

## SIZE-DEPENDENT NEURAL INTEGRATION BETWEEN GENETICALLY DIFFERENT COLONIES OF A MARINE BRYOZOAN

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### Summary

Although the formation of genetic chimeras is rare in the animal kingdom, it has long been known that colonial marine invertebrates fuse under natural conditions, forming genetic chimeras. I report here an example of selective, partial fusion. When small colonies of the encrusting marine bryozoan *Membranipora membranacea* grow into contact, they usually become behaviorally coordinated: if one colony is disturbed, both colonies will simultaneously retract their feeding structures (lophophores). As a first step towards understanding the fitness consequences of this type of apparent fusion, I examine its mechanistic basis. Using assays of zooid behavior, electrophysiological recordings and  $^{14}\text{C}$ -labeled metabolites, I demonstrate that physiological integration between *M. membranacea* colonies is both partial and

temporary. Specifically, this study demonstrates (1) that behavioral coordination is the result of neural integration between colonies, (2) that coordinated colony pairs do not exchange metabolites, and (3) that neural integration is a temporary phenomenon that is terminated as colonies grow larger. Additionally, I show that only those zooids at the middle of the intercolony border mediate neural integration. Partial physiological integration between *M. membranacea* colonies could be a highly specific interaction that increases the potential benefits of fusion (i.e. neural integration) while minimizing the potential costs (i.e. resource parasitism).

Key words: Bryozoa, *Membranipora membranacea*, neural integration, chimeras, behavioral coordination, coloniality, fusion, allorecognition.

### Introduction

Although fusion between genotypically different colonies has been documented in all major groups of marine invertebrates with colonial representatives (reviewed by Grosberg, 1988), very little is known about the fitness consequences of fusion between genotypes. Proposed benefits to fusion include an increase in colony size that could reduce the probability of colony mortality and accelerate the age of first reproduction (Buss, 1982). Conversely, costs of fusion could include the possibility of resource parasitism (Buss, 1982; Rinkevich and Loya, 1983b). In order to evaluate the potential for such costs and benefits, it is important that we understand the extent to which fusing colonies become physiologically integrated. As a first step towards understanding the fitness consequences of fusion, I test for physiological integration between apparently fused colonies of the encrusting marine bryozoan *Membranipora membranacea*.

There are three detectable ways in which modules *within* colonies can be physiologically integrated: (1) neurally (Horridge, 1957; Thorpe *et al.* 1975; Thorpe, 1982; Mackie, 1986); (2) metabolically, the ability to transport metabolites within a colony (Best and Thorpe, 1985; Miles *et al.* 1995); and, (3) structurally, the sharing of a common skeleton. It is also very likely that physiological integration involves various

forms of chemical communication in the form of hormones, growth regulators, etc. (Mackie, 1986); for example, chemical modulation may control the synchronous budding and onset of sexual reproduction seen in some ascidian colonies (Milkman, 1967). Presumably, these are all potential pathways by which physiological integration can occur following fusion between colonies.

Nevertheless, direct assessments of physiological integration between colonies are rare. Fusion is usually characterized only by the morphology of the intercolony border (e.g. coalescence of soft tissues). For some groups of colonial marine invertebrates, it is probably safe to assume that the morphological characteristics of fusion are indicative of physiological integration between colonies. For example, following fusion in compound ascidians, colonies share a common blood-vascular system and cells are freely exchanged between colonies (Oka and Watanabe, 1960; Katow and Watanabe, 1980; Taneda *et al.* 1985). For other groups, however, this relationship is tenuous. Rinkevich and Loya (1983a) used scanning electron microscopy to examine the borders between genotypically different colonies of the coral *Stylophora pistillata* that 'appeared' to have fused. Upon close examination, they found no evidence of physiological

connections between the colonies; tissues were actually separated by a narrow gap of less than 20–30  $\mu\text{m}$ .

The relationship between morphological fusion and physiological integration is particularly obscure in the Bryozoa. The highly compartmentalized nature of the zooids within a bryozoan colony makes identification of intercolony tissue coalescence difficult. Furthermore, the food translocation system (the funiculus) is not readily visible for observations of metabolite translocation between colonies. In the documented cases of fusion between bryozoans, the spatial alignment of zooidal compartments and subsequent formation of a common growing edge has been used to infer colony 'fusion' (Moyano, 1967; Stebbing, 1973; Humphries, 1979; Nielsen, 1981; Chaney, 1983; Gappa, 1989; Shapiro, 1992; Craig, 1994). In some cases, the presence of intercolony pore plates, circular groupings of pores located in the intercolony border, have then been used to infer physiological integration between colonies (Stebbing, 1973; Humphries, 1979; Chaney, 1983; Shapiro, 1992; Craig, 1994). It is through pore plates that the funicular system, long strands of mesenchymal cells that transport metabolites, metabolically link adjacent zooids within a colony (Thorpe *et al.* 1975; Bobin, 1977; Miles *et al.* 1995). Furthermore, the nervous system of each zooid within the colony is linked to the nervous systems of adjacent zooids through the pores in the pore plates (Lutaud, 1977, 1979).

Recent behavioral observations of the encrusting marine bryozoan *Membranipora membranacea* have cast doubt upon this inferred relationship between physiological integration and intercolony border morphology (i.e. alignment of zooids, formation of a common growing edge and the presence of pore plates). Following a disturbance, all the zooids within a bryozoan colony typically retract their feeding structures (lophophores) nearly simultaneously; this coordinated response is facilitated by a colonial nervous system (Thorpe *et al.* 1975; Lutaud, 1977, 1979). Shapiro (1992) found that following a disturbance to one colony of small juxtaposed pairs, *both* colonies retracted their lophophores simultaneously; behavioral coordination was not observed between large colony pairs. This behavioral coordination suggests that the colonies are neurally fused. However, not only did Shapiro (1992) find behavioral coordination between colony pairs that failed to align zooids and form a common growing edge, but he also found pore plates between colony pairs that lacked any evidence of behavioral coordination. These observations indicate that the presence or absence of morphological characteristics associated with fusion is not necessarily sufficient evidence for the presence or absence of physiological integration.

In order to gain a better understanding of the possible costs and benefits of fusion between colonies of *M. membranacea*, and of marine invertebrates in general, it is important to determine the extent of physiological integration between colonies. Interactions between colony pairs that share a nervous system but do not share metabolites are likely to be very different from interactions between colony pairs that do not share a nervous system but do share metabolites.

