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STUDY OF THE WATER BALANCE IN LARVAE OF *AESHNA CYANEA* (MÜLLER) BY MEANS OF MEASUREMENT OF CHANGES IN TOTAL BODY WEIGHT, WITH SPECIAL REFERENCE TO THE METHOD (ANISOPTERA: *AESHNIDAE*)

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As urine collection in the anisopteran larvae is very difficult, due to the respiratory function of the rectum, a study of the water balance is made by means of measurement of changes in the total body weight. A new, simple and accurate weighing method has been elaborated and tested. Drinking appears as an important factor in the regulation of water balance in larvae of *Aeshna cyanea* (Müll.).

INTRODUCTION

The water balance is one of the major problems in the study of the osmotic regulation in animals. In investigations on the water balance in larvae of *Aeshna cyanea* (Müll.), the fact that urine collection is quite impossible, due to the respiratory function of the rectum, raises a difficult problem. In spite of this, however, we succeeded in obtaining some interesting results by an analysis of the weight of normal specimens and also from animals which have been experimentally prevented from drinking during the time they spent in different experimental media. The fact that the only published method for the accurate weighing of aquatic animals is rather unwieldy, led us to improve a new technique which is more rapid, accurate and simple.

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MATERIAL AND METHODS

Only larvae of the last instar were used. Larvae in which imaginal characteristics had already become visible, were excluded, as they were in a stage approaching the last ecdysis (STRAUB, 1943). In order to avoid defecation during the experiments the larvae were starved for at least 3 days before and were not fed during the experiments. For all experiments a medium of continuously aerated tap- or diluted natural sea water was used. As cannibalism is not uncommon when no food is provided, the animals were kept strictly separated.

In the different experiments normal larvae and larvae which are prevented from drinking are used. To avoid any side effect resulting from a ligature placed around the neck (a possible deterioration in hormonal flow) it was decided to close the mouth with an inert dental cement. The anaesthetized larva (6 min. in fresh soda water) is fixed with the ventral side upwards. The mouth parts and ventral side of the head are carefully dried with filter paper in an air current. Dental cement (Ames The W.V.B. Ames C°, Fremont, Ohio) is applied and allowed to dry for 10 min. The larva is next placed in aerated tap water where it becomes active very rapidly.

In spite of the fact that the larvae can be dried easily, direct weighing is impossible, due to the unknown amount of water in the rectum. The only published method for the accurate weighing of aquatic animals (LOWNDES, 1942) avoids this problem. This method, however, which is based on the determination of the density and the volume of the animal, from which figures the weight is calculated is rather cumbersome. This led us to perfect a simple technique which is rapid and accurate. This method is based on the following principles. A vial, containing an exactly known volume of water is weighed; the animal is then placed in the vial which is weighed again. As the animal is not dried the difference between these two figures gives us the weight of the animal, increased by the weight of the water which adheres to the cuticula and which is present in the rectum. The volume of this unknown amount of water is then measured as follows: before placing the animal in the container, the larva is placed in a dye solution. After weighing, the concentration of the dye in the water in which the animal has been weighed is measured. From this figure it is possible to calculate exactly the amount of water which was brought into the container by the larva. Multiplying this volume by the density of the water which has been used in the experiment, the exact weight of the larva can be calculated by following simple formula:

$$W = G_2 - G_1 - (V \times D)$$

W = weight of the animal; G_1 = initial weight of the vial containing a known volume of water; G_2 = weight of the same vial after the animal has been put

into it; V = volume of the water which has been brought in with the animal; D = density of the water which has been used.

Procedure

To weigh *Aeshna* larvae, a flat-bottomed plastic vial (ϕ 2.5 cm) in which 10 cc of water is placed, is weighed. The larva is then put into a solution of 0.1 % Amaranth for 30 sec. Amaranth (Michrome N^{o} 37 Edw. Gurr Ltd.) is the dye of choice for it was found that it was not fixed to the tissues when used in the determination of the blood volume in insects (YEAGER & MUNSON, 1950; LEE, 1961). In order to prevent any dilution of the dye solution by the water which is brought in with the larva, the animal is subsequentlv allowed to stay 30 sec. in a second and third dish containing also 100 cc of the Amaranth solution. As the animals are used in experiments studying the water balance, any damage of the cuticula, which could result in a change of normal permeability, must be prevented. The tips of the forceps with which the animals are manipulated are therefore coated with Parafilm. The vial containing the animal is now weighed. After this, the volume of water, brought in with the larva, is determined by measuring the extinction of the water from the vial at 520 mμ. and comparing these figures to the extinction of known dilutions of the same stock solution of dye, in which the animal was placed before weighing. These dilutions were made by adding 5 to 40 cc dye solution to 1 l of water.

Evaluation of the weighing method

Table I gives the results when the same larva was weighed five times. From these figures it becomes clear that the method is very accurate, the standard errors being lower than 0.2%.

