Some morphological and histological effects of the poison of Lophopodella Carteri (Hyatt) on young Ambystoma Opacum (Gravenhorst) and Carassius Auratus (Linnaeus)

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SOME MORPHOLOGICAL AND HISTOLOGICAL EFFECTS OF THE POISON OF LOPHOPODELLA CARTERI (HYATT) ON YOUNG AMBYSTOMA OPACUM (GRAVENHORST) AND CARASSIUS AURATUS (LINNAEUS)

A Thesis
Presented to the faculty of the Graduate School of the University of Richmond in Partial fulfillment of the Requirements for the Degree of Master of Arts

by
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ABSTRACT

Larvae of the marbled salamander, *Ambystoma opacum*, and the goldfish, *Carassius auratus*, were subjected to poisoning by homogenates of *Lophopodella carteri*. Stock homogenates were prepared by grinding specimens of frozen *L. carteri* in a rotary blender and adding distilled water to give a 20 per cent concentration (wt./vol.); the stock was used to prepare working solutions.

Homogenates from bryozoans collected in 1964 were approximately twice as poisonous as those from the 1963 collection. It is possible that chemical changes during storage and bacterial action prior to freezing caused deterioration of the poisonous principle in the material from 1963. Stock homogenates were prepared immediately before testing as their toxicity declined over a period of several hours.

The stock homogenate from 1964 was used at the rate of 2.0ml per 50ml of spring water; that from the 1963 collection at the rate of 4.0ml per 50ml of spring water. Salamanders were capable of surviving for longer periods of time in concentrations that were lethal to fish in 60 minutes, and the stock homogenate from 1964 was used at the rate of 5.0ml per 50ml of water.

Test fishes showed an increase in respiratory movements accompanied by gaping opercles, a gradual loss of balance, and emission of mucus and blood from the gill regions prior to death. Size of the fish was not related to
the rate of poisoning. With the salamanders, there was a progressive increase in blistering of the gill filaments, sloughing of the epithelium, and exudation of blood and mucus from the gill region.

Histological preparations of the poisoned fishes and salamanders showed a hypertrophy of the epithelial lining of the gills, destruction of the lamellae and fimbriae respectively, a breakdown of the capillary walls, and an increase in the number of goblet cells in the pharynx.
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INTRODUCTION

Two species of freshwater bryozoans are known to be toxic to certain aquatic vertebrates, *Lophopodella carteri* (Hyatt) and *Pectinatella gelatinosa*. *L. carteri* has been reported to kill fish (Rogick, 1957; Tenney and Woolcott, 1964) and larval salamanders (Collins, Tenney and Woolcott, 1964). Oda has found that *P. gelatinosa* is poisonous to fishes (Rogick, 1957), and advances the hypothesis that the poisonous principle is centered in the coelomic fluid of the bryozoans (Oda, 1958). Preliminary studies by Collins, Tenney and Woolcott (1964) showed that the site of action of the poison on the salamanders was the gill region and that substantial disruption of the epithelium resulted in death.

The present study was undertaken to determine, in detail, the histological changes in gill tissues of poisoned goldfish, *Carassius auratus* (Linnaeus), and larvae of the marbled salamander, *Ambystoma opacum* (Gravenhorst).

MATERIALS AND METHODS

Colonies of *Lophopodella carteri*, collected from the Virginia State Bass Hatchery on August 1, 1963 and September 25, 1964 were placed in dialysis tubing and frozen in the deepfreeze compartment of a refrigerator as soon as possible after collection. The experimental animals used
were 32 larvae of *Ambystoma opacum* (total length 1.0-2.0 cm) that were hatched in the laboratory, and one hundred specimens of *Carassius auratus* (standard length 1.0-3.0 cm). All animals were maintained in aquaria until the tests were conducted.

Stock homogenates of *L. carteri*, made fresh immediately before each experiment, were prepared by grinding frozen colonies in a rotary blender, then diluting with distilled water to give a 20 per cent (wt./vol.) concentration. Preliminary tests showed that the homogenates of the more recently collected specimens were approximately twice as active as those from the earlier sample. Test solutions using bryozoans collected in 1964 were prepared by adding 2.0 ml of the stock homogenate to 50 ml of spring water in a finger bowl containing two fish, or 5.0 ml into a bowl containing two salamanders. Stock homogenate from the 1963 collection was used in the proportion of 4.0 ml stock to 50 ml of spring water.

