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Interactions between the Foot and Bud Patterning Systems in *Hydra vulgaris*

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In the freshwater coelenterate, hydra, asexual reproduction via budding occurs at the base of the gastric region about two-thirds of the distance from the head to the foot. Developmental gradients of head and foot activation and inhibition originating from these organizing centers have long been assumed to control budding in hydra. Much has been learned over the years about these developmental gradients and axial pattern formation, and in particular, the inhibitory influence of the head on budding is well documented. However, understanding of the role of the foot and potential interactions between the foot, bud, and head patterning systems is lacking. The purpose of this study was to investigate the role of the foot in the initiation of new axis formation during budding by manipulating the foot and monitoring effects on the onset of first bud evagination and the time necessary to reach the 50% budding point. Several experimental situations were examined: the lower peduncle and foot (PF) were injured or removed, a second PF was laterally grafted onto animals either basally (below the budding zone) or apically (above the budding zone), or both the head and PF were removed simultaneously. When the PF was injured or removed, the onset of first bud evagination was delayed and/or the time until the 50% budding point was reached was longer. The effects were more pronounced when the manipulation was performed closer to the anticipated onset of budding. When PF tissue was doubled, precocious bud evagination was induced, regardless of graft location. Removal of the PF at the same time as decapitation reduced the inductive effect of decapitation on bud evagination. These results are discussed in light of potential signals from the foot or interactions between the foot and head patterning systems that might influence bud axis initiation. © 1999 Academic Press

Key Words: **hydra; pattern formation; budding; axis induction; gradients.**

INTRODUCTION

Pattern formation is a fundamental aspect of development, and one of the earliest steps during pattern formation is the establishment of body axes. In some organisms axes are established during oogenesis (e.g., the anterior–posterior axis in *Drosophila* or the animal–vegetal axis in the sea urchin or *Xenopus*). In the freshwater coelenterate, hydra, a new apical–basal axis is established each time the parental animal reproduces asexually by budding (Clarkson and Wolpert, 1967; Webster and Hamilton, 1972). For this reason, hydra provide a convenient, simple model system in which to study axis formation.

The idea that gradients underlie the establishment of positional information specifying the axes has been a fundamental tenet of developmental biology for years. Indeed, in numerous, diverse organisms, graded signals have been shown to play a role in establishing pattern (Shenk *et al.,* 1993; Grens *et al.,* 1996; Burz *et al.,* 1998; Wikramanayake and Klein, 1997). Pattern regulation in hydra involves developmental gradients originating from two organizing centers, the apical head and the basal foot. Gradients of head activation and inhibition emanate from the head while gradients of foot activation and inhibition arise basally. Budding occurs between these two organizing centers, approximately two-thirds the distance from the head to the foot, at the base of the gastric region. A considerable understanding of the head and foot axial patterning processes has been achieved over the years and models have been devised to account for them (see Javois, 1992, for review; Sherratt *et al.,* 1995; Meinhardt, 1993, 1998). However, our understanding of bud initiation remains more rudimentary. The head clearly has an influence on budding. Budding is enhanced by removal of the head (Tardent, 1972) and suppressed by putting the head closer to the budding

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zone (Burnett, 1961). The head and bud patterning systems have been demonstrated to cross-react as the presence of heads and buds mutually inhibits each other (Shostak, 1974). Additionally, activating and inhibiting substances isolated from hydra which affect the head also affect budding (Schaller, 1973; Berking and Gierer, 1977). However, bud initiation is not regulated by a simple signal from the head. Analysis of budding in strains of hydra with mutant head activation and/or inhibition gradients suggests that neither head inhibition nor the ratio of head activation to inhibition controls budding (Sugiyama, 1982; Rubin and Bode, 1982; Takano and Sugiyama, 1983). Other factors must be involved. While earlier studies have alluded to the existence of interactions between the foot and bud patterning systems separate from that of the head and bud systems, such findings have yet to be definitely documented. This study was undertaken to investigate the role of the basal tissue in initiating bud evagination. Freshly dropped buds were isolated and maintained with feeding, and the onset of budding was monitored. Experiments were then performed on animals collected in this manner in which the peduncle/foot (PF) tissue was manipulated (removed, injured, or doubled) and the onset of first bud evagination and the time necessary to reach the 50% budding point (B_{50}) were then compared to unmanipulated or sham control animals. Depending on when the experiments were performed relative to the anticipated onset of budding, the onset of first bud evagination and/or B_{50} was induced or delayed relative to the doubling or removal of PF tissue, respectively. These results suggest that a signal emanating from the PF tissue is involved in initiating new axis formation. In addition, simultaneous removal of the PF and head suggests that opposing signals relative to bud axis initiation are emanating from each organizing center.

METHODS

Culture of animals. Asexual *Hydra vulgaris* (Clone 203) originally obtained from Pierre Tardent (Zurich, Switzerland) were used for all experiments. The hydra were maintained at 18°C in cultures of approximately 50–60 budding adults per tray containing 1 liter of hydra medium as described by Javois and Tombe (1991). Animals were fed *artemia* nauplii on Mondays, Wednesdays, and Fridays. Medium was changed daily and when animals were fed, 1 and 6 h after feeding. In preparation for the experiments, all nonbudding animals were removed from the cultures. Then, 16–24 h later, freshly dropped buds were collected and isolated in petri dishes at a density of 1 hydra/ml. This point was considered "time zero."

