Shahnawaz Kadir Shaikh. THE OSMOTIC BEHAVIOR OF ZYGOCOTYLE LUNATA (TREMATODA:PARAMPHISTOMIDAE). (Under the direction of Dr. James S. McDaniel) Department of Biology, July 1973.

Trematodes have been shown to be highly poikilosmotic and without the ability to osmoregulate, although there is a small, but direct, correlation between weight change and oxygen consumption. Other parameters of trematode osmotic behavior are unknown. My study was undertaken to compare the results of osmoregulatory studies utilizing weight change and oxygen consumption with patterns of carbohydrate consumption under osmotic stress.

Zygocotyle lunata (Dies. 1836) Stunkard 1917, a trematode of aquatic birds, was found to contain a considerable amount of polysaccharide (32-40% dry wt.) and glucose plus at least one other freely-extractable, alkali-stable, non-reducing sugar. Incubation of the worms in a range of sodium chloride concentrations between zero (deionized water) and 3% showed about 0.55% to be isotonic. A linear change in weight occurred rapidly in dilutions above and below isotonic. Further, the freely-extractable sugars leaked from the worms into the incubation media in each of the concentrations including that which was isotonic.

The total amount of freely-extractable sugar decreased as the salt concentration of the environment increased. At the same time, the polysaccharide showed a sharp increase. These observations support the conclusion that polysaccharide synthesis in Z. lunata proceeds at a high rate under osmotic stress.

In summary, Zygocotyle lunata, like other trematodes that have been investigated, does not regulate its water content in hypotonic and hypertonic environmental salt concentrations and can be said to be an osmoconformer. Alcohol-extractable carbohydrate and stored polysaccharide in Z. lunata change in a linear fashion with environmental salt concentration which would seem to establish observations on carbohydrate utilization as a measure of osmoregulatory activity.

# THE OSMOTIC BEHAVIOR OF

# ZYGOCOTYLE LUNATA

# (TREMATODA:PARAMPHISTOMIDAE)

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by

Shahnawaz Kadir Shaikh

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# INTRODUCTION

The osmotic behavior of endoparasitic worms has been little studied. Despite the lure of their exotic habitats and complex life histories, only a few worms have been investigated. The nematodes and cestodes have received more attention than the acanthocephalans and trematodes. General discussions of available information are found in von Brand (1966), Crompton (1970) and Bair and Peters (1971).

Some nematodes osmoregulate and some do not (see Rogers, 1962; Lee, 1965; Crofton, 1966; Arthur and Sanborn, 1969). The flatworms are sensitive to osmotic change in the environment and apparently can only adjust their internal osmotic pressure by varying their body volume (see Read and Simmons, 1963; Schwabe and Kilejian, 1968; Smyth, 1969; Erasmus, 1972). Acanthocephalans change weight readily with changes in the osmotic pressure of their surroundings and have been described as osmoconformers (Crompton, 1970).

Osmoregulatory studies among endoparasitic flatworms are rare. Much of the information available has been reviewed by Bair and Peters (1971). The trematodes that have been studied are Fasciola hepatica (Knox and Pantelouris, 1966), F. gigantica (Seddiqi and Lutz, 1966), Gastrothylax crumenifer (Goil, 1966), and Haematoloechus medioplexus (Bair and Peters, loc. cit.). All the studies of these worms except that of H. medioplexus utilized the measurement of weight change as an indicator of osmoregulatory response. The study of Bair and Peters (loc. cit.) on osmoregulation in H. medioplexus was expressed in terms of its oxygen uptake.

This study represents a part of a larger study on the biology of Zygocotyle lunata (Dies., 1836) Stunkard, 1917, a trematode parasite of birds and mammals, undertaken by Dr. James McDaniel and his students. There is no literature relating to osmoregulation in trematodes expressed in terms of their carbohydrate metabolism. The present study was undertaken to compare the results of osmoregulatory studies utilizing carbohydrate consumption with those using weight change, as well as to provide further information on the biology of Z. lunata.

## MATERIALS AND METHODS

Adult Zygocotyle lunata were maintained in laboratory mice of several strains, but mainly in an inbred strain of Swiss mice obtained from UNC-Chapel Hill (for details on this strain see Larsh et al., 1970). Laboratory infected Helisoma trivolvis served as a source of metacercariae. Information on the life history of this worm may be found in Willey (1941).

Infected snails were isolated in finger bowls during the daylight hours. Cercariae are not shed during the night in this species (Coggins and McDaniel, 1970). Mice were lightly etherized and infected by stomach tube with 10 to 12 cysts. This infection level produces the greatest number of and the largest sized worms at the proper time (Bailey and McDaniel, in preparation). Flukes used in these experiments were recovered from mice between days 30 and 32 after infection.

Flukes were removed to 0.9% sodium chloride (approximately isotonic to mammalian tissues) and then counted singly into a dish containing a fresh aliquot of the same solution. The worms were left in this solution over crushed ice for exactly 15 minutes to insure similar conditions for all experiments. About 30 worms were used in each experiment. At the end of the equilibration period the worms were pipetted (large diameter tubing) onto hard filter paper, blotted, weighed (to the nearest 0.1 mg.) on a torsion balance (Wet Weight I) and placed in 4 ml. of the appropriate medium for incubation. The time interval between each step was held to a minimum. The worms were placed in 25 ml. beakers lightly covered with parafilm for incubation.

The media for the experimental incubations were 0 (deionized water) and 0.5%, 1.0%, 1.5%, 2.0% and 3.0% NaCl.

Incubations were carried out at 38° C. in a controlled temperature water bath for periods of time from 15 minutes to 1 hour with constant shaking. Most experiments were done for periods of 30 minutes or less, since the worms became moribund after that time in the higher and lower medium concentrations.

One set of experiments determined the maximum and minimum weight change in this worm after a 20 hour incubation period in 3% NaCl and in deionized water. The worms were observed to determine how long they remained motile in these solutions.

Following incubation, the flukes were poured onto a polyvinyl-chloride membrane (Gelman VM-1) with a large pore size (5 microns). The fluid in which the worm had been incubated was saved for carbohydrate assay. The worms were weighed again on the torsion balance to determine if any change had occurred during the incubation period (Wet Weight II), and immediately transferred to a measured volume of 70% ethanol and extracted for at least 24 hours. Aliquots of the alcoholic extract were used for chemical and enzymatic analysis of "free" carbohydrates; that is, the ethanol-extractable monosaccharides and oligosaccharides that are not bound in cells.

The alcohol-extracted worms were digested with 20% KOH in a boiling water bath for 30 minutes. Alkali-stable, ethanol-precipitable polysaccharide was precipitated with 1.2 volumes of 95% ethanol. The

precipitate was washed with 70% ethanol containing 0.1% LiCl, and taken up in a measured volume of distilled water. Aliquots were used for determination of polysaccharides.

Several groups of worms were not incubated and served as controls. They were handled as the experimental groups except that after being equilibrated for 15 minutes and weighed they were removed directly to 70% ethanol for extraction.

The dry weights for all the worm groups were taken on alcohol-extracted worms which were dried in an oven at 95° C. for 20 hours.

Carbohydrate was determined by the phenol-sulfuric acid method of Dubois *et al.* (1956). This method is for the quantitative colorimetric microdetermination of sugars, their methyl derivatives, oligosaccharides, and polysaccharides. Glucose was specifically identified by the glucose oxidase method (Glucostat, Worthington Biochemical Corporation). Reducing compounds were determined by the Nelson method (Nelson, 1944).

