The Association of a Slime Bacterium with the Inner Envelope of the Egg of *Dytiscus marginalis* (Coleoptera), and the less common Occurrence of a similar Bacterium on the Egg of *D. semisulcatus*

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With two plates (figs. 1 and 4)

SUMMARY

The egg of *Dytiscus marginalis* L. is laid under water in the mesophyll of the leaf of various aquatic plants. The egg increases in size during development and this results in the splitting of the chorion, the outer envelope of the egg, usually into two halves. The inner envelope of the egg, the vitelline membrane, then forms the only complete shell of the egg in its later stages.

During the development of the embryo of *D. marginalis* the cysts of a slime bacterium (*myxobacteriale*) appear in great numbers on the vitelline membrane along the dorsal surface of the embryo and around each end of the egg. They are at first small and scattered, but by the time the larva hatches they are present in dense colonies. These bacterial cysts occur on eggs collected from water plants in ponds; but they develop just as readily on eggs obtained in the laboratory by placing the female *D. marginalis* in jars of tap-water with leaves that have never before been immersed in water.

Slime bacteria in the vegetative stage are carried in great numbers by the female *D. marginalis*, as has been shown by trailing the apex of the abdomen of a living beetle over an agar culture, when swarms of the characteristic rod-like cells have been obtained. Some of these bacteria will be transferred from the beetle to the egg during oviposition.

The bacterial cysts do not occur on unhealthy eggs and they rarely show normal development on eggs parasitized by the mymarid, *Caraphractus cinctus* Walker. This suggests that their development is dependent upon the healthy growth of the *Dytiscus* embryo.

In the egg of *D. semisulcatus* Mull. the chorion remains in close contact with the vitelline membrane throughout embryonic development and only occasionally shows small irregular cracks before hatching. On some eggs of this species a few scattered cysts of slime bacteria occur on the vitelline membrane. On occasional eggs they were more numerous, but they were never observed to be present in sufficient numbers to form the even covering which is so striking a feature of certain parts of the egg of *D. marginalis*.

INTRODUCTION

FOR some years I have been breeding the mymarid, *Caraphractus cinctus* Walker, on the eggs of various water beetles of the genera *Agabus*, *Ilybius*, *Colymbetes*, and *Dytiscus* (Jackson, 1958). I have thus had occasion to examine the eggs of all these species closely at all stages of their development and I have been surprised to find that, in eggs of *D. marginalis* in which the embryo
was well developed or from which the larva had hatched, minute greenish spheres were almost invariably to be found in great numbers in any microscopic preparation made of the egg envelopes (fig. 1, A, B). The minute spheres were restricted to certain parts of the inner membrane, the vitelline membrane, and they never occurred on the chorion. It was at first thought that these objects were algae, but they have now been identified by Dr. J. W. G. Lund and Miss V. G. Collins as cysts of one of the slime bacteria of the order Myxobacteriales.

These bacterial cysts occurring on the *Dytiscus* egg do not appear to be comparable with the various micro-organisms, usually believed to be symbionts, which occur intracellularly or intercellularly in a variety of insects, and which are transmitted from generation to generation in various ways (Buchner, 1953). Thus, the bacteria studied by Gier (1936) in roaches, and termed by him bacteroids, are present in certain cells (mycetocytes) in the fat-body. The bacteroids also occur in the ovary, between the oöcyte membrane and the follicle cells, and they later enter the egg cytoplasim. They retain their position against the vitelline membrane after the egg is laid, and, as the embryo develops, they move to the centre of the yolk. The slime bacterial cysts of the *Dytiscus* egg, on the contrary, have never been seen within the egg. They have only been found upon the vitelline membrane, where they appear during the later stages of embryonic development, and they persist on this membrane after the larva has hatched. In a variety of beetles which harbour yeasts or bacteria internally, the eggs become smeared with micro-organisms during oviposition, and the larvae on hatching eat a part of the contaminated shell and so become infected (Buchner, 1953). This could not happen with the *Dytiscus* larva since the larva is not in contact with the vitelline membrane before hatching, being completely enclosed in the embryonic cuticle (Jackson, 1957). When about to hatch the larva throws back its head and suddenly splits both the embryonic cuticle and the vitelline membrane, aided by the egg-bursters on the frons (Balfour-Browne, 1913). It then bursts out of the egg into the water to seek its prey, and since it sucks the juices of its victim through a groove in each mandible (after injection of a digestive fluid), there can be no question of contamination with slime bacteria during feeding. As will be discussed later (p. 442) an external source of infection of the female *Dytiscus* is indicated.