Peduncle/foot removal. The animals were fed once or twice after collection, and then the experiment was initiated 6 h after the feeding, at 24 or 72 h. Twenty-six to 30 animals were maintained as control animals while the lower PF were excised from the same number of experimental animals (Fig. 1a). All animals were maintained on the feeding schedule noted above. For approximately 10 days following the manipulation or until 100% of either the control or experimental animals evaginated their first bud, all animals were observed daily to monitor the onset of first bud formation. Onset of budding was defined in reference to the formation of first buds, and subsequent formation of second buds was not monitored. Budding rate was measured in reference to the time required to reach the 50% budding point (B_{50}). The PF removal experiment was performed four times each, 24 and 72 h after collection of animals.

Creation of animals with a second peduncle and foot. Buds for this experiment were collected as described above and all animals were fed once or twice before initiating the experiments at 48 or 72 h, respectively. Two approaches were taken to create an animal with a second PF. In the first, experimental animals were split from the basal disc up to the region just below the tentacles (Fig. 1d). After healing, only the lower region from the midpeduncle to basal disc remained separated, and the animals had two PF regions. As a "sham" control for potential injury to the budding zone, an incision was made from the area just below the tentacles through the budding zone (Fig. 1e). The onset of first bud evagination in sham animals that healed with a single head as well as experimental animals was compared to unmanipulated control hydra.

In the second approach, animals were taken at 48 h and lateral grafting was performed. This time point was selected because the animals had been fed once (and were somewhat larger and easier to manipulate), and the graft would be in place for at least 24 h before the preparatory stages of budding (see Discussion). Donor PF tissue was excised by cutting just beneath the border of the peduncle and gastric regions. Donor tissue was grafted into incisions made in host animals either basally, just below the junction of the gastric region and peduncle (Fig. 1f), or more apically, in the midbody column at the junction of the 2- and 3-regions (Fig. 1h). Monofilament fishline (DuPont, Wilmington, DE) was used and normal apical–basal polarity was maintained. Tissue pieces were held in place for 60 min to facilitate healing by inserting rings of polyethylene tubing over the ends of the fishline. As a control for the injury induced by the lateral grafting procedure, sham grafts were performed. For each grafting situation, an incision was made in the same spot as for grafting, fishline was inserted through the incision and out the mouth, and tubing was inserted onto the ends of the fishline (Figs. 1g and 1i). These animals were maintained on the fishline for 60 min and then removed. All manipulated animals were observed 24 h later to ensure that healing had occurred properly. Only experimental animals with two distinct PF were further maintained and monitored for the onset of budding. All animals were maintained on the feeding schedule described above for the duration of the experiment. The onset of first bud evagination in sham-grafted animals as well as in animals with two PF was compared to that in unmanipulated control animals. This experiment was repeated four times for the basally grafted PF and three times for the apically grafted PF. Sham controls were prepared twice each for the basal and apical incisions. Sample sizes ranged from 20 to 27 and monitoring was as indicated for the PF removal experiment.

Head versus head and peduncle/foot removal. Visible budding in isolated, unmanipulated hydra was consistently observed to begin at approximately 5 days following collection of freshly dropped buds. Animals that are continuously starved have been shown to lose their budding ability, and hydra that are decapitated are unable to ingest food. Given these facts, buds for this experiment were collected as described above, all animals were maintained on the feeding schedule through two feedings, and then the experiment was initiated 6 h after the second feeding at 72 h (48 h prior to the anticipated onset of budding). All animals including the unmanipulated controls were then maintained without feeding for the duration of the experiment, and for this reason the budding rate is reduced. In this experiment, one-third of the animals were

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decapitated just below the tentacle ring, one-third were decapitated and also cut midpeduncle to remove the PF, and the final third were left as unmanipulated control animals (Figs. 1b and 1c). This experiment was repeated three times; sample sizes and monitoring were as described for the PF removal experiment.

RESULTS

Two aspects of budding were analyzed during all the experiments. First, the onset of first bud evagination was monitored for each animal. Subsequent formation of second buds was not recorded, although it was observed that animals initiating first bud evagination sooner also formed second buds sooner. Second, the rates of first bud evagination in experimental and control groups were compared over the time course by assessing the time necessary to reach the 50% budding point (B_{50}).

Peduncle/foot removal delayed the onset of first bud formation and lengthened B_{50}. The lower PF was excised from experimental animals (Fig. 1a). Depending on when the PF was removed prior to the onset of first bud formation, B_{50} and/or the onset of first bud formation was delayed. In general, the effects were more pronounced when the manipulation was performed closer to the predicted onset of budding (5.0 ± 0.6 days for the 23 fed control groups monitored in this study).

When the PF was removed 24 h after the freshly dropped buds were collected, no delay in the onset of first bud evagination was observed. Both control and experimental animals began budding on the same day (Fig. 2, left column, arrows). However, in three of the four experiments, the B_{50} was slower in the experimental animals (Fig. 2, horizontal dashed line). Ultimately, budding in the experimental animals reached the same level as the controls. When the PF was removed 72 h after collection of the animals, much closer to the predicted onset of budding, first bud evagination was delayed in three of the four experiments (Fig. 2, right column, horizontal bars). The B_{50} was initially slower although the experimental animals eventually reached the same level of budding as the controls.

