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ZOOLOGICA

SCIENTIFIC CONTRIBUTIONS OF THE

NEW YORK ZOOLOGICAL SOCIETY

Contents

Published January 9, 1970

6

Intact Killifish *(Fundulus heteroclitus)* as a Tool for Medically Oriented Study of Marine Neurotoxins[1]

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(Plates I-II; Text-figure 1)

Using *Fundulus heteroclitus* (killifish), an abundant teleost fish in which aggregation-dispersion of melanophore pigment is rapid and predominantly nerve regulated, it was observed that systemic administration of tetrodotoxin or saxitoxin (purified shellfish poison or derived from *Gonyaulax catenella*) caused sectoral darkening, interpreted as following the previously reported pattern of peripheral pigment-motor unervation, indicating peripheral, central nervous system effects. The test can be used as a sensitive and specific assay for these toxins. Concurrent quantitative and qualitative analysis as well as screening for neuroactive toxins is possible using intraperitoneal or intragas-bladder injection of fish weighing 6-7 gm.(adv.). A consideration of our results and those of others indicates that *Fundulus herteroclitus* lacks the long postulated melanin dispersing nerve.

INTRODUCTION

THIS STUDY arose from work by Burke et al. (1960) and Ruggieri et al. (1962) with axenic cultures of *Gonyaulax* species and other dinoflagellate toxins, and from our discovery of a specific pigmentary response in *Fundulus* upon systemic administration of these toxins or tetrodotoxin. Using this response, we developed a sensitive and specific assay system for tetrodotoxin and saxitoxin. To do this, it was first necessary to further define *Fundulus* (1) by observing the effect of physical factors, and (2) through administration of drugs having known mechanisms of action. These were correlated with previously known facts regarding *Fundulus* and with subsequently published findings regarding the mechanism of action of saxitoxin and tetrodotoxin.

Relevant previously known facts are as follows:

1. *Fundulus heteroclitus* belong to that group of teleosts whose color changes are primarily under nervous control (Fingerman, 1963) as exerted through their melanin-aggregating (blanching or paling) sympathetic nerves (Wyman, 1924a) (Text-fig. 1).

2. There is no *direct* evidence that any opposing melanin-dispersing pigmentomotor nerves exist in *Fundulus* (Pye, 1964a).

3. A pituitary melanin-dispersing hormone (M.D.H.) must play a part in opposing sympathetic nerve effects, for although melanophores of intact *Fundulus* are nonresponsive to M.D.H. (Pickford & Kosto, 1957), denervated tail melanophones cannot expand to a black background in the absence of M.D.H. (Abramowitz, 1940; Pickford & Kosto, 1957).

4. *Fundulus* pituitary extracts contain, and their melanophores respond to, a melanin aggregating hormone (M.A.H.) (Pickford & Atz, 1957). Pineal substance aggregates melanophores of embryonic and larval *Fundulus* but not adult *Fundulus* (Wyman, 1924b) (Lerner's melanin aggregating hormone, melatonin, has since been isolated from the pineal gland [Lerner et al., 1958]).

5. *Fundulus* melanophores are neural crest derivatives and react directly to many neurotropic agents, as other investigators including Scheline (1963), Wyman (1924a), and Abbott (1968) have shown by work on isolated scales; however, many of these reactions are probably mediated by release of transmitter substance

from presynaptic membrane, as Fujii & Novales (1968) found for tetrodotoxin.

6. It has been stated by von Frisch (1911) and quoted by Brown (1957), Pye (1964a), Wyman (1924a), and others that the melanin-aggregating nerves of *Phoxinus phoxinus* L, a *Fundulus*-like teleost, emerge from the spinal canal at the 15th vertebra, bifurcating anteriad and posteriad (Text-fig. 1).

Tetrodotoxin (Narahashi et al., 1964) and saxitoxin (Kao & Nishiyama, 1965) were subsequently reported to interrupt neuronal function by blocking the rapid passive flux of sodium into nerve cells without affecting the sodium pump or the inward or outward shifts of potassium (Kao, 1966). No other agents are known to work precisely this way. In contrast, commonly employed pharmacological agents, such as local anesthetics and barbiturates, block both sodium and potassium ion passive flux (Frank & Sanders, 1963; Frank & Pinski, 1966; Narahashi et al., 1964; Narahashi et al., 1967). For reasons discussed in depth elsewhere (Down & McLaughlin, 1969), we believe the unique response of *Fundulus* to saxitoxin and tetrodotoxin reflects this difference, as well as the peculiarities of *Fundulus* melanophore innervation.

Materials and Methods

Toxin sources. Dinoflagellate toxins were obtained by growing *Gonyaulax catenella* and *G. tamarensis* in axenic culture. Cells were harvested, extracted, and the poison purified using techniques previously developed by Burke et al. (1960). Samples of purified shellfish poison were obtained from Edward J. Schantz, U.S.A. Chemical Corps Biological Laboratories, Ft. Detrick, Maryland. This shellfish toxin had been prepared as previously reported (Shantz, 1960). Although the dinoflagellate toxin has not been shown to be chemically identical to the shellfish toxin, for brevity these toxins will be called "saxitoxin" (Schuett & Rapoport, 1962), as no pharmacological distinctions have been observed previously or in this work. Crystalline tetrodotoxin prepared by Sankyo Co. was obtained from Dr. C. Y. Kao of the Downstate Medical Center, Brooklyn, New York, on May 12, 1964, and used within that month.

Potency standardization of toxins. One ml volumes of diluted saxitoxin were injected intraperitoneally (I.P.) into 20.7 − 27.1 gram white mice, and the concentration for an average 15-minute death time (1 Mouse unit, or M.U.) determined. Solutions were periodically restandardized with mice to eliminate error from loss of potency occurring over the seven month period of use. Because fish used were smaller than mice, volumes of 0.1 ml were almost always used for fish injection. Tetrodotoxin was standardized using the same technique (0.2 ug was found equal to 1 M.U.).

Saxitoxin; sodium concentration. Two samples of saxitoxin, one acid extracted, the other further purified and concentrated by passage through an ion-exchange column, were assayed for sodium and potassium content by the kidney-and-electrolyte laboratory of the Seton Hall College of Medicine and Dentistry. Except for 43 meq/L sodium in the acid extracted sample, these were present in negligible concentrations.

Preparation of solutions. Solutions were in water (a few in saline) and prepared with HCl or NaOH only if necessary to effect solution, then adjusted to pH 7. Fresh solutions were prepared daily, usually within two hours of use. No aseptic precautions were taken in preparing or injecting solutions.

