Int. J. Dev. Biol. 43: 817-821 (1999)

Original Article

In *Hydra magnipapillata* the activator of protein kinase C diC8 causes multiple head formation along the body axis only when accompanied by feeding, but heavy feeding alone is sufficient to cause multiple head formation

MICHAEL KROIHER*

Zoologisches Institut, Universität zu Köln, Köln, Germany

ABSTRACT In *Hydra magnipapillata* additional head structures can be induced to form by daily feeding accompanied by a daily treatment with diC8, an activator of protein kinase C. Based on these results, it was proposed that the PKC- pathway plays a central role in head formation in hydra. The results described here show that ectopic structures, as well as the ectopic localization of nerve cells, can be induced by heavy feeding alone. Furthermore, diC8 treatment does not induce ectopic head structures in starved animals. DiC8 reduces the rate of budding, leading to an unusual lengthening of the body column in reasonably fed animals.

KEY WORDS: *coelenterate, hydra, pattern formation, PKC, diacylglycerol*

Introduction

Body sections of the freshwater polyp hydra regenerate a head and a foot. Generally, one head and one foot is formed separated by a tube shaped gastric cavity. Under normal conditions, additional heads and feet are only produced by the vegetative process of budding, which takes place in a well defined region of the body column. Thus, there is a signaling system controlling the formation of only one head and one foot in the animal (for review see Berking, 1998).

One approach learning more about the molecular basis of this pattern forming system, is the treatment of animals with defined biochemical compounds and to look for a perturbation of the pattern of the animal. Various compounds implicated in the PI-system have an effect on pattern formation in hydra. Treatment of hydra with diC8 causes the formation of ectopic heads along the body column (Müller, 1989). DiC8 is an activator of PKC (Berridge, 1987). Treatment of animals with LiCl causes the formation of ectopic heads as well as ectopic feet depending on the treatment protocol (Yasugi, 1974; Hassel and Berking, 1990; Hassel *et al.*, 1993; Hassel and Bieller, 1996). LiCl is proposed to act as an inhibitor for enzymes in the recycling of IP_3 to PIP_2 (Berridge *et al.*, 1989). Recently, members of the PKC-family in hydra have been cloned. The local and temporal expression patterns of these genes in normal and experimentally manipulated animals support the idea of an important role of the PI- system in pattern formation (Hassel, 1998; Hassel *et al.*, 1998).

Here we show that diC8 by itself is unable to induce the formation of ectopic heads in *Hydra magnipapillata*. DiC8 can only induce such structures when combined with feeding. In addition, heavy feeding alone is sufficient to induce ectopic structures along the body column. Further, heavy feeding also causes the occurrence of RFamide immunopositive nerve cells in areas of the animal that are under normal conditions virtually free of such cells. Misexpression of RFamide immunopositive nerve cells was also induced by periodic treatment with diC8 combined with moderate feeding (Müller, 1991). The aim of this study was to better understand the influence of feeding and diC8 treatment on pattern formation.

Results

Müller (1989) reported the formation of ectopic head structures along the body column of *H. magnipapillata* WT 105 following several days of treatment with diC8, an activator of PKC. The

Abbreviations used in this paper: diC8, 1,2- dioctanoyl- sn- glycerol; HM, hydra medium; IP_3, inositoltrisphosphate; PI- system, phosphatidylinositol signal transduction pathway; PIP_2, phosphatidylinositolbisphosphate; PKC, protein kinase C.

***Address for reprints:** Zoologisches Institut, Universität zu Köln, Weyertal 119, 50923 Köln, Germany. FAX: XX49-221-470-5171.
e-mail: MKROIHER@NOVELL.BIOLAN.UNI-KOELN.DE

