

# Colony Integration and the Expression of the *Hox* Gene, *Cnox-2*, in *Hydractinia symbiolongicarpus* (Cnidaria: Hydrozoa)

PAULYN CARTWRIGHT<sup>1\*</sup> AND LEO W. BUSS<sup>2,3</sup>

<sup>1</sup>*Department of Biology, Yale University, New Haven, Connecticut 06520*

<sup>2</sup>*Department of Ecology and Evolutionary Biology, Yale University, New Haven, Connecticut 06520*

<sup>3</sup>*Department of Geology and Geophysics, Yale University, New Haven, Connecticut 06520*

**ABSTRACT** The stolonial mat is an anatomical feature correlated with increased colonial integration in several lineages of the cnidarian class Hydrozoa. *Cnox-2* is a *Hox* gene known to be expressed in the body column of the cnidarian polyp. We report the pattern of *Cnox-2* expression in both the stolonial mat and free stolons of the hydroid *Hydractinia symbiolongicarpus*. The gene is found to have high levels of expression in the mat similar to that found in the basal portion of the polyp, but it is not detectably expressed in those regions of free stolons where polyps are budded. These findings suggest that the stolonial mat arose via an expansion of the basal ectoderm of the polyp. *J. Exp. Zool. (Mol. Dev. Evol.)* 285:57-62, 1999. © 1999 Wiley-Liss, Inc.

The history of colonial metazoans is rich in trends of increasing colony integration, both within and between phyla. As may be expected from any pattern involving multiple independent evolutionary events, the processes whereby colonies become more integrated and hence increasingly individuated are diverse. Beklemishev ('69) reviewed this diversity and noted two features of particular importance: the evolution of colonial growth zones and of a colonial neural apparatus. Among the Cnidaria, where themes in colonial organization are particularly complex and intriguing, both features have arisen independently in several lineages.

The hydroid family Hydractiniidae is one such case. This family is comprised of three genera that differ in their colonial structure. *Stylactaria* and *Podocoryne* colonies are comprised of polyps connected by stolons from which they bud. Stolons are tube-like structures that lie adherent to the substratum and are composed of gastrodermal canals surrounded by ectoderm and encased in an acellular periderm (Fig. 1A). Via elongation, branching, and anastomosis, the stolons develop elaborate hydrorhizal networks (Fig. 1B). *Hydractinia*, a sister group of *Podocoryne* (Cunningham and Buss, unpublished data), shares these same features, but also displays an additional character, the stolonial mat (Fig. 1C). The stolonial mat is a network of branching endodermal canals in-

tercalated between two continuous layers of ectoderm (Fig. 1D). In the sister group to the Hydractiniidae, the sylasterine hydrocorals (Peterson, '79; Cairns, '87; Cunningham and Buss, unpublished data), the stolonial mat secretes an elaborate and organized calcium carbonate skeleton resulting in a highly integrated colony.

The configuration of the tissue layers in the stolonial mat of *Hydractinia* permits a mode of colony growth distinct from that of colonies which possess only free stolons. In free stolons, the ectoderm completely surrounds the endodermal canals (Fig. 1A), thus limiting growth to their long axes. By contrast, the continuous ectodermal layers of the mat (Fig. 1D) permit radial growth. Mat tissue bears a distinct histological complement as well. In particular, the upper ectoderm is invested with a continuous nerve net, which is lacking in free stolons (Stokes, '74b). The nerve net of the mat has been implicated in mediating certain coordinated behavioral responses amongst polyps (Josephson, '61; Stokes, '74a) and appears to me-

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\*Correspondence to: Paulyn Cartwright Department of Ecology and Evolutionary Biology, University of Kansas, Lawrence, KS 66045. E-mail: pcart@eagle.cc.ukans.edu