Similar effects were observed when the peduncle/foot was split to create two. In the first attempt to create animals with two PF, an incision was made through the basal disc and peduncle and up into the body column (Fig. 1d). When animals healed they had two distinct peduncles with feet, though each appeared to be half the width of a normal PF. As with the PF removal, the B_{50} was lengthened and/or the onset of first bud formation delayed. Again, the effect was more pronounced when the manipulation was performed closer to the predicted onset of budding. The B_{50} was slower in three of four trials regardless of whether the PF was split 48 h (Fig. 3, left column) or 72 h (Fig. 3, right column) after collection of the animals. The delay in the onset of first bud formation was clearly correlated with manipulations performed closer to the anticipated onset of budding. When the PF was split 48 h after collection, a delay occurred in one of four trials. However, when the PF was split 72 h after collection, much closer to the anticipated onset of budding, a delay in first bud formation was observed in three of four trials (Fig. 3, bars). To determine if the effects on budding were the result of injury from the cut, sham controls were performed four times 72 h after collection. A lengthy incision from just beneath the tentacles through the budding zone was made (Fig. 1e). The onset of first bud evagination in these animals was identical to control animals, and the B_{50} was the same (twice) or slightly slower (twice) (data not shown).

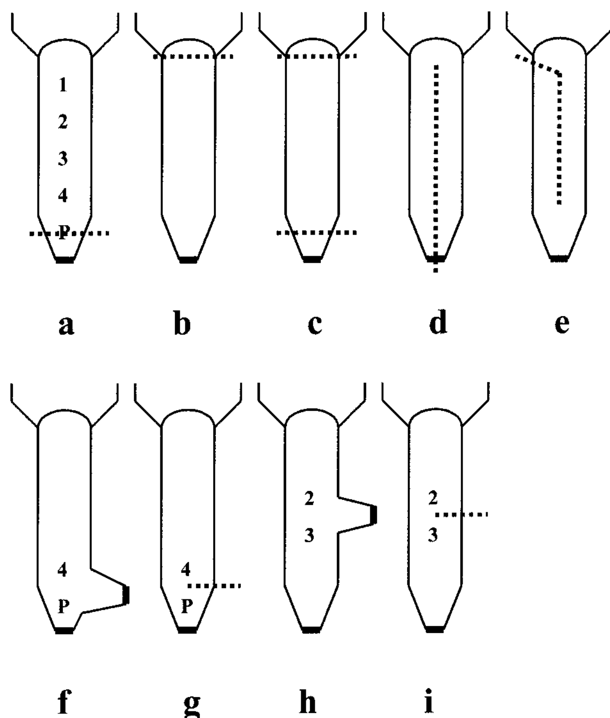

FIG. 1. Diagrammatic representations illustrating the experimental manipulations performed (dashed lines, sites of cuts). The head and tentacles are up, the gastric region (1–4) narrows at the peduncle (P), and the thick horizontal line at the bottom represents the foot. (a) Removal of lower peduncle and foot; (b) decapitation; (c) removal of head and lower peduncle/foot; (d) creation of animals with two peduncles and feet by cutting along the axis from the foot to the upper body column; (e) sham control for d in which animals were cut from below the tentacles through the budding zone; (f) lateral grafting of a second peduncle/foot to a basal position at the 4/P border; (g) sham control for f; (h) lateral grafting of a second peduncle/foot to an apical position at the 2/3 border; (i) sham control for h.

Doubling the peduncle/foot tissue by lateral grafting resulted in precocious budding. When the amount of foot tissue was doubled 48 h after collection of the animals by laterally grafting on a second PF to a basal position (Fig. 1f), both control and experimental animals began budding on the same day in three of four trials (Fig. 4, left column). However, in three of four trials the B_{50} in the experimental

