Evaluation of the risks posed by exogenous chemicals on the mechanisms of endocrine control in crustaceans.

(Contract number CDEP 84/5/294)

Watson, G.M, Depledge, M. H. & Bamber, S. D.

Plymouth Environmental Research Centre, University of Plymouth, Plymouth, Devon, PL4 8AA.

CONTENTS

EXECUTIVE SUMMARY

Little is known regarding the potential for chemicals to disrupt endocrine processes in invertebrate species. Investigations have thus far chiefly focussed on the potential influence of vertebrate type hormones and their analogues. There is however, little direct evidence that natural and anthropogenic compounds capable of modifying endocrine control in vertebrates have similar effects in invertebrates such as crustaceans. Investigations at the Plymouth Environmental Research Centre (PERC) carried out as part of the EDMAR programme did not show any alterations in the hormonally controlled processes of vitellogenesis, heart rate or osmoregulation in shore crabs (*Carcinus maenas*) following their exposure to various natural and synthetic vertebrate sex steroids. Preliminary findings suggest that the pathways and chemical messengers used by invertebrates differ from vertebrate species and little is known of the potential for exogenous contaminants to disrupt these pathways or mimic/antagonise the action of specific invertebrate hormones. Therefore, the aim of the present study was to shift the focus from vertebrate type hormones to the potential for exogenous invertebrate type hormones and their anthropogenic analogues to affect hormonally regulated processes in a model invertebrate (the decapod crustacean, *C.maenas*). The specific endocrine mediated functions under investigation were moulting, vitellogenesis and locomotor activity.

The hormone applied exogenously in this study was the ecdysteroid moulting hormone 20-hydroxyecdysone (20-HE). 20-HE is involved not only in moulting processes but also plays a part in reproduction and vitellogenesis and may have a role in behaviour and rhythmic locomotory processes. In order to identify any hormonally related disturbances in these processes, juvenile and adult (male/female) crabs were exposed to waterborne 20-HE in 3 different test systems. Waterborne exposures were of the semi-static renewal type with 20-HE dosed into seawater using methanol as a solvent vehicle (to increase its solubility).

The effect of exogenous 20-hydroxyecdysone exposure on vitellogenesis in females was investigated using a quantitative enzyme linked immunosorbent assay (ELISA) to measure the concentration of the yolk protein precursor vitellogenin present in haemolymph samples. Development, refinement and validation of the quantitative ELISA was carried out within the time frame of the project. Groups of female crabs were exposed to waterborne 20-HE at the nominal concentrations of 100µg/L, 5µg/L, 1µg/L and 100ng/L for a period of 16 weeks with appropriate controls run simultaneously. In order to establish the normal pattern of haemolymph Vg levels in vitellogenic females over time, a baseline study was run alongside the exposure trials. 40 female pre-moult crabs, collected and maintained as above, had haemolymph samples taken weekly over a period of 24 weeks, which were subsequently analysed using the ELISA described above.

The effect of 20-HE on moulting processes was studied by observing moulting frequency of juvenile (carapace width of <12mm) *Carcinus* in control and exposed individuals. To avoid cannibalism and allow identification of moulted individuals, crabs were kept separately in sealed “cages” suspended from glass frames, immersed in glass aquaria containing aerated seawater. Waterborne exposures were carried out at the nominal concentrations of 100µg/L, 5µg/L and 1µg/L 20-hydroxyecdysone with solvent and seawater controls over a period of 16 weeks. Mortality, moult frequency, moult interval and growth following moult were recorded over the exposure period.

Locomotor activity rhythms of freshly collected crabs were studied under constant conditions in tanks equipped with an actograph system. “Locomotory events” were plotted against time to produce characteristic peaks of activity coincident with times of expected high tide and darkness. In each of two separate exposure trials, 4 crabs were exposed to 100μg/L 20- hydroxyecdysone, alongside 4 solvent controls, for a period of 5 days.

Exposure to 20-HE appeared to have limited impact on the three processes investigated. There were no gross effects on moulting processes (moult frequency and growth following moult) besides increased mortality in the two highest exposure groups. No obvious effects of 20-HE exposure on locomotor activity or rhythmicity were observed. No obvious changes in vitellogenic events were observed in exposed individuals compared to controls, although it is possible that the timing of the trial was too late in the summer to include the peak in vitellogenic processes that became evident in the base line trial. However, the optimisation of the ELISA for quantification of vitellogenin in haemolymph provides us with an invaluable tool for monitoring Vg levels in wild populations as a potential biomarker of endocrine disruption. Before such an application however, a much more thorough understanding of vitellogenic events and their temporal progression in wild populations is required.

