Neurophysiological control of swimming behavior, attachment and metamorphosis in

black-footed abalone (*Haliotis iris*) larvae

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Abstract

Experiments were conducted to test the effect of a range of chemicals on larval responses in swimming behavior, attachment, and metamorphosis of the black-footed abalone (*Haliotis iris*). The effect of antibiotics on larval survival was first tested within negative (filtered seawater) and positive (GABA at 10^{-5}, 10^{-4}, and 10^{-3} mol L^{-1}) control assays over 3 days. This experiment corroborated the effectiveness of using antibiotics to improve survival of larvae without obvious synergistic interactions with the GABA inducer or confounding effects of potential bacterial interactions. Chemical treatments (acetylcholine, potassium chloride, dopamine, and glutamine) were then tested at various concentrations for their ability to modulate swimming behavior and induce larval attachment and metamorphosis over 14 days. Generally, larval state shifted from swimming to attached, and from attached to metamorphosed in the control and treatments over time. However, the peak percentage of attached and metamorphosed larvae varied in time among chemicals and concentrations. While overall percent metamorphosis was minimally enhanced after 14 days of exposure to some chemical treatments at certain concentrations, all treatments displayed significant capacities to down-regulate larval swimming and induce early attachment and metamorphosis. Mortality was recorded throughout the duration of the experiment, and was generally low (<20%) across controls and most treatments for exposures of less than 12 days. Interpretations of specific results from this study are used to elucidate neurophysiological control of larval activities for this abalone species. Comparisons with other marine

invertebrates highlight the specificities of chemical cues and endogenous regulatory mechanisms across relatively closely related taxa.

Introduction

Chemoreception is believed to facilitate larval settlement processes and metamorphosis of a great number of marine invertebrate taxa, including polychaetes (Chung et al. 2010), bryozoans (Yu et al. 2007), sea urchins (Swanson et al. 2006), corals (Birrell et al. 2008), barnacles (Khandeparker & Anil 2011), bivalves (Alfaro et al. 2006; Ganesan et al. 2010, 2012a,b) and gastropods (Williams et al. 2009). Numerous studies have been conducted to test the effects of 'natural' (algal surfaces and bacterial biofilms) and 'artificial' (neuroactive compounds) inducers of abalone larval settlement and metamorphosis (Wang et al. 2010; Yu et al. 2010; Tung & Alfaro 2011). Such studies have been used to generate several reviews on the topic (e.g., Roberts 2001; Daume 2006; Qian et al. 2007; Hadfield 2011), with the general consensus that abalone larvae settle and recruit on crustose coralline algae where bacterial and diatom biofilms are in abundance. However, the involvement of biofilms in the facilitation of settlement and metamorphosis by crustose coralline algae is questionable for some abalone species (Huggett et al. 2005; Roberts et al. 2007, 2010). Furthermore, specific settlement-inducing compounds within these natural surfaces have rarely been identified (but see Morse et al. 1980; Suenaga et al. 2004).

Another approach to investigate chemoreception and associated transduction mechanisms has been to test specific pharmacologically active compounds for their ability to induce settlement and metamorphosis. The amino acid and neurotransmitter γ-amino butyric acid (GABA) consistently has been shown to induce larval attachment and metamorphosis of several abalone species (Morse 1990; Roberts & Nicholson 1997; Bryan & Qian 1998; Moss

1999). While a pure-form of GABA is unlikely to be an environmental cue (Kaspar &

Mountfort 1995; Roberts & Watts 2010), abalone possess externally accessible chemosensory

receptors which recognize GABA analogues and GABA-mimetic peptides extracted from

crustose coralline algae (Morse et al. 1979, 1980; Trapido-Rosenthal & Morse 1986; Morse

1990). In *Haliotis asinina* larvae, Stewart et al. (2011) recently showed that expression of a

$GABA_A$ receptor is up-regulated during onset of settlement competency, and the authors

hypothesize a chemosensory function. A range of other neurotransmitters and hormones also

has been usefully employed as treatments to assist elucidation of endogenous molecular

mechanisms involved in the metamorphic transition and pre-metamorphic processes. Such

treatments most likely target receptors directly at sites downstream from primary

chemoreceptors, or act as precursors in the biosynthesis of neuroactive ligands (Young et al.

2011). Interestingly, for some marine invertebrate species, certain compounds (e.g., L-

DOPA) are specific inducers of attachment rather than metamorphosis, and *vice versa* (e.g.,

epinephrine) (Bonar et al. 1990). In abalone, Roberts and Nicholson (1997) found that KCl

(10 mmol L^{-1}) solutions induced between 50-70% of *H. iris* larvae to attach, while less than

10% of these larvae metamorphosed. Synergistic effects also may take place when

combinations of cues are used, especially when biofilms are involved (Roberts and Nicholson

1997; Roberts et al. 2007, 2010). Thus, induction of abalone larval attachment and

metamorphosis has been suggested to be triggered by separate cues (Roberts and Nicholson

1997; Bryan and Qian 1998) acting on independent or interacting biochemical pathways

(Morse 1990; Roberts 2001).

The black-foot abalone, *Haliotis iris*, is endemic to New Zealand, and accounts for all

current commercial abalone cultivation in the country. Aquaculture production of abalone in

New Zealand relies entirely on land-based operations, within about 14 farms. Hatchery and

nursery facilities are used to develop juveniles, which are fed diatom biofilms and then

weaned onto artificial feeds (Dyck et al. 2011). While some work has been conducted to identify chemical inducers of larval attachment and metamorphosis, more research is needed to determine the mechanism by which environmental cues are interpreted by chemosensory organs to generate behavioural responses and initiate regulatory developmental programs. Such information could maximize the potential use of inducers throughout the commercial larval rearing process and provide insight into the evolution of the neural circuitry involved in these conserved larval traits. Thus, the aim of this paper is to identify the effect of compounds (GABA, acetylcholine, potassium chloride, dopamine, and glutamine) on swimming behavior, attachment and metamorphosis of *H. iris* larvae.

Methods and Materials

Larval Supply

Abalone (*Haliotis iris*) larvae (7 days post-fertilisation) were obtained from OceaNZ Blue abalone farm, Ruakaka, northern New Zealand. The larvae were transported moist in a cold container to the Auckland University of Technology (AUT) laboratory, Auckland, New Zealand. To allow time for the organisms to equilibrate to their new conditions, the larvae were placed in a 2 L beaker with natural fresh filtered (0.22 µm) seawater (FSW) and left to stand for 1 hr. Healthy swimming larvae were then decanted into another beaker, and the volume was adjusted to obtain a larval concentration of approximately 50 larval mL^{-1}.

Antibiotics

To avoid the potential confounding effects of bacterial interactions, antibiotics were used in this study after testing for their effects on larval survival/mortality and any synergistic interactions with a neurotransmitter under different concentrations. To this end, a pilot

experiment was conducted with larvae exposed to and not exposed to antibiotics (150 µg mL^{-1} each of penicillin G sodium and streptomycin sulphate) in sterile polystyrene Petri plates (60 mm in diameter, 14 mm in depth) over 72 hrs. The pilot experiment contained a set of controls (FSW) and three treatments (GABA at 10^{-5}, 10^{-4}, and 10^{-3} mol L^{-1} concentrations), which were tested with antibiotics and another set of controls and three GABA treatments, which were left without antibiotics. Ten replicate Petri plates were used for each of the controls and treatments. The experiment was conducted at $17 \pm 1°C$ and under constant ambient light conditions in a multi-purpose aquaculture lab. Petri plates treated with antibiotics contained 1 mL antibiotics solution, and plates that were treated with GABA had a 1 mL concentrated ($10\times$) treatment solution added. All Petri plates were filled with FSW to 9 mL before adding 1 mL of larval solution (ca. 50 larvae plate^{-1}) for a final assay volume of 10 mL. After 72 hrs, larval mortality was recorded as described below.

