77-9963

LEDERHENDLER, Israel Izja, 1947-
REPRODUCTIVE BEHAVIOR OF *Aplysia dactylomela* (Rang, 1828) (Opisthobranchia: Gastropoda).

City University of New York, Ph.D., 1977
Psychology, psychobiology

Xerox University Microfilms, Ann Arbor, Michigan 48106

REPRODUCTIVE BEHAVIOR OF *Aplysia dactylomela*

(Rang, 1828) (Opisthobranchia: Gastropoda)

by

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A dissertation submitted to the Graduate Faculty in Psychology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, the City University of New York.

February 1, 1977

This manuscript has been read and accepted for the Graduate Faculty in Psychology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

The reproductive and inter-individual behavior of Aplysia dactylomela (Gastropoda: Opisthobranchia) was investigated to understand social organization and bond-formation in these simultaneous hermaphrodites. Contact and copulation, and egg-laying, were studied in four experiments to answer the following questions: Can members of the species respond to each other at a distance? Is conspecific responsiveness related to immediately prior social exposure? Are egg-laying and copulation related in any way?

To answer whether members of the species can respond to each other at a distance, 16 reproductively-mature A. dactylomela were observed in a unidirectional stream in the presence of conspecific stimulation or plain sea water. Streams containing conspecific stimulation were significantly more effective in eliciting positive taxis toward the stimulus source.

To determine the effect of the Aplysia's immediately prior social exposure on subsequent behavior, animals with four levels of exposure (alone, no contact, contact, copulation) were paired in various combinations with different animals, and their behavior during successive thirty-minute observations was studied. The behavior of a pair was predictable on the basis of each partner's preliminary experience. Animals which were paired during a preliminary observation were most likely to initiate contact and become the sperm donor. Individuals usually reversed the sperm donor and

recipient roles when paired with a different partner, as well as with the same partner over a 24-hour session of observations. Preliminary social exposure was also found to be a factor in the influence of experienced animals on the locomotion of "alone" animals.

The relationship of copulation and egg-laying was studied by comparing several egg-laying characteristics before and after a 24-hour pairing. The inter-spawn interval, which was consistently three days, was shorter in a significant number of Aplysia following copulation. Egg-laying recency did not have any effect on copulation latency and duration.

The social interactions of pre-copulatory A. dactylomela was studied in successive observations of five pairs. Copulation occurred in only one pair, and the reversal of sperm donor and recipient roles was seen.

A certain amount of contact may be necessary before juveniles can copulate. Egg-laying need not follow the first instances of copulation but copulation might initiate a series of morphological and physiological changes which are necessary for egg-laying at a later stage of development.

ACKNOWLEDGEMENTS

This thesis is in part the product of the support, confidence, and guidance of Drs. Ethel Tobach, Howard R. Topoff, and H. Philip Zeigler, to whom I would like to express my sincerest appreciation.

Appreciation is also expressed for the comments, criticism, and helpful suggestions of Drs. Wayne P. Aspey, Edward S. Hodgson, and to Dr. Carl Berg, Jr., for his encouragement in the early stages of this study.

I am particularly grateful to Ms. Kate Herriges for her assistance in the preparation of the data and the final manuscript, and to Mr. Louis Medvene, for his personal support and friendship.

Special thanks are due to David Ballantine and Ron Larson of the University of Puerto Rico for the identification of algae and assistance with collection of the animals.

The research was supported by the Research Foundation of The City University of New York, Training Grant MH-13051, and the Department of Animal Behavior, American Museum of Natural History.

This thesis is dedicated to my teacher, Dr. Ethel Tobach; my parents, Herschl and Anya Lederhendler; and my friend, Kate Herriges.

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INTRODUCTION TO THE THESIS

Thirty-five different species of Aplysia, or sea hares, mostly inhabit warm seas throughout the world. A. dactylomela, A. juliana, and A. parvula are very common and have been collected circumglobally. They are opisthobranchs, and together with the pulmonates and prosobranchs make up the three subdivisions of snails, or gastropods. As a genus, Aplysia have been found in all kinds of marine habitats, varying from intertidal splash zones to shallow grass and sandbeds, to subtidal sponge beds. Sea hares have been of interest to biologists since the early 19th century, largely because of their habit of releasing purple fluid when disturbed (Cuvier, 1803).

Recently Aplysia and other marine invertebrates have become important to neurobiological and biomedical researchers because the nervous system contains large and conveniently accessible cells for studies of intracellular and intercellular phenomena (Kandel and Kupfermann, 1970; Kandel, 1974). In general, the justification for this research is that in evolution, neuronal and other cellular processes are conservative and therefore similar across many species. Thus, the mechanism of the nerve impulse was finally worked out using the giant axons of squid, and was then found to have very general application to other systems (Young, 1964). The aspects of nervous system organization which are conservative is not entirely obvious however, in that some very significant differences appear to exist between even closely-related "simple" systems (Kennedy, 1975).

On the other hand, such relatively less complex organisms have become very interesting for the comparative problem of relating behavioral and neural levels of organization (Hoyle, 1975; Fentress, 1976).

Preliminary field investigation with Aplysia dactylomela revealed two types of association. They were often found concentrated in one area. Some were only in close proximity, but others were in contact or copulating. For the research, it was important to find large number of animals. This led to issues of immediate practical significance. It became important to know the circumstances which led to aggregation and the behavioral processes which might account for its occurrence. Very little was available in the literature to account for these aggregations although other workers also noted their occurrence.

Consequently, the general objectives for this study became the investigation of reproductive behavior and inter-individual activity in relation to the level of bond formation. These terms require some explanation, and Beer's (1971) critique of students of social behavior is particularly germane. Beer pointed out that studies of the social behavior of animals persistently focus on behavioral problems in isolation from the overall species context. Since adaptation may occur at various stages in the life cycle (de Beer, 1951) theories of the evolution of social organization must take into account the total diversity of social phenomena in any one species. Such an attempt was made here in that inter-individual processes were studied in juveniles and adults, and in relation to egg-laying.

In the broadest sense, social organization is ubiquitous because the members of each species are organically related by the nature of reproduction. The corollary, that sufficient members or populations of each species manifest typical patterns of life history changes which ensure reproductive success, also ensure ubiquity. Social bonds, in the sense of reciprocal stimulation between individuals, are not necessarily a pre-condition for social organization. Many species which do not demonstrate reciprocal patterns of stimulation participate in coordinated reproduction at a level of individual responsiveness to external stimuli, as well as by modes of response available at different stages of individual development (Tobach, 1963). Nonetheless, they remain of considerable interest for the problem of the evolution and development of social organization. Allee, for example, compared "infrasocial" and "social" animals and drew attention to the special position of sex in the comparative analysis of social evolution:

> "In its earliest forms aggregations formed without sexual stimuli and at the lowest level of group integration may have survival value for members...These aggregations are important in social evolution...Sex may have evolved from the mutual stimulation which has been demonstrated to occur under certain conditions when two similar asexual cells are crowded together within a limited volume of medium...Sex, once originated, became one of the integrating factors in further social development..." (Allee, 1931, p. 4)

Animal aggregations may occur passively, as planktonic forms which become concentrated in a bay by the action of currents (Thorson, 1950; Crisp, 1973) or they may be active, occurring through the taxic or kinetic reactions of individual organisms to stimulation from an external source (Fraenkel and Gunn, 1961).

To compare different phyletic levels, Tobach and Schneirla (1968) distinguished responses to stimulation by other animals as biotaxis: a qualitative distinction focussing attention on groupings based on reciprocal processes in bond formations. Social behavior was further divided into biosocial and psychosocial levels which is of particular interest to this study because it is particularly useful in comparisons of vertebrates and invertebrates. Bond formation at the biosocial level refers generally to groups where members are dominated by "behavior resulting from reciprocal stimulation such as pheromone production. Aspects of their neural equipment hold behavioral modification within distinct limits, so that patterns of action typically are determined rather directly by structural and physiological factors. Such factors limit the storage of the trace effects of experience and restrict the use of such trace effects in new situations. The type of group bond formed in such animals is of a situationally determined character, " (Tobach and Schneirla, 1968, p. 70). In contrast, psychosocial levels of social behavior are based on taxic and biotaxic responses which "become modified through the integration of maturation and experience by virtue of such processes as conditioning, learning, and concept formation (among others), introducing a plasticity of behavior" and where, " the meanings rather than the immediate effects of stimuli are functional," (p. 71). This differentiation between biosocial and psychosocial and the accompanying mesolevels has been illustrated in a classic paper by Schneirla and Rosenblatt (1960) comparing social insects and mammals.

When beginning an investigation of the social processes in a mollusk, it seemed appropriate to consider whether even biotaxic levels of bond formation existed. It seemed that reproductive behavior in Aplysia was readily accessible to observation and could be investigated from this perspective.

Evidence for parallel and analogous forms of neuroendocrine regulation in vertebrate and invertebrate reproductive processes has been growing steadily (Scharrer and Scharrer, 1963). As different components of the mating patterns become less determined by immediate situational variables, plasticity in other aspects also increases. One could suppose, based primarily on the vertebrate literature (Young, 1961; Aronson, 1959) that the relative dominance of endocrine and neural processes in invertebrates would also change in the direction of increased plasticity in the behavior. Since sexual behavior is frequently the only, or minimal point of interaction in the life cycle of many species of invertebrates, the distinction between biosocial and psychosocial levels in reproduction is helpful in comparing levels of social organization across species in general.

Associative phenomena, including social bond formation, have been studied in invertebrates. However, investigations have largely been restricted to the arthropods and the social insects (Isoptera and Hymenoptera) in particular. Considering the diversity in the forms of behavioral and ecological adaptation which are found even within closely-related groups of invertebrates, their value in the comparative study of social processes warrants considerably more attention by comparative psychologists than they have afforded (See Wilson, 1971, for review).

Non-reproductive social phenomena in crustacea have received considerable attention, including pair-formation in shrimp (Johnson, 1969; Wickler and Seibt, 1972; Seibt, 1973); aggregation in lobsters and crabs (Bovbjerg, 1959; Atema and Engstrom, 1971; Stevcic, 1971; McLeese, 1973; Berrill, 1975); and other behaviors reviewed by Schone (1961). Inter-individual aspects of settling and habitat selection of invertebrate pelagic larvae have been reviewed by Meadows and Campbell (1972). Aggregation and other social behaviors have been discussed for flatworms (Reynierse *et al.*, 1969; Reynierse and Gleason, 1975); polychaetes (Evans, 1971); echinoderms (Reese, 1968); and cephalopods(Wells, 1966). Reviews by Fraenkel and Gunn (1961), Carthy (1971), Evans (1968), Winn and Olla (1972), Corning et al. (1973) reveal a wealth of information for the study of invertebrates social behavior and comparative psychology in general. Among others are included problem areas such as learning, orientation, stimulus filtering, and changes in responsiveness.

The behavior of *Aplysia* can conveniently be organized into three major categories: locomotion, feeding, and reproduction and social behavior. Burrowing, which has recently been described by Aspey and Blankenship (1976a, 1976b), and inking, described by Tobach et al.(1965), will be discussed with reproduction because of the special implications of these behaviors for social processes. Where information is lacking or particular facts need further illumination, studies of other opisthobranchs will be incorporated into the discussions.

The relatively large number of publications on habituation and sensitization of the gill and siphon reflexes do not bear directly on the central issues of this study (For reviews see Corning et al., 1973, volume 2; Kandel, 1974). The studies of Carew et al. (1971; 1972) and Carew and Kupfermann (1974) are notable because they showed that Aplysia have a capacity for some long-term storage of the effects of experience, and that the laboratory-induced modifications parallel adjustments to naturally-occurring conditions.

A. Life History of Aplysia

The distribution of Aplysia seems to be limited by life history, feeding habits, and temperature relations (Eales, 1960). Thus, since it is an herbivore it is usually found in the littoral and sub-littoral zones. Aplysia are considered to live approximately one year (Miller, 1960; Carefoot, 1967; Nishiwaki, 1976). During this time they tend to increase in size, reach a peak, and then decrease in size until death. These authors found that spawning occurred during a restricted period of several months when the animals had attained a large size.

Originally Eales (1921) proposed that the life history of Aplysia punctata was organized around a spawning migration from the sublittoral to the littoral. Miller found egg masses of A. punctata in the sublittoral and concluded that such a migration was not a necessary part of the life cycle. These findings were replicated in Carefoot's study in a different location. Miller and Carefoot's work also provided strong evidence that the life cycle of A. punctata consisted of non-overlapping generations. Eales, on the other hand, found a longer spawning period in A. punctata and suggested that the pattern was bi-generational. She was supported in this finding by Usuki (1970) from studies of several species of Aplysia in Japanese waters. The finding of seasonal changes in the neurohormonal induction in A. californica of egg-laying (Strumwasser et al., 1969) would suggest that this species also has a more active spawning period in the late summer months. As Smith and Carefoot (1967) found, the occurrence of spawning was closely correlated with an increase in temperature to between 9 C. and 14 C. One could suppose, therefore, that the same species might have both a bi-generational cycle, depending on the temperature characteristics of the waters in which it was studied. Comparisons of egg-laying patterns in different species from tropical and temperate waters would illustrate this question. A. brasiliana from the Gulf of Mexico do not seem to have a breeding season (W.P. Aspey, 1976).

Kriegstein et al. (1974) found that A. californica will metamorphose only when provided with a specific algae which is also eaten by adults. Strength and Blankenship (1976) found the same for A. brasiliana. This suggests that at least some sea hares settle and grow on the same algae.

B. Locomotion

Locomotion in Aplysia received attention from Jordan (1901) and Parker (1917) as a convenient animal in which to investigate gastropod locomotion. Aplysia always locomote forward through a single wave which travels the length of the foot in a retrograde fashion.

Several specializations in locomotion have also been described (Bebbington and Hughes, 1973; Hamilton and Ambrose, 1975). Some species of Aplysia swim by means of parapodial flapping or possibly undulations of the body. In species which swim, this method of locomotion appears to be a more efficient means of local movement. Hamilton and Ambrose (1975) and Aspey, Cobbs, and Blankenship (1976) measured swimming efficiency in relation to current strength and direction which indicated that it was an oriented movement. Lederhendler et al. (1975) reported that the locomotion of Aplysia dactylomela was negative with respect to the "net" direction of currents and tides and reported evidence that certain interspecific relationships with sea cucumbers, or sessile jellyfish could influence their movements. (Aplysia dactylomela do not swim.)

A circadian pattern of locomotion has been descirbed in A. californica using a mechanical recorder (Kupfermann, 1968), a television tracking (Strumwasser, 1971), and a running wheel (Jacklet, 1972). Under a 12/12, light/dark cycle, A. californica begins locomoting shortly before light-onset and enters a relatively quiescent period just before dark. The amount of locomotion was greater under higher light intensities. Tactile stimulation or sea water agitation inhibited locomotion.

A circadian rhythm in the eye was discovered by Jacklet (1969a, 1969b) in the frequency of compound action potentials recorded directly from the optic nerve when the eye and nerve complex are isolated in organ culture. At first, it was believed that the locomotor rhythms were controlled by the optic rhythms. Further investigations showed that the activity in the eye itself was not necessary for the maintenance of this rhythm, but that the process was integrated through synchronized relationships between several photoreceptors and circadian oscillators within different ganglia in the nervous system (Eskin, 1971; Lickey and Zack, 1973; Block and Lickey, 1973; Block and Smith, 1973; Block et al., 1974; Jacklet, 1974).

Carefoot (1970) reports that A. juliana becomes active at night when it feeds, while A. dactylomela is more active in the late afternoon. These patterns changed within a week when individuals were removed to the laboratory, suggesting the influences of unknown environmental factors in the maintenance of activity rhythms.

In related opisthobranchs, Alkon (1974) found that a positive phototaxis in Hermissenda crassicornis could be modified by rotational stimulation.

Crozier and Arey (1919) reported a negative rheotaxis with respect to the tidal currents in Chromodoris zebra. They considered the response to be related to local movements of these animals. Removal of the rhinophores eliminated these movements.

Chemical stimuli from food and other sources have been implicated in chemotactic responses. These will be discussed in the following section.

At present it can be concluded that Aplysia are clearly capable of directed locomotor activity. The sensory influences of light, current, temperature, and food probably interact with internal rhythmic changes in producing the observed effects.

C. Feeding

In the field, different species of Aplysia feed on a variety of red and green algae depending on whether a particular population lives in the littoral or sublittoral zone and possibly the stage of development (Eales, 1921; Winkler, 1963; Carefoot, 1970; Krakauer, 1971). In general, *Aplysia* do not appear to consume brown algae.

Carefoot's (1967a, b; 1970) studies of *A. punctata*, *A. dactylomela*, and *A. juliana* showed that growth rates depend on the species of algae eaten. He found that the algae eaten in the field was the one that yielded the most rapid growth when tested in the laboratory. However, *A. dactylomela* preferentially ate a different species of food when it was provided. Various species of algae were eaten if other options were not available, though it is noteworthy that some were never eaten.

The chemosensory basis of feeding behavior has received considerable attention. It has generally been agreed that chemosensitivity must be assumed for the entire exposed surface of gastropods (Charles, 1966; Hyman, 1967). Kohn (1961) in a review of chemoception in gastropods, concluded that the chemosensory and mechanosensory functions are the major systems through which information about changing environmental conditions are integrated.

A number of field and laboratory studies have shown that *Aplysia* sense and find food at a distance through chemotaxis. *A. juliana* showed a highly selective response to the alga *Ulva lactuca*. Other potential foods were not effective in producing approach responses unless induced by solutions of the prepared food. Both the anterior tentacles and mouth region were effective in chemosensory responses, and the dorsal tentacles were implicated in rheotaxis under the condition of a gentle current (Frings and Frings, 1965).

Preston and Lee (1973) found evidence in *A. californica* for chemical sensitivity at the dorsal tip of the buccal tentacles to food stimulation. They also found that this species will reliably turn toward a steeper gradient of food stimulation presented in a unidirectional flow of water. Audesirk (1975) could not confirm the chemosensory role for the rhinophores in A. californica, but did support the distance chemoception findings and the consistent role of the buccal tentacles in food finding.

As Preston and Lee found that stimulating the sea hares with food solution prior to a test also produced a negative rheotaxis, it seems likely that both types of response are effective (to current and to chemical). The role of the rhinophores can therefore be thought of as two separate but interdependent levels, orientation to the stream and identification of attractive substances. Of considerably more interest to the present study is that Preston and Lee found the dorsal tentacle response to tactile stimulation was different depending on its prior experimental history (previously stimulated with food stimuli or not previously stimulated). Jahan-Parwar (1972) found that very small concentrations of certain amino acids introduced at a distance resulted in typical feeding responses in the mouth.

From these reports, it seems that feeding in Aplysia consists of several behavioral phases: detection of food-stimulation, orientation and approach, and the feeding response itself. In further laboratory experiments, Kupfermann (1974a) found that A. californica do not feed continuously. After a large meal, locomotion stopped and it became difficult to elicit biting response with food stimuli compared to nonsatiated individuals. Non-satiated animals took less time to respond to a second food stimulus presented 30 seconds later. After the second presentation, the bite latency and intervals remained constant through successive presentations, although Kupfermann reports considerable individual variation in these variables. The latency for biting responses was reduced during the light rather than dark period.

In further experiments, Kupfermann (1974b) was able to demonstrate that the biting and approach phases of feeding could be dissociated by lesions which severed the buccal and cerebral connectives, and pedal and cerebral connectives respectively; and Susswein and Kupfermann (1975) demonstrated that bulk alone was sufficient for satiation.

Studies of feeding in other opisthobranchs have generally supported the notion that further analysis of feeding and its regulatory properties can follow the classical research in vertebrates; that is, related to the phases of detection, orientation and approach, and ingestion (Lee *et al.*, 1974; Lee and Palovcik, 1976; Davis *et al.* 1973; Davis and Mpitsos, 1971; Paine, 1963), and the regulation of these processes by various neural and hormonal levels of organization.

