Induction of larval settlement by KCl in three species of *Bugula* (Bryozoa)

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Abstract. Elevation of the K^+ concentration in seawater (added as KCl) induces larval settlement and metamorphosis in *Bugula simplex*, *B. stolonifera*, and *B. turrita*. All three of these bryozoan species have similar bell-shaped dose-response curves: 5 mM excess K^+ is sufficient to increase settlement and metamorphosis significantly over seawater controls in all the species and optimal responses to excess K^+ concentrations occur at 10–25 mM, 5–10 mM, and 10–15 mM for *B. simplex*, *B. stolonifera*, and *B. turrita*, respectively. Percent settlement in all three species is decreased at levels greater than 25 mM excess KCl. Similar bell-shaped curves have been observed for KCl-induced settlement of larvae from other, but not all, invertebrate groups. Larvae of *B. turrita* do not settle at a significantly higher level than seawater controls when exposed to excess K^+ concentrations >25 mM even in the presence of a second favorable artificial inducer, filmed chicken eggshell membrane, indicating that K^+ at high concentrations interferes with the normal settlement process. Furthermore, pulse exposures for 5 min, 15 min, and 30 min to 10 mM excess K^+ are ineffective at inducing settlement over levels in seawater controls. When larvae of *B. turrita* were induced to settle by 10 mM excess K^+ , completion of metamorphosis was not significantly different from that of controls lacking excess KCl (86% and 91%, respectively). However, if larvae induced by 10 mM excess K^+ are not removed from the 10 mM excess K^+ after settlement, the success ratio of metamorphosis is significantly lower than in controls (72% and 91%, respectively). Our results confirm previous studies demonstrating that settlement of bryozoan larvae can be induced by excess K^+ . Bryozoan larvae examined to date become responsive around 5–10 mM excess K^+ . These data suggest that shared underlying features exist in the mechanism triggering settlement and metamorphosis in bryozoans and that excess K^+ is a useful experimental tool for inducing settlement of bryozoan larvae.

Additional key words: metamorphosis, Cheilostomata, *Bugula simplex*, *B. stolonifera*, *B. turrita*

Many benthic marine invertebrates possess a larval stage in their life cycle. The larval stage is concluded with metamorphosis to the juvenile stage, a process often mediated by cues from the environment (Crisp 1974; Scheltema 1974; Chia & Rice 1978; Chia 1989). The isolation and characterization of such cues triggering this transformation have motivated much research (for a review see Pawlik 1992; Gibson & Chia 1994). However, with few exceptions, the identification of natural inducers has remained elusive. As a result of this difficulty, researchers have used various artificial means for triggering settlement and/or metamorphosis in order to gain insight into these processes. Organic compounds are one such category of inducers (e.g., Morse et al. 1979; Baloun & Morse 1984; Pawlik 1986, 1990; Rittschof et al. 1986; Yool et al. 1986; Pennington & Hadfield 1989; Tegtmeyer & Rittschof 1989; Zimmer-Faust & Tamburi 1994). In addition to

organic compounds, certain inorganic cations such as K^+ are effective in initiating settlement and/or metamorphosis of many invertebrates (Pawlik 1992; Pechenik & Gee 1993; Pearce & Scheibling 1994). K^+ , however, is not a universal trigger, as elevated K^+ apparently does not affect settlement and metamorphosis in anthozoans (Morse et al. 1988), some bivalves (Eyster & Pechenik 1987; Gustafson et al. 1991), and ascidians (Grave & Nicoll 1939). Indeed, elevated levels of K^+ apparently inhibit settlement of barnacle cyprids (Rittschof et al. 1986) and some ascidian tadpoles (Lynch 1961). Present data do reveal an overall phylogenetic pattern to the KCl response. For example, in molluscs, although all gastropod larvae examined metamorphose in response to elevated K^+ levels, larvae of most bivalves do not (an exception is *Saccostrea commercialis* [Nell & Holiday 1986]). Similarly, in cnidarians, settlement can be triggered by K^+ in hydrozoans, but not in anthozoans.