Test animal. Killifish were trapped from October 1963 through May 1964 in estuaries and tidal pools of Cape May County, New Jersey, where water temperatures as low as −1.5C° were recorded. Occasionally, when this source of supply was unavailable, fish were obtained from the Osborn Laboratories of Marine Sciences, New York Aquarium, Brooklyn, New York. Transported in polyethylene bags, fish showed hypoxia-induced darkening only when shipments were delayed, and such fish were used only for experiments on hypoxia damage. *Fundulus* were maintained in aerated sea water aquaria, kept on a neutral-shaded background in an air conditioned room (24-28°C). Unless otherwise stated, light-adapted fish over a light background were used. All experiments were performed at normal room illumination.

Fish were generally used within 48 hours of collection. They were placed on a light or dark background for one-half hour before injection, and after treatment were placed in culture dishes over light (white porcelain pans) or dark (wet carbon paper) backgrounds.

Injection of test animals (13 mm long, 27 gauge needles). With practice, capture and restraint of fish in a bare, moistened hand took no longer than 10 seconds. For preliminary or quantitative work, the injection method of choice proved to be intra-gas-bladder (i.g.b.). Subcutaneous and intramuscular injections gave variable results, probably reflecting chance injection near nerves or blood vessels. Intraperitoneal (i.p.) injections were easier but lacked the advantage of i.g.b. injections of entry confirmation by gas aspiration. Aspiration attempted before i.p. injection ruled out gas bladder or blood vessel entrance. To avoid damaging major vessels

during i.g.b. injection, the needle was not permitted to contact the vertebral bodies. Occasionally, i.g.b. injection caused vascular damage visible through the body wall, and fish so effected were discarded.

A minimum of three fish were injected for each concentration of each solution tested.

Timing of melanophore responses, melanophore recoveries, and death times. Times of earliest visible melanophore changes were recorded. Death times represent the irreversible cessation of visible opercular movements.

Weighing Fundulus. All fish were drained of excess water and weighed immediately.

Photography. Kodak Panatomic 35-mm film was exposed by an electronic flash unit.

RESULTS

Only the responses of dispersion and aggregation of melanin are dealt with. Experiments were too brief for observable morphological effects, *i.e.* pigment mass change.

Temperature sensitivity of fish. Local temperature change-induced melanophore effects have been described in *Phoxinus* (Pye, 1964c). We found that transfer of *Fundulus* from seawater at −1.5°C to room temperature or vice versa had no immediate or delayed visible effect except transient equilibrium loss, without visible melanophore reaction.

Hypoxia. Fifty fish held in a water-filled polyethylene bag until hypoxic became dark, and most died. Most survivors regained ability to respond to a white background, but three remained dark. To test melanophore integrity in permanently dark, damaged animals, L-arterenol (norepinephrine) 0.01 mg/g was injected i.p. two days after the hypoxic episode. Within two minutes total blanching occurred, as with normal dark-adapted animals, indicating melanophore integrity of hypoxia-damaged fish.

Size and species differences. We determined the response of various size-ranged animals. *Fundulus* were graded into three size-groups: small (1.3 to 2.2 g.); medium (5.5 to 6.5 g.); and large (9.1 to 18.8 g.). Mice ranged from 24 to 27 g. Saxitoxin was administered i.p. to mice and i.g.b. to fish. Separate tests showed no difference between time or intensity of effects upon i.g.b. versus i.p. injection. Results are shown in chart (Table I). Dosages given to small and medium sized fish and to mice gave death times

TABLE I

TIME/DOSE RELATIONSHIPS, SAXITOXIN SAMPLE CONTAINING 1.0 MOUSE UNIT/
0.6 ml (INTRA AIR- SAC INJECTION)
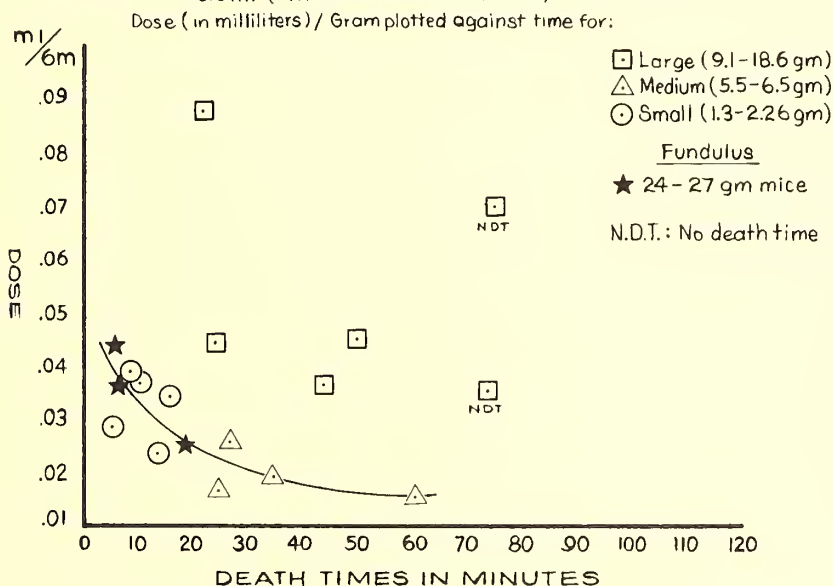
which fell along a single curve. Large fish gave consistent results only with more concentrated solutions, probably because their smaller area of internal absorbing surface per gram of animal and per ml of injected solution vis-a-vis smaller fish resulted in slower toxin absorption. Thus, when animal size is approximately the same, toxin sensitivity of mice is greater than that of *Fundulus*. These results dictated our use of *Fundulus* weighing 7-g or less, to allow use of saxitoxin in the same dilution as for standardization by mouse test. Results were consistent and no correction factor for size was needed.

Peculiarties of Fundulus *vasculature.* We assumed tourniquets might serve to sort out nerve- vs. blood-mediated melanophore responses. Rubber band tourniquets were tried and found ineffective.

Peculiarities of Fundulus *melanophore innervation.*

A. Cholinergic (dispersing) pigmentomotor nerves.

Melanin aggregating nerves of *Fundulus* are known to be adrenergic (Pye, 1964a; Wyman, 1924a). Melanin dispersing nerves when postulated have been assigned cholinergic activity (Pye, 1964c; Fujii and Novales, 1968). We attempted to unmask melanin dispersing nerve action by blocking the sympathetic nervous system with a sympatholytic agent before injecting an acetylcholinesterase inhibitor to increase the concentration of acetylcholine.