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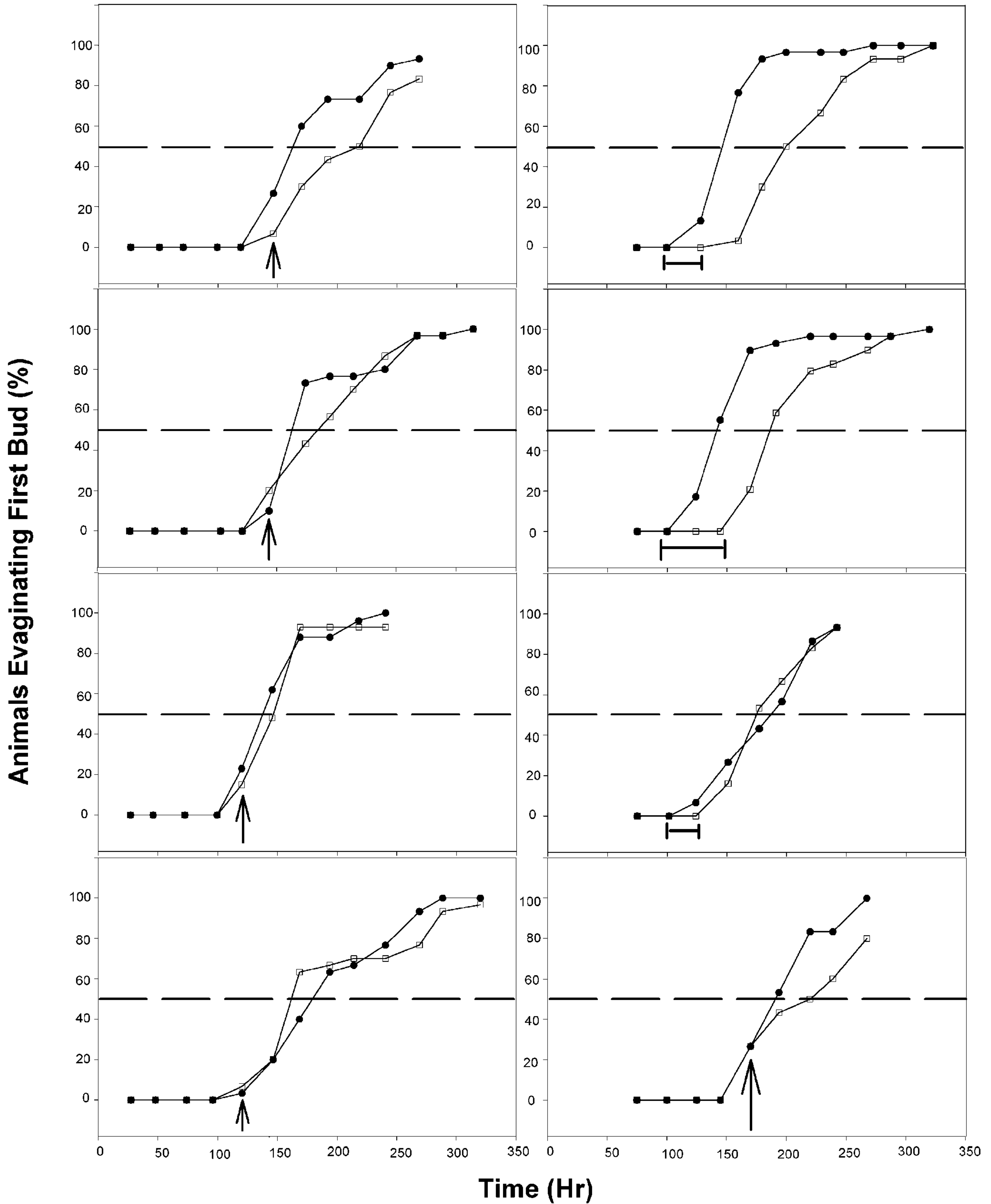

FIG. 2. First bud evagination in control animals (closed circles) and animals in which the lower peduncle/foot was removed (see Fig. 1a) (open squares) 24 h (left column) and 72 h (right column) after collection of hydra. The horizontal dashed lines indicate the 50% budding point (B_{50}). Arrows indicate when the onset of budding began simultaneously in control and experimental animals. The horizontal bars indicate a delay in the onset of budding. Each graph represents an independent experiment in which 26–30 control or experimental animals were analyzed/experiment.