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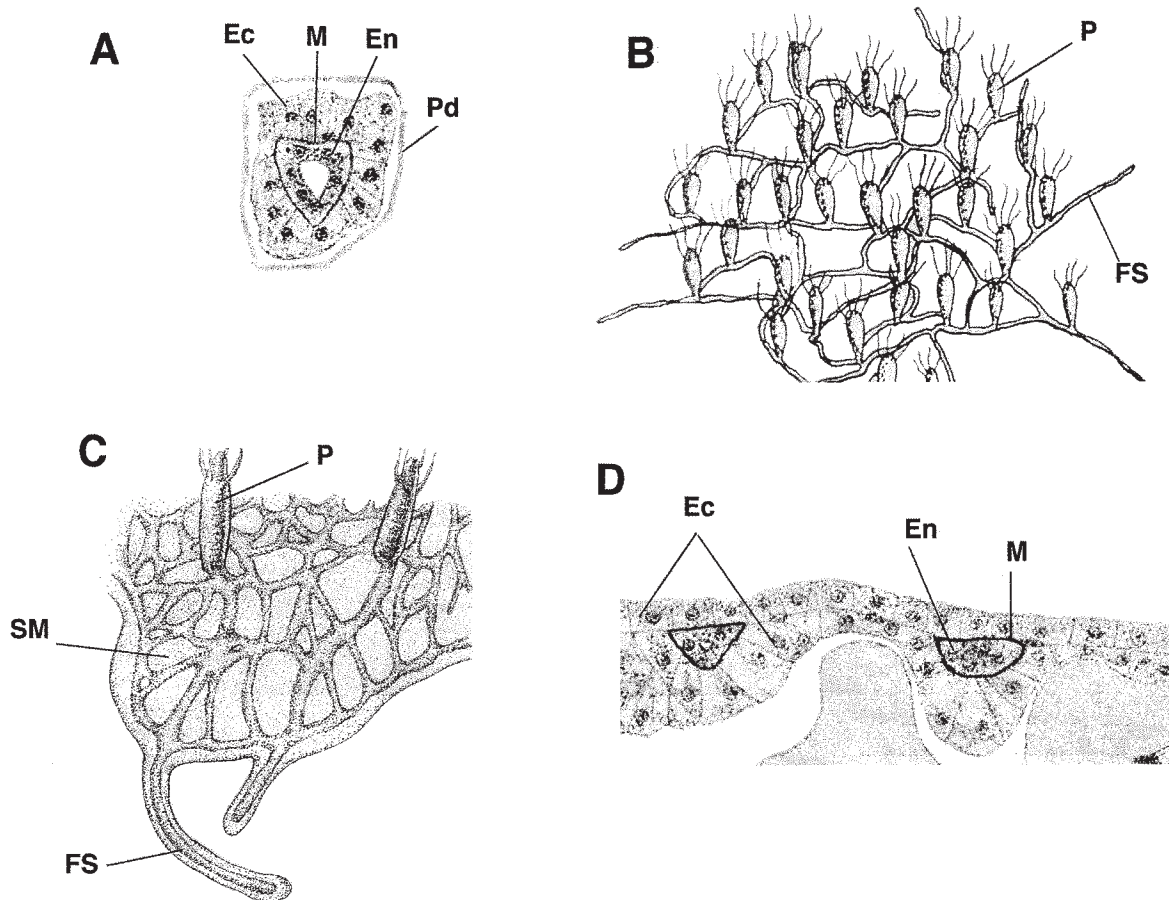


Fig. 1. Schematic diagram of (A) a cross section of a free stolon and (B) the hydrorhizal system characteristic of *Podocoryne* and *Sylactaria*. (C) A *Hydractinia* colony with a stolon mat and free stolons, and (D) cross section of a

stolon mat. Modified from Collcutt (1897), Blackstone ('96), Buss and Blackstone ('91). Ec, ectoderm; En, endoderm; FS, free stolon; M, mesoglea; P, polyp; Pd, periderm; SM, stolon mat.

diate certain colony-wide features of gastrovascular flow (Blackstone and Buss, '93; Dudgeon and Buss, '96).

