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Some experiments on the osmoregulation and respiration of Eristalis larvæ.

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The fly larvæ belonging to the genus *Eristalis* (and allied genera) are adapted to a very peculiar environment, living as they do in water and mud charged with organic impurities and generally devoid of dissolved oxygen. The reaction of the water may vary between wide limits, since it will sometimes contain appreciable amounts of free ammonia and at other times an excess of CO_2 and methane.

From the beautiful researches of Réaumur (1738) it is well known that the larvæ breathe air from the atmosphere which they reach from depths up to 10 cm by a telescopic tail-like appendage (Queu de rat, Réaumur) bearing spiracles at the tip, but their respiratory mechanisms are still rather obscure, and this applies especially to the beautiful set of "gills" which occasionally protrude from the anus and make beating movements in the water, about once per second, for several seconds. There is a great deal of disagreement between the writers dealing with these animals, concerning both the time of appearance and the function of these organs.

Réaumur clearly understood that they are forced out by blood pressure and states that they come out only when the larvæ defecate.

Trybom (1875) describes 10—12 filaments on each side of the anus. He saw the retractor muscles and the fairly rich supply of tracheæ and says that the filaments are often put out when a larva is sinking down through the water. They will then make powerful movements and act as an organ of swimming. Possibly they may function also as tracheal gills.

Chun, writing at about the same time (1876), considers them as glands acting within the hind gut, but admits it as very probable that they also function as gills when protruding. "Zumeist konnte ich sie bemerken, wenn die Larven in reines Wasser gebracht wurden".

Wahl (1900) gives an elaborate description of the tracheal system in *Eristalis*, without, however, paying any attention to the fact that certain elements in it and especially the very large tracheal sacs have an elliptic cross section and are easily compressed by contraction of the cutaneous musculature to flat bands, thereby effecting an expiration. Within the anal filaments Wahl observed not only the system of tracheæ with spiral thread, but also a network of tracheal capillaries branching out from the "end cell". He does not doubt the function as tracheal gills. He once saw them come out simultaneously in a large number of larvæ in very foul water.

Wesenberg-Lund (1915) also considers the filaments as gills and states that they are utilized by the larvæ when the water contains a minimum of oxygen.

Alsterberg (1934) in a paper specially dealing with the anatomy and biology of *Eristalis* larvæ denies that the "gills" are ever brought out when the larva is not in contact with the atmosphere through the spiracles at the end of the "tail". He is positive that the habitat of the larvæ is very often oxygen free and comes to the conclusion that the gills serve for the eli-

mination of CO_2. In my book: Comparative Physiology of Respiratory Mechanisms (Philadelphia 1941) I expressed doubts regarding this function.

It can be stated as a summary of this account that the anal filaments are protruded very occasionally for short periods at a time and apparently without any fixed correlation with oecological conditions. I agree with Alsterberg that they cannot be of any use for the absorption of oxygen, also because the surface area is too small, but the idea that they take over, at least partly, some elimination of CO_2 is worth an experimental test.

The experiments to be described in the following were started on the assumption that the anal filaments might bear some relation to the osmoregulation of the animals, like the anal papillæ of Chironomid larvæ, and contain a mechanism for active ion uptake. The experiments were made mostly on material kindly supplied by professor K. Berg from the neighbourhoods of Hillerød and Suserup respectively, while a small number of larvæ were taken by myself in Halsnæs. I have not attempted to identify species, but I believe that most of the material and probably the whole belongs to a single species, and it certainly shows very uniform reactions. One specimen was reared to the imago stage and identified by Mr. S. L. Tuxen as *Eristalis arbustorum* L. I shall number the experiments I to X, but in reality each experiment described is representative of a small series which were usually slightly varied, but gave substantially the same results.

The first experiment started was to try and wash out salt from a small number of larvæ by keeping them in slowly running distilled water. This they would stand for a surprisingly long time.

7 larvæ, weighing 60—100 mg each, were put into ca 10 cc distilled water, renewed every 3—6 minutes.

On 6' day 1 larva dead, probably during moulting.

On 12' day 5 living larvæ, 3 have the filaments protruding. These are taken out and used for a special experiment, as described below.