Since the myxobacteria are probably little known except to the specialist, it may be of interest to mention that these organisms, called myxobacteria or slime bacteria, consist in the vegetative stage of flexible rod-like cells which form a colony or swarm and multiply by transverse fission. According to

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**Fig. 1 (plate).** A, photomicrograph of a portion of the vitelline membrane of a hatched egg of *D. marginalis* under low magnification, to show the distribution of the bacterial cysts. The V-shaped base of the rent made by the larva in hatching shows at the top right-hand side. From a glycerine jelly mount.

B, photomicrograph of an area of the vitelline membrane from an egg of *D. marginalis* containing an embryo larva, to show the bacterial cysts. Mounted in polyvinyl lactophenol.
Bergey and others (1948) the cells may form a group which moves as a unit by a crawling or creeping motion away from the centre of the colony, and the moving cells pave the substrate with a thin layer of slime on which they rest. In the fruiting-stage the rods ordinarily associate in clumps to form cysts, and in the cysts, in some forms, the rods may become ovoid or cylindrical, functioning as spores.

Most of the species have been obtained from soil and from the dung of various animals, sometimes also from lichens or decaying leaves, bark, &c., while some species of *Cytophaga* are marine. Most slime bacteria are saprophytic but some are believed to be facultative parasites on various bacteria. One species, *Chondrococcus columnaris* (Davis) Ordal and Rucker, is parasitic on fish, while *Polyangium parasiticum* Geitler is parasitic on an alga (*Cladophora*) growing in fresh water and forms its cysts always under water (Geitler, 1925). As far as I am aware this is the first record of the occurrence of these bacterial cysts on the egg of a water-beetle, and, though Blunck (1914) has described the egg of *D. marginalis* in much detail, he makes no reference to them. I have found similar cysts present, usually in very small numbers, on some eggs of *D. semisulcatus*, but I have never found them on the eggs of water-beetles belonging to other genera, though the eggs of *Agabus* and *Ilybius* may be laid on the same aquatic plants, those of *Ilybius* being similarly buried in the leaf tissue. These bacterial cysts are more numerous on some eggs of *D. marginalis* than on others, but they show a fairly constant orientation on the vitelline membrane. Before describing their position it will be necessary to refer to the principal changes occurring in the envelopes of the egg of *D. marginalis* from oviposition to hatching. The condition of these envelopes in the egg of *D. semisulcatus* will be dealt with later.

**Dytiscus marginalis** L.

Oviposition. As is well known, the female *D. marginalis* inserts her egg by means of her powerful cutting ovipositor in the tissues of various plants growing under water. The process of laying has been described and figured by Blunck (1913) and his observations are included in Korschelt's monograph (1924). The long egg is so inserted in the mesophyll of the leaf that it is hidden from view, but, as the egg swells during its development, the epidermis of the leaf becomes raised and the narrow slit through which the egg is laid widens appreciably. It is thus usually possible in the later stages of development to see the anterior end of the egg through this opening in the leaf (fig. 2). The egg is so inserted that the dorsal surface of the embryo is uppermost (on the outer side of an upright leaf), while the lower part of the head with the mandibles and other mouth-parts faces the inner part of the leaf. This is in accordance with Haliez's law (1886), the orientation of the egg corresponding to that of the mother, for, when laying, the beetle clasps the plant with its legs and projects the egg into the tissues of the leaf in line with its body, so that the head of the egg, dorsal side up, comes to lie under the slit made by the ovipositor.
The egg envelopes. In the newly laid egg of *D. marginalis* the chorion is so closely applied to the vitelline membrane that the two membranes can only be distinguished in parts by transmitted light. The micropylar area shows distinctly in the chorion as a round white spot at the anterior end of the egg. When newly laid the egg is surrounded with gelatinous cement which stains pale green with methylene blue. The egg undergoes a great increase in size during development, and Blunck (1914, pp. 88–89) records that the egg swells from 1.2 to 2.25 mm in diameter, and increases in length by more than a millimetre. He states that in the course of embryonic development the chorion becomes entirely detached from the vitelline membrane and cracks. I have found that, as the egg increases in size, the chorion splits, usually into two equal halves, by a long slit down the dorsal and ventral surface of the embryo. The two halves of the chorion closely invest the sides of the egg, as is seen in fig. 2, where the chorion of the right side shows. The two halves of the chorion are readily overlooked, but when stained with methylene blue they show as two elongated, pale, greenish-blue, concave plates which together retain the original shape of the egg. Sometimes they remain united posteriorly. The micropyle may remain attached to one side of the split chorion, or else it may break away from the chorion and adhere only to the vitelline membrane (fig. 3, A).