The test animals were removed from the bowls at intervals of 15, 30, and 60 minutes and immediately placed into a fixative. Control animals were kept in 50 ml of spring water for 60 minutes, then fixed. One-half of the total number of animals tested were fixed in Zenker’s fluid and the others in Bouins fixative. After fixation, the organisms were stored in 70 per cent ethanol. In preparation for a histological study, the heads, including the gill
regions, were removed and embedded in paraffin. The heads of the fish were serially sectioned at 8, 12, or 15 microns and those of the salamanders at 7 microns. The mounted sections were stained with Delafield's hematoxylin and eosin Y (Humason, 1962).

**EXPERIMENTS AND RESULTS**

Experiments were conducted to determine the effects of the homogenate on fish and salamander larvae with respect to the gross changes in their behavior and structure, and histological modifications of the gills.

**Experiment 1.** To compare responses of goldfish in two different size groups, eight specimens ranging from 1.0-1.4 cm st. lg. and eight from 1.6-2.5 cm were exposed to the homogenate from the 1963 collection. Two fish from each size group were maintained as controls. These specimens were fixed in Zenker's fluid; a replication of this experiment using Bouins fixative produced similar results.

Observable behavioral changes began 8-10 minutes after introducing test organisms into the solutions. There was increased activity of the fishes, followed by an increased rate of respiratory movements, loss of balance, and exudation of mucus from the gill regions, as described by Tenney and Woolcott (1964). No significant differences were noted between size of fish and influence of the poison. Study
of prepared slides showed Zenker’s fixative to be superior
to Bouins fluid, in that more detail was preserved in the
epithelial regions.

Figure 1 is a photomicrograph of a section showing the
normal gill structure of one of the goldfish used as a con-
trol. Four aortic arches pass dorsoventrally in the pha-
ryngeal region, and are situated in such a way that No. 1
lies somewhat anterior and external to No. 2, followed by
Nos. 3 and 4. Figures 2 and 3 are more highly magnified
views of a gill arch, revealing the pairs of trailing gill
filaments with numerous lamellae, giving each filament a
"rake-like" appearance. The epithelial lining of each fil-
ament is a uniseriate layer with oval nuclei; where this
lining extends around a lamella the nuclei and cells are
extremely flattened (Figs. 4 and 5). Within the matrix of
each gill filament are an afferent and an efferent vessel
separated by a bar of hyaline cartilage (Fig. 4); normally
the nucleated red blood cells from these vessels fill the
lamellae forming capillary beds for respiratory activity
(Fig. 6).

Sections of treated animals revealed a progressive
disintegration of gill tissue and changes in the appearance
of the epithelial lining of the pharyngeal wall (Figs. 7 and
8). In specimens treated for 15 minutes there was a swell-
ing of the epithelial layer of the gills, giving them a
blistered appearance; the lamellae appeared to be distort-
ed and folded onto the filament, making them indistinct (Fig. 9). After 30 minutes there were degenerative changes of the matrix, the epithelial lining of the filaments was swollen; the lining of the lamellae was separated, and at 60 minutes had broken down, allowing the blood cells to flow into the pharyngeal cavity and become mixed with the abundant mucus found in the mouth region (Fig. 11). The afferent and efferent filamental arteries were swollen but remained intact except for some which were opened at the extremities. The epithelial lining of the pharynx was pronouncedly swollen and there was evidence of an increase in the number of goblet cells correlated with increases in exposure time to the homogenate.

In sections of fish exposed for 60 minutes the gills had lost their normal appearance due to destruction of the lamellae and appeared more as a loose association of disorganized cells (Figs. 10 and 12). The lamellae were destroyed and the disassociated cells were scattered throughout the pharynx, some clinging to the gill filaments. The epithelium of the filaments was blistered and separated from the disrupted underlying matrix.

**Experiment 2.** Goldfish were used as experimental animals to test the effects and relative toxicity of *L. carteri* collected in 1963; the procedures used were the same as for experiment 1.
The behavioral pattern and anatomical effects on external gill structures of test animals were the same as those described for experiment 1. However, the homogenate from the 1963 collection took almost twice as long to produce comparable results at the same concentration.

To check these results, eight fish were treated with homogenate from the 1963 collection at the rate of 4.0ml to 50ml of water. Another eight were subjected to homogenate from the 1964 collection at the rate of 2.0ml per 50ml of water. Samples were taken at 15, 30, and 60 minutes from each concentration; controls were collected after 60 minutes in spring water. Half the specimens from each interval were fixed with Zenker's and the rest with Bouins fluid.