0214-6282/99/$15.00

Printed in Spain
www.ehu.es/ijdb

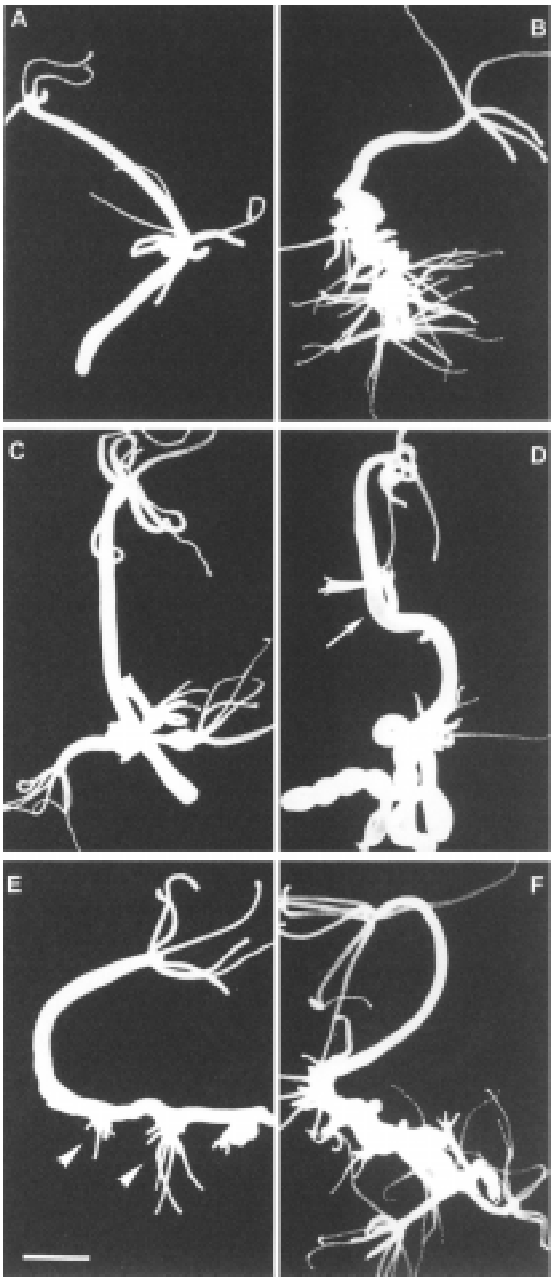

Fig. 1. Ectopic structure formation in *H. magnipapillata* caused by a treatment with diC8 at different feeding rates and by heavy feeding only. *Moderately fed animals* **(A)** *and heavily fed animals* **(B)** *were treated periodically with diC8 for 14 days. Note the ring of ectopic tentacles (A) and the multiple ectopic structures (B). Animals were fed heavily* **(C-F)**. *Normal shaped animal with multiple buds (C), extreme elongated animal (note the plait like structure) with additional budding regions (arrow; D), animal with two additional ectopic heads (arrowhead; E) and animal with multiple ectopic structure formation (F). Pictures were taken after 14 days (A- C) or 30 d (D- F). Bar 2 mm.*

substance was applied every day immediately after feeding. We confirmed this result (Fig. 1A,B) but found that the heavily fed animals of the control group produced ectopic heads as well (Fig. 1D-F). The structures formed in both groups looked similar. In both groups, the body column elongated prior to the appearance of the first additional head structures (Fig. 1; also observed by Müller, 1989). Further, the sequence of events was similar in both groups. It starts with the formation of single tentacles at the body column followed by the development of complete extra heads or ectopic structures or a little tip formed, which immediately develops the hypostome and tentacles as observed in multiheaded hydra *mh-1* (Sugiyama, 1982). The formation of this ectopic structure is clearly distinct from the development of a bud (Fig. 1E; Fig. 3C). In addition, secondary heads develop, which arise like buds in a position outside of the budding region (Fig. 1D). In both groups, ectopic structure formation is also correlated and preceded by the formation of RFamide+ nerve cells (Fig. 2; see also Müller, 1991).

However, one strong difference was observed. In diC8 treated animals the elongation is faster and the formation of ectopic head structures starts earlier than in the heavily fed but untreated animals (Fig. 3). Moderately fed animals produced ectopic head structures only if they were treated with diC8 simultaneously (Fig. 3) and these animals develop fewer ectopic heads than heavily fed animals treated with diC8 (Fig. 1A,B; Fig 4). Moreover, the periodic treatment of starved animals with diC8 failed to cause extra head structures (Fig. 3). It failed to cause an elongation (not shown), and it failed to cause the differentiation of additional RFamide+ nerve cells. Budding is inhibited by the treatment with diC8, as previously reported by Müller (1995), whereas feeding stimulates budding (Table 1).

Hydra vulgaris responds in a different way. With or without diC8 treatment, ectopic head structures were not observed following heavy feeding. Rather, these animals responded with a proportional growth in size followed by the insertion of new tentacles into the existing tentacle ring as already described by Müller (1995).

Discussion

In Hydra, the formation of ectopic head structures and the formation of additional nerve cells in the body column is correlated with an unproportional elongation of the body column but not with the treatment of diC8. The responding group includes heavy fed *H. magnipapillata* and moderately fed *H. magnipapillata* treated with diC8. The non responding group includes moderate fed *H. magnipapillata*, unfed treated and untreated *H. magnipapillata* and further starved or heavy fed and treated or untreated *H. vulgaris*.