Priscoline (tolazoline), an alpha adrenergic blocking agent, upon i.p. injection of 0.02 mg caused a generalized light to dark gray reaction in light adapted animals (4 g) in ~ 10 minutes, with return to normal (light) color at ~ one hour. Eserine (physostigmine), an acetylcholinesterase inhibitor injected in a 2 mg dose in other dark adapted animals caused paling in ~ 10 minutes. This was soon followed by respiratory arrest (cessation of movements of gill operculae) usually preceded by tail spasms and/or convulsions.

Individual drug effects thus established, we gave light adapted *Fundulus* ranging from 1.8-6.8 g priscoline 0.02 mg i.p. and again the partial uniform darkening occurred in ~ 10 minutes. Twenty minutes after i.p. priscoline injection, eserine 2.0 i.g.b. was given each animal. Within 10 minutes respiratory arrest occurred without deepening of the priscoline-induced partial darkening. In a similar experiment light-adapted animals on a light background given doses of priscoline 10x larger (0.2 mg) survived, but melanin dispersal lasted up to five hours. When fish had again become partially light, each was given eserine 2.0 mg and the results were

the same, *i.e.,* they progressed to respiratory arrest in ~ 10 minutes without intervening change in degree of melanin dispersion.

B. Adrenergic (aggregating) pigmentomotor nerves.

It has been reported by von Frisch (1911) and widely quoted (Brown, 1957; Pye, 1964a; Wyman, 1924a) that the melanin-aggregating nerves of *Phoxinus phoxinus* L., a *Fundulus*-like teleost, emerge from the spinal canal at the fifteenth vertebra, bilaterally, and bifurcate anteriad and posteriad (Text-fig. 1).

We found the certain minimum lethal dose (M.L.D.) of saxitoxin, given i.p. or i.g.b. to *Fundulus* weighing not over 6 g, to be 0.15 M.U./g (~ 0.03 ug/g). Half this amount (or more) evokes darkening by quadrants. Usually within one minute, 1-4 sectors (usually right or left anterior) begin to darken (Pl. I, fig. 1). The darkening increases and appears in other sectors (Pl. I, fig. 2), proceeding at an independent rate within each sector until all but the midsection is fully dark (Pl. II, fig. 1). With lethal doses, total body darkening and death ensue (Pl. II, fig. 2). Tetrodotoxin (M.L.D. = 0.075 MU/g = ~ 0.015 ug/g) also causes this sectoral darkening. We believe this illustrates a melanin-aggregating nerve distribution in *Fundulus* similar to that demonstrated in *Phoxinus.*

However, it would be surprising to find that these sympathetic pigmentomotor nerves always emerge from the spinal cord and pass into the sympathetic chain at precisely the fifteenth vertebra. We observed that dark sectors in hundreds of fish, elicited by i.p. or i.g.b. injection of saxitoxin, usually resulted in the pale midzone being centered at or near the fifteenth vertebral level bilaterally. Rarely, the sharply demarcated posterior boundary of one anterior sector and the contralateral sector in a given fish varied in location by as much as four myotomes. Also in some fish, the pale midzone centered several myotomes anteriad, and in others several myotomes posteriad to the fifteenth vertebra. This indicates variability in the level of emergence of the pigment motor nerve fibers.

Discussion

Temperature sensitivity. Even though sudden temperature change had no visible effect upon the shade of intact *Fudulus,* we kept fish at room temperature for 12 hours or longer before using them. This precaution proved worthwhile, for it has since been found (Guttman & Barnhill, 1968) that the excitability of tetrodotoxin-treated axons is more temperature-dependent than that of normal axons.

Hypoxia. That total-animal hypoxia resulted
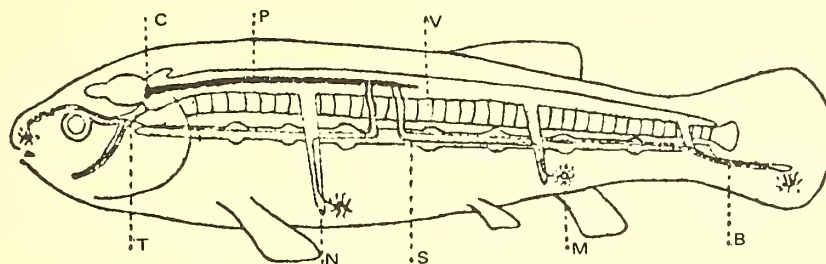
TEXT-FIG. 1. Diagram of the course of the pigment-motor nerve fibers in the minnow. Pigment-motor centers and fibers in heavy black. Nervous system stippled. (Modified after von Frisch, '11; pl. 4, fig. 6.) C, pigment-motor center in medulla; P, pigment-motor center in spinal cord; T, trigeminal nerve; N, spinal nerve; M, melanophore; V, vertebral column; S, sympathetic system with pigment-motor fibers; B, spinal nerve to tail.

in complete generalized melanin dispersal contrasts with statements by Wyman (1924a), who cut vessels and nerves, and Spaeth, who used isolated *Fundulus* scales, as related by Wyman (1924a), indicating the effect of anoxia upon *Fundulus* melanophores is aggregation. Although the "anemia contraction" of melanin as observed by Wyman (1924a) could be due to loss of endocrine control, alone or reinforced by loss of oxygen supply, the same reasoning cannot be applied to results with isolated scales as these remain dark in Ringer's solution until oxygen is withdrawn. However, this direct paling effect of anoxia upon melanophores can be explained if melanin dispersal is associated with maintenance of melanophore polarization, and if oxygen is required for this energy-consuming task. Then, if melanophores of hypoxic, intact *Fundulus* still obtain enough oxygen from surrounding water even after depression of the more hypoxia-sensitive nervous system, release from pigment-motor nerve control would allow them to maintain polarity and the accompanying melanin dispersion. Animals remaining permanently dark following an hypoxic episode probably incurred nervous system damage and thus, permanent melanophore release from pigment-motor nerve control—a potentially valuable preparation for other toxin or melanophore research.