Chemical Treatments

The chemical treatments in this study included the amino acid and neurotransmitter γ-aminobutyric acid (GABA), the neurotransmitter acetylcholine, excess potassium ions (as KCl), the hormone and neurotransmitter dopamine, and the amino acid glutamine. Stock solutions of each treatment were prepared immediately prior to settlement assays by dissolving the compounds in FSW to produce $10\times$ concentrates of each treatment level for the final assay concentrations. The final assay concentrations were 10^{-6} mol L^{-1} for GABA, 10^{-5}, 10^{-4}, and 10^{-3} mol L^{-1} for acetylcholine, 10, 20, and 30 mmol L^{-1} for KCl, 10^{-6}, 10^{-5}, and 10^{-4} mol L^{-1} for dopamine, and 10^{-6}, 10^{-5}, and 10^{-4} mol L^{-1} for glutamine. These specific concentrations were chosen based on previous experiments (e.g., Roberts 2001; Roberts & Watts 2010). GABA at 10^{-6} mol L^{-1} was chosen as a positive comparative control, since this

concentration has been shown to optimally induce attachment and metamorphosis in this abalone species (Roberts et al. 2007; Roberts & Watts 2010)

Larval Assays

Haliotis iris larvae from a single batch were used to test the effect of five different chemical treatments and a control on swimming behavior, attachment and metamorphosis over a period of 14 days. For each treatment and control, 70 replicate Petri plates were established with the appropriate chemical (GABA, acetylcholine, potassium chloride, dopamine, and glutamine) or FSW (control). The experiment was conducted at 17 ± 1°C, and under constant ambient light conditions with plates being randomly distributed to minimize experimental biases. Based on results of a pilot experiment to test the suitability of using antibiotics within the larval assays, penicillin G sodium and streptomycin sulphate were used in this study to ensure good survival of larvae. Thus, each plate contained 1 mL larval solution (ca. 50 larvae), 1 mL of antibiotic solution, 1 mL concentrated ($10\times$) treatment solution or FSW (controls), and 7 mL of FSW. A different set of ten replicate plates from each of the five treatments and control were examined every 2 days, with the last set of plates being examined after 14 days. This procedure ensured that larvae were not disturbed until they were sampled at their corresponding time from the start of the experiment.

At each sampling event, the number of larvae which were swimming, attached, and metamorphosed in each plate, was recorded under a dissecting microscope at 20–$45\times$ magnification. Larvae that were attached were oriented vertically so that the shell opening was in direct contact with the surface of the Petri plate (Fig. 1) and resisted gentle suction by pipette. Individuals were considered metamorphosed when distinct fan-shaped growth of the peristomal shell was visible (Fig. 1). The number of larvae swimming, attached, and

metamorphosed was converted to percents based on the initial number of larvae introduced in each plate. The total percent of larvae within these categories do not add to 100% because individuals at the bottom (not swimming), but not attached (lying horizontally), are not reported herein.

Mortality was identified by staining the larvae with neutral red vital stain (Platter-Rieger & Frank 1987; Jacobson et al. 1993). A neutral red stock solution prepared in FSW was diluted in each plate to a final stain concentration of 20 ppm. After 30 min, larvae were viewed under the microscope at $20\times$ magnification. Larvae that did not have stained tissues were considered dead.

Statistics

To test the effects of antibiotics and GABA on larval survivability over 3 days, percent mortality data were transformed (arcsine square root) and analyzed using 2-way ANOVA. To determine within-treatment exposure duration effects of other chemical treatments on swimming behavior, attachment and metamorphosis, percent data were similarly transformed and analyzed using 1-way ANOVAs. To assess the effects of treatments compared to the controls, percent data were linear transformed (square root) and analyzed using a series of ANCOVAs within a general linear model with exposure duration as the covariate. All tests were evaluated at a significance level of 0.05. Univariate data analyses were conducted in Minitab version 16 statistical software package. To identify effect similarities among treatments, average linkage hierarchical agglomerative cluster analyses (Euclidian Distance) were performed on raw percent responses (swimming, attachment and metamorphosis) to each treatment level and time point. Two-D Multi-Dimensional Scaling (MDS) plots were created and overlaid with distance clusters to provide spatial representation of data, facilitate

interpretation and give statistical fit to the data. Multivariate data analyses were conducted in The Plymouth Routines In Multivariate Ecological Research statistical software package (PRIMER-E v.6).

Results

Antibiotics

The pilot experiment to test for the efficacy of using antibiotics resulted in clear improved survival of larvae exposed to antibiotics compared to those not exposed to antibiotics for the control and all concentrations of GABA (Fig. 2). Results of a two-way ANOVA revealed a main effect of antibiotics ($F_{1,\,72} = 135.92$, $p < 0.001$), indicating that larvae treated with streptomycin and penicillin had substantially improved survival compared to untreated larvae (Table 1). There also was a main effect of GABA concentration revealing, as expected, that higher concentrations were more toxic. However, the interaction between antibiotic treatment and GABA concentration effect revealed that the toxic response only occurred in larvae which did not receive antibiotic treatment (Fig. 2).

Larvae exposed to antibiotics had mortalities of less than 5% across the control and treatments. Conversely, the percent mortality for larvae not exposed to antibiotics increased from $17.7 \pm 11.5\%$ in the control to 83.5 ± 8.5 in the GABA at 10^{-3} mol L^{-1} treatment. Based on these results and the fact that no obvious toxic effects (e.g., velar abscission, abnormal swimming behavior) were observed when the larvae were exposed to antibiotics across the control and treatments, antibiotics were used throughout the rest of the study.

Larval Swimming Behavior, Attachment and Metamorphosis

A general shift in larval state from swimming to attached and from attached to metamorphosed was observed across the control and the treatments (GABA, acetylcholine, potassium chloride, dopamine, and glutamine) in all concentrations over time (Figs. 3–7; Tables 2-3). However, the peak in percent attached and metamorphosed varied in time for the different chemicals and their concentrations. Negative control plates had near 100% swimming larvae after 2 days, and about 30 and 20% attached and metamorphosed larvae, respectively, after 14 days (Fig. 3). For the positive control (10^{-6} mol L^{-1} GABA), the peak percent attachment was observed after just 2 days, with a generally increasing percent of metamorphosed larvae, which peaked at about 90% after 14 days (Fig. 3). However, by day14, many of those larvae which had undergone metamorphosis in the preceding few days had died (i.e., ~50% mortality). Excess potassium ions (KCl) at concentrations of 10 and 20 mmol L^{-1} induced larvae to attach relatively early, with sustained percentages of just under 40% from 4 to 14 days (Fig. 4). Less than 40% swimming larvae were recorded in treatments with potassium ions at all concentrations. While the lowest concentration (10 mmol L^{-1}) appeared to slightly hasten metamorphosis, relatively low numbers of post-larvae were observed throughout the experiment at the higher concentrations tested (20 and 30 mmol L^{-1}).

Application of the neurotransmitter acetylcholine at low concentrations (10^{-5} mol L^{-1}) resulted in similar larval response patterns as the negative controls (Figs. 3, 5). However, at higher concentrations (10^{-4} and 10^{-3} mol L^{-1}), the attachment and metamorphosis processes were accelerated (Fig. 5), with significant differences detected between untreated larvae (Table 3). A lower percent of swimming larvae was observed at the beginning of the experimental period in the two highest acetylcholine concentrations compared to those in the 10^{-5} mol L^{-1} concentration and the negative control.

Exposure to dopamine resulted in generally high swimming activity at the beginning of the experiment for the lowest concentrations (10^{-5} and 10^{-6} mol L^{-1}), and almost complete cessation of swimming at 10^{-4} mol L^{-1} (Fig. 6). Similar to the other treatments tested, exposure of larvae to dopamine resulted in an increase of attachment rate with the highest percent attachment occurring after 14, 10, and 10 days for concentrations of 10^{-6}, 10^{-5}, and 10^{-4} mol L^{-1}, respectively. No effect on metamorphosis was detected at the two lower concentrations compared to negative controls, but significant inhibition of metamorphosis was observed at the highest concentration (Table 3).