## RESULTS

The physical, chemical and enzymatic determinations made on the trematode tissues and fluids and the incubation media are summarized in Table I. The wet weights of the worm groups were established just after their removal from the host and at the termination of the experimental incubations; dry weights were determined from heat-dried alcohol-extracted worms. These flukes contained large quantities of polysaccharide and glucose plus at least one other freely-extractable, alkali-resistant, non-reducing sugar. Carbohydrates leaked from the worms during incubation and were found in the saline medium.

### Weight Change

This worm underwent a change of weight that had a direct relation to the incubation medium (Fig. 1). The change was inversely proportional to the concentration of the medium. The curves indicate that the zero change in weight would occur in concentrations of sodium chloride approximately between 0.5% and 0.6%. Weight gain in hyposmotic media appeared to be much greater than the corresponding weight loss in hyperosmotic media. Long-term experiments in hyposmotic and hyperosmotic media indicated a similar situation (Table II, Fig. 2). Placed in deionized water, the worms gained just over 200 percent in weight and equilibrated in about 2 hours. Worms in 3% NaCl lost about 50% of their original weight within the first 30 minutes of the experiment and slowly regained weight throughout the remainder of the time

of the experiment. It was not possible to tell how long the worms survived in these solutions. Movement stopped within 3 hours in deionized water and within 30 minutes in the 3% saline.

The change in weight was quite rapid in the initial stage of each experiment except for the 0.5% NaCl (Fig. 3). From experiments continued for 1 hour, it appeared the worms reached a state of weight equilibrium within 30 minutes. In deionized water the worms gained weight rapidly and continuously, whereas the weight gain in 0.5% saline was not pronounced. The concentration of dilute saline in which the worms could survive and maintain a steady weight was not determined, but it does lie between zero and 0.5%. Observations on the behavior of the worms and of their weight loss, would indicate the greatest concentration of saline in which the worms could survive and maintain a steady weight was about 1.5%.

Carbohydrate Change

This worm contained a large amount of alcohol-extractable carbohydrate, consisting of glucose and at least one other alkali-stable, non-reducing sugar (Table I). Both fractions were in the various incubation media (Table I). Their presence in the media indicates they leaked from the worms during the experimental incubations.

Both fractions in the alcohol-extractable pool decreased as the salt content of the medium increased (Fig. 4). The trend for the appearance of both fractions in the experimental medium paralleled that

of their occurrence inside the worm (Fig. 4). These sugars decreased both inside the worm and in the incubation medium as the time of the incubations was lengthened except for glucose which increased (Fig. 5, Table I).

This worm contained a large quantity of alkali-stable, ethanol-precipitable polysaccharide (Table I). Although there was fluctuation, the tendency was for the fraction to increase as the salt concentration of the medium increased (Fig. 6). A comparison of short and long term experiments showed this fraction to increase with time except for the experiments in 0.5% sodium chloride (Fig. 7).

TABLE I

Physical, Chemical and Enzymatic Determinations
Values are means ± standard error

| Medium (% NaCl) | Time (min.) | Exps. No. | Wet Wt. I (mg.) | Wet Wt. II (mg.) | Change Wt. (%) | Dry Wt. (mg.) | ETOH-extractable (µg/mg dry wt.worm) | | | | | KOH-stable polysaccharide (µg/mg dry wt.) | NaCl Medium (µg/mg dry wt.wm.) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Carbohydrate | | | Reduc. Comp'nds | | | | | |
| | | | | | | | Glucose | Total | KOH boiled | Total | KOH boiled | | Glucose | Total | KOH boiled |
| Control | 0 | 4 | 217.7 | --- | --- | 46.9±5.8 | 2.9 ±0.2 | 6.5±0.2 | 3.4±0.5 | 6.1±1.9 | 0.3±0.1 | 352.1±18.4 | --- | --- | --- |
| Deion. water | 15 | 1 | 112.8 | 171.5 | +52.0 | 21.9 | 4.2 | 25.6 | 13.5 | 25.4 | 0 | 326.2 | 1.8 | 6.8 | 3.7 |
| | 30 | 4 | 176.3 | 281.9 | +60.4±1.51 | 33.3±3.3 | 4.5±0.9 | 14.8±0.2 | 8.6±1.1 | 9.2±0.2 | 0 | 344.1±14.7 | 1.9±0.6 | 7.8±0.6 | 3.3±0.4 |
| 0.5 | 30 | 2 | 117.8 | 125.2 | +6.83 | 22.3 | 1.1 | 13.6 | 8.1 | 5.6 | 0 | 364.6 | 0.4 | 4.0 | 2.9 |
| | 60 | 5 | 176.6 | 174.7 | -1.66±4.0 | 33.3 | 2.1±0.4 | 9.2±1.4 | 8.0±2.9 | 7.7±1.4 | 0 | 341.4±11.8 | 2.2±0.2 | 4.7±0.1 | 1.2 |
| 1.0 | 30 | 1 | 154.9 | 115.9 | -25.2 | 29.6 | 0.5 | 10.4 | 7.5 | 3.6 | 0 | 335.6 | 0.5 | 5.5 | 3.7 |
| | 60 | 5 | 176.6 | 134.5 | -24.0±1.1 | 33.4 | 1.3±0.4 | 8.4±0.8 | 7.4±1.9 | 3.9±0.2 | 0 | 374.4±17.0 | 0.9±0.3 | 5.2±0.7 | 2.1±0.1 |
| 1.5 | 30 | 1 | 149.9 | 99.6 | -33.6 | 30.5 | 0.4 | 8.1 | 5.8 | 2.7 | 0 | 372.2 | 0.3 | 2.9 | 2.9 |
| | 60 | 5 | 180.9 | 117.0 | -35.1±1.4 | 35.8 | 1.0±0.3 | 7.9±0.4 | 5.4±0.5 | 3.8±0.6 | 0 | 388.2±8.1 | 0.9±0.6 | 3.9±0.3 | 2.8±0.1 |
| 2.0 | 30 | 6 | 174.9 | 106.2 | -39.2±0.7 | 35.4 | 0.6±0.1 | 6.9±0.8 | 4.9±0.6 | 2.9±0.6 | 0 | 362.6±7.9 | 0.8±0.2 | 2.9±0.3 | 2.3±0.2 |
| 3.0 | 20 | 1 | 175.7 | 100.6 | -42.7 | 36.2 | 0.9 | 7.0 | 4.6 | 1.8 | 0 | 405.1 | 0.2 | 2.4 | 1.5 |