One may object that gastric tissue of starved animals may be unable to produce a head. However, body sections of starved animals regenerate a head while the treatment of intact starved animals with diC8 failed to cause extra head formation. Thus, repetitive treatments with diC8 have to be combined with feeding in order to cause extra head formation.

In order to understand the results obtained, it is necessary to understand the unusual elongation and how a treatment with diC8 is able to cause this elongation even at moderate feeding conditions.

Upon feeding the body of a detached bud, it elongates until it starts to produce its own buds. A bud recruits tissue from the parent (Shostak and Kankel, 1967; Otto and Campbell, 1977a). This is a fast process which causes the bud to grow at the expense of the parent. Therewith, the rate of budding and its concurrent tissue loss delimits the length of the parent's body. The loss of tissue at the base and at the tentacles is comparatively low (Campbell, 1967). Heavy feeding increases both cell proliferation and budding (Otto and Campbell, 1977b; Bode *et al.*, 1977). When the increase of

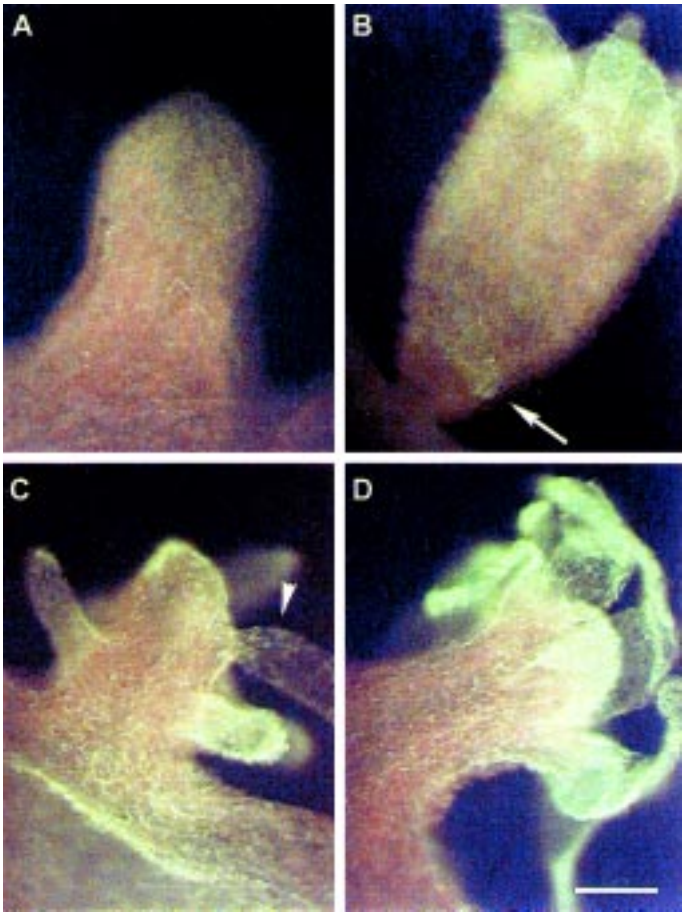

Fig. 2. Localization of RFamide immunopositive nerve cells in *H. magnipapillata*. *Early budding stage without RFamide positive perikaryons in the bud and in the surrounding gastric tissue.* **(A)** *Late budding stage with RFamide positive cells in the tentacles and the head region* **(B)***, note the very few positive cells arising in the foot region (arrow); early stage of ectopic head formation after heavy feeding with a very high number of labeled cells in the ectopic head as well as in the surrounding gastric tissue* **(C)***, note the ectopic tentacle within the ectopic head (arrowhead); late stage of ectopic head formation* **(D)**. *Bar, 175 μm.*

tissue by cell proliferation exceeds the loss of tissue caused by budding the body elongates. In heavily fed *H. magnipapillata,* the body elongates while the head remains of almost constant size. In *H. vulgaris,* the proportions of the animal are maintained (see also Müller, 1995).

Shiba (1981) and Müller (1995) found that both TPA and diC8 inhibit budding (the latter result could be confirmed here). Furthermore, diC8 treatment causes smaller buds to detach (Müller, 1995). Obviously, due to diC8 treatment less tissue is lost. The simplest conclusion would be that this reduced loss caused the observed elongation at moderate feeding conditions at which untreated animals do not change their length.