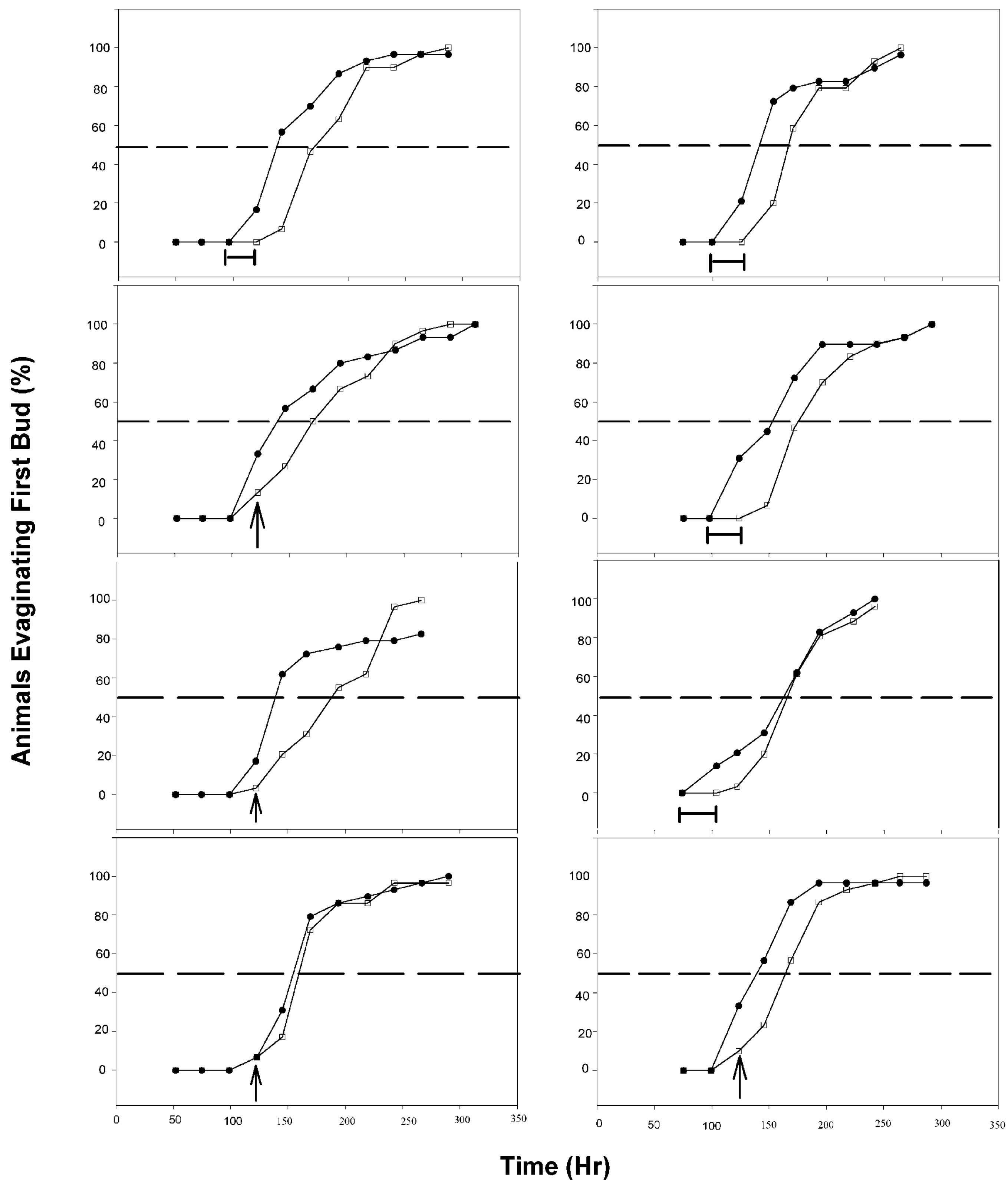

FIG. 3. First bud evagination in control animals (closed circles) and animals in which the peduncle was split (see Fig. 1d) 48 h (left column) or 72 h (right column; open squares) after collection of the hydra. Other symbols as in Fig. 1. Each graph represents an independent experiment in which 29–30 (left column) or 26–30 (right column) control or experimental animals were analyzed/experiment.

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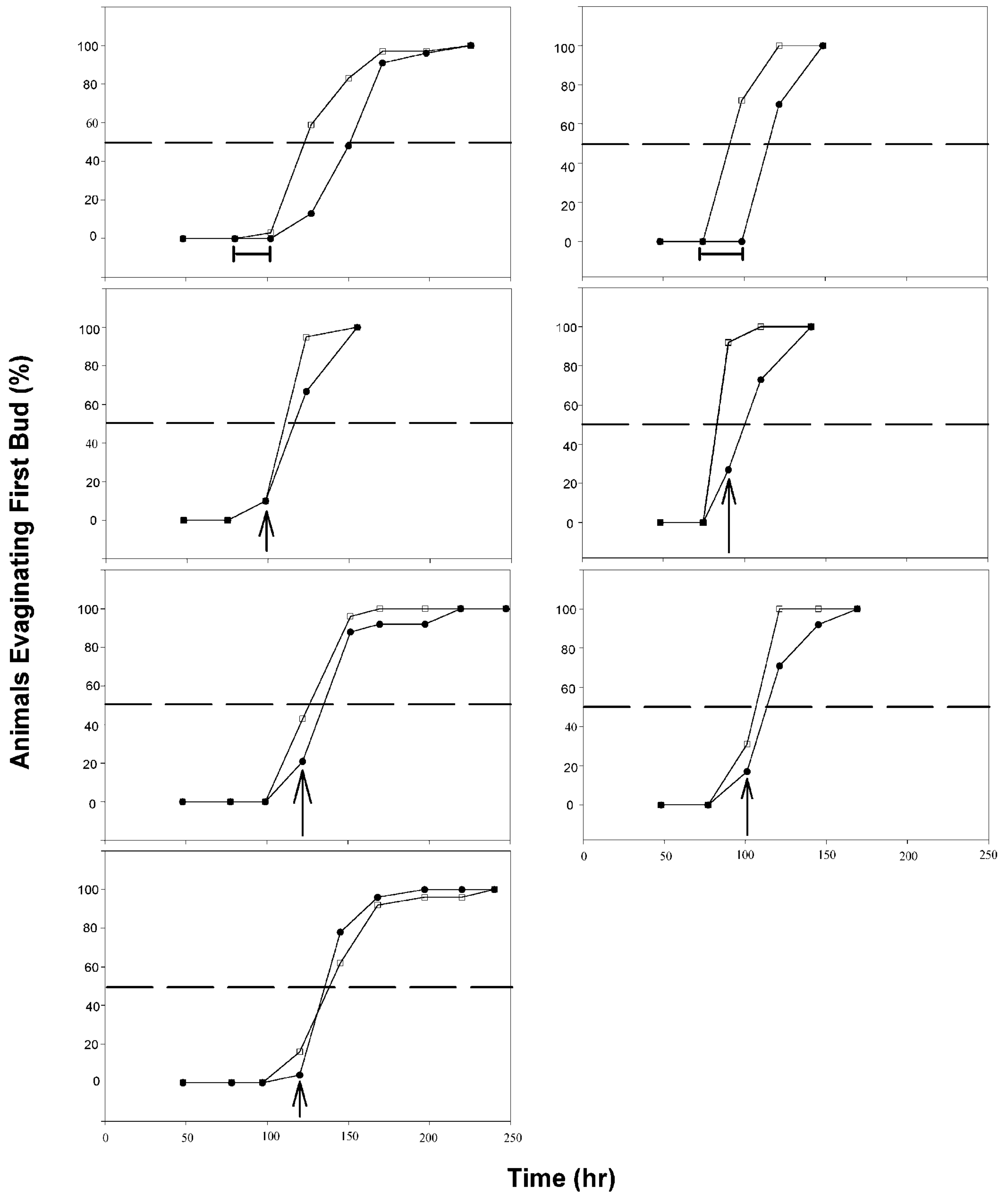