Glutamine treatments reduced larval swimming activity during the first two days of exposure to about 35, 55, and 30% for concentrations of 10^{-6}, 10^{-5}, and 10^{-4} mol L^{-1}, respectively (Fig. 7). Early larval attachment was promoted at all concentrations compared to negative controls, with highest percent attachment observed at 10^{-4} mol L^{-1}. Limited effects of glutamine were observed on metamorphosis within all concentrations compared to negative controls (Fig. 3, 7, Table 3).

Multivariate cluster analyses revealed that all tested compounds had higher capacities to differentially modulate larval swimming behavior and attachment compared to metamorphosis (Figure 8). Generally, larval swimming activity was down-regulated across all treatments compared to negative control assays, except for acetylcholine at 10^{-5} mol L^{-1}, which clustered with the control (Fig. 8 A, B). Exposure of larvae to acetycholine (10^{-3} mol L^{-1}) and excess potassium ions (10 mmol L^{-1}) resulted in most similar swimming responses to the positive control (GABA). High concentrations of glutamine, dopamine (10^{-4} mol L^{-1}) and potassium (30 mmol L^{-1}) formed clusters due to substantial reduction in swimming activity. Larval attachment was less affected by acetylcholine and dopamine (Fig. 8 C, D). Clusters formed by effects of potassium (10 and 20 mmol L^{-1}) and glutamine (10^{-4} mol L^{-1}) could be attributed to early attachment induction compared to negative controls. Potassium at 30

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mmol L^{-1} almost completely inhibited attachment, whereas GABA was a strong and fast-acting inducer of attachment. Although slight stimulatory and/or inhibitory metamorphic responses were observed in some treatments, these effects were minimal compared to the strong inductive effects of GABA (Fig. 8 E, F).

Mortality

Results from the larval mortality assays indicate a generally low mortality ($< 20\%$) across control and treatments for exposures of less than 12 days, except for KCl at 20 mmol L^{-1} after 2 days, dopamine at 10^{-5} mol L^{-1} after 10 days, and glutamine at 10^{-4} after 8 and 10 days (Fig. 3-7). Conversely, mortality was generally high after 10 days, especially for the control, GABA, KCl at 30 mmol L^{-1}, and acetylcholine at 10^{-5} and 10^{-3} mol L^{-1}.

Discussion

Application of GABA, acetylcholine, potassium ions, dopamine, and glutamine on *Haliotis iris* larvae resulted in varied responses with regard to larval behavior and mortality. The most distinctive result was a general decrease in swimming activity across all treatments and concentrations compared to the control, except for acetylcholine at 10^{-5} mol L^{-1}, which did not differ from the control. This reduction in swimming activity is interpreted as a decrease in ciliary movement caused by exposure to the treatments. While this is the first evidence of such an effect on larval behavior for this species, previous studies have investigated the neuronal control of larval ciliary movement in a number of mollusc species and considerable efforts have been made to characterize and map the neural circuit architectures involved (Jékely et al. 2008). Results from these studies frequently reveal that

patterns of cilia-based locomotion, velar ciliary beat frequency and the rate and duration of

ciliary arrests are under dual innervation by excitory serotonergic and inhibitory

dopaminergic mechanisms (Croll & Dickinson 2004; Braubach et al. 2006; Croll 2009). Our

results demonstrate that swimming of *H. iris* larvae is quickly down-regulated by dopamine

within the first two days, which infers a similar inhibitory dopaminergic role. However, for

some species it appears that alternative dopamine function and/or involvement of other

neurotransmitters may exist. For example, in larvae of the polychaete *Spirobranchus*

giganteus, agonisation of dopaminergic receptors has no effect on normal swimming

behavior; whereas, reversible selective blocking of β-adrenergic receptors leads to fast ciliary

arrest (Marsden & Hassessian 1986). These observations indicate an active participation of

noradrenalin and/or adrenalin in neuronal control of larval swimming for *S. giganteus*.

Supportive evidence of an adrenergic involvement for other species also has been reported

(e.g., mussels [Alfaro et al. 2011]). In addition, GABAergic (Katow et al. 2013), peptidergic

(Conzelmann et al. 2011), and cholinergic (Grazia Aluigi et al. 2012) mechanisms also have

been implicated in the regulation of marine invertebrate larval ciliary activities.

Based on the results of the pilot experiment to test the effect of antibiotics on larval

mortality, the application of antibiotics significantly reduced mortalities over 72 hrs. Some

authors have highlighted the fact that there is high variability in larval responses to antibiotics

(Baloun & Morse 1984; Searcy-Bernal et al. 1992; Roberts 2001). Such variability may be

due to a range of factors, including different experimental conditions (Searcy-Bernal et al.

1992), variations in the abundance of antibiotic-resistant microbes (Roberts 2001), and/or

larval batch variability (Baloun & Morse 1984; Trapido-Rosenthal & Morse 1986). In the

present study, such variations were reduced by using the same batch of larvae and testing

simultaneously across the treatments and control. While it is clear from our data that the use

of antibiotics is necessary for extended duration (>3 days) settlement and metamorphic

assays, experimental biases due to the use of antibiotics cannot be discounted, but no obvious adverse effects were observed.

Another important, but unexpected, finding of this study was that mortalities in controls were typically higher than those observed under treatment conditions. This could be due a combination of factors.   For example, abalone are known to delay metamorphosis when there is an absence of an appropriate environmental cue (Hooker & Morse 1985) and, in such cases, over-consumption of yolk reserves leads to negative impacts on growth, metamorphic success and post-metamorphic survivial (Roberts & Lapworth 2001). While non-feeding haliotid larvae can uptake dissolved organic matter in seawater as an additional source of energy (Jaeckle & Manahan 1989; Shilling et al. 1996), the small volume of water supplied (10ml) may not have provided sufficient dissolved nutrients to balance the energy used for respiration, organogenesis, movement, biomineralization, growth and tissue remodelling during metamorphosis. In addition, it is possible that the down-regulation of swimming activities caused by treatments in the first 4 days of exposure reduced total metabolic costs. Thus, since larvae retained their yolk reserves longer they may have been able to re-partition their energy-wealth to other critical processes, such as growth and metamorphosis. In bryozoans, larvae expend as much as 1.6% of their initial energy reserve per hour whilst actively swimming (Wendt 2000). Also, in barnacles, larvae lose their competence to metamorphose successfully at the same time as the larval energy supply for swimming and substrate exploration is depleted (Lucas et al. 1979).

We used GABA as a positive control in our study because it previously had been shown to induce larval settlement, attachment and/or metamorphosis in *H. iris* (Roberts 2001; Roberts et al. 2007; Roberts & Watts 2010) and numerous other haliotids, including *H. asinina* (Gapasin & Polohan 2004; Stewart et al. 2008, 2011), *H. refescens* (Trapido-Rosenthal & Morse 1985; Searcy-Bernal et al. 1992), *H. diversicolor* (Bryan & Qian 1998),

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H. diversicolor supertexta (Li et al. 2006; Yu et al. 2010), *H. discus hannai* (Yu & Bao-ling 1995; Takami et al. 2002), *H. gigantea* (Fukazawa et al. 2001), *H. discus discus* (Fukazawa et al. 2001), *H. midae* (in Roberts 2001), *H. virginea* (Roberts & Nicholson 1997) and *H. australis* (Moss 1999). However, the effective doses for these species vary between 0.5–10 µmol L^{-1}, implying species specificity. With approximately 55% attachment after 2 days exposure, our results are lower than those (98%) found by Roberts and Nicholson (1997) for the same species. However, after 4 days exposure to GABA, similar percentages of metamorphosis were recorded in both studies (ca. 40%). It is possible that our larvae were more developmentally advanced and we may have missed high attachment rates within the first 2 days. Compared to other compounds we tested, GABA induced the highest percentage of metamorphosis (ca. 90%), and also induced fast attachment (55% within 2 days). It has been suggested that the activity of GABA is due to its close structural relationship with naturally occurring inducer molecules (Morse & Morse 1984a,b). The GABA isoforms γ-hydroxybutyric acid and δ-aminovaleric acid are present in some crustose coralline algal species, and have been shown to be active inducers of *Haliotis* spp. settlement and metamorphosis.

