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On the occurrence of symbiotic micro-organisms in the Cecidomyiidae or Gall Midges (Diptera) with special reference to the larvae of *Rhabdophaga saliciperda* Duf.[1]).

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Historical Introduction.

In India practically no work has been undertaken, so far, on this highly important group of insects, the Cecidomyiidae, with the exception perhaps of a few stray systematic notes, although some of these insects are long known to cause heavy depredations on agricultural and orchard products almost in every country. This tempted me to take up this much-neglected group while working in England in order to get myself acquainted in as many ways as possible with the various aspects of the subject. Accordingly a very common midge, *Rhabdophaga saliciperda* was selected as a first measure on account of the ease with which it could be procured, and the present note is only an attempt to tackle one of the outstanding problems, viz., the nutrition in the group.

The presence of micro-organisms could well be expected in the gall midge *Rhabdophaga saliciperda* since as we know it has been a common belief during recent years that all wood-infesting insects,

[1]) Part of this work was carried out at the Entomological laboratory of the Imperial College of Science and Technology, London and the rest was done at the laboratories of the Zoological Survey of India, Indian Museum, Calcutta. I am grateful to the authorities of both these Institutions for the facilities they have afforded to me.

the present insect being one of them, as a general rule, are associated with some sort of micro-organisms which assist them in the act of nutrition of the food matters. In other words, the micro-organisms act as true symbionts in these cases.

This theory of symbiosis has been amply substantiated by the classical experiments of CLEVELAND (1924) who has clearly demonstrated that the termites are unable to digest wood if deprived of their symbionts, in this case some flagellates (Protozoa). BUCHNER (1928) goes even further, he does not limit himself within the wood-boring insects only, as according to him the sap-sucking insects also, such as *Homoptera* and *Heteroptera* may carry symbiotic micro-organisms, and very recently WIGGLESWORTH (1929) has pointed out that micro-organisms in the form of intracellular bacteroids may as well occur in the blood-sucking insects such as the *Glossina*, Culicidae, Pupipara, *Cimex* and Pediculidae. These organisms in several cases take the form of fungus spores or bacteria as observed by PORTIER (1911) in *Nonagria typhae* (*Lepidoptera*).

Micro-organisms as stated above are certainly useful in the matter of digestion to the insects but then they may be used as actual larval food also. Thus HUBBARD (1897) went as far as to design a special name i. e., Ambrosia beetle for the timber-boring beetles just because of their feeding on some fungus called Ambrosia fungus. These beetles and their larvae have an amazing way of cropping the fungus. In *Asphondylia prunorum*, a Cecidomyiid, NEGER (1908, 1910) detected the larvae feeding on a fungus *Macrophoma* sp. Again *Miastor* larvae living under rotting bark, and according to WINNERTZ (1853), the larvae of sub-genus *Diplosis* feed not on decaying wood but on the micro-organisms present under such circumstances; this has been conclusively shown by HARRIS (1923) who succeded in rearing the larvae of *Miastor* in an agar culture of the micro-organisms but not on sterilised bark. Such behaviour of insects has also been noted in the mycetophagous Diptera, *Sciara* for instance, as also in several others including *Drosophila* and *Musca* (BAUMBERGER, 1919).

Habits and Habitat of the species.

I do not find any record of the genus *Rhabdophaga* from the oriental countries but it occurs freely in the continents of Europe and America, being mostly restricted to willows producing various types of galls on their branches, flower buds or leaves.

The species *Rhabdophaga saliciperda* is no exception, it also in common with the other members of the genus inhabits the galls on various kinds of willows; my observations here however, are based on those from willows *Salix fragilis* in England. The mother insects of this species lay their eggs usually in some old burrows of the trees, and the larvae on hatching crawl inwards underneath the bark, ultimately damaging the wood. The species, thus, is a major pest to the willow-growing districts causing considerable damage to the willow wood which is of no less economic importance especially in cases where the wood is used for bat-making.

Although the insects under consideration are of European origin the association of the species and particularly of the genus *Rhabdophaga* with the willow trees should arouse considerable interest to the entomologists in India, since we also grow a lot of willows in Kashmir and bordering places for our newly sprung industry of cricket-bat manufacturing and the like of it. That being so it is more the imperative that we should keep an eye on this group of insects here, and see that the indeginous willows of this country are kept free from pests.