On 14' day blood is taken from the 3 remaining larvæ for determination of the osmotic concentration in comparison with fresh larvæ*). These latter showed concentrations corresponding to about 135, 130 and 120 millimol (mM) NaCl. The 3 which had been treated showed 110, 105 and <50 respectively. It is assumed that the last one has brought out the filaments and lost a large quantity of salt through them.

The slight loss shown by two larvæ, amounting to some 20 mM, is remarkable and suggests that the integument is probably almost impermeable to water. Into most fresh-water animals water penetrates osmotically at a fairly rapid rate, because the internal concentration is much higher than outside. This water is again excreted as urine and, since the urine cannot be made salt free, a continuous loss of salt is unavoidable and is normally made good by active absorption of ions. If the anal filaments of *Eristalis* are such organs their protrusion in distilled water will cause an increased loss. Almost all fresh-water organisms will lose salt at a fairly rapid rate in distilled water and many have been shown to take up salts from ordinary fresh water (Compare Krogh 1939).

In exp. II 5 larvæ were tested for water permeability by means of heavy water (Krogh and Ussing 1937). When a larva is placed in about its own volume of water containing D_2O any diffusion into the larva will diminish the concentration of D_2O in the surrounding fluid. The results of determinations, kindly made for me by dr. H. Ussing, are summarized in table I.

*) The method was a modification of that of Ursprung and Blum (1930) and will be described elsewhere.

Duration hours	Weight Larva mg	Weight water mg	Conc. of D_2O % initial	Conc. of D_2O % final	Conc. decrease %
1	103	200	2.035	2.048	— 0.013
1	177	200	2.035	2.048	— 0.013
6	137	200	2.035	2.016	+ 0.019
24	127 (2 larvæ)	200	2.035	1.596	+ 0.441

The first 3 changes are inside the limits of error of the determination and indicate impermeability for water. The fourth shows a definite decrease and may probably be explained by protrusion of the anal filaments for a brief period.

Impermeability for water generally means impermeability for all substances dissolved in water and insoluble in fatty substances and this, of course, will protect the larvæ against many harmful substances. Wesenberg-Lund states that they will stand concentrated mercuric chloride for more than 24 hours. I have found that they will live indefinitely in water alkaline from ammonia at p_H 10 or acid from HCl at p_H 3. From one natural habitat prof. Berg reports to me a p_H between 9 and 10, while another was about neutral.

In a fairly large number of aquatic arthropods special organs have been demonstrated which will stain brown or black when the living animals are treated with dilute silver nitrate. H. Koch in 1934 put forward the suggestion that these were the organs responsible for active salt uptake, and in the few forms tested (Koch 1938) this suggestion was found to be correct or at least extremely probable. The decisive proof was brought by K. Schmidt-Nielsen (1941) who placed crayfish in so dilute $AgNO_3$ that even AgCl would remain in solution. This solution is 0.01 mM and contains about 1 mg silver per liter. After 24 hours a certain part of the gills would have reached a considerable concentra-

tion of silver. So long as the animals were kept in the dark this would be invisible, but when the gills were exposed to light the silver compound would be reduced and the gills stained by metallic silver.

That the uptake of silver is a vital function, depending upon oxidation processes, was shown by the fact that no uptake took place in the absence of oxygen. We must assume that silver, which is chemically related to the alkali metals, is taken up by the same osmoregulatory mechanism which deals with these, but becomes chemically combined within the active cells which are presumably thereby blocked.

In exp. III it was attempted to stain the anal filaments of *Eristalis* larvæ by treatment with 0.01 mM $AgNO_3$.

Larvæ which had their filaments more or less permanently protruding and were usually rather weak, including the 3 from exp. I, were put into $AgNO_3$, usually in the dark and afterwards exposed to light. Those which were obviously weak at the start died without becoming stained, and it has been definitely established that after death no uptake of silver occurs from this very dilute solution. Two which were more lively became stained, one just visibly brown when dead after 24 hours; the other, still living after a few hours, slightly brown and when dead after 20 hours definitely brown.