Furthermore, both of these interactions will probably differ from ones in which colonies share both a nervous system and metabolites. Additionally, it is important to assess whether fusion is temporary or permanent; fusion followed by disconnection will have different consequences from permanent fusion. With these possibilities in mind, the present study was designed to answer three questions. (1) Is behavioral coordination between bryozoan colonies the result of intercolony neural fusion? (2) Is behavioral coordination permanent or temporary? (3) Do behaviorally coordinated colonies also exchange metabolites?

## Materials and methods

### *Animal collection and identification of coordinated colony pairs*

This research was conducted at the Friday Harbor Laboratories (FHL) on San Juan Island, Washington, USA. Colonies of *Membranipora membranacea* L. were collected offshore from Turn Island by selecting bryozoan-encrusted blades of kelp in the genus *Laminaria*. The kelp blades were placed in seawater-filled vessels and transported by boat to FHL where they were immediately hung from the floating dock. Collection and transportation did not appear to stress the colonies as they could be observed feeding immediately after being hung from the dock. Colonies were removed as needed by cutting off pieces of kelp with attached colonies.

Two types of colony pairs were used in the study: genotypically different and genotypically identical colony pairs. To ensure that pairs consisted of genotypically different colonies, I only used pairs for which I could identify the ancestrula of each colony; the ancestrula is a distinct pair of zooids that develops directly from the sexually produced larva after settlement and metamorphosis. Genotypically identical colony pairs were created by scraping a 3 mm wide strip along the middle of a colony, thus separating it into two semi-circular sub-clones of equal size. The sub-clones were then allowed to grow until they had re-established contact and formed a common growing edge. This generally took about 2 weeks.

Either an electrical or a mechanical stimulus was used to test for behavioral coordination between colonies. For electrical stimuli, single square pulses of 5–10 V were applied for 5–10 ms through an electrode placed on the surface of one colony of each pair. All stimuli were at or just above the threshold required to cause all zooids in the stimulated colony to retract their lophophores. Mechanical stimuli were applied by using a blunt dissecting probe to gently depress the uncalcified growing edge of the colony at the point furthest from the neighboring colony. A pair was considered to be behaviorally coordinated if all the zooids in the non-stimulated colony also retracted their lophophores.

### *Electrophysiological recording*

Using electrical stimuli, I tested genotypically different pairs of approximately equal-sized (approximately 1–2 cm<sup>2</sup>) colonies until I had obtained five coordinated and five non-

coordinated colony pairs. For each pair, extracellular electrophysiological recordings were made simultaneously from each colony by attaching fine polyethylene suction electrodes (50–75  $\mu\text{m}$  i.d.) to the frontal membrane of a single zooid along the periphery of each colony. Signals were captured within the 1 Hz to 1 kHz waveband, amplified and displayed on a digital oscilloscope and recorded using a chart recorder. The preparation was shielded to reduce electrical interference. For one of the five coordinated colony pairs, conduction velocities of electrical signals traveling within and between colonies were determined. The positions of both the stimulating and recording electrode were changed for each measurement, with the two electrodes being either in the same colony ( $N=5$ ) or in different colonies ( $N=5$ ).

Additionally, I identified one colony triplet in which all three colonies were behaviorally coordinated (a stimulus applied to any one of the colonies resulted in all three colonies retracting their lophophores). The colonies were arranged in a straight line, and electrophysiological recordings were made from the two end colonies.

#### *Permanence of behavioral coordination*

To determine whether behavioral coordination is permanent once established, 80 colony pairs transplanted from their algal substratum onto acrylic panels were monitored for 6 weeks. To establish the transplants, blades of the red alga *Iridea* with newly settled *M. membranacea* colonies were collected from Turn Island and Reuban Tart State Park, San Juan Island. Small (<10 mm<sup>2</sup>), individual colonies were removed from the *Iridea* blades by stretching the algal blade until the colony detached. The colonies were placed in pairs on acrylic panels and kept in an aquarium with running sea water. After the colonies had attached (24 h), the acrylic panels were hung from the FHL floating dock. Starting at the beginning of the second week, the acrylic panels were brought into the laboratory once a week. In the laboratory, behavioral coordination of colony pairs was tested using mechanical stimuli. Colonies were also videotaped so that colony size could later be determined using video-integrated image analysis. Colony pairs were never in the laboratory for more than 2 h, and during this time they were kept in an aquarium supplied with running sea water. For each colony pair, the size of the larger colony when coordination terminated was estimated by taking the mean of the colony size measured at the time when the colony was (1) last coordinated and (2) first non-coordinated; this represents an approximate estimate since coordination could have stopped at any time during the intervening week.

#### *Identification of zooids mediating behavioral coordination*

Two types of cuts were made with a scalpel blade along intercolony borders to determine which zooids mediate behavioral coordination. The first type of cut was started at the *middle* of the intercolony border, herein defined as the portion of the intercolony border intersected by a line drawn between the ancestrulae (the founding zooids of each colony). After making an initial cut of approximately 2–4 mm long

(approximately the width of 3–6 zooids), an electrical stimulus was applied to one colony. If the colonies were still coordinated, the initial cut was increased in approximately 1–2 mm increments towards each edge of the intercolony border (2–4 mm total). The colony was then tested again for behavioral coordination. I repeated this procedure until the colonies were no longer coordinated; the total length of the cut was then measured. The second type of cut followed the same procedure except that cuts were started at the *edges* of the intercolony border (see Fig. 2). All colony pairs used in this experiment ( $N=28$ , approximately 1–2 cm<sup>2</sup> each) were completely surrounded by other colonies and so were no longer growing; this ensured that all zooids along the intercolony border were mature and had been in contact with the other colony long enough for behavioral coordination to be established (Shapiro, 1992).

#### *Metabolite translocation experiments*

##### *Intracolony translocation experiment*

To determine whether translocation of metabolites could be detected within a colony, I fed solitary colonies algal cells that had been labeled with <sup>14</sup>C (following the methods of Miles *et al.* 1995). I used a microinjection syringe to introduce the labeled algal cells (*Dunaliella* sp.) into the top of a 3.5 mm high feeding containment ring (diameter 3 mm) placed on the surface of the colony. Immediately after the feeding, each colony was rinsed with sea water, placed in an aerated aquarium and allowed to translocate the metabolites for either 0 ( $N=6$ ) or 24 h ( $N=6$ ).