Experiment 3. This experiment was designed to determine the effects of the homogenate on another aquatic vertebrate. Preliminary tests showed that larval salamanders were able to survive as long as 3 hours in concentrations that were lethal to fish within 60 minutes. The tests involved 2.0ml, 4.0ml and 5.0ml concentrations of the 1964 stock homogenate per 50ml of spring water. The animals died within 60 minutes in the 5.0ml solution but not in the lower concentrations; although they eventually succumbed in all of the test solutions. On this basis, the 5.0ml concentration was used in further testing involving larval salamanders.
Sixteen larvae were exposed to the test solution, as in the experiments previously described for fishes. Four were removed at each time interval; two from each interval were fixed in Zenker's fluid and two in Bouins fluid. Four larvae were kept as controls in spring water for 60 minutes.

Observations on the test larvae under the binocular dissecting microscope revealed both behavioral and gross morphological changes beginning approximately 10 minutes after introducing the homogenate. The ends of the gill fimbriae developed a blistered appearance; the swelling of the gills became progressively more pronounced and spread throughout the gill fimbriae; mucus secretions were noted in the gill region after 15 minutes. Hemorrhaging began from capillaries located in the tips of the fimbriae and spread to their bases within 45 minutes. The salamanders, which at first were motionless in the bowls, began jerking movements about 35 minutes after introduction of the homogenate, followed by loss of balance as disintegration of the gills progressed. Movement of fluid by the cilia on the gills ceased in treated larvae at 40 minutes. At 60 minutes vesiculation of the gill fimbriae, secretion of mucus, and emission of blood from the gill region was extensive.

Figure 13 is a low power view of a normal salamander larva showing the three pairs of gills, each consisting of
a ramus with its bar of hyaline cartilage and trailing gill filaments. A more detailed view of the normal gill structure may be seen in Fig. 14. Figure 15 shows the uniseriate epidermis with its flattened nuclei, and the network of the inner connective tissue with mesenchymal cells having numerous interconnecting processes may be seen in Fig. 16.

Histological sections of poisoned larvae revealed gross overall morphological changes in the gill structure (Fig. 17). At 15 minutes the epithelial layer was swollen and there was evidence of slight blistering (Fig. 19) which increased in severity with length of exposure to the homogenate. Specimens treated for 30 minutes showed an increase in swelling of the epithelial layer of the gills extending to the walls of the pharynx (Fig. 18). At this stage the nuclei of the epithelial cells were rounded and enlarged and many of the cells were cytolyzed, allowing the nuclei to pass into the pharyngeal cavity (Fig. 20). Also, abnormalities of the inner connective tissues were evident by this time. Sections secured from test animals just prior to death showed extensive cellular breakdown and much debris was present (Fig. 21). There was a general disintegration of the inner connective tissue, with a retraction of the protoplasmic processes of the cells and an enlargement of their nuclei. A repetition of this experiment gave results of the same magnitude.
DISCUSSION

Zenker's fixative proved more suitable than Bouins for this investigation, producing more finely detailed outlines of the epithelium and permitting uniform staining of fish tissues. In the fish, the cell nuclei and epithelial linings were easily overstained, especially in specimens fixed in Bouins fluid. Finer details of the inner connective tissues and less overall swelling of the salamander gills was evidenced with Zenker's fixative. With both fish and salamanders, Bouins fluid, due to its tendency to swell tissues, appeared to damage the epithelial layer.

In these experiments, the size of the goldfish did not appear to be related to their ability to withstand the toxic effects of the homogenate; this observation is in agreement with the work of Tenney and Woolcott (1964). This effect may be due to the ratio of body weight to the respiratory surface of the gills, which remains constant within a species but varies considerably among species (Lagler, 1962). Due to this the homogenate might affect various species of fish at different rates.

Young larvae of A. opacum not only possess a relatively large gill surface but have an epithelium that is thin and mucous covered, and functions in cutaneous respiration (Noble, 1931), whereas the scales of fishes make cutaneous respiration of minimal importance. These factors
may explain why the larvae can withstand greater concentrations of poison, as there were no visible effects on the body epithelium.