More definite results were obtained by exposing larvæ for a long time to 0.01 mM Ag in distilled water, and in the final test this was done by keeping a considerable number in the dark in a flat basin with 1 cm cotton wool just covered with the solution which flowed through at the rate of 10 l in 24 hours. As the filaments of some individuals are of a light yellow colour, as observed through the integument, care was taken to select specimens in which they were invisible. Some

darkly pigmented larvæ were included, because they had been seen with outstretched filaments which also were unpigmented. Many larvæ pupated during the experiment and could not yield any information.

After 6 days several unpigmented larvæ showed filaments definitely brown when seen through the integument, and two were found dead with filaments protruding and, after exposure to light, of a dark brown colour.

After 9 days the filaments were forced out by pressure from 3 darkly pigmented larvæ after they had been exposed to sunlight. One of these had the filaments quite clear, one light brown and one dark brown, especially the tips. In most of the clear larvæ the filaments as seen through the integument were definitely brown, but in a few they had remained clear.

There can be no doubt therefore that the filaments when protruded take up and concentrate silver from extremely dilute solution, and by analogy with a number of other arthropods this is taken as an indication — but not a definite proof — that they can act as osmoregulators by active absorption of salts.

When the integument is practically impermeable to water the need for salt absorption cannot be considerable and may be present only just after a moult when the size of the organism is rapidly increased. It is conceivable therefore that the anal filaments may have other functions besides the hypothetical, but probable, one of absorbing salts, and it becomes worth while to test the one suggested by Alsterberg viz. the elimination of CO_2. The first step was to test the permeability of the integument (excluding the filaments) to O_2 and CO_2. When it is impermeable to water the permeability to gases may possibly be very low or even absent.

In exp. IV one larva (61 mg) was put in a closed vessel in 7 cc distilled water saturated with air (6.3 cc

O_2/liter at 20.5°) and after a suitable time a sample of the water was analysed for O_2. In one period of 0.75 hour 1.75 mm³ O_2 disappeared out of a total of 44 mm³. In a second period of 2.2 hours 5.45 mm³ disappeared. These figures correspond to an uptake of 2.3—2.5 mm³/hour, or a very low permeability.

The oxygen pressure inside the animal must have been zero during most of the time. The surface area of the larva was about 100—150 mm². Assuming arbitrarily that the layer of low permeability is 0.01 mm thick and taking the pressure difference as 20 % of an atmosphere the diffusion constant works out at about 2×10^{-7} or less than 2 % of that for chitin (Krogh 1919).

In a similar exp. V CO_2 was determined in the water before and after a larva of 112 mg had been enclosed for 1 hour. The CO_2 given off amounted to 28.4 mm³/hour. The internal CO_2 pressure is unknown, but there can be no doubt that the larval integument is several times more permeable to CO_2 than to oxygen, but even so the permeability must be considered as very low.

Even after 2 hours without access to the atmosphere the larvæ are in good condition and most of the CO_2 eliminated is produced anaërobically, as will appear from determinations given below.

Exp. VI. In order to find the relation between the CO_2 eliminated in exp. V and the normal metabolism this was determined roughly in a micro respirometer and found to be about 250 cc O_2/kg/hour, increasing in an active animal to double this figure or more. This corresponds to 25 mm³/hour in an animal weighing 100 mg, and unless the ventilation of the tracheal system is excessive we shall expect a considerable fraction of the CO_2 produced in the metabolism to diffuse out through the integument to the water, even without any participation by the anal filaments.

Alsterberg found that by putting the animals in water saturated with CO_2 he could bring about a protrusion of the "gills"; if I understand him rightly, after the CO_2 treatment. I have varied this procedure in several ways, but without any success, and the most convincing conclusion is reached from respiration expe-