Unlike the chorion, the vitelline membrane is able to stretch considerably, and it thus comes to form the only envelope entirely enclosing the egg in its later stages (fig. 2). The egg then projects widely from the chorion, especially at the front end, and at this stage it is firm to touch, though the newly laid egg is soft. According to Blunck (1914, p. 86) the vitelline membrane becomes of firm consistency owing to the secretion by the serosa of chitin-like lamellae which form a secondary vitelline membrane. This is the serosal cuticle (Wigglesworth, 1950). The vitelline membrane is entirely smooth except for...
the presence of the numerous bacterial cysts in certain areas at the time when
the embryo is well formed. The chorion, on the other hand, has a slightly
granulated texture owing to a faint embossing of very minute adjacent spots.
The chorion stains more deeply than the vitelline membrane with aceto-
carmine or methylene blue, and sometimes under natural conditions it takes
up a brown colour from withered leaves surrounding the egg and so appears
slightly darker than the rest of the egg. If one removes an egg with an advanced
embryo from the cavity in the leaf, the two halves of the chorion are often left
behind adhering to the leaf tissue.
When the larva hatches, it is the vitelline membrane that it ruptures, forming a rent at the anterior pole of the egg, the rent being V-shaped on the dorsal surface (figs. 1, A; 3, B). The embryonic cuticle which previously enclosed the embryo, surrounding the antennae, mouth-parts, and legs in individual sheaths, is left partially projecting from the egg as a delicate transparent membrane (fig. 3, A, B). This has already been described (Jackson, 1957). The larva then emerges through the widened slit in the epidermis of the leaf left by the beetle in laying. The shell of the egg, distended by the larva, contracts after hatching, and the leaf tissue, raised up by the swelling of the egg, collapses, so that hatched eggs are readily overlooked.

It is hoped that the above account, based on the examination of many eggs of *D. marginalis*, will make clear the principal facts regarding the more obvious changes in the envelopes of the egg during development, for some confusion has arisen on this subject in the literature. Wesenberg-Lund (1912) refers to the outer envelope as sticking close to the wall of the egg cavity (*Eiloge*) and tearing as one opens the hole. He looks upon it merely as a lining of the cavity, but remarks that at one end it bears a small knob. I believe this supposed lining to be the chorion and the small knob the micropylar area.

The eggs under observation were laid from the end of March till the end of June in 1957 and 1958. Oviposition in this species appears to be restricted to spring and early summer. The incubation period during May was 17 to 19 days in an unheated north room.