The feeding habit of the Cecidomyiid larvae differs considerably in the different groups; they may be seen feeding for all apparent purpose on rotton wood, on decaying plants and bulbs, on the bark of trees, on fungi or on living plants and may ultimately feed their way into the growing wood as in the case of *Rhabdophaga saliciperda.* Larval food thus, is mainly of vegetable character and they are mostly monophagous living exclusively on certain species of plant or at least on closely allied plants. Some live however, as guests or parasites on galls formed by other Cecidomyiidae, or by Acari or Agromyzidae, some live in the society of Aphids.

Demonstration of micro-organisms in the species.

For this purpose recourses were taken of culture methods, dissection of the larvae, and microtomy.

Culture Method. Two sets of experiments were carried on. The larvae after being removed from the galls in one set were sterilised either by dipping in 90 % alcohol for about ten to fifteen minutes, or in $HgCl_2$ solution (1 in 1000) for about five minutes, this method however only sterilised the exterior of the larvae, and in the other set no sterilisation was followed. The larvae in both the sets were then crushed and transferred to culture tubes seperately

containing potato agar, BROWN'S medium or prune agar. The culture tubes were then introduced in an incubator at a temperature of about 22° C. In the instances where the larvae were sterilised, a heavy growth of bacteria was found after three or four days stay inside the incubator, whereas in the other set, where the larvae were not sterilised, a crop of fungus was noticed within a short time of insertion into the incubator; in most cases however, a mixed crop of fungi and bacteria was obtained, the bacteria appearing later in sequence.

The absence of fungus from the tubes containing sterilised larvae points to show that the fungus spores were not present in the interior of the larvae but were associated with the outer skin only, that being the reason why they germinated from the unsterilised larvae. On the basis of this finding, a natural suspicion was aroused as to the presence of fungus spores in the larval gallery. Accordingly, scrapings from the larval burrows were incubated in the culture media mentioned before and from these, copious fungal growths were invariably obtained. Controls were kept which gave negative results as could be expected. The fungi obtained from different samples belonged to the genera *Botrytes*, *Penicillium*, *Cladosporum* and *Hormodendrum*.

It is very interesting to note, that the spores, although present in the larval burrow, could not germinate as long as the insect remained there. It is only after the emergence or the death of the midge that they germinate, the hyphae of the fungus protruding out conspicuously as white threads. As long as the larva is active there, the spores are kept in check possibly by the secretion of its anal glands. For once the spores germinate the larva would be killed as was found by placing the larvae on several occasions in cultures of these fungi. In a few days, the larvae were contaminated and engulfed by the growing fungi which now become instrumental in causing death to the insects.

NEGER (1908, 1910), who worked on another Cecidomyiid, *Asphondylia prunorum* and PORTIER (1911) working on *Nonagria* also observed similar phenomenon. Both these authors recorded in their individual cases that if the symbiotic fungi were allowed to sporulate they chocked the larvae much in the same way as in the present case. PORTIER (1911), however, thought that the fungus (*Isaria*) in *Nonagria* larvae was held in check by the secretion of the labial glands.

Dissection. Since it was definitely demonstrated that bacteria could be had from a culture of the crushed larvae with steri-

lised skin, my next problem was to dissect out the gut of the larva and take the infusion of the gut only in the culture media to be certain about the region of lodgement of the micro-organisms concerned. But unfortunately the larvae being very minute (measuring about 3 mm. when mature) the attempt was unsuccessful, some amount of perivisceral fluid and other extraneous matters always got mixed up with the gut extract thus affecting the accuracy of the observations.

Microtomy. Microtome sections both transverse and longitudinal of 6 u thickness and smears of the larval gut also revealed that the fungus spores were absent from the interior of the larval body; the presence of bacteria was, however, clearly demonstrated from inside the gut, thus confirming the results obtained from culture methods. The studies in sections of the larvae achieved the end which the somewhat crude method of taking the gut infusion after dissecting out the gut mentioned above could not do, by exhibiting the larval mid-gut as the true seat of bacteria; whereas in the whole mounts and sections through cuticle of the larvae, fungus spores were often noticed clinging to the larval skin.

The sections were stained in Haematoxylin, often being counterstained with Eosin, and the smears either in Giemsa or in Heidenhain's Iron Alum Haematoxylin.

Mode of Infestation with Micro-organisms.

Regarding the way the infestation of the fungus in the larval bed takes place, Hubbard (1897) thinks that the female consciously carries the spores to the new gallery and sows them, whereas Neger (1908, 1910) believes that the spores become accidentally attached to the wing cases of the female when she leaves the larval gallery the walls of which are coated with the microscopic plants. The latter mode of emination probably occurs in *Rhabdophaga saliciperda.* The spores once having found an access in the vicinity where the eggs are laid by this midge would find an easy passage to the larval galleries being carried presumably unconsciously on the minute spines of the crawling larvae to their new environments.

