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Galley, Thomas; Beaumont, Andrew; Le Vay, Lewis; King, Jonathan

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Title: Influence of exogenous chemicals on larval development and survival of the king scallop *Pecten maximus* (L.)

Authors: Thomas H. Galley[a]*, Andy R. Beaumont[a], Lewis Le Vay[a], Jonathan W. King[a]

Affiliations: [a] Centre for Applied Marine Sciences, College of Natural Sciences, Bangor University, Menai Bridge, Anglesey LL59 5AB, UK.

*Corresponding Author: Centre for Applied Marine Sciences, College of Natural Sciences, Bangor University, Menai Bridge, Anglesey LL59 5AB, UK, Tel: (44) (0)1248 388603, e-mail: t.h.galley@bangor.ac.uk

Abstract

The commercially important king scallop, *Pecten maximus* (L.), is the focus of ongoing research to optimise hatchery culture practices. At present, difficulties in the culture of this species are commonly associated with the transition from the larval to juvenile stages, with protracted metamorphosis, low development synchronicity and variable survival. The use of exogenously applied chemical agents, as demonstrated in other bivalve species, has been viewed as a means of resolving these issues. The present study evaluated the effects of the previously untested chemicals KCl, NH_4Cl, acetylcholine chloride and GABA, in addition to the previously tested chemical L-DOPA, on the induction of larval metamorphosis and larval survival in *P. maximus*. A range of concentrations of each chemical, applied over a 48 hour period, were assessed. Larval metamorphic response, based upon the development of functioning gill filaments or secondary shell growth (dissoconch), was low and concentration-dependent. Larval survival was also concentration-dependent, with all chemicals becoming toxic to larvae at high test concentrations. . Among the tested chemicals, KCl at 20mM and L-DOPA at 10^{-6}M induced significantly ($P<0.05$) higher rates of larval metamorphosis, which was improved by 208% and 128% respectively, compared to the controls after 7 days. However, whilst the KCl treatment was toxic, reducing survival by 33% compared to the control ($P<0.05$), the L-DOPA treatment significantly increased survival by 49% compared to the control ($P<0.05$). Furthermore, the influence of these two chemicals on larval development varied, with KCl promoting dissoconch growth and L-DOPA promoting gill development, suggesting that the pathways influenced by these two chemicals maybe different. In contrast, NH_4Cl, acetylcholine chloride and GABA proved ineffective at inducing metamorphosis over the range of concentrations tested. The results of this study provide further evidence supporting the potential use of chemical agents, though further work is required to fully realise the ability to completely synchronise larval metamorphosis in *P. maximus* for hatchery application.

Keywords

Pecten maximus, Larvae, Chemical Induction, Metamorphosis, Survival

Abbreviations

ACH	Acetylcholine chloride
ANOVA	Analysis of variance
GABA	γ-aminobutyric acid
L-DOPA	L-3,4-dihydroxyphenylalanine
KCl	Potassium chloride
M	Molar
NH_4Cl	Ammonium chloride

Introduction

The king scallop *Pecten maximus* is a commercially important species in Europe. Annual total production (capture and aquaculture) in Europe reached 65,701 tons in 2013, almost doubling over the previous ten years, although notably production only reached 55,764 tons in 2014 indicating a potential slowdown (FAO, 2017). However, nearly all of production is made up of wild capture fisheries which provide over 99% of the supply, equivalent to 65,632 tons in 2013 and 55,726 tons in 2014 (Millican, 1997; Spencer, 2002; FAO, 2017). Nevertheless, the risk of unpredictable spat fall, and seasonal constraints on availability have led to recognition of a need for hatcheries to support the expansion of cultivation and ensure the provision of a regular supply of seed (Millican, 1997; Spencer, 2002). Culture of *P. maximus* is well documented (Gruffydd and Beaumont, 1970; Gruffydd and Beaumont, 1972; Beaumont and Budd, 1983; Millican, 1997; Bergh and Strand, 2001; Spencer, 2002; Torkildsen and Magnesen, 2004), with recent studies examining the impact of variables including water recirculation on larval production (Magnesen and Jacobsen, 2012), the treatment of broodstock with erythromycin on larval survival (Holbach *et al*., 2015) and the use of probiotics to combat pathogenic threats (Kesarcodi-Watson *et al*., 2016). However, it has been demonstrated that the mean yield of spat from eggs is as little as 1% (Andersen *et al*., 2011). Whilst hatchery production is still considered challenging for this species, it has been commercially produced since the 1980's and is currently produced in several European countries (Bergh and Strand, 2001; Robert and Nicholas, 2000; Spencer, 2002; Torkildsen and Magnesen, 2004; Anon, 2012; FAO, 2017).

Difficulties in the culture of bivalves have often been associated with settlement and metamorphosis, the period during which mature larvae undertake exploratory behaviour as they search for a suitable substratum, before undergoing permanent morphological changes allowing them to adapt to their new benthic habitat (Bayne, 1965; Pawlik, 1990; Lutz and Kennish, 1992; Gosling, 2003). In the hatchery environment, *P. maximus* exhibits a protracted development over several weeks, leading to a lack of synchronous development and variable levels of survival within larval batches (Nicolas *et al*., 1998; Robert and Nicholas, 2000). In the natural environment, settlement and metamorphosis of marine invertebrates is controlled by a range of interrelated biotic and abiotic factors operating over different temporal and spatial scales (Jackson, 1986; Pawlik, 1990; Pawlik, 1992; Rodríguez *et al*., 1993). Chemical cues and triggers have been identified as major influential factors on

settlement and metamorphosis in marine invertebrates (Pawlik, 1992), and have been associated as originating from conspecifics (Pearce and Scheibling, 1990a; Zhao and Qian, 2002), biofilms (Satuito *et al*., 1995; Zhao and Qian, 2002; Zhao *et al*., 2003), bacteria (Bonar *et al*., 1990; Fitt *et al*., 1990; Satuito *et al*., 1995), specific habitats (Pearce and Scheibling, 1990b; Swanson *et al*., 2004; Cob *et al*., 2010) and food sources (Steneck, 1982; Ritson-Williams *et al*., 2009). Whilst a number compounds have been isolated, including histamine from the red algae *Delisea pulchra* (Swanson *et al*., 2004), ammonia from bacteria (Coon *et al*., 1988; Bonar *et al*., 1990) and jacaranone from the red algae *Delesseria sanguinea* (Yvin *et al*., 1985), the identity of many of these naturally-derived cues remains unknown.