Davis et al., (1974a, 1974b) found that feeding may occupy a "hierarchically higher" position in relation to righting response, head withdrawal, copulation, and resting state, but seems to be suppressed by the action of hormones during egg-laying.

The significance of these investigations for the present study are that feeding and locomotion are related, and that *Aplysia* can detect, orient to, and approach a source of chemical stimulation.

D. Reproduction and Social Behavior

Aplysia, in common with the opisthobranchs in general, is a simultaneous hermaphrodite. They are different from other genera in that they have not been reported to copulate reciprocally and are commonly thought not to do so.(Fretter and Graham, 1966; Thompson, 1976). These characteristics allow a unique pattern of copulating chains in which groups of three or more are coupled so that some members of the chain are in position to receive and transfer sperm at the same time. Occasionally, these chains have been observed to form a circle such that all members can occupy both roles (Eales, 1921). Copulating dactylomela are most frequently found as pairs in the field and laboratory.

In a pair, the sperm recipient has its foot in contact with the substrate while the sperm donor is positioned over the visceral hump. Eales (1921) continues the description for A. punctata as follows:

> "B then attaches the anterior portion of its foot to the mantle of A and grasps it with considerable tenacity. The posterior part of the foot of B, however, remains free and curls around the "tail" of A. The parapodial lobes of A open widely and embrace the body of B, but the anal spount protrudes. The penis of B is then thrust from its sheath and curves downwards and backwards in the form of an inverted U, to be inserted into the common genital aperture of A. The whole of the penis is thrust into the vagina and reaches to the base of the vaginal pocket close to the spermatocyst... Sperms pass along the spermatic groove of B along the penis groove, and into the vaginal cavity of A." (p. 251)

Coupling can last for many hours; Eales (1921), however, found that the spermatic groove was empty during the period. It is possible, therefore, that sperm were not transferred, or that sperm are not transferred continuously.

The reproductive morphology has been well covered (See Thompson, 1976, for review) and provides a reasonable basis for discussion of three major functions of reproductive behavior: namely, sperm transfer, sperm receipt, and egg-laying. If the detection of and approach towards conspecifics is included, a relatively complete framework for the study of reproductive processes can be described.

Behavior associated with copulation or social processes in general have not been studied to any extent in Aplysia. Several preliminary reports have been published and together with data from other gastropods are suggestive that a rather rich area for behavior, physiology, and ecology is available for exploration.

Lederhendler et al. (1975) found that in the field, pairs were found to contact or copulate more frequently in the morning than in the afternoon. Laboratory patterns of copulation confirmed the field data, but contact, as such, was not a sensitive variable as far as morning and afternoon factors were concerned (Lederhendler et al., 1977). It would appear that contact behavior may be an aspect of social processes which under certain circumstances is independent of copulation. A similar pattern has been reported by Thompson and Bebbington (1969) and Newby (1972) but they did not provide data for their observations. Newby also stated that the sperm recipient role seemed to be assumed by the larger individual of a pair, and animals less than 40 grams were not observed to copulate.

In a 1976 study, Lederhendler et al. found that most animals assumed the sperm donor or recipient roles with no particular bias, but a small number were observed in the same roles consistently. Of these, the sperm recipients copulated more frequently with different partners than sperm donors.

Although the finding of large numbers of sea hares in one place is documented (Eales, 1921; Hamilton and Ambrose, 1975), the nature of this association is not known. Because Aplysia will orient and locomote in response to currents and food, aggregations may well form on the basis of these factors. On the other hand, conspecific responsiveness has been demonstrated in a number of gastropods including the opisthobranchs; in relation to trail-following in periwinkles (Hall, 1973; Dinter, 1974); and helix (Farkas and Shorey, 1976); spacing in Biomphalaria gldbrata (Simpson et al., 1973), fighting behavior in Hermissenda crassicornis (Zack, 1975).

Crozier's (1918) study of the reciprocal copulator Chromodoris zebra (a nudibranch) suggested that animals paired and copulated by actively responding to each other. When individuals were within 10 cm the genital papillae were frequently protruded. Although a copulating pair intromit the penises reciprocally and more or less simultaneously, if they were separated only one had sperm flowing from it. Mutual fertilization was the outcome of a copulatory bout; thus it is possible that the emission of sperm may be under the control of interactive processes in the two individuals involved.

Observations of the sexual behavior of the carnivorous opisthobranch Pleurobranchia californica led Davis and Mpitsos (1971) to distinguish four phases of interaction: 1. detection; 2. approach; 3. body orientation (reverse aposition of gonophores); 4. simultaneous engagement of sexual organs. The coupling posture could be elicited from an unpaired animal by stimulating it with sea water which recently had contained mating animals.

In other gastropods, Wolper (1950) indicated processes of distance chemoreception between sexual pairs of Paludina vivipara. And the investigations of Lind (1973) and Jeppeson (1976) on Helix pomatia suggested a role for the partner and internal factors in the progress of a complex mating sequence

Egg-laying is reported to follow the onset of copulation in maturing A. punctata. (Smith and Carefoot, 1967). Crozier (1918) observed that egg-laying followed copulation in the nudibranch Chromodoris zebra. Aplysia store sperm and are capable of laying a number of fertile egg masses when maintained alone (Thompson and Bebbington, 1969; Thompson, 1976; MacGinitie, 1934). However, the precise influence if any, of copulation on spawning has not been systematically investigated.

Fecundity in Aplysia is influenced by diet (Carefoot, 1967b) and appears to be related to season and/or temperature in non-tropical species (Strumwasser, et al., 1969; Carefoot, 1967; Nishiwaki, 1976).

Egg-laying can be induced by extracts from a symmetrical group of cells (bag cells) of the abdominal ganglion which are neurosecretory (Kupfermann, 1967, 1970; Strumwasser, et al., 1969; Toevs and Brackenbury, 1969; Arch, 1972a, 1972b and 1976 for review). The induction of egg-laying is not uniformly effective in all individuals (Dudek and Pinsker, 1976) and the naturally-occuring stimuli for the activation of the bag cells is not known. Coggeshall (1972) found that bag cell extract seems to cause the small muscle cells which wrap around each follicle of the ovotestis to contract and expel a ripe oocyte.

The oocyte is transported to the accessory genital mass (Coggeshall, 1972) where fertilization occurs. An egg mass is usually deposited within one or two hours. Vicente (1966) reports that ablation of the cerebral ganglion also induces egg-laying, which suggests the possibility of a role for other influences in addition to bag cell hormone in the induction of egg-laying.

The available evidence is clearly indicative of active processes in the social organization of *Aplysia* but the mechanisms have not been investigated systematically. When *Aplysia* are found in contact or copulating in the field, their history preceding and following aggregation, contact, and copulation, and egg-laying are not known. It is important to know the pattern of sex role reversal, the individual variables which lead to the assumption of copulatory roles, and how this can be accomplished. It is also important to know when and for how long various roles are maintained. A start in this direction has been made by Aspey and Blankenship (1976), Carew and Kupfermann (1974), Hamilton and Ambrose (1975), and Lederhendler, et al. (1975, 1977), but it has only served to indicate how much work there is to be done.

Four studies are reported which have examined reproductive behavior in relation to several aspects of the life history of *A. dactylomela*:

The first study reports observations of the development of conspecific interaction for pre-copulatory animals.

The second experiment investigated the effectiveness of conspecific stimulation to elicit orientation and approach.

The third study observed paired interactions to illuminate several aspects of conspecific interaction: 1. the predictability of individual social behavior in relation to prior exposure to different conspecifics; 2. changes in the interactions within a pair observed continually for 24 hours; 3. differences in locomotion which might be related to ongoing or prior social interactions.

The last study examined patterns of egg-laying in individually-maintained animals and in relation to the potential influence of copulation on these patterns.

II. METHODS AND PROCEDURES

A. Collection

188 Aplysia dactylomela were collected from mid-January through August, 1975. The size range of the collected animals varied between 3 grams (2 cm in length) and 520 grams (about 22 cm long). Collections took place off the southwest coast of Puerto Rico near La Parguera.

Three collection zones were defined (Figure 1); Plajita Rosada, Enrique, and Collado. Most (85%) of the animals in the study were collected within these zones; the remaining animals were collected at nearby islands, always in very small numbers. As Figure 1 shows, the southwest coast of Puerto Rico is typically dotted with small reefs and mangrove islands. On the leeward side of many of these islands are shallow Thalassia flats which extend to about 3 meters depth after which the sandy bottom descends rapidly to great depth.

Sea hares were usually collected in water 0.3 meters to 5 meters deep in areas where the algae Laurencia obtusa, Acanthophora spicifera, and Ulva lactuca were the major apparent foods. The Plajita Rosada area was mostly a rocky intertidal area marked by the presence of L. papillosa as the apparent food source. Of these foods, A. spicifera was the most common and abundant during all stages of the study in most areas. Except for two weeks in June, each zone was visited at least once a week for the duration of the study

Aplysia were found while snorkelling, wading, or poling. The method used depended on the depth, the water clarity, the bottom quality, as well as the wind and tide conditions. When an animal was sighted, the time, location, and field conditions were recorded. Most field trips took place early in the morning or late afternoon because of a pattern of increased wind activity beginning late morning and continuing through the afternoon, which made boating and visibility difficult.

When an animal was sighted, its location and social circumstances (alone, in contact, or copulating) were recorded. A plastic bag was passed between the animal and substrate and it was carried to the boat and placed in a numbered bucket containing about two litres of sea water. The water in the bucket was changed as often as possible and shaded from the sun in order to maintain fairly constant temperatures and oxygen levels. When the animal was on a mangrove root, or between or underneath rocks and coral, the individuals were removed to the bucket by hand.

In the laboratory the animals were weighed and tagged and then placed in individual 20-litre aquaria. To weigh the animal, a mesh beg was drawn between the sea hare and the substrate, and the bag and sea hare suspended from an Ohaus Dial Spring Scale (no. 8014) for 10 to 15 seconds, before the value was recorded. To tag an animal, a color-coded number 1 stainless steel safety pin was inserted through the left parapodium. The viscera of the sea hares were protected from injury by placing two fingers between the parapodium and mantle area, to guide the pin back out.

B. Maintenance

Aplysia were maintained in individual 20-litre all-glass aquaria (30 cm long x 30 cm wide x 30 cm deep) with constantly-running sea water.

Each tank had a constant-level siphon designed to draw water from the center of the tank, and to prevent blockage by the sea hares themselves, which normally move along the bottom and sides.

The tanks were kept on outdoor water tables in a semi-open area which was partially protected from the rain by an overhang about 20 meters above the ground. This meant there was no direct sunlight after about 11 a.m. As sea hares were frequently found in shady spots under grass or mangroves, or behind rocks, the laboratory arrangement was considered typical of naturally-occurring light conditions. In addition, no aquaria were without some shading during most of the morning.

Algae grew rapidly in the aquaria, so to maintain visibility for routine work they were scrubbed every three days with a plastic scouring pad. When an inhabitant was changed, the tank was washed thoroughly in hot water and then sun-dried.

The sea hares were fed fresh algae daily. At first, algae were gathered from the immediate location of collection. It quickly became apparent that only certain algae were being eaten. From then on, *Acanthophora spicifera* was the main foodstuff, with occasional supplements of *Laurencia obtusa* or *L. papillosa* as available. Sources of *A. spicifera* were found which were reliable and relatively free of epiphytes, reducing preparation time. Large amounts of this food could be maintained in excellent condition for several days under constantly-running sea water. Food was always available except while the tanks were being cleaned. The general amount of food was monitored, but served only as an indication of the health of each animal.

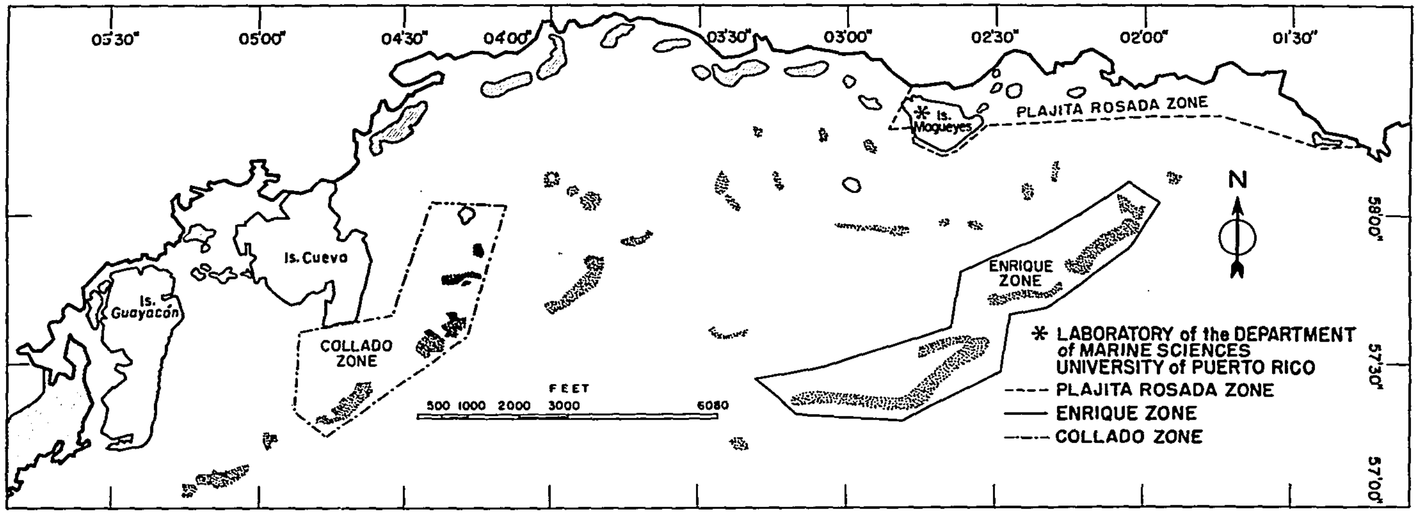
Each day the tanks were carefully searched for eggs laid and the spawn location was recorded. The egg mass was removed intact, and labelled by placing it in a separate mesh bag, which was suspended in a special tank with other egg masses for subsequent observation. When an animal was observed to be laying eggs, special note was made of the time; otherwise checks usually occurred within eight hours to help determine when an egg mass was laid. The egg masses themselves were monitored under a dissecting scope until early rotatory movements were observed or until their non-viability could be established.

Temperatures increased gradually from January to mid-September, from 25°C. to 30°C. The maintenance (stock) tanks had a slightly greater range, from 23.5°C. to 32°C. over the same time period.

C. Experimental Procedures

The details of the procedures are reported in connection with each of the appropriate studies. A general procedure which was followed as often as possible was to weigh the animal each time its location was changed.

Figure 1. Southwest coast of Puerto Rico, near La Parguera. Three major collection zones are indicated by solid, dash, and dot-dash lines.



05'30"
05'00"
04'30"
04'00"
03'30"
03'00"
02'30"
02'00"
01'30"
58'00"
57'30"
57'00"
Is. Cueva
Is. Guayacón
Is. Magueyes
PLAJITA ROSADA ZONE
COLLADO ZONE
ENRIQUE ZONE
N
FEET
500 1000 2000 3000 6080
* LABORATORY of the DEPARTMENT of MARINE SCIENCES UNIVERSITY of PUERTO RICO
PLAJITA ROSADA ZONE
ENRIQUE ZONE
COLLADO ZONE

SECTION I

The Development of Conspecific Interactions in Juvenile *Aplysia dactylomela*: An Observational Study

FOREWORD

The purpose of this study was to describe developmental changes in the interactions of "juvenile" Aplysia dactylomela. It became possible with the discovery of an apparently "juvenile" population in the Plajita Rosada collection on the southwest coast of Puerto Rico during April and May 1975. (See Figure 1, page 24.) This was an unusual research opportunity in that the capability of raising sea hares through metamorphosis has been restricted to only a few laboratories in which behavioral development was, to date, not investigated. The methods and procedures used were peculiar to an opportunity such as this, in that they depended on the continued success of finding "juveniles".

I. INTRODUCTION

Aplysia californica has been raised through metamorphosis and some of the morphological and behavioral changes related to metamorphosis have been described (Kriegstein et al., 1974). Further progress in the establishment of laboratory cultures of Aplysia has been reported by Hadfield (1975) and Strength and Blankenship (1976). Recently studies of morphological development have become available for some related opisthobranchs (Thompson, 1958, 1962, 1967; Tardy, 1970; Bonar and Hadfield, 1975). But there are no published studies of behavioral development in the opisthobranchs.

The first occurrence of copulation in Aplysia seems to be related to size. Although no data were provided, in a frief report Newby (1972) stated that copulation was never observed in animals less than 40 grams body weight. Smith and Carefoot (1967) collected small sea hares (1-8 grams) and kept these animals together as a group. Copulation was not observed until the sixteenth day in the laboratory. The body weights at the time of copulation were not reported; however, copulation was observed at about the time the gonads became mature.

Thus, it appears that the reproductive behavior develops some time after metamorphosis. The morphological studies of the bag cells by Frazier et al.(1967) support the notion that the reproductive system as a whole may develop after metamorphosis.

The bag cells are a neurohormonal group of the abdominal ganglion which contribute to egg-laying (Arch, 1976). The cells are fewer in number and may be non-secretory in small animals (2 grams). In sea hares larger than 50 grams, however, secretory granules are present and the number of cells in the cluster increases markedly. Thus, some neuroendocrine aspects of egg-laying become functional around the stage where sea hares begin to copulate.

Although the data are scanty, it appears from the above studies that copulation and size might be related. In this study the same sea hares were observed in regular repeated pairings. Systematic observations of their behavior were made and careful measurements of their weights were taken.

II. METHODS AND PROCEDURES

A. Subjects and Maintenance

A sample of 56 sea hares was collected off the southwest coast of Puerto Rico at Plajita Rosada. This is a rocky inter-tidal beach marked by the presence of *Laurencia papillosa* and sargassum. (For details of collection and further sample characteristics, see page 20.)

The sea hares were maintained in an indoor laboratory in 1-litre aquaria with running sea water and constant daylight illumination. Food was always available and consisted of *Acanthophora spicifera* which was thoroughly rinsed before being placed in the tanks. Wet weights of the animals were measured every other day on a *Mettler* balance.

To carry out observations of the interspecific behavior, the members of a pair were matched for size. As a result, at the time of collection, the smallest animals were paired to permit a maximum amount of observation during a long period of growth and development.

Five pairs were chosen for observation. These were all from the April and May collections (see Figure 1). A failure in the sea water supply system cut the period of observation short, resulting in only 15 to 22 days in which a total of 22 paired observations were made.

B. Apparatus and Procedure

Observations occurred between 5:00 p.m. and 7:30 p.m. under a 60-watt fluorescent bulb. The animals were paired by their size. Two pairs were observed for 30 minutes every six days; two pairs were observed every other day. The fifth pair was observed as individuals on experimental day 1 and then every six days (Table 1). A circular bowl 30 cm in diameter and 10.2 cm high was filled with fresh sea water to a depth of 8 cm. The animals were taken from their home tanks and weighed. The observation began immediately upon the introduction of the second animal.

The frequency and duration of contact and copulation as well as more detailed aspects of the interactions were recorded. Contact was defined as any part of an individual touching any other part of another individual. A sea hare initiated contact if it approached the other and touched it with any part of the body. Approach was defined as any locomotion which decreased the interindividual distance while oriented toward the second individual. Reciprocal contact occurred when both animals initiated the contact. Copulation was said to occur upon the intromission of one animal's penis into the common genital opening of the partner. Under the observational conditions described above, this could be determined exactly. If a pair was still in contact or copulating by the end of the observation, spotchecks every five minutes continued until the animals separated.