The origin of the stolon mat in *Hydractinia* not only satisfies both of the conditions that Beklemishev ('69) identifies as important in colonial integration, acquisition of a colonial growth zone and nerve net, but also appears to be essential to the more advanced form of integration displayed by the stylasterine hydrocorals. It is less clear, however, how such an innovation might have arisen. Two hypotheses are immediate candidates. Either the innovation involved a capacity of stolon ectoderm to fuse along their lateral margins, or the innovation is attributable to an expansion of the polyp base over the stolon hydrorhiza. The former would require at least two modifications: loss of the periderm that encases free stolons and the acquisition of a nerve net. The latter scenario requires neither modification as a precondition,

due to the fact that the polyp lacks a periderm and possesses a continuous nerve net. This hypothesis does, however, demand the existence of a genetic system capable of regulating the extent of the polyp base.

*Hox* genes are plausible candidates for such a role, as they are involved in specifying axial positional information in diverse metazoan taxa (Slack et al., '93). Recent work has extended these findings to the Phylum Cnidaria. The *Hox* gene, *Cnox-2*, has been shown to be expressed at high levels along the aboral end of the *Hydra* polyp and at low levels at the oral end (Shenk et al., '93a,b). In addition, *Cnox-2* is found to be expressed at high even levels throughout developing gastrozoid polyps in *Hydractinia* (Cartwright, '97), and subsequently becomes differentially expressed in *Hydractinia* mature gastrozoids in a manner similar to that found in *Hydra* (Cartwright et al., '99).

*Cnox-2*'s apparent role in axial patterning in cnidarian polyps makes it a good candidate for investigating the origin of the stolonal mat. Specifically, *Cnox-2* may be used as a marker to explore whether or not the stolonal mat represents an expansion of the polyp base or a fusion of stolonal ectoderm. We therefore documented the expression of *Cnox-2* in *Hydractinia symbiolongicarpus* in stolonal mat tissues and regions of free stolons where polyps are budded.

## METHODS

### *Polyclonal antibody production*

*Cnox-2* antigen was produced from a 19 AA HPLC purified synthetic peptide (W.M. Keck Biotech. Resource Center, Yale University) which comprised amino acids 112–130 (PREGEEAAPSQ-KIYPPFGRDS) located outside the homeodomain, towards the amino terminus. The synthetic peptide was coupled to ovalbumin (Pierce, Rockford, IL) as a carrier protein and purified over a size-exclusion column (Pierce). Rabbits were immunized with 200  $\mu$ g of antigen and boosted twice with 100  $\mu$ g. Crude sera recognized synthetic peptide alone and synthetic peptide conjugated to an alternate carrier protein, KLH (Pierce) on a dot blot (not shown). IgG fractions were prepared by protein A chromatography (BioRad, Hercules, CA) according to manufacturer's instructions. Protein A purified antibodies were found to bind to a protein of the predicted size on a western blot from *Hydractinia* total protein fractionated by SDS/ Page (not shown).

### *Whole mount immunolocalization*

*Hydractinia* colonies grown on coverslips were relaxed in menthol and fixed in 4% paraformaldehyde at 4°C overnight. Colonies were washed 3  $\times$  10 min in phosphate-buffered saline with 0.25% TritonX-100 (PBST) and incubated for 2 hr at room temperature (r.t.) in 10% goat normal serum/PBST. Protein A (BioRad) purified anti-*Cnox-2* antibodies were diluted to 20  $\mu$ g/ml in 10% goat normal serum/PBS and added to colonies for incubation overnight at 4°C. Colonies were washed 3  $\times$  10 min at r.t. in PBST. FITC conjugated goat anti-rabbit antibody (Sigma, St. Louis, MO) was diluted 1:100 in 10% goat normal serum/PBS and added to colonies for incubation at r.t. for 2 hr. Colonies were washed in PBST for 3  $\times$  10 min, counterstained in 0.05% Evans Blue for 5 min, and washed 10  $\times$  10 min in PBS. Colonies were mounted in 1:10 PBS:glycerol containing 2.5 mg/

ml octane and 1.0  $\mu$ g/ml of 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI). All observations reported here were repeated in multiple replicate trials.

