

# Ultraviolet irradiation initiates ectopic foot formation in regenerating hydra and promotes budding

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We have studied the effects of ultraviolet-C (UVC) and Ultraviolet-B (UVB) on growth and pattern formation in *Pelmatohydra oligactis*. UVC brings about a significant increase in budding in intact hydra while UVB does not exhibit such an effect. Excessive budding could be a response for survival at wavelengths that damage biological tissues. If the head or base piece of a bisected hydra is irradiated and recombined with the unirradiated missing part, regeneration proceeds normally indicating that exposure of a body part with either an intact head or foot to UVC does not influence pattern formation. Most significantly, in the middle piece, but not in the head or the base piece of a trisected hydra, UVC leads to initiation of ectopic feet formation in almost one third of the cases. Thus, UV irradiation interferes with pattern formation in regenerating hydra, possibly by changing positional values, and promotes budding in intact hydra. This is the first report on induction of ectopic feet formation by UV in regenerating hydra and opens up the possibility of using UV irradiation as a tool to understand pattern formation in the enigmatic hydra.

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## 1. Introduction

Hydra, a member of the phylum Cnidaria, has an oral-aboral body axis and exhibits a remarkable ability to regenerate. It has been a favorite model of developmental biologists because a large number of developmental phenomena occur continuously in the polyp as a consequence of both sustained growth in the polyp and pattern formation in its regenerating body parts (Berking 1998; Galliot and Schimid 2002; Bode 2003). Correct formation and maintenance of the oral-aboral polarity in hydra has been explained on the basis of morphogenetic gradients (Meinhardt 1997; Bode 2003). The validity of this approach has gained strength in recent years as homologs of a large number of vertebrate pattern forming genes and signalling cascades have been detected in hydra (reviewed in Galliot 2000; Bosch and Khalturin 2002). Studies with hydra are likely to provide insights into the

evolution of anteroposterior and dorsoventral axes in bilaterians. In amphibian embryos, ultraviolet (UV) irradiation is known to interfere with the formation of the dorsoventral axis. Irradiation of an early frog embryo with ultraviolet-C leads to ventralization of the embryo. This is believed to occur as a consequence of inhibition of grey crescent formation due to blocking of cortical rotation by UV that, in turn, inhibits formation of the Nieuwkoop center (Gimlich and Gerhart 1984). We have studied the effects of UV radiation on growth of intact polyps and pattern formation in regenerating body parts of *Pelmatohydra oligactis*. We find that UV irradiation of intact polyps promotes budding while that of certain regenerating body parts induces ectopic feet formation. To our knowledge, this is the first report of UV affecting pattern formation in hydra. The study shows that UV irradiation can be employed as a powerful tool in understanding molecular regulation of pattern formation in hydra.

**Keywords.** Ectopic foot formation; pattern formation; *Pelmatohydra oligactis*; promotion of budding; regeneration; ultraviolet radiation

## 2. Materials and methods

### 2.1 Culture of *P. oligactis*

A clonal culture of *P. oligactis* was maintained in hydra medium (Hassel *et al* 1993) at room temperature (22–26°C). Hydra were fed on alternate days with *Cyclops* (Crustacea). Four hours after feeding, the cultures were washed and cleaned. The same feeding schedule was followed everyday.

### 2.2 Regeneration experiments

Full-grown hydra were selected, starved for 48 h and each individual was cut into 3 approximately equal pieces with sharp tungsten needles along the oral-aboral axis. Regeneration of the fragments was monitored for 5 days. The cultures were not fed during regeneration. Regeneration of head structures was monitored by morphological and functional criteria while that of the foot was confirmed by foot-specific staining. Hydra were stained with 2,2-azino-di (3-ethyl-benzthiazoline-sulfonic acid-6) ammonium salt (ABTS, Sigma) for the detection of foot-specific peroxidase (Hoffmeister and Schaller 1985). About 10 hydra were incubated in 3 ml of freshly prepared staining solution containing citric acid (65.5 mM), trisodium citrate (34.5 mM), ABTS (0.1%) and hydrogen peroxide (0.003%) for 20 min at room temperature. The reaction was terminated by addition of sodium azide (0.4 mM). After microscopic observation, the hydra were preserved in sodium azide until photographed.