It has been proposed that budding starts when the distance to the head exceeds a certain limit, indicating a bud inhibiting signal emerging from the head region (Tripp, 1928). The same signal would prevent the formation of additional heads in that region. Therewith, an unproportional elongation should cause a pattern instability in particular some distance away from the head above the budding region. Just this was observed, in both, heavily *H. magnipapillata* and in moderately fed *H. magnipapillata* treated with diC8.

Based on current models, the formation of head structures as well as the increase of the positional value is caused by short range activation coupled to a long range inhibition (Meinhardt, 1993). Upon heavy feeding and also moderate feeding coupled to diC8 treatment, the increase of the positional value occurs in patches along the body column indicated by the formation of RFamide+ nerve cells. Finally, heads are formed separated from each other by gastric tissue. Further, this increase of the positional value does not occur in an all-or-none way but rather stepwise.

This fits the proposition that both regimes interfere with the pattern forming system in such a way that activation becomes rhythmically enhanced, somewhere exceeding a certain threshold. The threshold is given by the local level of inhibition. The inhibition of the head, buds and of the basic production contribute to this local level of inhibition. Due to the different range of the activation and the inhibition, the increase of the activation remains locally restricted and thus the positional value increases locally. Due to the coupling between activation and inhibition, the activation is transient, which causes the positional value to increase by one step only (Berking, 1998). Patches of increased positional value contribute to the basic level of inhibition. Therewith, budding is antagonized and the body column becomes longer. However, this only takes place in the case of enhanced cell proliferation. In starved and moderate fed animals and also in *H. vulgaris* the threshold is obviously not passed. In starved *H. magnipapillata* repetitive treatments with diC8 are unable to either stimulate activation or to reduce the threshold in such a way that the positional value increases locally.

In intact *H. magnipapillata* the rhythmic heavy feeding –simply due to a well supply of energy or due to the supply of specific substances (from the Artemia food)– is sufficient to cause the patchy increase of the positional value in a certain body region

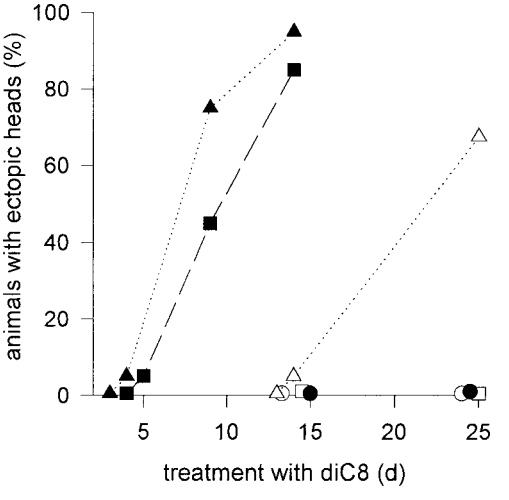

Fig. 3. Development of ectopic heads in *H. magnipapillata* in relation to different feeding rates and diC8 treatment. *Untreated (white symbols) and diC8 treated (black symbols) groups of non fed (circle), moderate fed (square) and heavy fed (triangle)- samples were observed for the formation of ectopic heads for up to 25 days. N= 40.*

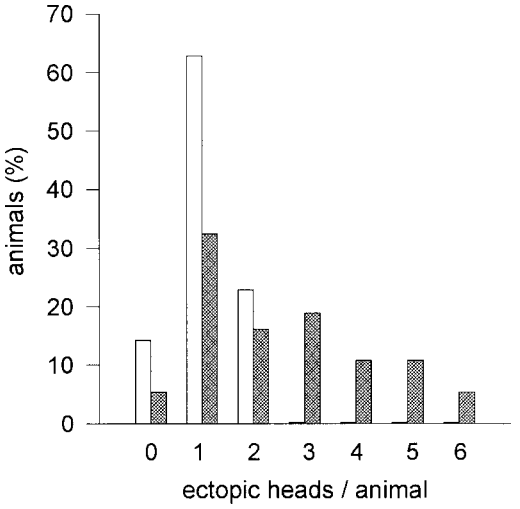

Fig. 4. Ectopic head formation in *H. magnipapillata* caused by a treatment with diC8 and different feeding rates. *Moderate fed- (white bars) and heavy fed- (hatched bars) samples were treated periodically with diC8 for 14 days. The number of additional ectopic heads was counted at the end of the treatment. Note that without feeding, no ectopic structures developed during the treatment with diC8 (not shown). N= 40.*

which finally leads to extra heads. The accompanying rhythmic diC8 treatment supports this effect. It alone is unable to cause the effect. Thus, diC8 is unable to replace the compounds contained in the food. This does not exclude that at other conditions and in other animals such a treatment increases the positional value directly. In intact moderately fed *H. magnipapillata,* one cannot decide yet whether the treatment primarily causes the extra elongation due to a stimulation of the formation of patches of high positional value, which in turn reduces the rate of budding (see above), or if budding is reduced, which in turn causes an increase of the length of the animal.