The apparent decrease noted in toxicity of homogenates from bryozoans collected in 1963 possibly was due to either bacterial action prior to freezing (Tenney and Woolcott, 1964) or to a chemical decomposition over the extended period of storage. Specimens stored frozen for long periods tend to dry and become yellow-brown in color; in any case, stock homogenates must be used immediately after preparation as toxicity declines over a period of several hours. Until the poisonous principle is isolated and chemically defined, however, the exact nature and causes of deterioration cannot be determined.

The behavioral and morphological changes that occur in poisoned animals indicate death by asphyxiation. Continued surfacing for air accompanied by gradual loss of balance of test animals lends support to this hypothesis. Also, the secretion of large amounts of mucus into the gill region in response to exposure to the homogenate may hinder normal respiratory activity. The initial changes in gill structure that lead to asphyxiation were observed in test specimens fixed at 15 minutes; slight swelling and erosion of the gill extremities had already begun (Figs. 9 and 10). At 30 minutes vesiculation and edema of the gill tissues occurred along the entire surface of the fimbriae and lamellae;
the gross behavioral changes, such as loss of balance, may be related to the histological changes, and are indicative of a lack of oxygen and an accumulation of waste products within the tissues of the test animals. Figures 12 and 20, of fish and salamander respectively, show destruction of the epithelial linings and capillary beds of the gills to the extent that these structures could no longer function efficiently, if at all. At this stage in salamander larvae, the ciliated epithelial cells were destroyed thus decreasing flow of water over the gill surfaces, and further diminishing the efficiency of gill respiration. The anoxic animals could gain relief only temporarily by gulping air. Secretions of the gills of test animals after 60 minutes revealed extreme distortion of the epithelial linings as well as the inner matrix tissues and endothelial linings of capillaries. By this time they were dead or near death.

The secretion of large amounts of mucus that covers the exposed surfaces may be a defense mechanism against irritants, but this is largely ineffective in the case of *L. carteri* poisoning. Much of the mucus comes from the pharynx where there is an increase in the number of goblet cells of the mucosal layer. However, once the homogenate has adversely affected the gill tissues the pathological changes proceed irreversibly and the test animals slowly suffocate.

Several organisms other than freshwater bryozoans produce substances poisonous to fishes and other animals; how-
ever that of *L. carteri* appears to be unique in its action. The saponin from the starfish is a neurotoxin and has been reported also to have a strong hemolytic effect (Hashimoto, 1960). Holothurin is produced in the body wall and the cuvierian tubules of sea cucumbers (Hyman, 1955), and acts in a manner similar to the poison from starfish. Neither of these substances is known directly to affect the gill tissues of poisoned animals. A number of organic compounds toxic to various animals have been found, including taricha-toxin from the eggs of several species of the salamander *Taricha* (Mosher et al., 1964). This substance is identical to tetrodotoxin, a neurotoxin previously reported from puffer fish (Suborder; Tetraodontoidae). Several other organic neurotoxins are known to occur in various salamanders, including one in the skin of *Triturus pyrroghaster*, which is a respiratory depressant in mammals. The nemato-cyst toxin of *Physalia physalis*, isolated by Læne and Jodge (1958) affects the nervous system of fishes and frogs, particularly the respiratory centers, but does not cause hemolysis of fish erythrocytes.

Several chemical compounds and ions are toxic to fish. Sodium hypochlorite and ammonia affect the rate of respiration (Marchetti, 1960); lead, zinc, and sodium penta-chloropenate affect organs such as the liver and gonads in a manner suggesting that the secondary effects or inanition and/or stress are the most prominent features of the chronic
intoxications produced (Crandall, 1963).

Neuhold and Sigler (1960) have found that sodium fluoride kills trout and carp in a way somewhat similar to the poison of \textit{L. carteri}. The gills are affected and erratic movements and muscular contraction follow introduction of this compound. There is a proliferation of the mucous gland cells of the epithelium of the gills, an effect not noted with \textit{L. carteri} poisoning because of destruction of the cells of the epithelium.

In this preliminary study, no attempts have been made to examine other tissue components of the fish for possible pathological changes; therefore it is not known whether or not \textit{L. carteri} attacks tissues other than those of the gills and pharynx. A general histological survey of other body parts would be desirable in order that other pathological changes might be noted. The chemical isolation and identification of the poisonous principle will facilitate studies at the biochemical level of pathological effects.
LITERATURE CITED


Fig. 1. Portions of three gill arches of a normal goldfish. Anterior gill rakers (A) and posterior paired filaments bearing the lamellae (B); parts of the bony arch support (C) and a branchial artery (D) are seen. (100X).