Size and species differences. The ratio of internal absorbing surface to animal body mass is important when saxitoxin, tetrodotoxin, and other rapidly absorbable compounds are injected i.p. or i.g.b. Small animals have greater area of visceral plus parietal peritoneal surface per gram than large animals. We observed that saxitoxin m.l.d. per gram for mice (20-plus g) and *Fundulus* in its most abundant sizes (1.5 to 6 g) is nearly the same: the interspecies difference in sensitivity cancels out the difference in sensitivity due to size when animals of these

sizes are employed. This is fortuitous, and may or may not hold true for any other given toxin.

Peculiarities of Fundulus *vasculature.* It is probable that our results using tourniquets were negative due to collateral circulation via vessels within vertebral canals. These results supplement fruitless attempts of others (Wyman, 1924a) to sort out endocrine vs. nerve mediated melanophore effects by ligating or otherwise modifying *Fundulus* circulation.

Peculiarities of Fundulus *melanophore innervation.*

A. *Cholinergic (dispersing) pigmentomotor nerves.*

While direct evidence for melanin-dispersing nerves in *Fundulus* is wanting, many have argued for their existence on the basis of indirect evidence (Fingerman, 1963; von Geili, 1942; Mills, 1932a,b; Parker, 1948; Waring, 1963). Like Scott (1965), we consider the interpretation of the results upon which this indirect evidence is based to be open to question. Electron microscopy has shown but one type of end-plate on *Fundulus* melanophores (Bikle, 1966). Others (Pye, 1964b; Healey, 1954; Healey & Ross, 1966; Abbott, 1968) found no evidence of melanin dispersing nerves in *Phoxinus* or *Fundulus*. Wyman (1924a) concluded that there is no indication of a double innervation to *Fundulus* melanophores. Our results support this view.

Pye (1964c) showed that "regetine appears to suppress all activity in chromatic (paling) nerve tracts while leaving the melanophores free to respond to the humoral influence of large doses of pituitary extracts." He also showed that rogentine does not influence in any way the normal melanin aggregating action of adrenalin directly upon melanophores. Thus, in our attempts to unmask melanin dispersing nerve activity, we propose that the alpha adrenergic blocking agent, priscoline, similar to regetine, causes

melanin dispersal by blocking the melanin aggregating nerves with little if any direct effect on melanophores. If then, fish are made partially dark by priscoline-induced alpha adrenergic nerve blockade, eserine-induced buildup of acetylcholine should cause further melanin dispersal by stimulation only of unblocked cholinergic melanin dispersing nerves, if any exist. But no such effect, nor any other indication of melanin dispersing nerve action, was seen in our priscoline-eserine experiments. We have not yet performed trials substituting beta adrenergic blocking or anti-adrenergic compounds for priscoline.

B. Cholinergic (aggregating) pigmentomotor nerves.

Anatomic features of the pigment-motor nervous system were mentioned under RESULTS (see Text-fig. 1). We believe that darkening by sector, whether induced by saxitoxin or tetrodotoxin, etc., is explainable only on the basis of the anatomic features of the peripheral melanin aggregating nervous system of *Fundulus*. No other known system within the animal (retinal elements, afferent sensory nerves, pigment-motor centers in medulla or spinal cord, etc.) offers an adequate explanation. The elicitation by known neurotoxins supports this interpretation. The midsection nearest the point of nerve emergence from the spinal cord is last to darken, indicating differential nerve sensitivity reminiscent of the ascending paralysis which saxitoxin and tetrodotoxin induce in mammals, including man. Results indicating the probable ionic mechanism for peripheral pigmentomotor system > central nervous system sensitivity to saxitoxin and tetrodotoxin and further confirming specificity of sectoral darkening is given in detail elsewhere (Down & McLaughlin, 1969).

SUMMARY

Using *Fundulus heteroclitus,* a hardy and abundant teleost bait fish in which aggregation-dispersion of melanophore pigment is rapid and predominantly nerve regulated, we found that systemic administration of tetrodotoxin, or saxitoxin cause sectoral darkening, interpreted as following the previously undocumented (in *Fundulus*) pattern of peripheral pigment-motor innervation, indicating peripheral > central nervous system effect, and constituting a sensitive and specific assay system for the toxins. Concurrent quantitative and qualitative analysis of as well as screening for neuroactive toxins is possible using i.p. or i.g.b. *Fundulus* injection.

A combination of our findings and old and new published information leads us strongly to doubt the existence of long-postulated melanin dispersing nerves in *Fundulus heteroclitus.*

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EXPLANATION OF THE PLATES
Effects of saxitoxin (M.L.D.)

PLATE I

FIG. 1. Darkening in right anterior sector begin-
ning.
FIG. 2. Both anterior sectors totally or near to-
tally dark.

PLATE II

FIG. 1. All four sectors dark, midsection still
light, fish listing.
FIG. 2. Postmortem, showing body-wide darken-
ing and muscular atony (held by one gill
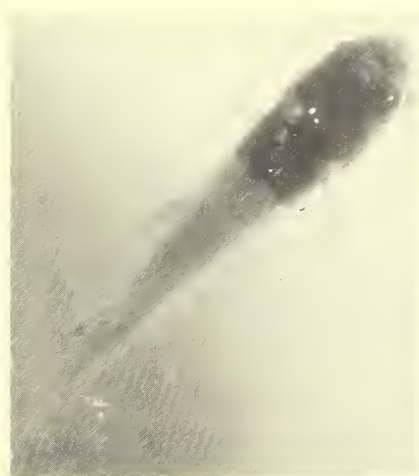cover, head down).

FIG. 1

FIG. 2

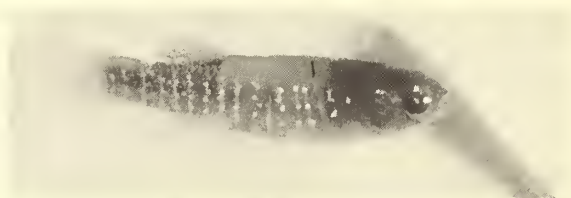INTACT KILLIFISH (FUNDULUS HETEROCLITUS) AS A TOOL FOR
MEDICALLY ORIENTED STUDY OF MARINE NEUROTOXINS

FIG. 1

FIG. 2

INTACT KILLIFISH (FUNDULUS HETEROCLITUS) AS A TOOL FOR
MEDICALLY ORIENTED STUDY OF MARINE NEUROTOXINS

7

Studies on the Biology of Barnacles: Parasites of *Balanus eburneus* and *B. balanoides* from New York Harbor and a Review of the Parasites and Diseases of Other Cirripedia[1]

Lucie Arvy[2] and Ross F. Nigrelli[3]

(Plate I)