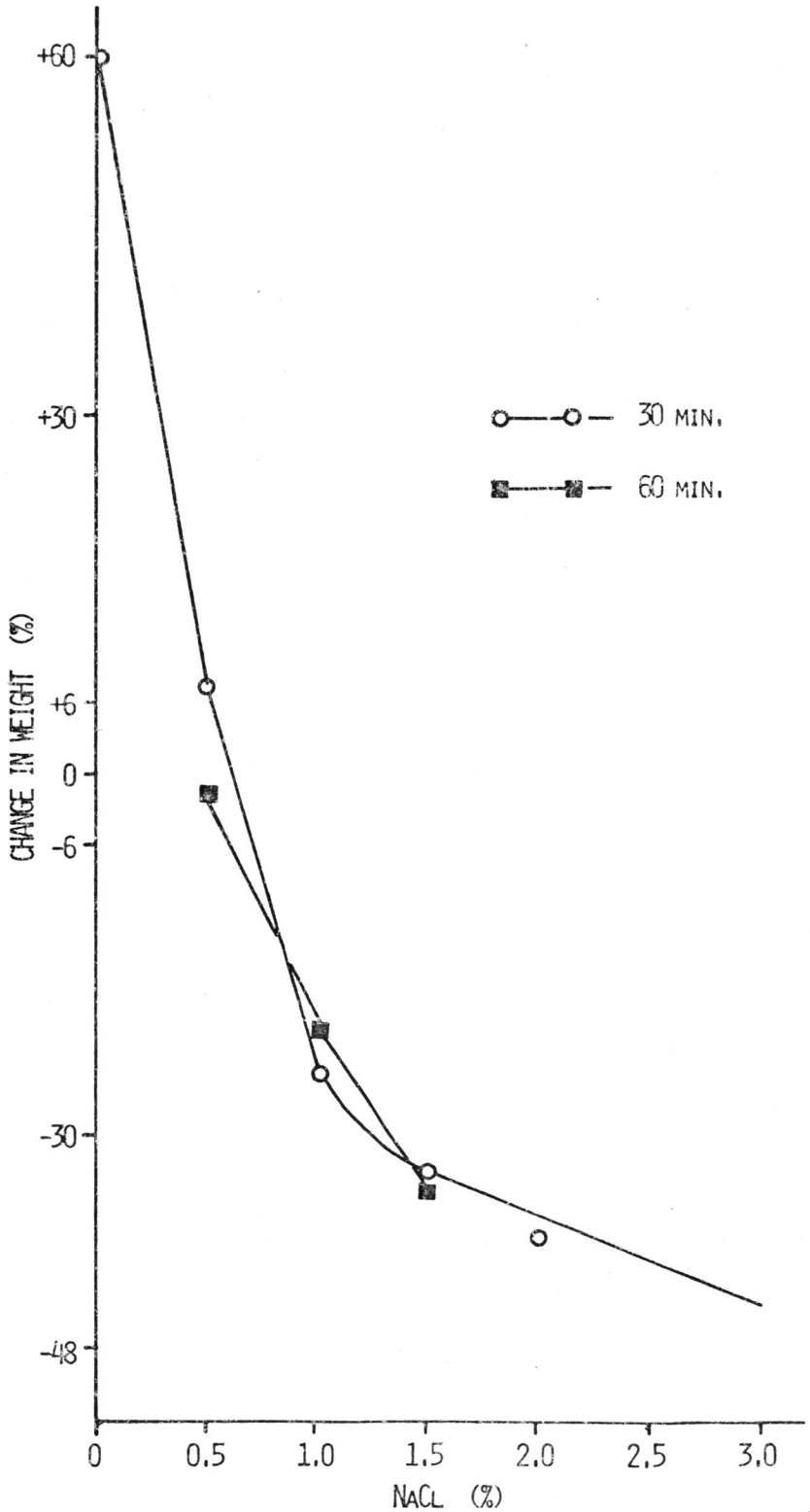


Fig. 1. Percent wet weight change in Zygocotyle lunata upon exposure to several dilutions of sodium chloride

TABLE II

Long-term Incubations of Zygocotyle lunata
in Deionized Water and Three Percent Sodium Chloride

| Time (hrs) | Deionized Water | | 3% NaCl | |
|---|---|---|---|---|
| | Wet Weight Worms (mg) | Wt. Change (%) | Wet Weight Worms (mg) | Wt. Change (%) |
| 0 | 18.7 | 0 | 16.6 | 0 |
| 0.25 | | | 7.8 | 53.1* |
| .50 | 28.6 | 53* | 8.1 | 51.2 |
| .75 | 39.8 | 112.8 | 8.7 | 47.6 |
| 1.00 | 46.2 | 147.1 | 9.6 | 42.2 |
| 1.25 | 52.9 | 182.9 | 9.6 | 42.2 |
| 1.50 | 56.1 | 200.0 | 9.6 | 42.2 |
| 1.75 | 56.2 | 200.5 | | |
| 2.00 | | | 10.7 | 35.5 |
| 2.25 | 57.8 | 209.1 | | |
| 2.50 | | | 12.9 | 22.3 |
| 2.75 | 58.3 | 211.8 | | |
| 3.00 | | | 12.7 | 23.5 |
| 3.25 | 58.2 | 211.2 | | |
| 3.50 | | | 12.7 | 23.5 |
| 3.75 | 57.7 | 208.6 | | |
| 5.50 | | | 12.1 | 27.1 |
| 5.75 | 56.9 | 204.3 | | |
| 6.50 | | | 12.6 | 24.1 |
| 7.75 | 53.8 | 187.7 | | |
| 20.75 | 53.8 | 187.7 | 12.6 | 24.1 |