KCl induces larval attachment and/or metamorphosis in various *Haliotis* spp. with high specificity to the optimal inductive concentration across taxa (e.g., 6 mmol L^{-1} for *H. asinina* [Gapasin & Polohan 2004], 20 mmol L^{-1} for *H. diversicolor* [Bryan & Qian 1998] and 40 mmol L^{-1} for *H. discus hannai* [Yang & Bao-ling 1995]). Our results reveal that KCl induces attachment at a concentration of 10 mmol L^{-1}, but has limited effect on metamorphosis after 4 days exposure. Roberts & Nicolson (1997) previously tested the effect of 10 mmol L^{-1} KCl on settlement and metamorphosis of *H. iris* larvae, and also observed moderate attachment (50-70%) and negligible metamorphosis (< 10%) after 2 days exposure. It is important to note that while total metamorphosis over the duration of the experiment

with 10 mmol L^{-1} KCl was similar to the negative control assays in our study, the general trend in the data suggests an effect of KCl on the morphogenic pathway by reducing the time to onset of metamorphosis (i.e., first observable metamorphic responses occurred after 6 and 10 days for treatment and control assays, respectively). Furthermore, differences in swimming behavior, attachment and metamorphosis between treatment and control assays were detected by ANCOVA and/or comparative analyses of regression intercepts and slopes (Table 3). Based on the range of optimal inductive KCl concentrations reported for other species, we also tested the effects of KCl at 20 and 30 mmol L^{-1}. At these higher concentrations, almost no metamorphosis was observed and only attachment was enhanced at 20mmol L^{-1}. These results differ from those of Kang et al. (2004), who found that KCl induced metamorphosis, but not attachment in *H. discus hannai*. In addition, KCl induced both attachment and metamorphosis in *H. diversicolor supertexta* (Li et al. 2006) and *H. asinina* (Gapasin & Polohan 2004). The high variability of developmental responses to KCl across these closely related species may indicate the presence of diverse regulatory mechanisms. Potassium ions are universal regulators of cell membranes and it has been suggested that KCl acts by depolarising externally accessible excitable cells connected to the peripheral or central nervous system (Baloun & Morse 1984). However, treatment of molluscan neural tissues with KCl also can result in initiation of synthesis or release of serotonin, dopamine, norepinephrine and acetylcholine (Stefano et al. 1981, 1997; Wieland et al. 1989; Salánki & Hiripi 1990).

As with KCl, acetylcholine did not appear to enhance overall metamorphosis, but it did seem to influence the morphogenic pathway by hastening commencement of velar abscission and peristomal shell growth. It is unclear whether this effect was caused by a coupling of the metamorphic gene regulatory network to the induced pre-metamorphic behavior/process prior to the event, or whether acetylcholine is directly involved in the

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morphogenic signalling pathway during the event. While acetylcholine is a potent inducer of larval settlement in some bivalve species (Yu et al. 2008; Alfaro et al. 2011; Young et al. 2011; Sánchez-Lazo et al. 2012), no effect on attachment or metamorphosis has been observed in the five *Haliotis* spp. tested thus far (*H. discus* [Fukazawa et al. 2001], *H. discus hannai* [Akashige et al. 1981], *H. diversilcolor supertexta* [Yu et al. 2010], *H. gigantea* [Fukazawa et al. 2001], *H. rufescens* [Morse et al. 1979]). However, recent analysis of acetylcholine-binding proteins (analogous sequences to ligand-binding domains of nicotinic acetylcholine receptors) during larval development of *H. discus hannai* revealed substantial up-regulation during onset of settlement/metamorphic competency (Huang et al. 2009). Furthermore, spatial expression patterns showed positive signals for acetylcholine-binding proteins in the prototroch ciliary band of trocophore-stage larvae, and high expression in the prototroch and foot of competent veliger larvae (Huang et al. 2009). These results suggest that acetylcholine is involved in regulation of ciliary activity and likely has an active role in settlement behavior through modulation of swimming, pedal exploration of substrates and subsequent larval attachment. Indeed, our results clearly demonstrate for the first time that prolonged exposure to acetylcholine causes cessation of swimming and early initiation of attachment in a haliotid gastropod.

Our results showed no induction of *H. iris* metamorphosis with dopamine, but this chemical did cause down-regulation of swimming activity over the first two days of exposure. In addition, there were latent effects on promotion of larval attachment (8-10 days post-treatment). In contrast, Yu et al. (2010) found a much faster and stronger effect of dopamine (and its precursor metabolite L-DOPA) on attachment of *H. diversicolor supertexta*, which may indicate a putative involvement of dopaminergic signalling in the critical pre-metamorphic process. While conclusive comparative data are lacking for other *Haliotis* spp., Wang et al. (2010) investigated the effects of the neuroactive dopamine

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derivatives norepinephrine and epinephrine in *H. asinina*. Metamorphosis was marginally

enhanced by epinephrine, but neither compound induced larval attachment (Wang et al.

2010). On the other hand, dopamine and related compounds are potent inducers of larval

settlement in a wide range of bivalve molluscs, such as mussels (Alfaro et al. 2011; Sánchez-

Lazo et al. 2012; Young et al. 2012), oysters (Coon et al. 1985; Grant 2009; Teh et al. 2012),

clams (Sumin et al. 2006) and scallops (Martinez et al. 1999). However, while many bivalves

seem to rely on dopaminergic and adrenergic signalling throughout the settlement process,

the presence of a functional role for dopamine specifically during gastropod larval attachment

is more elusive. Nevertheless, due to dopamine's involvement in regulation of ciliary activity

across diverse organisms and cell-types (reviewed by Croll 2009; Jékely 2011), dopaminergic

signalling likely coordinates swimming behavior across marine invertebrate taxa, and at least

plays a facilitatory role during initiation of attachment in *Haliotis* spp.

Resembling the effects of GABA, KCl, acetylcholine and dopamine, glutamine also

reduced swimming activity in *H. iris* larvae within the first two days of exposure. Although

there was some indication of induced attachment at high concentrations (10^{-4} mol L^{-1}), high

mortalities (ca. 25%) were simultaneously observed. Glutamine generally had little or no

effect on larval attachment and metamorphosis at the lower concentrations tested. Similarly,

Morse et al. (1979) found that the amino acid also had no effect on larval settlement in *H.*

refescens. On the other hand, the glutamine derivative and GABA intermediate, L-glutamate,

does induce settlement in some *Haliotis* spp. (*H. refescens* [Morse et al. 1979], *H. discus*

hannai [Kang et al. 2003] and *H. asinina* [Stewart et al. 2008]), acting either *via* its direct

ability to function as an excitory neurotransmitter or through endogenous decarboxylation to

GABA. These results may imply that endogenous levels of glutamine are not rate-limiting in

the presynaptic release of glutamate and/or GABA, and it is unlikely to be involved in

primary regulation of the metamorphic transition in haliotid gastropods. In stark contrast,

settlement of barnacle larvae is not induced by glutamate, but is enhanced by glutamine (Mishra & Kitamura 2000). Furthermore, glutamine also induces fast synchronous metamorphosis in five species of sea urchins (*Strongylocentrotus intermedius* [Naindenko 1996], *Scaphenchinus mirabilis* [Naindenko 1996], *Pseudocentrotus depressus* [Yazaki & Harashima 1994], *Hemicentrotus pulcherrimus* [Yazaki 1995], and *Anthocidaris crassispina* [Sato & Yazaki 1999]). These variable responses to glutamine and its derivatives highlight the specificities of chemical cues and chemosensory mechanisms in particular taxa.