Three species of organisms previously reported as predators or as parasites of barnacles and two commensal peritrichs have been found in populations of *Balanus eburneus* and *Balanus balanoides* occurring on the rock jetties from Sea Gate and immediately adjacent to the Osborn Laboratories of Marine Sciences at Seaside Park, Coney Island, New York City, respectively. The species found were *Cephaloidophora communis* (Protozoa: Sporozoa: Gregarinida: Eugregarinina: Cephalinoidea: Cephaloidophoridae) from the intestine of *Balanus eburneus; Epistylis horizontalis* and *Epistylis nigrellii* (Protozoa: Ciliophora: Ciliata: Peritrichida) from the branchial lamellae of *Balanus balanoides* and *B. eburneus,* respectively; *Stylochus ellipticus* (Platyhelminthes: Turbellaria: Polycladia: Acotylea: Stylochidae) from the internal wall of the opercular valves of *Balanus eburneus* attached to *Mytilus edulis;* and metacercariae, possibly of *Maritrema arenaria* (Trematoda: Digenea: Microphallidae), on the external gut wall and other tissues of *Balanus balanoides.* The presence of these organisms in local barnacles represents a new geographical record. These and their effects on the host are briefly described together with a review of the literature on other parasites and diseases of barnacles.

Introduction

THERE is very little information in the literature on the diseases and parasites of Cirripedia, and apparently only the members of the Balanidae have been investigated with any degree of thoroughness. The most common group of parasites found in these sessile crustaceans are the Gregarinida (Protozoa: Sporozoa), fungal parasites, larval digenetic trematodes (metacercariae), and a single species (*Hemioniscida balani*) of parasitic isopod have also been reported. Some of these parasites may be more common than indicated in the literature. Studies in our laboratory have shown that one species of gregarine (*Cephaloidophora communis*) and the metacercaria of a microphallid trematode, together with a predatory turbellarian (*Stylochus ellipticut*) and a commensal peritrich ciliate (*Epistylis horizontalis*), occur in bar-

nacles (*Balanus eburneus* and *B. balanoides*) in the New York area. These are reported below together with a review of the literature.

Gregarinida

Gregarines have been repeatedly observed in barnacles since they were first described by Kölliker in 1847; the most recent report deals with electron microscope studies on *Cephaloidophora communis* in the intestine of *Balanus tintinnabulum* (Reger, 1966). *C. communis* was first described in *Balanus improvisus, B. eburneus,* and *B. amphitrite* by Mawrodiadi in 1908, and has since been reported by several authors in other Balanidae. This species, together with *Pyxinioides bolitoides* in *Balanus nubilis* from the Pacific coast (see Table I), was recently discovered by the present investigators in *Balanus eburneus* collected on the jetties adjacent to the Osborn Laboratories of Marine Sciences. Since development occurs when the sporozoites penetrate the cells of the gut epithelium, some pathological effects (*e.g.* necrosis and desquamation) must occur in spite of the seemingly innocuity of the bioassociated sporadins (Pl. I, fig. 1). Barnes (1955) suggested that infections with gregarines have some profound effect on the

TABLE I

PROTOZOA: GREGARINIDA

Host	Parasite	Author
Balanus pusillus	*Gregarina balani*[2][6]	Kölliker, 1847
Pollicipes polymerus	*Gregarina valettei*[7]	Nussbaum, 1890
Balanus improvisus	Unnamed gregarine	Solger, 1890
Balanus perforatus	*Nematoides fusiformis*[3]	Mingazzini, 1891
Balanus perforatus	*Nematoides fusiformis*	Labbé, 1899
Pollicipes cornucopia	*Nematoides fusiformis*	Labbé, 1899
Balanus improvisus	*Cephaloidophora communis*[7]	Mawrodiadi, 1908
Balanus eburneus	*Cephaloidophora communis*	Mawrodiadi, 1908
Balanus amphitrite	Unnamed gregarine	Mawrodiadi, 1908
Chthamalus stellatus	*Frenzelina chthamali*[4]	Léger and Duboscq, 1909
Chthamalus stellatus	*Cephaloidophora communis*	Léger and Duboscq, 1909
Balanus eburneus	Unnamed gregarine	Budington, 1910
Balanus amphitrite	*Pyxinioides balani*	Trégouboff, 1912
Balanus eburneus	*Pyxinioides balani*	Trégouboff, 1912
Balanus improvisus	*Cephaloidophora communis*	Trégouboff, 1912
Balanus tintinnabulum	*Cephaloidophora communis*	Trégouboff, 1912
Balanus amphitrite	*Cephaloidophora communis*	Ball, 1937
Balanus crenatus	*Cephaloidophora communis*	Henry, 1938
Balanus glandula	*Cephaloidophora communis*	Henry, 1938
Balanus cariosus	*Cephaloidophora communis*	Henry, 1938
Balanus nubilis	*Pyxinoides bolitoides*[5]	Henry, 1938
Balanus nubilis	*Gregarina spissa*	Henry, 1938
Balanus nubilis	*Cephaloidophora magna*	Henry, 1938
Balanus balanus pugetensis	*Cephaloidophora multiplex*	Henry, 1938
Balanus rostratus heteropus	*Cephaloidophora multiplex*	Henry, 1938
Balanus balanus pugetensis	*Pyxinoides pugetensis*[5]	Henry, 1938
Mitella polymerus	*Gregarina valettei*	Henry, 1938
Balanus eburneus	*Cephaloidophora communis*	Ball, 1950
Balanus balanoides	*Gregarina balani*	Ouspenskaia, 1960
Balanus amphitrite	*Cephaloidophora communis*	Heckman, 1961
Chthamalus stellatus	*Bifilida rara*	Tuzet and Ormières, 1964
Chthamalus stellatus	*Pyxinioides chthamali*	Tuzet and Ormières, 1964
Balanus tintinnabulum	*Cephaloidophora communis*	Reger, 1966
Balanus eburneus	*Cephaloidophora communis*	Arvy and Nigrelli (Present paper)
Balanus nubilis	*Pyxinioides bolitoides*	Arvy and Lacombe (recorded in present paper)
Balanus balanoides	*Epistylis horizontalis* Chatton[1]	Arvy and Batisse, 1968
Balanus eburneus	*Epistylis nigrellii*	Arvy and Batisse, 1968

[1] Arvy, and Batisse (in press) recently rediscovered the peritrich ciliate *Epistylis horizontalis Chatton* among the ovarian follicles in *Balanus balanoides* collected off the rock jetty adjacent to the Osborn Laboratories of Marine Sciences; they also reported a new species, *E. nigrellii*, from the gills of *B. eburneus* in the same locality.