It may be that the extra nerve cells are simply the response to a changed positional value following elongation of the body column. However, feeding stimulates nerve cell formation. The observed effect is fast. Stem cells which are just in the middle of their S- phase become committed to develop into nerve cells (Berking, 1979). TPA also stimulates nerve cell formation. Here the observed effect is not fast. During treatment the commitment to become a nerve cell is prevented, following treatment commitment is enhanced. Stem cells which start their S-phase following treatment become committed to the nerve cell pathway (Greger and Berking, 1991). Whether diC8 causes the same dynamics as TPA treatment or feeding is unknown. However, one has to keep in mind that the extra RFamid+ nerve cells (a fraction of all nerve cells) are not detected in starved animals treated with diC8. As yet, there is no good argument that a (repetitive) treatment with diC8 is sufficient to directly cause the ectopic formation of these nerve cells.

TABLE 1

THE BUDDING RATE IS INFLUENCED BY DIC8 TREATMENT AND BY FEEDING

Type of Feeding	Total Number of Buds		Buds/Animal		p-value
	untreated	diC8-treated	untreated	diC8-treated	
Non Fed	69	59	1.72	1.47	0.86
moderate fed	298	168	7.46	4.22	0.004
heavy fed	546	445	13.67	11.13	0.308

The treatment with diC8 was 10^{-5}M, the budding rate was scored for 14 days. N= 40

Materials and Methods

To investigate whether food (*Artemia salina*) by itself or in combination with diC8 treatment has an influence on pattern formation in hydra, 40 animals were selected from a mass culture, transferred in a glass petri- dish and kept in 100 ml hydra medium (10^{-3}M $CaCl_2$, $5x10^{-4}$ M $MgCl_2$, $2.5x10^{-5}$ M EDTA, $7.5x10^{-5}$ $Na+$, 10^{-4} M $KHCO_3$, $3.5x10^{-4}$ M $NaHCO_3$ in millipore water, pH 7.6). Animals were kept without food, were fed daily at 9.30 a.m. with up to 5 nauplii/hydra (moderate feeding) or with more than 20 nauplii/hydra (heavy feeding). The HM of the petri- dish was adjusted to 20 ml for feeding and each animal was fed by hand with the specified number of shrimps. After 60 min the remaining shrimps were washed out and the solution was adjusted to 100 ml. Treatment with diC8 was done in 7 ml HM at a concentration of 10^{-5}M (Müller, 1989). DiC8 was dissolved in dimethylsulfoxid at a concentration of 10^{-2}M and stored as a freezer stock. To prepare treatment solution, 7 µl aliquot of the freezer stock was dissolved in 500 µl HM by sonification and added to 6.5 ml HM containing the animals. Treatment with diC8 was performed in glass bowls in the dark with little agitation starting 3 h after the beginning of the feeding. The duration of the treatment on the first day was 60 min, the treatments on the following days were performed for 2 h. Thereafter the animals were washed 3 times and kept in 100 ml HHM. Treatment of animals with diC8 started on the 4th day after the beginning of the distinct feeding regime. In the experiments without diC8 the animals were handled just like in the experiments with the diC8 treatment. In most cases, the distinct feeding regime lasted for over 3 weeks. In all experiments, detached buds were removed after feeding and before treatment with diC8 was started.

Staining of RFamide immunopositive nerve cells in whole-mount preparation was done according to Grimmelikhuijzen (1985). Statistical significance was calculated with the fisher exact test.

Acknowledgments

I thank Dr. S. Berking for stimulating discussions and Dr. D.G. Grosskopf-Kroiher and Alysia Runde for critical reading of the manuscript.

References

BERKING, S. (1979). Control of nerve cell formation from multipotent stem cells in Hydra. *J. Cell Sci. 40*: 193-205.

BERKING, S. (1998). Hydrozoa metamorphosis and pattern formation. *Curr. Top. Dev. Biol. 38*: 81-131.