There is another possible way of dispersal of spores as in the case of the micro-organisms-feeding insects, where the spores devoured often come out undigested with the faeces, and the excreta under favourable conditions produces a bountiful crop of fungus food.

Role of Micro-organisms.

As regards the exact role of micro-organisms found in association with the insects there is a great deal of dispute. By the presence of micro-organisms, BAUMBERGER (1919) contends, the insects overcome the disadvantages of the chemical and mechanical composition of wood. The micro-organisms are of value not because of their digestive but because of their synthetic power. Ample evidence in support of this theory is on record. Mention has already been made of the convincing experiments of Cleveland in the termites. Such insects although seemingly feeding on wood are not actually doing so, as they are incapable of digesting wood as such. The food-wood must be partially digested by the micro-organisms or some secretory and excretory products of theirs before it can be taken by these insects. In other cases the micro-organisms serve for the protein deficiency from which the larvae living in plant tissues suffer, the plant tissues possess less than 2 % of protein only, while the protein contents of the micro-organisms are over 10 % (UVAROV, 1928). Somewhat similar explanation is given for the plant-sucking insects such as the Aphids and Coccids. BUCHNER (1921) however, holds that these insects utilise their symbiont in assimilating nitrogen from the air, thus supplementing the deficiency of nitrogen of normal food of insects. BUCHNER based his hypothesis as gathered from UVAROV (1928) on the assumption that the insects in question ingest only the soluble carbohydrates in cell-sap and not protein, while UVAROV himself thinks that protein and other nitrogenous substances of plant-sap are probably more completely utilised by sucking-insects than are the carbohydrates.

GLASER (1923) suggests that the presence of micro-organisms may be one of the principal sources of the accessory growth factors of the larvae of some flies. He succeded in rearing two generations of *Drosophila* under perfectly sterile conditions proving that the micro-organisms in these flies are of little importance compared to those in the wood-feeding insects. Practically the same view has been put forth in explaining the role of micro-organisms in the blood sucking insects by WIGGLESWORTH (1929) who suggests the possibility of these organisms to be of some accessory food-value comparable or even identical with the vitamins necessary for mammalian growth, although previous to him ROUBAUD (1919) thought that these organisms are analogous to those found in the wood-feeding insects and that they play an essential part in the act of

digestion. But the former author refutes this on the ground that the digestion of cellulose is far apart from the digestion of blood and the same function for the micro-organisms cannot therefore, be attributed in the two widely seperated groups of insects, one feeding on wood and the other feeding on blood.

Discussion.

From what I have said of the midge *Rhabdophaga saliciperda*, it may be gathered that a rather complicated affair exists as to the fate of the micro-organisms in this species. That the micro-organisms both fungi and bacteria, are in some sort of symbiotic relation with the larva can well be accepted, but what rôle the micro-organisms play amongst themselves is not yet satisfactorily proved. Again another anomalous position is created by the fact that the fungus spores although so closely associated with the larvae should not be detected in the gut of the insects. One would be inclined to think that larvae living in such a surrounding would unavoidably ingest fungus cells as was observed by Baumberger (1919) in *Drosophila*. This surprising behaviour cannot be explained at the moment with our inadequate knowledge of insect nutrition and the techniques involved therein. Although I am aware that one cannot be so dogmatic at this stage that the fungus spores could not as well live in the gut, nevertheless, their presence in close association with the larvae was definitely established, this being of real importance to me.

At least in another insect *Nonagria typhae* (Lepidoptera) the presence of both a fungus and a bacterium has also been recorded, but there the relationship of the micro-organisms is different from that of the present case. In *Nonagria*, Portier (1911) observed, that the larvae feeding within the stems of marsh plants *Typha* have an abundance of motile conidia of a fungus (*Isaria*) in the intestine. This fungus is always found to be accompanied by a micrococcus which by dissolving cellulose, a necessary condition for the fungus to thrive, helps the latter immeasurably. Here is a case of typical symbiosis between the two micro-organisms, both being obtained from the intestine of the larva unlike that in the *Rhabdophaga*.

In conclusion, I wish to acknowledge my indebtedness to Miss Hall of the Royal College of Science, London for the identification of the fungi and many other valuable helps. My thanks are also due to Dr. Ram at that time working in the same College for his readiness to discuss bacteriological matters.

Summary.