## RESULTS

Free stolons of *Hydractinia* extend beyond the periphery of the stolonal mat and occasionally bud polyps (see Fig. 1C). Figure 2A shows *Cnox-2* expression in such a polyp detected by a polyclonal antibody to the *Hydractinia* gene product. *Cnox-2* expression is undetectable in the region of free stolon from which the polyp budded. When the *Cnox-2* expression pattern (Fig. 2A) is compared to a generalized nuclear stain (Fig. 2B), it is clear that the limits of *Cnox-2* expression in the body column of the polyp define a sharp boundary between the base of the polyp and the stolon. Thus, *Cnox-2* expression delineates the base of the polyp in the absence of mat tissues.

By contrast, *Cnox-2* is expressed at uniform and high levels throughout the stolonal mat (Fig. 3A). The level of expression in the mat is comparable to that found in the polyp body column and base (Fig. 3B). *Cnox-2* expression is continuous from the ectoderm of the polyp base to the upper ectodermal layer of the mat, with no apparent boundary between the base of the polyp and the stolonal mat (Fig. 3C).

The expression of *Cnox-2* in the base of the polyp and in the stolonal mat, but not in free stolons, is consistent with the stolonal mat of *Hydractinia* arising via a modification in the limits of the basal ectoderm of the polyp. This hypothesis is further supported by the observation that when mat isolates form in the periphery of some *Hydractinia* colonies, they only arise at the base of polyps originally budded on free stolons ( $n = 23$  mat isolates at base of stolons,  $n = 0$  mat isolates in free stolons without polyps). Thus, the close proximity of polyps appears to be a requisite condition for mat tissue formation.

## DISCUSSION

The origin of the stolonal mat in *Hydractinia* appears to be essential for increased colonial integration as found in the stylasterine hydrocorals. Understanding how this innovation may have arisen is thus key to understanding an important evolutionary trend within this group. *Cnox-2* is a cnidarian *Hox* gene previously implicated in polyp axial patterning in two hydroid taxa (Shenk et al., '93a,b; Cartwright et al., '99). Our investigations of *Cnox-2* expression in stolons and mat tissue of *Hydractinia* are consistent with the mat