LIST OF FIGURES

INTRODUCTION

Endocrine disruption can be defined as the interaction of exogenous xenobiotics with the endocrine systems of organisms. The phenomenon is well documented in vertebrate species including man (Sharpe and Skakkebaek 1993, Colborn *et al* 1996). The effect of vertebrate type hormones on aquatic organisms has received considerable investigation, with reports of sex changes in riverine fish (Sumpter 1995) and marine snails (Matthiessen and Gibbs 1998) and abnormalities in the reproductive organs of alligators (Guillette *et al* 1994).

Less is known regarding the potential for chemicals to disrupt endocrine processes in invertebrate species (see Depledge and Billinghurst 1999 for review). Since invertebrates account for 95% of all described animal species and are vital components of marine ecosystems (deFur *et al* 1999), it is pertinent to study the potential effects of anthropogenic contaminants on their hormonally regulated functions. Investigations into endocrine disruption in invertebrates have thus far chiefly focussed on the potential influence of vertebrate type hormones and their analogues. There is little direct evidence that natural and anthropogenic compounds capable of modifying endocrine control in vertebrates have similar effects in invertebrates such as crustaceans (see Pinder *et al* 1999 for review). Investigations at the Plymouth Environmental Research Centre (PERC) carried out as part of the EDMAR programme did not show any alterations in the hormonally controlled processes of vitellogenesis, heart rate or osmoregulation in shore crabs (*Carcinus maenas*) following their exposure to various natural and synthetic vertebrate sex steroids. Despite the gaps in our knowledge regarding invertebrate endocrine sytems, preliminary findings suggest that the pathways and chemical messengers used by invertebrates differ from vertebrate species. Thus, exogenous sources of oestrogen and its mimics together with androgens do not appear to have any significant effects on the endocrine controlled physiological or reproductive processes investigated in this decapod species.

Little is known of the potential for exogenous contaminants to mimic or antagonise the action of specific invertebrate hormones. Therefore, the aim of the present study is to shift the focus from vertebrate type hormones and instead concentrate on the potential of exogenous invertebrate type hormones and their anthropogenic analogues to effect hormonally regulated processes in a model invertebrate, (the decapod crustacean, *C.maenas*). The specific endocrine mediated functions under investigation are moulting, vitellogenesis and locomotor activity.

The hormone being applied exogenously in this study is the ecdysteroid moulting hormone 20-hydroxyecdysone. The ecdysteroid moulting hormone ecdysone is produced endogenously in the Y organ (a paired epithelial endocrine gland) (Fingerman 1987) of decapod crustaceans. The Y organ converts dietary cholesterol into ecdysone and secretes it into the haemolymph. It is then transported to peripheral tissues where it is converted to the bioactive haemolymphatic form, 20-hydroxyecdysone (or β-ecdysone), titres of which increase just prior to moulting (Lachaise *et al* 1993). Ecdysteroids exert their effect by binding to an intracellular receptor protein within the target tissue. Synthesis of ecdysteroids by the Y organ is under inhibitory control from MIH (moult inhibiting hormone). MIH is a member of a different group of hormones, the neuropeptides, produced from the X-organ/sinus gland complex associated with the eyestalk in decapods. Ecdysteroid synthesis is also under stimulatory control from another steroid hormone, the sesquiterpenoid methyl farnesoate (MF), produced by the mandibular organ (Tamone and Chang 1993).

20-hydroxyecdysone, and the other ecdysteroids are not only involved in the moulting process but have also been shown to be involved in reproduction and vitellogenesis and may have a role in behaviour and rhythmic locomotory processes. It has been suggested that ecdysteroids may stimulate vitellogenesis (Fingerman 1997) and ovarian maturation and protein synthesis (Chan 1995, Oberdorster and Cheek 2001), although this is still poorly understood. Indeed, in some species, administration of ecdysteroids has inhibited vitellogenesis (Chang 1989). It appears that the ability of ecdysteroids to promote vitellogenesis in the hepatopancreas is species dependent (Loeb 1993) as is the need for ecdysteroids for the completion of vitellogenesis (Pinder *et al* 1999). Correlations between haemolymph ecdysteroid titres and vitellogenesis have been reported (Chang 1993, Okumura *et al* 1992, Young *et al* 1993a,b). However, it is still unclear as to whether ecdysteroids directly influence vitellogenesis, or that their levels during vitellogenesis are simply indicative of the corresponding stage of the moult cycle.

In order to identify any hormonally related disturbances in the processes of moulting, vitellogenesis and locomotor activity in crustaceans, it is important to understand the functioning of these systems under normal conditions.

MOULTING.

Crustaceans must shed their exoskeleton (ecdysis) periodically for growth to occur, a process known as moulting. Ecdysis is in fact only a small part of the moult cycle, which can take up to a year or more and involves profound physiological and biochemical changes (Chang 1995).