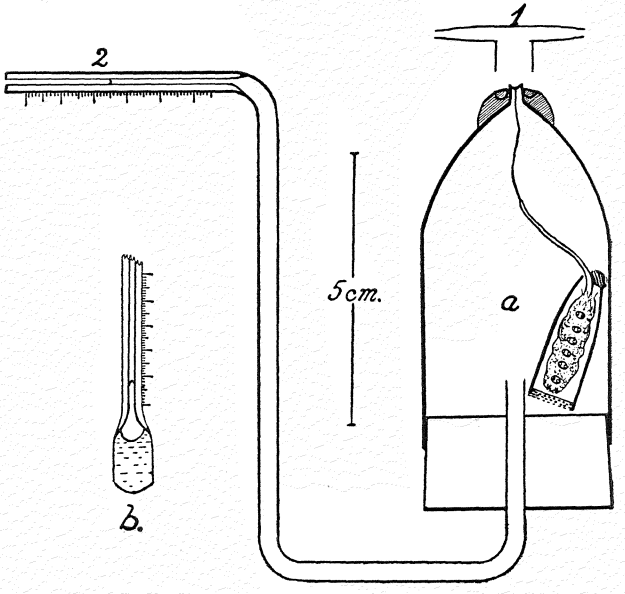

Fig. 1.

riments with various gas mixtures. If Alsterberg is right breathing of mixtures with a high CO_2 percentage should cause protrusion of gills, while I find that instead an increased tracheal ventilation is brought about.

Exp. VII was made by means of the arrangement shown in fig. 1 a which is a slight modification of a similar apparatus formerly employed (Krogh 1920). The spiracles of the animal come in contact with the atmosphere in a very small opening. The water meniscus here is scarcely displaced by the respiratory move-

ments, but these cause the meniscus in the horizontal graduated tube (2) to move back and forth, and the volume of each respiration can be read or, if desired, recorded. *Eristalis* larvæ are so restless and have such a strong urge to creep into any narrow openings that it is necessary to enclose the experimental animal in a cage made from a piece of glass tubing, closed at one end with gauze and at the other, after the animal has crept in, almost closed with a small lump of plasticine, but leaving just enough space for the breathing tube. The cage must be so narrow that the enclosed animal cannot turn round. Atm. air is breathed directly from the meniscus, but when the animal is to be exposed to other gas mixtures these are passed in a slow current through the T tube (1), fixed on top of the apparatus by a small lump of plasticine.

An animal weighing 153 mg was first allowed to breathe ordinary air for 15 minutes. During this period the respiration was fairly regular with about 4 breaths per minute of 5 mm^3 each. As in other insects the expiration is active and fairly rapid, the inspiration passive and, on account of the resistance in the long breathing tube, quite slow. At the equilibrium position of inspiration an *Eristalis* larva is lighter than water when the cutaneous musculature is completely relaxed, but slightly heavier than water when it shows a normal tonus.

The animal was now given pure oxygen. The frequency became reduced to 2 and the depth also. After 18 m. the respiration ceased entirely for 2 m. During a pause the spiracles may remain open, and if so the meniscus by which the movements are measured remains quiescent. When the spiracles are closed a regular diminution in volume is observed, due to the absorption of O_2. Reëstablishment of connection with the atmosphere brings about a volume increase followed by an expiration.

Atm. air was now given. The ventilation remained low and irregular for 10 m. Thereupon the animal struggled, with numerous small respiratory movements. After the struggle the respiration again became regular, but very slow, 1 per min., and somewhat deeper, 6 mm^3. This appears to be the most common type of breathing when the animal remains in undisturbed connection with the atmosphere.

In almost pure nitrogen there was first a series of very deep respirations, up to 10 mm^3, but then the respiration stopped almost completely. This observation was repeated several times.

In nitrogen, containing about 4.5 % O_2, on the other hand the breathing became after 1 m. deep and regular, averaging 8 mm^3 in depth 4 times per minute.

A mixture of 10 % CO_2 in air may cause the respiration to cease completely for up to a few minutes, but when it starts again it soon becomes fairly regular and slightly deeper than with atm. air, and this effect persists for some time after the medium has been changed to ordinary air. These reactions are even more pronounced with 20 % CO_2 which will gradually increase the frequency to 4 per min. and the depth to 9 mm^3 and maintain an increased ventilation for at least 10 m. in ordinary air.

This experiment shows conclusively that both oxygen lack and CO_2 excess can act as respiratory stimulants, but CO_2 excess begins by inhibiting respiration and shows a definite stimulating after effect; while the effect of a relative oxygen lack is immediate and ceases simultaneously with the stimulus. No effect of CO_2 upon the anal filaments has been observed in experiments on five animals.