### 2.3 UV irradiation

**2.3a Irradiation of whole hydra:** Groups of 10 hydra were irradiated at either 254 (ultraviolet-C; UVC) or 312 (ultraviolet-B; UVB) nm at 0, 100, 200, 500 and 1000 J/M<sup>2</sup>. These were closely monitored for 8 days to detect effects of UV, if any. Each experiment was replicated 8 times. Thus 80 hydra were used for each dose of UV.

**2.3b Irradiation and rejoining of parts of hydra:** A single hydra was cut into two, approximately in the middle, giving a head (H) and a base (B) fragment of roughly equal length. Either one or both were irradiated with 500 J/M<sup>2</sup> UV at one of the two wavelengths and the irradiated half was manually rejoined to its unirradiated counterpart. Thus, in all, there were six treatment groups and a control group; each group made up of 30 hydra monitored individually in five replicate experiments, as follows:

Group I: Unirradiated fragments H and B joined (control).

Group II: Fragment H irradiated with UVC and joined to unirradiated fragment B.

Group III: Fragment B irradiated with UVC and joined with unirradiated fragment H.

Group IV: Both, fragments B and H irradiated with UVC and joined.

Group V: Fragment H irradiated with UVB and joined with unirradiated fragment B.

Group VI: Fragment B irradiated with UVB and joined with unirradiated fragment H.

Group VII: Both, fragments B and H irradiated with UVB and joined.

Each joined individual hydra was maintained separately, in 24 well plates and monitored for 15 days. The hydra were fed during this period.

**2.3c Irradiation and regeneration of parts of trisected hydra:** In a third experiment, the 3 pieces of a trisected hydra were irradiated separately at 500 J/M<sup>2</sup> UV at one of the two wavelengths and regeneration was monitored over a period of 5 days by using the criteria mentioned above. Corresponding unirradiated body pieces served as controls. The cultures were not fed during this period.

### 2.4 Statistical analysis

All the statistical analyses were performed using SPSS version 11.0 under Windows. Growth of irradiated hydra was compared with the control at both 254 and 312 nm using one-way ANOVA with post-hoc Dunnett's multiple comparison test. Time dependent dose response was also studied using one-way ANOVA and further by curve fitting to identify the nature of the response curve. Curvilinear regression analysis was carried out to study the nature of growth of hydra with increasing UV dose at both 254 and 312 nm.

## 3. Results

### 3.1 Effects of irradiation of whole hydra

Whole hydra irradiated either with UVC or UVB were monitored for a period of 8 days. The polyps continued to feed normally and did not exhibit any detectable deleterious effects of UV. Polyps exposed to UVC showed acceleration of growth as evident from increased budding resulting in more polyps (table 1). One-way ANOVA with post-hoc Dunnett's multiple comparison test indicated occurrence of significant acceleration of growth at 500 J/M<sup>2</sup> UVC ( $P = 0.0001$ ) as compared to unirradiated controls. The slight acceleration of growth observed due to UVB

(table 1), however, was not statistically significant. A dose-dependent response of hydra to UV irradiation was noted. The response to both UVC and UVB was curvilinear (4th degree polynomial with  $R^2 = 0.98$ ), with peaks at 100 and 500 J/M<sup>2</sup> and a trough at 200 J/M<sup>2</sup>. A trough was also observed in the case of 1000 J/M<sup>2</sup> UVC while a plateau was seen at 500 J/M<sup>2</sup> UVB (not shown). Time-dependent dose response was also studied. One-way ANOVA indicated that UV irradiation did not have any effect on growth as compared to controls at day 2. Thereafter the response increased in a parabolic fashion. Highest response was observed for 500 J/M<sup>2</sup> UVC. Polyps exposed to different doses of UVB showed growth comparable to the control. Thus, the results show that irradiation of whole polyps with 500 J/M<sup>2</sup> UVC leads to significant acceleration of growth in *P. oligactis*.