Endocrine control of moulting.

During the moult cycle, circulating levels of ecdysteroids vary considerably. At postmoult, haemolymph titers of ecdysteroids are negligible and remain so throughout intermoult. Ecdysteroid levels increase dramatically in premoult and then drop steeply prior to ecdysis (Chang 1989). During intermoult, production and secretion of ecdysteroids by the Y-organ is under inhibitory control from MIH, produced by the x-organ/sinus gland complex, keeping circulating titers low. High affinity binding of MIH to Y organ membrane bound receptors has been demonstrated in *C.maenas* (Webster 1993). During early premoult, the Y-organ is freed from inhibition and ecdyteroid levels rise, triggering the latter stages of premoult, before dropping prior to ecdysis. Postmoult sees ecdysteroid production inhibited and haemolymph titers returned to basal levels. Removal of Y-organ inhibition by eyestalk ablation leads to a rapid and dramatic increase in the levels of circulating ecdysteroids and therefore to precocious moulting (Chang 1989,1995, Skinner 1985). This shortening of the moult cycle has been observed in many crustaceans (Chang 1989 & 1995, Skinner 1985).

The present study seeks to determine if such precocious moulting could be triggered by exogenous sources of ecdysteroids or compounds which mimic their action. This would be distinctly disadvantageous for field exposed crustaceans. Such individuals might prematurely enter into stages of the moult cycle whilst being subject to unfavourable environmental conditions or be at increased risk of predation. The present study will address this issue and *C.maenas* is to be exposed to exogenous sources of 20-hydroxyecdysone and the effect on moulting observed.

VITELLOGENESIS

Female decapods develop a large number of heavily yolked eggs (oocytes) in the ovary. *C.maenas* for example, produces an average of 185,000 eggs in each reproductive cycle (Crothers 1967). The yolk, containing proteins, lipids and carbohydrates provides nourishment for the developing embryos and nauplii which must subsist on it for up to several weeks following hatching (Tom *et al* 1992). The process of yolk synthesis and deposition is termed vitellogenesis (Subramoniam 1999) and the major yolk protein that accumulates in the oocytes is vitellin (Vt) (Lee *et al* 1996). The primary translation product and precursor of vitellin is a high density lipoprotein called vitellogenin (Vg), present in the haemolymph of vitellogenic females. Vg is immunologically identical to Vt (Lee *et al* 1996). Quantitative and semiquantitative correlations between haemolymph Vg and ovarian Vt concentrations in developing oocytes and have been observed in many crustaceans (Adiyodi 1985, Okumura and Aida 2000). It is suggested that vitellogenin is synthesised in the ovary and also in extraovarian tissues (specifically the hepatopancreas) and transported in the haemolymph to the ovary, where it is converted to vitellin and incorporated as yolk globules into developing oocytes (Lee *et al* 1996, Chen and Chen 1994).

Endocrine control of vitellogenesis.

Vitellogenesis is under the strict control of a number of antagonistic hormones. There are four main hormones involved in the control of vitellogenesis - the neuropeptide gonad inhibiting hormone (GIH), (also known as vitellogenesis inhibiting hormone -VIH), neuropeptide gonad stimulating hormone (GSH), the sesquiterpenoid methyl farnesoate (MF), and ecdysteroid(s). This study is concerned primarily with the role of 20-hydroxyecdysone in vitellogenesis and attempts to elucidate the effect of exogenous application of this hormone on Vg levels in female crabs.

There is evidence in the literature that in some crustaceans, ecdysteroids play a role in vitellogenesis. It has been suggested that high ecdysone titres related to premoult processes may promote the early stages of vitellogenesis (Skinner 1985). Ecdysteroids (ecdysone, 20-hydroxy ecdysone and ecdysteroid conjugates) have been detected in follicles, oocytes and embryos of several species of shrimp, crab and amphipod (Chang 1989), and the available evidence indicates that these ecdysteroids are sequestered into the ovary by binding to yolk precursor proteins (Subramoniam 2000). The specific function of these ovarian ecdysteroids is, however, unclear. Increased ecdysteroid titres in the haemolymph have been correlated with the progression of vitellogenesis in certain species, including *C.maenas* (Lachaise et al 1981), the spider crab *Acanthonyx lunulatus* (Chaix *et al* 1982) and the freshwater prawns *Macrobrachium nipponense* (Okumara *et al* 1992) and *M.rosenbergii* (Young *et al* 1993a). However, other studies have reported decreasing levels of haemolymph ecdysteroids during vitellogenesis eg: *Panaeus monodon* (Young *et al* 1993b). Young *et al* (1993a) suggest the differences observed may indicate that the roles of ecdysteroids in vitellogenesis in these two species differ. ie: ecdysteroids stimulate vitellogenesis in *M.rosenbergii*, but are not directly involved in *P.monodon* vitellogenesis.