FIG. 4. First bud evagination in control animals (closed circles) and animals in which a second peduncle/foot was laterally grafted either basally (left column) or apically (right column; open squares) (see Figs. 1f and 1h) 48 h after collection of the hydra. Other symbols as in Fig. 1. Each graph represents an independent experiment in which 20–27 control or experimental animals were analyzed/experiment.

animals was earlier (Fig. 4). In the one trial where control onset of budding was delayed a day relative to the experimental animals, this effect was most pronounced.

Grafting the second PF to a more apical position gave the same results (Fig. 4, right column). In two trials, the onset of first bud formation began on the same day for both experimental and control animals; however, the B_{50} was shorter in the experimental animals. In the third trial, the experimental animals began budding the day before the control animals and the difference in B_{50}s was most pronounced.

To determine if the precocious onset of budding was the result of injury from the lateral grafting procedure, sham grafts were prepared twice for each graft site. The onset of first bud evagination and B_{50} in apical sham animals was the same as control animals. For basal sham animals the onset of first bud evagination was 1 day earlier than control animals, but the B_{50}s were the same (data not shown).

Simultaneous peduncle/foot removal counteracted the induction of budding resulting from decapitation. In this experiment, one-third of the animals were decapitated just below the tentacle ring, one-third were decapitated and also cut midpeduncle to remove the PF, and the final third were an unmanipulated control group (Figs. 1a–1c). The overall level of budding in these experiments was reduced because once animals were decapitated, none of the animals were fed for the duration of the experiments. In general, the level of budding varied among the three trials, but the overall relationship between the control and two experimental groups remained the same (Fig. 5). The control group always had the lowest level of budding, with the onset of first bud evagination occurring 24 h after the experimental groups in two of three trials. In all three trials, decapitated animals had the highest overall rate of first bud evagination as well as the largest number of animals developing buds. Animals whose head and PF were simultaneously removed showed a reduction in the rate of first bud evagination and number of animals evaginating buds compared to decapitated animals. To evaluate for significant differences in number of buds evaginated between manipulated and control animals, the two-way χ^2 test for goodness of fit was employed. Only in trial 3 did decapitated animals show a significant induction of budding compared to control animals (Fig. 5, asterisk; $P < 0.05$). No significant differences in budding were observed between head PF-excised and control animals.

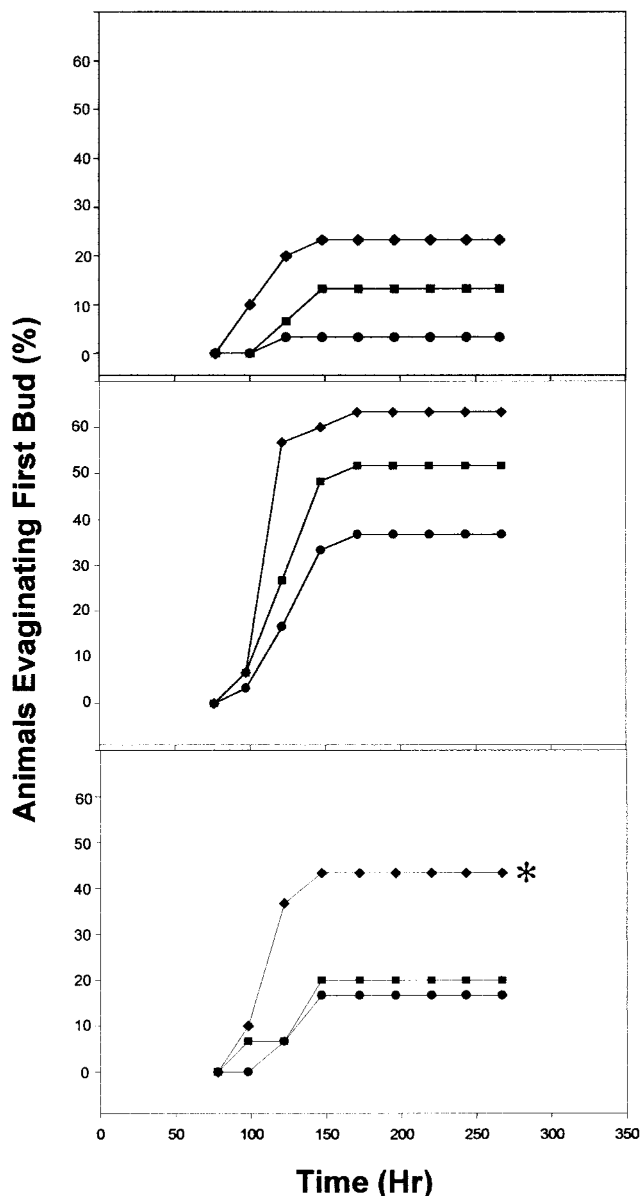

FIG. 5. Data from three independent experiments are graphed to illustrate budding in control hydra (circles), hydra which were decapitated (see Fig. 1b) (diamonds), and hydra which were decapitated and had the lower peduncle/foot removed (see Fig. 1c) (squares) 72 h after collection of the animals. Twenty-nine to 30 control and experimental hydra were analyzed/experiment. Asterisk indicates experimental animals whose budding rate was significantly higher than that of control animals (χ^2 test; $P < 0.05$).