C. Sample Characteristics

Figure 1 shows the distribution of weights for the population from which the experimental subjects were selected. The median weight of the entire group was 56 grams. The experimental sea hares were all collected in April and May when most of the population (15 out of 28) were below 60 grams. The median weight of the experimental animals was 16 grams.

Only one pair of the 56 animals from Plajita Rosada were found copulating and no other animals were found in contact even though most were collected near each other, within approximately 75 meters of coastline.

III. RESULTS

Figure 2 shows the growth curves and duration of contact and copulation with repeated pairings for each pair in the study. As these data indicate, copulation occured in only one pair (5). There appears to be a tendency for longer contact interaction with increasing size wherever there is a consistent tendency to contact at all.

Pairs 4 (Figure 2d) and 5 (Figure 2e) each had seven and six observations respectively. This permitted somewhat more detailed statements about the interactions. As Table 2 shows, Pairs 4 and 5 were different in their patterns of contact. In Pair 4, a single contact, initiated by B, occurred early during the initial pairing and lasted for 35 seconds. The animals weighed 11 and 20 grams respectively during this observation. During the first three pairings, two contact were initiated mutually; six other contacts were initiated only by Partner B (one in the first pairing, three in the second, and two in the third). In the fourth observation B initiated six out of nine contacts. In the fifth, B initiated two contacts, A initiated one, and two were mutually initiated. In the sixth pairing, out of eight separate contacts, B initiated only two, four were initiated by A, and two were mutual. But in the seventh pairing, B initiated three out of five contacts where two were mutually initiated. Thus, out of 27 separate contacts which were not initiated by both animals (total of eight), B initiated 19 and A initiated 8.

In pair 5, contact occurred during the first observation when the pair weighed 28 and 34 grams respectively. There was no contact for the second pairing. Considering all six observation, six contacts were initiated reciprocally and four were not. These four were all initiated by partner A, who was also the sperm donor in three of the four observation periods where copulation occurred. Partner B became the sperm donor only after A had assumed that role three times. The animals contacted shortly after the third pairing and began to copulate after 90 seconds of the observation period had elapsed. At this time, they weighed 53 and 58 grams respectively. Copulation occurred in each of the next three pairings. The four copulations lasted for 43, 13, 21 and 49 minutes respectively. It is noteworthy that when partner B assumed the sperm donor role the duration increased to a level equivalent to the first copulation when A was the sperm donor.

IV. DISCUSSION

The *Aplysia* observed in this study were assumed to be precopulatory because of the following: 1. Of 56 animals collected from the same location, only one pair was found copulating in the field; these two individuals weighed 47 and 55 grams respectively. In addition, none of the animals found in the field were in contact, although many were near each other. 2. Most of the sea hares did not copulate with repeated pairings in the laboratory. 3. None of the animals laid eggs while they were in the laboratory. Together with their small size when collected, these observations suggest that they were not reproductively mature.

Although only one of the five pairs copulated, it shared some characteristics with those that did not copulate such as change in weight, duration of contact, etc. However, it is useful to examine this pair in detail.

For example, Frazier *et al*. (1967) found that the bag cells of *A. californica* began to contain neurosecretory granules when animals reached 50 grams in weight. Copulation occurred after each animal of Pair 5 passed the 50-gram mark in body weight, indicating that this may be a necessary but not sufficient condition for copulation to occur, as all pairs reached that weight before observations ended.

In the one pair which copulated, the animal which was the sperm recipient was heavier at the start (B). Following the first copulation there was a spurt in its growth rate in comparison with its partner's (A's), and their congruent precopulatory pattern of weight change. If the spurt in weight proves to be reliably associated with copulation, the onset of egg-laying (which presumably depends on bag-cell hormones) could be related to copulation and the contacts which precede it.

Lederhendler _et al._ (1976) found that in reproductively mature _A. dactylomela_, animals which behave more consistenly in the sperm recipient role copulate with more partners than the consistent sperm donors. They suggest that this may be related to a hormonal feedback system related to amount of sperm received. In the observations recorded here, role reversal occurred after several copulations in which the same roles were assumed. This is consistent with the function of such a feedback system.

These observations are considered preliminary but indicate directions for further research on the possible inter-dependence of social stimulation and hormones in development of reproductive processes in this species.

V. SUMMARY

Successive observations of five pairs of juvenile *Aplysia dactylomela*. Copulation occurred in one pair. Reversal of sperm donor and recipient roles was seen.

TABLE 1

Characteristics of Five Pairs of Pre-Copulatory *A. dactylomela* Observed Repeatedly in Different Frequencies

	Initial Weight (g)	Number of Pairings	Interval Between Pairings
Pair 1			
Partner A	14	4	6 days
Partner B	16		
Pair 2			
Partner A	25	3	6 days
Partner B	26		
Pair 3			
Partner A	13	2	6 days[1]
Partner B	16		
Pair 4			
Partner A	6	7	2 days
Partner B	11		
Pair 5			
Partner A	21	6	2 days
Partner B	24		

[1] unpaired on first experimental day

TABLE 2

Patterns of Contact and Copulation of "Juvenile" Aplysia dactylomela

Paired Every Two Days

	Pairing						
	1	2	3	4	5	6	7
Pair 4							
Number of Contacts Initiated By Partner A	0	0	0	3	1	4	0
Number of Contacts Initiated By Partner B	1	3	2	6	2	2	3
Number of Contacts Initiated Reciprocally	0	1	1	0	2	2	2
Pair 5							
Number of Contacts Initiated By Partner A	2	0	0	2	0	0	
Number of Contacts Initiated By Partner B	0	0	0	0	0	0	
Number of Contacts Initiated Reciprocally	1	0	1	1	1	2	
Sperm Recipient	-	-	B	B	B	A	

FIGURE 1

Frequency Distribution of Initial Weights of *A. dactylomela* Collected At Plajita Rosada, Puerto Rico (N=56)

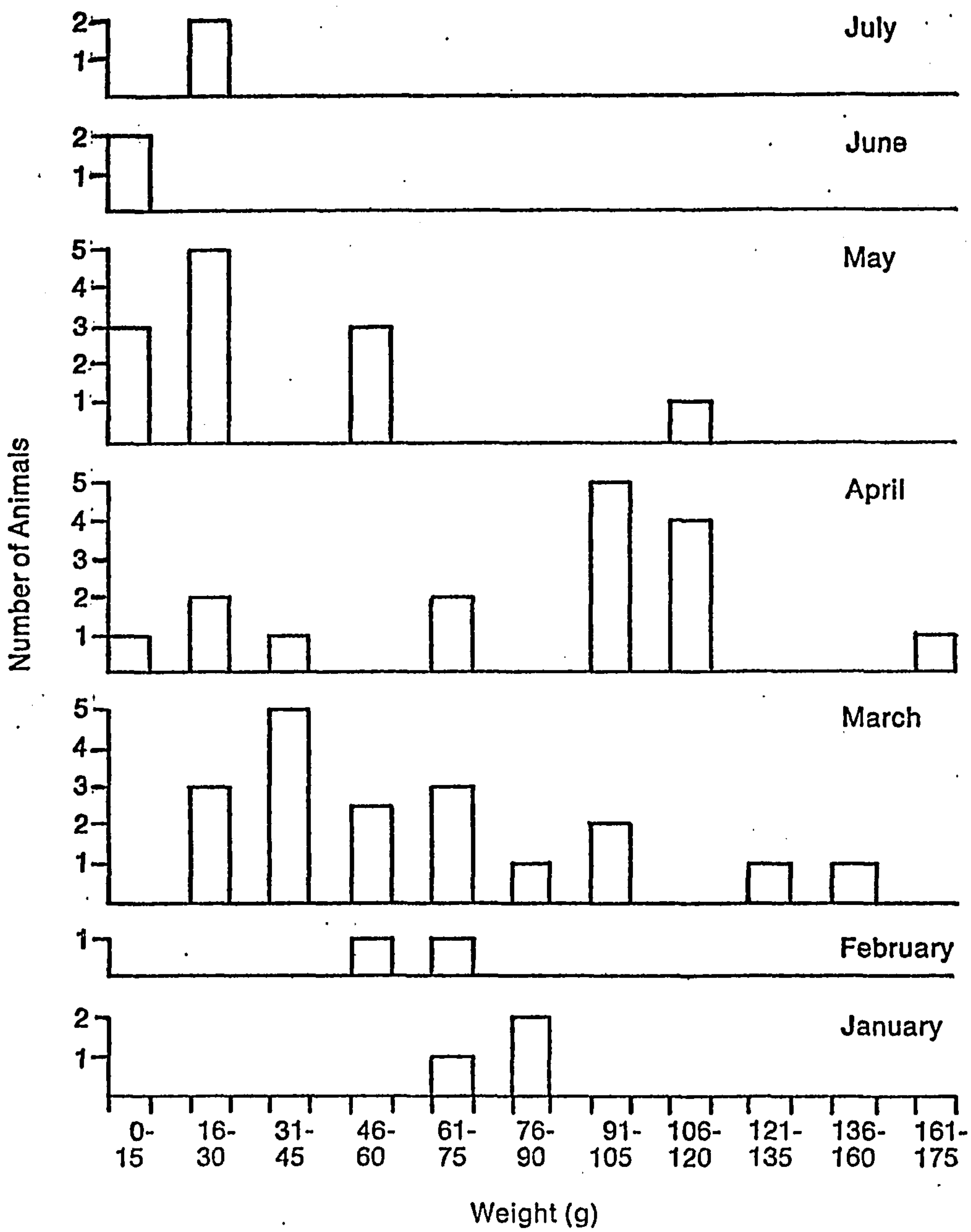


Figure 2 (a-e). Growth curves and duration of contact (black bars) and copulation (white bars) for five pairs of "juvenile" _Aplysia dactylomela_.

FIGURE 2A

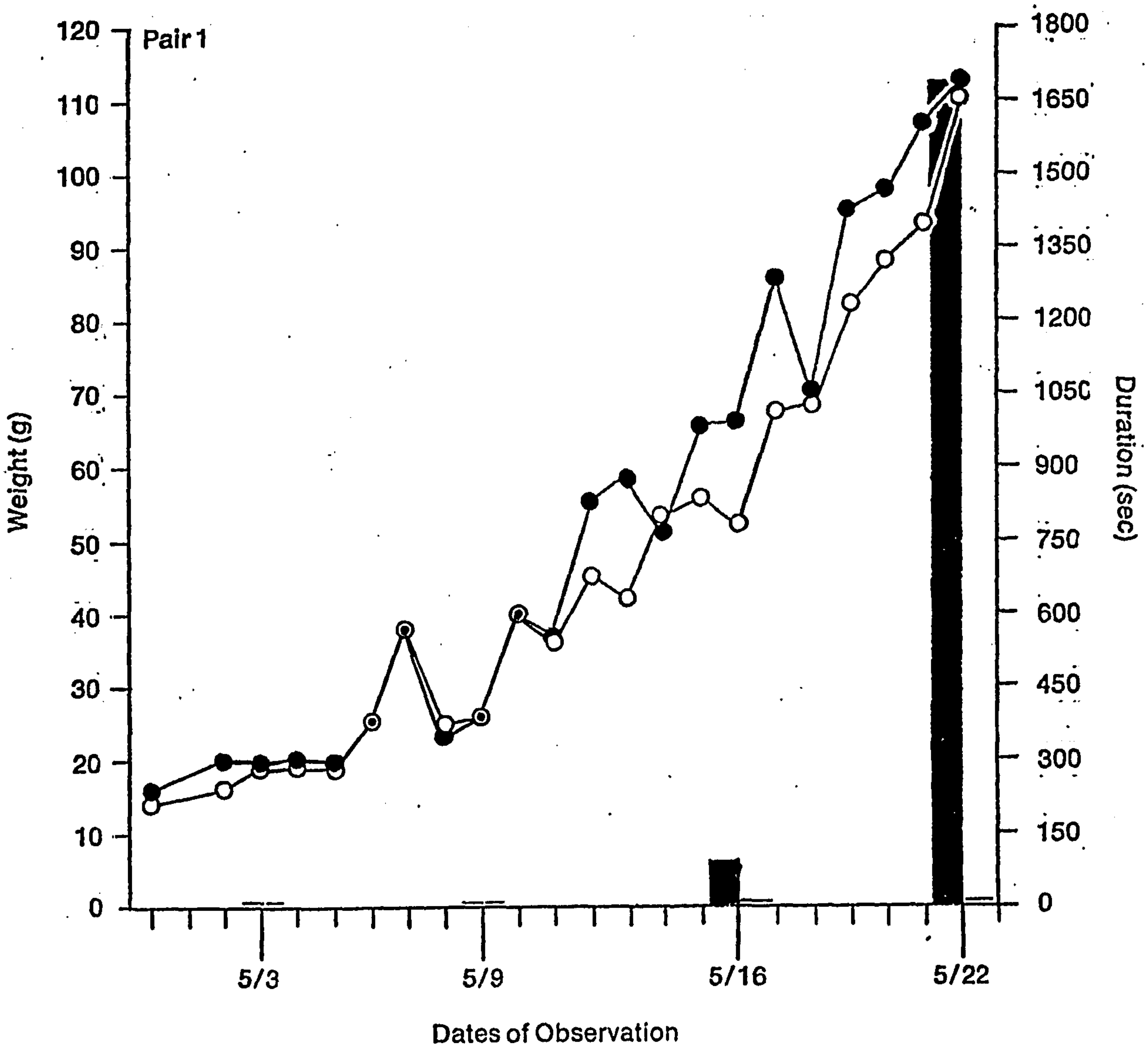

Pair 1
Weight (g)
Duration (sec)
0
10
20
30
40
50
60
70
80
90
100
110
120
0
150
300
450
600
750
900
1050
1200
1350
1500
1650
1800
5/3
5/9
5/16
5/22
Dates of Observation

FIGURE 2b

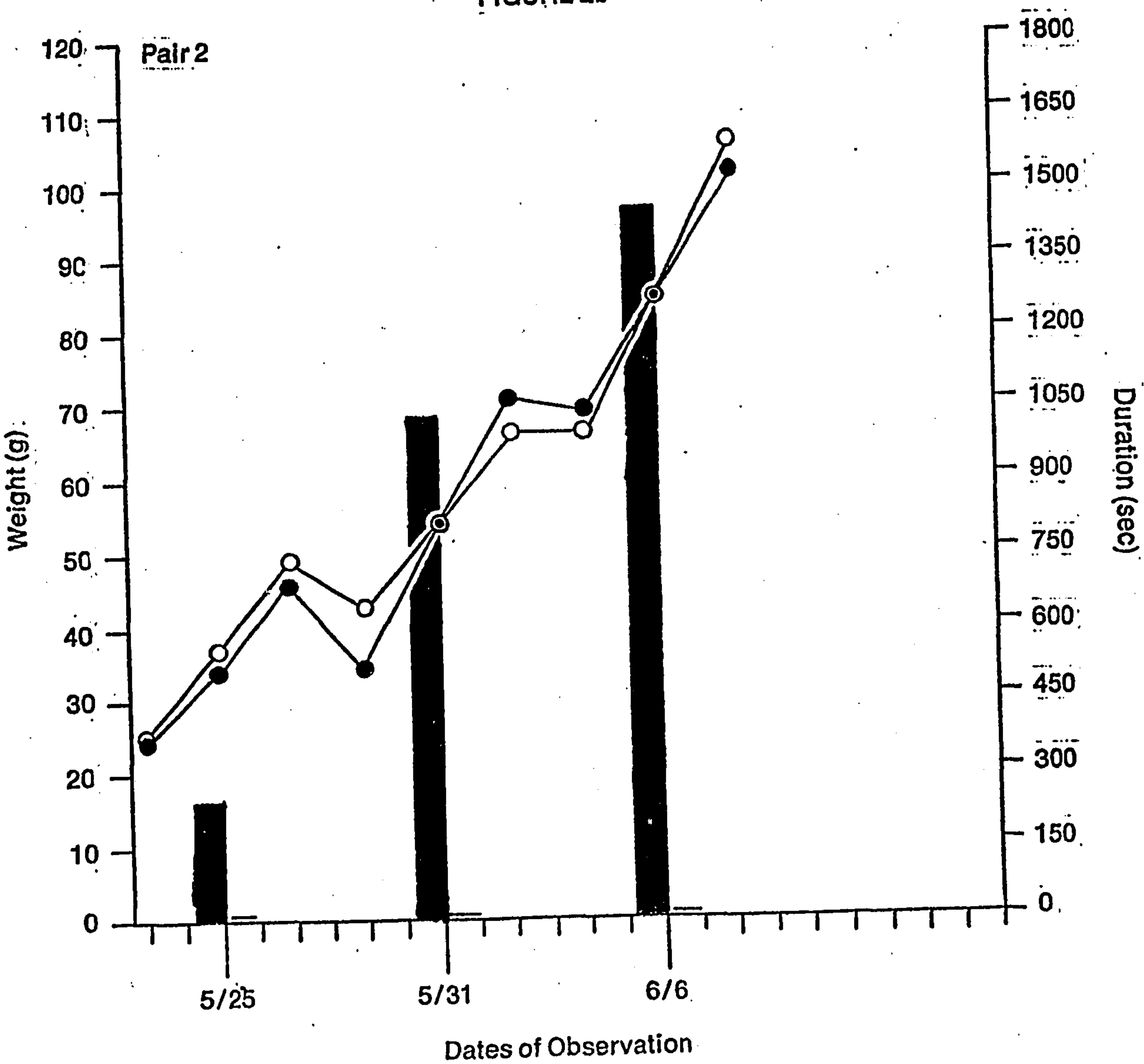

Pair 2
Weight (g)
120
110
100
90
80
70
60
50
40
30
20
10
0
Duration (sec)
1800
1650
1500
1350
1200
1050
900
750
600
450
300
150
0
5/25
5/31
6/6
Dates of Observation

FIGURE 2c

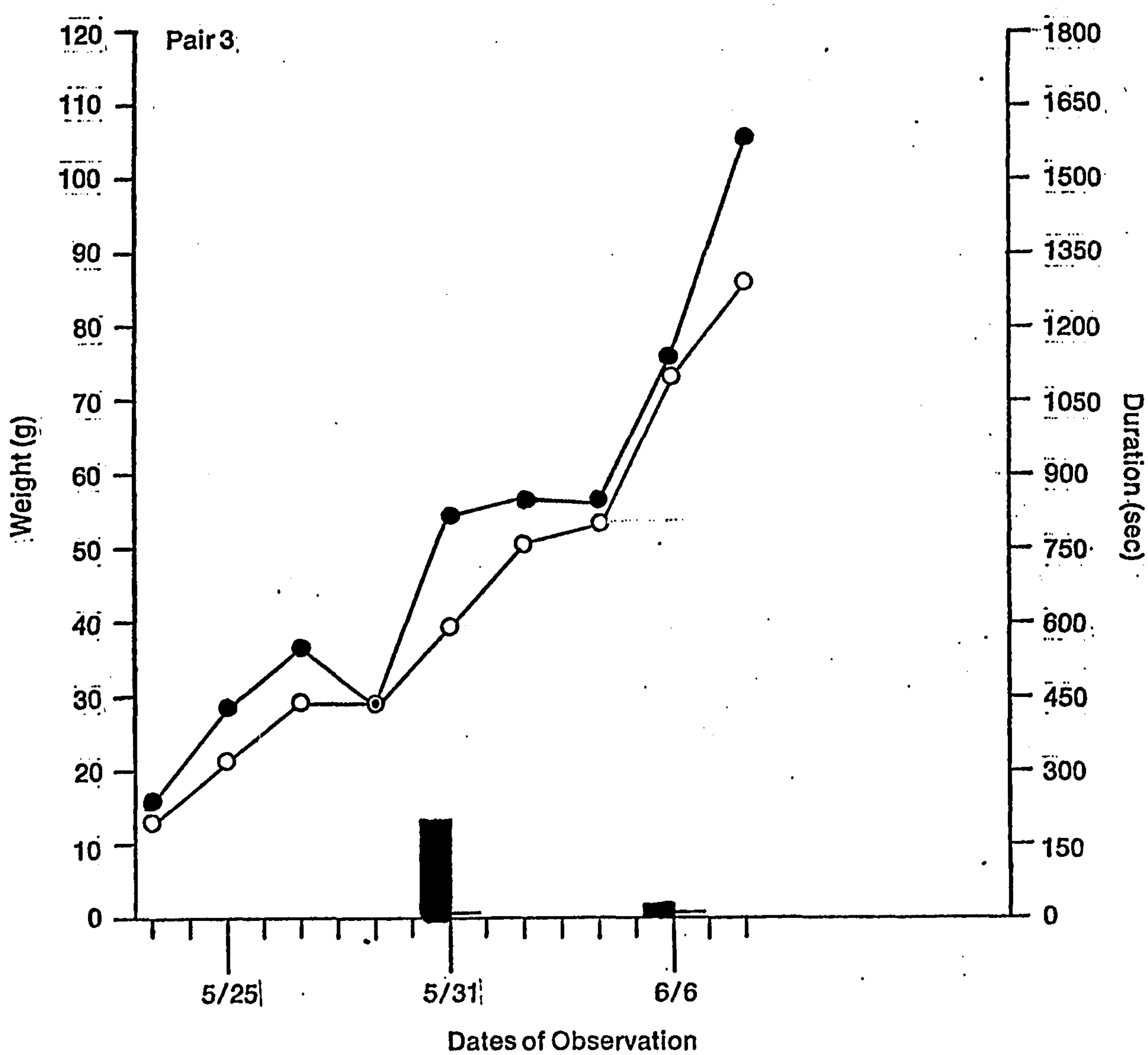

Pair 3
Weight (g)
0
10
20
30
40
50
60
70
80
90
100
110
120
Duration (sec)
0
150
300
450
600
750
900
1050
1200
1350
1500
1650
1800
5/25
5/31
6/6
Dates of Observation