Exp. VIII. By means of the arrangement shown in fig. 1 b, put on instead of the T tube, an *Eristalis* larva can be made to breathe from a bubble of gas of any

desired composition and volume, and the bubble can be withdrawn into the graduated tube and analysed by a technique described many years ago (Krogh 1904).

A few determinations were made in which a larva, after being cut off from the atmosphere for about ½ m. was allowed to breathe from an air bubble for 40 sec. In a typical determination the final composition of the bubble would be 4 % CO_2, 14 % O_2 and 82 % N_2. If this is the normal composition of the air in the tracheal system the animal, when ventilating at the rate of 6 mm^3/min., would use up about 0.4 mm^3/min., corresponding to 200 cc/kg/hour.

The apparent respiratory quotient is very low, because a large fraction of the CO_2 is given off through the skin to the water.

When now such a larva is cut off from the atmosphere for 3 m. and then allowed to breathe for a few m. from a bubble of oxygen a certain quantity of nitrogen will afterwards be found in the bubble, and if a complete mixing with the gas in the tracheal system is assumed it becomes possible to calculate the volume of that system. In one such case a larva of 204 mg. was given an O_2 bubble of 27.3 mm^3. After 6 m. the volume was reduced to 13.2 mm^3 containing 5.2 % CO_2, 46.6 % O_2 and 48.2 % N_2. Taking the initial N_2 percentage in the tracheal system as 82 a calculation gives the tracheal volume 18.9 mm^3 or 9 % of the animal volume. ½ of this can be renewed by a deep breath. The tracheal system is therefore very effective, and no accessory respiratory mechanism is likely to be of any use, the more so as the fluid in which the larvæ live is generally irrespirable.

Tracheal volumes can be determined also by experiments utilizing the fact that the pressure of a gas quantity multiplied by its volume at that pressure remains constant when the pressure is varied. To do this a larva

was in exp. IX sealed up in a small glass vessel, filled with water and provided with a horizontal tube of narrow bore in which the displacement of the water meniscus could be read off under a low power microscope. The tube was connected with an arrangement to apply pressure which could be read on a mercury manometer. It is necessary to allow for the water vapour tension and there is also a small correction for the compressibility of the water in the container.

In one determination we had the reduced barometric pressure of 736 mm and an increase of 96 mm produced a volume reduction (corrected) of 2.06 mm^3. Assuming that the tracheal walls offer no resistance to the compression the tracheal volume x can be calculated from $736\ x = (736 + 96)(x - 2.06)$ or $x = 17.8\ mm^3$ in a larva of 122 mg.

The assumption made can be verified by a series of determinations at increasing pressures. If there is a resistance it must increase with the compression and a decreasing volume will be found. Determinations with 191, 287 and 381 mm excess pressure gave the values 17.8, 18.1 and 17.7 mm^3. At the last named pressure the volume of the tracheal system was reduced by 6 mm^3, and further experimentation showed that it could be reduced by 10 before any definite resistance appeared. This shows that the ventilation tracheæ had at least this volume and offered practically no resistance to compression.

When a larva is kept enclosed in such a pressure chamber without access to the air the volume will at first show a decrease, because oxygen is used up, but it will soon begin to increase again, and the larva becomes lighter than water. This agrees with an observation first made by Alsterberg and repeatedly confirmed by myself: When a larva is kept in water, so deep that the breathing tube cannot reach the surface,

it will float up to the surface after a period of $^1/_2$ to 2 hours. It will breathe there for some time and may then sink down again and repeat the performance. It follows from these observations that some gas is accumulated in the tracheal system when the animal is cut off from the atmosphere and the nature of this gas must be ascertained.