From an aquaculture perspective it has been recognised that the identification of chemical agents with inductive properties could provide an effective means of controlling settlement and metamorphosis in commercially important species (Cooper, 1982; Baloun and Morse, 1984; Mesías-Gansbillar *et al*., 2008; Teh *et al*., 2012; Kang *et al*., 2013). To this end a wide assortment of chemical agents have been tested in bivalves, with effects ranging from increased induction of normal settlement and metamorphosis, to abnormal development, death or no effect at all (Cooper, 1982; Coon *et al*., 1985; Doroudi and Southgate, 2002; Zhao *et al*., 2003; García-Lavandeíra *et al*., 2005; Yang *et al*., 2008; Mesías-Gansbiller *et al*., 2013). These chemicals are thought to act either as functional analogues of natural inducers, as precursors, or as active components within a signalling pathway (Yool *et al*., 1986; Pawlik, 1990). In bivalves a number of compounds have proven effective at stimulating settlement, metamorphosis, or both (Table 1).

Table 1: Chemical agents effective at inducing settlement and metamorphosis in marine bivalve larvae. X denotes a significant inductive effect.

Chemical	Bivalve species	Effective concentration (M = Moles, mM = Millimoles)	Settlement	Metamorphosis	References
γ-aminobutyric acid (GABA)	*Chlamys varia*	10^{-6}M	X		Mesías-Gansbiller *et al*., 2008
	Mytilus galloprovincialis	10^{-4}M	X	X	García-Lavandeira *et al*., 2005
	Ostrea edulis	10^{-4} / 10^{-5}M	X	X	García-Lavandeira *et al*., 2005; Mesías-Gansbiller *et al*., 2013
	Pinctada fucata martensii	10^{-4}M	X		Yu *et al*., 2008
	Pinctada margaritifera	10^{-4}M	X		Doroudi and Southgate, 2002

	Pinctada maxima	10^{-3}M	X		Zhao *et al*., 2003
	Ruditapes philippinarum	10^{-4}M	X	X	García-Lavandeira *et al*., 2005
	Venerupis pullastra	10^{-4}M	X	X	García-Lavandeira *et al*., 2005
L-3,4-dihydroxy-	*Crassostrea gigas*	10^{-4} / 10^{-5}M		X	Coon *et al*., 1985; Nicolas *et al*., 1998
phenylalanine	*Crassostrea iredalei*	10^{-6}M	X		Teh *et al*., 2012
(L-DOPA)	*Mytilus edulis*	10^{-5}M	X	X	Cooper, 1982; Dobretsov and Qian, 2003
	Ostrea edulis	10^{-4}M	X		Mesías-Gansbiller *et al*., 2013
	Patinopecten yessoensis	10^{-4}M		X	Kingzett *et al*., 1990
Potassium	*Argopecten purpuratus*	10mM	X	X	Martinez *et al*., 1999
	Mytilus coruscus	50mM		X	Yang *et al*., 2013
	Mytilus galloprovincialis	20 / 30mM	X	X	Yang *et al*., 2008; Sánchez-Lazo and Martínez-Pita, 2012
	Pinctada fucata martensii	20mM	X		Yu *et al*., 2008
	Pinctada maxima	10 to 30mM	X		Zhao *et al*., 2003
Ammonium	*Mytilus coruscus*	1mM		X	Yang *et al*., 2013
	Mytilus galloprovincialis	10mM		X	Yang *et al*., 2008
	Pinctada fucata martensii	10mM	X		Yu *et al*., 2008
	Patinopecten yessoensis	5mM		X	Kingzett *et al*., 1990
Acetylcholine	*Mytilus coruscus*	10^{-5}M		X	Yang *et al*., 2013
	Mytilus edulis	10^{-6}M	X		Dobretsov and Qian, 2003
	Mytilus galloprovincialis	10^{-2}M	X		Sánchez-Lazo and Martínez-Pita, 2012
	Pinctada fucata martensii	10^{-4}M	X		Yu *et al*., 2008
	Pinctada maxima	10^{-3}M	X		Zhao *et al*., 2003

Effectiveness of these compounds is influenced by concentration, length of exposure and by variations in larval sensitivity (Doroudi and Southgate, 2002; Zhao *et al*., 2003; García-Lavandeira *et al*., 2005; Yu *et al*., 2008; Teh *et al*., 2012; Mesías-Gansbiller *et al*., 2013). Therefore extensive testing of chemical agents with candidate bivalve species is essential.

In the case of *P. maximus* previous studies have shown that competent larvae have been induced to metamorphose in the presence of several agents (Table 2). However, this species has poor sensitivity to the few chemicals tested to date and induction levels remain relatively low. Further investigation is essential to assess the potential of alternative chemical agents and identify an effective application method.

Table 2: Chemical agents effective at inducing settlement and metamorphosis in *Pecten maximus*.

Chemical Agent	Effective concentration (mg litre^{-1})	Settlement	Metamorphosis	References
Epinephrine	1 - 10	X	X	Chevolot *et al*., 1991; Nicolas *et al*., 1996; Nicolas *et al*., 1998
Homogentisic acid	1		X	Chevolot *et al*., 1991
Jacaranone	0.5		X	Chevolot *et al*., 1991
L-DOPA	1 - 5		X	Chevolot *et al*., 1991; Nicolas *et al*., 1998

The aim of this study was to assess the effectiveness of several chemical agents, including four previously untested agents, on their ability to induce metamorphosis in competent *P. maximus* larvae, as well as assessing their impact on larval survival. This study tested the hypothesis that the effect of exogenous chemical agents on *P. maximus* larvae was chemical and concentration dependent.

