence heart rate. In the mussel Mytilus edulis, an oxygen tension below 1% caused a gradual decrease in respiration (Remane and Schlieper, 1971). Prosser et al. (1957) showed that the goldfish Carassius auratus was acclimated to reduced oxygen levels and its metabolism was affected in two ways: 1) the standard rate of oxygen consumption decreased, and 2) the critical oxygen pressure shifted to lower values (from about 3.1 to 1.5 ml O₂/l). In the Atlantic cod, Gadus morhua, reduction of the ambient oxygen level from 10 to 3 ml O₂/l lowered the rate of oxygen consumption, but increased the respiratory volume (Saunders, 1962).

An increase in the cardiac rate for developing L. sardina embryos (Fig. 14) was correlated to increased salinity. The heart beat rate for those embryos fertilized and incubated under field conditions (at increased temperatures) produced heart rates significantly greater than those embryos incubated in the laboratory (at a constant 18.5°C). The possibility of decreased oxygen content in the sand during fertilization and incubation seemed to have little effect on the cardiac rates. As the salinity increased, so did the heart beats/minute, thus suggesting an increased energy consumption at an increasing difference in osmotic
concentration between the blood and the medium (Spaargaren, 1973). To maintain an increased rate of metabolism, the embryo consumes its yolk storage at a faster rate and decreases its potential to survive upon hatching.

**Hatching Success**

Hatching success for the Gulf grunion at different salinities showed that with an increase in salinity the percent of viable hatched prolarvae decreased. This conclusion agrees with the work performed by several authors (Lasker et al., 1972; Kinne and Kinne, 1962; Holliday and Blaxter, 1960; Holliday, 1965).

Embryos incubated in the field at 35 o/oo and 37 o/oo had the highest hatching percentage. This may be due to fluctuating environmental temperatures. Hubbs (1961, 1966) hypothesized that short daily exposures to sublethal temperatures are not necessarily deleterious and that under certain circumstances (lower salinities) cyclic changes of temperature increases survival.

Under field conditions, the incubation period for the Gulf grunion varies from 9 days (January to March) to 7 days (April to May), depending upon ambient seasonal temperatures. Reynolds and Thomson (1974) showed that grunion eggs were capable of hatching up to 32 days postfertilization, but after 28 days the percentage hatched decreased greatly.

**Natural Conditions During Incubation: A Scenario**

After the grunion spawn their eggs in the beach on a descending high spring tide, the eggs essentially remain high and dry (Fig. 4) until the next spring tide series, at which time the turbulence of the
surf slowly erodes the beach sand from around the eggs and total release (hatching) normally occurs on the highest high tide of the next spring tide series. During the period of incubation, the eggs are maintained in an optimal condition due to the formation of a salty crust layer of sand (by evaporation) on the beach surface (0 to 2.5 cm deep). This crusty layer of sand provides a natural incubator for the developing grunion embryos.

From the time the eggs are spawned in the beach (2.5 to 10 cm deep), temperature and moisture content play major roles in the development of the eggs. Vertical beach temperatures obtained after grunion runs (Fig. 8) showed the effects of ambient air temperatures of the beach strata to a 50-cm depth. The temperatures of the pod levels varied from 11.0 to 33.5°C after four grunion spawning runs and the vertical temperature variation for each run did not exceed 1.0°C. As the wetting front of interstitial water flowed down into the ground water table, the vertical temperatures became more diverse near the surface (Fig. 9). This agrees with data obtained by Janssen (1967b) and Johnson (1965) on the temperature regime of beach infauna of Swedish and California beaches, respectively. During the incubation period, the harsh fluctuating ambient temperatures are buffered, thus providing a more stable temperature regime for embryonic development. Pod level temperatures (4/25/70 to 5/8/70) during incubation ranged from 20.5 to 34.6°C, while the surface temperatures were 16.2 to 38.9°C. The air temperatures for the same period were 14.8 to 30.3°C.
The moisture content of the beach ranged from saturation values obtained during spawning to a water content of less than 5% during incubation (Fig. 10). A reduced moisture content was definitely necessary for proper embryonic development of embryos incubated under laboratory or field conditions. Increased moisture contents caused rapid deterioration of the eggs by bacteria. This fact coincides with data obtained by Ehrlich and Farris (1971) on incubation of Leuresthes tenuis eggs.

The effects of increased salinities and decreased oxygen content in the beach strata were considered but monitoring of these parameters proved futile. Johnson (1967) has shown that salinities of interstitial water found within 10 cm of the surface increased (34 o/oo to 41 o/oo) as a result of evaporation. Jansson (1967a) has demonstrated that the vertical distribution of oxygen (to 1 meter) on a Swedish beach decreased slightly. The oxygen content was found to be more dependent upon the physical and chemical properties of the sand instead of depth. With these references, assumptions were made that increased salinities in the pod levels did occur at El Golfo and that the oxygen content was adequate for embryonic development.

The last factor to consider was the effect of parasitism on developing grunion eggs in the beach. It was discovered that large numbers of dipteran larvae and adults, Fucellia rejecta, parasitized the eggs (the 50 o/oo group and the natural pods) during incubation. Malloch (1923) and Coll (1969) found Fucellia rejecta consuming eggs of Leuresthes tenuis near San Diego, California. A survey of Gulf grunion pods from Punta Machorro showed that 60% of the embryos had been devoured. The
dipteran larvae and adults were found infesting only those eggs in close proximity to bird resting sites. From these results, the population of *Fucellia rejecta* found near El Golfo de Santa Clara, Sonora, Mexico, might be considered a disjunct population.

After establishing the effects of various biotic influences on the incubation of Gulf grunion, the maximum probable hatch can now be interpolated for the eight different salinities (Figs. 16 and 17). The laboratory data (mean of 3 replicates) clearly demonstrates the hatching success obtained two days after the hatch date in all salinities. As the salinity increased from 35 o/oo to 60 o/oo, the percent of hatch decreased from 82% in the control group to 39% for the 60 o/oo group. Only those embryos incubated at 66 o/oo produced a marginal quantity of viable pro-larvae (2%) after the second day of hatch (Fig. 17). The hatch results obtained from the field experiment reinforced the trend of increased salinities decreasing the maximum hatch potential. Under field conditions, the maximum hatch achieved was found in the 35 o/oo group (97%) and this compared favorably with a 91% hatch ($\bar{x}$ of 25 naturally spawned pods) found in the 37 o/oo group. At 60 o/oo, the hatching potential was 59%, while in 66 o/oo a meager 8% hatched live. This agrees with Hubbs' (1966) hypothesis that fluctuating environmental temperatures seem to enhance the maximum probable hatch found in the field.

In conclusion, the Gulf grunion eggs utilized in this study were fertilized, incubated, and hatched at higher salinities which produced a decreased hatching rate, an increased metabolic rate, and a greater consumption of the embryo's yolk reserve. With this knowledge, the grunion fisheries could be severely affected by the construction of a desalination
plant (producing salinities exceeding 40 o/oo) near El Golfo de Santa Clara. The species' only means of averting extinction would be through migration to areas of lesser salinities.
LITERATURE CITED