*$\frac{\text{Experimental-Original Wt.}}{\text{Original Wt.}} \times 100$

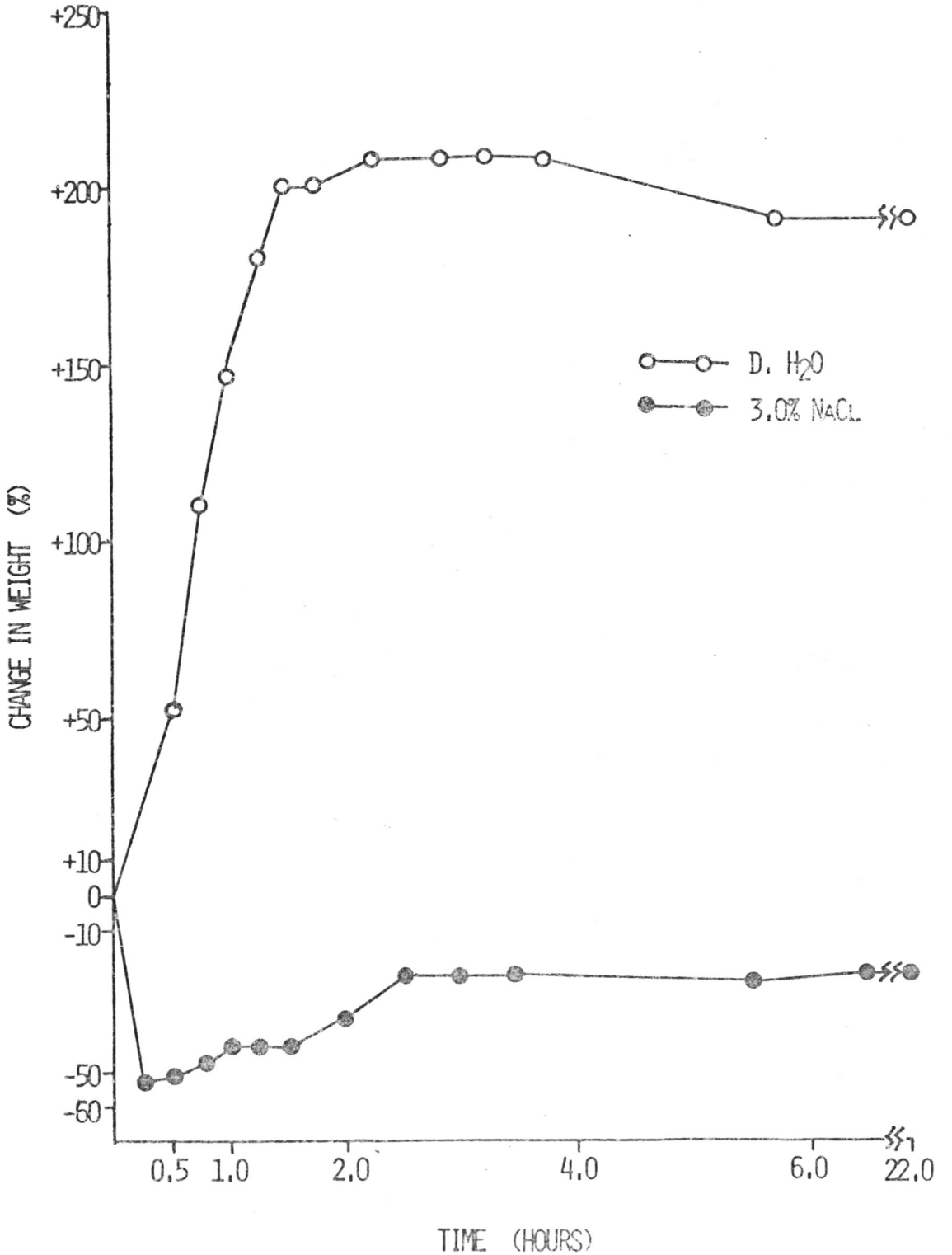


Fig. 2. Percent wet weight change in Zygocotyle lunata held for twenty hours in deionized water and three percent sodium chloride

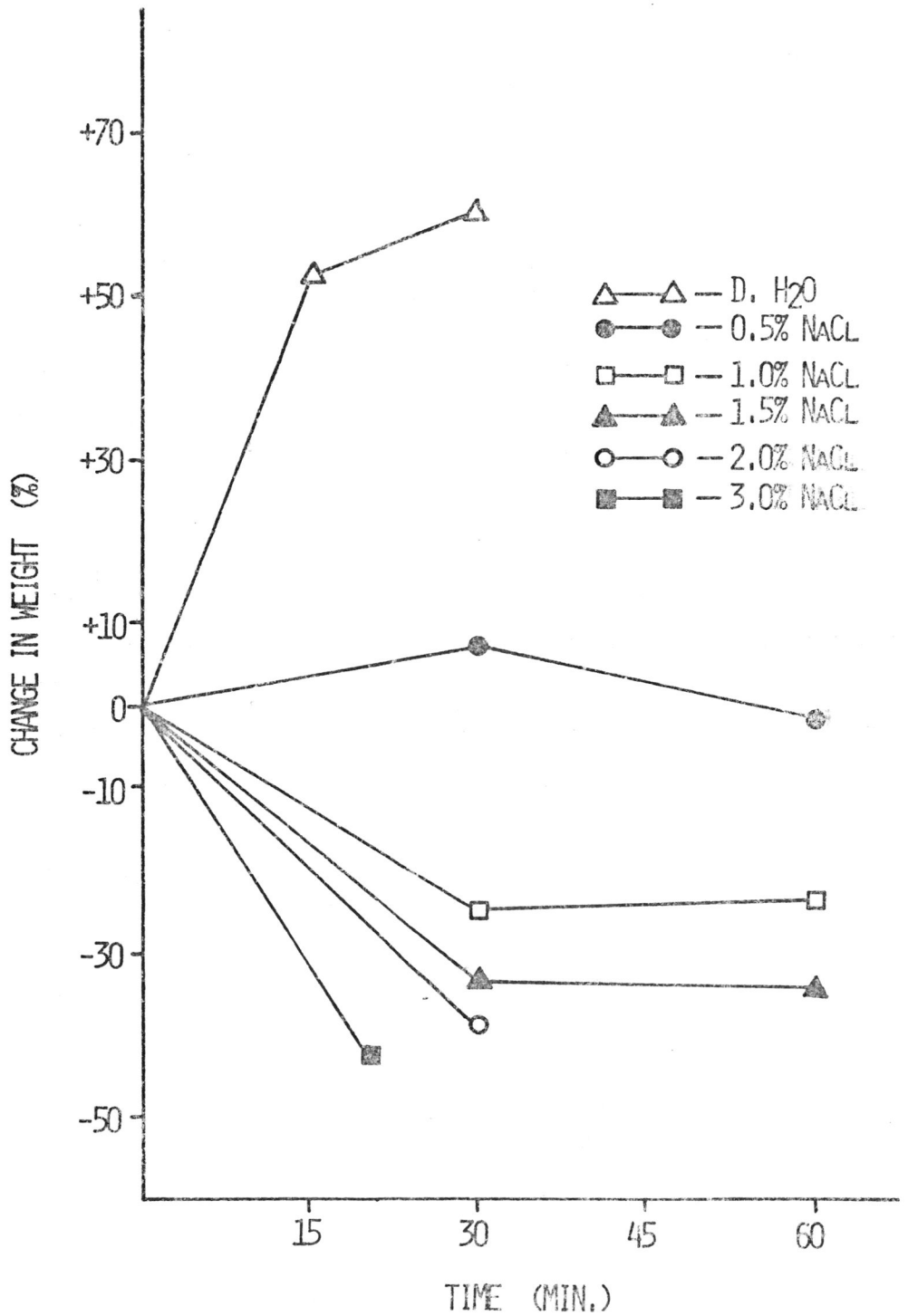


Fig. 3. Percent wet weight change in Zygocotyle lunata in incubations from 15 to 60 minutes in various dilutions of sodium chloride