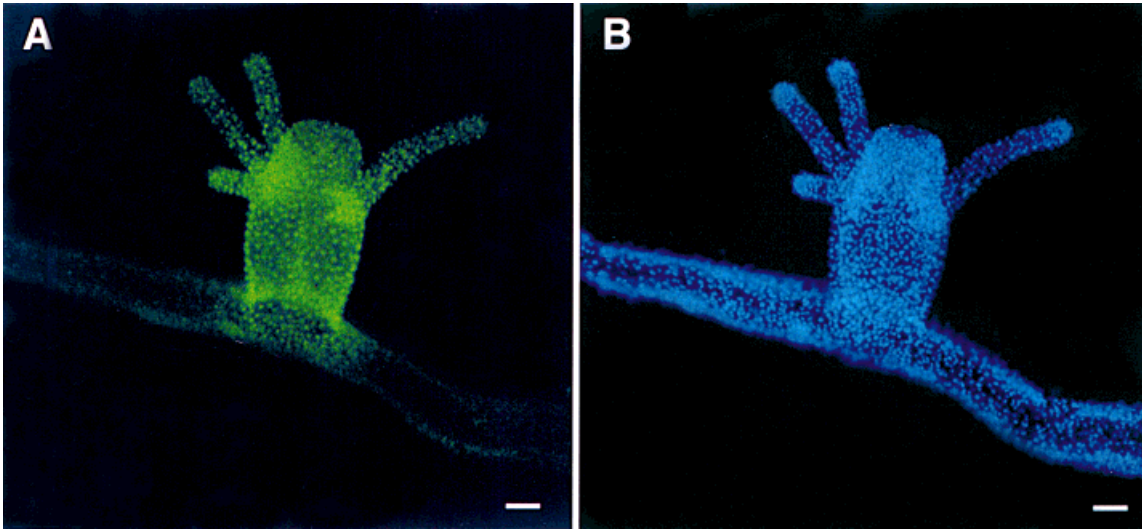


Fig. 2. **A:** *Cnox-2* expression in a young polyp arising from a free stolon in *Hydractinia*. Expression detected using polyclonal antibodies generated to the *Cnox-2* gene product

and visualized by immunofluorescence. Nuclei expressing *Cnox-2* appear green. **B:** Same specimen showing nuclei stained with DAPI, which appear blue. Scale bars = 0.1 mm.

arising through the extension of the polyp base. The histological similarities between polyp body column and mat tissue, in addition to our findings that mat tissue is only found in proximity to polyps, all lend further support for this hypothesis. The mat, it seems, is a colonial foot.

The alternative hypothesis, that the stolonial mat originates through fusion of the stolonial ectoderm, predicts that free stolons and mat tissue would have the same pattern of *Cnox-2* expression. Since mat tissues express *Cnox-2* at high even levels and *Cnox-2* is not detectably expressed in stolons, our findings do not support a stolonial origin for mat tissue.

Our results utilize *Cnox-2* as a marker of the basal polyp ectoderm and so do not, alone, imply that alterations in expression were directly responsible for either initiating or maintaining the stolonial mat. The possibility, however, that *Cnox-2*, or related genes, may have been directly involved should not be discounted given the increasing body of comparative data implicating homeobox genes in the major evolutionary innovations (for some recent examples see: Panganiban et al., '95; Averof and Patel, '97; Cartwright et al., '99). Since neither genetic transformation nor antisense technologies are yet available for hydroids, any test will be necessarily comparative. Fortunately, alterations in the rela-

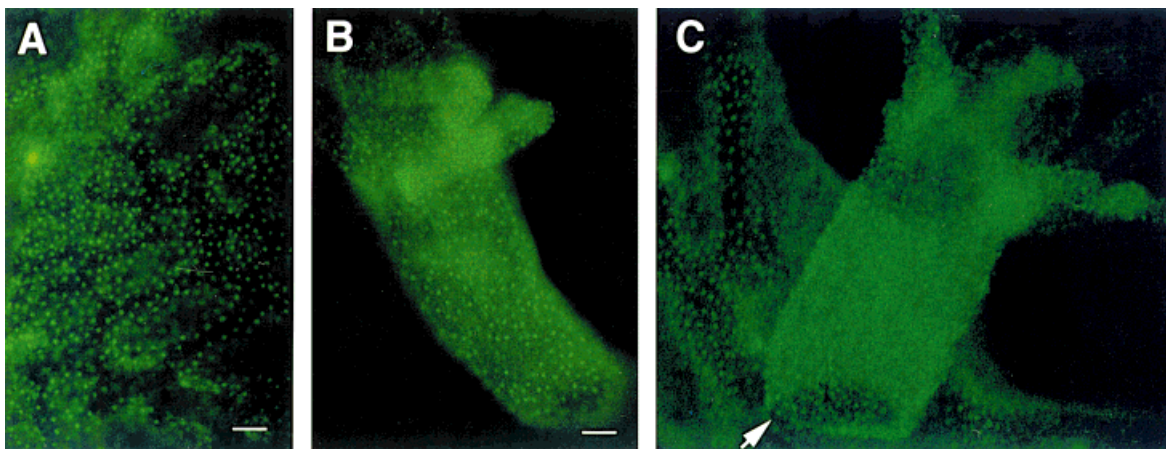


Fig. 3. *Cnox-2* expression in (A) the stolonial mat and (B) a polyp of *Hydractinia*. Polyp and stolonial mat (C) showing continuous *Cnox-2* expression (arrow). Scale bars = 0.1 mm.