DISCUSSION

Developmental gradients of activation and inhibition emanating from the two organizing centers, the head and foot, have been postulated to control the initiation of new axis formation during budding in hydra (Sugiyama, 1982; Rubin and Bode, 1982; Takano and Sugiyama, 1983). Numerous experiments have demonstrated that removal of the head or relocation of the head closer to the budding zone results in induction or inhibition of budding, respectively (Burnett, 1961; Tardent, 1972; Shostak, 1974). The experiments reported here were undertaken in order to determine whether similar manipulations of the foot would influence bud evagination. In general, the results indicate that manipulating the foot influences bud evagination. Additionally, the effects emanating from the head and foot can

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counteract each other. Because the effect of adding a PF did not depend on the location of the second PF, the possibility also exists that the foot effect is mediated via changes to the head patterning system.

The removal or injury of the peduncle/foot has two distinct effects on the budding process. The patterning processes that result in bud evagination are underway well before there are physical signs of budding. This was originally apparent from the work of Berking and Gierer (1977) who analyzed the budding process in detail using a purified endogenous inhibitor of budding. From their experiments, they defined four overlapping preparatory phases that occur 24 h prior to the physical onset of budding. In addition to this approach, several recently cloned genes have been shown to be expressed in the budding zone and/or "bud primordia." These findings confirm that molecular changes are occurring well before bud evagination and the physical initiation of a new axis (Martinez *et al.,* 1997; H. Bode, personal communication).

In this study, two effects on budding were observed as the result of removing or injuring the PF. The time necessary to reach the 50% budding point (B_{50}) was lengthened regardless of whether the PF had sufficient time to heal or regenerate prior to the anticipated onset of budding. Second, if the PF was removed or injured close to the preparatory stages occurring 24 h prior to bud evagination, the actual onset of budding was postponed 24–48 h. The injury effect was specific for the PF region as injuring by cutting at the midgastric region or the gastric/peduncle border did not affect budding.

Using the peroxidase staining method of Hoffmeister and Schaller (1985), feet were seen to regenerate 24–48 h after their removal (data not shown). Therefore, bud evagination did not begin until the foot had regenerated or healed. This result correlates with those of Ando *et al.* (1989) who demonstrated that a foot "helps" a bud to form. Following tandem grafting of apical pieces of body tissue, a nearest-neighbor analysis revealed that the instances of a bud forming adjacent to a foot were significantly higher than expected, while the number of times a head was found next to a bud was significantly lower than predicted. In addition, the formation of secondary feet preceded that of buds, implying that the presence of the foot "helped" bud formation.

Doubling the peduncle/foot tissue by lateral grafting induced bud evagination regardless of the graft position. If the foot has a positive influence on budding, it can be hypothesized that creating an animal with two feet might amplify this effect. The initial attempt at creating two PF by splitting the animals longitudinally did not give the expected result. Rather, the effect was the same as removing the PF. In retrospect, these animals did not have two full-sized PF. Since injury to the midgastric region or the gastric/peduncle border (control experiments for the lateral grafting procedures) did not affect budding, it can be concluded that both injury to the foot region and its removal can delay budding.

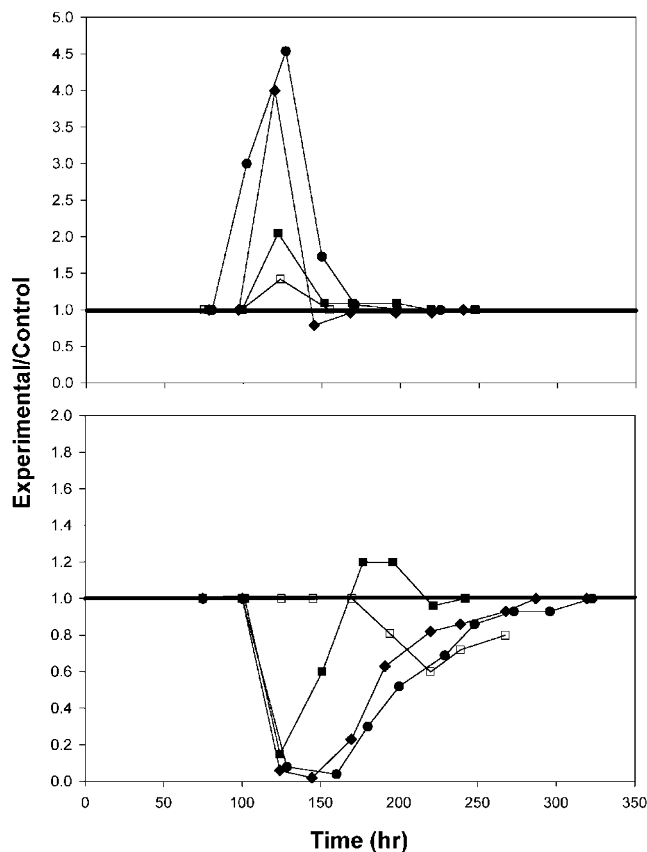

FIG. 6. Comparison of the ratio of experimental to control bud evagination in hydra with a second peduncle/foot grafted basally (top; data from Fig. 4, left column) and hydra with the lower peduncle and foot removed 72 h after collection (bottom; data from Fig. 2, right column). Different symbols arbitrarily represent the four trials of each experiment. A ratio greater than 1 indicates an induction of budding while a ratio less than 1 indicates a delay in the onset of budding.