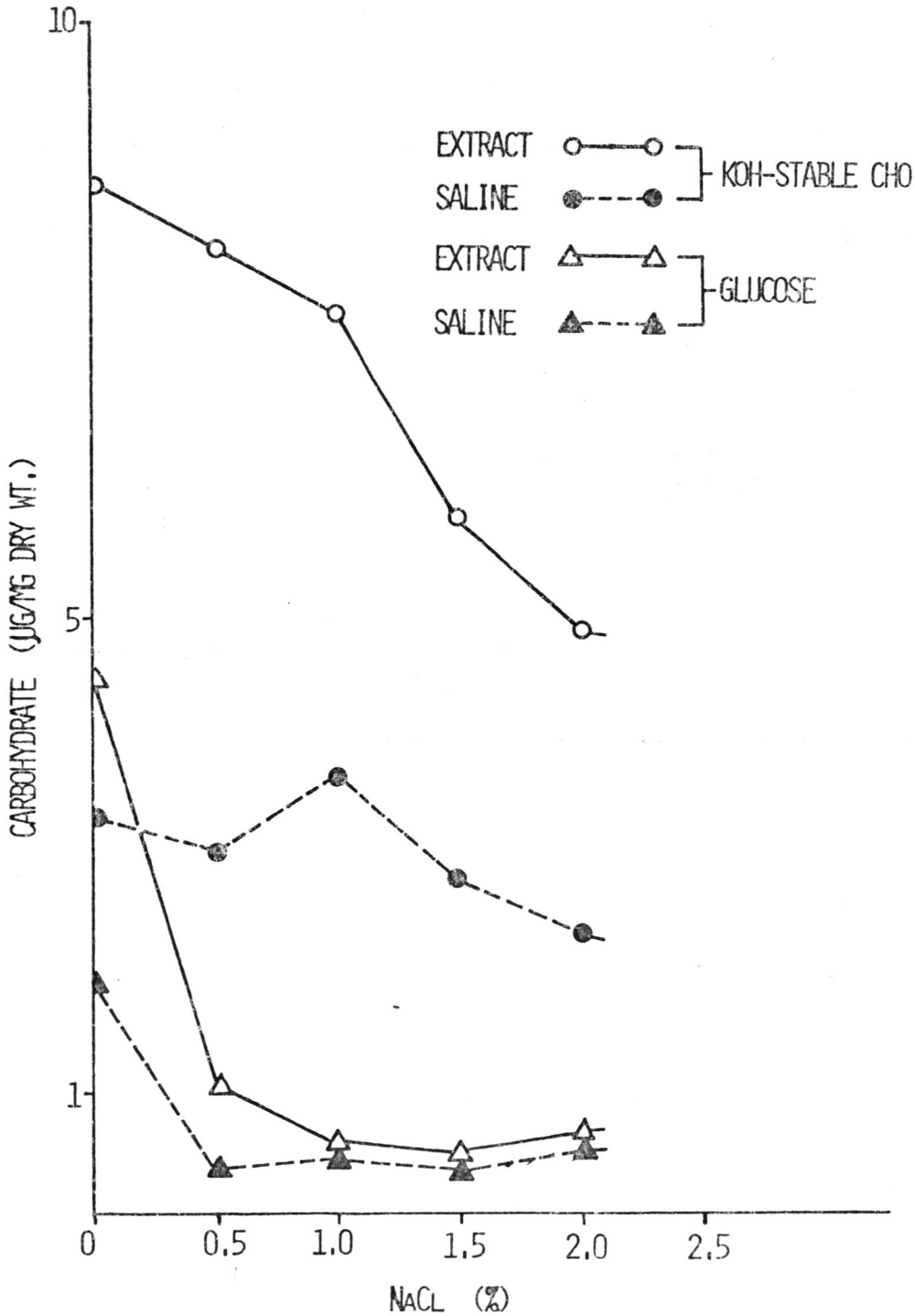


Fig. 4. Average change in alcohol-extractable carbohydrate inside the worm and in the incubation media

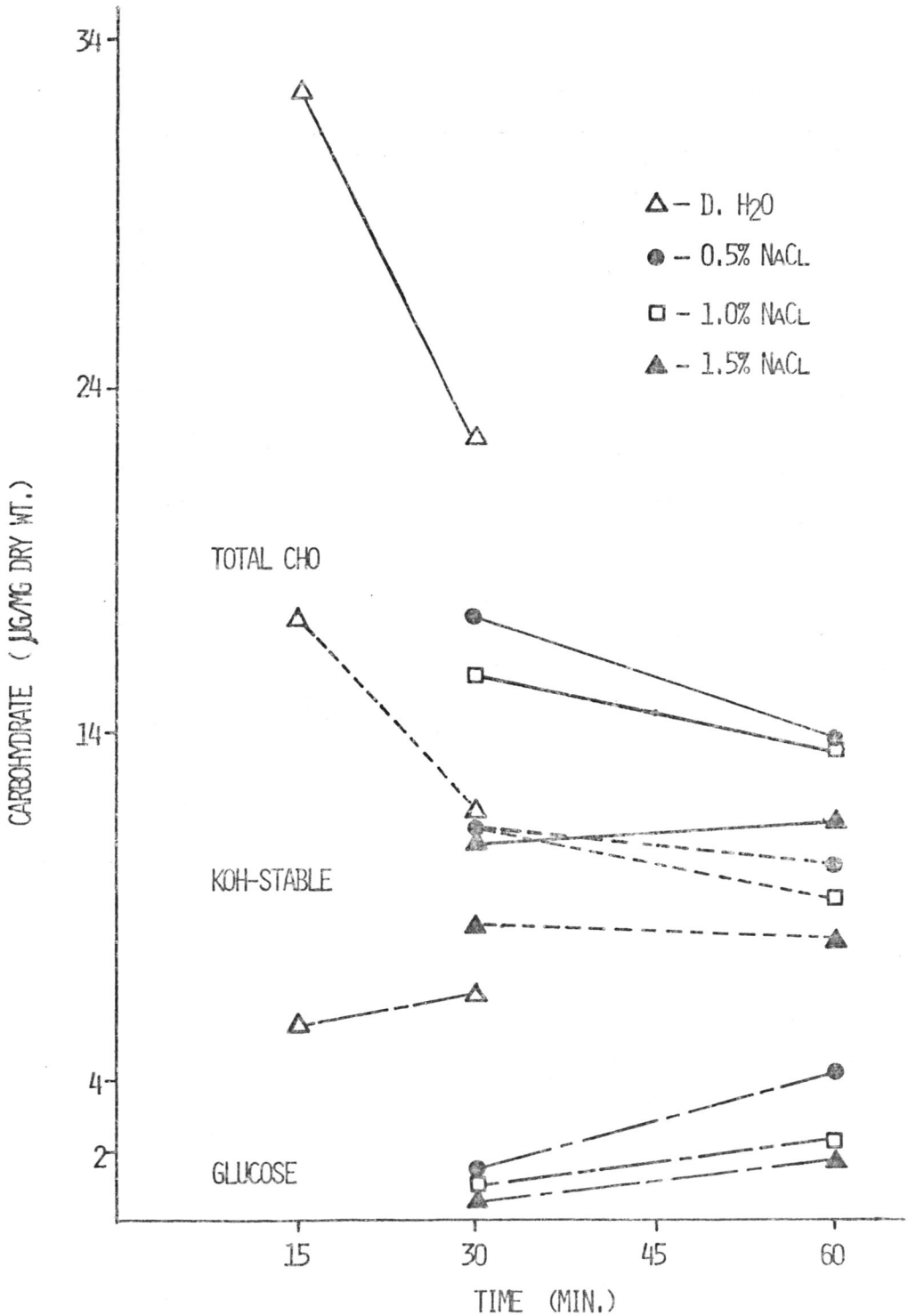


Fig. 5. Average change in alcohol-extractable carbohydrate inside the worm and in the incubation media in incubations of from 15 to 60 minutes in various dilutions of sodium chloride