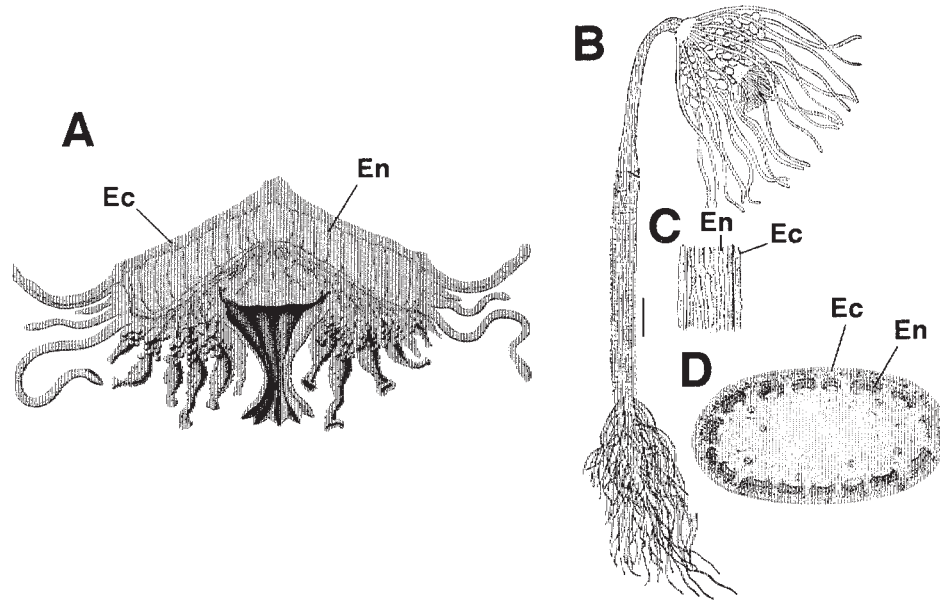


Fig. 4. **A:** Cross section of a free living porpitiid (modified from Haeckel [1888]). **B:** *Corymorpha* (scale bar = 1 cm) [modified from Hyman ('40)]. **C:** Detail of its internalized hydro-

zoan system. **D:** Cross section of *Tubularia* [modified from Warren ('06)]. Ec, ectoderm; En, endodermal canal.

tive position of the polyp base and the hydrorhizal network have arisen independently in several hydrozoan lineages.

Two cases, in particular, are fertile test beds. Just as the stolonial mat of *Hydractinia* was essential to the subsequent advanced integration of stylasterine hydrocorals, an equally striking instance is known to have occurred independently. The hydrozoan family Zancleidae, like the Hydractiniidae, is a colonial taxon with representatives displaying both a creeping stolonial network and a stolonial mat. Prevot ('59) suggested, and molecular analysis has recently corroborated (Bridge, '94), that the Zancleidae are a sister group to the free-living porpitiids (i.e., the 'chondrophores' *Vellela* and *Porpita*, Fig. 4A). The porpitiids possess a high degree of colonial integration, with continuous ectodermal layers surrounding endodermal canals.

The converse trend also exists. The capitate superfamily Tubulariodea is comprised of both solitary and colonial members and is striking by virtue of the extraordinary sizes attained by solitary polyps (Fig. 4B). Key to attaining such dimensions in an animal where respiration is limited by diffusion has been the internalization within the polyp of the hydrorhizal system (Fig. 4C and D). Thus, both routes to increasing physiological integration—the elaboration of the polyp as in Tubulariodea and elaboration of the colony as in Hydractiniidae and Zancleoidea—appear cor-

related with alterations in the demarcation of the polyp base and the hydrorhizal system. Our results with *Cnox-2* suggest that exploration of the expression of this gene in these taxa would prove illuminating.