The goal of the present study is to assess the efficacy of K^+ as an artificial inducer of settlement and metamorphosis in the cheilostomes *Bugula simplex*, *B. stolonifera*, and *B. turrita*. Effective inducers of settlement are important agents in studies investigating larval energetics, metamorphic competence, and effects of larval swimming duration on subsequent juvenile success, as these types of studies depend on the ability to induce metamorphosis reliably. Excess potassium ions potentially offer one such experimental tool.

Settlement of bryozoan larvae is tightly coupled to metamorphosis in that settlement is irreversible and is marked by eversion of the metasomal (internal) sac, the first morphogenetic movement of metamorphosis in larvae of *Bugula* spp. and other bryozoans (Zimmer & Woollacott 1977). Thus, settlement in bryozoans is not exclusively a behavioral change associated with substratum exploration; instead, it is a clearly defined process whereby larvae permanently attach to the substratum. We considered a larva settled only after attachment to the substratum via eversion of its metasomal sac.

Eiben (1976) observed that KCl induced larval settlement in the ctenostome *Bowerbankia gracilis*, as did Lynch (1947) with the cheilostome *Bugula neritina*, and Stricker (1989) with the cheilostome *Membranipora membranacea*. In contrast, Lynch (1949) reported that larvae of *B. simplex* (as *B. flabellata* RYLAND 1958) from Woods Hole, Massachusetts, were inhibited by concentrations of excess K^+ greater than 100 mM. In the present study, we: (1) re-examine, at lower concentrations, the effectiveness of excess K^+ in triggering settlement of larvae of *B. simplex* from Woods Hole; (2) extend these investigations to larvae of its sympatric congeners *B. stolonifera* and *B. turrita*; (3) assess the effect of elevated concentrations of K^+ on inhibiting settlement in the presence of a second artificial inducer; (4) examine the effectiveness of pulse vs. continuous exposure of larvae of *B. turrita* to excess K^+ ; and (5) determine the effect of exposure to excess K^+ on the ability of individuals to complete metamorphosis.

Methods

Gravid colonies of *Bugula simplex* HINCKS 1886, *B. stolonifera* RYLAND 1960, and *B. turrita* DESOR 1848 were collected over three reproductive seasons (summers 1993, 1994, 1995) from the undersides of floating docks in Eel Pond, Woods Hole, Massachusetts (41°26' N, 70°40' W). Colonies were kept in plastic aquariums at 20° C in darkness with continuous aeration and they were re-used as a source of larvae over a 5-day period. No food was provided.

To obtain larvae, colonies were removed from the dark, placed in glass bowls (about 1.5 l), and exposed to a combination of natural and fluorescent light. Several parent colonies were used simultaneously to increase the genetic heterogeneity in the population of larvae used for experiments. Within 15 min of illumination, larvae appeared and by 1 h, often hundreds of positively phototactic larvae were released. The phototactic behavior of larvae facilitated their collection, as they aggregated on the illuminated side of dishes. Groups of 15 to 75 larvae were pipetted into each treatment vessel. Experiments were started within an hour after the appearance of larvae; therefore, larvae differed in age by at most 60 min within a treatment.

Experiments were conducted in 30-ml acid-washed glass Stendor dishes at 20° C under constant fluorescent illumination provided by ceiling lamps. For all experiments with *B. simplex* and *B. turrita*, we used MBL artificial seawater (see Cavanaugh 1956; K^+ is 9 mM in MBL seawater); with *B. stolonifera*, we used Eel Pond water, as preliminary tests demonstrated that larvae of *B. stolonifera* behaved abnormally in MBL artificial seawater. Excess K^+ was added as KCl. Concentrations from 0 mM to 40 mM excess KCl were used to generate dose-response curves. Each trial also included providing larvae with a substratum of filmed chicken eggshell membrane, a known artificial inducer of settlement in bryozoans (Hasper 1911). In the absence of the artificial substratum, larvae settled on the walls and bottom of the glass dishes and on the air-water interface. Experiments were ended when settlement had reached about 50% settlement in the eggshell-membrane condition. Experiments with *B. simplex* and *B. stolonifera* continued for approximately 3–4 h, while those with *B. turrita* required 12 h.