Similar conflicting results are reported in studies where ecdysteroids are administered *in vivo* or *in vitro*, with the effect being either inhibitory or stimulatory

(Chang 1993, Chang 1989). Clearly further research is required to determine the roles of ecdysteroids in vitellogenesis and the present study aims to determine the effect of exogenous ecdysteroids on vitellogenesis in the shore crab, be it inhibitory or stimulatory.

LOCOMOTOR ACTIVITY AND ENDOGENOUS RHYTHMICITY.

In addition to the processes of moulting and reproduction in *C.maenas*, the present study will investigate the hormonal control of locomotor activity in this species. At present, information on the hormonal regulation of locomotor activity in crustaceans is limited. Presented below is a general overview of *C.maenas* locomotor activity patterns relevant to the present research and a brief description of what is presently known about their hormonal regulation.

Locomotory activity patterns in *C.maenas*.

The locomotory activity exhibited by *C. maenas* is regulated by interlinked endogenous circadian and circatidal rhythms. The resultant locomotor pattern consists of peaks of activity around times of high tide (circatidal) overlaid with periods of increased activity at times of nocturnal high tides as compared to diurnal high tides (circadian) (Williams 1985). Essentially, the level of activity associated with a tidal peak is modulated by the circadian rhythm (Webb 1983). This rhythmicity in locomotor activity allows *C. maenas* to undertake daily migrations into inter-tidal areas where food and mating sites can be found. Increased activity at the time of nocturnal high tides also affords crabs increased foraging opportunities with limited risk of predation. Cessation of activity at low tide allows crabs to seek shelter, avoiding desiccation and avian predation (Naylor 1985).

The characteristic pattern of locomotor activity is maintained in the laboratory under constant conditions for 4-6 days before starting to break down (Williams 1985) with crabs kept in normal light-dark, non-tidal conditions exhibiting a “daily rhythm” overlaid with a weak approximate tidal component (Webb 1983). Crabs kept for a month or more under constant conditions, whilst subject to constant illumination, showed a circadian rhythm with no tidal component (Webb 1983).

Under natural conditions, circadian/tidal rhythms are continually entrained (synchronised with predictable patterns of environmental change) by a number of exogenous variables (salinity, hydrostatic pressure, temperature, immersion and wave action) to ensure the expressed behaviour is in phase with the environmental regime (Bolt and Naylor 1986, Naylor 1985, Reid and Naylor, 1985). Under constant laboratory conditions, in the absence of environmental cues, the accuracy of endogenous free running rhythms slowly decreases and rhythmic patterns of behaviour become imprecise (Naylor 1985). Tidal rhythms in *C. maenas* can, however, be entrained in the laboratory by simulated tides with peaks of high salinity (Bolt and Naylor 1986), and subtle changes in temperature and pressure associated with tides in the normal habitat (Williams and Naylor 1969, in Naylor 1985). The entrained rhythmicity is then maintained when the animal is returned to constant conditions.

Endocrine control of locomotor activity and rhythmicity.

Little is presently known regarding the endocrine regulation of locomotor activity and endogenous rhythms in decapod crustaceans. Experiments involving eyestalk ablation identified the presence of an eyestalk factor responsible for the regulation of locomotor activity in crustaceans. Further research determined this factor to be a neuropeptide, termed neurodepressing hormone (NDH). NDH, secreted by the sinus gland, depresses the responsiveness of motor and sensory neurons and decreases the spontaneous firing of motor neurons (Fingerman 1987). It is thought to have a role in the modulation of circadian activity (Williams 1985, Arechiga *et al* 1974, 1979) and its release is rhythmic, reflecting the rhythmic activity patterns seen in crustaceans (Webb 1983). The cyclical nature of its release also suggests it is under the partial control of a biological clock mechanism elsewhere in the CNS (Naylor 1985). Removal of this inhibitory influence by eyestalk ablation results in prolonged heightened, arhythmic locomotory activity in *C. maenas* (Williams 1985, Bolt and Naylor 1986). Eyestalk ablation has also been shown to abolish entrainability in *C.maenas*, as the ability to entrain to high salinity episodes in the laboratory is lost in eyestalkless crabs (Bolt and Naylor 1986). This is consistent with the view that the eyestalk neurosecretory complex is the site of a possible component of the crab's physiological clock (Bolt and Naylor 1986).