[2] The barnacle parasites included in the *Gregarina* must be reclassified since the members of this genus are exclusively found in insects (Tuzet and Ormières, 1964).

[3] Sporocysts of *Gregarina* and *Nematoides* in Cirripedia are unknown.

[4] The name *Frenzelina* is now used for a testate sarcodina of the family Difflugiidae; *F. chthamali*, however, is a true gregarine that has been reclassified as *Cephaloidophora* and/or *Pyxinioides* (see Henry, 1938, for discussion of this species).

[5] Misspelling for *Pyxinioides,* the generic name created by Trégouboff (1912).

[6] Kamm (1922) believes that *Gregarina balani* is a synonym of *Pyxinioides balani* and redescribes *Cephaloidophora communis, Pyxinioides chthamali, Gregarina valettei,* and *Nematoides fusiformis* from various species of barnacles.

[7] Henry (1938) redescribes *Cephaloidophora communis* and *Gregarina valettei* in more detail; the former was also redescribed by Trégouboff (1912) and by Tuzet and Ormières (1964).

physiology of the developing barnacle larvae. Apparently, in some unknown way, the infection causes a delay in the liberation of the nauplii; the latter, however, continue to develop within the mantle to a physiological state that shortens the transformation time into the second naupliar stage during its free existence.

Trematoda

As may be noted from Table II, the metacercariae of only three species of digenetic trematodes of the family Microphallidae have been reported in barnacles. Lebour (1908-1911) was apparently the first to describe metacercariae from a species of barnacles on the North-umberland coast of England, which she named *Cercaria balani.* More recently, Ouspenskaia (1960) reported similar type metacercarial cysts in *Balanus balanoides* from the Barents Sea. However the description and figures are not detailed enough for us to make a comparison with our species, although they were described as the metacercariae of *Maritrema linguilla* (L. A. Jägerskiöld) and *Maritrema gratiosum* (W. Nicoll). The latter are sexually mature stages that occur in the intestine of gulls, terns and other marine birds.

In our routine studies on the biology of the barnacles, similar type metacercariae were also found in *Balanus balanoides* collected in local waters. The worms are contained in thin-walled, yellowish, more often white, and refringent spherical cysts measuring 0.3 mm in diameter; the body wall of the parasite is covered with delicate spines, except for its posterior third; oral and ventral suckers are approximately of equal size; the cecae are relatively long, each branch measuring on the average 0.5 mm in length, and easily demonstrated when stained

vitally with neutral red (Pl. I, figs. 2-4). Attempts at excystment by feeding isolated cysts to killifish, *Fundulus heteroclitus,* were unsuccessful. Further experiments to induce this process were dropped in view of the failures reported by Hadley and Castle (1940) when they fed cysts from infested *Balanus balanoides* collected from Woods Hole, Massachusetts, and from the coast of Maine to young *Larus argentatus, Sterna hirundo hirundo, Trigonoides macularius,* white mice, white rat, kitten, and domestic fowl. On the basis of circumstantial evidence, *i.e.,* the discovery of sexually mature adults together with metacercarial cysts in the intestine of the turnstone, *Arenaria intrepes morinella,* a bird that is known to feed on barnacles, Hadley and Castle concluded that the metacercariae and the adults were the same; they considered this to be a new species for which the name *Maritrema arenaria* was given. The striking similarities of our form with those figured by Hadley and Castle lead us to conclude that the cysts from *Balanus balanoides* taken from local waters are also the metacercariae of *Maritrema arenaria.*

The pathological effects of metacercarial infestations in barnacles are not too well known. Our observations show that in light infestations, the cysts are usually localized on or near the gut; when the infestations are exceptionally heavy, all parts of the body, except for the appendages and the lumen of the gut are involved. In such instances, the metacercariae are firmly embedded in a relatively thick connective tissue formed around the external gut wall. Barnacles showing such extreme conditions have ovaries that are reduced to filiform cords of follicular tissue and entirely devoid of oocytes. Whether or not this is a consistent pathological feature remains to be established.

Table II

Trematoda

Host and Locality	Parasite	Author
Balanus sp. (Northumberland coast)	*Cercaria balani* (metacercariae)	Lebour, 1908/11
B. balanoides (Woods Hole)	Metacercariae of *Maritrema arenaria*	Hadley and Castle, 1940
B. balanoides (Barents Sea)	Metacercariae of *Maritrema gratiosum* W. Nicoll	Ouspenskaia, 1960
B. balanoides (Barents Sea)	Metacercariae of *Maritrema linguilla* L. A. Jägerskiöld	Ouspenskaia, 1960
B. balanoides (Coney Island)	Metacercariae of undetermined *Microphallidae;* in all probability *Maritrema arenaria*	Arvy and Nigrelli (present paper)

Isopoda

Infestations of barnacles by the protandrus hermaphrodite *Hemioniscus balanus* (Crustacea: Isopoda: Ipicaridea: Hemioniscidae) have been known since the latter part of the 19th century and have since been reported in these hosts from various parts of the world. In its developmental cycle, the males (cryptoniscus stage) become transformed into females when they take up the parasitic existence. The female is accompanied by grotesque changes in form, eventually becoming an enlarged star-shaped egg-sac (abdomen), with evidence of its crustacean characteristics indicated by the retention of certain head and thoracic appendages. In the early stages of the transformation processes, the parasitic female sucks the body fluids of the barnacle, which are stored into two large "liver" lobes; this nutrient material is eventually transferred to the ripening eggs as reserve food material (see Wimpenny, 1966).

Each barnacle may harbor one or more parasitic females; Perez (1923) found as many as seven individuals in a single *Balanus balanoides.* It has been suggested that such a heavy infestation inhibits the development of the gonads as the result of mechanical pressure, or may actually cause a destruction of the ovaries. However, such ovariectomized barnacles are still capable of carrying on most life functions as indicated by the continued rhythmic movements of the cirri of the parasitized animals.

Fungus and Lichens

Three species of fungi have been reported as infecting barnacles. Two of them, namely *Didymella balani* and *Pharcidia marina* from the tests and shell of *Balanus balanoides* and *Chthamalus stellatus* were originally classified as ascomycetes by Hariot (1887) and Bommer (1891), but have since been recognized (Santesson, 1939) as marine lichens of the genera *Arthropyrenia* and *Didymella* (for clarification of the taxonomy, see Johnson and Sparrow, 1961).