This method is applicable to animals from fresh water and sea water, the stock solution of Amaranth being made with the same water as the medium in which the animals are allowed to stay. The accuracy depends on different factors which can easily be controlled, such as the precision in measuring the 10 cc of water which is present in the vials; the cleanliness of the forceps which are used to manipulate the animals. As mentioned above, the original dye solution in which the animal to be weighed, is allowed to stay 30 sec. is slightly diluted. However, as the animal is passed three times into another dye solution, we calculated that the dilution of the third solution is about 3.10^{-6} mg%, which is in fact negligible.

Table I

Testing the weighing method (The same larva is weighed five times; – $\frac{\Sigma X}{n}$: mean, s: standard deviation, s_x: standard error; for other abbreviations cf. text)

Larva 1

G_1	G_2	V.D.	W.	
23,1741	24,6983	0,3508	1,1734	$\frac{\Sigma X}{n}$ = 1,1721
23,2222	24,7261	0,3344	1,1694	
23,2367	24,7427	0,3352	1,1708	s = 0,0027
22,9011	24,3469	0,2760	1,1698	
23,1396	24,6519	0,3352	1,1771	s_x = 0,0012

Larva 1, 24 h. later

23,4503	24,9185	0,3015	1,1667	$\frac{\Sigma X}{n}$ = 1,1702
22, 9877	24,4626	0,3085	1,1709	
23,0695	24,5650	0,3245	1,1710	s = 0,0023
23,0040	24,4903	0,3175	1,1689	
23,2112	24,7612	0,3764	1,1736	s_x = 0,0013

Larva 2

G_1	G_2	V.D.	W.	
23,4162	24,7710	0,2775	1,0773	$\frac{\Sigma X}{n}$ = 1,0742
23,1959	24,5343	0,2654	1,0730	
22,9368	24,2267	0,2171	1,0728	s = 0,0034
23,1479	24,5725	0,3547	1,0699	
23,1448	24,5664	0,3434	1,0782	s_x = 0,0015

RESULTS

A first group of 5 mouth sealed larvae is placed in tap water. The animals are weighed before and after sealing their mouths and then every 24 hours. Each weighing is repeated three times. The results are shown in Figure 1A. The change of weight is expressed as mg $^{o}/_{oo}$, i.e. mg pro g. body weight. From this it is clear that no appreciable change of body weight occurs. The total osmotic pressure of the hemolymph (170 mM NaCl/l) is a little lower as in normal animals (180 mM NaCl/l).

Figure 1B shows the results of a second experiment during which a group of 3 normal individuals and 3 larvae, prevented from drinking, are kept in a hypotonic medium of diluted sea water (osm. press.: 119 mM NaCl/l). In the normal larvae the body weight stays constant, the hemolymph osmotic pressure is normal: 185 mM NaCl/l. In mouth sealed larvae the weight decreases regularly until the 50th hour, however, when it stays constant. The mean osmotic pressure of the hemolymph of these animals has markedly increased: 224 mM NaCl/l.

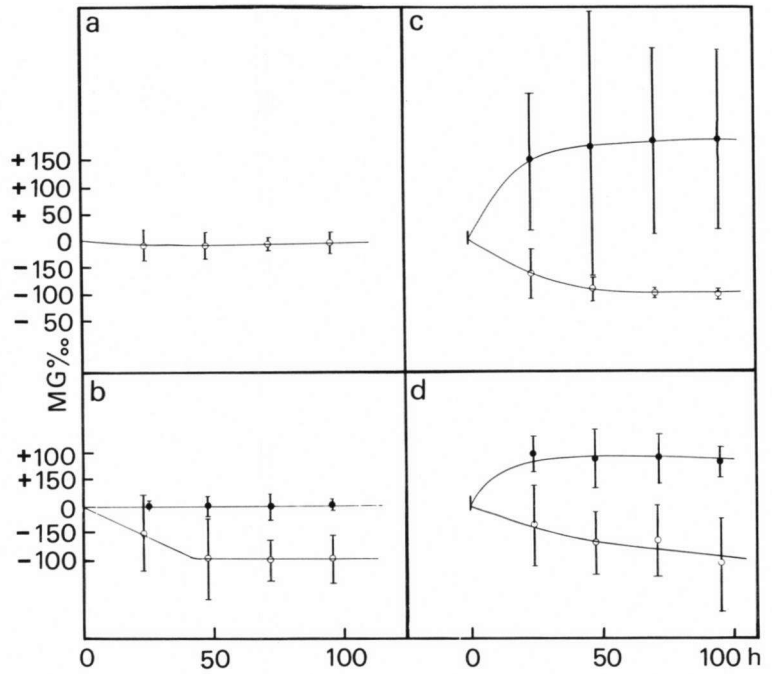

Fig. 1. Changes in total body weight in larvae of *Aeshna cyanea* (Müll.) during the time they spent in different external media, viz.: (a) Tap water; – (b) Diluted sea water, O.P.: 119 mM NaCl/l; – (c) Diluted sea water, O.P.: 235 mM NaCl/l; – (d) Diluted sea water, O.P.: 304 mM NaCl/l. The change of body weight is expressed in mg %. (Closed circles: normal animals; – open circles: mouth sealed larvae. Mean values ± 2 s).