**Location of the bacterial cysts.** The bacterial cysts do not show at all on a newly laid egg or in an egg within 4 days of laying. The exact time of their appearance has not been ascertained, but by the time the embryo is well formed, showing legs and ocelli, they are usually present (fig. 1, B). It is not easy to determine whether they appear on the vitelline membrane before or after the chorion has split. Since the *Dytiscus* egg is so closely enveloped by the plant tissue it will readily be understood that it is by no means easy to ascertain at what moment the chorion splits, for however carefully one removes the epidermis of the leaf to uncover the egg, there is a danger of splitting the chorion in the process. Moreover the bacterial cysts in their early stages are very small and inconspicuous and are not readily seen in a living egg because of the opacity of the embryo, so that it is necessary to kill the egg and to mount the vitelline membrane for microscopical examination. However, in the few young eggs with intact chorion which I have examined I have failed to find the cysts, and I consider that it is most likely that the rod-shaped bacteria gain access to the vitelline membrane after the chorion has begun to crack, and that they then start cyst formation. The bacterial cysts are usually largest and most numerous on the part of the vitelline membrane forming the head of the egg, the area which is exposed by the widening of the initial cut in the epidermis of the leaf and which is left bare when the chorion splits. They also occur all along the dorsum of the embryo and round the posterior pole. They are thus chiefly located on the upper surface of the living egg exposed by the dorsal split in the chorion to the surrounding water and in
a position to receive some light from the splitting of the leaf tissue as the egg swells. They are not present on the ventral surface of the vitelline membrane which is closely pressed to the leaf tissue. Towards the sides of the eggs, where the bacterial cysts are few, they are much smaller (3 to 5 \( \mu \) in diameter), while at the anterior pole of the egg they usually attain their largest size (10 \( \mu \) in diameter), and often occur together in twos or threes or fours (measuring up to 36 \( \mu \) in length), and they may also form irregular groups (fig. 4, A, B). During their early period of development on an egg they are small and widely scattered. After the Dytiscus larva has hatched, the bacterial cysts persist on the empty shell of the egg, and indeed appear to increase in size, for it is on the hatched eggs that I have found the densest aggregates (figs. 1, A; 4, A, B). In preparations examined with an oil-immersion lens, Miss Collins has recognized the rod-like bacteria between the cysts.

Often a female D. marginalis will drop her eggs in the bottom of the jar instead of inserting them in plant tissue. Such dropped eggs would appear ideal to use for the study of the development of the bacterial cysts, but on such eggs the slime bacteria never develop. These dropped eggs invariably decay and become mouldy. They are probably infertile, as Joly (1945) suggests.

Conditions under which cysts of the slime bacteria develop on the egg of D. marginalis. I have found these cysts present on eggs of D. marginalis collected from a small pond at Gilston, Largoward, Fife. The eggs had been laid on Juncus articulatus L. under water. However, the cysts develop just as readily on eggs laid in the laboratory. For my breeding experiments with Caraphractus it was essential to get the Dytiscus females to lay eggs on plants that had not previously grown in ponds, since plants collected from water might already contain some stage of the mymarid. I have therefore used plants of f. articulatus lifted from grassy areas where no water was lying and have grown them indoors in tap water. Moreover I have frequently used the leaves of tall grasses, or of Carex otrubae Podh., which had never been in water, and have plunged them in jars of tap-water and introduced to each jar a laying Dytiscus female. No soil was placed in the jars. The beetles were fed principally on earthworms. Eggs were deposited in the leaves under these conditions and usually developed normally, provided the leaf was not too stiff to allow for the swelling of the eggs. The eggs so obtained showed just as abundant colonies of slime bacterial cysts as the eggs collected from ponds. It seemed to me probable that the bacteria were carried by the beetle herself; so in early December I took a living female to Miss Collins for investigation, with interesting results.

Occurrence of bacteria on female D. marginalis. It was found by Miss Collins that slime bacteria are present in enormous numbers on the female beetle. They have been recovered in the rod stage by merely dipping the

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**FIG. 4 (plate).** A, photomicrograph of an area of the vitelline membrane from the hatched egg of D. marginalis shown in fig. 1, A, with the cysts more highly magnified. B, photomicrograph of an area of the vitelline membrane of a hatched egg of D. marginalis showing some bacterial cysts in large groups. Preparation stained in aceto-carmine and mounted in euparal.
posterior extremity of the living beetle in sterile water. Moreover, by trailing the apex of the abdomen of the female over an agar plate Miss Collins obtained within 24 h swarms of the rod-shaped vegetative cells characteristic of slime bacteria. She obtained the highest numbers of slime bacteria from brushings of the posterior dorsal extremity of the beetle’s abdomen into sterile tap-water and the subsequent plating of these on an agar plate. The posterior segments of the beetle are normally covered by the elytra and bear dense setae, but the beetle protruded these segments on being touched. Few rod stages of the bacteria were recovered from the head and prothorax, which are smooth apart from punctures, and devoid of bristles.