### 3.2 Irradiation and rejoining of parts of hydra

Experiments were designed to study whether growth promotion in UV irradiated polyps is due to preferential effects of UV on positional information in the anterior or posterior halves of the polyp. UVC or UVB irradiation of either fragment H or B and its rejoining with an unirradiated counterpart as well as irradiation and rejoining of both fragments was carried out and growth of the resulting polyps was monitored (table 2). Although irradiation of one or the other or both fragments with UVC appeared to lead to an increase in growth as compared to the controls, the effects were not statistically significant in any of the experiments using the two wavelengths. This was surprising since when both the head and base parts are irradiated and combined, one would expect an increase in budding

similar to that seen in an intact irradiated polyp. However, when the budding in control (unirradiated) intact polyps and in control (unirradiated) bisected and rejoined polyps was compared (table 3), it was found that the experimental procedure of cutting and rejoining of polyps itself induced significantly higher budding. This effect seems to have masked the effect of UVC on budding. The observation, on the other hand, supports our belief that budding in hydra is accelerated as a natural response to adverse environmental influences.

### 3.3 Effects of irradiation on parts of trisected hydra

In another experiment, effects of UV irradiation on regeneration of parts of trisected polyps were studied to see if the treatment interferes with pattern formation. Trisection of a hydra generated 3 fragments, a head fragment, a middle fragment and a basal fragment. UVC and UVB had no effect on pattern formation in the regenerating head and base fragments of a trisected hydra. Irradiated head fragments regenerated feet in 3 days as in unirradiated controls while irradiated basal fragments regenerated complete functional heads in 3 days like in the controls. Remarkable results were obtained in the UVC-irradiated middle pieces. Out of 24 middle pieces irradiated with UVC, seven showed the formation of ectopic feet (29%: figure 1). Out of these seven, two showed duplicated feet at the usual location (figure 1a,b), two showed 'twin' feet at the usual location (figure 1c,d), while the remaining three showed beginning of foot formation in the middle of the body column (figure 1e-g). Out of the last three, one had an unusually large area showing foot-specific staining in the middle of the body column in

**Table 1.** Growth of hydra in response to UV irradiation at 254 and 312 nm. A group of ten hydra was irradiated at either wavelength and their growth was observed over a period of eight days.

UV dose J/M <sup>2</sup>	Number of hydra				
	Day 0	Day 2	Day 4	Day 6	Day 8
254 nm					
0 (control)	10	10 ± 0	10.9 ± 0.83	13.9 ± 3.1	18.3 ± 3.3
100	10	10 ± 0	12.9 ± 2.30	18.9 ± 1.98	22.3 ± 3.06
200	10	10.1 ± 0.35	14.1 ± 3.23	18.5 ± 2.33	22.8 ± 4.03
500	10	10.4 ± 0.52	14.8 ± 4.1	22.9 ± 4.2	26.9 ± 6.2
1000	10	10.4 ± 0.52	14.9 ± 4.36	18.4 ± 3.6	22.6 ± 5.45
312 nm					
0 (control)	10	10 ± 0	10.9 ± 0.83	12.4 ± 1.85	14.0 ± 2.51
100	10	10.1 ± 0.35	11.3 ± 0.88	14.3 ± 3.85	16.6 ± 3.02
200	10	10.1 ± 0.35	11.5 ± 1.60	13.5 ± 3.11	15.3 ± 3.33
500	10	10.3 ± 0.70	11.8 ± 1.28	13.4 ± 1.19	15.5 ± 2.5
1000	10	10 ± 0	11.6 ± 0.74	13.5 ± 1.69	15.9 ± 1.55

Values are expressed as mean ± SD for eight replicates.