Additional experiments with larvae of *B. turrita* tested the effects of elevated levels of K^+ on the responses of larvae to a known second inducer of settlement, eggshell membrane. Membranes were filmed by placing them in Eel Pond seawater for at least 24 h. In these experiments, larvae were exposed to 25 mM and 35 mM excess K^+ in the presence of eggshell membrane under the same experimental conditions outlined above except that Eel Pond seawater was used in place of MBL artificial seawater.

We also tested the efficacy of pulse exposures to excess K^+ at inducing settlement in larvae of *B. turrita*. Larvae were exposed to 10 mM excess K^+ for 5 min, 15 min, or 30 min. After the pulse, larvae were taken through two 50-ml washes in MBL seawater and transferred to a 30-ml acid-washed glass Stendor dish containing a polystyrene substratum in 20 ml MBL seawater. The maximum final concentration of excess

Fig. 1. Induction of settlement in three bryozoan species (*Bugula*) on a substratum (S; filmed eggshell membrane) without added KCl, or at concentrations of 0–40 mM excess KCl in the absence of a filmed substratum. Error bars = 1 Std. Err.

K⁺ was less than 0.1 mM. A control condition was identical to the above treatments except that the larvae were not exposed to excess K⁺. Settled larvae were counted 24 h after the pulse exposure.

To assess potential deleterious effects of excess K⁺ on metamorphosis, we induced settlement of larvae of

B. turrita using 10 mM excess K⁺ and then (1) transferred them to MBL artificial seawater; or (2) transferred them to MBL seawater containing 10 mM excess K⁺. In an additional control treatment, larvae initiated metamorphosis in the absence of excess K⁺ and were then transferred to MBL seawater. In all treatments, larvae were offered polystyrene, a known effective artificial substratum (Woollacott et al. 1989). Settlement was allowed to occur for 5 hours, after which time the attached larvae were transferred to their respective solutions. After 4 days, the feeding ancestrulae were counted and this number was expressed as a fraction of the total number that initiated metamorphosis. The number of individuals in each treatment ranged from 14 to 189.

In all of our experiments, the number of larvae settled in each treatment was converted to percent settlement. Data from within treatments were found not to differ significantly from a normal distribution. A factorial ANOVA was performed to identify heterogeneity of variances within the data set. Fisher's PLSD, a post hoc test, was used to identify the sources of such variation among the data.

Results

In all three species of *Bugula* we studied, induction of settlement showed a positive trend at low concentrations of excess K⁺ and a negative trend at high concentrations (Fig. 1). The negative trend most likely reflects inhibition at higher concentrations, as larval behavior was drastically altered at levels above 25 mM excess K⁺. At these concentrations and higher, larvae sank to the bottom of the dish and moved in a sluggish manner. Although development was not consistently followed in these dose-response experiments, we generally observed that the larvae which initiated metamorphosis at high concentrations (35–45 mM) did not complete it; rather development was arrested sometime shortly after eversion of the internal (metasomal) sac. Dose-response curves of the three species were generally similar, but exhibited some subtle differences. Analysis of variance revealed significant heterogeneity of variances among treatments for each species (Table 1). For *B. simplex*, the maximum response occurred at 10–25 mM excess K⁺; for *B. stolonifera*, 5–10 mM excess K⁺; and for *B. turrita*, 10–15 mM excess K⁺ (Table 2). These data indicate that *B. stolonifera* reached its maximum response at lower concentrations; however, the absolute maximum response for *B. stolonifera* at 5 mM was not greater than that of *B. simplex* or *B. turrita*. Indeed, at 10 mM, it was lower than both *B. simplex* and *B. turrita*. Additionally, inhibition of settlement was found for all three species

Table 1. One factor ANOVA results of the effect of various KCl concentrations on the initiation of settlement in *Bugula simplex* (n = 6), *B. stolonifera* (n = 5), and *B. turrita* (n = 6).