To determine whether placing a cut through the colony would stop translocation, additional solitary colonies ( $N=3$ ) were selected. Using a razor blade, a cut was placed through the colony, isolating approximately a quarter of the zooids from the rest of the colony (see Fig. 4C). During feeding, the containment ring was placed within a few millimeters of the cut, and colonies were allowed to translocate metabolites for 24 h. Movement of metabolites within the colony was visualized using autoradiography following the methods of Miles *et al.* (1995).

##### *Intercolony translocation experiment no. 1*

To determine whether coordinated colony pairs exchange metabolites, four types of colony pairs composed of approximately equal-sized colonies were used (electrical stimuli were used to assay behavioral coordination): (1) genotypically different, coordinated colony pairs ( $N=9$ ); (2) genotypically different, non-coordinated colony pairs ( $N=12$ ); (3) genotypically identical, coordinated colony pairs ( $N=13$ ); and (4) genotypically identical, coordinated colony pairs with a cut placed along the colony border ( $N=9$ ). This final treatment served to control for any metabolites that could have entered the unfed colony as a result either of leakage of algae from the containment ring during feeding or from secondary uptake of labeled metabolites excreted by the fed colony during the translocation period. The experiment was run in three blocks. Each block had an approximately equal

number of colonies from each of the four pair types (2–5 colonies per pair type per block). For all colony pairs, the feeding containment ring was placed entirely within one colony within a few millimeters of the intercolony border. After feeding, all colonies were washed and allowed to translocate metabolites for 24 h.

In addition to autoradiography, a scintillation counter was used to quantify levels of radioactivity within the fed and unfed colony of each pair. After the autoradiography, individual colonies were scraped from the algal blade and placed in a scintillation vial. To determine background levels of radiation, scintillation counts were also determined for each colony of five colony pairs in which neither colony was fed. On the basis of an external chemical-standards ratio quench curve, all counts  $\text{min}^{-1}$  recorded from the scintillation counter were converted to disintegrations  $\text{min}^{-1}$ . For a more detailed description of the methods, see Miles *et al.* (1995).

The middle colony of a triplet was also fed labeled algae. This colony was coordinated with one neighboring colony, but not coordinated with the other neighboring colony. Following the feeding, the colony triplet was treated identically to the other colonies used in the experiment.

#### Intercolony translocation experiment no. 2

This experiment was designed to address two possible problems associated with the first intercolony translocation experiment. First, if significant amounts of  $^{14}\text{C}$  were lost during the autoradiography procedure, subsequent scintillation counts could be inaccurate. To determine the amount of  $^{14}\text{C}$  lost during the autoradiography procedure, approximately half of the colonies ( $N=11$ ) in each of the four treatments (same as in intercolony translocation experiment no. 1) were placed directly into the scintillation vials after the translocation

period. The remaining colonies ( $N=10$ ) were dried and used for autoradiography before being placed in scintillation vials.

A second potential problem is that translocation patterns between colonies in which only about 30 zooids within a colony are fed (as in intercolony translocation experiment no. 1) could differ from translocation patterns between colonies in which all the zooids within a colony are fed. Thus, instead of using the containment ring, I placed a  $2\text{ cm} \times 2\text{ cm}$  piece of acrylic panel over one colony of each pair to prevent the zooids in the unfed colony from extending their lophophores and feeding. The labeled *Dunaliella* culture ( $50\ \mu\text{l}$ ) was then added using a microinjection syringe over the uncovered colony. During feeding, the position of the syringe was moved over the surface of the colony so that algae were released uniformly over all zooids in the colony.

## Results

### Electrophysiological recording

From the peripheral zooids of the colonies, I detected pulses that were conducted throughout the colony. Pulses occurred spontaneously (i.e. no obvious zooidal behavior was correlated to the observed electrical signals) at a frequency of approximately  $1\ \text{pulse s}^{-1}$ . These pulses were typically about  $10\ \mu\text{V}$  in amplitude with a duration of about 3 ms. Lophophore retraction in colonies disturbed electrically or mechanically was accompanied by a rapid burst of pulses ( $>50\ \text{pulses s}^{-1}$ ) lasting for a few seconds.

For all non-coordinated colony pairs ( $N=5$ ), electrophysiological recordings made simultaneously from each colony demonstrated that electrical signals were not conducted between colonies; both colonial nervous systems displayed independent activity (Fig. 1A). In contrast, recordings from

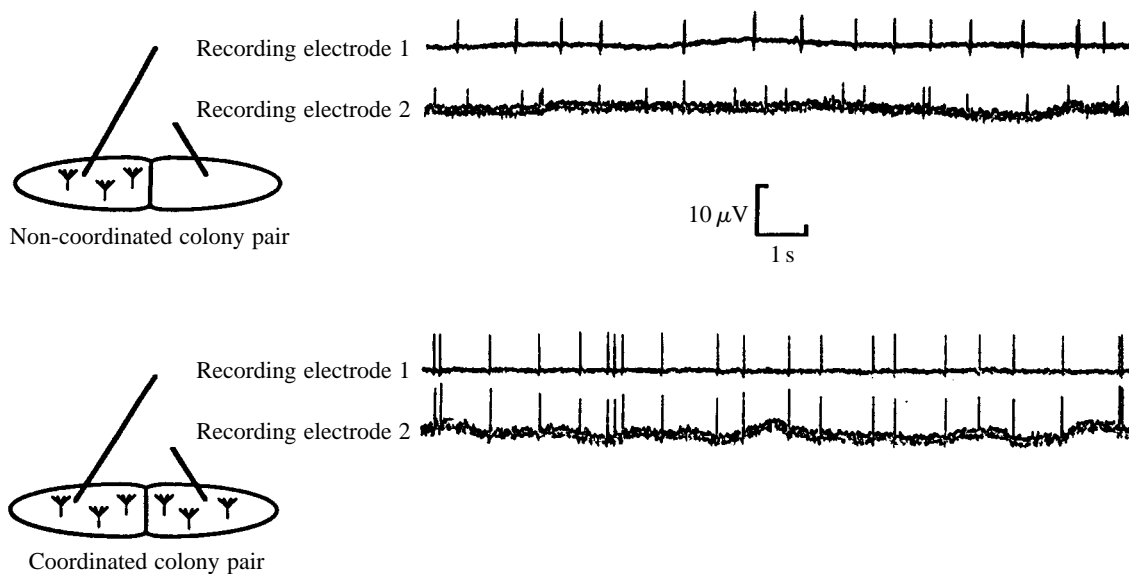


Fig. 1. Electrophysiological recordings from non-coordinated and coordinated colony pairs. (A) Recordings of spontaneous activity of the colonial nervous system made simultaneously from each colony of a non-coordinated pair. (B) Recordings of spontaneous activity of the colonial nervous system made simultaneously from each colony of a coordinated pair.