In summary, results from the effects of GABA, potassium chloride, acetylcholine, dopamine, and glutamine on larval swimming behavior, attachment and metamorphosis reveal that all these chemicals have the ability to down-regulate larval swimming and induce early attachment and metamorphosis with relatively low mortality for exposures of less than 12 days. These findings provide novel information on the neurophysiological control of larval activities in Haliotid gastropods, which may lead to future commercial application for enhancing larval production.

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Figure Captions

Figure 1 *Haliotis iris* larvae in A, swimming, B, unattached, C, attaching, and D, metamorphosed stages.

Figure 2 Mean percent (±SE) mortality of abalone larvae in control plates (C) and treatments (GABA at 10^{-5}, 10^{-4}, and 10^{-3} mol L^{-1} concentrations) after 3 days exposure. Black bars denote trials without antibiotics and white bars indicate trials with antibiotics.

Figure 3 Mean percent (±SE) swimming, attachment, metamorphosis (A, C) and mortality (B, D) of abalone larvae in negative (filtered seawater) and positive (10^{-6} mol L^{-1} GABA) control assays over 14 days exposure.

Figure 4 Mean percent (±SE) swimming, attachment, metamorphosis (A, C, E) and mortality (B, D, F) of abalone larvae over 14 days exposure to 10, 20 and 30 mmol L^{-1} potassium chloride.

Figure 5 Mean percent (±SE) swimming, attachment, metamorphosis (A, C, E) and mortality (B, D, F) of abalone larvae over 14 days exposure to 10^{-5}, 10^{-4} and 10^{-3} mol L^{-1} acetylcholine chloride.

Figure 6 Mean percent (±SE) swimming, attachment, metamorphosis (A, C, E) and mortality (B, D, F) of abalone larvae over 14 days exposure to 10^{-6}, 10^{-5} and 10^{-4} mol L^{-1} dopamine hydrochloride.

Figure 7 Mean percent (±SE) swimming, attachment, metamorphosis (A, C, E) and mortality (B, D, F) of abalone larvae over 14 days exposure to 10^{-6}, 10^{-5} and 10^{-4} mol L^{-1} glutamine.

Figure 8 Agglomerative hierarchical clustering of larval swimming (A and B), attachment (C and D) and metamorphic (E and F) responses to chemical treatments and controls. Plots on left show average linkage dendrograms obtained from the Euclidian distance between percent larval responses and 2D-MDS plots on right represent the same data overlaid with distance clusters, where solid lines indicate distances of B, D and F, ≤30, and dotted lines indicate distances of B and D, ≤50. The analysis included −ve control (C), +ve control (GABA), acetylcholine (A), potassium chloride (K), dopamine (D), and glutamine (G) at various concentrations ($3 = 10^{-3}$, $4 = 10^{-4}$, $5 = 10^{-5}$ and $6 = 10^{-6}$ mol L^{-1}, and 10, 20 and 30 mmol L^{-1}). Note differences in dendrogram scales.
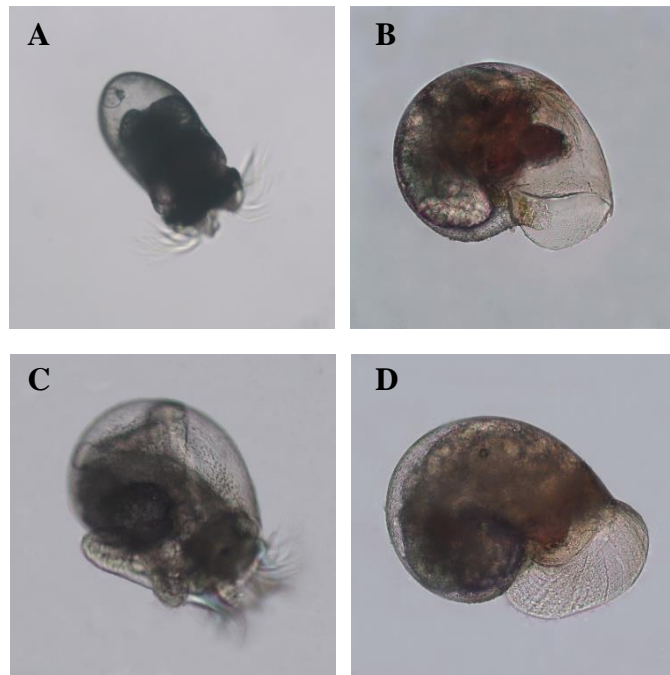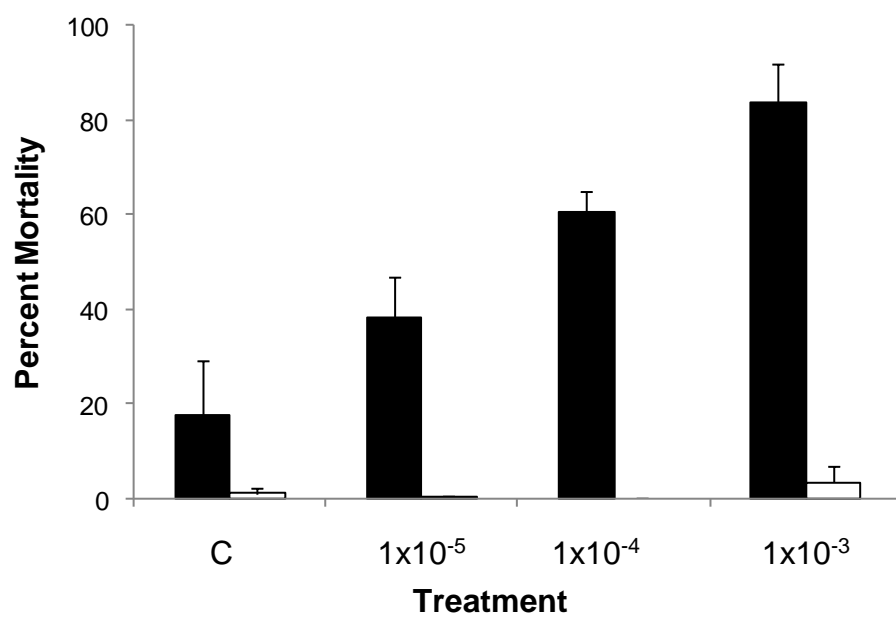
Figure 1