1. Micro-organisms both fungus spores and bacteria have been noted from the larvae of the gall midge *Rhabdophaga saliciperda.*

2. From cultures in different media and microtome sections it has been revealed that the fungus spores lie on the outside of the larvae, whereas the bacteria are lodged within the mid-gut.

3. Probable way of infestation and the role of micro-organisms in the different insects have been fully discussed.

Bibliography.

BAUMBERGER, J. P. (1919): A nutritional study of insects, with special reference to micro-organisms and their substrata. Journ. exp. Zool. Vol. 28 p. 1—81.

BUCHNER, P. (1921): Studien an intracellularen Symbionten. III. Die Symbiose der Anobiinen mit Hefepilzen. Arch. f. Protistenk. Bd. 42 p. 319—336.

— (1921): Tier und Pflanze in intracellularen Symbiosen. 460 p. Berlin.

— (1922): Hämophagie und Symbiose. Die Naturwissenschaften Bd. 10 p. 703—710.

— (1928): Holznahrung und Symbiose. 84 p. Berlin.

CLEVELAND, L. R. (1923): Correlation between the food and morphology of termites and the presence of intestinal Protozoa. Amer. Journ. Hyg. Vol. 3 p. 444—461.

— (1924): Physiological and symbiotic relationship between intestinal Protozoa of termites and their hosts. Biol. Bull. Woods Hole Mass. Vol. 46 p. 178—227.

— (1925): Ability of termites to live perhaps indefinitely on a diet of pure cellulose. Ibid. Vol. 48 p. 289—293.

— (1926): Symbiosis among animals with special reference to termites and their intestinal flagellates. Quart. Rev. Biol. Vol. 1 p. 51—60.

— (1928): Further observations and experiments on the symbiosis of termites and intestinal Protozoa. Biol. Bull. Woods Hole Mass. Vol. 54 p. 231—237.

FELT, E. P. (1908): The economic importance and food habits of American Gall midges. Rep. Ent. Soc. Ontario p. 43—46.

— (1911): Summary of food habits of American Gall midges. Am. Ent. Soc. Amer. Columbus, Ohio Vol. 4 p. 55—62.

GLASER, R. W. (1923): The effect of food on longevity and reproduction in flies. J. Expt. Zool. Philadelphia Vol. 38 p. 383—412.

HARRIS, R. G. (1923): Sur la culture des larves de Cecidomyies paedogenetiques (Miastor) en milieu artificiel. C. R. Soc. Biol. Vol. 88 p. 256—258.

HUBBARD, H. G. (1897): The ambrosia beetles of the United States U. S. Dep. Agric. Div. Entom. Bull. Vol. 7 p. 9—30.

KUSKOP, M. (1923): Bakteriensymbiosen bei Wanzen (Hemiptera heteroptera). Arch. f. Protistenk. Bd. 47 p. 350—384.

MERCIER, L. (1907): Recherches sur les bacteroides des Blattides. Arch. f. Protistenk. Bd. 9 p. 346—358.

Neger, F. M. (1908): Ambrosiapilze. Berl. Deutsch. Bot. Ges. Bd. 26a p. 735—754.
— (1910): Ambrosiapilze. III. Weitere Beoabachtungen an Ambrosiagallen. Ibid. Bd. 28 p. 455—480.
Portier, P. (1911): Developpement complet deslarves de Tenebris molitor obtenu an moyen dune nourriture sterilisee a hante temperature. C. R. Soc. Biol. Vol. 82 p. 59—60.
Reichenow, E. (1922): Intracellulare Symbionten bei blutsaugenden Milben und Egeln. Arch. f. Protistenk. Bd. 45 p. 95—116.
Roubaud, E. (1919): Les particularites de la Nutrition et de la vie Symbiotique chez lez Muches Tsetses. Ann. de l'Inst. Past. T, 33 p. 489—536.
Schneider-Orelli, O. (1923): Untersuchungen über den pilzzüchtenden Obstbaumborkenkäfer Xyleborus (Anisandrus) dispar und seine Nährpilze. Zentralbl. f. Bakt. usw. 2. Abt. Bd. 38 p. 25—110.
Uvarov, B. P. (1928): Insect Nutrition and Metabolism. A summary of the literature. Trans. Ent. Soc. Vol. 2 p. 255—343. London.
Wigglesworth, V. B. (1929): Digestion in the tsetse fly: a study of structure and function. Parasitology Vol. 21 No. 3 p. 288—321.
Winnertz (1853): Beiträge zu einer Monographie der Gallmucken. Linn. Ent. Bd. 8 p. 154—522.