FIGURE 2d

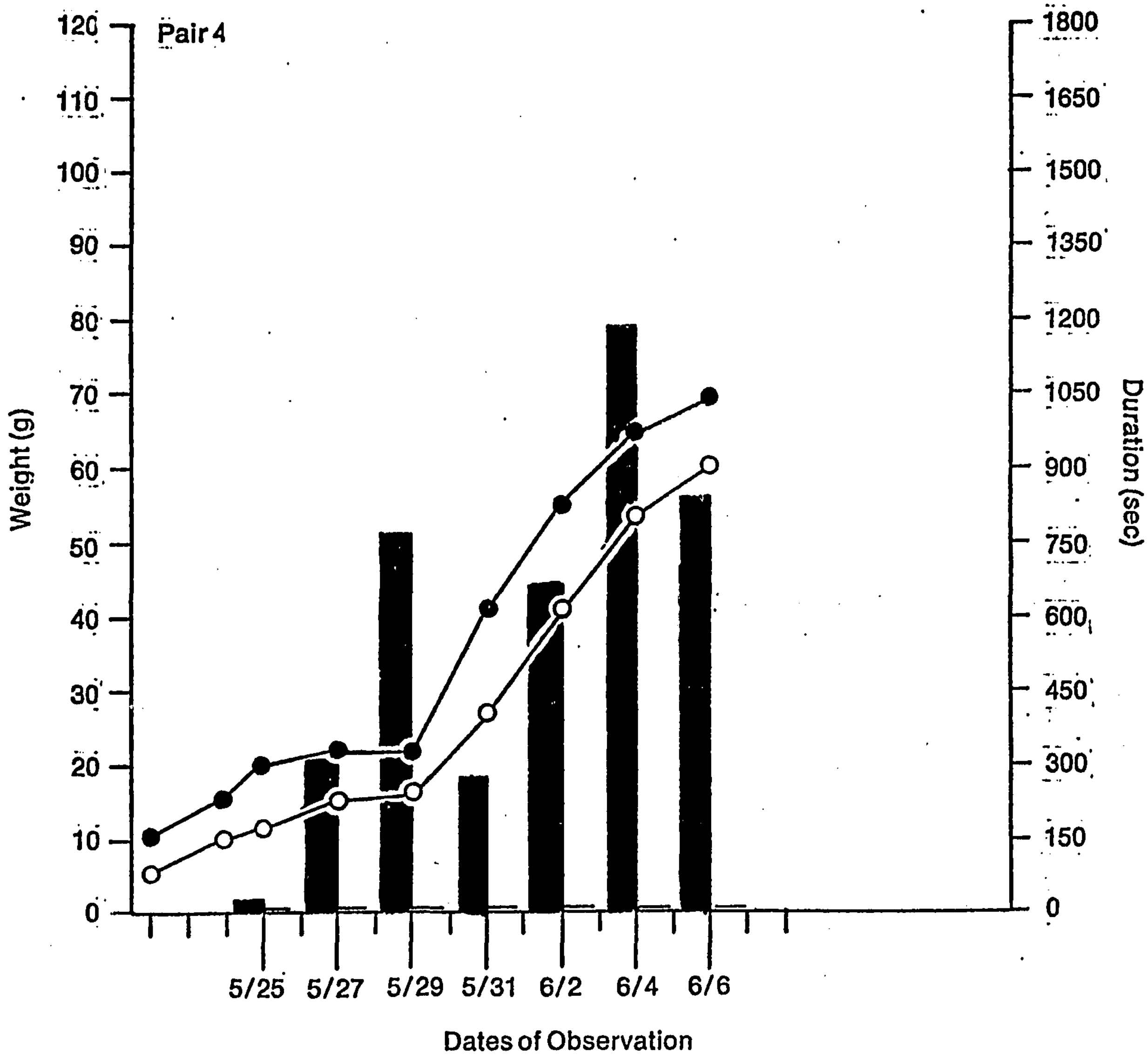
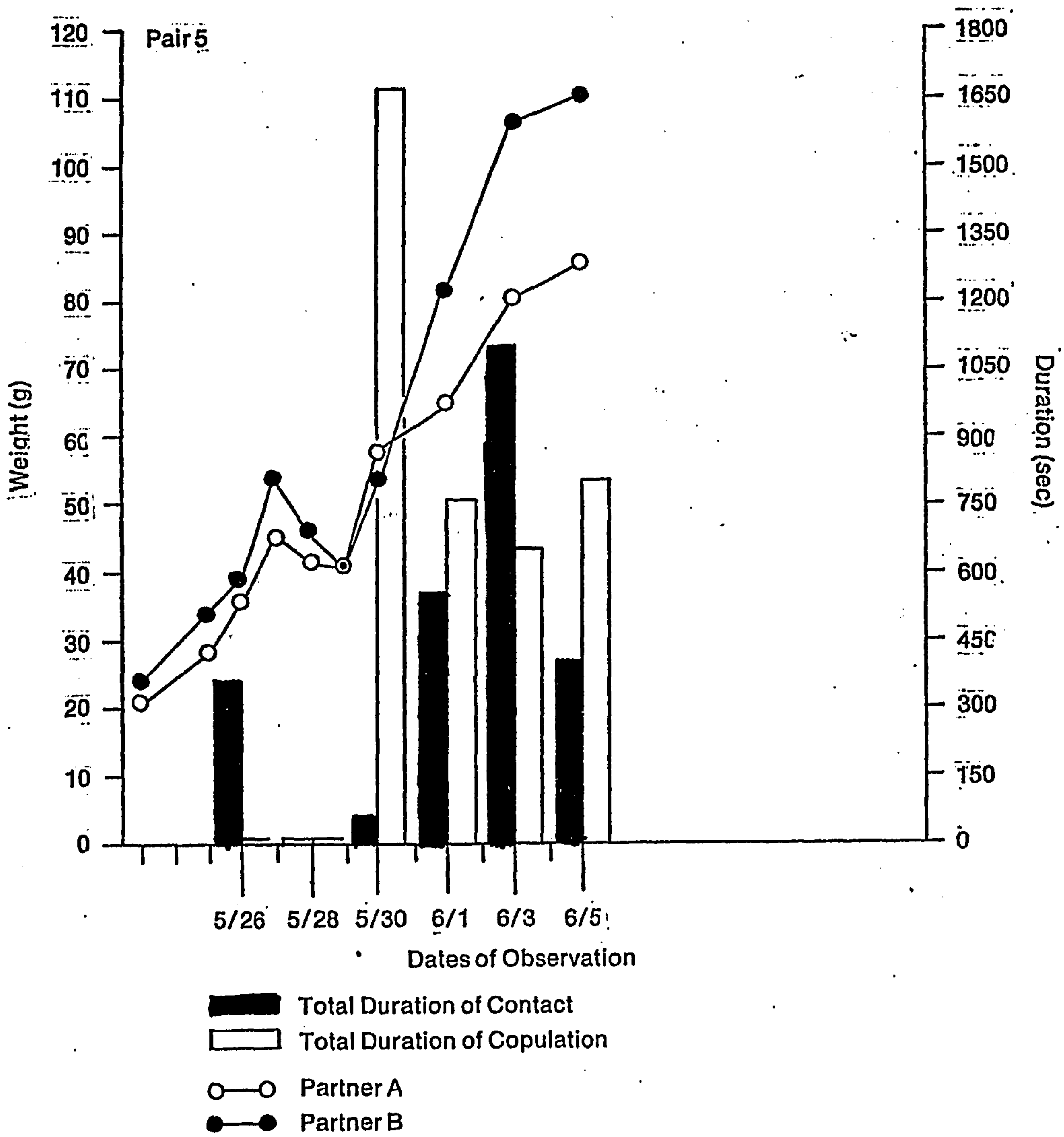

Pair 4
Weight (g)
0
10
20
30
40
50
60
70
80
90
100
110
120
Duration (sec)
0
150
300
450
600
750
900
1050
1200
1350
1500
1650
1800
5/25
5/27
5/29
5/31
6/2
6/4
6/6
Dates of Observation

FIGURE 2e


Pair 5
120
110
100
90
80
70
60
50
40
30
20
10
0
Weight (g)
1800
1650
1500
1350
1200
1050
900
750
600
450
300
150
0
Duration (sec)
5/26
5/28
5/30
6/1
6/3
6/5
Dates of Observation
Total Duration of Contact
Total Duration of Copulation
Partner A
Partner B

SECTION II

Taxis in *Aplysia dactylomela* (Rang, 1828) To Water-Borne Stimuli From Conspecifics

I. INTRODUCTION

The finding of large numbers of sea hares in one place has been documented by many naturalists. Another kind of grouping also occurs in relation to contact or copulation. But the process by which either of these patterns occurs or their relationship to each other is not known.

One theory concerning the formation of the larger groupings was proposed by Eales (1921). She suggested that as the animals grow they pass from the sublittoral to the littoral zone in relation to the different algae found at varying depths. However, Miller (1960)and Carefoot (1967a) found that the type of migration proposed by Eales was not necessary to complete the life cycle of a population; but their studies did not exclude the possibility of its occurrence. On the other hand, recent studies of feeding behavior in *Aplysia* (Frings and Frings, 1965; Kupfermann, 1974; Preston and Lee, 1973; Jahan-Parwar, 1972; Audesirk, 1975) have demonstrated that *Aplysia* respond to food stimuli at a distance, and that they will move negatively with respect to current in doing so. Thus, it is possible that in the field an aggregation can be formed in relation to food sources. Although not well understood, other factors such as current (Lederhendler *et al.*, 1975; Hamilton and Ambrose, 1975) may also influence the movements of *Aplysia* and therefore the aggregation process.

One of the variables which may be of considerable significance for aggregation is the role played by conspecific stimulation, which would be a critical factor in further comparative discussions of social organization in Aplysia (Tobach and Schneirla, 1968). Conspecific responsiveness has been implied for opisthobranchs in the work of Crozier (1918), Davis and Mpitsos (1971), and Zack (1975). Aspey and Blankenship (1976b) found that burrowing in A. brasiliana appeared to be induced by the presence of burrowed individuals. Egg-laying conspecifics seemed to affect the behavior of burrowed animals by increasing the probability of aggregation and copulation (Aspey and Blankenship, 1976c).

This experiment was designed to determine whether A. dactylomela responds to stimuli from conspecifics. Consequently, an apparatus was chosen which allowed animals to respond to a source of stimulation by approach or withdrawal. By "approach" is meant any process which results in a decrease in the distance between the head of the animal and the source of stimulation. "Withdrawal" was defined by an increase in this distance.

Current seemed to be a convenient vehicle for the presentation of stimulation as it has been used with experiments involving food stimulation. However, as many animals show rheotaxis (Fraenkel and Gunn, 1961) it was necessary to carry out preliminary studies which established a current velocity which in itself would not bias the behavior in question.

Approach was measured in terms of total distance moved towards or away from the source of stimulation. The orientation of the test animals was recorded every 15 seconds in terms of the head of the animal's being either toward, away from, or neither, with respect to the source of stimulation. This was considered to be an important variable because the entire surface of the gastropod body may be chemically sensitive (Charles, 1966; Kohn, 1961). An orientation toward the source of stimulation would suggest that the principal sensory structures related to the conspecific approach are in the anterior part of the body.

In order to ensure that an adequate degree of stimulation was being used, two "levels" of conspecific stimuli were used (a single conspecific or six conspecifics in the same volume of water). In addition, a copulating pair was included as a condition to see if a qualitatively unique substance was or was not given off during copulation, as evidenced by the behavior of test animals.

II. METHODS AND PROCEDURES

A. Subjects

Sixteen *Aplysia dactylomela* were obtained in July and August, 1975, from the shallow-water thalassia beds on the leeward side of Enrique reef on the southwest coast of Puerto Rico. Following collection, animals were kept in outdoor 20-litre glass tanks which were continuously supplied with fresh sea water. The weights (Table 1) ranged between 185 grams and 510 grams at the time of testing; all had laid at least one egg mass and had been observed copulating in the sperm donor or sperm recipient role. (For further details regarding collection and maintenance, see page 20.)

B. Apparatus

The influence of water-borne stimulation was tested in a narrow compartment 75 cm (length) x 15 cm (height) x 15 cm (wide), made of pine boards coated with fiberglass resin (Figure 1). Sea water entered through a 1.25 cm hole at one end, located 10 cm above the bottom. A nylon mesh screen covering the hole and a row of plastic baffles with their front line 5 cm in front of the entering water had the effect of spreading the stream evenly across the width of the container. Water flowed out at the other end through a hole of the same size set at the same height. The bottom and sides of the runway were marked by seven lines, one every 10 cm. These were used to indicate the location of an animal with respect to the current source.

Preliminary experiments were undertaken to set the flow rate. In these pilot studies plain sea water was in the source tank. Different flow rates were produced by varying the depth of water in the source tank.

Six animals which had been maintained alone for seven to ten days were individually placed in a perforated plastic container (the "start" box) with its head toward or away from the source of the stream and observed in four ten-minute trials at different flow rates. The start box was located in the middle of the test chamber 30 cm from the baffles. It enclosed the individual in a space 10 cm (length) x 15 cm (width). To start a trial the start box was raised clear of the animal, and positioned in front of the outflow hole to prevent the animal from stopping the stream.

The responses of the animals to different flow rates was observed until there was no bias in their response, regardless of the orientation at the start. This rate proved to be 4 litres/minute. The rate was obtained by maintaining the distance between the height of the water source and the height of the water in the compartment at 41-44 cm.

Additional evidence of the hydraulic characteristics of the situation was obtained through dye studies. Non-nutritive food coloring was placed in the "source tank" and would progress down the runway evenly without differential rates along the sides or the middle. The gradient of stimulation and the duration of a consistent gradient depended on the concentration of dye used. The greater the concentration, the less time it took for a concentration of dye to become noticeable at the start barrier. With one ml of dye in 20 litres of water, it took 2.5 minutes, but with 10 ml of dye in 20 litres of water it took only nine seconds.

C. Methods and Procedures

1. Design

A modified Latin Square design was used. (Edwards, 1950) Every animal was observed in four five-minute trials during which it was in water siphone from each of the four types of source water ("stimulus conditions"). The conditions in the source tank were: (a) sea water; (b) one sea hare; (c) six sea hares; (d) one pair of copulating sea hares.

2. Procedure

Animals were kept alone for seven to ten days in 20-litre stock tanks. Before an animal was tested, it was weighed and brought to the experimental situation in a plastic carrying bucket. To start a five-minute observation, an animal was placed in the start box. A tap started the current, but the start box was not removed for another 60 seconds to ensure a uniform stream of stimulation. When the gate was raised the location and behavior of the test animal was recorded every 15 seconds for five minutes. Animals were started in the center of the runway to allow the possibility for both approach and withdrawal with respect to the inlet. Between trials of a sequence all apparatus was rinsed thoroughly and wiped with a cloth.

3. Data Recorded

The animal's location and orientation was recorded on a check sheet. Displacement was measured as positive or negative values of the distance between the animal's location at the start of a trial and the final location. For purposes of analysis, displacement was converted to positive integers by adding 35 cm to every value. Thus, a displacement of 70 cm indicates that an animal reached the baffles in front of the eanty point. A displacement of 0 cm indicates that the animal reached the maximum distance from the inlet; that is, the outlet point.

Twenty locations per trial were recorded; but generally the direction of locomotion was constant and the animal's ultimate location was reached early and maintained.

III. RESULTS

The experimental arrangement, pre-observation weights and the displacement data for each of the four conditions is shown in Table 1. There was no bias between size of the animals and sequence of presentation of the four conditions. (Kruskal-Wallis One-Way Analysis of Variance, $X^2 = 0.3$; Siegel (1956)). A Latin Square analysis of variance (Edwards, 1950) shown in Table 2, indicates that the displacement was influenced by the treatment conditions. ($F = 2.89$, $p < .05$). The direction of these differences is indicated by a comparison of the mean displacement towards the water source under each of the four conditions. (Condition A: 31.3 cm (-3.7); Condition B: 45.0 cm (+10); Condition C: 48.8 cm (+13.8); Condition D: 46.9 cm (+11.9).

This displacement toward the source is greater when the current carries any form of conspecific stimulation (Cohen's Power Test, $p < .01$ (Cohen, 1969)) than if it is only plain water; i.e., A < B, C, D. In addition, the current carrying stimuli from six *Aplysia* produces a greater displacement towards the source than Condition B, containing only one *Aplysia* , i.e., B < C. (Cohen's Power Test, $p < .05$). The copulating pair was of intermediate effectiveness as compared to a single individual or six animals in that there were no differences among them on a Cohen Power Test.

A McNemar Test of Change of the test animals' final orientation indicates that a significant change in individuals occurred

[1]Numbers in parentheses represent the mean of the displacement values actually obtained.

only when the plain water condition was compared to water containing six A. dactylomela ($X^2 = 4.17$, $p < .05$). Table 3 shows that changes in orientation did not occur for any other comparison.

IV. DISCUSSION

The data presented here indicate that A. dactylomela will move into a current and toward a source of conspecific stimulation. When a greater number of conspecifics were in the source tank (six adults in 20 litres) a distinct movement toward the stream's origin was evident. It would appear, therefore, that the approach process may depend on the intensity of stimulation. The observational situation was designed to present a current flow that would not bias the response of the animals ("neutral rheotaxis"). It is possible, however, that the chemical stimulation from conspecifics changes the response. Lederhendler et al. (1975) reported that in the field sea hares will move negatively with respect to currents formed by tidal changes and topographic characteristics in a grass flat, but the chemosensory context of these changes is not known.

There is no evidence in this study that copulating individuals differentially influence the approach processes of a third animal.

The results of this study are consistent with the hypothesis that groupings of A. dactylomela can occur by an active approach response to conspecifics in the absence of food stimulation. However, since Aplysia approach food stimulation and possibly approach eggs as well, it is reasonable to suggest that aggregations of sea hares are formed in relation to a complex pattern of stimulation from conspecifics (including the eggs), food, and currents.