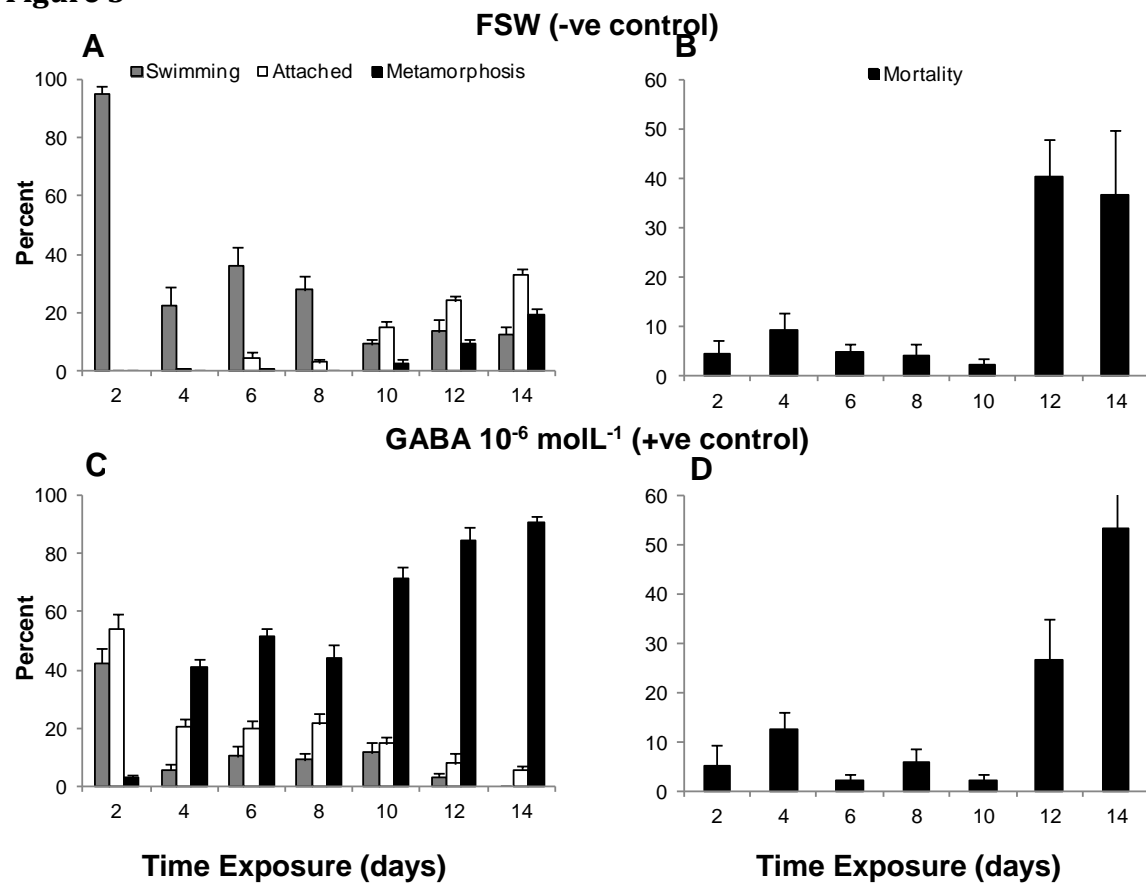**Figure 2**

Figure 3

FSW (-ve control)

A — Swimming, Attached, Metamorphosis (Percent vs Time)

B — Mortality

GABA 10^{-6} molL^{-1} (+ve control)

C — Percent vs Time Exposure (days)

D — Time Exposure (days)

34

Figure 4

Figure with six panels (A–F) showing KCl exposure effects.

KCl 10 mmol L^{-1} (A, B)
KCl 20 mmol L^{-1} (C, D)
KCl 30 mmol L^{-1} (E, F)

Panels A, C, E: Percent vs Time Exposure (days) with Swimming, Attached, Metamorphosis.
Panels B, D, F: Mortality vs Time Exposure (days).

Figure 5

Figure 6

Dopamine 10^{-6} mol L^{-1}

Dopamine 10^{-5} mol L^{-1}

Dopamine 10^{-4} mol L^{-1}

A — Swimming, Attached, Metamorphosis
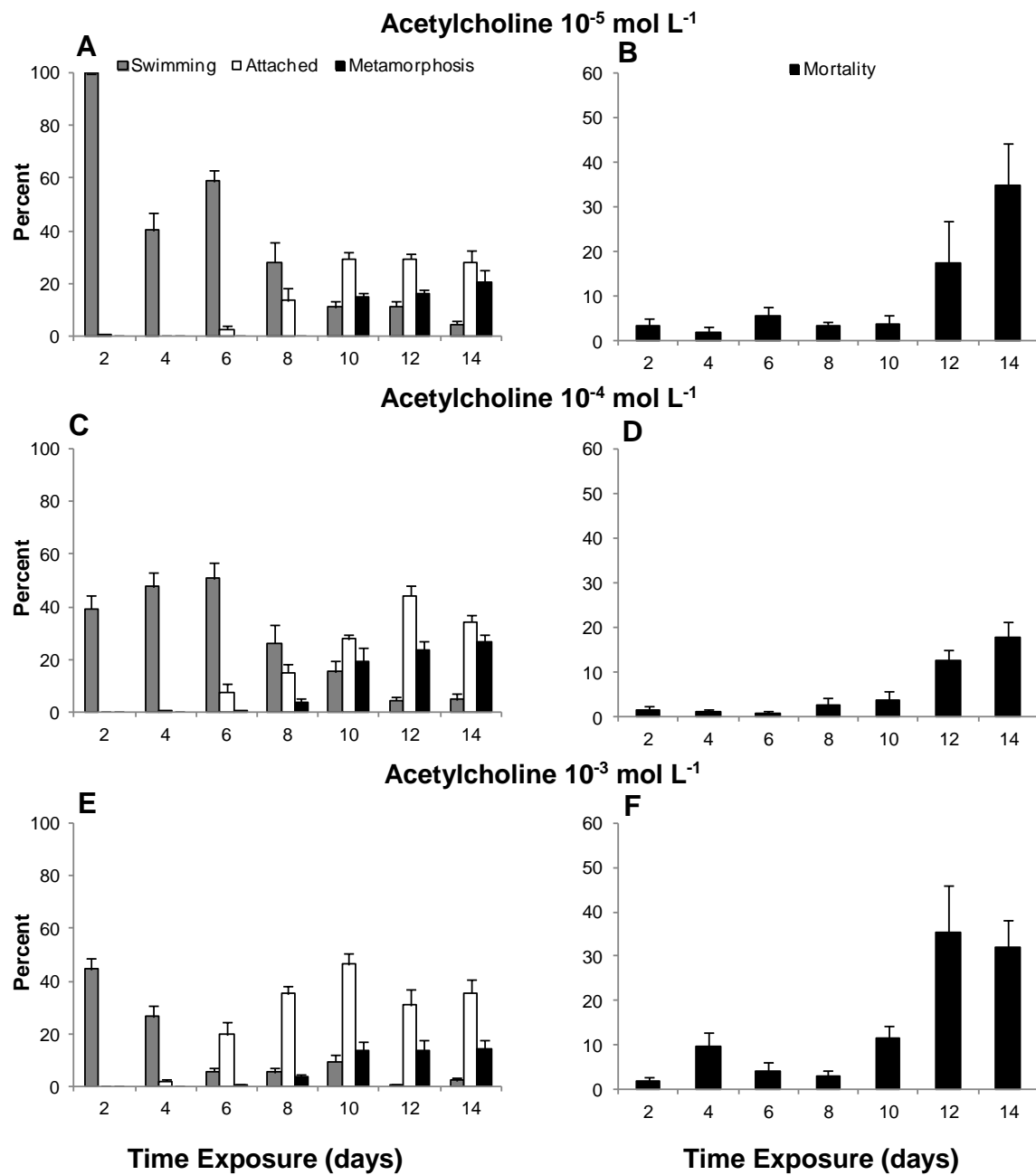
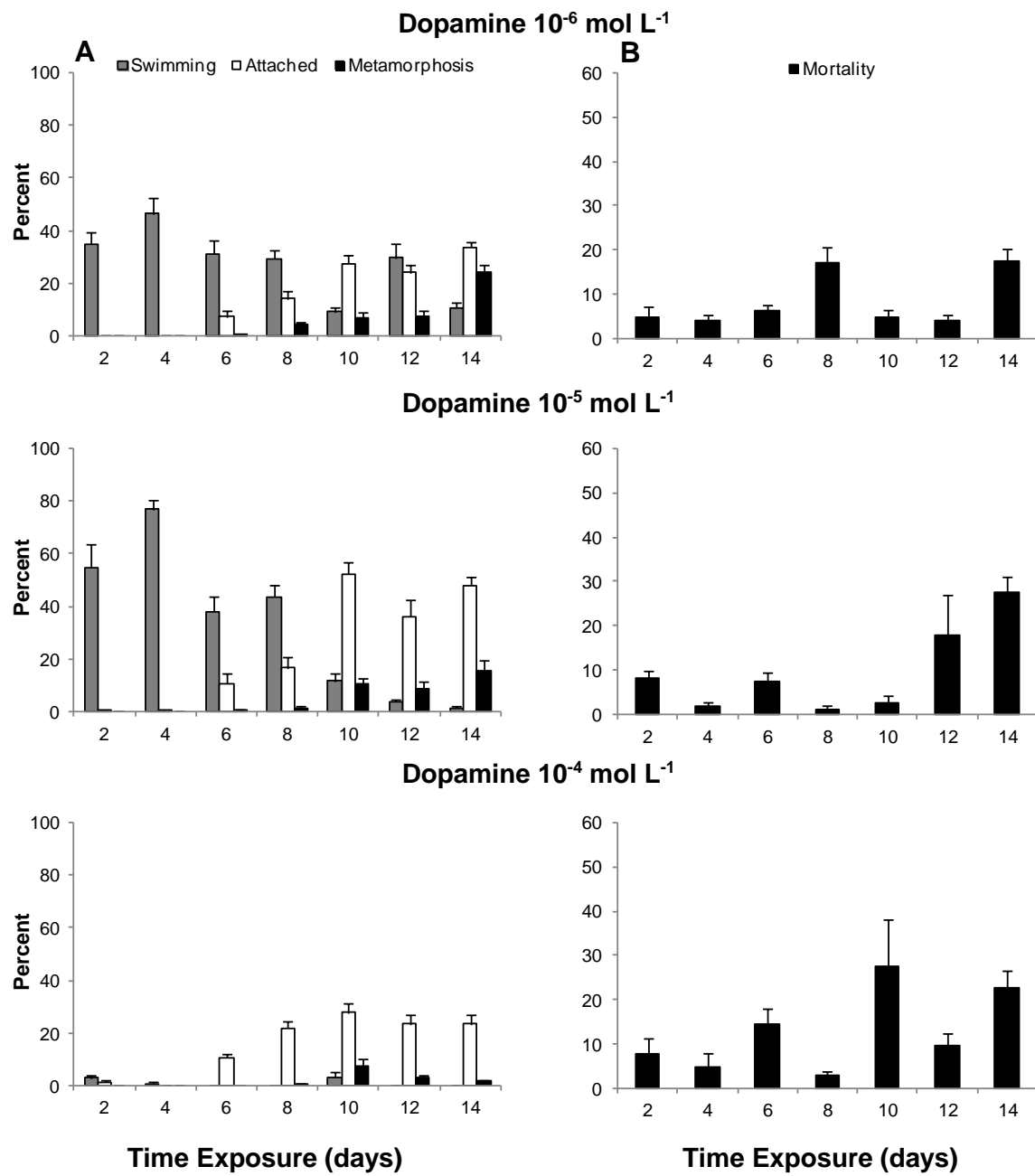B — Mortality