Materials and Methods

Larval culture

Veliger larval *P. maximus* (202 ±19µm in shell length) were obtained from the Scalpro AS hatchery (Rong, Norway) and sent to the Centre for Applied Marine Sciences (CAMS) at Bangor University, Wales. On arrival, imported larvae were checked to determine quantity and condition, based upon survival, before stocking at a density of 5 larvae ml^{-1} in 65-litre static polyethylene tanks. Tanks were operated as static batch systems, and were filled to a volume of either 35 or 45 litres with 1µm filtered, UV-light irradiated seawater (FSW), at a salinity of 33‰. Seawater was sourced via a sub-surface pump in the Menai Strait, from where it was pumped into settlement tanks before being piped to the research laboratory for fine filtration using GE hytrex filter cartridges and irradiation with a 110W Commercial UV steriliser, before use. Culture temperature was maintained at 16±1˚C. Three times a week the larvae were sieved onto a 45µm mesh screen and inspected, and the containers cleaned before the larvae were restocked. Larvae were fed with a mixed microalgae diet

equivalent to 30 cells μl^{-1} day^{-1}, consisting of *Pavlova lutheri* (PLY75), *Isochrysis* sp. (clone T-ISO) (PLY506A) and *Chaetoceros calcitrans* (PLY537) at a ratio of 1:1:1. Veligers were reared until they reached competence to metamorphose, which was 7 to 8 days from when they arrived in the laboratory.

Competence of pediveligers to undertake metamorphosis was assessed based upon the presence of eye-spots. Experiments were initiated once the eye-spot ratio reached approximately 35-50%. Two batches of larvae were used for the assays carried out in this study. Notably, no size grading of larval cultures was undertaken. Assessment of mean larval size (µm) and survival percentage at competence, immediately prior to use, were also determined in order to provide additional reference indicators for this species of bivalve. Size was based on the shell length (µm) of approximately 30 larvae, with measurements made from digital analysis of photomicrographs using the image analysis software Image J. Survival was estimated by comparing stocked number of imported larvae and final numbers within cultures using sub-sample counts. In larval batch 1, larvae measured 236.8±25.5µm at competence. In batch 2 larvae measured 233.3±20.3µm at competence. Survival to this point was estimated at 100% for both batches.

Chemical agents

All chemicals, potassium chloride (KCl), ammonium chloride (NH_4Cl), acetylcholine chloride (ACH), γ-aminobutyric acid (GABA), and L-3,4-dihydroxyphenylalanine (L-DOPA), were obtained from Sigma-Aldrich (Poole, UK). Concentrated stock solutions of KCl (1Molar), NH_4Cl (1M), ACH (10^{-1}M), GABA (10^{-2}M) and L-DOPA (10^{-2}M) were prepared by dissolving the chemicals in FSW. All stock solutions were freshly prepared on the same day as the assay. Stock solutions were diluted into the FSW containing the larvae to achieve the experimental test concentrations. KCl was assayed at concentrations of 10, 20, 30 and 40mM; NH_4Cl at 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}M; ACH at 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}M; and GABA and L-DOPA at 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}M.

Chemical assays

Assays were carried out in 1000ml Pyrex-glass beakers containing 500ml of static FSW, stocked at a density of 5 larvae ml^{-1} and maintained at 16±1˚C. Each chemical treatment

concentration was carried out in triplicate in each assay. All assays were conducted in the light. Larvae were exposed to each test compound for 48 hours, after which they were thoroughly rinsed on a 45µm mesh screen to remove residual chemicals and restocked in clean 1000ml beakers containing FSW. Further water changes were conducted every 2-3 days. During the experiments, larvae were fed with a mixed microalgae diet as described for larval rearing. Larval batch 1 was utilised for assays of KCl, NH_4Cl and ACH, whilst larval batch 2 was used for assays of GABA and L-DOPA. Triplicate control treatments were included within each assay. . The beginning of the assay is defined as the point at which the chemical treatments were added to beakers containing larvae.

Three sub-samples of larvae were taken from each replicate beaker to assess larval metamorphosis and survival after the 48 hour induction period and at the conclusion of the experiment, after a total experimental period of 7 days (5 days post-treatment). Immediately prior to sampling a small brush was used to dislodge any attached post-larvae and the culture vessels were agitated to suspend all animals within the water column. Utilising a Leica DME binocular microscope the number of metamorphosed post-larvae was assessed and the total remaining number of live individuals counted to determine survival. Percentage metamorphosis and percentage survival were both calculated relative to the initial stocking density. Two criteria were used to assess larval metamorphosis; the appearance of elongated and functional gill filaments (gill formation) and the growth of secondary shell (dissoconch) (Nicolas *et al*., 1998).

Statistical analyses

All results are expressed as the percentage of larvae that have undergone metamorphosis, based upon both gill filament development and secondary shell growth, and larval survival. Before analysing, all percentage data sets were converted by arcsine square root transformation. The data presented in all figures is untransformed. Mean data sets were tested using the Anderson-Darling test to investigate departure from normality and Bartlett's test to assess heteroscedasticity before applying any test of comparison (Sokal and Rohlf, 1995). ANOVA tests were used to determine if there was any significant difference among treatments, followed by pairwise comparisons between treatments using Fisher's Least Significant Difference test (LSD). All results were considered to be significantly different when $P<0.05$. Analyses were undertaken using the statistical package Minitab®.

Results

Potassium chloride

Figure 1 shows the mean percentage of metamorphosed and surviving larvae after 48 hours and 7 days, in response to testing a range of KCl concentrations over an exposure period of 48 hours. At the beginning of the assay 46.9 ±6.0% of larvae possessed eye-spots, with 2.5 ±2.3% showed some signs of early gill formation and none showed any secondary shell growth.