The biogenic amine 5-HT has been shown to stimulate release of NDH and produces hyperglycaemia in crayfish, presumably by stimulating release of CHH from the sinus gland (Fingerman 1995). GABA (gamma amino butyric acid) has been shown to inhibit the release of NDH (Fingerman 1995). The regulation of release of these two peptide hormones has implications for locomotor activity in crustaceans. Locomotor activity may also be affected by the cardioexcitatory influence of 5-HT, dopamine and octopamine, released from the pericardial organ (Pinder *et al* 1999), which increase the frequency and amplitude of the heartbeat (Fingerman 1987).

Preliminary experiments conducted with *C.maenas* during the summer here in Plymouth have also suggested a possible role for ecdysteroid mediated perturbations to locomotor activity. Exposure to waterborne 20-hydroxyecdysone abolished the characteristic locomotor activity in several test animals under laboratory conditions. These preliminary experiments will be resumed with crabs being exposed to waterborne 20-hydroxyecdysone whilst contained within an actograph system designed to record locomotory activity over a period of 5-6 days.

In summary, the present study aims to determine the effect of waterborne exposure to the steroid moulting hormone 20-hydroxyecdysone on three endocrine mediated processes in *C.maenas* - moulting, locomotor activity and vitellogenesis.

METHODS

DEVELOPMENT OF QUANTITATIVE ELISA FOR VITELLOGENIN

Details of the development of the quantitative ELISA can be found in the appendix.

COLLECTION OF EXPERIMENTAL ANIMALS AND LABORATORY CONDITIONS

For the vitellogenesis experiments, female individuals of the species *Carcinus maenas* were collected on incoming tides using a baited drop-net from Jenkins Quay on the Avon estuary at Bantham, South Devon, England. On return to the laboratory, crabs were maintained for an acclimation period of 1 week in holding tanks containing well aerated 34ppt, 15+/-1°C seawater, under a 12hr light: 12hr dark regime. During exposure experiments laboratory conditions were unchanged from those of the acclimation period.

For the locomotor activity experiments, green intermoult male crabs of carapace width (<65mm) were collected in the same way as above, but transferred immediately to the actograph test system on return to the laboratory. During the experimental period, crabs were kept in glass aquaria containing 8L of aerated, prepared seawater (Instant Ocean, salinity 34ppt), at 20°C under constant low level light.

For the moulting experiments, juvenile crabs (of carapace width <5mm) were collected by hand at low tide from a rocky shoreline (Jennycliff Beach, Plymouth). On return to the laboratory they were size classed, transferred to the moulting "cages" described below (see moulting methods), and immersed in glass aquaria containing 10L of aerated, 34ppt, 15+/-1°C seawater, under a 12hr light: 12hr dark regime.

WATERBORNE 20-HYDROXYECDYSONE EXPOSURE

Waterborne exposures were of the semi-static renewal type. Regular water changes were immediately followed by the redosing of exposure water with the test chemical.

The ecdysteroid moulting hormone 20-hydroxyecdysone (Sigma cat no: H-5142) was dosed into seawater using methanol as a solvent vehicle (to increase its solubility). Firstly, a 10mg/ml stock solution of 20-hydroxyecdysone in methanol was prepared. A "dosing solution" was then prepared by dissolving a 250µl aliquot of the stock solution in 24.75ml of filtered seawater. This avoided the addition of small volumes of stock solution into the large volume of seawater in each aquaria.

VITELLOGENESIS

The effect of exogenous 20-hydroxyecdysone exposure on vitellogenesis in females was investigated using the quantitative enzyme linked immunosorbent assay (ELISA) for the yolk protein precursor vitellogenin in haemolymph samples.

Pre-screening of female crabs for exposure experiments

Before exposures could begin, it was imperative that the vitellogenic status of the experimental animals be known. Knowledge of the level of haemolymph vitellogenin at time zero is needed so that each animal can be used as it's own control. Changes, if any, in vitellogenin titres can then be placed in the context of the animals starting point and then compared to control animals whose vitellogenic history is also known.

Initially, it was hoped that all crabs used would be at the same stage of vitellogenesis. The best way to do this is to collect females which are in the same stage moult stage and (hopefully) at the beginning of the vitellogenic cycle. This would allow the comparison of like with like and measurement of the whole reproductive cycle, which is clearly advantageous. Green (intermoult) females usually fit the above criteria as they are recently moulted, mated and ready to start vitellogenesis. Sampling trips however yielded much fewer green females than was hoped. Red (premoult) females are much further into vitellogenesis and show greater variability with respect to haemolymph Vg, but were much more plentiful at the time of sampling (June/July 2001).

Therefore, the decision was made to pre-screen all crabs for Vg and assign them to classes according to their approximate vitellogenic state. Each treatment group would then contain representatives from each class, whose Vg levels were known at the time of exposure. Future measurements of Vg would not be compared directly as a mean for each group but be presented as a value relative to the starting point of each crab. Each crab is essentially assigned a position on the vitellogenesis continuum and effects of exogenous hormone would be measured as deviations from the norm at each position (determined by the control group).