Time Exposure (days)
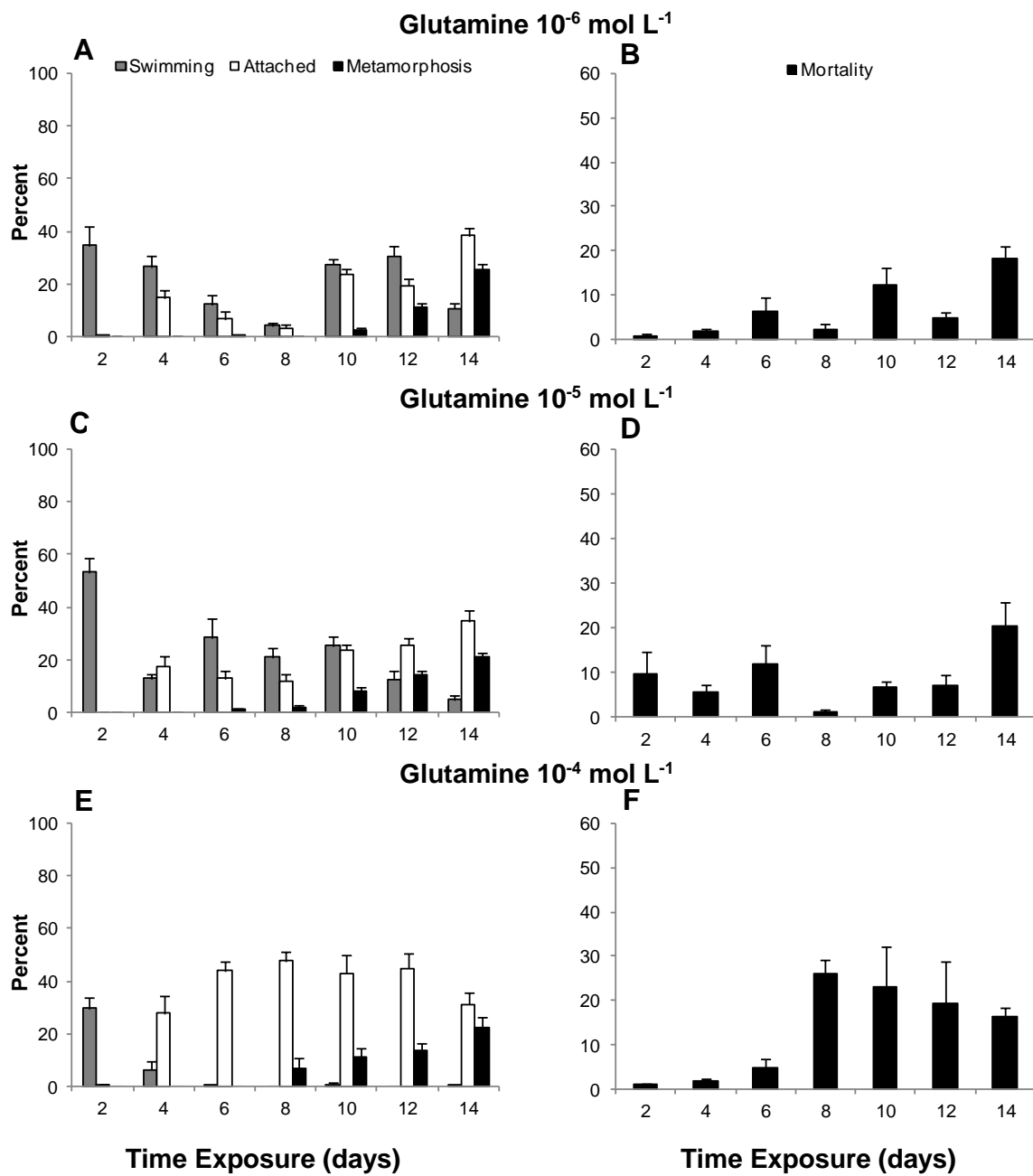
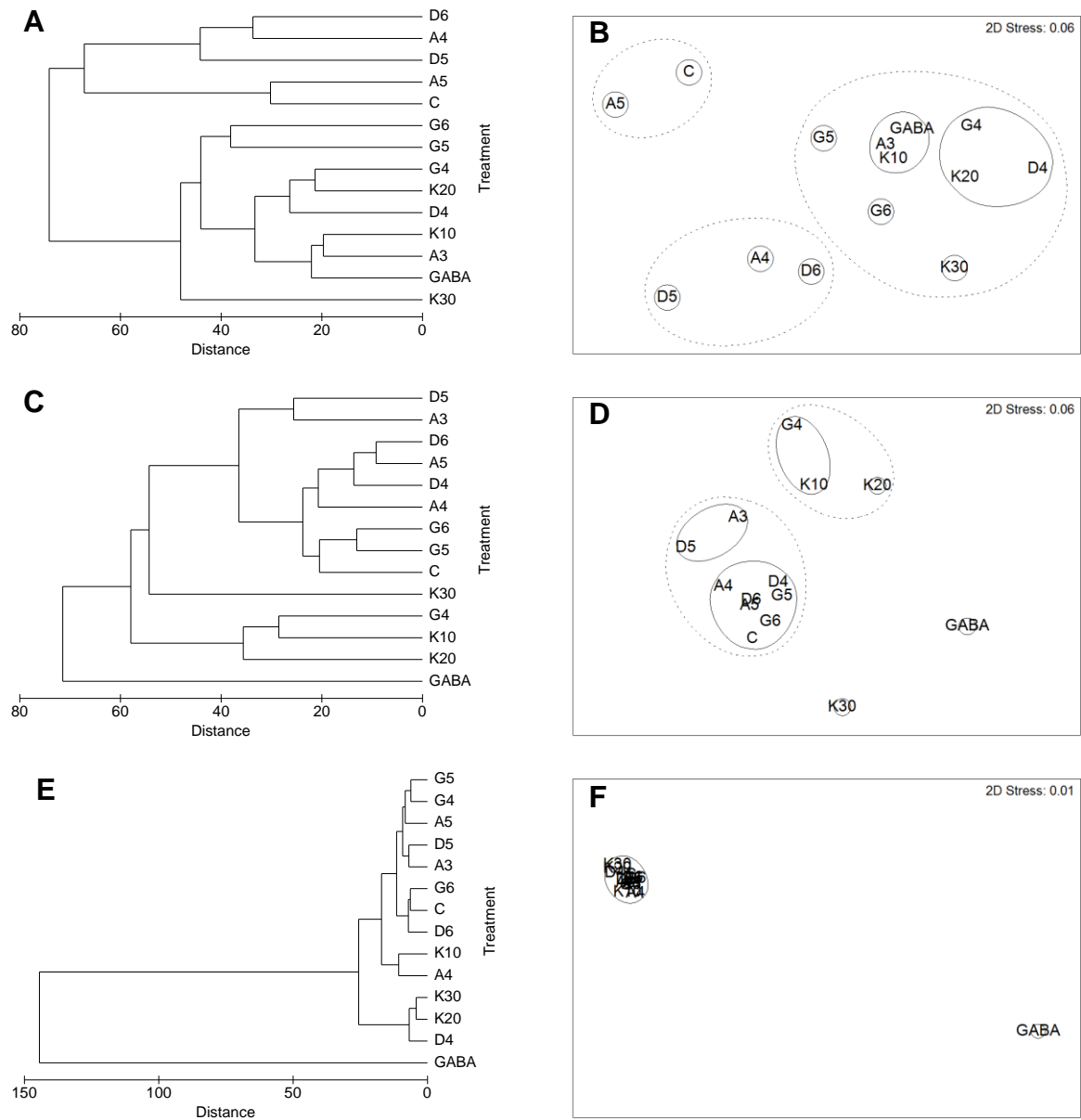Percent