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#### LITERATURE CITED

- Averof M, Patel NH. 1997. Crustacean appendage evolution associated with changes in Hox gene expression. *Nature* 388:682–686.
- Beklemishev WN. 1969. Principles of comparative anatomy of invertebrates, volume 1. Chicago: The University of Chicago Press.
- Blackstone NW. 1996. Gastrovascular flow and colony development in two colonial hydroids. *Biol Bull* 190:56–68.
- Blackstone NW, Buss LW. 1993. Experimental heterochrony in hydractiniid hydroids: why mechanisms matter. *J Evol Biol* 6:307–327.
- Bridge DM. 1994. Phylogeny and life cycle evolution in the Phylum Cnidaria. PhD thesis, Department of Biology, Yale University.
- Buss LW, Blackstone NW. 1991. An experimental exploration of Waddington's eigenetic landscape. *Phil Trans R Soc Lond B* 332:49–58.
- Cairns SD. 1987. Evolutionary trends in the Stylasteridae (Cnidaria, Hydrozoa). In: Bouillon J, Cicogna F, Cornelius PFS, editors. Modern trends in the systematics, ecology, and evolution of hydroids and Hydromedusae. Oxford: Clarendon Press.

- Cartwright P. 1997. Characterization of a HOM/HOX homeobox gene, *Cnox-2*, and the evolution of coloniality in the Hydrozoa (Phylum Cnidaria). PhD thesis. Yale University. 107 p.
- Cartwright P, Bowsher J, Buss LW. 1999. Expression of Hox gene, *Cnox-2*, and the division of labor in a colonial hydroid. Proc Natl Acad Sci USA (in press).
- Collcutt MC. 1897. On the structure of *Hydractinia echinata*. Q J Microsc Sci 40:77–100.
- Dudgeon S, Buss LW. 1996. Growing with the flow: on the maintenance and malleability of colony form in the hydroid *Hydractinia*. Am Nat 147:667–691.
- Haekel E. 1888. Report on the Siphonophorae collected by H.M.S. *Challenger* during the years 1873–76. London: The Challenger Reports.
- Hyman LH. 1940. The invertebrates: protozoa through Ctenophora. New York: McGraw-Hill Book Company, Inc.
- Josephson RK. 1961. Colonial responses of hydroid polyps. J Exp Biol 38:559–577.
- Panganiban G, Sebring A, Nagy L, Carroll S. 1995. The development of crustacean limbs and the evolution of arthropods. Science 270:1363–1366.
- Peterson KW. 1979. Development of coloniality in Hydrozoa. In: Larwood G, Rosen BR, editors. Biology and systematics of colonial organisms, volume 11. New York: Academic Press. p 105–159.
- Prevot E. 1959. Morphologie et evolution des structures tentaculaires chez les hydres gymnoblastes Capitata. Recueil des Travaux de la Station Marine d'Endoume 29:91–126.
- Shenk MA, Bode HR, Steele RE. 1993a. Expression of *Cnox-2*, a HOM/HOX homeobox gene in hydra, is correlated with axial pattern formation. Development 117:657–667.
- Shenk MA, Gee L, Steele RE, Bode HR. 1993b. Expression of *Cnox-2*, a HOM/HOX gene, is suppressed during head formation in *Hydra*. Dev Biol 160:108–118.
- Slack JMW, Holland PWH, Graham CF. 1993. The zootype and the phylotypic stage. Nature 361:490–492.
- Stokes DR. 1974a. Physiological studies of conducting systems in the colonial hydroid *Hydractinia echinata*. J Exp Zool 190:1–18.
- Stokes DR. 1974b. Morphological substrates of conduction in the colonial hydroid *Hydractinia echinata*. J Exp Zool 190:19–46.
- Warren E. 1906. On *Tubularia solitaria* sp. n., a hydroid from the Natal coast. Anals Natal Gov Mus 1:83–96.