Species	df	F-test	p-value
<i>B. simplex</i>	59	9.88	0.0001
<i>B. stolonifera</i>	49	24.16	0.0001
<i>B. turrita</i>	59	12.19	0.0001

Table 2. Means and standard errors of percent settlement in controls and excess KCl concentrations for *B. simplex*, *B. stolonifera*, and *B. turrita*. Any pair of means enclosed by the range of any one bar are not significantly different at the 95% confidence level (Fisher PLSD post hoc test).

Condition	Mean settlement	Standard error
<i>Bugula simplex</i> (n = 6)		
20mM	84.7	7.0
15mM	80.9	8.1
10mM	75.5	11.5
25mM	62.0	6.8
5mM	58.0	11.0
Eggshell	33.6	12.0
Eel Pond	34.1	15.3
30mM	30.3	3.9
0mM	27.5	11.0
35mM	16.5	6.2
40mM	2.8	1.5
<i>Bugula stolonifera</i>		
10mM	62.6	5.6
5mM	52.0	6.5
Eggshell	51.8	8.6
0mM	19.6	6.2
15mM	26.8	5.6
20mM	9.4	1.1
25mM	3.6	1.4
30mM	2.8	.97
35mM	5.0	.84
40mM	4.4	1.3
<i>Bugula turrita</i> (n = 6)		
10mM	71.2	7.5
15mM	64.0	5.8
Eggshell	58.7	3.8
5mM	52.0	11.3
20mM	45.2	8.5
Eel Pond	26.5	9.1
0mM	25.8	5.8
25mM	23.8	5.7
30mM	16.7	5.3
35mM	16.0	3.1
40mM	9.2	2.0

at higher concentrations of excess K⁺. *Bugula stolonifera*, which reached its maximum response at 5 mM excess K⁺, exhibited a precipitous decline in settlement at concentrations greater than 10 mM. *Bugula simplex* and *B. turrita* showed significant declines at concentrations greater than 25 mM and 15 mM, respectively (Fig. 1).

Continuous exposure of larvae of *B. turrita* to 10 mM excess K⁺ increased settlement significantly over seawater controls (t=4.8; p=0.001) (Fig. 2). However, pulse exposures to 10 mM excess K⁺ for 5 min, 15 min, and 30 min were ineffective at triggering settlement over the seawater control condition (ANOVA; F=0.198, p=0.896; N=5) (Fig. 2).

In the presence of eggshell membrane (em) alone, settlement of *B. turrita* was significantly greater (p<0.05) than in controls (Eel Pond seawater without filmed substratum), an indication that larvae were competent to settle and were responsive to this artificial substratum. However, at excess K⁺ concentrations of 25 mM and 35 mM, the presence of eggshell membrane did not have a significant effect on triggering settlement—mean settlement $\pm$ 1 S. E.: 25 mM, 18.0 $\pm$ 5.6; 25 mM & em, 18.6 $\pm$ 4.8; 35 mM, 14.1 $\pm$ 7.7; 35 mM & em, 12.0 $\pm$ 2.5 (ANOVA; F=1, p=0.3530; N=6).

Larvae of *B. turrita* that settled on polystyrene substrata in the presence or absence of 10 mM excess K⁺ did not differ significantly in completing metamorphosis (86% and 91%, respectively; Fisher PLSD, p=0.495). However, if larvae induced by 10 mM excess K⁺ were not removed from 10 mM K⁺ solution and placed in seawater lacking excess KCl, the percent completing metamorphosis was significantly lower than that in the control condition (72% and 91%, respectively; Fisher PLSD p=0.013). ANOVA showed significant heterogeneity of variances between the three conditions (F=4.08, p=0.035; N=7 [N=6 for continuous K⁺ condition]).