After the initial 48 hour treatment period no KCl treatment induced higher levels of larval metamorphosis than the control, although larvae subjected to concentrations of 10, 30 and 40mM presented significantly lower gill formation (Figure 1a, Fisher's $P<0.05$). At this point there was no significant difference in survival compared to the control (ANOVA $F=0.37$, $P=0.872$, $DF=4$) (Figure 1b). After 7 days, both gill formation and survival rate were significantly impaired at concentrations of 20mM and above (Figures 1c and 1d, Fisher's $P<0.05$). Growth of secondary shell occurred at a significantly higher frequency (208% higher) in larvae exposed to 20mM KCl compared to the control (Fisher's $P<0.05$), and survival at this level was approximately 33% lower than the control.

L-3,4-dihydroxyphenylalanine

Figure 2 shows the mean percentage of metamorphosed and surviving larvae after 48 hours and 7 days, respectively, in response to testing a range of L-DOPA concentrations over an exposure period of 48 hours. At the beginning of the assay 39.7 ±3.4% of larvae possessed eye-spots, with 2.8 ±1.8% showing some signs of early gill formation and none showing any secondary shell growth.

L-DOPA was found to significantly influence larval metamorphosis and survival at the different concentrations assayed. By 48 hours L-DOPA treatments had decreased larval metamorphosis, with concentrations of 10^{-5}M and above showing significantly reduced gill formation (Figure 2a, Fisher's $P<0.05$). However, after 7 days significantly greater gill formation was observed in larvae exposed to 10^{-6}M compared to all other treatments (Figure 2c, Fisher's $P<0.05$). At this level, metamorphosis was 128% higher than observed in

the control. No other treatment significantly increased gill formation, whilst no treatment significantly encouraged secondary shell growth above that seen in the control group. Higher concentrations proved toxic, with concentrations of 10^{-4}M and 10^{-3}M significantly reducing survival rate after 48 hours compared to the control (Figures 2b, Fisher's $P<0.05$), with few or no larvae surviving at these concentrations by the 7th day (Figure 2d). In this larval batch survival rate after 7 days was relatively low, with a 63% drop in survival rate between 48 hours and 7 days in the control group. However, survival rate was significantly improved by L-DOPA at 10^{-6}M, with a 49% improvement over the control group by the 7th day (Figure 2d, Fisher's $P<0.05$).

Ammonium chloride

Figure 3 shows the mean percentage of metamorphosed and surviving larvae after 48 hours and 7 days, respectively, in response to testing a range of NH_4Cl concentrations over an exposure period of 48 hours. At the beginning of the assay the proportion of larvae possessing eye-spots and showing early gill formation was the same as for the KCl assay.

NH_4Cl did not elicit a significant improvement in larval metamorphosis in terms of either gill development or secondary shell growth over the study period (Figures 3a and 3c). Concentrations of 10^{-3}M and above significantly decreased larval gill formation and secondary shell growth at both sample points, compared to the control (Fisher's $P>0.05$). A concentration of 10^{-1}M NH_4Cl was highly toxic within 48 hours (Figure 3b, Fisher $P<0.05$). After7 days, concentrations 10^{-3}M and above resulted in significantly lower survival compared to the control (Figure 3d, Fisher's $P<0.05$).

Acetylcholine chloride

Figure 4 shows the mean percentage of metamorphosed and surviving larvae after 48 hours and 7 days, respectively, in response to testing a range of ACH concentrations over an exposure period of 48 hours. At the beginning of the assay the proportion of larvae possessing eye-spots and showing early gill formation was the same as for the KCl assay.

ACH over the tested concentrations did not elicit a significant improvement in either gill formation or secondary shell growth over the study period (Figure 4a and 4c). After 7 days gill development by larvae at all concentrations of ACH was significantly lower than that

seen in the control group (Fisher's P<0.05), although secondary shell growth was only significantly lower at the highest concentration of 10^{-2}M (Figure 4c), with almost total mortality experienced at this concentration (Figure 4d). Exposure to acetylcholine significantly reduced larval survival at concentrations of 10^{-4}M and above, but only after 7 days, (Fisher's P<0.05), with the level of toxicity increasing with concentration (Figure 4d).

γ-aminobutyric acid

Figure 5 shows the mean percentage of metamorphosed and surviving larvae after 48 hours and 7 days, respectively in response to testing a range of GABA concentrations over an exposure period of 48 hours. At the beginning of the assay the proportion of larvae possessing eye-spots and showing early gill formation was the same as for the L-DOPA assay.

Over the tested concentrations GABA inhibited larval development in most cases (Figures 5a and 5c). As with ACH, the toxicity of GABA was not apparent at 48 hours, but survival rate after 7 days was reduced in treatments exposed to concentrations of 10^{-5}M and above (Fisher's P<0.05), with the level of toxicity increasing with concentration (Figure 5d). Only a concentration of 10^{-6}M did not depress larval development, using either measure, or survival.

Discussion

P. maximus is known to exhibit protracted metamorphosis over a 2 to 3 week period, during which 35 to 70% of larvae metamorphose, with metamorphosis in the first week often not exceeding 5% (Nicolas *et al*., 1996; Nicolas *et al*., 1998; Robert and Nicholas, 2000).

We found no positive influence of any chemical treatment over metamorphosis within the first 48 hours, this was only detected after 7 days, reflecting the slow larval development progression of this species (Robert and Nicholas, 2000). Progress of larval development in control treatments ranged from 5.6 to 19.1% with functional gills appearing in the first 48 hours, reaching 12.7 to 50.9% with functional gills and 2.2 to 2.9% with dissoconch growth after 7 days. The rate of metamorphosis in our control groups, based upon dissoconch

growth, is similar to that witnessed in previous studies (Nicolas *et al*., 1996; Nicolas *et al*., 1998; Robert and Nicholas, 2000).