Fig. 2. A portion of the first gill arch of a normal fish (A) with a branchial artery (B) and the pairs of hemibranchs (C) arising from the arch. (100X).
Fig. 3. A more detailed view of the normal gill filaments showing their arrangement and the pattern of lamellae on each filament, (A) lamellae, (B) arch. (300X).

Fig. 4. A close-up of a hemibranch of a normal goldfish showing the bar of hyaline cartilage(A), the epithelial layer(B) and the lamellae(C). (500x).
Fig. 5. A gill filament with an associated blood vessel(A), the thin epithelium(B) and the blood filled lamellae(C). (500X).

Fig. 6. The detailed structure of the gill lamellae showing the uniseriate epithelial layer(A) in a control animal. (1000X).
Fig. 7. A cross-sectional view of two gill filaments of a test fish after 30 minutes in the homogenate. At (A) the lamellae are ruptured, and the epithelium of the filaments appears swollen at (B). (300X).

Fig. 8. A pair of hemibranchs from a test fish of 30 minutes exposure showing the disorganization of the lamellae(A) and sloughing of the epithelium(B). (300X).
Fig. 9. A gill filament from a test fish after 15 minutes in *L. carteri* homogenate showing the initial swelling of the epithelium (A) and the infolding of the lamellae (B). (300X).

Fig. 10. A cross-sectional view of a hemibranch of a fish exposed to the homogenate for 15 minutes showing the swollen epithelial layer (A) and the general disorganization of tissues. (500X).
Fig. 11. The appearance of a hemibranch of a fish after 60 minutes in L. carteri homogenate. The gill filament is almost totally disrupted with only the cartilage bar(A) and main blood vessels(B) remaining. Cytolysis is evident and the gill is apparently functionless. (500X).

Fig. 12. A gill filament of a fish subsequent to a 30 minute exposure to homogenate. Blistering of the epithelium is evident(A) as is the presence of mucous secretions(B). Distortion has reached such proportions that normal respiratory activities have probably ceased. (1000X).
Fig. 13. A cross-sectional view through the hindbrain region of a normal salamander. Three pairs of external gills (A) with gill bars of hyaline cartilage are shown (B). (100X).

Fig. 14. A higher magnification of the gills of a control salamander showing the gill bars (A) below which is one of the larger blood vessels (B). (300X).
Fig. 15. The normal gill of a salamander with its covering of simple squamous epithelium(A) and ovoid, flattened nuclei(B). The inner connective tissue appears as a network. (500X).

Fig. 16. The fine details of the normal gill of a salamander showing the close association of the uniseriate epidermis(A) to the underlying connective tissue(B). (1000X).
Fig. 17. A cross-sectional view of a test salamanders gills exposed to homogenate for 30 minutes. (100X).

Fig. 18. A section of the fimbriae of a 30-minute test salamander showing the swollen epithelium(A) with its enlarged, rounded nuclei(B). (300X).
Fig. 19. A cross-section of *A. opacum* after a 15 minute exposure. Swelling and slight blistering of the epithelial layer is evidenced (A). (500X).

Fig. 20. The appearance of gill fimbriae of *A. opacum* following 30 minutes in *L. carteri* hemogenate. Extensive swelling of the epithelium is seen at (A) as is some cytolysis. The processes of the inner connective tissue cells (B) have retracted, causing this region to appear in disarray. (500X).
Fig. 21. The gill fimbriae of a test larva of *A. opacum* exposed to *L. carteri* homogenate for 60 minutes. The nearly total destruction of the epithelial layer is evident (A) and much cellular debris (B) is present. The inner connective tissue (C) is completely disrupted and the cell processes extensively shrunken. (1000X).
VITA

Eric Justin Collins was born in Roanoke, Virginia on April 24, 1941, and attended elementary and secondary school there. He was graduated from Jefferson Senior High School in June 1959.

He entered Roanoke College in Salem, Virginia where he majored in Biology. While at Roanoke College he was a member of Beta Beta Beta National Honorary Biological Society and Vice President of the Baptist Student Union. He was graduated on June 3, 1963 with a Bachelor of Science degree.

He entered the Graduate School of the University of Richmond in September 1963. While there, he became a member of the Virginia Academy of Science, was historian of Beta Beta Beta, and presented two papers at the 1964 and 1965 meetings of the Academy. He was graduated on June 7, 1965 with the degree of Master of Arts.