Figure 7

Figure 8

Table 1: Two-way ANOVA results of larval mortality between control and GABA treatments with and without antibiotics.

Source	DF	MS	F	p-value
Antibiotics (A)	1	33330.3	135.92	0.000
Treatment (T)	3	2776.5	11.32	0.000
A x T	3	2513.3	10.25	0.000
Error	72	245.2		
Total	79			

Table 2: One-way ANOVA results of larval responses to different treatments over time (measured at 2 day intervals over 14 days).

Treatment	Concentration	Swimming				Attachment				Metamorphosis			
		df	MS	F	p-value	df	MS	F	p-value	df	MS	F	p-value
Control	N/A	7	10101	85.27	<0.001	7	1872	68.19	<0.001	7	933	55.99	<0.001
		72	118			72	28			72	17		
GABA (mol L^{-1})	10^{-6}	7	10330	118.31	<0.001	7	2068	30.24	<0.001	7	7168	103	<0.001
		72	87			72	68			72	69		
KCl (mmol L^{-1})	10	7	8367	164.24	<0.001	7	2101	34.62	<0.001	7	1308	37.82	<0.001
		72	51			72	61			72	35		
	20	7	9519	206.67	<0.001	7	2432	36.51	<0.001	7	50	2.09	0.055
		72	46			72	67			72	24		
	30	7	10795	183.64	<0.001	7	328	5.60	<0.001	7	2	1.22	0.304
		72	59			72	59			72	2		
Acetylcholine (mol L^{-1})	10^{-5}	7	11292	118.11	<0.001	7	2239	36.89	<0.001	7	1421	49.03	<0.001
		72	96			72	61			72	29		
	10^{-4}	7	8579	71.47	<0.001	7	2902	75.03	<0.001	7	1912	51.57	<0.001
		72	120			72	39			72	37		
	10^{-3}	7	10283	194.81	<0.001	7	3008	37.07	<0.001	7	938	21.88	<0.001
		72	53			72	81			72	43		
Dopamine (mol L^{-1})	10^{-6}	7	6864	94.20	<0.001	7	2199	68.25	<0.001	7	1046	33.99	<0.001
		72	73			72	32			72	31		
	10^{-5}	7	9970	103.73	<0.001	7	3716	43.49	<0.001	7	744	14.90	<0.001
		72	96			72	86			72	50		
	10^{-4}	7	12014	567.00	<0.001	7	1854	50.20	<0.001	7	218	9.18	<0.001
		72	21			72	37			72	24		
Glutamine (mol L^{-1})	10^{-6}	7	7761	109.78	<0.001	7	1928	45.37	<0.001	7	1268	136.33	<0.001
		72	71			72	43			72	9.3		
	10^{-5}	7	8001	88.72	<0.001	7	1724	32.50	<0.001	7	1121	66.30	<0.001
		72	90			72	53			72	17		
	10^{-4}	7	12018	388.83	<0.001	7	3134	26.15	<0.001	7	1174	20.11	<0.001
		72	31			72	120			72	58		

Table 3: ANCOVA results between treatment and control assays with comparisons of regression intercepts and slopes. Dashes (-) indicate analyses which were not conducted due to data not meeting the ANCOVA assumptions.

Treatment	Concentration	Swimming			Attachment			Metamorphosis		
		Intercept	Slope	ANCOVA	Intercept	Slope	ANCOVA	Intercept	Slope	ANCOVA
GABA (mol L^{-1})	10^{-6}	<0.001	0.298	<0.001	<0.001	<0.001	-	<0.001	<0.001	-
KCl (mmol L^{-1})	10	<0.001	0.008	-	<0.001	<0.001	-	0.045	0.182	<0.001
	20	<0.001	<0.001	-	<0.001	<0.001	-	0.001	<0.001	-
	30	<0.001	0.079	<0.001	<0.001	<0.001	-	<0.001	<0.001	-
Acetylcholine (mol L^{-1})	10^{-5}	0.019	0.034	-	0.779	0.699	0.162	0.797	0.139	0.018
	10^{-4}	0.986	0.532	0.227	0.870	0.050	<0.001	0.417	0.001	-
	10^{-3}	0.005	0.954	<0.001	0.006	0.919	<0.001	0.301	0.823	-
Dopamine (mol L^{-1})	10^{-6}	0.026	0.010	-	0.003	<0.001	-	0.753	0.514	0.041
	10^{-5}	0.020	0.008	-	0.708	0.040	-	0.394	0.500	0.576
	10^{-4}	<0.001	<0.001	-	0.003	0.104	<0.001	0.003	0.000	-
Glutamine (mol L^{-1})	10^{-6}	<0.001	<0.001	-	0.004	0.073	0.005	0.505	0.185	0.245
	10^{-5}	0.020	0.084	0.075	<0.001	0.018	-	0.784	0.131	-
	10^{-4}	<0.001	0.135	<0.001	<0.001	0.016	-	0.927	0.201	-