**Effect of parasitism by Caraphractus cinctus on the bacteria.** When I first observed these organisms on healthy eggs of *Dytiscus*, I was puzzled not to have noticed them on various *Dytiscus* eggs which I had mounted when parasitized with *Caraphractus*. From an examination of many parasitized eggs, in preservative or mounted, I have now found that the bacteria fail to develop normally on such eggs. A successfully parasitized *Dytiscus* egg may contain from 25 to 50 imagines of *Caraphractus*, and usually the entire contents of the egg are consumed. Moreover, in eggs with many parasites the vitelline membrane swells so that the chorion splits, just as in an egg in which a *Dytiscus* embryo is developing. It would appear that the bacteria begin to develop on these eggs but find conditions unsuitable and perish. I have frequently found the remains of colonies of bacterial cysts on parasitized eggs, but they are brown and abnormal in appearance. Often only a sprinkling of brown dots may show at the anterior pole of the egg, while at other times the cysts are certainly present, but each cyst is bordered with a brown ring and has not the translucent, greenish appearance of healthy colonies. Only in one parasitized egg I have examined did the organisms appear more nearly normal and this egg contained only 5 parasites of *Caraphractus*; so perhaps the conditions were more suitable to the development of the bacteria.

**Dytiscus semisulcatus Müll**

I collected a single female of this species at Tents Muir in Fife on 24 October 1958. It was placed with *Juncus articulatus* and began laying from the end of October till February 1959. The eggs were inserted in the sheathing basal portion of the *Juncus* leaf and were orientated in the same way as those of *D. marginalis*, with the dorsal surface upwards and the anterior end under the slit made by the ovipositor. It was surprising to find that, contrary to what occurs in *D. marginalis*, the chorion remained in close contact with the vitelline membrane throughout development. In some eggs small irregular longitudinal cracks were observed in the chorion, in others even these did not show, and only rarely in a hatched egg did a piece of chorion become partially detached. Hatching occurs by the larva forcing an opening in the anterior end of the egg, through the combined vitelline membrane and chorion. The egg just before hatching measures from 7-8 to 8-6 mm in length. After hatching the shell contracts by more than 1 mm in length. The larva about an hour
after hatching measures 18 mm, excluding the cerci. The embryonic cuticle remains within the egg but a small portion of it may project from the hole. The incubation period of eggs laid in late October was about 3 weeks.

**Bacterial cysts.** Many eggs laid by this female during the winter have been examined before and after hatching, but only on some eggs were bacterial cysts found and usually these were sparingly present and widely scattered. In no case did they form the even and regular covering as on the egg of *D. marginalis*. Since the vitelline membrane in *D. semisulcatus* is so closely covered by the transparent chorion, the location of the cysts could only be determined by peeling off a strip of the chorion, when the cysts were found on the vitelline membrane below, just as in *D. marginalis*. The few cysts found showed a more definite wall than did the cysts on the egg of *D. marginalis*, but Miss Collins considers that they belong to the same group of bacteria.

**DISCUSSION**

It has been shown that in the egg of *D. marginalis*, in which the chorion splits widely long before the larva hatches, the vitelline membrane is closely covered in certain areas with cysts of a slime bacterium. In the egg of *D. semisulcatus*, on the contrary, the chorion closely surrounds the vitelline membrane throughout development, showing usually only a few small cracks; and bacterial cysts, when they are present, are usually few and widely scattered. From this it seems probable that the vegetative stage of the bacterium gains access to the vitelline membrane as the chorion cracks. It is known that the female of *D. marginalis* carries these bacteria in great numbers at the apex of her abdomen and it seems most likely that they are transferred during laying to the gelatinous cement with which the female fastens the egg into the plant tissue. As the chorion splits the bacteria will make their way on to the vitelline membrane and there start cyst formation. This association between slime bacteria and the vitelline membrane is strikingly developed in the egg of *D. marginalis*, probably because the vitelline membrane in this species is so much exposed.