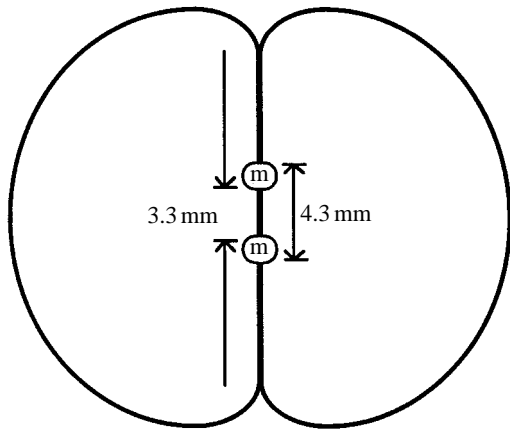


Fig. 2. Diagrammatic summary of results from experiments in which cuts were placed across the intercolony border. For cuts started from the edges of the intercolony border (shown on the left), intercolony coordination stopped after all but 3.3 mm of the intercolony border had been cut. For cuts started in the middle of the intercolony border (shown on the right), intercolony coordination stopped after 4.3 mm of the intercolony border had been cut. These results indicate that only those zooids flanking the zooids located at the very center of the intercolony border mediate neural integration (the locations of these zooids are indicated by 'm').

coordinated colony pairs ( $N=5$ ) indicated that electrical signals were conducted across the intercolony border (Fig. 1B).

All measured inter- and intracolony conduction velocities were between 59 and 81  $\text{cm s}^{-1}$ . However, intracolony conduction velocities ( $70.6 \pm 2.8 \text{ cm s}^{-1}$ , S.E.M.) were significantly faster than intercolony conduction velocities ( $61.4 \pm 1.5 \text{ cm s}^{-1}$ ; two-tailed  $t$ -test:  $t=2.9$ ,  $P<0.02$ , d.f.=8).

In the one colony triplet tested, simultaneous recordings made from the two end colonies indicated that signals were being conducted from one colony, through the middle colony and into the third colony at the other end.

#### *Permanence of behavioral coordination*

Of the 80 colony pairs transplanted onto the acrylic panels, 69 pairs became established (in the other pairs, one or both colonies fell off or suffered significant damage from either abrasion or predation). Of the established colonies, 61 (88.4%) became behaviorally coordinated. At the end of the 6 week sampling period, 49 (80.3%) of these coordinated colonies were no longer coordinated; of these colonies, only those that had suffered no damage ( $N=37$ ) during the study period were used to determine colony size at the time that coordination was terminated. The mean area ( $\pm 1$  S.E.M.) of the larger colony of each pair was  $3.32 \pm 0.32 \text{ cm}^2$  (range 0.713–8.469  $\text{cm}^2$ ;  $N=37$ ) when coordination was terminated. The mean area of the larger colony of the 12 pairs that were still coordinated at the end of the 6 week period was  $1.06 \pm 0.212 \text{ cm}^2$  (range 0.248–2.845  $\text{cm}^2$ ) (two-tailed  $t$ -test,  $t=3.65$ ,  $P<0.001$ , d.f.=47).

#### *Identification of zooids mediating behavioral coordination*

When cuts were started at the *middle* of the intercolony

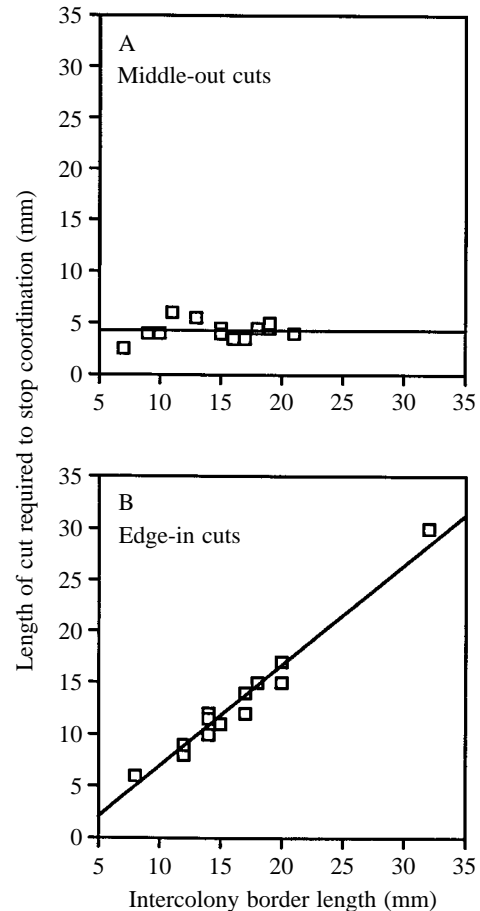


Fig. 3. Length of cuts along intercolony border required to stop intercolony behavioral coordination of zooids as a function of intercolony border length. (A) Intercolony border length and the length of cut required to stop intercolony coordination for cuts started at the middle of the intercolony border. The slope of the regression line was not significantly different from zero (simple linear regression,  $F_{1,11}=0.385$ ,  $P=0.56$ ,  $r^2=0.03$ ). (B) Intercolony border length and the length of cut required to stop intercolony coordination for cuts started at the edges of the intercolony border (see Fig. 2). The slope of the regression line was significantly different from zero (simple linear regression,  $F_{1,13}=387.081$ ,  $P<0.0001$ ,  $r^2=0.97$ ).

border, the average cut length ( $\pm$  S.E.M.) required to stop behavioral coordination was  $4.3 \pm 0.251 \text{ mm}$  (mean border length was  $14.6 \pm 1.2 \text{ mm}$ ,  $N=13$ ). In contrast, when cuts were started at the *edge* of the intercolony border, the mean cut length required to stop behavioral coordination was  $13.0 \pm 1.4 \text{ mm}$  (mean total border length  $16.3 \pm 1.4 \text{ mm}$ ,  $N=15$ ). Behavioral coordination was terminated by edge cuts before the entire border was cut. For cuts started at the edge, the mean length of border left uncut at the middle was  $3.3 \pm 0.257 \text{ mm}$ ; this was significantly shorter than the length of the cut required to stop coordination when cuts were started in the middle of the intercolony border (two-tailed  $t$ -test:  $t=2.68$ ,  $P<0.05$ , d.f.=26) (Fig. 2).