The third species, *Lagenidium chthamalophilum* (Phycomycete), is a virulent fungal agent that was found to be the cause of an epizootic in 1957 in the barnacle *Chthamalus fragilis denticulata* from Beaufort, North Carolina, with an incidence that ranged from 12.5% to 100% (Johnson, Jr., 1958). This highly

Table III

Isopoda: *Hemioniscus balanus*

Species	Locality	Author
Balanus sp.	German coast, North Sea	Buchholz, 1886
Balanus sp.	Wimereux, French coast, English Channel	Caullery and Mesnil, 1899
Balanus improvisus	Gironde estuary, France	Perez, 1900
Balanus perforatus	Roscoff, Brittany, France	Perez, 1923
Balanus balanoides	Roscoff, Brittany, France	Prenant, 1923
Chthamalus stellatus	Roscoff, Brittany, France	Prenant, 1923
Balanus balanoides	Atlantic coast north to Tromsø (Norway)	Crisp, 1951
Balanus amphitrite	English estuaries	Crisp and Molesworth, 1951
Balanus sp.	South Africa	Sandison, 1954
Balanus balanoides	Southwest coast of England	Crisp and Southward, 1954
Balanus porcatus	?	Crisp, 1954
Chthamalus dalli	North American Pacific coast	Cornwall, 1955
Elminius modestus	?	Crisp and Davies, 1955
Balanus balanoides	Atlantic French coast	Crisp and Fischer-Piette, 1959
Balanus perforatus	?	Crisp and Patel, 1960
Eliminius modestus	Roscoff	Bourdon, 1963
Balanus balanoides	Roscoff	Bourdon, 1963
Balanus balanoides	Halifax area (New Scotland)	Crisp, 1968
Balanus balanus	From Labrador to Massachusetts	Crisp, 1968
Balanus glandula	Friday Harbor	Crisp, 1968
Chthamalus dalli	Friday Harbor	Crisp, 1968
Balanus balanus	Irish Sea, Faroe, Shetlands	Crisp, 1968
Balanus hameri	Irish Sea	Crisp, 1968

Remarks: Crisp (1968) states that barnacles such as *B. improvisus, B. algicola, Chthamalus dentatus,* and *Elminius modestus,* may also be parasitized by *Hemioniscus balani.* Forms such as *Verruca stroemia, Balanus crenatus, B. perforatus,* and *B. stellatus* are never infested; Perez (1900) has made a similar observation, *e.g.* in the Gironde estuary *Balanus improvisus* is heavily infested by *Hemioniscus balani* but *Chthamalus stellatus* are never parasitized; the reason of this apparent immunity remains unexplained.

TABLE IV

LICHENS AND FUNGUS

Host	Locality	Parasites	Author
		Lichen	
Chthamalus stellatus	test	*Didymella balani*	Hariot, 1887
Balanus balanoides	shell	*Pharcidia marina*	Bommer, 1891
		Fungus	
Chthamalus fragilis var. *denticulata*	ova	*Lagenidium* *chthamalophilum*	Johnson, 1958

pathogenic fungus infects the ova and apparently is specific for *Chthamalus fragilis*, since *Balanus amphitrite* in the same waters is resistant to infection both under natural and experimental conditions.

Johnson reported that the fungus develops in the ova of the barnacle at any time between gastrulation and the emergence of the nauplii; neither the released nauplii nor the somatic tissues are involved. The degree of infection varies with the stage of development of the egg mass. Thus, embryos with three or more appendage buds are most often infected; earlier stage embryos, i.e. with one or more appendage buds, are completely destroyed, leaving only clusters of egg membranes filled with fungus mycelium. Lamellae with more mature embryos apparently are more resistant, since some embryos escape invasion by the fungus and develop into normal nauplii. The infection is initiated by laterally biflagellate planonts that become transformed into spores when they settle on the egg. Within three minutes after attaching to the egg membrane, the spore protoplasm penetrates the membrane, increases in size into a hyphal rudiment, and grows along the embryo. The infection is visible as pallid grey or grey-green lamellae. The infection spreads rapidly through the entire cluster so that within two days all the embryos are invaded. There can be little doubt that *Lagenidium chthamalophilum* attacking the ova of the barnacle *Chthamalus fragilis* may have caused a reduction in population density of this species in Beaufort, North Carolina, during and after the epizootic. As pointed out by Johnson (1958), further studies are needed to establish the importance of this fungus. Studies must be undertaken on distribution and severity of infection; conditions favoring the development and spread of the infection; the factors responsible for host susceptibility; and, whether or not the epizootics are cyclic.

TURBELLARIA

Members of the genus *Stylochus*, sometimes called the oyster "leech," (Platyhelminthes: Turbellaria: Polycladia: Acotylea: Stylochidae) are predators occurring free or "encapsulated" (walled-off by chitinous secretions of the host) in oysters, barnacles, pangurid crabs, and in other invertebrates (see Hyman, 1951; Cheng, 1967). Those in the barnacles are usually found free or encapsulated on the internal wall of the opercular valves, and sometimes closely associated with the ovaries (Skerman, 1960). *Stylochus ellipticus*, which according to Loosanoff (1956) may be responsible for the destruction of large numbers of oysters on the flats at Milford, Connecticut, was found locally in a population of *Balanus eburneus* attached to *Mytilus edulis*. The worms were found free on the internal wall of the opercular valves or deep within the host on which they were feeding. There was no evidence that the flatworms were feeding on the *Mytilus*.

It has been estimated that a single oyster-inhabiting turbellarian lays about 22,000 eggs in a month, which at 28° C hatch in a few days into pelagic ciliated larvae, become transformed into adults in two months, live a free existence in the littoral zone for about a year, and eventually encysting in great numbers on all parts of the oyster spat, causing heavy mortality. It remains to be seen whether or not *Stylochus*

TABLE V

TURBELLARIA

Host	Predator	Author
Balanus sp.	*Stylochus neapolitanus*	Lang, 1884
Balanus sp.	*Stylochus zanbibaricus*	Skerman, 1960
Balanus eburneus	*Stylochus ellipticus*	Arvy and Nigrelli (present paper)

ellipticus is as important a predator for young, newly set barnacles as it is for the spats. The abnormal arrangement of the plates of *Balanus eburneus* attacked by the *Stylochus ellipticus*, which we observed, may be indicative of an invasion early in its growth.