**Table 2.** Growth of *P. oligactis* in response to UV irradiation (either at 254 and 312 nm) of specific body parts. Hydra were cut into two fragments [0–50% (fragment H) and 50–100% (fragment B) body length] and were rejoined. Each hydra was maintained separately and followed for next fifteen days for growth in culture.

	Number of hydra						
	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13	Day 15
254 nm							
HB	1.3 ± 0.53	1.6 ± 0.65	2.0 ± 0.83	2.8 ± 1.02	3.2 ± 1.25	3.9 ± 1.25	4.5 ± 1.35
HuvB	1.3 ± 0.44	1.7 ± 0.56	2.3 ± 0.96	3.1 ± 1.28	4.0 ± 1.57	4.7 ± 1.57	5.4 ± 2.18
HBuv	1.3 ± 0.46	1.8 ± 0.65	2.8 ± 1.06	3.1 ± 1.17	3.8 ± 1.18	4.4 ± 1.47	5.1 ± 1.98
HuvBuv	1.3 ± 0.46	1.8 ± 0.56	2.5 ± 0.88	3.1 ± 0.89	3.9 ± 0.99	4.3 ± 1.37	5.1 ± 1.58
312 nm							
HB	1.6 ± 0.65	2.0 ± 0.69	2.7 ± 0.99	3.5 ± 1.22	4.5 ± 1.50	5.1 ± 1.62	5.9 ± 1.74
HuvB	1.6 ± 0.65	2.1 ± 0.68	2.8 ± 1.20	3.4 ± 1.50	4.0 ± 1.43	4.4 ± 1.64	4.9 ± 1.51
HBuv	1.6 ± 0.72	2.3 ± 0.68	3.0 ± 1.24	3.5 ± 1.53	3.9 ± 1.59	4.3 ± 1.93	5.0 ± 1.88
HuvBuv	1.7 ± 0.70	2.3 ± 0.70	2.9 ± 0.62	3.4 ± 0.88	4.1 ± 1.43	4.7 ± 1.87	5.5 ± 1.79

Each time point represents data of 30 individual hydra used in five replicates. HB, Neither fragment H nor B was irradiated; HuvB, fragment H was irradiated but not fragment B; HBuv, fragment B was irradiated but not fragment H; HuvBuv, both fragments H and B were irradiated.

**Table 3.** Comparison of budding in unirradiated intact polyp and bisected and rejoined polyps of *P. oligactis*.

	Number of hydra		
	Day 3	Day 5	Day 7
Intact polyp	1.0375 ± 0.01548	1.2563 ± 0.06122	1.5000 ± 0.08563
Bisected and rejoined polyps	1.3667 ± 0.07504	1.7500 ± 0.08774	2.3000 ± 0.12866

Bisection followed by rejoining stimulated budding. Stimulation of budding was statistically significant by Mann-Whitney test ( $P < 0.05$ ) for day 5 and 7. Data expressed as mean ± SE.

addition to the normal foot (figure 1g). The remaining 17 irradiated middle pieces did not exhibit ectopic foot formation (figure 1h). In none of the unirradiated middle pieces, ectopic foot formation was noted. The results show that UVC interferes with pattern formation in a significantly large proportion of regenerating middle pieces and tilts the positional value in favour of foot formation.