The effectiveness of chemical induction appears to be related to the competence of larvae to undergo metamorphosis (Coon *et al*., 1990; Chevolot *et al*., 1991); if larvae are immature then the chemical is unlikely to stimulate a response. There is a correlation between competence and morphological features, such as size and the formation of eye-spots, though considerable variability in responses in batches of larvae have been reported (Bonar *et al*., 1990). In *P. maximus* competence has previously been related to the presence of double shell rings at the margin of the shell, which corresponds to the peripheral groove to which the dissoconch shell attach (Chevolot *et al*., 1991; Nicolas *et al*., 1998). Competence of *Pecten maximus* to undertake settlement has also been associated with a larval size exceeding 212µm (Robert and Gérard, 1999) and size grading with 150µm mesh screens selects for a higher percentage of competent larvae (Chevolot *et al*., 1991; Nicolas *et al*., 1998). In this study the mean size of each batch of *P. maximus* larvae was over 200µm, in line with previous studies. Eye-spot formation has been used to characterise the end of larval stages in other bivalve species including *Crassostrea gigas* (Coon *et al*., 1990), *Pinctada margaritifera* (Doroudi and Southgate, 2002), *Pinctada maxima* (Zhao *et al*., 2003) and *Mytilus edulis* (Eyster and Pechenik, 1987; Dobretsov and Qian, 2003). In the present study the level of larval competence was based upon the presence of pigmented eye-spots, rather than the presence of a peripheral groove.

Potassium chloride

Our results demonstrated for the first time that excess K^+ acts as a positive metamorphic agent in *P. maximus*, promoting a significant increase in dissoconch growth. The widespread effect of excess K^+ in the metamorphosis of some invertebrates has led to the suggestion that there may be a shared sensitivity to K^+ as an inductive cue in marine invertebrates (Yool *et al*., 1986). The exact mode of action remains to be ascertained although it is thought to be a consequence of the depolarisation of external cell membrane caused by the influx of K^+ (Baloun and Morse, 1984). Whilst K^+ has previously been shown to be effective in a number of bivalves in each case metamorphosis has been observed following a lag period of 2 to 4 days after a treatment window of 24 to 48 hours (Martinez *et al*., 1999; Yang *et al*., 2008; Yang *et al*., 2013). In contrast continuous exposure, in the case of *M. galloprovincialis* for 48

to 96 hours, has been shown to inhibit metamorphosis (Yang *et al*., 2008). This indicates shorter exposure periods may prove more beneficial. In the scallop *A. purpuratus* KCl was effective at a concentration of 10mM over an exposure of 48 hours, utilising a similar protocol to the present study (Martinez *et al*., 1999). However, whilst Martinez *et al*. (1999) induced approximately 45% of larvae to metamorphose, the level of synchronous larval development in the current study remains low. In other marine invertebrates sensitivity to K^+ as a metamorphic inducer has ranged from approximately 15% in the gastropod *Haliotis diversicolor (*Bryan and Qian, 1998) to 100% effective in the gastropods *Crepidula fornicata* (Pechenik and Heyman, 1987), and *Strombus canarium* (Cob *et al*., 2010), whilst it has notably failed to induce a response in *M. edulis* (Eyster and Pechenik, 1987; Dobretsov and Qian, 2003).

Assessment of metamorphosis in *P. maximus* has typically relied upon the presence of post-larval shell growth (dissoconch) as an indicator (Chevolot *et al*., 1991; Nicolas *et al*., 1998), however the differentiation of functional gill bars has also been used as an indicator in Pectinidae and other bivalves (Bonar *et al*., 1990; Fitt *et al*., 1990; Kingzett *et al*., 1990; Martinez *et al*., 1999). In addition to dissoconch growth, gill formation represents a clear, distinctive change involving the degeneration of the velum and the formation of ciliated gill buds which become the feeding organ of the animal (Gruffydd and Beaumont, 1972; Gosling 2003; García-Lavandeira *et al*., 2005). The current study utilised both measures to assess metamorphosis. However, this has shown that if gill formation had been taken as the sole indicator of metamorphosis this would have shown that exposure to KCl does not induce a metamorphic response. In fact, KCl notably inhibited gill formation at concentrations of 20mM and above. It is therefore clear that the indicators of metamorphosis do not necessarily correlate and can have a high impact upon perceived influences.

Prolonged exposures to excess K^+ at concentrations of 50mM and above are known to be toxic to some bivalve larvae (Martinez *et al*., 1999; Zhao *et al*., 2003; Yu *et al*., 2008). In the current study any positive influence on metamorphosis must be offset against a drop in survival of approximately 33% at an effective induction concentration (20mM), with concentrations of 20mM and above proving toxic to some degree. Whilst showing promise, further testing is required to establish the full potential of KCl as an inductive metamorphosis agent to use with *P. maximus*.

L-3,4-dihydroxyphenylalanine

L-DOPA has proven to be effective on inducing metamorphosis in a number of bivalve species, including *P. maximus*, although larval response is concentration and exposure time dependent. We found that L-DOPA has a positive influence on both larval gill formation and survival. This is the reverse of the result seen with KCl, meaning that it would not have been observed if only dissoconch growth had been measured. No other treatment provided a beneficial influence on *P. maximus* larvae, although concentrations of 10^{-4}M above were detrimental to both development and survival.

Comparison of our results and previous studies with *P. maximus* (Chevolot *et al*., 1991; Nicolas *et al*., 1998) showed that overall induction levels with L-DOPA remain low. However, previous studies found L-DOPA induced significantly higher rates of dissoconch growth, at concentrations of 5.1 x 10^{-6}M and 2.54 x 10^{-5}M (1 to 5mg/l) over exposures of 24 to 48 hours, with optimum metamorphosis ranging from 6 to 12% by 1 week (Chevolot *et al*., 1991; Nicolas *et al*., 1998). This is in contrast to our findings, since no observable improvement was seen in dissoconch growth across similar concentrations. In this study dissoconch growth was highest in the 10^{-6}M treatment at 2.4±0.8%, slightly lower than in other studies. Concentrations equivalent to 10^{-6}M (0.2 mg/l) and below reportedly have no inductive metamorphic effect (Nicolas *et al*., 1998), however previous studies did not record gill formation for comparison.

L-DOPA concentrations of 5.07 x 10^{-5}M (10 mg/l) have previously been reported to reduce metamorphosis and be toxic to *P. maximus* larvae (Nicolas *et al*., 1998). However, this study demonstrates the effect of L-DOPA concentration on survival in this species to a degree not previously shown. At 10^{-5}M there is no detrimental influence according to either measure of larval development or survival, however at 10^{-4}M and above L-DOPA is highly toxic to larvae and reduces development. This is in line with studies on other species of bivalve (Coon *et al*., 1985; Zhao *et al*., 2003; Yu *et al*., 2008).