Alsterberg attempted to show by calculations that the gas in question must be nitrogen, diffusing in from the water, but this contention is untenable, because the negative pressure necessary for such diffusion and postulated by Alsterberg does not and cannot exist, when the air sacs are so easily compressed as I have found. Moreover I have seen the volume increase in a larva, enclosed in a very small compression vessel, much more than all the nitrogen dissolved in the surrounding water could account for. There can be no doubt therefore that the gas is produced within the animal, and I can find only two possibilities. One is a production of CO_2 by the metabolic processes going on after deprivation of oxygen, and the other is a production of methane + CO_2 by bacterial fermentation within the gut. The second possibility can be realized only when the animals are feeding and the gut filled with mud, while the first can act also in starving animals. Starving animals will float up to the surface just as well as animals having mud in the gut, and CO_2 production must therefore be taken to be the normal mechanism for increasing the buoyancy.

This is borne out by a respiration experiment X in which a larva was brought in contact with an air bubble after 1.5 hours stay in 5 cc water. After a couple of respirations this bubble was analysed and found to contain 20.7 % CO_2 and 3.5 % O_2. The CO_2 content in the tracheal air must have been higher and perhaps even 40 %. In a large body of water a considerable

proportion of the CO_2 formed must diffuse out through the integument, but enough is left evidently to cause the animals to float.

A bacterial formation of methane in the gut appears quite possible, since methane formation in the mud which the animals devour is an extremely common occurrence, and the gas, when formed, will no doubt diffuse into the tracheal system, just as the methane produced in the paunch of cows finds its way into the air expired from the lungs, but no definite evidence that such formation actually takes place in the larvæ has been obtained. In one observation in a sealed vessel the gas production amounted to 15.6 mm^3 in 5 hours which makes a bacterial methane production rather likely. On the other hand no gas production was found in one exp. with isolated feces from several larvæ in a sealed vessel.

Summary.

The biological conclusions to be drawn from the preceding experiments and observations are briefly as follows.

The *Eristalis* larvæ are protected against noxious influences from the surrounding medium by a highly impermeable integument. The practical impermeability to water affords protection against loss of salts, and the animals can live in a salt free fluid or in highly acid or alkaline solutions for prolonged periods.

Respiration takes place only from the atmosphere through the posterior spiracles. There is an effective mechanical ventilation of the large air sacs which offer only the slightest resistance against compression to flat bands. A certain proportion of the CO_2 produced in the metabolism will diffuse out through the integument. Practically no oxygen is lost to the medium, although this is as a rule oxygen free.

The filaments which are occasionally, and usually for brief periods only, made to protrude from the anus have no respiratory function whatever. They contain a mechanism which will actively absorb silver from an extremely dilute solution and is probably concerned with osmoregulation by active ion uptake, but even this does not seem to be of much use to the animal.

When the larvæ are cut off from the surface the metabolism becomes anaërobic and part of the surplus of CO_2 produced accumulates in the tracheal system and may cause the larvæ to float up to the surface. This is facilitated by relaxation of the musculature. When the muscular tonus is normal and the spiracles in contact with the air the larvæ are slightly heavier than water.

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Dansk Oversigt.

*Eristalis*larver er beskyttede mod mange skadelige paavirkninger fra omgivelserne ved at deres hud er næsten fuldstændigt uigennemtrængelig for vand og vandopløselige stoffer. Herved beskyttes de ogsaa vidtgaaende mod salttab, og de kan leve længe saavel i destilleret vand som i stærkt sure og basiske opløsninger.

Aandedrættet finder udelukkende sted fra atmosfæren gennem spiraklerne i spidsen af den lange „rottehale". De store luftsække i kroppen ventileres mekanisk og lader sig ved udaandingen let sammenpresse til flade baand. En del af den ved stofskiftet dannede kulsyre diffunderer ud gennem huden, men der sker ikke ad den vej noget kendeligt ilttab, skønt dyndet, hvori de lever, i reglen er iltfrit.

De traadformede legemer, som lejlighedsvis og i reglen kun kort tid ad gangen presses ud fra endetarmen, har ingen gællefunktion. De indeholder en mekanisme, som aktivt optager sølv fra yderst tynde opløsninger og fungerer antagelig osmoregulatorisk ved aktiv ionoptagelse.

Naar larverne ikke kan naa overfladen, bliver stofskiftet anaërobt. En del af den derved dannede kuldioxyd ophobes i trachesystemet og bringer dyrene til at flyde op til overfladen.