When cuts were started at the *edges* of the intercolony border, the length of the cut required to stop coordination was

Fig. 4. Autoradiographs from intracolony metabolite translocation experiments.

Diagrammatic representations of colonies are shown below the autoradiographs; the black circles in the diagrams represent the sites at which colonies were fed and the outer ring represents the colony border. Light areas of autoradiographs correspond to the presence of  $^{14}\text{C}$ -labeled metabolites. (A) Solitary colony that was killed immediately after feeding (translocation time 0h). The small light spot to the left of the feeding site is  $^{14}\text{C}$  that leaked from the containment ring during feeding. (B) Solitary colony that was allowed to translocate metabolites for 24h after feeding. (C) Solitary colony in which a cut (indicated on the lower diagram) was placed in the colony prior to feeding (translocation time 24h). Scale bars, 5 mm.

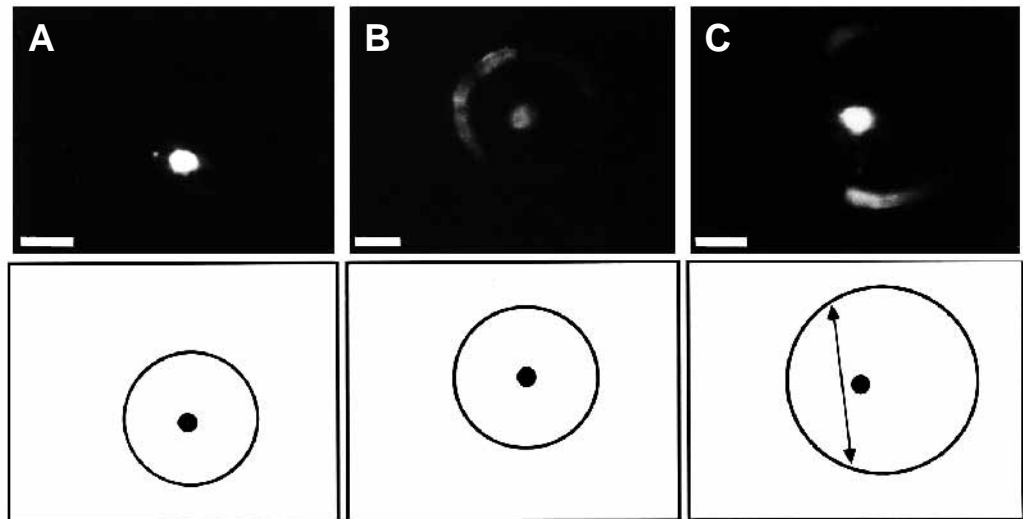
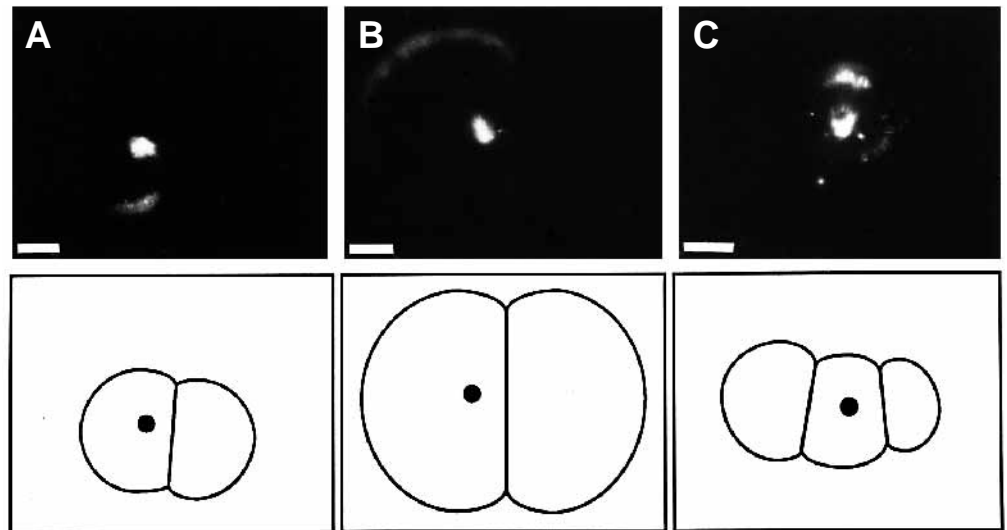


Fig. 5. Autoradiographs from intercolony translocation experiment no. 1. Diagrammatic representations of colonies are shown below the autoradiographs; the black circles in the diagrams represent the sites at which zooids were fed and the outer rings represent the colony boundaries. Light areas of autoradiographs correspond to the presence of  $^{14}\text{C}$ -labeled metabolites. (A) Genotypically different, coordinated colony pair. (B) Genotypically different, non-coordinated colony pair. (C) Autoradiograph of a colony triplet; the middle colony was coordinated with the colony on the left and not coordinated with the colony on the right. Scale bars, 5 mm.



independent of the length of the intercolony border (Fig. 3A; simple regression of cut length *versus* border length:  $P=0.56$ ,  $r^2=0.03$ ). In contrast, there was a strong correlation between the length of the intercolony border and the length of the cut required to stop coordination (Fig. 3B; simple regression of cut length *versus* border length:  $P<0.0001$ ,  $r^2=0.97$ ). This indicates that neural integration was mediated by a fixed number of zooids located at the middle of the intercolony border.

#### Metabolite translocation experiments

##### Intracolony translocation experiment

All autoradiographs from solitary colonies that were dried immediately after feeding showed that most of the  $^{14}\text{C}$  was restricted to the feeding site within the containment ring (Fig. 4A). In contrast, metabolites were observed at the growing edge of all colonies given a 24h translocation period

(Fig. 4B). Also, cuts placed within a colony immediately before feeding prevented metabolites from being translocated to those zooids beyond the cut (Fig. 4C).