Before vitellogenin screening, all crabs were first numbered and labelled (with labels glued to the carapace) according to their approximate moult stage. Pre-moults were identified with the prefix "R" eg:R2 and intermoults with "G" eg:G11. These colour classifications can only be considered approximate since subtle differences in moult stage in similarly coloured crabs are common. Pre-screening of female crabs was then carried out to determine their vitellogenic status. Following the pre-screen individuals were assigned to groups according to their vitellogenic status. Representatives from each group were then included in each treatment set and were identifiable by their colour-number for repeated sampling.

Effects of exogenous ecdysteroids on vitellogenesis in *C.maenas*

Prior to 20-hydroxyecdysone exposure, all crabs were kept under control conditions (as described above) for two weeks, and haemolymph sampled weekly. These two "pre-exposure" samples were then included in the sample set for each crab.

Groups of 10 pre-screened female crabs were then exposed, in glass aquaria containing 10L of filtered, aerated seawater, to waterborne 20-hydroxyecdysone at the nominal concentrations of 100μg/L, 5μg/L, 1μg/L and 100ng/L for a period of 16 weeks. Two groups of pre-screened females were also included as solvent (n=8) and seawater controls (n=10). Haemolymph samples were taken weekly from each crab, from the arthrodial membranes at the base of the walking legs, using a glass capillary

and plunger. Samples were immediately snap frozen in liquid nitrogen and stored at -80°C until analysis.

Crabs were fed every three days with irradiated whole cockle and their water changed and redosed within 12hrs of feeding. Moulted crabs were removed wherever possible to avoid cannibalism from other individuals. These crabs were then mated and their exoskeleton allowed to fully harden before being returned to the exposure trial. Dead crabs were also removed to avoid fouling of the exposure water.

Baseline data set for non-exposed crabs.

In order to establish the normal pattern of haemolymph Vg levels in vitellogenic females over time, a baseline study was run alongside the exposure trials. 40 female pre-moult crabs, collected and maintained as above, were haemolymph sampled weekly over a period of 24 weeks. Samples were then quantitatively assayed for total protein and then Vg as described below.

Quantitative ELISA.

Each weekly haemolymph sample was first assayed for determination of total protein (by the Bradford method). This was required to calculate the volume needed to yield a concentration of 400ng/100µl for use in the quantitative Vt ELISA. All samples from each individual crab were run on the same microplate, so as to avoid any interindividual variation in binding affinity between plates.

For analysis of haemolymph vitellogenin levels, 400ng/100µl of haemolymph protein was added to triplicate wells of a 96 well microplate, alongside a triplicate series of Vt standards (from 2ng/100µl to 100ng/100µl). Coating buffer was then used as the diluent for both standards and samples. As before, all samples from each individual crab were run on the same microplate.

The plate, containing standards and diluted samples in triplicate was then incubated overnight at 4°C or for two hours at room temperature. Following incubation the plate was washed three times with TPBS to remove any unbound samples/standards. The remaining sites on the well walls were then blocked using 200µl of a bulk protein mixture (dried milk powder dissolved in PBS) which was then left to incubate at room temperature for 1 hour.

Following incubation with blocking buffer, the plate was again washed three times and 100µl of primary antibody (conjugated with milk powder dissolved in PBS) was added to each well. This primary antibody was raised against vitellin and binds to any Vg bound to the well walls. The plate was then incubated at 37°C for 1 hour, washed 3 times with TPBS and then 100µl of secondary antibody (conjugated with milk powder in PBS), was then added to each well. This secondary antibody binds to the primary antibody. The secondary antibody was left to incubate for 1 hour at room temperature. The plate was then washed 4 times with TPBS and 100µl of developer solution added to each well. The absorbance of each well of the plate was then read at 405nm at minute intervals using a microplate reader (see figure 1). Concentrations of Vt in the samples were then interpolated from the standard curve.

MOULTING

The effect of exogenous hormones and mimics on the process of moulting can be studied by observing moulting frequency of *Carcinus* in control and exposed individuals. Juveniles of the species (carapace width of <12mm) are used since the frequency of moulting at this life stage is highest. To preclude cannibalism of conspecifics (a common occurrence following moult in *Carcinus*) and to allow identification of moulted individuals, crabs were kept separately in sealed "cages" suspended from glass frames, immersed in glass aquaria of aerated seawater (see figure 2). In this way, up to 15 individuals per tank were exposed to the test chemical simultaneously and under identical conditions of constant temperature, light:dark cycle and salinity, without loss of newly moulted individuals.