Discussion

Continuous exposure to excess K⁺ induced larval settlement in *Bugula simplex*, *B. stolonifera*, and *B. turrita* optimally at 10–25 mM, 5–10 mM, and 10–15 mM, respectively. Our findings with *B. simplex* confirm an earlier report by Lynch (1949) indicating that settlement was inhibited by excess K⁺ at concentrations in excess of 100 mM. Our data (Fig. 1) demonstrate that concentrations of excess K⁺ above 25 mM are inhibitory and that the 100 mM concentration used by Lynch is well beyond the maximum sensitivity of *B. simplex*. At the same time, our results do not support Lynch's (1949) conclusion that potassium inhibits

Fig. 2. *Bugula turrita*. Pulse or continuous exposure of larvae to 10 mM excess K^+ . Pulses were for 5, 15, or 30 min; larvae were then washed twice in 50 ml MBL seawater and transferred to 30-ml glass Stendor dishes containing a polystyrene substratum in MBL. S=seawater control in which larvae were *not* exposed to 10 mM excess KCl; after 30 min they were washed and transferred as in the pulse conditions. C=continuous exposure: larvae were exposed to 10 mM excess KCl for 30 min, washed twice in 50 ml of MBL seawater containing 10 mM excess KCl and transferred to 30-ml Stendor dishes containing 10 mM excess KCl and a polystyrene substratum. Error Bars = 1 Std. Err.; N = 5.

metamorphosis; 10–25 mM excess K^+ clearly promoted settlement in *B. simplex* (Fig. 1).

Our data show that at low levels of excess K^+ (5 mM), larval settlement of all three species is significantly higher than in control treatments (Table 2). Thus, larval settlement is induced by elevated KCl in all species of bryozoans examined to date. Moreover, all larvae respond maximally around 10 and 20 mM excess KCl: *Bowerbankia gracilis*, 11.6 mM (Eiben 1976); *B. simplex*, 10–25 mM (this study); *B. stolonifera*, 5–10 mM (this study); *B. turrita*, 10–15 mM (this study); and *Membranipora membranacea*, 10 mM (Stricker 1989). That bryozoan larvae respond so consistently to elevated K^+ suggests a commonality in the underlying mechanism by which excess K^+ induces settlement.

We also document that pulse exposures of 5, 15, or 30 min to 10 mM K^+ are not sufficient to trigger settlement, but that continuous exposure is required (Fig.

2). Pechenik & Heyman (1987) similarly found that larvae of the gastropod *Crepidula fornicata* did not metamorphose in response to “brief” (1–5 h) exposures to 5–50 mM excess KCl. These data suggest that the mechanism of K^+ induction is not analogous to the irreversible resetting of a switch or other such transient event.

Our results further demonstrate that concentrations of KCl greater than 25 mM prevent settlement in the presence of a favorable artificial inducer. Yool et al. (1986) obtained similar results with the gastropods *Phestilla sibogae* and *Haliotis rufescens* when they exposed larvae simultaneously to known natural inducers and K^+ (>25 mM excess). These data suggest that elevated K^+ interferes with settlement and/or metamorphosis. Moreover, 20 mM excess K^+ and a natural inducer, when presented simultaneously, acted antagonistically in 4 out of 6 cases with *Phestilla sibogae* (Pechenik et al. 1995).

Although we did not explicitly test whether settlement can be induced by increased Cl^- or changes in osmotic pressure, previous studies have examined and eliminated these possibilities in a wide variety of invertebrate taxa (Baloun & Morse 1984; Rittschof et al. 1986; Yool et al. 1986; Todd et al. 1991). Furthermore, an increase of 50 mM excess KCl, a level 10 mM in excess of the maximum used in our experiments, increases osmolarity of seawater by <2% (Pechenik & Heyman 1987).