##### Intercolony translocation experiment no. 1

All of the autoradiographs from each of the four treatments showed labeled metabolites at the site of feeding and at the growing edges of the fed colony (Fig. 5A,B). For nearly all colonies in the four treatments (40 out of 43), absolutely no labeled metabolites were observed in the unfed colony. However, in two out of the 13 genotypically identical coordinated colony pairs, and in one of the nine genotypically different coordinated pairs, faint traces of  $^{14}\text{C}$  were observed in the unfed colony near the section of the intercolony border closest to the feeding site.

In the one colony triplet, labeled metabolites were found

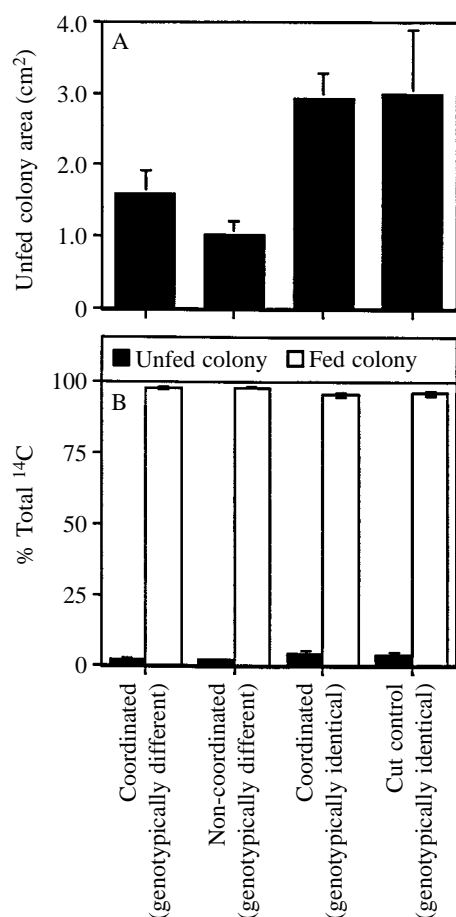


Fig. 6. Unfed colony size and percentage of total <sup>14</sup>C (disintegrations min<sup>-1</sup> fed colony + disintegrations min<sup>-1</sup> unfed colony) found in unfed and fed colonies of intercolony translocation experiment no. 1. (A) Mean area (+1 S.E.M.) of unfed colonies for each pair type. Colony area differed significantly among treatments (one-way ANOVA on ln-transformed data:  $F_{3,39}=7.15$ ,  $P<0.0001$ ). (B) Mean percentage (+1 S.E.M.) of <sup>14</sup>C found in unfed and fed colonies of each pair type. After controlling for size, there was no effect of colony pair type on the percentage of <sup>14</sup>C found in the unfed colony (Table 1).

only in the fed colony; there was no evidence of translocation to either the coordinated or the non-coordinated neighbor (Fig. 5C).

Scintillation data were analyzed using analysis of covariance (ANCOVA). The dependent variable used was the arcsine-transformed percentage of total <sup>14</sup>C (disintegrations min<sup>-1</sup> fed colony + disintegrations min<sup>-1</sup> unfed colony) detected in the unfed colony of each pair. The size of the unfed colony was used as a covariate because there were significant differences in unfed colony size among treatments (one-way analysis of variance, ANOVA, on ln-transformed area of unfed colony:  $F_{3,39}=7.15$ ,  $P<0.0001$ ; Fig. 6A). However, the mean size of unfed colonies in genotypically identical, coordinated and cut colony pairs were not significantly different ( $t$ -test,  $P>0.05$ ). Likewise, the mean size of unfed colonies in genotypically different, coordinated and non-coordinated colony pairs were not significantly different ( $t$ -test,  $P>0.05$ ). Since colony pairs were composed

Table 1. Analysis of covariance (ANCOVA) results for the effects of experimental replicate, colony size and colony pair type on the percentage of total radioisotope found by scintillation counting the unfed colony of each pair

Source	d.f.	Sums of squares	<i>F</i>	<i>P</i>
Block	2	0.0040	0.98	0.382
Colony size	1	0.0244	11.91	0.001
Colony pair type	3	0.0045	0.73	0.534
Model error	36	0.0737		

None of the interaction terms was significant.

of approximately equal-sized colonies, the same pattern was seen for the fed colonies of each pair.

The results from scintillation counting also indicated that metabolites were not being translocated between colonies (Table 1). Since none of the interaction terms was significant, they were removed from the model. There was a significant effect of colony size, independent of treatment. However, colony pair type had no significant effect on the level of <sup>14</sup>C found in the unfed colony (Table 1). Levels of <sup>14</sup>C found in the unfed colonies were uniformly low (Fig. 6B); for all treatments, the mean proportion of <sup>14</sup>C found in the unfed colony was less than 5%.

#### Intercolony translocation experiment no. 2

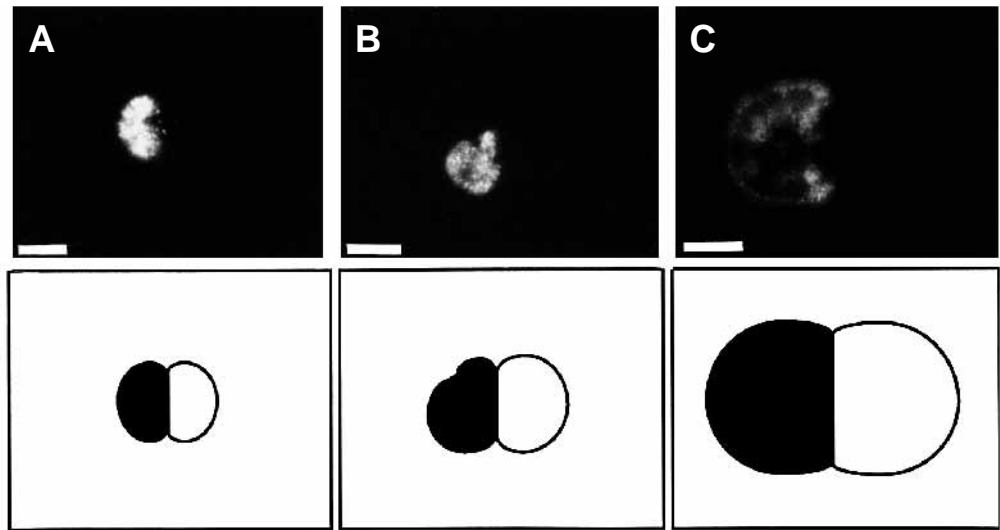
The mean total radioactivity ( $\pm 1$  S.E.M.) for colony pairs that were dried for autoradiography prior to being placed in scintillation vials ( $3892 \pm 746$  disintegrations min<sup>-1</sup>;  $N=11$ ) was not significantly different from the mean value for colonies that were placed directly in scintillation vials (mean  $4622 \pm 1140$  disintegrations min<sup>-1</sup>;  $N=10$ ; two-tailed  $t$ -test:  $t=0.545$ ,  $P=0.59$ , d.f.=19). Thus, while it appears that some of the radioisotope could have been lost during the autoradiography procedure, these losses were not significant.