#### 4. Discussion

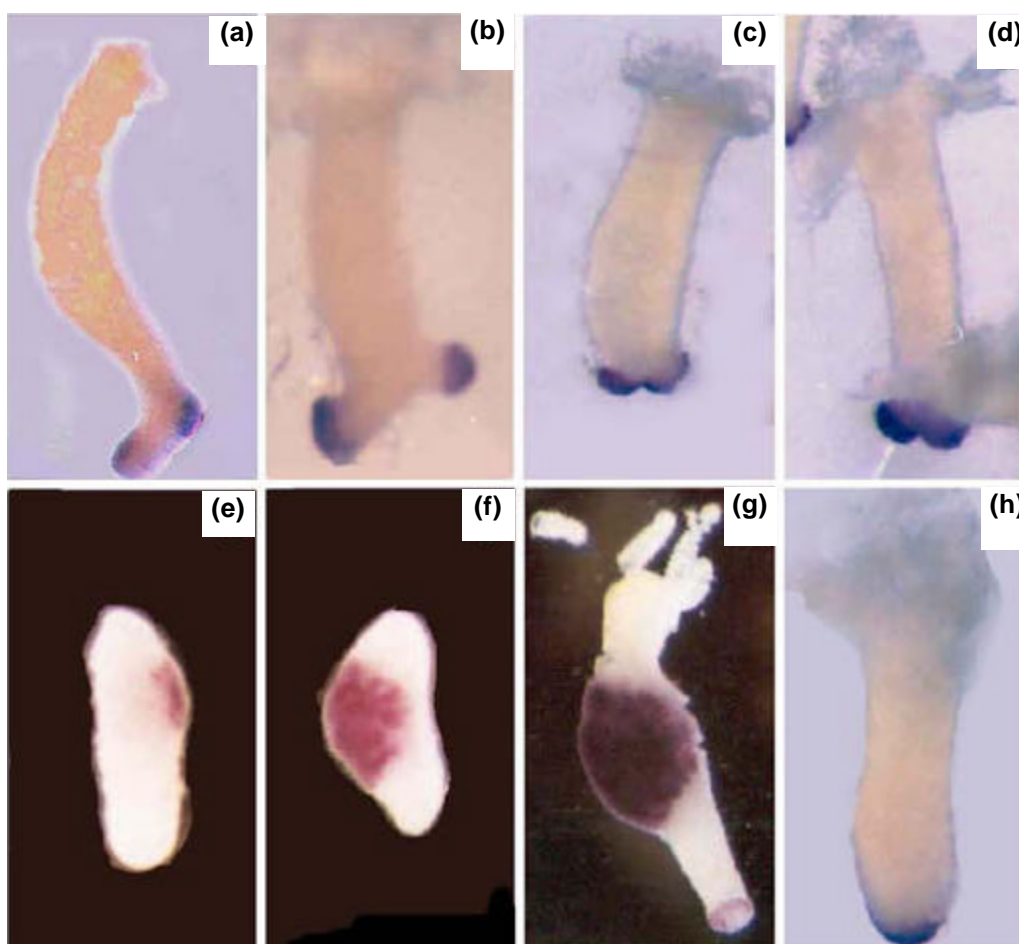
Cnidaria is the most 'elementary' animal phylum, which contains members having a defined body plan including an axis, a nervous system and tissue layers. Hydra, a member of Cnidaria, has been extensively used to study diverse aspects of development of multicellular organisms. The reasons for this are primarily two. First, cells are continuously sloughed off from the oral and aboral ends of hydra and are replaced by other cells arising from stem cell populations present in the body column, thereby

permitting us to study tissue dynamics and cell differentiation. The second reason is that body parts of hydra possess a remarkable ability to regenerate. This provides an ideal situation in which to examine pattern formation, normally an embryonic phenomenon, in the adult (Okada 1996; Berking 1998; Bode 2003). Moreover, the hypostome of hydra is an organizer: upon transplantation it can induce a new body axis (Browne 1909). A large number of exogenous agents have been studied for their effects on pattern formation in hydra, and this has helped to elucidate biochemical pathways involved in the generation and action of morphogens (Berking 1998). We studied the effects of UV irradiation in intact and regenerating hydra with a view to gaining new insights into pattern formation in hydra.