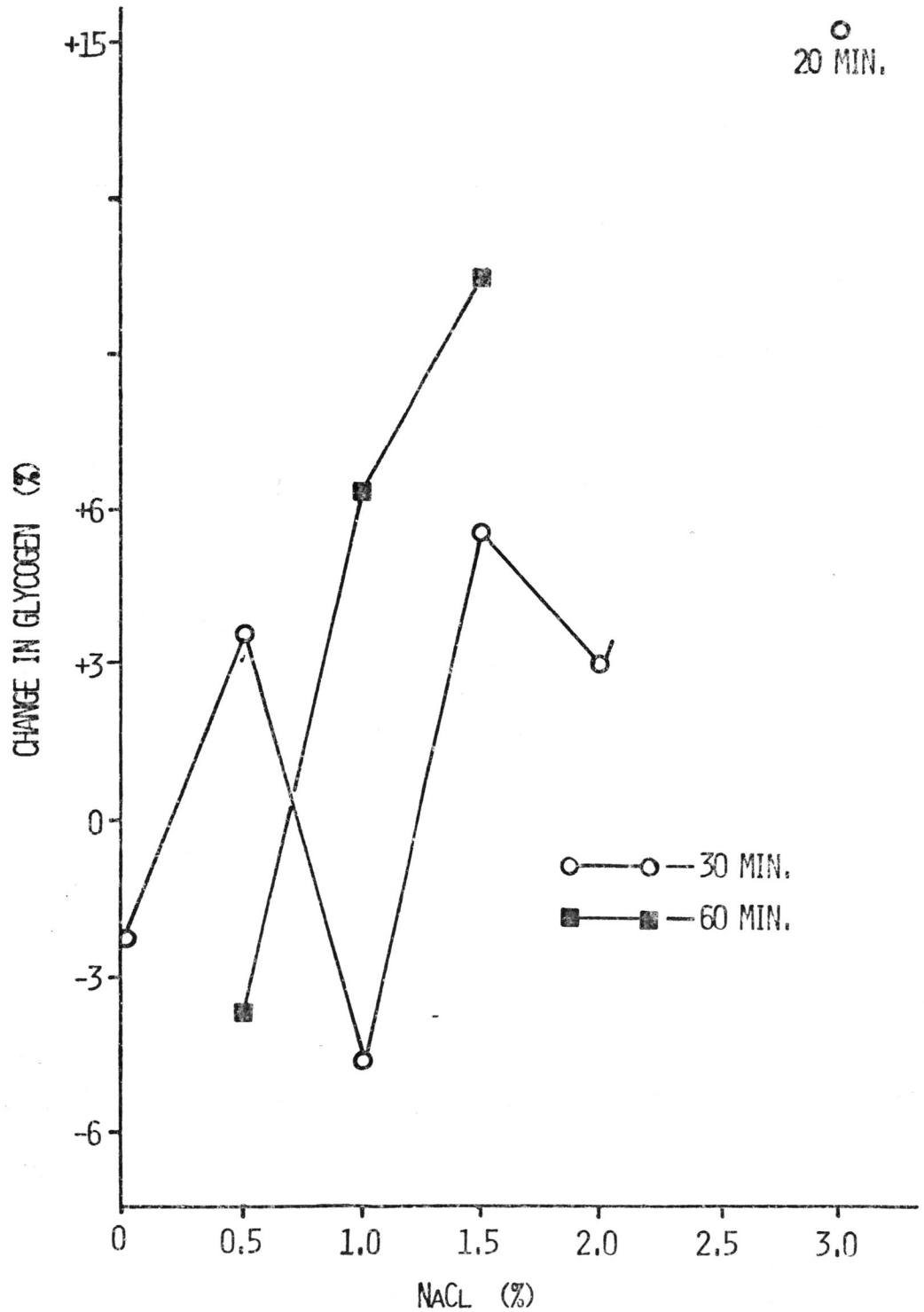


Fig. 6. Percent change in polysaccharide in Zygocotyle lunata incubated in various dilutions of sodium chloride

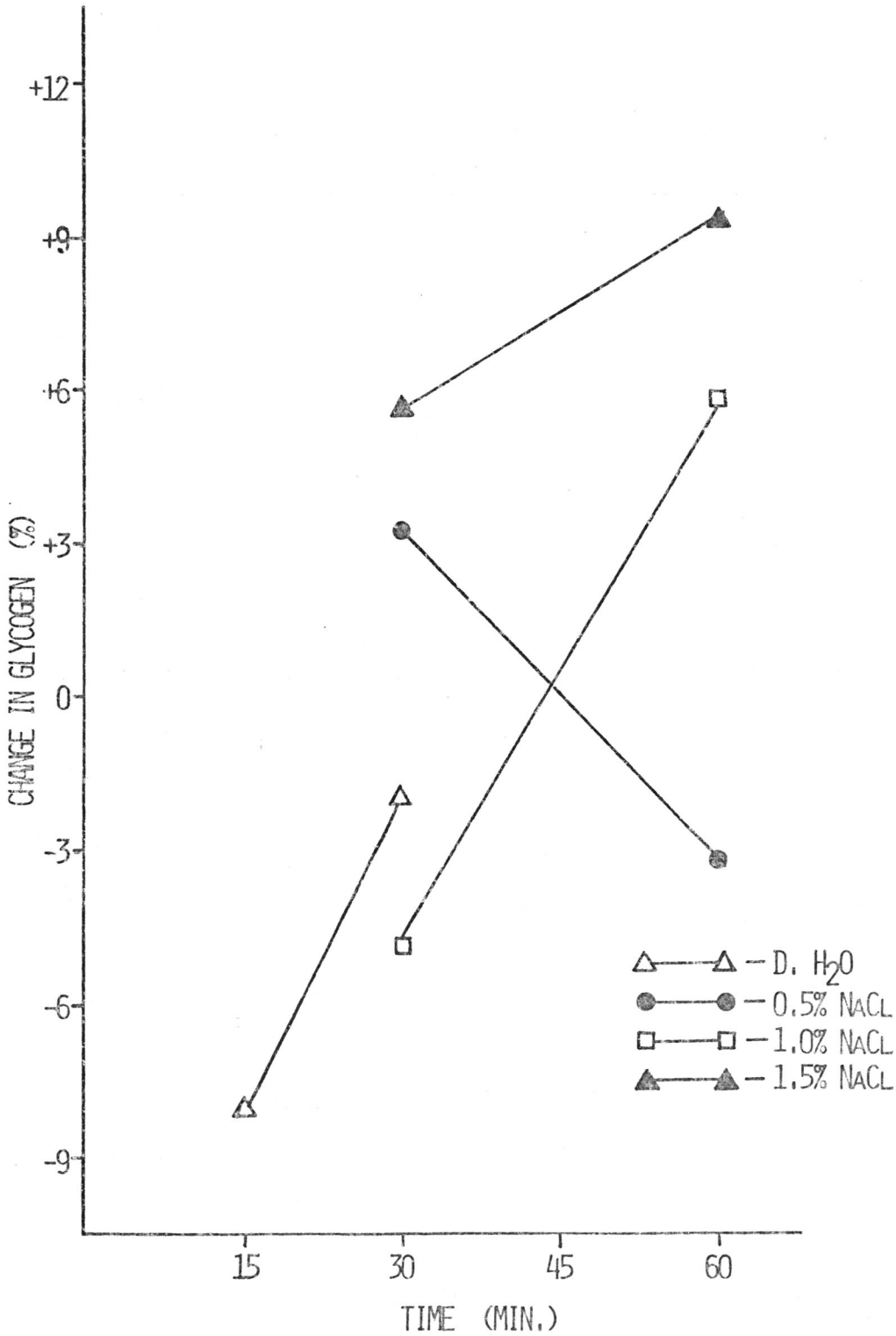


Fig. 7. Percent change in polysaccharide in Zygocotyle lunata in incubations of from 15 to 60 minutes in various dilutions of sodium chloride

## DISCUSSION

The parasitic nematodes are on the whole more resistant to variations in environmental osmotic concentration than flatworms or acanthocephalans. Ostertagia survive equally well in complex media having osmotic concentrations equivalent to the range of 0.4 to 1.3% NaCl (Davey, 1938). The optimum survival of Ascaris lumbricoides is at about 1% NaCl, but limited survival occurs in concentrations ranging from 3 to 5% NaCl (Cavier and Savel, 1952, 1953, as interpreted by von Brand, 1966). The most resistant nematode so far studied is the larva of Eustrongylides ignotus (von Brand and Simpson, 1942). It survives for several months in media containing, besides organic components, NaCl concentrations varying from 0.5 to 1.0%, and up to 16 days in 3% NaCl.

The Acanthocephala are quite sensitive to changes in osmotic pressure. In isotonic or hypertonic solutions they are flat and ribbonlike, but rapidly swell up becoming round and turgid in hypotonic solutions. The most suitable molecular concentration for maintenance in vitro of Neoechinorhynchus emydis (a parasite of turtles) is 0.5 to 0.7% NaCl (Gettier, 1942). Dunagan (1962) found a Tyrode's solution with 0.9% NaCl a satisfactory culture medium for N. emydis and N. pseudemydis.