Autoradiographs showed no evidence of intercolony translocation of metabolites in genotypically different coordinated ( $N=3$ ), genotypically different non-coordinated ( $N=3$ ), genotypically identical coordinated ( $N=3$ ) or genotypically identical cut colonies ( $N=2$ ). When all zooids of a colony were fed, labeled metabolites were observed over the entire area of the colony; in none of the pairs were labeled metabolites observed in the unfed colony (Fig. 7). Thus, it did not matter if all or only a few zooids within a colony were fed; in neither case were metabolites translocated to the neighboring colony. Scintillation data were not analyzed for a treatment effect because data on colony size were only available for half of the colonies.

#### Discussion

This is the first study to provide electrophysiological evidence of neural fusion between genotypically different colonies for any group of colonial marine invertebrate. Studies

Fig. 7. Autoradiographs from intercolony translocation experiment no. 2. All zooids within one colony were fed algae labeled with  $^{14}\text{C}$ . Diagrammatic representations of colonies are shown below the autoradiographs. The black areas in the diagrams represent the sites at which zooids were fed; light areas of autoradiographs correspond to the presence of  $^{14}\text{C}$ -labeled metabolites. (A) Genotypically different, coordinated colony pair. (B) Genotypically different, non-coordinated colony pair. (C) Genotypically identical, coordinated colony pair. Scale bars, 5 mm.



documenting fusion between cnidarian colonies have used only intercolony coordination of polyp retraction as evidence of neural fusion between genotypically different colonies (Potts, 1976; Hidaka, 1985; Chornesky, 1991). Although several studies have described morphological fusion (alignment of zooids and/or the presence of pore plates) between bryozoan colonies (Moyano, 1967; Stebbing, 1973; Humphries, 1979; Nielsen, 1981; Chaney, 1983; Gappa, 1989; Shapiro, 1992; Craig, 1994), only Shapiro (1992) has provided behavioral evidence of neural fusion; none provided electrophysiological evidence.

The electrical pulses I detected in the present study appear to represent activity of the colonial nervous system. These pulses were similar in amplitude (approximately  $10\ \mu\text{V}$ ) and duration (approximately 3 ms) to the Type I (T1) pulses described by Thorpe *et al.* (1975); T1 pulses are believed to be nerve potentials associated with the colonial nervous system of *Membranipora membranacea*. Additionally, as also observed by Thorpe *et al.* (1975), when I disturbed colonies electrically or mechanically, lophophore retraction was accompanied by a single larger pulse (similar to the T2 pulses described in Thorpe *et al.* 1975) followed by a rapid burst of T1 pulses ( $>50\ \text{pulses s}^{-1}$ ) lasting for a few seconds.

Although conduction velocities of electrical signals traveling within colonies were somewhat faster than conduction velocities of electrical signals traveling between colonies, there is no evidence for fundamental differences between intra- and intercolony neural connections. The conduction velocities of the intracolony pulses detected by Thorpe *et al.* (1975) ranged from 50 to  $100\ \text{cm s}^{-1}$ ; all intra- and intercolony conduction velocities measured in the current study were within this range. The observed differences in mean conduction velocity probably resulted because only those zooids at the mid-region of the intercolony border mediate neural connections. Conduction velocities were calculated by measuring the length of a straight line between the stimulating and recording electrodes; unless this line also passed through the middle of the intercolony border, the length of the neural

pathway between the two electrodes would have been underestimated. This, in turn, could have led to an underestimate of actual conduction velocities.

The results of this study also demonstrate that neural integration is a temporary phenomenon. Although 12 colonies were still coordinated at the end of the 6 week period of investigation, these colonies were significantly smaller than the mean size at which neural integration was terminated in the remaining 37 colonies for which size data were available. Thus, it is likely that these 12 colonies had not yet reached the size at which at which coordination is terminated. I propose that termination of intercolony coordination as colonies age is the result of normal deterioration of interzooidal neural connections. Although zooids are capable of regeneration, senescence of zooids still occurs in older parts of the colony (Palumbi and Jackson, 1983). Assuming that colony pairs grow as expanding circles at approximately equal rates, the middle of the intercolony border will correspond to the point at which the two colonies first came into contact. By this logic, the middle of the intercolony border also corresponds to the oldest part of the intercolony border, with younger portions of the intercolony border flanking the middle. Since coordinated colonies are small at the time of first contact, the zooids mediating intercolony coordination will be among the oldest zooids in the colony. If only those five or 10 zooids at the middle of the intercolony border mediate neural integration, then once these zooids deteriorate, behavioral coordination will be terminated.

Two observations suggest that the termination of coordinated behavior is the result of zooid senescence. First, there is evidence that neural connections between zooids within a colony deteriorate as zooids age. In larger, solitary colonies, while peripheral zooids are typically healthy, older, central zooids are frequently unhealthy (as evident by little or no feeding behavior) or dead (Palumbi and Jackson, 1983; D. F. Shapiro, personal observation). Second, the progressive deterioration of zooids from youngest to oldest would explain why those zooids located at the exact midpoint of the



intercolony border did not appear to transmit electrical signals. Prior to the cessation of intercolony coordination, there should be a period during which only the central zooids mediating coordination have deteriorated, with the younger, flanking zooids still neurally fused with the neighboring colony (Fig. 2). An alternative explanation is that neural connections were disrupted because the repeated cuts traumatized the colonies. This hypothesis can be rejected, however, on the basis of the correlation between border length and cut length for intercolony borders cut from the edge; such a correlation should not exist if colonies become traumatized after a given amount of cutting.