Although the site of action of L-DOPA remains unclear, our results suggest the pathway influenced by L-DOPA maybe distinct from that of excess K^+ in *P. maximus,* whilst both were effective at inducing a larval response, L-DOPA induced increased gill formation compared to increased dissoconch growth induced by KCl.

Ammonium chloride, Acetylcholine chloride and γ-aminobutyric acid

Each of these chemicals has proven to be an effective metamorphosis inducer in other bivalve species, yet they have also produced only at best inconsistent responses in several other ones (Cooper, 1982; Coon *et al*., 1985; Eyster and Pechenik, 1987; Dobretsov and Qian, 2003; Zhao *et al*., 2003).

In the current study, examination of the effect of NH_4Cl, ACH and GABA on the metamorphosis of larval *P. maximus* showed that there was no positive inductive influence either after 48 hours or by 1 week. However each chemical proved potentially detrimental to larvae development and survival. Concentrations of NH_4Cl at or above 10^{-3}M clearly impaired both larval development and survival, although this was not necessarily evident until after 7 days. Whilst a negative impact of ACH on larvae was seen at each tested concentration, although this was only fully evident after 7 days. ACH reduced gill formation at each concentration tested and reduced larval survival at concentrations of 10^{-4}M and above, although dissoconch growth was only reduced at the highest concentration (10^{-2}M). For GABA, only at a concentration of 10^{-6}M was gill formation, dissoconch growth and survival rates equal to the control after 1 week. All other treatments proved detrimental to development and toxic to larvae, but this was only apparent after seven days exposure.

Potential interaction of induction stimuli

The influence of chemical cues cannot be considered in isolation to other factors. Several studies have demonstrated that bivalves settle or metamorphose more readily in the presence of seawater turbulence (Eyster and Pechenik, 1987; Nicolas *et al*., 1996; Nicolas *et al*., 1998). In *P. maximus* cultures in still water conditions and without chemical induction, the metamorphosis rate after 1 week rarely exceeds 5%, however turbulence alone has increased metamorphosis of this species by as much as 16% (Chevolot *et al*., 1991; Nicolas *et al*., 1996; Nicolas *et al*., 1998). Additionally the provision of adequate nutrition, both in terms of quantity and quality, is also critical to larval development in *P. maximus* (Nicolas and Robert, 2001; Tremblay *et al*., 2007). Tremblay *et al*. (2007) determined that in *P. maximus* the composition of microalgae diets influenced the rate of metamorphosis, and that the incorporation of *Rhodomonas salina* or the replacement of *Chaetoceros gracilis* by

Skeletonema costatum in traditional hatchery diets (*Isochrysis aff. galbana, P. lutheri and C. gracilis*) could lead to an improved metamorphosis rate. However, it is the combining of multiple factors which is possibly of the greatest interest. Previous studies have demonstrated a cumulative effect on settlement and metamorphosis by several factors, in addition to the use of combinations of chemical agents (Baxter and Morse, 1987). Nicolas *et al*. (1998) found that combining seawater turbulence with the chemical agent L-DOPA increased metamorphosis over each variable individually, although the reverse was true for the chemical epinephrine. Therefore there is potential to investigate the combination of stimuli in an effort to further improve induction.

Conclusion

In conclusion this study assessed the effect of five chemical agents on larval metamorphosis and survival, four of which had never previously been examined in *P. maximus*. The newly tested chemical KCl can be added to the list of agents effective at inducing a metamorphic response in *P. maximus*, alongside the chemical L-DOPA which was re-examined in this study. In contrast NH_4Cl, ACH and GABA all proved ineffective, although their lack of influence in this bivalve species was not previously known. However, it is apparent from the results of this study that alternate methods of assessing metamorphosis can give differing results. Reliance on just one method of assessment would have missed a positive effect by one of the chemicals we tested. Future studies should consider carefully which criteria to apply, especially in species with slow protracted development, such as *P. maximus*, where treatment effects may be small. The positive effect of KCl on metamorphosis must also be off-set against a drop in survival, which was not observed with L-DOPA. The induction of synchronous larval development in *P. maximus* remains to be achieved. Recommendations for application within a hatchery environment cannot therefore be made at this point. Further investigation of these and other chemical agents, as well as their method of application, is recommended to determine whether a sufficiently effective and economically viable method for induction of synchronised metamorphosis in *P. maximus* can be found.

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List of Figure Legends

Figure 1: Percentage metamorphosis and larval survival in *Pecten maximus* at 48 hours (a, b) and after 7 days (c, d), following exposure to 10mM, 20mM, 30mM and 40mM of KCl for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control ($P<0.05$).

Figure 2: Percentage metamorphosis and larval survival in *Pecten maximus* at 48 hours (a, b) and after 7 days (c, d), following exposure to 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}M of L-DOPA for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control ($P<0.05$).

Figure 3: Percentage metamorphosis and larval survival in *Pecten maximus* at 48 hours (a, b) and after 7 days (c, d), following exposure to 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}M of NH_4Cl for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control ($P<0.05$).

Figure 4: Percentage metamorphosis and larval survival in *Pecten maximus* at 48 hours (a, b) and after 7 days (c, d), following exposure to 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}M of acetylcholine chloride for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control ($P<0.05$).

Figure 5: Percentage metamorphosis and larval survival in *Pecten maximus* at 48 hours (a, b) and after 7 days (c, d), following exposure to 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}M of γ-aminobutyric acid for 48 hours. All points represent the mean ±standard deviation. An asterisk (*) represents a result significantly different to the untreated control ($P<0.05$).