In the other two experiments two groups of 3 larvae (one normal, the other mouth sealed) are exposed to a hypertonic external medium of diluted sea water.

Figure 1C shows the results for ± 1/2 sea water (O.P. = 235 mM NaCl/l). The weight of the normal larvae increases to a constant level which is reached after app. 70 hours. The osmotic pressure of their hemolymph has become slightly hypertonic: 251 mM NaCl/l. In the mouth sealed larvae there is a marked decrease of weight to a constant level: the hemolymph osmotic pressure is slightly higher than in the normal individuals: 262 mM NaCl/l. The last experiment offers analogous results. The external osmotic pressure is 304 mM NaCl/l (Fig. 1D). The normal larvae show an increase of weight whereas in the mouth sealed larvae the total body weight decreases. There is no marked difference between the hemolymph osmotic pressure of both groups, they are slightly hypertonic: 316 mM NaCl/l in normal larvae, 317 mM NaCl/l in mouth sealed individuals.

DISCUSSION

The effect of starvation on the osmotic pressure of the hemolymph is very different according to the species of aquatic insects. In larvae of *Aeshna cyanea* there is no change in hemolymph composition nor osmotic pressure after starvation for 240 h. in tap water (personal results which will be published later).

As shown by the first experiment (Fig. 1A) there is no evidence of drinking in these animals. It can be stated that the osmotic influx of water is compensated by the excretory system, whereas the outflux of salts, due to diffusion, is compensated by an active absorption, probably by the basal pads of the rectal gills (KOCH, 1934; KROGH, 1939). Larvae of *Chironomus thummi* do not drink in tap water either (HARNISCH, 1934), whereas *Notonecta glauca* and *N. marmorea* are obliged to drink in the same conditions as the water outflux, due to excretion, is only partially compensated by osmotic influx (STADDON, 1963). Larvae of *Limnephilus affinis* (SUTCLIFFE, 1961) and larvae of several species of Diptera (WIGGLESWORTH, 1933; SCHALLER, 1949) do not drink when kept in tap water. In larvae of *Aedes aegypti*, a continuous intake of a certain amount of external medium occurs, as the larvae are filter feeders (STOBBART, 1971). In these animals however, a marked increase of body volume is observed when neck-ligatured animals are kept in tap water.

When our larvae are allowed to stay in a hypotonic medium of diluted sea water the animals are obliged to drink. This is clearly shown by the difference between the two experimental groups (Fig. 1B). In normal larvae the weight and hemolymph osmotic pressure stay constant; in mouth sealed larvae the decrease of the body weight and increase of the hemolymph osmotic pressure led us to suppose that under these conditions the larvae must drink. The fact that after a certain time, the weight of the animals, prevented from drinking, stays constant, suggests that their higher osmotic pressure leads to an increased water entry which again compensates the excretory outflux. These results compare favorably with those obtained by STOBBART (1971) for larvae of *Aedes aegypti* when these are kept in an isosmotic sucrose solution.

In hypertonic media our normal larvae are observed to drink large amounts of water at the start of the experiment. This results in a marked weight increase during the first 48 hours. The increase stabilises after 80 hours.

Animals prevented from drinking show a remarkable initial loss of weight which also stabilises around the 80 hour period. Increased drinking is brought about by dehydratation due to the hypertonic external medium. When the hemolymph becomes slightly hypertonic to the medium there is no further change in weight. It is probable that a fall in urine production takes place due to the fact that water uptake by osmosis is lower. In *Sialis* larvae SHAW (1955) demonstrated, by ligature experiments, that urine production depends on the osmotic water influx. As demonstrated by STOBBART (1971) in larvae of *Aedes aegypti* the

regulation of the body volume and urine production are closely related. In these larvae an amount of the fluid, produced by the Malpighian tubules, is moved forward in the midgut by retroperistaltic movements of the pyloric chamber and reabsorbed in the hemolymph. As stretch receptors regulate these retroperistaltic movements, the rate of urine production depends partially on the body volume, which in turn depends on osmotic water inflow.

The fact that, as mentioned above, a marked increase of body volume occurs in the neck-ligatured larvae of *Aedes aegypti,* when these are kept in tap water, can be explained by the supposition that the information from the stretch-receptors can not reach the head region, the ligature interrupting the nerve cord (STOBBART, 1971). As only normal and mouth-sealed larvae were used in our experiments we have no information as to the existence of a similar mechanism in larvae of *Aeshna cyanea.* That other factors are involved in the body volume regulation is suggested by the observation that body weight increases to a constant higher level in normal larvae when they are kept in hypertonic media. Finally the fact that in normal individuals the weight stays constant at a higher level when they are allowed to stay in hypertonic media, is due to an increased hemolymph volume in these larvae (personal results).

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