Normal deterioration of neural connections between colonies does not preclude the possibility that cessation of behavioral coordination at a particular colony size is adaptive. If there is some cost to neural integration between larger colonies, restricting the number of zooids mediating neural integration could be a mechanism by which colonies limit the duration of physiological integration with neighbors. However, specific studies on the fitness consequences of neural integration between small and large colonies are needed before the adaptive significance of the cessation of neural integration can be determined.

The metabolite translocation experiments suggest that there is no significant translocation of metabolites between colonies. However, if only very small amounts of  $^{14}\text{C}$  are translocated between colonies, it could be argued that the methods used to detect intercolony translocation of metabolites were not sensitive enough. There were at least two possible sources of error. First, algae could have leaked from the containment ring during feeding and been taken up directly by the neighboring colony; using a nearly identical feeding technique, Miles *et al.* (1995) found that up to 10% of the total  $^{14}\text{C}$  leaked from the containment ring during feeding. Because it was necessary to place the containment ring near the intercolony border, leakage could have extended to the unfed colony. In fact, this is the most likely source of the  $^{14}\text{C}$  observed in the three unfed colonies previously mentioned. The second source of error would only affect the results of the scintillation counts. When scraping the colonies from the algal substratum into the scintillation vials, small amounts of  $^{14}\text{C}$  from the fed colony could have contaminated the unfed colony. The higher levels of  $^{14}\text{C}$  measured in larger colonies could be a reflection of this type of error; because larger colonies share longer intercolony borders, they could have received higher levels of contamination. Thus, given these sources of error, it is possible that the scintillation counts alone are not sensitive enough to detect intercolony translocation if translocation rates between colonies are extremely low. However, the autoradiographs are quite sensitive and capable of detecting any radioactivity above background levels (Miles *et al.* 1995). Thus, the scintillation counts, in conjunction with the autoradiographs, indicate that metabolites are not being translocated between colonies.

There are at least two proximate reasons why metabolites are not transferred between colonies despite the presence of pore plates (Shapiro, 1992). First, it is possible that there are

functional constraints involved in the formation of intercolony pore plates. Pore plates found along intercolony borders are morphologically distinct from those found between zooids within a colony (Shapiro, 1992); thus, they could also differ functionally. Second, known patterns of metabolite translocation in bryozoans indicate that metabolites are not necessarily translocated in the direction of the neighboring colony. Miles *et al.* (1995) hypothesize that distally directed translocation in *M. membranacea* (Bobin, 1977; Best and Thorpe, 1985; Miles *et al.* 1995; this study) is controlled by a source-sink process similar to that observed in plants; areas of active growth create 'sinks' that 'pull' metabolites from areas of active feeding. Since no active growth is occurring along the intercolony border, it would not be expected to act as a 'sink'; consequently, metabolites should not be translocated towards the neighboring colony. Thus, under normal conditions, metabolites would be expected to move within and not between the colonies, as observed. This second possibility is supported by the observation that metabolites were not translocated between genotypically identical colonies. Data from this study do not eliminate the possibility that transfer of metabolites between colonies could occur if one colony is starving or growing more actively than the other colony. However, unless the food supply for these colonies is extremely patchy on a very fine scale, such a situation seems unlikely under natural conditions.

Together, the results from the electrophysiological recordings and metabolite translocation experiments indicate that morphological characteristics indicative of tissue coalescence between colonies do not necessarily imply full physiological integration. While morphologically fused colonies are assumed to be physiologically integrated, direct assessments of physiological integration are rarely performed. Exceptions include histological studies and the injection of dye into incurrent sponge apertures to demonstrate that fused sponges share common water-vascular canals (Mukai and Shimoda, 1986; Wulff, 1986; Ilan and Loya, 1990), visual observations of food translocation and polyp retraction to demonstrate that fused cnidarians share a common gastrovascular and nervous system (Hidaka, 1985; Chornesky, 1991; Shenk and Buss, 1991), and visual observations of the movement of blood cells to demonstrate that fused ascidians share a common blood-vascular system (Katow and Watanabe, 1980; Taneda *et al.* 1985; and references therein). Previous examples of fusion in the Bryozoa have used the presence of pore plates between colonies as evidence for full physiological integration between colonies (Stebbing, 1973; Humphries, 1979; Chaney, 1983; Shapiro, 1992; Craig, 1994). The results of the current study, however, combined with the results of Shapiro (1992), indicate that, although pore plates located along the intercolony border may or may not be indicative of neural integration, there is no evidence that they are ever involved in metabolite translocation.

Partial physiological integration between *M. membranacea* colonies could be a highly specific interaction that increases the fitness of small colonies. If there is a benefit to neural

integration of zooids within a colony, there should also be a benefit to neural integration of zooids between colonies. Concomitantly, the lack of metabolite movement between colonies precludes the possibility of resource parasitism. Rinkevich and Loya (1983b) found that metabolites were translocated unidirectionally between grafted colonies of the coral *Stylophora pistillata*; they suggested that in this case the relationship between the coral colonies was competitive in nature. Since *M. membranacea* colonies do not appear to exchange metabolites, the potential costs of physiological integration are minimal.

It has been suggested that fusion among juvenile colonies is a cooperative interaction (Knight-Jones and Moyses, 1961; Jackson, 1985, 1986; Ilan and Loya, 1990; Shenk and Buss, 1991). Although the fitness consequences have not been investigated, size-specific fusion (fusion among small, but not large, colonies) has been demonstrated in sponges (Ilan and Loya, 1990), cnidarians (Hidaka, 1985; Shenk and Buss, 1991), freshwater bryozoans (Mukai *et al.* 1984) and algae (Tveter-Gallagher and Mathieson, 1980; Maggs and Cheney, 1990). Fusion results in an instantaneous increase in size. Since mortality rates are typically disproportionately higher for small colonies (Loya, 1976; Ayling, 1980; Hughes and Connell, 1987; Yund *et al.* 1987; Buss and Yund, 1988; Davis, 1988; Harvell *et al.* 1989), fusion should decrease a colony's probability of mortality. Thus, the possibility exists that size-specific fusion is a general mechanism for increasing the fitness of juvenile colonial marine invertebrates.

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