V. SUMMARY

Sixteen reproductively mature *Aplysia dactylomela* were observed in a unidirectional stream under each of four stimulus conditions: sea water; one sea hare; six sea hares; and a copulating pair. Streams containing conspecific stimulation were significantly more effective in eliciting a positive taxis toward the stimulus source. The sea hares showed a distinct orientation toward the source of stimulation. A copulating pair was not different from one or six animals in producting the approach. The sea hares showed a distinct final head orientation to six sea hares when compared with sea water only; final orientation did not differ in any other comparisons.

The data are interpreted as support for the notion that groupings of sea hares can occur by active approach processes to conspecifics in the absence of food stimulation. Large aggregations in the field may be related to a complex pattern of stimulation from conspecifics, food, and physical factors such as current.

TABLE 1

Modified Latin Square Arrangement to Test Response of *A. dactylomela* To Conspecific Stimuli

Conditions[1] Latin Square	Sequence	Animal	Weight(g)	Displacement in Centimeters[2] Trial 1	Trial 2	Trial 3	Trial 4
ABCD	I	1	190	0	70	70	70
BDAC	II	2	270	30	60	25	50
CADB	III	3	230	70	30	25	70
DCBA	IV	4	245	50	35	35	30
ABCD	I	5	410	50	50	60	60
BDAC	II	6	185	70	35	70	65
CADB	III	7	380	70	60	70	45
DCBA	IV	8	400	60	0	40	40
ABCD	I	9	185	5	65	50	40
BDAC	II	10	500	35	30	0	35
CADB	III	11	370	60	70	60	35
DCBA	IV	12	300	40	70	70	45
ABCD	I	13	510	0	10	0	30
BDAC	II	14	240	50	40	35	50
CADB	III	15	235	30	0	40	45
DCBA	IV	16	200	40	70	0	40

[1]A: Sea water only
B: One *A. dactylomela*
C: Six *A. dactylomela*
D: pair of copulating *A. dactylomela*

[2]0 to 35 = -35 to 0; 35 to 70 = 0 to +35

TABLE 2

Results of Latin Square Analysis of Variance: Displacements of *A. dactylomela* With Respect to Conspecific Stimuli

Source of Variation:	Sum of Squares	df	Mean Square	F
Independent Observations:				
Order of Presentation	776.18	3	258.73	
Residual Between Individuals	11198.43	12	933.21	
Total Between Individuals	11974.61	15	---	
Correlated Observations:				
Conditions	3101.18	3	1033.73	2.89*
Trials (Sequence)	382.14	3	127.38	0.36
Residual Error from Latin Square	1677.61	15	111.84	0.23
Residual Within Individuals	13345.32	27	494.28	
Total Within Individuals	18506.25	48	---	
Total For Experiment	30480.86	63	---	

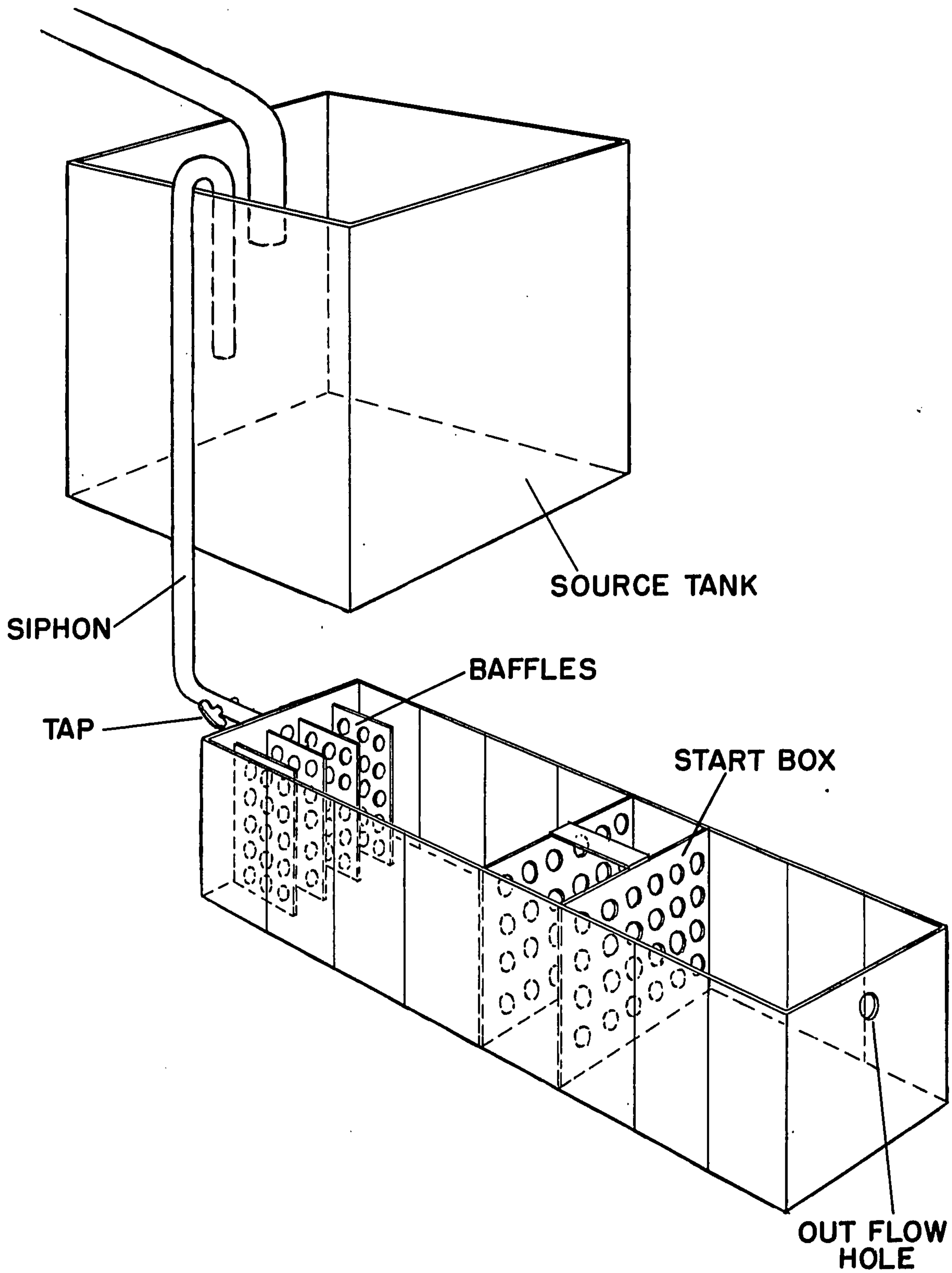
* $p < .05$

TABLE 3

Number of *A. dactylomela* Whose Final Orientation Was Toward Or Away From Source of Stimulation

	Condition: One Sea Hare		Condition: Six Sea Hares	
	Away From	To	Away From	To
Sea Water Only				
Away From:	6	1	5	0
To:	5	4	6	5
One Sea Hare				
Away From:			4	1
To:			5	6

Figure 1. Apparatus used to test response of _A_. _dactylomela_ to conspecific stimulation.


SOURCE TANK
SIPHON
BAFFLES
TAP
START BOX
OUT FLOW
HOLE

SECTION III

Patterns of Contact and Copulation in Paired *Aplysia dactylomela* (Rang, 1828)

I. INTRODUCTION

Little is known about copulation in *Aplysia*. In sexually dimorphic species, including consecutive hermaphrodites, the behavior of each partner of a copulating pair is typically assigned in terms of male and female functions. However, as simultaneous hermaphrodites, the sex behavior of *Aplysia* requires a different designation of the roles which can be assumed during copulation. As Thompson (1976) points out, in present-day opisthobranchs three aspects of sexual functioning are present simultaneously: oviposition, the receipt of sperm, and the transfer of sperm. In Ghiselin's (1969) survey of simultaneous hermaphrodite he proposed that the reproductive system of the opisthobranchs probably was derived from sexually-dimorphic ancestors. Thompson (1976) concluded that the ancestral system proposed by Ghiselin, "probably could not permit the efficient execution of male and female roles simultaneously, i.e., reciprocal copulation was not at this stage possible, nor could oviposition and copulation occur at one and the same time. The three aspects of sexual functioning must therefore have been rigidly delineated in terms of behavior (p. 53)."

Reciprocal copulation is the most common pattern of sperm exchange in opisthobranchs. In the Aplysiamorpha, however, pairs are thought not to do so. In pairs, individuals occupy only the sperm donor or recipient roles at any one time. In addition, a unique pattern of chain-copulation is found in which three or more individuals couple such that all but the first and last members of the chain occupy both roles simultaneously. This pattern has been proposed by Thompson (1976) as a mechanism which could favor full use of the potential gamete production and gamete utilization of the sexually mature members of a population.

The phases of the interaction may be defined as: (1) approach; (2) contact; (3) pre-copulatory contact; (4) intromission; (5) separation. Paired interactions can also be characterized as singly or reciprocally initiated depending on whether one or both partners approached the other. During copulation the penis of one partner is everted and intromitted into the common genital opening located between the parapodia and anteriorly to the mantle (Appendix, Table A). Following copulation, the animals may separate or reverse roles, at which time the original sperm recipient becomes the sperm donor.

Little is known about the processes which determine the assumption of the donor or recipient role, or the pattern of role reversal. In the laboratory, most individual _A. dactylomela_ assumed the sperm donor role as often as the sperm recipient role when groups were observed (Lederhendler _et al._, 1976). When animals are found copulating or in contact in the field, the nature of the interaction leading to the roles which were assumed is not known. It is important to know if the roles assumed were a function of the behavior which preceded it, and if the same or different roles can be expected to occur in subsequent interactions with different individuals.

This study was undertaken to analyze if any orderly relations existed for an individual's behavior with a conspecific in terms of its previous social experience. An experiment was designed to permit comparisons among sea hares with different levels of exposure to conspecifics.

In each observation locomotion, contact, and copulation were studied. Special attention was focused on the different roles which could be assumed at any point in the paired observation; namely, the contact initiator, the initiator of copulation, the sperm donor and the sperm recipient. Locomotion was chosen as a potentially sensitive dependent variable because it has diel properties (Kupfermann, 1970; Strumwasser et al., 1969; Jacklet, 1972) and is responsive to chemosensory manipulations (Audesirk, 1975; Preston and Lee, 1973; Jahan-Parwar, 1972; Frings and Frings, 1965).

II. METHODS AND PROCEDURES

A. Collections

90 *Aplysia dactylomela* (sea hares) were used in paired observations during the seven-month period of the study. The animals were collected off the southwest coast of Puerto Rico near La Parguera from mid-January through August, 1975.

Three major collection zones are shown in Figure 1 (Page 24). From west to east they are: Collado, Enrique Reef, and Plajita Rosada. The area is marked by many small mangrove islands and coral reefs. Typically, shallow water *Thalassia* flats and sandy bottom regions are found on the leeward side of these islands.

When an animal was found, it was collected by drawing a plastic bag between the foot and the substrate. Then it was placed in a bucket and taken to the laboratory. In the laboratory the animals were weighed and tagged before being placed individually in aquaria.

B. Subjects

The weights of the 90 sea hares at the time of experimental observation ranged between 80 and 590 grams, reflecting the weights of different groups of animals collected in the field during different months (Table 1). 74 of the animals had weights which ranged between 150 and 590 grams.

Because of varying collection success, animals had to be used as they became available, or they had to be maintained in the laboratory until others were found, during which time egg masses were laid frequently. For the experiment, pairs were matched for similarity of size and recency of egg-laying.

Thirty pairs differed from each other by 5-65 grams in weight; five pairs differed by 80-110 grams; and eight pairs differed by 140-250 grams. 18 pairs did not differ at all as to interval of last egg mass and time of pairing (0-5 days); 20 pairs differed from each other by one day (0-1; 1-2; 2-3; 3-4); five pairs differed more widely (0-2; 1-3; 0-10 days).

C. Maintenance Conditions

The dimensions of each aquarium were 30 x 30 x 30 cm. Each had a single inlet hose and a constant-level siphon designed to draw water from the center of the aquarium, and maintain a water level approximately 10 cm below the top. This arrangement minimized blockage of water flow by the sea hares, which move only on the walls or bottom. It also prevented the escape of the animals, as they usually did not move higher than the water surface. The volume of water in each tank was normally 18 litres with an exchange rate of approximately one to two litres/minute.

Animals were fed fresh algae (Acanthophora spicifera)daily. The general amount of feeding was monitored, but served only as an indication of the health of each animal.

The aquaria were kept on outdoor water tables in an area which was partially protected from rain by a concrete overhang. Tanks were cleared of encrusting algae by scrubbing the walls with plastic scouring pads every three days. When an inhabitant was changed, the tank was thoroughly washed with hot water and then sun-dried.

D. Methods and Procedures

An experimental session consisted of five 30-minute observations within 24 hours, one preliminary observation and four experimental observations. The preliminary observation was followed immediately by the first experimental observation; the second, third and fourth followed at six-hour intervals. Figure 2 diagrams the method for assigning pairs based on an individual's preliminary experience as a paired or unpaired animal.

There were four possible "levels" of exposure to conspecifics, constituting a hypothetical continuum, with unpaired animals (alone) representing "least" exposure, and pairs which contacted and copulated representing the other extreme. All of these "levels" were represented in the preliminary observation; in the first experimental observation all animals were paired and these pairs were maintained through the successive observations.

Table 2 shows the experimental groups which resulted from different combinations of preliminary conspecific exposure. These combinations yielded treatment groups determined by the behavior of the animals themselves, thus it was not always possible to obtain the same number of pairs in each combination.

The ten possible combinations were assigned to broader treatment categories (A, B, and C). Treatment A pairings are defined by neither partner having had any contact. In Treatment B, one of the partners had either contact or copulation experience. Both partners in Treatment C had some contact or copulation experience.

At the end of a 24-hour session, the animals were weighed and returned to their home tanks.

E. Observation Procedures

Animals were observed initially in plexiglas tanks 45 cm long x 30 cm wide x 30 cm deep, containing about 25 litres of continually-flowing sea water at approximately four litres/minute. Water flowed out of both ends, to avoid the complication of currents. Pairs were introduced into 75-litre plexiglas tanks 90 cm long x 30 cm deep x 30 cm wide for the experimental observations.

The observation area, which was outdoors, was protected on four sides by canvas tenting. A single 15-watt red incandescent bulb, which hung 100 cm above the middle of the area, was turned on for all observations, and permitted observations during the night.

Preliminary observations were made between 8:00 a.m. and 10:30 a.m., followed by the first experimental observation between 8:30 a.m. and 11:00 a.m.; the second occurred between 2:30 p.m. and 5:00 p.m.; the third between 8:30 p.m and 11:00 p.m.; and the fourth between 2:30 a.m. and 5:00 a.m.

F. Behavior Recorded

Behavioral data were recorded on a 20-key Esterline Angus event recorder, and were supplemented by handwritten notes as well as by verbal reports entered on tape. Definitions of the behavior items are found in Table 3.

TABLE 1

Number and Size of Aplysia dactylomela Collected in

La Parguera, P. R. January-August, 1975

Collado Zone

Month	N	Median Weight (g)	Range
January	3	80	80-145
February	0	--	--
March	1	150	0
April	0	--	--
May	1	190	0
June	0	--	--
July	19	240	120-380
August	0	--	--

Enrique Zone

Month	N	Median Weight (g)	Range
January	1	110	0
February	1	95	0
March	1	250	0
April	0	--	--
May	0	--	--
June	0	--	--
July	11	270	175-590
August	32	310	140-535

Plajita Rosada Zone

Month	N	Median Weight (g)	Range
January	0	--	--
February	3	85	80-105
March	7	115	100-210
April	9	155	85-225
May	1	105	0
June	0	--	--
July	0	--	--
August	0	--	--

TABLE 2

EXPERIMENTAL OBSERVATION:

Number of Pairs of *Aplysia dactylomela* Observed in Different Combinations

During Experimental Observations[1]

Animal B: Behavior During Preliminary Observation	Animal A: Behavior During Preliminary Observation — Alone 30 Individuals	Paired: 60 Individuals — Did Not Contact	Paired: 60 Individuals — Contact Only	Paired: 60 Individuals — Copulated
Alone (A) Paired With	6[A]*	8[A]	5[B]	5[B]
Did Not Contact (NC) Paired With	---	2[A]	9[B]	0[B]
Contact Only (C) Paired With	---	---	2[C]	4[C]
Copulated (Cp) Paired With	---	---	---	2[C]

[1]Four sea hares were included in the preliminary observation which were not observed in experimental observations.

*Combinations in treatment categories A, B, and C.

TABLE 3

Behavioral Items Used in Paired Observation

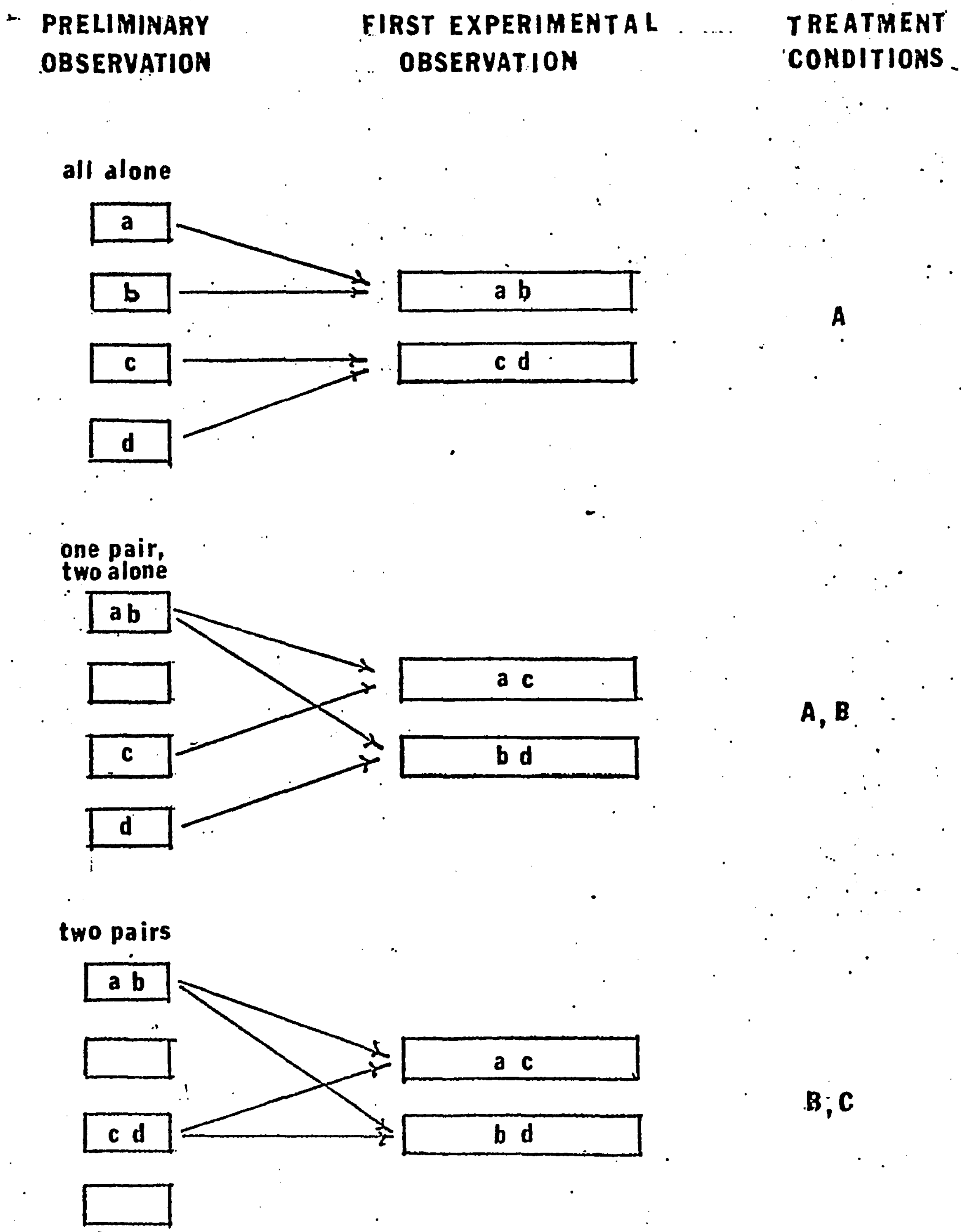
Of *Aplysia dactylomela*

1.	Oriented towards:	With respect to longitudinal axis of the body, the head of the animal is directed at the second animal.
2.	Locomotion:	A displacement of the sea hare in space in association with wavelike contractions of the foot.
3.	Contact:	Any part of an animal touching any part of another individual.
4.	Initiates contact:	An animal initiates contact if it approaches the other and touches it with any part of the body.
5.	Approach:	Any locomotion which decreases inter-individual distance while oriented towards the individual.
6.	Mutual or Reciprocal contact:	Both individuals initiate the contact.
7.	Passing contact:	Contact occurs as one or both individuals are locomoting. The duration of contact is relatively brief, depending on rate of locomotion, and there is no change in the activity pattern or direction of locomotion during the contact or later.
8.	Copulation Initiator (C.I.):	An animal which initiates a contact leading to copulation.
9.	Typical copulatory posture: (tcp):	An animal has its head between and under the parapodia of the other and to the right of the shell.