BERRIDGE, M.J. (1987). Inositol trisphosphate and diacylglycerol, two interacting second messengers. *Annu. Rev. Biochem. 56*: 159-193.

BERRIDGE, M.J., DOWNES, C.P. and HANLEY, M.R. (1989). Neural and developmental actions of lithium: a unifying hypothesis. *Cell 59*: 411-419.

BODE, H.R., FLICK, K.M. and BODE, P.M. (1977). Constraints on the relative sizes of the cell population in *Hydra attenuata. J. Cell Sci. 24*: 31-50.

CAMPBELL, R.D. (1967). Tissue dynamics of steady state growth in *Hydra littoralis.* II. Patterns of tissue movement. *J. Morphol. 121*: 19-28.

GREGER, V. and BERKING, S. (1991). Nerve cell production in Hydra is deregulated by tumor- promoting phorbol ester. *Roux´s Arch. Dev. Biol. 200*: 243-236.

GRIMMELIKHUIJZEN, C.J.P. (1985). Antisera to the sequence Arg-Phe-amide visualise neuronal centralisation in hydroid polyps. *Cell Tissue Res. 241*: 171-181.

HASSEL, M. (1998). Upregulation of a *Hydra vulgaris cPKC* gene is tightly coupled to the differentiation of head structures. *Dev. Genes Evol. 207*: 489-501.

HASSEL, M. and BERKING, S. (1990). Lithium ions interfere with pattern control in *Hydra vulgaris*. *Roux's Arch. Dev. Biol. 198*: 382-388.

HASSEL, M. and BIELLER, A. (1996). Stepwise transfer from high to low lithium concentrations increase the head forming potential in *Hydra vulgaris* and possibly activates the PI cycle. *Dev. Biol. 177*: 439-448.

HASSEL, M., ALBERT, K. and HOFHEINZ, S. (1993). Pattern formation in *Hydra vulgaris* is controlled by lithium sensitive processes. *Dev. Biol. 156*: 362-371.

HASSEL, M., BRIDGE, D.M., STOVER, N.A. and STEELE, R.E. (1998). The level of expression of a protein kinase C gene may be an important component of the patterning process in Hydra. *Dev. Genes Evol. 207*: 502-514.

MEINHARDT, H. (1993). A model of biological pattern formation of hypostome, tentacles and foot in Hydra: how to form structures close to each other, how to form them at a distance. *Dev. Biol. 157*: 321-333.

MÜLLER, W.A. (1989). Diacylglycerol-induced multihead formation in Hydra. *Development 105*: 309-316.

MÜLLER, W.A. (1991). Stimulation of head-specific nerve cell formation in Hydra by pulses of diacylglycerol. *Dev. Biol. 147*: 460- 463.

MÜLLER, W.A. (1995). Competition for factors and cellular resources as a principle of pattern formation in Hydra. I. Increase of the potential for head and bud formation and rescue of the regeneration-deficient mutant *reg-16* by treatment with diacylglycerol and arachidonic acid. *Dev. Biol. 167*: 159-174.

OTTO, J.J. and CAMPBELL, R.D. (1977a). Budding in *Hydra attenuata*. Bud stages and fate map. Tissue economics of Hydra: regulation of cell cycle, animal size and development by controlled feeding rates. *J. Exp. Zool. 200*: 417-428.

OTTO, J.J. and CAMPBELL, R.D. (1977b). Tissue economics of Hydra: regulation of cell cycle, animal size and development by controlled feeding rates. *J. Cell Sci. 28*: 117-132.

SHIBA, Y. (1981). Modulatory action of 12-O-Tetradecanoylphorbol-13-acatate on bud production in Hydra. *Roux's. Arch. Dev. Biol. 190*: 305-307.

SHOSTAK, S. and KANKEL, D.R. (1967). Morphogenetic movements during budding in Hydra. *Dev. Biol. 15*: 451-463.

SUGIYAMA, T. (1982). Roles of head- activation and head- inhibition potentials in pattern formation of Hydra: Analysis of a multi- headed mutant strain. *Am. Zool. 22*: 27-34.

TRIPP, K. (1928). Regenerationsfähigkeit von Hydren in verschiedenen Körperregionen nach Regenerations- und Transplantationsversuchen. *Z. Wiss.Zool. 132*: 470-525

YASUGI, S. (1974). Observations on supernumerary head formation induced by lithium chloride treatment in the regenerating hydra, *Pelmatohydra robusta*. *Dev. Growth Differ. 16*: 171-180.

Received: July 1999
Accepted for publication: August 1999