Waterborne exposures were carried out at the nominal concentrations of 100μg/L, 5μg/L and 1μg/L 20-hydroxyecdysone (n=15) with solvent and seawater controls (n=9) over a period of 16 weeks. Crabs were fed every 2 days and water changed and redosed within 12hrs of feeding. Tanks were checked daily and exuviae were counted, removed and measured following moult. Any mortalities were also recorded. The interval (days) between moults and size increase following moult was calculated for all crabs with more than one recorded moult within the exposure period.

LOCOMOTOR ACTIVITY.

Locomotor activity rhythms of freshly collected crabs were studied under conditions of constant low level light, temperature and salinity using tanks equipped with an actograph system. The activity of crabs in individual glass tanks was monitored using a system of infrared light beams passing across the width of the tank. As a crab passes between the emitter and detector, the beam is broken and this is logged as a "locomotory event" by an attached computer (see figure 3). Beam breaks over a period of 4-5 days were plotted against time to produce characteristic peaks of activity coincident with times of expected high tide and darkness. Such a system is ideally suited to observe the effect of exogenous chemicals and hormones on this predictable expression of locomotory behaviour in *C.maenas*.

Before any exposure experiments were performed, the activity of 8 crabs (kept in control conditions) was monitored in the actograph system over a period of 6 days. This was to provide a seasonal measure of expected activity in crabs collected in winter. In each of two separate exposure trials, 4 crabs were exposed to 100μg/L 20-hydroxyecdysone, alongside 4 solvent controls, for a period of 6 days. Crabs were kept in glass aquaria containing 8L of aerated, prepared seawater (Instant Ocean, salinity 34ppt), at 20$\pm$ 3°C under constant low level light.

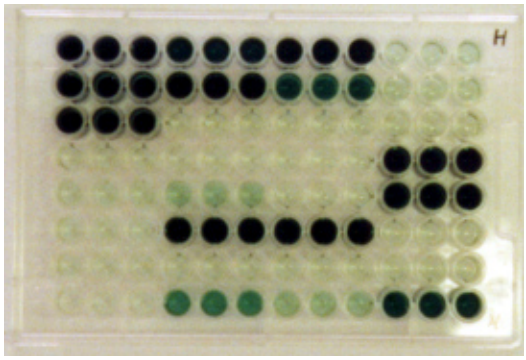


Figure 1- ELISA microplate showing developed samples in triplicate wells.

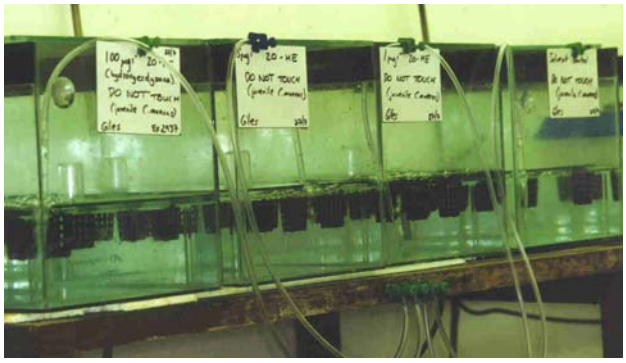


Figure 2- Moulting racks deployed in exposure tanks

Figure 3- Actograph system showing tanks, infrared emitters/detectors and control computers.

RESULTS

MOULTING

The results from the moulting trial can be seen below in figures 4-14.

Figure 4 shows cumulative mortality over the length of the exposure period for each treatment. Mortality for the two highest exposure groups (100 and 5µg/l) is clearly elevated over the remaining three treatments. The majority of these mortalities were amongst the smallest size class of crabs (5-6mm carapace width).

Mean number of moults and number of successful subsequent moults at each treatment level can be seen in figures 5-9. In these figures only those crabs which survived a moult were included in the data set for the subsequent moult. No clear differences can be seen between the treatments. The number of crabs undergoing third and fourth moults is clearly reduced from the number undergoing their first and second moults, since the time interval between successive moults (or moult frequency) decreases with increasing moult number. This is a natural phenomenon and is not an effect of the test chemical.

Figures 10-15 show the mean moult interval and mean size increase following moult at each treatment, for moults 1-2, 2-3 and 3-4. No significant differences between the treatments can be seen, as indicated by the standard deviation of the mean. Exposure to 20-hydroxyecdysone at the concentrations used has had no discernible effect on moult frequency or on growth following moult.

The above results illustrate that aside from an increase in mortality in the smallest size classes, 20-hydroxyecdysone has not had a gross effect on moulting processes in juvenile *C.maenas*.