The reactions of tapeworms to changes in the osmotic pressure of the surrounding medium have not been studied in a systematic comparative fashion. Much of the data available suggests that many of them behave like osmometers and have little capacity to regulate the internal osmotic pressure except by volume regulation of the body. An almost

linear change in weight as a function of salt concentration has been observed in Moniezia, Schistocephalus, Hymenolepis, and Calliobothrium (see reviews of Read and Simmons, 1963; Smyth, 1969). However, Read and Simmons (loc. cit.) point out that as in most cases in which generalizations would make for a happy situation, there may be exceptions to this pattern of response. There is some data to indicate that Lacistorhynchus tenius, a trypanorhynchid from elasmobranchs, may have some capacity for osmoregulation. There is a range of salt concentration (215-250 mM NaCl) in which this worm apparently neither gains nor loses weight.

To date, the osmotic behavior of four trematodes has been investigated in some fashion. Knox and Pantelouris (1966) studied the osmotic behavior of Fasciola hepatica in several concentrations of modified Hedon-Fleig (H-F) medium. They determined that there was little change in weight in this fluke during several hours of culture in normal H-F and concluded the medium was suitable from an osmotic standpoint. The fluke carried out no water regulation under their experimental conditions and changed weight as a function of the concentration of the medium in H-F solutions from 0.5 to 2.0 normal. The worms either gained or lost weight in the concentrations of H-F below and above 1.0 normal and within 1 to 4 hours had equilibrated at this greater or lesser weight in concentrations of H-F between 0.75 and 1.25 normal. In 0.5 and 2.0 normal H-F, this fluke dies within one hour. This worm survives equally well in salt solutions containing from 58 to 230 mM NaCl (Stephanson, 1947). A related observation would be that of Bueding (1950) who determined the metabolic activity of

Schistosoma mansoni was not altered in complex media containing 60 to 160 mM NaCl. These latter two observations have been cited as evidence to indicate trematodes are fairly resistant to changes in environmental osmotic concentration.

Osmotic and ionic regulation in Fasciola gigantica was studied by Siddiqi and Lutz (1966). Using several concentrations of Tyrode's solution, they determined F. gigantica to be poikilosmotic. The worms did not change weight in normal Tyrode's solution and these investigators concluded it was approximately isotonic to the osmotic pressure of the worm's body fluids. This worm rapidly gains and loses weight in concentrations of Tyrode's ranging between zero (deionized water) and 2.0 normal. In 75% Tyrode's there is a 10% gain in weight; in 50% it is three times, in 25% six times and in deionized water 11 times that in 75%. In hypertonic media the change is not so marked. It is about 10% in 125, 13% in 150 and 22% in 200% Tyrode's. Within 15 minutes, this worm equilibrated its weight above or below what it was in 1.0 normal Tyrode's, in concentrations from 0.5 to 2.0 normal. Knox and Pantelouris (1966) found that F. hepatica showed about an 18% increase in weight in 75% Hedon-Fleig medium and a loss of about 11% in 125% H-F, which is similar but a more marked response than that of F. gigantica in Tyrode's solution. Further, F. hepatica did not long survive in 0.5 and 2.0 normal H-F so, therefore, appears less tolerant of concentration changes than F. gigantica.

Weight change in response to different concentrations of saline media by Gastrothylax crumenifer was investigated by Goil (1966). This

trematode proved poikilosmotic and changed weight in a direct relationship with the concentration of saline media containing between 0.4 and 0.8% NaCl. The approximate point of isotonicity is between 0.4 and 0.5% NaCl.

Knox and Pantelouris (loc. cit.), Seddiqi and Lutz (loc. cit.) and Goil (loc. cit.) were doing their experiments in different parts of the world and published within months of each other in 1966. All utilized the measurement of weight gain or loss as a potential indicator of osmoregulation. It was not until 1971 that a further observation on the osmotic behavior of a trematode appeared.

Since oxygen is used in metabolism, Bair and Peters (1971) felt oxygen uptake would be a convenient measure of energy expenditure necessitated by osmoregulation. They determined weight change and oxygen consumption for Haematoloechus medioplexus, a trematode parasite of the lungs of frogs, in five concentrations of sodium chloride--zero (distilled water), 0.4, 0.7, 1.4 and 3.0%. The 0.7% NaCl is isotonic with amphibian blood. This trematode did not control its weight in the several changes of environmental salt concentration. In the medium approximately 0.5 normal (0.4% NaCl) the weight gain was 31%; the loss in 1.5 normal (1.4% NaCl) was 33%. The oxygen uptake varied only slightly in all concentrations except 3.0% in which the worms did not survive. The greatest amount of oxygen was consumed in the isotonic medium, 0.7% NaCl. In distilled water the average oxygen consumption was down 10%; in 0.4% and 1.4% NaCl it was down 2.5% and 0.5% respectively. The authors concluded that these data "show a direct correlation between weight change and oxygen consumption studies and helps to

establish the latter as reliable measures of osmoregulatory activity." It appears that H. medioplexus is relatively metabolically resistant to differences in environmental salt concentration (as is S. mansoni, Bueding, 1950).

Observations in this laboratory (Renn and McDaniel, 1973) show oxygen consumption in Z. lunata to vary in a direct fashion with changes in environmental salt concentration. Its consumption is 2.47 $cm^3O_2/gm$ dry worm tissue/hour in 0.5% NaCl, 2.0 in 1.0%, and 1.74 in 1.5%. This represents a variation of at least 30%. Zygocotyle lunata appears poikilometabolic while H. medioplexus maintains a relatively stable metabolism through considerable change in environmental salt concentration. It was this observation that indicated a possible difference in response between these two trematodes that indicated the possibility for my study. An effort was made to standardize the experimental procedures with those of Bair and Peters (loc. cit.) so that comparisons ultimately could be made between the osmotic behavior of H. medioplexus and Z. lunata.

Adult trematodes have a well developed carbohydrate metabolism that is almost wholly anaerobic. This information has been subjected to extensive review (see von Brand, 1966; Erasmus, 1972; Read, 1961, 1968; Smyth, 1966). Trematodes can utilize glucose and their endogenous carbohydrate reserve is glycogen. The glycogen content is usually high (1.6-30% dry wt., Cheng, 1973). The rate of carbohydrate consumption is similar under aerobic and anaerobic conditions. The rate is usually high but can be quite variable. Schistosoma mansoni utilizes

an amount of glucose equivalent to one-sixth to one-fifth of its dry weight in one hour (Bueding, 1950). The process of carbohydrate degradation is phosphorylative glycolysis and the initial steps follow those of the classical Embden-Meyerhof sequence.

Apparently Zygocotyle lunata carried out no water regulation under the conditions of these experiments. It behaved as an osmometer increasing in weight in hypotonic solutions and becoming elongate and turgid, while losing weight in hypertonic solutions and becoming shrunken and wrinkled. The data indicate that approximately 0.55% NaCl would be isotonic to this worm (Table I, Fig. 1). This is well below the blood and tissue fluids of its avian and mammalian hosts (approximately 1.0% NaCl). Goil (1966) found a similar situation for G. crumenifer, a trematode parasite of water buffalo in India, in several concentrations of sodium chloride (isotonic between 0.4 and 0.5%). However, F. hepatica and F. gigantica did not change weight in normal Hedon-Fleig or Tyrode's mammalian physiological salts media (Knox and Pantelouris, 1966; Siddiqi and Lutz, 1966). The frog lung fluke, H. medioplexus, did not change weight in 0.7% NaCl, which is also approximately isotonic with the fluids of the host (Bair and Peters, 1971).