When grafting a second PF basally created two feet, first bud evagination occurred sooner, and the B_{50} was reached sooner. The difference between delaying first bud evagination by removing the PF and precociously inducing first bud evagination by doubling the PF tissue was most obvious when the ratio of experimental to control budding animals was plotted over the time course and compared. Faster experimental budding rates plot as values greater than 1, whereas slower experimental budding rates appear as values less than 1 (cf., Fig. 6, top and bottom).

The fact that a second PF grafted apical to the budding zone has the same effect on bud initiation and the B_{50} time as a second PF grafted basally places constraints on the potential role of the foot patterning system in bud initiation. This is further discussed below.

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Peduncle/foot removal counteracts the inductive effect of decapitation on budding. The PF and head were removed simultaneously to investigate potential interactions between the influence of the foot on budding and previously documented head influences on budding. When animals are decapitated, budding is induced, an observation that has been well documented. The experiment reported here showed that the induction in budding caused by decapitation could be counteracted by simultaneous PF removal. In all three trials the inductive effect due to decapitation was reduced when the PF was simultaneously removed, and in trial 3 this effect was all but eliminated.

In a similar manipulation, Berking and Gierer (1977) compared the effects of decapitation and foot removal on bud formation by pretreating with endogenous inhibitor and then decapitating one set of animals, removing the lower peduncle and foot from another group, and allowing a third group to serve as unmanipulated controls. Their results showed that the number of animals with newly formed buds 15–24 h after pretreatment with inhibitor was highest in decapitated animals and lowest in hydra with excised feet. Taken together, the results of these two studies suggest that interactions between the head, foot, and bud patterning systems exist. The presence of a head has an inhibitory effect on budding while the presence of a foot permits budding.

The role of the foot patterning system on bud initiation. Different models based on diffusion have been proposed to account for the establishment and maintenance of axial polarity within hydra (Goodwin and Cohen, 1969; Wilby and Webster, 1970; Wolpert *et al.,* 1972; Gierer and Meinhardt, 1972; Meinhardt and Gierer, 1974; MacWilliams, 1982; Meinhardt, 1993, 1998; Sherratt *et al.,* 1995). The presence of a budding zone midway between the head and foot organizing centers has been explained in terms of the ranges of the developmental gradients of head and foot inhibition (Meinhardt, 1993). Budding has been proposed to occur in a region outside the influence of either inhibitory signal. Clearly, with regard to head inhibition, the numerous data support this conclusion. However, the results of this study suggest that the role of the foot in bud initiation is that of a positive influence. If the foot is injured or removed, a bud will not evaginate until the foot has healed or regenerated. When there is insufficient time to heal or regenerate a foot before the initiation of budding, budding is delayed. In addition, doubling the foot tissue enhances this positive influence, causing precocious bud initiation. Interestingly, the origin of this foot signal in relation to the budding zone is not relevant. Placing the extra foot basally or apically has the same effect. This finding suggests that the positive signal emanating from the foot might be related to the foot activation potential. Recently, Hoffmeister (1996) has isolated two peptides with foot-activating properties. However, exogenous application of one of these factors, pedibin, to animals does not enhance bud evagination (H. Shimizu, personal communication). These findings place constraints on the role of the foot patterning system with regard to bud axis initiation. Either a separate signal unrelated to foot activation or foot inhibition influences budding, or the foot effect is mediated via the head patterning system.

Recent studies investigating interactions between the foot and head patterning systems have demonstrated that moving the PF closer to the head or doubling of PF tissue by basal lateral grafting leads to rapid shifts in both apical and basal gene expression (Forman and Javois, in press). Knowing that the foot and head patterning systems interact in this manner raises the possibility that the effects on bud evagination observed following manipulation of the foot might be mediated through alterations of the head patterning system. At present this possibility cannot be ruled out. Doubling the amount of foot tissue by basal lateral grafting rapidly downregulates gene expression associated with the head (Forman and Javois, in press). It could also reduce the effects of head inhibition allowing the precocious onset of budding. Theoretically, this type of effect would be initiated regardless of where the grafted PF is placed. Likewise, injury or removal of the foot might reduce or eliminate its effect on the head. This in turn might enhance the effects of head inhibition on the budding process. Budding would then be delayed until the foot healed or regenerated and reexerted its effect on the head patterning system.

Regardless of how the results of the experiments presented here are interpreted, the overall finding is that manipulation of the foot patterning system influences initiation of budding in a positive way. This confirms the roles of both organizing centers in the initiation of new axis formation. As more genes expressed during the budding process are identified, it will be possible to analyze the effects of manipulating the head and foot patterning systems on axis initiation at the molecular level. In particular, these studies will provide more detailed information on global aspects of patterning in a simple organism beyond what we currently know about head or foot patterning as isolated events.

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