The most significant finding of the present study is the ability of UVC to induce ectopic foot formation in a significant proportion of regenerating middle pieces of trisected hydra. Head formation in regenerating hydra has been studied extensively and is believed to result from a

head-activator gradient (Berking 1998). Much less is known about foot formation and regeneration and the relationship between foot formation and the morphogenetic gradient is unclear (Bode 2003). Even today the regulation of foot formation is poorly understood. An intriguing possibility for a mechanism through which UVC could induce ectopic foot formation involves meprins. Meprins are complex, multidomain, oligomeric metalloproteinases that are highly regulated at the transcriptional and post-translational levels and have an important role in cell-extracellular matrix interactions (Norman *et al* 2003). Matrix metalloproteinases have been implicated in foot formation in hydra. The signal peptide pedibin that stimulates foot formation and regeneration (Hoffmeister-Ullerich 2001) has been shown to downregulate Farm1, a matrix metalloproteinase expressed in the gastric region (Kumpfmüller *et al* 1999). Two matrix metalloproteases,

hydra metalloproteinase 2 (HMP 2, Yan *et al* 2000) and a hydra matrix metalloproteinase (HMMP, Leontovich *et al* 2000) have been shown to be essential for foot formation. Inhibition of HMP-2 and HMMP by introduction of antisense constructs leads to inhibition of foot formation suggesting that their effects on foot formation may be through their action on matrix components. Interestingly, UV brings about upregulation of metalloproteinases in the human skin (Brenneisen *et al* 2002, Brennan *et al* 2003). It is therefore likely that UVC exposure of regenerating middle pieces leads to an upregulation of metalloproteinases ultimately leading to ectopic foot formation. It is not clear why the anterior (head) and the posterior (base) parts of the trisected hydra do not respond to UVC exposure in a similar manner. It could be due to the influence of organizing centers, a hypostome or a base, in these pieces, which the middle pieces lack.



**Figure 1.** Pieces of trisected hydra were irradiated separately at  $500 \text{ J/M}^2$  UV at 254 nm and regeneration was monitored over a period of 5 days. UV irradiation was found to induce ectopic foot formation in the middle pieces. Out of seven such cases, two showed duplicated feet at the usual location (a, b), two showed 'twin' feet at the usual location (c, d), while the remaining three showed beginning of foot formation in the middle of the body column (e–g). Note an unusually large area showing foot-specific staining in the middle of the body column in addition to the normal foot in one case (g). The remaining 17 irradiated middle pieces did not exhibit ectopic foot formation (h).

Another possible mechanism of promotion of foot formation concerns Wnt signalling. Wnt signalling is responsible for the formation of the dorsoventral axis in *Drosophila* and vertebrates (reviewed in Cadigan and Nusse 1997) and it is inhibited by Dickkopf-1, a secreted product of the *Dkk-1* gene (Glinka et al 1998). *Dkk-1* is heavily upregulated by UV in the chick limb bud (Grotewold and R  ther 2002). The Wnt signalling pathway is active in hydra and participates in head formation (Hobmayer et al 2000). A *Dickkopf-3*-related gene, *HyDkk-3*, has recently been found in hydra (Fedders et al 2004) although so far it has been shown to be expressed only in the nematocysts in the gastric region. If *HyDkk-3* product is an inhibitor of Wnt signalling in hydra, it is conceivable that its upregulation by UV would suppress head formation in the regenerating middle pieces, thus favouring foot formation. It will be interesting to test if one or both these possible mechanisms of UV action on pattern formation in regenerating hydra are active.

Irradiation of intact polyps of *P. oligactis* with UVC leads to stimulation of growth as seen from the significantly greater number of hydra arising out of the irradiated ones (table 1). This may be a natural survival response of hydra. Even the polyps that were bisected and rejoined exhibited a similar response indicating that adverse environmental influences elicit a natural budding response from these animals.

The present study demonstrates novel effects of UV irradiation on pattern formation in regenerating hydra that lead to ectopic foot formation at multiple sites. UV treatment can thus be fruitfully employed as a tool in dissecting out the cellular and molecular regulation of pattern formation in the enigmatic hydra.

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