Read and Simmons (1963) and Smyth (1969) point out there are serious pitfalls in accepting such variable environmental osmotic pressure data unequivocally. Animals living in a mixture of substances, as the intestinal contents or body fluids, are permeable to some of the constituents of the medium and not others. Therefore, the actual osmotic pressure of a solution as measured by physico-chemical means

may not be as significant to a worm as the actual content of materials to which the tegument of the worm is permeable. De Rycke (1972) pointed out that such data would indicate that many parasites are living at non-optimal osmotic pressures that undoubtedly result in sub-optimal development which may not always be recognized as such. He showed that the mammalian bile duct cestode, Hymenolepis microstoma keeps steady weight at freezing point depressions of 0.50 and 0.46° C. in saline and sucrose solutions respectively. However, these media could only be used during limited periods of time since the worms started to lose weight after 30 minutes in any dilution. De Rycke and Evans (1972) found that H. microstoma developed optimally in culture at a freezing point depression of 0.60° C. approximately, and quasi normally (their words) from 0.50 to 0.73° C. The higher and lower water content did not cause death but inhibited development.

The rate of weight change in Z. lunata was rapid in those concentrations of salt above and below isotonic. In those experiments that were continued for one hour the worms had equilibrated their weight within 30 minutes (Fig. 3). In comparative experiments, F. hepatica took longer to equilibrate, roughly 1 to 4 hours (Knox and Pantelouris, 1966), while F. gigantica was equilibrated within 15 minutes (Siddiqi and Lutz, 1966). These latter two experiments were both done in complex salts media yet show considerable difference in their effect on the rate of weight change in these worms.

Zygocotyle lunata contains a large amount of alcohol-extractable carbohydrate, consisting of glucose and at least one other alkali-stable and non-reducing sugar (Table I). The non-glucose fraction is undoubtedly the disaccharide trehalose, which is widely distributed among invertebrates. My method for recovering this fraction was essentially that of McAlister and Fisher (1972). Fairbairn (1958) reported glucose and trehalose to be the only free sugars in 71 species representing the major invertebrate phyla and including the trematode, F. hepatica. In Z. lunata the ratio of trehalose to glucose increases as the environmental salt concentration decreases. At the same time, the total amount of alcohol-extractable sugar is decreasing (Fig. 4).

Both fractions of the alcohol-extractable pool were recovered from the various incubation media (Table I, Fig. 4). Their presence there would indicate that both sugars leaked from the worms during incubation. The appearance of both fractions in the medium paralleled their occurrence inside the worms, which indicates the mechanism may be diffusion. The significance of carbohydrate leakage is unknown; however, von Brand (1966) states it is most likely that it is a pathological process in response to unphysiological in vitro conditions (see p. 26).

The total amount of alcohol-extractable carbohydrate inside and outside the worm decreased with time in all of the several concentrations of salt (Table I, Fig. 5). Both fractions of the pool are not

decreasing, however. The alkali-stable, non-reducing fraction (trehalose) decreases sharply during the first 30 minutes (in deionized water) and continues downward through the next 30 minutes more gradually (in 0.5%, 1.0% and 1.5% NaCl). Glucose continues to rise gradually over the 60 minutes. Thus, it can be inferred that it is the trehalose fraction that represents the greatest loss from the free pool under osmotic stress. Perhaps this pattern fits a reversible metabolic pathway from trehalose to glucose to glycogen. Trehalose represents a ready supply of glucose for energy metabolism and, perhaps, helps maintain a glucose gradient as in insects (see discussion in Cheng, 1973).

The storage polysaccharide, glycogen, shows a sharp increase as the sodium chloride content of the medium increases (Table I, Fig. 6). Both the 30 minute and 60 minute experiments show the increase although the former have considerable more variation. It appears that polysaccharide may be mobilized, for awhile at least, between 0.5 and 1.0% NaCl. When the amount of glycogen from worms in each saline medium is plotted with time at intervals between 15 and 60 minutes, all are seen to be increasing except in the 0.5% NaCl solution (Table I, Fig. 7). The reason for this is not readily apparent unless, perhaps, normal catabolic mechanisms do not function under osmotic stress. If this is the case, it can be assumed that the catabolic metabolism of Z. lunata functions normally in environmental solutions that are roughly isotonic but dysfunctions in hyposmotic and hyperosmotic media.

Comparisons between osmotic behavior experiments that were done using complex Ringer's-type solutions and those using simple monomolecular solutions (NaCl, sucrose, etc.) are, perhaps, impossible.

Certainly, it has been known for decades that simple sodium chloride solutions do not serve as adequate culture media for animals. Even more complex non-nutrient culture media are suspect in some cases. A number of observations show that cestodes leak carbohydrates even when maintained in complex solutions that should be physiologically satisfactory (see von Brand, 1966). However, my study does make possible some comparisons between Z. lunata and other trematodes treated in a similar manner.

Zygocotyle lunata contains an amount of alcohol-extractable carbohydrate and polysaccharide that relates closely with data obtained for other parasitic worms and trematodes (see von Brand, 1966). According to a recent tabulation, Z. lunata possesses a slightly greater amount of polysaccharide than other worms for which data are available (Cheng, 1973).

Zygocotyle lunata does not change weight in sodium chloride solutions between 0.5 and 0.6%, concentrations far below that of the fluids of its avian and mammalian hosts. However, it loses a considerable amount of alcohol-extractable carbohydrate to media at those concentrations (Table I). Similarly, Gastrothylax crumenifer, a parasite of water buffalo, does not change weight between 0.4 and 0.5% NaCl even when other molecules are added in concentrations approximating those of the fluids of the host (Goil, 1966). For Haematoloechus medioplexus, a lung parasite of amphibians, a 0.7% sodium chloride solution is isotonic. This fluke, then, does not change weight in a salt concentration approximating that of the fluids of its hosts (Bair and Peters, 1971).

Zygocotyle lunata leaked both fractions of its alcohol-extractable carbohydrate into the incubation media (Fig. 4). The worm contained less, and less entered the media as the salt concentration of the environment increased. It appears that instead of mobilizing stored polysaccharide for energy mechanisms in answer to osmotic stress, biochemical reactions that resulted in the greater production of polysaccharide were, in fact, uninhibited or stimulated (Fig. 6). If endogenous molecules were being increased or decreased inside the worm as a mechanism for osmotic balancing, it would be advantageous to form many small molecules under the conditions of these experiments. Further, the thermodynamics of reversible chemical reactions should flow toward the production of glucose and trehalose as these compounds diffuse into the environmental media and endogenous supplies decrease. However, locking up valuable molecules in storage products inside cells could be considered a short-term method of combating environmental osmotic change.

## SUMMARY

1. *Zygocotyle lunata*, like other trematodes that have been investigated, does not regulate its water content in hypotonic and hypertonic environmental salt concentrations. It gains and loses weight to a stable change in weight, at least, in solutions containing between 0.5% and 1.5% NaCl. Therefore, *Z. lunata* can be said to be an osmoconformer.

2. Observations on alcohol-extractable carbohydrate and stored polysaccharide in *Z. lunata* show them to respond in a linear fashion with changes in environmental salt concentration. Therefore, perhaps its sugar metabolism is related to the osmotic pressure of its environment.

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