TABLE 3, Continued

10. Copulation:	The intromission of an animal's penis into the common genital opening. As this was not readily confirmed during the observation period, an operational definition was adopted whereby a pair which assumed the typical copulatory posture (above) for 60 seconds was considered to be copulating. This was changed to a contact if the intromission was not subsequently confirmed by closer visual inspection, or upon separation at the end of the 30-minute initial pairing.
11. Typical Initiator (T.I.):	The individual which initiated all the contacts before copulation; or, where at least one contact was initiated by each animal, the individual which initiated more contacts and at least 60 seconds longer than those initiated by the partner.

Figure 2. Procedural design for derivation of experimental pairs of *Aplysia dactylomela* based on preliminary levels of exposure to conspecifics. Treatment Conditions: (A) Neither member of pair made contact; (B) One member of pair made contact or copulated; (C) Both members of pair made contact or copulated.


PRELIMINARY OBSERVATION
FIRST EXPERIMENTAL OBSERVATION
TREATMENT CONDITIONS
all alone
a
b
c
d
a b
c d
A
one pair, two alone
a b
c
d
a c
b d
A, B
two pairs
a b
c d
a c
b d
B, C

III. RESULTS

A. Behavior During Preliminary Pairing

1. Contact and Copulation

Of sixty sea hares which were paired, seven pairs copulated, 11 made contact for different amounts of time, and 12 never made contact at all. The first contact occurred within 15 minutes in 14 out of 18 pairs and copulation never occurred in less than 10 minutes. Pairs which made contact were not different from those which copulated in the latency to first contact or in the duration of contact (Appendix, Tables B and C). But the total time spent interacting was greater in pairs which copulated (Mann-Whitney "U" Test: $U = 13$, $p < .05$, two-tailed test; Table 4).

In pairs which copulated, the latency to copulation was unrelated to the latency of first contact (Appendix, Table D). However, once contact occurred, copulating pairs remained in contact, whereas contact pairs did not (Fisher's Exact Probability Test, $p = .025$, Table 5).

2. Locomotion

Animals which were paired spent more time locomoting than animals which were alone (Kruskal-Wallis Analysis of Variance, $H = 10.0$, $p < .025$, Table 6.)

Alone animals were not significantly different from no-contact pairs ($z = 1.37$, $p = .18$) but locomoted for less time than those which contacted ($z = 2.64$, $p < .01$) and those which copulated ($z = 2.07$, $p < .04$; all of the above are Mann-Whitney "U" two-tailed tests). It is noteworthy that of the 30 animals alone, eight did not locomote, while all of the no-contact animals locomoted, suggesting that although they were not statistically different in the total time spent locomoting, qualitative differences might be present.

The contact and copulation pairs were not different in the total time locomoting. However, since the interactions were longer for copulating pairs (Table 4) the proportions of non-interactive time spent locomoting were compared and showed that pairs which copulated actually did locomote more (Mann-Whitney "U" Test, $z = 2.49$, $p < .012$, two-tailed test).

Most of the animals spent a greater proportion of time locomoting before the first contact than afterwards (21 out of 22 pairs which contacted, 8 out of 11 pairs which copulated).

Contact initiators locomoted more than non-initiators only in pairs where contact occurred (Mann-Whitney "U" Test, $U = 16$, $p < .02$, two-tailed test) but not in those pairs which copulated (Table 7).

B. Behavior During First Experimental Pairing

1. Contact and Copulation

Table 8 shows the distribution of pairs which copulated (14), contacted (8), or did not contact (21) during the first experimental observation in terms of treatment categories (A, B, C) and specific pairing types. This distribution did not differ significantly from the behavior of paired animals during the preliminary observation (Chi-square = 3.08, df = 2). The latency to first contact and latency to copulation were not different among any of the nine pairing types (Kruskal-Wallis Analysis of Variance; contact: $H = 7.52$, df = 8; copulation:

TABLE 4

Total Duration of Time in Contact and Copulating

In Pairs of *Aplysia dactylomela*[1]

	Pairs Which Contacted	Pairs Which Copulated
N	11	7
Median (sec)	505	1476
Range (sec)	80-1584	465-1658

[1]Mann-Whitney "U" Test: U = 13, $p < .05$

TABLE 5

Behavior Following First Contact in Pairs of A. dactylomela During Preliminary Observation[1]

	Remained In Contact	Did Not Remain In Contact
Pairs Which Contacted	1	10
Pairs Which Copulated	5	2

[1]Fisher's Exact Probability Test, $p = .025$

TABLE 6

Total Duration of Locomotion by Individual *A. dactylomela* During Preliminary Observation[1]

	Alone	Paired: Did Not Contact	Paired: Contact Only	Paired: Copulated
Total Number of Sea Hares	30	24	22	14
Number Locomoting	22	24	22	13
Median (sec)	185	242	495	410
Range (sec)	0-1505	30-847	144-1453	0-1608

[1]Kruskal-Wallis One-Way Analysis of Variance, $H = 10.0$, $p < .02$

TABLE 7

Non-Interactive Locomotion In Pairs of *Aplysia dactylomela* During Preliminary Observation

	Pairs Which Contacted[1]		Pairs Which Copulated[2]	
	Contact Initiator	Contact Non-Initiator	Contact Initiator	Contact Non-Initiator
Number of Sea Hares[3]	10	10	6	6
Median (sec)	445.5	180	347	181.5
Range (sec)	168-772	31-498	88-800	0-915

[1] Mann-Whitney "U" Test: $U = 16$, $p < .01$, one-tailed test

[2] Mann-Whitney "U" Test: $U = 18$, not significant

[3] Pairs in which the initiator and non-initiator roles could be clearly identified

H = 2.69, df = 8; Appendix, Tables E and F).

In contrast to the behavior of pairs during the preliminary observation, pairs which contacted or copulated during the following observations were not different from each other in tendency to remain in contact (Chi-square = 1.72; df = 1; Appendix, Table G).

Table 9 shows a tendency in the preliminary observation, and within treatment groups in the first experimental observation, for the typical contact initiator to be both the initiator of the copulation and the sperm donor. When the data for the experimental treatment groups are combined, a significant pattern of role consistency is apparent within pairs. However, individuals did not consistently assume the same roles in the first experimental observation and the preliminary observation (Table 10).

It seemed likely, therefore, that the roles assumed during the experimental observation might be related to an individual's specific type of preliminary exposure to conspecifics. Tables 11A and 11B show that where one experimental partner had been alone and the other paired, it was the paired animal which initiated the contacts and the copulation (Two-tailed binomial tests: T.I.: p = .004; C.I.: p = .032). However, the role during copulation was not reliably related to the preliminary experience.

TABLE 8

Behavior of *Aplysia dactylomela* During First Experimental Observation

Behavior During Preliminary Observation

Treatment Category:

	Pairing Type	A			B				C			
	Partner A:	A	A	NC	A	A	NC	NC	C	C	Cp	
	Partner B:	A	NC	NC	C	Cp	Cp	Cp	C	Cp	Cp	Total
Behavior During First Experimental Observation	Number of Pairs Which Did Not Contact (NC)	2	4	1	3	2	3	0	2	3	1	21
	Number of Pairs Which Made Contact (C)	2	2	0	1	1	1	0	0	1	0	8
	Number of Pairs Which Copulated (Cp)	2	2	1	1	2	5	0	0	0	1	14

TABLE 9

Number of Pairs in Which Contact and Copulation Were

Initiated by the Same or Different Individual

	Preliminary Observation			First Experimental Observation											
				Treatment Category:											
				A			B			C			Total		
Roles	Same	Diff	p[1]	Same	Diff	p	Same	Diff	p	Same	Diff	p	Same	Diff	p
T.I.=C.I.	6	0	.032	4	1	*	8	0	.008	1	0	--	13	1	.002
C.I.=S.D.	5	1	*	5	0	.062	7	1	.070	0	1	--	12	2	.012
T.I.=S.D.	5	1	*	4	1	*	7	1	.070	0	1	--	11	3	.058

[1] Two-tailed binomial test

T.I.: typical initiator
C.I.: copulation initiator
S.D.: sperm donor
=: same as

* $p > .10$

TABLE 10

Number of Times Initiator and Copulation Roles Were Assumed

By *Aplysia dactylomela* with Different Partners

Role During First Experimental Observation	Role During Preliminary Observation					
	T.I.	Non T.I.	C.I.	Non C.I.	S.D.	S.R.
Same	5	4	2	1	4	1
Different	4	1	1	0	2	1

T.I.: Typical initiator
C.I.: Copulation initiator
S. D.: Sperm donor
S. R.: Sperm recipient

TABLE 11A

Relationship of Initial Experience of Individual *A. dactylomela*

To Roles When Paired With Different Partners

Initial Experience Of Pair	Number of Pairs in Which Indicated Roles Are Assumed In a Second Pairing					
			Sperm Donor			
	T.I.	C.I.	Within 30 Min.	Within 45 Min.	Within 60 Min.	S.D. Total
Alone	0	0	0	0	0	0
Paired, No Contact	4	3	2	1	0	3
Alone	0	0	0	1	0	1
Paired, Contact	2	1	1	0	0	1
Alone	0	0	0	1	0	1
Paired, Copulation	2	1	1	0	0	1
Paired, No Contact	3	3	3	0	1	4
Paired, Contact	3	2	2	0	0	2
Paired, Contact	1	0	0	0	0	0
Paired, Copulation	0	0	0	0	1	1

TABLE 11B

Total Number of Pairs in Which Indicated Roles Are Assumed in Second Pairing

Pairing Type	T.I.	C.I.	S.D.
Initially Alone	0	0	2
Initially Paired	9	6	6
p[1]	.004	.032	*
Initially Paired	4	3	4
Initially Paired	3	2	3
p	*	*	*

[1]Two-tailed binomial test

* $p > .10$

T.I.: typical initiator
C.I.: copulation initiator
S.D.: sperm donor

2. <u>Locomotion</u>

The data on locomotion behavior reported in the following sections support the contact and copulation findings. They point to a consistent relation between an individual's behavior and its own and its partner's preliminary experience. As locomotion may have been affected by whether the animals made contact or copulated, the data were analyzed further in terms of locomotion which took place when pairs were not in contact or copulating (non-interactive locomotion). Differences in this behavior among all nine pairing types were statistically significant (Kruskal-Wallis One-Way Analysis of Variance: $H = 20.57$; p .01; Tables 12A and 12B). Statistically significant differences were also found within the treatment conditions B and C, but not A (Table 12B). The C/Cp comparison could not be treated statistically because of the small sample size; however, the data were not overlapping and were in the same direction as in the other comparisons.

These analyses showed that during the first experimental observation, copulating animals locomoted less than animals that did or did not make contact, when one or both members of the pair had social contact experience (Treatments B and C), but not when neither of them had such experience (Treatment A). During the preliminary observation, however, the animals that copulated, locomoted more than those that did not. The only comparison appropriate to an investigation of this difference was between the animals that had been alone during the preliminary observation and copulated during the first experimental observation. All animals had been in individually-maintained "home tanks" before the preliminary observation, and this condition might be considered equivalent in some ways to the condition of the alone animals during the preliminary observation. The other pairs that copulated during the first experimental observation had had mixed social experience during the preliminary observation and thus could not be used in the comparison.

Among the alone (during the preliminary observation) animals paired with other alone animals (six pairs), only two pairs copulated. These four animals locomoted 0, 512, 2 and 72 seconds during the first experimental observation and copulated for 906 and 958 seconds respectively. The seven pairs that copulated during the preliminary observation, locomoted more than the two pairs described above (median = 263 seconds; range = 0-915 seconds) and copulated for a shorter duration (median = 274 seconds; range = 98-1076 seconds). The difference between the two groups of copulating pairs is not statistically significant, but it should be noted that five of the seven pairs copulating during the preliminary observation did so for shorter durations (98-468) than the two pairs which copulated during the first experimental observation.

In combining sea hares with different experience for the first experimental observation, it became possible to compare animals that had similar social experience during the preliminary observations, but were paired with animals with different experience. Thus, animals that had been alone but were paired with other alone animals could be compared with alone animals that were combined with animals that had been paired but did not make contact, made contact or copulated. In this way, six comparisons could be made for each of the four types of experience during the preliminary observations;

e.g., A with A compared with A with NC

A with A compared with A with C

A with A compared with A with Cp

A with NC compared with A with C

A with NC compared with A with Cp

A with C compared with A with Cp.

Of all the possible comparisons, only five were significant at less than the .10 level of probability. Four of these were related to the animals that had been alone during the preliminary observation. (Table 11). The only other comparison that was statistically reliable also involved an alone animal. In all five instances, the alone animal locomoted more when it was paired with an animal which had had "less" social stimulation, if a continuum were seen starting with alone (no social stimulation) and ending with copulation ("most" social stimulation).

During the preliminary observation, contact initiators generally locomoted more than non-initiators. As Table 14 shows, this relationship

tionship was also found during the first experimental observation both in Treatment Category A and B (Mann-Whitney "U" tests: A: U = 17, p = .05; B: U = 13, $p < .01$; two-tailed tests). The sample size for Treatment C was too small to be analyzed.

When the locomotion of individuals was compared across the preliminary and first experimental observations, there was no consistent tendency for an increase or a decrease in the amount of locomotion (Appendix, Table H).

C. Behavior During Four Successive Observations

1. Contact and Copulation

Twenty-seven pairs of A. dactylomela were observed during all four experimental periods and Table 15 shows their behavior during each observation. A Cochran-Q test showed that all pairs copulated more during the second, third, and fourth observations than during the first (Q = 8.58, $p < .05$). Further analysis (Table 16) showed that pairs which did not copulate at first were more likely to copulate during the second observation (McNemar Test of Change, H = 5.79, $p < .02$). After the second observation pairs were always more likely to be copulating, with a small number changing equally in either direction.

Tables 17 and 18 show that complementary increases also occurred in the frequency of separate contacts and number of changes in copulation roles.

Associated with an increase in role reversals during the second, third, and fourth observation was the separate observation of six instances of a reciprocal copulation posture in six different pairs. Three occurred during the second observation, two during the third observation, and one during the fourth observation. This is the first report of reciprocal copulation in Aplysia. Although reciprocal intromission was confirmed by inspection, sperm transfer was not examined. Therefore, it should be referred to as a "reciprocal copulatory posture". Durations of the confirmed reciprocal copulatory posture ranged from 30 seconds to 1460 seconds, with an average of 349 seconds. They usually occurred in relation to a role reversal--that is, a pair might reverse roles when the recipient twists around and intromits. Shortly after that the original donor withdraws the penis and the roles become reversed without a break in contact. However, in two pairs this was not the case and these had the two longest durations of the group (352 seconds, 1460 seconds).

It has been shown (Table 10) that individual sea hares do not maintain the sperm donor or recipient roles when their partner is changed. Table 19 shows that this holds consistently for the same pair when they are together for 24 hours. It also appears that the more often pairs were observed copulating the stronger the tendency to find the partners occupying different roles.

The tendency for the typical initiator to assume the roles of copulation initiator and sperm donor was also found to be true in the second and third observations, but not in the fourth (Table 20).

2. Locomotion

A series of sign tests on 42 individuals observed during successive observations showed that there was no tendency for individuals to increase or decrease the time spent locomoting (Appendix Table I).

TABLE 12A

Locomotion During Non-Interactive Time of Individual *Aplysia dactylomela*

During the First Experimental Observation

Behavior During First Observation	Treatment A			Treatment B			Treatment C		
	NC	C	Cp	NC	C	Cp	NC	C	Cp
Number of Sea Hares	14	8	10	16	6	16	12	2	2
Median (sec)	437	313	364	624	220	79	705	380	24
Range (sec)	0-1310	0-733	0-905	0-1441	0-536	0-444	80-1680	290-470	0-48

NC = No Contact
C = Contact Only
Cp = Copulation

TABLE 12B

Statistical Comparisons of Non-Interactive Locomotion of Individual *Aplysia dactyomela* (Table 12A) by Treatment Group and Behavior During First Experimental Observation

Kruskal-Wallis Analysis of Variance

Comparison:	H Value	p
Among All Treatments:		
Treatment A (NC;C;Cp)		
Treatment B (NC;C;Cp)	20.59	$< .01$
Treatment C (NC;C;Cp)		
Within each treatment:		
Treatment A:	2.58	$> .20$
Treatment B:	6.14	$< .05$
Treatment C:	6.59	$< .05$

Mann-Whitney "U" Test

Comparison:	U Value	p
Treatment B		
NC : C		n.s.
NC > Cp	73.0	$< .05$
C > Cp	20.5	$< .05$
Treatment C		
NC > C		n.s.
NC > Cp	0	$< .05$
C : Cp		--

TABLE 13

Comparisons of Non-Interactive Locomotion of *A. dactylomela*

During the First Experimental Observation (Medians, sec)

Alone with Alone	Alone with No Contact	Alone with Contact	Alone with Copulating	Contact with Alone	Contact with No Contact
226[a,b]	0[a,c]	202[d]	0[b,c,d]	448[e]	38[e]

	Mann-Whitney One-Tailed Test: U	p
a:	25	.03
b:	5	$<$.01
c:	12.5	.08
d:	5	.04
e:	4	.01

TABLE 14

Non-Interactive Locomotion Of *A. dactylomela* During The First Experimental Observation

	Treatment A		Treatment B[1]	
	Initiator	Non Initiator	Initiator	Non Initiator
Number of Sea Hares	9	9	9	9
Number Locomoting	9	5	9	4
Median (sec)	511	2	188	0
Range (sec)	72-733	0-905	47-536	0-250

[1]Two pairs could not be characterized by a clear initiator.