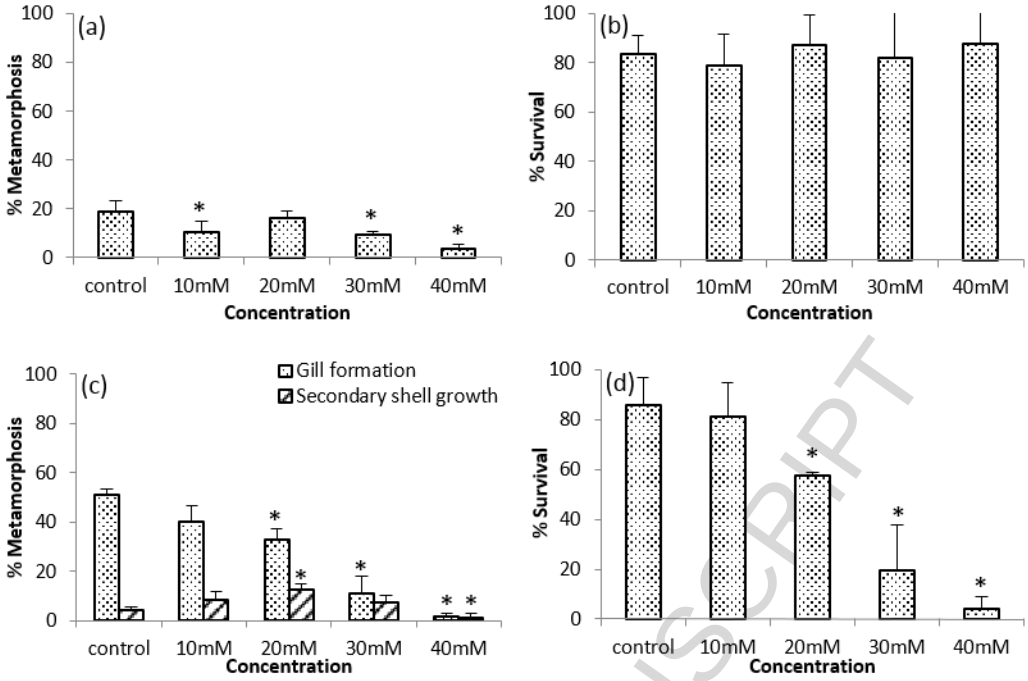

Figure 1

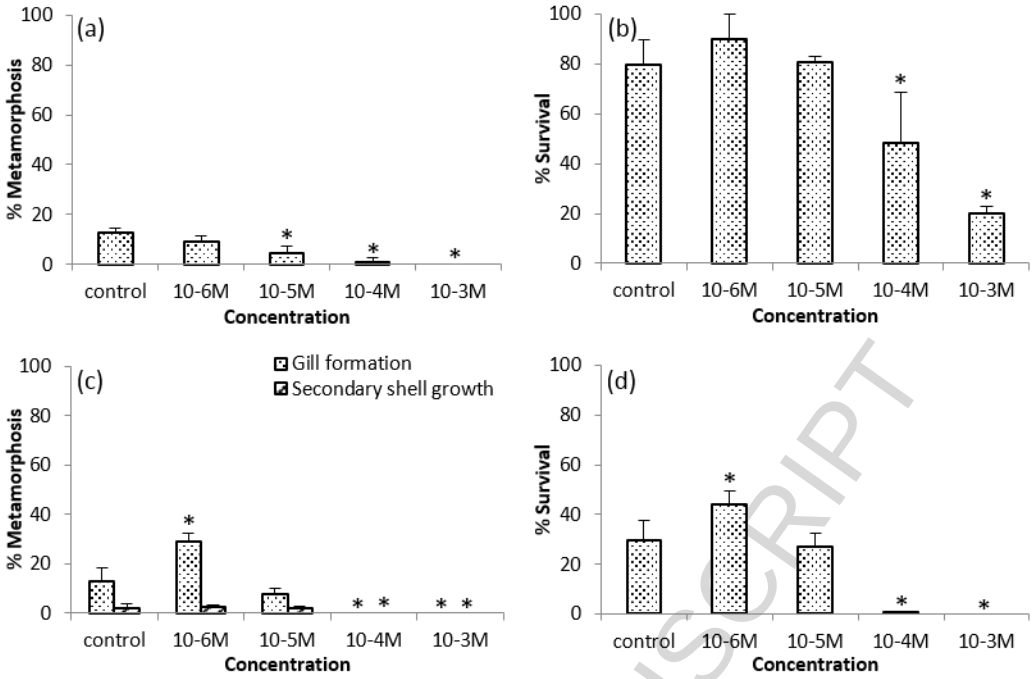

Figure 2

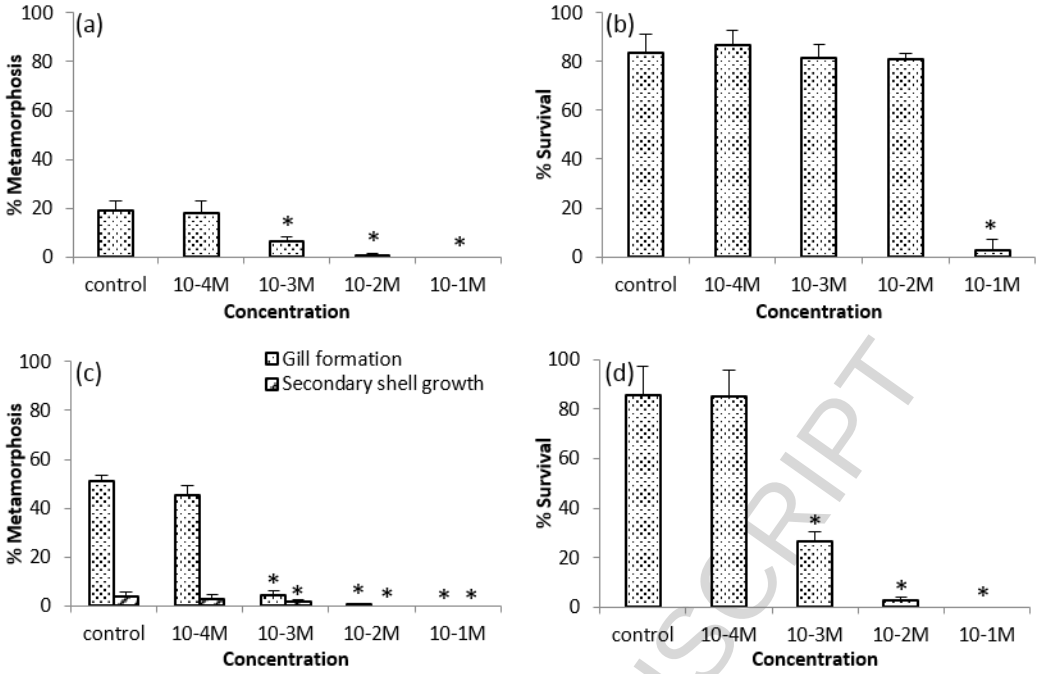

Figure 3

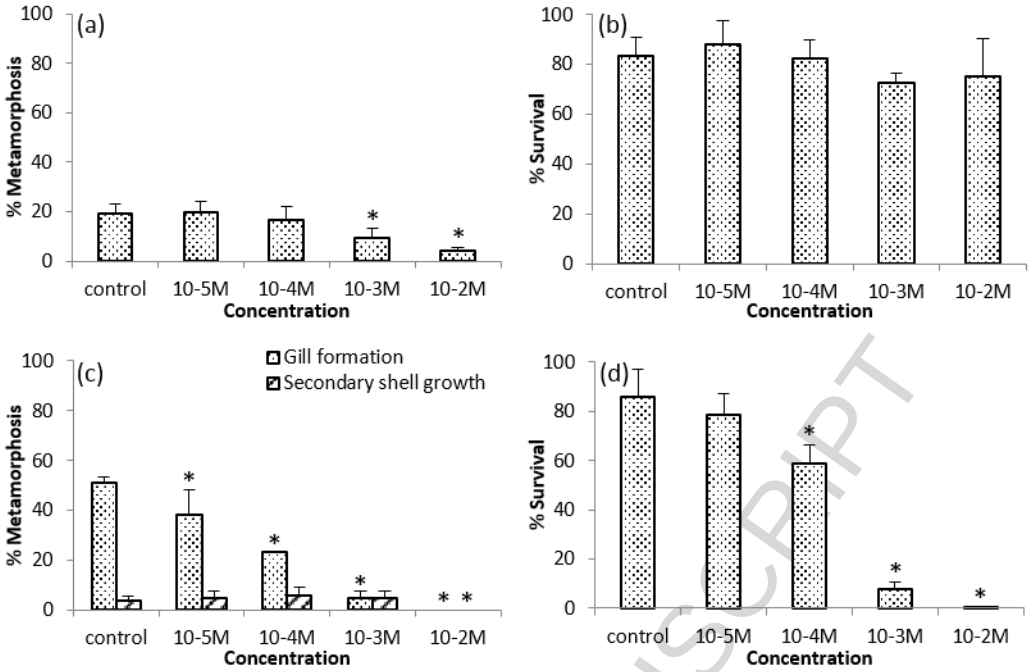

Figure 4

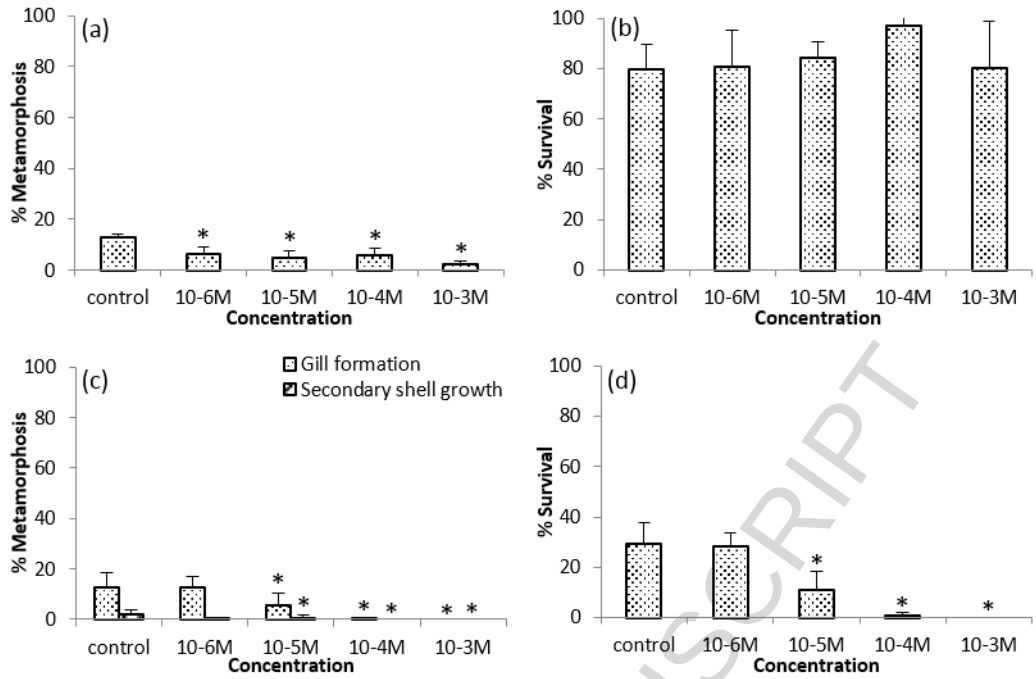

Figure 5

Statement of Relevance

Influence of exogenous chemicals on larval development and survival of the king scallop *Pecten maximus* (L.)

The results of this study provide important advances in our understanding and knowledge of the use of chemical agents in the commercially important bivalve *Pecten maximus*. This study has identified a new inductive chemical and highlighted considerations for future investigators.

Highlights

- Assessed effect of chemical agents on larval metamorphosis and larval survival
- Metamorphic response was low, and concentration-dependent
- KCl and L-DOPA at concentrations of 20mM and 10^{-6}M, respectively, increased the rate of metamorphosis, the final stage of larval development
- Effective KCl treatment reduced survival by 33%, whilst L-DOPA increased survival by 49%
- KCl promoted dissoconch growth and L-DOPA promoted gill development
- Different methods of assessing metamorphosis can give different results