It is too early to make any suggestions regarding the biological significance of this association between *Dytiscus* eggs and slime bacteria. It may or may not be a symbiotic association. In the egg of *D. semisulcatus* the bacterial cysts may not always be present, and when they occur they are usually few and widely scattered; yet the eggs develop normally. The egg of this species is protected throughout development by the chorion, while in the egg of *D. marginalis* much of the vitelline membrane becomes exposed as the chorion splits. According to Bergey and others (1948) the slime bacteria often appear to live in close association with various true bacteria and are probably parasitic upon them. There is a possibility that the presence of the slime bacteria on the exposed areas of the vitelline membrane may afford protection against harmful bacteria. It is at least certain that their presence is in no way injurious to the developing embryo.
The fact that the bacterial cysts do not congregate on the chorion, the outer envelope of the egg, even in *D. semisulcatus* where the chorion remains in position, is evidence that the bacteria do not merely use the egg as a convenient surface to squat upon, but that their stance upon the vitelline membrane provides the conditions necessary to them for survival and cyst formation. In this position they will be in close contact, through the vitelline membrane, with the fluid surrounding the developing embryo, and they may benefit from the gases diffusing from the water into the egg during the respiration of the growing embryo. In the egg of *D. marginalis* the cysts occur principally on the vitelline membrane of the dorsal surface of the egg and especially at the anterior pole, and these are the parts of the membrane that are exposed by the splitting of the chorion and which may be better supplied with oxygen than the membrane of the under-surface of the egg, buried in the plant tissue. When the embryo is killed by parasitism of *Caraphractus*, the conditions for the survival of the bacteria are no longer suitable. After the larva of *D. marginalis* hatches, the cysts are still present on the egg and actually occur in denser colonies. Miss Collins has found the rod cells of a myxobacterium present on an old hatched egg of this species recovered from dead leaves of *Juncus articulatus* collected from a pond in December, when the egg would be at least 6 months old.

There can be no doubt that the female beetle herself transmits these bacteria in the vegetative stage to the eggs; how the next generation acquires them remains an unsolved problem. The female *Dytiscus*, according to my observations on captured specimens, may not begin oviposition until probably a year old, so that during many months she will be swimming about amongst aquatic plants where she will doubtless become infected with the rod-like vegetative cells. Were the bacteria carried by the female internally and introduced into the egg during its development within the ovary, one would expect a uniform and constant distribution on each egg, and this is not the case in the egg of *D. semisulcatus*, and not always so even in the egg of *D. marginalis*. The evidence points to an external infection of the egg from the mother, and the degree of infection will be influenced largely by the extent to which the vitelline membrane becomes exposed by the splitting of the chorion, and hence will be low in the egg of *D. semisulcatus*.

It is clear that the relationship of the slime bacteria to the *Dytiscus* egg raises many problems which remain unsolved. One does not know why the exposed vitelline membrane provides these bacteria with suitable conditions for cyst formation nor what stimulus is required to cause the encysted cells ultimately to germinate. One point that could be readily ascertained by entomologists is whether these cysts are equally common on the eggs of *D. marginalis* from other localities. The eggs I have found bearing them were laid by various females collected in Fife, by one specimen from Surrey, and by two from Westmorland. It would also be interesting to know whether they are present on the eggs of all species of *Dytiscus* and how far their occurrence depends on the degree of the splitting of the chorion, for it would seem
from my observations on the eggs of \textit{D. marginalis} and \textit{D. semisulcatus} that the extent of the breakdown of the chorion is a specific character.

The identification of the organisms present on the vitelline membrane of the \textit{Dytiscus} egg was primarily due to Dr. J. W. G. Lund of Windermere Laboratory, who first suggested that they might be bacterial zoogloea. I am much indebted to him for his diagnosis. I am especially grateful to Miss V. G. Collins of the same laboratory for all the trouble she has taken in determining their identity more closely, and for culturing the vegetative cells obtained from the female beetle. She has supplied me with much information and given me references to the literature, and I greatly appreciate all the help she has so kindly given me. I wish to express my thanks to the specialists on algae who most kindly examined some of the material before its identification as a slime bacterium; namely, Dr. H. Blackler of St. Andrews University, Dr. Francis Drouet of New Mexico Highland University, Mr. R. Ross of the British Museum (Natural History), and Professor Gilbert M. Smith of Stanford University.

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