TABLE 15

Number of Pairs of *A. dactylomela* Copulating, In Contact,

Or Not in Contact During Each of Four Observations[1]

	Experimental Observations			
	1 8:30-11:00 a.m.	2 2:30-5:00 p.m.	3 8:30-11:00 p.m.	4 2:30-5:00 a.m.
Copulation	10	19	18	17
Contact	4	5	8	7
No Contact	13	3	1	3
Total	27	27	27	27

[1]Cochran-Q Test: $Q = 8.58$, $p < .05$

TABLE 16

Changes in Copulation Within Pairs

A: First and Second Observations

Number of Pairs In First Observation	Number of Pairs in Second Observation: Did Not Copulate	Copulated
Copulated	2	8
Did Not Copulate	5	12

B: Second and Third Observations

Number of Pairs In Second Observation	Number of Pairs in Third Observation	
Copulated	7	12
Did Not Copulate	2	6

C: Third and Fourth Observations

Number of Pairs In Second Observation	Number of Pairs in Fourth Observation	
Copulated	5	13
Did Not Copulate	5	4

TABLE 17

Frequency of Separate Contacts in 27 Pairs of *Aplysia dactylomela* Observed During Four Observations[1]

	Observation: 1	2	3	4
	8:30-11:00 a.m.	2:30-5:00 p.m.	8:30-11:00 p.m.	2:30-5:00 a.m.
Median	1	2	3	1
Range	0-3	0-8	0-5	0-6

[1] Friedman Two-Way Analysis of Variance: $X_r^2 = 17.39$, $p < .001$

TABLE 18

Frequency of Changes in Copulation Role in 27 Pairs of *Aplysia dactylomela* During Four Observations[1]

	Observation			
	1 8:30-11:00 a.m.	2 2:30-5:00 p.m.	3 8:30-11:00 p.m.	4 2:30-5:00 p.m.
Median	0	1	1	1
Range	0-1	0-4	0-4	0-2

[1] Friedman Two-Way Analysis of Variance, $X_r^2 = 9.52$, $p < .01$

TABLE 19

Copulation Role Relationship in Same Pair of *Aplysia dactylomela*

During Four Observation Periods

Number of Observations in Which Pairs Copulated[1]	Maintained Role Relationship	Did Not Maintain Role Relationship	Two-tailed Binomial test: p
2	4	3	*
3	1	8	.04
4	0	5	.06
Total Number of Pairs	5	16	.026

[1]Five pairs were observed copulating only once and one pair was never observed copulating.

TABLE 20

Copulation Roles Assumed by Typical and Copulation Initiators

In the Same Pair of *Aplysia dactylomela*

Observation		Sperm Donor	Sperm Recipient	Total	Two-tailed Binomial test: p
First	Typical Initiator	11	3	14	.058
	Copulation Initiator	12	2	14	.012
Second	Typical Initiator	20	3	23	.002
	Copulation Initiator	18	0	18	.002
Third	Typical Initiator	15	4	19	.02
	Copulation Initiator	13	1	14	.002
Fourth	Typical Initiator	9	4	13	*
	Copulation Initiator	8	2	10	*

* not statistically significant.

IV. DISCUSSION

This experiment was undertaken to gain an understanding of copulatory behavior in a simultaneous hermaphrodite. To determine whether any aspects of the social behavior were predictable from the *Aplysia*'s prior behavior, the experiments were designed to record the occurrence and patterns of sex role assumption as well as individual changes in locomotion.

The observations made possible several statements about both copulatory and non-copulatory interactions. In most pairs of *A. dactylomela* the probability of contact and copulation is fairly well-assured after they are placed in a tank and individuals will assume both the donor and recipient roles in the paired situation. The social behavior of an individual could not be predicted on the basis of its own behavior with a different partner during a prior pairing. Paired individuals spent more time locomoting before contact than did unpaired individuals in a preliminary observation, but individual activity was not related to their own prior experience in the subsequent experimental pairing. The latencies to contact and copulation were variable and did not seem to be related to either the outcome of an encounter or the prior social experience of the individuals.

The absence of individual consistency in role assumption across pairings might have been the effect of uncontrolled variables in the experimental situation, or it might have been due to factors which increased the probablity of fortuitous contact. The evidence, however, has argued for a degree of inter-individual organization in the interactions of A. dactylomela. However, a number of factors may have contributed to the variability of the social responses in these experiments. Foremost among these is the uncertain history of the adults when they were collected. All the experimental animals were alone for at least five days before the experiment and most were alone for two or three weeks. It was hoped that this pre-experimental procedure would make the animals more similar in their social responsiveness. However, animals at different stages of development may have responded differently to the effects of individual maintenance. This seems to be true for their spawning behavior (page 112). Unfortunately, in the absence of developmental information, it is impossible to assess the influence of the differences in individual histories.

Although individual social patterns were not predictable across different partners, they pointed to an important influence of the prior social experience of each partner on their behavior together. In most cases, the animal which initiated contact was likely to become the sperm donor. In the first experimental observation the contact initiator was likely to become the sperm donor if it had been paired initially and if its partner was socially inexperienced (alone). The basis for these interactions appears to be a chemosensory orientation to conspecifics. In a previous experiment, (page 40) it was found that conspecific stimuli can lead to a decrease in interindividual distance in that test animals approached a source of conspecific stimulation. In the present study, before any contact occurred, and in the absence of unidirectional current, individuals spent more time locomoting in the presence of other sea hares than if they were alone.

Pairs which copulated locomoted more than those which only contacted. However, when copulating pairs were separated and put together with a different partner, they locomoted less than previously-paired animals which did not copulate. Since animals which copulated still locomoted more than initially-unpaired animals, there may have been a confounding of the effects of prior social experience and the handling of a copulating pair.

Within a 24-hour session, the sperm donor and recipient roles reversed frequently during the second, third, and fourth observations, but never during the preliminary or first observations. As the frequency of copulating bouts within a session increased the duration of each bout may have decreased; as a result, the quantity of sperm transferred may also have decreased. When animals are limited in the options available for sperm transfer, as in a paired situation, there might be some advantage to the species, in terms of genetic variation, for some mechanism which limits the number of eggs fertilized by the same donor (Lederhendler, 1977). Thompson (1976) suggested that chain copulation could have the effect of maximizing the use of gametes. The frequent alternation of copulatory roles in pairs is an additional mechanism where a maximum use of gametes occurs and may be of particular importance where low density conditions exist.

In order to assess the possible influence of circadian locomotor rhythms on paired interactions, observations were made every six hours. Although this design does not test for circadian rhythms directly, the absence of any differences in overall activity levels across observations is surprising in view of the well-known rhythms of *A. californica*

(Kupfermann, 1968; Strumwasser, et al., 1971; Jacklet, 1972), although this may have also been related to conditions in this study which facilitated contact and copulation. Once the initial contacts occurred, animals were more likely to be seen in contact than apart. Since Kupfermann (1968) reported that tactile stimulation inhibited locomotion, perhaps in the present situation the overall level of locomotion was reduced sufficiently to mask any rhythmic patterns of locomotion.

Comparisons of locomotion during the preliminary and first observations provide some other clues about the processes which may be involved in contact and copulation. Initially unpaired animals locomoted less in the presence of experienced animals than in the presence of other initially-unpaired sea hares. These lower levels of locomotion seemed to be related to the kind of exposure (no contact, contact, copulation) which the other partner had initially. It would appear that something changed in the paired animals related to their experience and that these changes can differentially affect the locomotion of inexperienced animals.

One way in which the differential effects of locomotion may be understood is through a hypothesis that the three levels of exposure represented three stages of the social facilitation of locomotion. Thus, pairs which contacted would be in a state of "high" facilitation, pairs which copulated would be in a state of "refractory" responsiveness, and no-contact pairs would be in a state of "low" facilitation. When paired with inexperienced animals, the "high-facilitation" state could provide more intense stimulation which would, in turn, facilitate the locomotion of the partner. The other two "states" could operate by either actively inhibiting the locomotion of the inexperienced animals or possibly by not providing any stimulation at all. In either case, a strong implication arises in which the integration of inter-individual behavior occurs in relation to chemosensory processes related to prior social experience.

These kinds of relationships between social experience and locomotion can be very significant in species adaptation. Under field conditions, the locomotion of animals which have been alone can be regulated by socially-active conspecifics which are within the stimulus-effective range. Neighboring animals can then become more active and initiate contacts with the lone individual. Also, it appears likely from the present data that in the first interaction with a new animal a socially-experienced animal will assume a different role from that which it had last assumed.

Such social processes can act to increase the probability of genetic variation. The significance of the ancillary characteristics of chain copulation and frequent role reversals have already been indicated for maximizing the number of fertile eggs. It is expected that differences in the reproductive behavior in other species of _Aplysia_ would represent differing strategies of adaptation around the theme of genetic variability and fecundity. Confirmation of this hypothesis depends on comparative and developmental studies of reproductive processes.

SECTION IV

Egg-laying In Individually-Maintained *Aplysia dactylomela*

I. INTRODUCTION

Many characteristics of any population of *Aplysia* are likely to be determined directly by the fecundity and temporal pattern of egg-laying in the previous generation (MacGinitie, 1934; Miller, 1960; Usuki, 1970; Nishiwaki, 1975). Many of the probable factors related to egg laying behavior in *Aplysia* have not been studied. However, some environmental variables are known, and one neurohormonal factor (bag cell hormone) has been studied in detail.

Among the environmental variables seasonality has been found in species from the temperate waters of Japan, the United Kingdom, and California (Nishiwaki *et al.*, 1975; Miller, 1960; Carefoot, 1967a; Strumwasser *et al.*, 1969) but tropical species have not been studied. The seasonal effect may be related to the influence of temperature on the maturation of gonads (Smith and Carefoot, 1967). Fecundity may be directly affected by diet or influenced indirectly through its influence on individual growth (Carefoot, 1967b).

Among the physiological variables, egg-laying has been reliably induced by injections of a hormone from the bag cells (Kupfermann, 1967; 1970; Toers and Brackenbury, 1969; Strumwasser *et al.*, 1969; Arch, 1972; 1976). The bag cells are a cluster of neurosecretory cells of the abdominal ganglion. The hormone which they produce makes the muscles of the ovotestis contract and expel oocytes (Coggeshall, 1972). Seasonal and size variables seem to play a role in the effectiveness of bag cell hormone to induce egg-laying (Strumwasser *et al.*, 1969). In related morphological studies, Frazier *et al.* (1967) found that the bag cell clusters are very small in two-gram A. californica when neurosecretory granules are absent. In sea hares greater than 50 grams, neurosecretory granules were present, and the number of units in the bag cell clusters had increased to almost adult levels.

Egg-laying induction by bag cell hormone injections is not uniformly effective in all individuals (Strumwasser et al., 1969; Dudek and Pinsker, 1976.) Davis et al. (1974) indicated that this was also true for Pleurobranchea, a related species of opisthobranch. Other variables are probably important and not known, and as oxygenation, food supply, and population density have been identified as factors in other gastropods (Lusis, 1966; Mooij-Vogelaar et al., 1973) these factors may also be relevant for Aplysia egg-laying.

In adults, Aspey and Blankenship (1976) suggested that egg-laying conspecifics or the eggs themselves may be a source of stimulation for sexual behavior in A. brasiliana. Perhaps conspecific interaction can also influence the egg-laying. Support for this notion comes from Crozier (1918) who observed that in numerous matings of the opisthobranch Chromodoris zebra spawn were usually deposited following copulation.

In order to study social factors in relation to egg-laying, this study was designed to establish baselines for several parameters of the behavior while A. dactylomela were maintained individually for different amounts of time. Following these preliminary observations the animals were paired and their egg-laying before, during, and after the pairing was compared.

Three parameters of individual egg-laying were recorded:

the latency to first egg mass after collection, the interval between egg masses, and the total number of egg masses laid. These are discussed in more detail below.

II. METHODS AND PROCEDURES

A. Collection and Subjects

122 *Aplysia dactylomela* were collected for this study between January and September 1975, in the waters off the southwest coast of Puerto Rico near La Parguera. Three major collection zones are indicated in Figure 1, page 24. Each zone was visited at least once a week for the duration of the study.

The number of animals collected at each location, the months in which they were collected, and the number of days kept alone are shown in Table 1.

B. Maintenance

When sea hares were found their location was noted and they were brought back to the laboratory where they were weighed, tagged, and invidually placed in outdoor 20-litre all-glass aquaria with continuously-running sea water. The exchange rate of the sea water was kept at approximately 2 litres/minute. The tanks were cleaned every three days by scrubbing with plastic scouring pads. When an inhabitant was changed, the tank was washed with hot water and then sun-dried.

Sea hares were fed fresh algae (*Acanthophora spicifera*) daily. Food was available at all times, except when tanks were being cleaned. Further details on the collection and maintenance procedures have been described earlier(pages 20 to 24).

C. Procedure

Each day, early in the morning, and at dusk, all tanks were

TABLE 1

Initial Size and Number of *Aplysia dactylomela* Collected

During Different Months

Number of Days Maintained Individually	Collection Zone	Number of Sea Hares	Weight (g)		Months Collected
			Median	Range	
4-9	Plajita	7	85	55-150	Jan, Feb, Mar, April
	Enrique	20	290	130-485	May, August
	Collado	2	225	220-230	May
10-15	Plajita	9	71.5	25-100	Jan, Feb, Mar, April
	Enrique	8	247.5	60-485	Jan, Feb, July, August
	Collado	4	202.5	45-235	Jan, Mar, July
16-21	Plajita	8	62.5	40-105	Mar, April, May
	Enrique	15	365	135-520	July, August
	Collado	17	185	80-370	May, July
22-27	Plajita	3	40	30-100	May, April
	Enrique	5	185	130-435	July, August
	Collado	5	160	75-250	Jan, July
28-33	Plajita	1	110	---	May
	Enrique	2	60	15-105	Jan, Feb
	Collado	1	250	---	July
34-66	Plajita	7	100	35-175	Mar, April, May
	Enrique	3	125	125-250	Jan, March
	Collado	5	210	115-335	Mar, May, July

carefully searched for egg masses. An egg mass was removed intact, placed in a separate bag of nylon mesh, and suspended with other egg masses in an aquarium supplied with continually-flowing sea water.

The egg masses have been described extensively (MacGinitie, 1934; Hyman, 1966; Thompson and Bebbington, 1969). A typical mass is a long continuous tangled strand which has been coiled upon itself during spawning; the mass is held together by adhesive material secreted continually as the eggs are laid. A mass is attached to the wall of the tank or to algae provided as food. Within the strand, capsules containing 5-20 eggs each are arranged helically along its length.

Viability was determined for each egg mass by daily observation under a dissecting microscope. Rotatory movements described by Sanders and Poole (1910) were used as the indicator that the eggs were fertile and that the embryos had developed at least to the veliger stage.

D. Procedure for 24-Hour Pairing

Pairs were matched for recency since last spawning and size. They were removed from the stock tanks, weighed, and placed in plexi-glass tanks 90 cm (length) x 30 cm (depth) x 30 cm (width) supplied by continuously-running sea water. The 24-hour period was divided into four quarters; a half-hour observation every six hours. Further details of the pairing may be found on pages 60 to 68 .

E. Data Analysis

The possible confounding of the variables time alone, weight, and location and month collected had to be sorted out before the effects of copulation on the relevant parameters could be assessed.

Three parameters of egg-laying were recorded: the latency to first egg mass after collection, the interval between egg masses, and the total number of egg masses laid. The latency to first spawning was considered a potentially important variable because it seemed possible that it might be sensitive to the history of each sea hare before it was found. The interval between egg masses and the total number laid over time were parameters which can indicate how often eggs are being laid as well as any regularities in individual patterns of spawning.

III. RESULTS

Inter-Spawn Interval

Table 2 shows that in general the sea hares laid more egg masses the longer they were in the laboratory. The median inter-spawn interval, however, remained constant for the initial three-week period; between 22 and 45 days the interval appeared to increase slightly. Four animals were alone longer than 45 days (one each: 47 days, 52 days, 61 days, and 66 days). The sample was too small to analyze and there was no orderliness related to the interval.

In Table 3 the number of egg masses laid and the inter-spawn interval before pairing were organized in relation to the month in which animals were collected. As the table shows, the number of days alone, the weights, and the number of egg masses covered a wide range within each collection period. However, the median inter-spawn interval was consistently three days, regardless of the month in which animals were collected.

Effects of Pairing

Only one pair was never observed to copulate. The other pairs were observed copulating at least once. Only animals which were observed copulating were used in the analysis of egg-laying patterns before and after copulation.

The influence of the 24-hour pairing was assessed using the median inter-spawn interval as the dependent variable because as shown above it proved to be a stable baseline. Of 32 seahares which laid at least two egg masses before and after pairing, eight did not change, six had a greater median interval, and 18 sea hares had a smaller median

TABLE 2

Egg-Laying Characteristics of Individually-Maintained *Aplysia dactylomela* Before Pairing

Number of Days Alone	Number of Sea Hares	Number of Egg Masses: Total	Number of Egg Masses: Median	Number of Egg Masses: Range	Two or More Egg Masses: Number of Sea Hares	Number of Egg Masses	Number of Intervals	Inter-Spawn Interval (Days): Median	Inter-Spawn Interval (Days): Range
4-9	29	55	3	0-4	17	38	29	3	1-4
10-15	21	59	3	1-6	16	66	35	3	1-6
16-21	40	146	4	0-8	34	142	108	3	1-8
22-27	13	40	3	0-6	13	40	29	4	1-14
28-33	4	8	1.5	0-5	2	8	6	4.5	1-7
34-39	6	55	9.5	7-11	6	55	48	4	1-9
40-45	3	19	9	0-10	2	19	17	3	2-6

TABLE 3

Characteristics of Egg-Laying in Aplysia dactylomela Before Pairing: Maintained Individually and Collected in Different Months

Month Collected[1]	Number Collected	Location	Weight (g)		Days Alone		Number of Egg Masses		Inter-Spawn Interval	
			Median	Range	Median	Range	Median	Range	Median	Range
January	11	PR, E, C	75	15-160	26	7-47	4	0-11	3	1-14
March	18	PR, E, C	60	25-235	17	4-61	1	0-19	3	1-7
April	12	PR	100	55-175	19	10-39	5.5	2-11	3	1-10
May	10	PR, E, C	192.5	55-300	17	5-66	2	0-17	3	1-10
July	32	E, C	195	80-520	17	14-52	4	0-8	3	1-9
August	34	E	295	130-520	9	5-26	3	0-6	3	1-4

[1]Five sea hares were collected in February--only one laid more than one egg mass; no sea hares were found in June.

[2]PR: Plajita Rosada
E: Enrique
C: Collado

interval after pairing (Sign test, $p = .022$, two-tailed test). However, if all 32 animals were considered, their median inter-spawn interval was three days.

18 sea hares laid eggs during the 24-hour pairing; copulation was first observed in the first quarter in seven cases (and one of these laid eggs as it was acting as a sperm recipient). The rest of the animals laid eggs in the second and third quarters. The remaining 11 cases were first observed copulating in the second quarter and they all laid eggs in that quarter but after copulation had occurred for the first time.

Seasonality

The earlier finding that the median inter-spawn interval did not change in different months suggested that egg-laying in *A. dactylomela* may not vary seasonally. This was confirmed in Table 4 which shows that almost all the animals laid eggs during each month they were in the laboratory. However, an interesting pattern was suggested when twelve sea hares which never laid eggs were studied more closely.

Some members of this group were present in every month, but eight were present in March, and six of these were collected at Plajita Rosada. The overall size of Plajita Rosada animals was smaller than animals which were collected from two other collection zones (Table 5). The six non-egg-layers from Plajita Rosada had a median weight of 40 grams. In addition, 17 other animals, with a median weight of 26 grams, collected at Plajita Rosada were too small to maintain in the 20-litre tanks and so were maintained separately in another facility using one-litre aquaria (see pages 29 to 31). None of these 17 small animals

TABLE 4

Number of Sea Hares Laying One or More

Egg Masses in Any Month[1]

Month	Number in Lab	Number Laid Eggs
February	15	12
March	19	11
April	15	11
May	23	18
June	13	12
July	25	23
August	47	45
September	16	14

[1]Twelve sea hares never laid eggs.

laid any eggs during the two to three week period in the laboratory.

Latency to First Laboratory Spawning

The median latency to first spawning was 5.5 days and ranged between one and 52 days for 110 of the animals which laid eggs. 90 animals laid two or more egg masses before pairing. When the latency to first spawning for individuals was compared against their own median inter-spawn interval, the latency was longer than the interval in 64 cases, smaller in 19 cases, and the same in seven cases (Sign test, $z = 4.84$, $p < .001$, two-tailed test).