In all bryozoan larvae examined to date (Eiben 1976; Stricker 1989; this study), a decline in settlement occurs beyond KCl concentrations of 15–25 mM. Yool et al. (1986) observed similar declines in inductive responses with *H. rufescens* (gastropod), *P. sibogae* (gastropod), *Astraea undosa* (gastropod), and *Phragmatopoma californica* (polychaete); as did Todd et al. (1991) with *Adalaria proxima* (gastropod), and Pearce & Scheibling (1994) with *Echinarachnius parma* (echinoid).

The shape of the K^+ dose-response curve may be conceptualized as two superimposed curves; an upward inducer curve at lower concentrations which is dominated at higher concentrations by a downward inhibitory curve. We speculate that at a given concentration of excess K^+ , some individuals in the population are induced to settle while others, which are more sensitive to the injurious effects of K^+ , are inhibited. This model provides a framework in which to interpret the response curves of bryozoans and other invertebrates. For example, the model explains the relatively broad maximal response (a plateau) of *B. simplex* (10–25 mM) as a low sensitivity to the deleterious effects of excess K^+ , whereas *B. stolonifera* (5–10 mM), which has a much narrower maximal response curve

(a peak), is likely more sensitive to K^+ . Indeed, our data suggest the dose-response curves of the three bryozoan species are the result of species-specific sensitivities to the deleterious effects of excess K^+ . That is, the three species become responsive to K^+ at the same concentrations (5 mM), but they develop inhibition at differing concentrations: >25 mM for *B. simplex*, >10 mM for *B. stolonifera*, and >15 mM for *B. turrita*. The mechanism by which K^+ exerts its injurious effects is unknown, but may involve the inability of larvae to maintain ionic balance over their epithelial surfaces. Baloun & Morse (1984) suggested that the settlement response is due to selective depolarization of receptor cells by K^+ influx through calcium-mediated potassium-ion channels. Presumably, at pre-inhibitory K^+ concentrations, the frequency of depolarization of these cells is increased such that the normal settlement event is elicited. It is possible, however, that inhibition develops when the gradient between external and internal K^+ concentrations is such that the animal cannot maintain normal ionic balance over its epithelial surface. Thus, the negative slope of the dose-response curve may be a result of K^+ toxicity.

Larvae of *Bugula turrita* induced to settle by 10 mM excess K^+ and then transferred to MBL seawater completed metamorphosis 86% of the time, compared to 91% for larvae induced to settle without exposure to excess K^+ . These percentages are not significantly different and are similar to those observed by Woollacott et al. (1989) with larvae of *B. stolonifera* which were induced to settle in the absence of K^+ ($>90\%$). In contrast, individuals of *B. turrita* exposed to 10 mM excess K^+ for the duration of metamorphosis are significantly affected (only 72% completion). This result is not surprising in that successful completion of metamorphosis in *Bugula* spp. depends on an intricate series of coordinated muscular and microfilament-generated movements (Woollacott & Zimmer 1971; Reed & Woollacott 1982, 1983), processes likely affected by elevated exogenous cations. All juveniles that did complete metamorphosis, however, appeared anatomically normal and began feeding. This observation is in accord with the findings of Eyster & Pechenik (1988) that the growth, respiration, and feeding rates of juveniles of the gastropod *Crepidula fornicata* did not differ significantly following natural or KCl-triggered metamorphosis. Our data suggest that excess K^+ is not detrimental to development when newly metamorphosed individuals are removed from the excess K^+ solution after settlement. Thus, exposure to 10 mM excess K^+ followed by transfer to normal seawater after settlement is an effective method to control the duration of the swimming stage in larvae of *B. turrita* and possibly other bryozoans. Such ability to control

larval swimming duration is necessary when investigating the energetics of swimming and metamorphosis and the effects of larval swimming duration on juvenile fitness.

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