Discussion

Much has been written on the biology of barnacles, especially on their distribution, nutrition and growth, factors affecting mortality of natural populations, and particularly the role of temperature on the life cycle. These topics have been reviewed by several authors (Henry; Bookhout and Costlow, Jr.; Connell; Barnes) in a symposium on "Marine Boring and Fouling Organisms" held at the Friday Harbor Laboratories in 1957 (edited by Dixy Lee Ray and published in 1959 by the University of Washington Press). Very little information is included on the enemies of barnacles; it is apparent from the present paper that more studies are needed to establish the possible role of predators, parasites, and diseases in barnacle ecology. It is possible that one or more of these agents, under certain specific conditions, may be important for the biological control of these economically significant fouling organisms. More studies are especially needed on possible fungal and bacterial infectious agents that may play such a role.

Summary

Three species of organisms previously reported as predators or as parasites of barnacles and two commensal peritrichs, have been found in populations of *Balanus eburneus* and *Balanus balanoides* occurring on the rock jetties from Sea Gate and immediately adjacent to the Osborn Laboratories at Seaside Park, Coney Island, New York City, respectively. The species found were *Cephaloidophora communis* (Protozoa: Sporozoa: Gregarinida: Eugregarinina: Cephalinoidea: Cephaloidophoridae) from the intestine of *Balanus eburneus; Epistylis horizontalis* and *Epistylis nigrellii* (Protozoa: Ciliophora: Ciliata: Peritrichidae) from the branchial lamellae of *Balanus balanoides* and *B. eburneus*, respectively; *Stylochus ellipticus* (Platyhelminthes: Turbellaria: Polyclada: Acotylea: Stylochidae) from the internal wall of the opercular valves of *Balanus eburneus* attached to *Mytilus edulis;* and metacercariae, possibly of *Maritrema arenaria* (Trematoda: Digenea: Microphallidae), on the external gut wall and other tissues of *Balanus balanoides*. The presence of these organisms in local barnacles represents a new geographical record. These and their effects on the host are briefly described together with a review of the literature on other parasites and diseases of barnacles.

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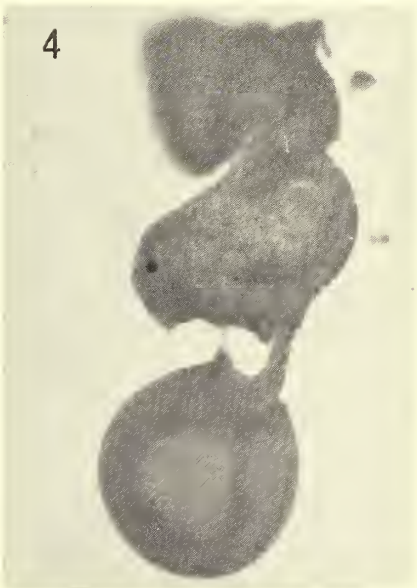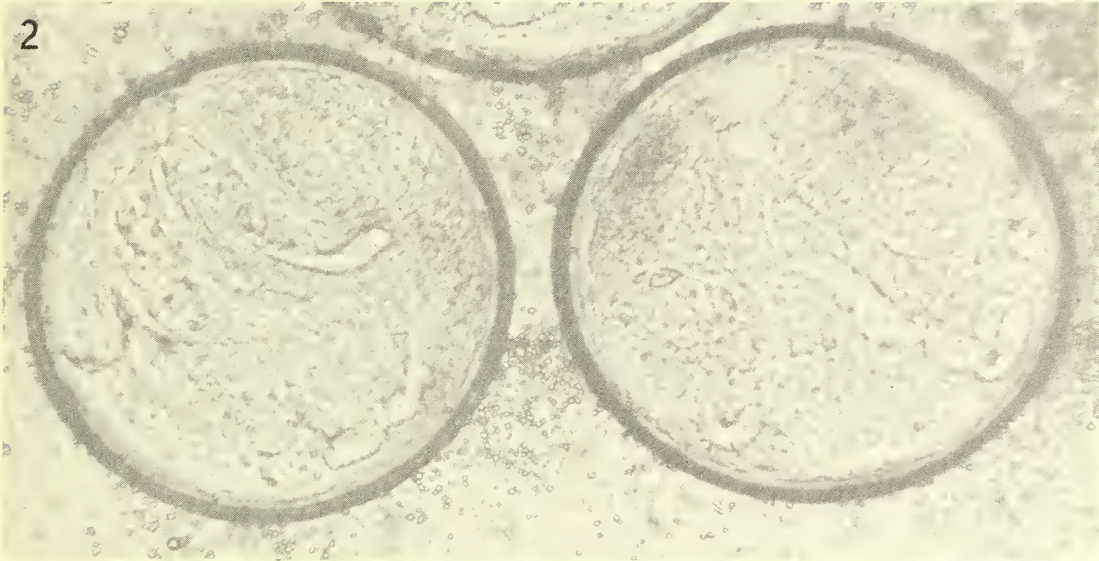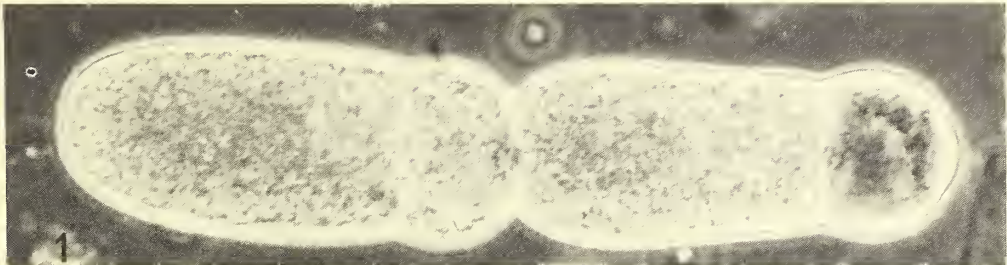
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EXPLANATION OF THE PLATE

Fig. 1. Sporadin of *Cephaloidophora communis* Mawrodiadi (1908) from the intestine of *Balanus eburneus* collected on rock jetties adjacent to Sea Gate, Coney Island, Brooklyn, New York.

Figs. 2-4. Metacercariae of Microphallid digenetic trematode, probably *Maritrema arenaria* Hadley and Castle (1940), from *Balanus balanoides* collected on rock jetties on Coney Island Beach; Fig. 2: unstained living specimens; Fig. 3: cyst stained with neutral red; Fig. 4: excystment.

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