The latency to first spawn was significantly related to location (Chi-square test = 10.01, df = 4, $p < .05$; Table 5). That is, referring to Table 5, the animals from Plajita Rosada were most likely to have a longer latency. Further examination of the table shows that the populations were not biased in relation to month; rather, size appeared to be most relevant. In other words, the longer latency to first spawning appears to be related to the population collected at Plajita Rosada which was smaller. As shown earlier, this group of sea hares also contained many small individuals which did not lay eggs.

TABLE 5

Latency to First Egg Mass Laid of *Aplysia dactylomela*

From Start of Individual Maintenance

	0-4 Days			5-6 Days			7 Days		
Collection Location	Enrique	Plajita	Collado	Enrique	Plajita	Collado	Enrique	Plajita	Collado
Number of[1] Sea Hares	21	5	12	9	3	9	14	16	10
Collection Month	February April May July August	February March April	May July	February April July	March April	January May July	January February April July August	January May April March	January March May July
Weight:									
Median (g)	300	65	212.5	250	65	190	250	92.5	185
Range (g)	90-500	25-105	125-280	105-285	45-115	45-370	60-520	30-110	80-235

[1]Chi-square test: = 10.1, df = 4, $p < .05$

IV. DISCUSSION

The data in this study showed that once *A. dactylomela* begin laying eggs in the laboratory, spawning proceeds with some regularity. Spawn were deposited every three days by most of the individuals.

When sea hares were paired and copulated, significantly more individuals had a shorter median inter-spawn interval following copulation. In a group of 18 animals which laid eggs during the pairing, eggs were reliably deposited after copulation occurred rather than before or during copulation.

The *A. dactylomela* from this area in Puerto Rico did not show seasonality during an eight-month period (February-September) in egg-laying. Species from temperate waters do show seasonality. Since temperature can influence the growth and maturation of the gonads (Smith and Carefoot, 1967) it may be that the less variable temperature conditions in Puerto Rico result in reproductively-mature animals throughout the year.

In the present study, data were presented that showed individual size to be related to egg-laying in two ways: a minimum size appears to be necessary for egg-laying to occur at all, and the latency to first observed spawning is longer in animals which were smaller when collected (Table 5).

The latency to first spawning may be related to an individual's history prior to collection. Lederhendler (page 25) found that in juvenile animals at least some interactions involving non-copulatory contact occur prior to first copulation. In the field, when these juveniles were collected only one copulating pair of intermediate size (greater than 40 grams) was found. Although collected near each other within 200 meters of coastline, small animals (less than 40 grams) were never found in contact. It seems possible therefore that at least some of the critical variables in the development of egg-laying behavior depend on social stimulation. The relationship between small size and longer latency to first spawn when alone may be indicative of fewer copulations in the field in the intermediate-sized animals. These conclusions also suggest research on the relationship between social stimulation and the development of the bag cell cluster.

V. SUMMARY

Individual <u>Aplysia dactylomela</u> were observed systematically before and after a 24-hour pairing. Pre-pairing observations revealed that most egg masses were laid every three days, and that the latency to the first spawning was significantly longer than the intervals after egg-laying began. There was no evidence for seasonal changes in any of the observed parameters of egg-laying. The latency to first spawning seemed to be largely a function of the size of the sea hare. This appeared to be related to the amount and kind of social interactions <u>A. dactylomela</u> may have had prior to collection in the field.

GENERAL DISCUSSION AND SUMMARY

The goal of this thesis was to study how inter-individual responsiveness in *Aplysia dactylomela* might affect reproductive behavior. This was examined in relation to egg-laying, juveniles, and adults. As an initial step the observations and experiments were aimed at determining the predictability of reproductive behavior based on an individual's prior exposure to conspecifics, and the probability of responses to conspecifics at a distance through chemoceptive processes. The findings emphasize that each of the behavioral phenomena investigated here is not discrete; they fit together in a general theme, albeit preliminary, of behavioral processes contributing to reproductive success in a simultaneous hermaphrodite.

Major Findings of the Four Studies

Copulation by adults has been observed frequently in the natural environment as well as laboratory settings, but there have not been any systematic investigations of the processes contributing to its initiation and maintenance.

Five pairs of field-collected juveniles were observed systematically and provided the first descriptions of changes in conspecific interactions in pre-copulatory *Aplysia*. In the laboratory, the juveniles increased rapidly in size and seemed to increase the duration of contacts with repeated pairings and accelerating growth. Only one of the five pairs copulated; animals in this size range never laid eggs.

When reproductive behavior of adults was studied, under these experimental conditions, the behavior of individuals could not be predicted on basis of their own prior social behavior. However, social experience was found to play a role in that subsequent interactions depended on the prior experience of both partners and through ancillary processes such as locomotion. Adults responded to conspecific stimuli in a stream by approaching the source of stimulation, although the copulatory activity of the conspecifics was not differentially effective in the approach process. Conspecific responsiveness in adults was also shown in the absence of unidirectional current in that the time spent locomoting by paired animals was significantly greater than by unpaired animals.

Almost all the adults laid eggs without any apparent seasonality. Of 436 egg masses which were monitored, only 49 were not viable to the stage of rotatory movement. Viable egg masses were laid by animals before and after non-viable masses with no apparent orderliness. A potentially important parameter of egg-laying was found to be the inter-spawn interval, which was three days. This interval was characteristic of sea hares maintained individually for up to three weeks. It decreased in a significant number of animals following a 24-hour pairing.

Small sea hares (< 40 grams) rarely laid eggs Intermediate-sized animals (40-85 grams) had a longer latency to the first spawning following collection, but they also showed a three-day interval between subsequent spawnings.

Reconstruction and Suggestions for Further Research

The studies of the juveniles suggested a certain amount of growth is requisite for copulation. When copulation first occurs, the individuals assume a predominant role of sperm donor or recipient. In the case of the only copulating "juvenile" pair, the sperm donor role was assumed by the smaller animal. Following copulation the recipient showed a spurt in growth which was not shown in the other pairs. Perhaps the role assumed affected the subsequent growth rate. Differences in body weight or related factors may dispose animals to assume one or the other role predominantly in early stages of development. Following the initial experience, both roles become more probable.

Clearly, further research with the juvenile stages is necessary, and any statements at this point are tentative. It would be preferable to use laboratory-raised animals with known developmental histories. The relatively rare field-collected juveniles are a feasible alternative.

As mentioned above, the behavior of individual adults could not be predicted from their social behavior immediately prior to pairing with another animal. Further consideration of their behavior clearly indicated, however, that the contact and copulatory roles depended on the previous social experience of <u>both</u> members of a pair. Differences between initially-paired and initially-alone animals were significant in relation to activity and contact initiation, but not for copulatory role. Experienced animals locomoted more with initially-unpaired animals, but not more than other experienced animals when paired with them. These changes are reminiscient of those found by Preston and Lee (1973) for feeding behavior. They found that pre-stimulated animals locomoted more in response to food stimulation than other animals which were not stimulated prior to the test.

The outcome of any encounter between two animals may or may not be copulation for reasons that are not completely understood. Since the contact initiator most often became the sperm donor, it seems likely that certain factors prior to the contact affected whether or not they copulated. The preliminary social experience may have altered the "state" of the animal so that the contact initiator was at a different level of receptivity to conspecific stimulation. At the same time, the locomotion of socially-inexperienced animals was differentially influenced by the level of social experience of their partners. It is possible, therefore, that the contact, no-contact, or copulation animals were different in the kind of stimulation which they provided.

The implications of the above discussion for the inter-relationship of copulation and locomotion are interesting. This study found that Aplysia can aggregate in response to each other. Thus, aggregations can be based both on food and conspecific stimuli as well as other factors, such as current. The data discussed above suggest that if an individual sea hare, which has been alone, approaches or comes within the stimulus-effective distance of a group of socially-active conspecifics, its locomotion will become reduced or inhibited. Animals which have just copulated may then approach the less active animal and most probably copulation will take place. As the more active animal is likely to be the contact initiator and sperm donor, the likelihood is that later the recipient will locomote more and become the sperm donor.

The effect of being both a sperm donor and a recipient may have further implications as seen in the egg-laying studies. Copulation can have a direct effect on egg-laying as it reduces the inter-spawn interval. Analysis of the effects of copulation is complicated by the sperm storage properties of _Aplysia_, which has not been studied. It is not known whether the sperm which were used in fertilizing a particular egg mass were stored or recently transmitted. Recently-intromitted sperm may be at a physiological advantage in comparison to previously stored sperm. Parker (1970) has reviewed aspects of this issue in insects. He pointed to the significance of direct sperm competition as a significant phenomenon in selection processes.

A further implication for the relationship between social experience and reproductive behavior is based on a reconstruction of several scattered bits of information available in the literature. A cohesive pattern emerges when these are combined with some of the present findings within a developmental framework.

In different laboratories, it has been reported that _Aplysia_ do not copulate if they are below a certain size (Newby, 1972; Smith and Carefoot, 1967). The observations in this thesis support those findings. Morphological evidence for a relationship between growth and egg-laying has been reported by Frazier, et al. (1967).

In the egg-laying studies of this thesis, size was found to be related to egg-laying in two ways: very small animal ($<$ 40 grams) did not lay eggs even if they had some paired experience; and intermediate-sized animals (40-85 grams) had a longer latency to first spawning. It seems as if smaller animals which have had less contact and have not copulated as frequently do not lay eggs as often as larger animals which presumably have had more social experience. In early developmental stages, social stimulation may provide some of the necessary experience for egg-laying to occur.

Individual egg-laying patterns in the laboratory do not necessarily reflect the typical pattern in the field, where the interval between egg-laying may be greater or less than the three-day interval found in this study. A primary difference between the two conditions is the absence of social stimulation in the laboratory, which also provides more homogeneous stimulation. Other differences between the field and laboratory could be a greater variety of algae constituting the diet in the field, or the stimulation from changing wave or tidal activity. In the field, there may not be any regular pattern of egg-laying. Instead, the laboratory conditions may have produced a situation in which an underlying process emerged which was related primarily to *A. dactylomela*'s neuroendocrine functions, which at some level relates to a three-day interval.

The data and findings of this thesis indicate further research in both field and laboratory on the relation between social experience and the development of reproductive processes in *Aplysia*. The frequency of contact and copulation in relation to the occurrence of egg masses and size of individuals could be studied in the field as well as in the laboratory, where sperm exchange in relation to patterns of contact and copulation can be studied. Sex role reversal studies are indicated as well. Field data based on continuous observations of tagged animals can be complemented by laboratory studies where the pattern of role options is manipulated. Comparative studies of *Aplysia* and other groups of simultaneous hermaphrodites are necessary to determine the generality of the forms of behavioral adaptation proposed in this investigation.

APPENDIX

TABLE A

Qualitative Description of Copulation in *A. dactylomela*

The purpose of this section is to add and modify the description of copulation published by Eales in 1921 so that further quantitative studies of the copulatory pattern can be made to analyze the relationship among different aspects of the behavior. This description is based on repeated observations of 43 pairs in Puerto Rico (30-minute observations) and 21 other pairings during preliminary studies at the Lerner Marine Laboratory in Bimini, Bahamas (60-minute observations). During these pairings numerous opportunities occurred where *A. dactylomela* pairs contacted and copulated, or where ongoing copulation ended and the partners separated.

Most frequently, interactions leading to copulation began with with one individual approaching the second. Copulation also occurred, but less frequently, when both individuals approached one another. In this second case, an interaction followed which was either a "passing" contact or a "mutual" contact. In a "passing" contact, neither individual changed its orientation or slowed its rate of movement. When locomotion slowed or came to a halt a reciprocal pattern of contacts ensued; the pair was considered to be in the "mutual" or "reciprocal" phase of the interaction.

This interactive phase continued for a variable amount of time, ranging from a few seconds to about 30-45 minutes. During this phase a frequent and apparently significant pattern consisted of oral-mantle "brushing"; that is, the mouth and buccal tentacles would move anteriorly, then caudally, to the siphon, along the edge of the parapodia and mantle surface. The oral region would contact the lateral edge formed by the inside of the parapodium and mantle skin and "brush" its oral tentacles along its partner's mantle perimeter. When the head behaved this way relative to the edge formed by the right parapodium (i.e., on the side where the genital opening is located) the pattern was considered to have moved into the next qualitative phase--the "typical copulatory posture", or tcp.

This phase is operationally useful because the parapodia obstruct observation of the genital opening so it is usually difficult to tell whether intromission has actually occurred by observation alone. It was usually, but not always, predictable that a pair which entered the tcp phase would copulate, and that the animal which placed its head between the other's parapodia would be the sperm donor. If the sea hare's parapodia were open and if locomotion slowed or stopped, it was more likely to assume the recipient role.

During these pre-copulatory phases, the muscular sheath surrounding the penis could be seen everted. Several times, the penis sheath itself was observed to perform a series of discrete contacts of the mantle area and body wall in the area of the genital opening.

The occurrence of a typical copulatory posture was not followed invariably by copulation, although this was typical. Certain pairs would enter tcp phase, separate or resume mutual contact patterns after which the same or different roles might be assumed by the partners. This type of behavior seemed to occur more often where mutual contact followed mutual approach.

When only one individual (contact initiator) approached its partner, the contact which followed was to any part of the body with the anterior part of the locomoting individual. At this point one of two things could happen. The animal which was inactive could remain inactive, or it could become increasingly active as the unidirectional contact continued.

For tcp to occur, the parapodia of the non-initiator must be relaxed and at least slightly separated. If these conditions are met, the contact initiator will most probably become the sperm donor. If the non-initiator becomes active, either partner could engage the other in a tcp. Although in general the contact initiator will become the sperm donor, this is not predictable on the basis of which animal approached the other.

Once intromission occurs, copulation could last anywhere from four minutes to six or more hours. The pattern, duration, and nature of sperm transfer is not precisely known in *Aplysia*.

Copulations of shorter duration seemed to correlate with the start of locomotion activity by the sperm recipient, while in those of longer duration the recipient tends to remain inactive.

The break of a copulation of short duration was followed by role reversal while those of longer duration seemed to be followed by a copulation in which the same roles were assumed.

During copulation, regular pulsations of a large part of the neck regions as well as bobs of the head were frequently noticed in the sperm donor; at the same time, changes in the anterior margins of the parapodia of the recipient could be observed. These parapodial changes were also seen in non-copulating individuals which were not locomoting. They are distinct from the relatively intense and rapid closure of the parapodia associated with the expulsion of water from the siphon. The difference may be described as lower in amplitude and of greater frequency.

It has commonly been assumed that *Aplysia* do not copulate reciprocally. Under the conditions of this study, reciprocal intromission was observed and confirmed six separate times in six different pairs. It is not clear why reciprocal copulation is not normally observed in larger groups or in the field; its occurrence in this study may be the result of keeping the same pair together in a confined volume for a 24-hour observation period, following a separation from other sea hares. Frequently, reciprocal intromission seemed to be a short transitional phase when a pair reversed roles but did not separate during the reversal.

Two different sperm recipients were observed to lay eggs at the same time that the respective partners were intromitting their penises. On one occasion in a grouped situation, two sperm donors were literally pulled out of a single sperm recipient.

TABLE B

Latency to First Contact in Pairs of *Aplysia dactylomela* During Preliminary Pairing[1]

Interval of Observation In Seconds	Number of Pairs Which First Made Contact During Interval: Pairs Which Copulated	Pairs Which Made Contact
0-100	1	5
101-200	2	1
201-300	1	1
301-400	0	0
401-500	0	2
501-600	0	0
601-700	0	0
701-800	1	0
801-900	0	0
901-1000	0	0
1001-1100	0	0
1101-1200	0	0
1201-1300	1	0
1301-1400	1	1
1401-1500	0	0
1501-1600	0	1
1601-1700	0	0
1701-1800	0	0

[1]Mann-Whitney "U" Test: U = 28.

TABLE C

Total Duration of Contact in Pairs of *Aplysia dactylomela* During Preliminary Observation[1]

	Pairs Which Copulated	Pairs Which Contacted
Median (sec)	256	80
Range (sec)	79-1335	6-1584
Number of Pairs	7	11

[1]Mann-Whitney "U" Test: U = 31, not significant.

TABLE D

Latency (sec) to Copulation in *Aplysia dactylomela* During the Preliminary Observation[1]

Pair	Latency to First Contact	Latency to Copulation
1	79	724
2	143	1332
3	188	1702
4	256	672
5	747	924
6	892	1695
7	1335	1568

[1]Spearman-Rho = -0.32.

TABLE E

Latency to First Contact in *Aplysia dactylomela* During and After

The First Experimental Observation[1]

	Treatment Category								
	A			B			C		
During Observation	AA	ANC	NCNC	AC	ACp	NCC	CC	CCp	CpCp
Number of Pairs	4	4	1	2	3	6	0	1	1
Median (min)	9	12.5	--	5.5	3	3	--	--	--
Range (min)	1-13	4-17	22	4-7	2-4	1-8	--	12	2
After Observation									
Number of Pairs	2	3	1	3	2	3	2	3	1
Median (min)	60.5	82	--	83	288.5	66	157.5	101	--
Range (min)	56-65	32-395	47	36-335	31-546	64-363	38-277	79-122	38
Total Number of Pairs	6	7[2]	2	5	5	9	2	4	2

[1] Kruskal-Wallis One-Way Analysis of Variance: $H = 7.52$, $df = 8$, p not significant.

[2] No data available for one pair which did not contact within 30 minutes.

TABLE F

Latency to Copulation in *Aplysia dactylomela* During and After

The First Experimental Observation[1]

	A			B			C		
During Observation	AA	ANC	NCNC	AC	ACp	NCC	CC	CCp	CpCp
Number of Pairs	2	2	1	1	2	5	--	--	1
Median (min)	15	16.5	--	--	15.5	18	--	--	--
Range (min)	14-16	11-22	26	12	6-25	8-25	--	--	6
After Observation									
Number of Pairs	3	4	1	1	1	4	1	4	1
Median (min)	80	106	--	--	--	99	--	112	--
Total Number Of Pairs	5[2]	6[3]	2	2[4]	3[3]	9	1[2]	4	2

[1] Kruskal-Wallis One-Way Analysis of Variance: H = 2.69, df = 8.
[2] One Pair
[3] Two Pairs   No Spotcheck Data Following Observation
[4] Three Pairs

TABLE G

Behavior Following First Contact in Pairs of *Aplysia dactylomela* During First Experimental Observation

	Number That Remained In Contact	Number That Did Not Remain In Contact
Treatment A: Number of Pairs:		
Which Made Contact	4	4
Which Copulated	6	4
Treatment B: Number of Pairs:		
Which Made Contact	2	4
Which Copulated	12	4
Treatment C: Number of Pairs:		
Which Made Contact	2	0
Which Copulated	2	0
Total Contact	8	8
Total Copulated	20	8

TABLE H

Changes in the Locomotion of Individual *Aplysia dactylomela*

During First Experimental Observation

Animals Initially Alone		Number Increased	Number Decreased	Number No Change
Paired With:				
	A	5	6	1
	NC	2	5	1
	C	3	1	1
	Cp	0	4	1
Animals Initially No Contact				
Paired With:				
	NC	2	2	0
	A	6	2	0
	C	6	3	0
Animals Initially In Contact				
Paired With:				
	C	2	3	0
	A	4	0	0
	NC	1	8	0
	Cp	2	1	1
Animals Initially Copulated				
Paired With:				
	Cp	1	3	0
	A	3	2	1
	C	2	2	0

A = Alone; NC = paired, no contact; C = paired, made contact; Cp = paired, copulated.

TABLE I

Amount of Locomotion for 42 *Aplysia dactylomela*

During Four Observations

Successive Observations	Number of Sea Hares:		
1 : 2	1 > 2	1 < 2	1 = 2
	18	22	3
2 : 3	2 > 3	2 < 3	2 = 3
	24	17	1
3 : 4	3 > 4	3 < 4	3 = 4
	15	24	3

No differences were statistically significant.

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