LOCOMOTOR ACTIVITY

A typical mean locomotor activity trace for 8 crabs kept under control conditions can be seen in figure 16. Times of high water and approximate light and dark periods are shown. Peaks of activity can be seen around the time of expected high tide, with the amplitude of these peaks greatest at times of nocturnal high tides. Over time, in the absence of environmental cues, the rhythmicity of the activity pattern slowly breaks down and amplitude is slightly decreased.

Locomotor activity of both the 20-hydroxyecdysone exposed and control crabs in the first exposure trial can be seen in figures 17 and 18. Mean activity of both treatment groups is shown in figure 19. No gross deviations from the expected pattern are observed in the exposed group. No discernible loss of rhythmicity is seen in the earlier stages of the exposure period and amplitudes of activity are broadly similar for both exposed and control groups. Breakdown of the characteristic pattern of rhythmic behaviour is consistent with the results of the initial control trial. The amplitude of mean activity increases towards the end of the experimental period in control crabs. This can be attributed to the increased locomotor activity of two crabs in particular (see fig 18).

Locomotor activity of 20-hydroxyecdysone exposed and control crabs in the second exposure trial can be seen in figures 20 and 21. Mean activity of both treatment groups is shown in figure 22. No gross deviations from the expected pattern are observed in the exposed group. No discernible loss of rhythmicity is seen in the earlier stages of the exposure period and amplitudes of activity are broadly similar for both exposed and control groups. Breakdown of the characteristic pattern of rhythmic

behaviour occurs earlier than expected in both treatment groups and in control crabs, activity levels are greatly decreased after 48hrs. The mean activity pattern for exposed crabs (figure 22) is largely attributable to the contribution of one particularly active crab in the treatment group, thus creating the impression that locomotor activity has been greatly increased above control levels by exposure to 20-hydroxyecdysone. A closer look at the results would reveal this not to be the case, as without the contribution of the aforementioned individual, control and exposed crabs are again broadly similar.

It would therefore appear that 20-hydroxyecdysone has not caused gross perturbations to rhythmic locomotor activity patterns in *C.maenas* at the time of year the present study was undertaken.

DEVELOPMENT OF QUANTITATIVE ELISA FOR VITELLOGENIN-

Results from the various steps in the development of the quantitative ELISA can be found in the appendix.

BASELINE VITELLOGENESIS STUDY

The haemolymph vitellogenin (ngVg/100µl) levels of selected baseline individuals can be seen presented in figure 23. Data sets that come to an end before the completion of the observation period are the result of mortality or moulting and subsequent mortality (caused by cannibalism by conspecifics).

A clear pattern of changing vitellogenin titre can be seen in certain individuals, with a sharp increase culminating in an initial large peak at the beginning of the baseline sampling period and a smaller, secondary peak towards the end. The presence of these “twin peaks” has been verified by repeated analysis of the same samples and is reproducible. This general pattern has a few exceptions. For example, crab 32 exhibits a very large secondary peak of Vg, similar in shape and amplitude to the initial peak. Individuals such as crab 6, 20 and 35 have elevated levels of Vg in their haemolymph which are maintained following the initial increase. Other baseline crabs (9, 40) show a very low level of Vg throughout the sampling period (for the purposes of the present study, a vitellogenin concentration of less than 10ng/100µl was considered a basal or background level).

VITELLOGENESIS EXPOSURE TRIAL

The haemolymph vitellogenin levels of 20-hydroxyecdysone exposed and control individuals can be seen presented in figures 24-29. Data sets that come to an end before the completion of the exposure period are the result of mortality or moulting and subsequent mortality (caused by cannibalism by individuals in the same tank). Data sets that end prematurely and then resume later in the exposure period are from moulted individuals which were successfully removed from the trial, mated and returned when suitably hardened. Missing data points for certain weeks are a result of loss or exhaustion of haemolymph samples.

Haemolymph Vg titres of the majority of individuals included in the exposure trial remain at a basal level throughout the exposure period and their vitellogenin profile lacks the large peaks seen in the baseline. Certain individuals exhibit the large peaks similar to those seen in the baseline crabs (for example R2 and G11 seen in the 100ug/l and 5ug/l treatment groups respectively). Other crabs also show less marked

peaks (eg: G21 and R27 from the 100ng/l exposure group) whilst others exhibit subtle increases over longer time scales eg: G30 (solvent control group). Overall, however, there seems to be little discernible pattern to the haemolymph Vg levels in the majority of the crabs in the exposure trial. There are also no clear differences between the treatment groups, with the majority of individuals in all groups exhibiting background levels of haemolymph Vg throughout (with the exception of those mentioned above).

Therefore, despite the inclusion of the Vg pre-screen step, it would appear the bulk of crabs assayed did not subsequently undergo the events of vitellogenesis so clearly evidenced in the baseline study. Potential explanations for this will be covered in the discussion.

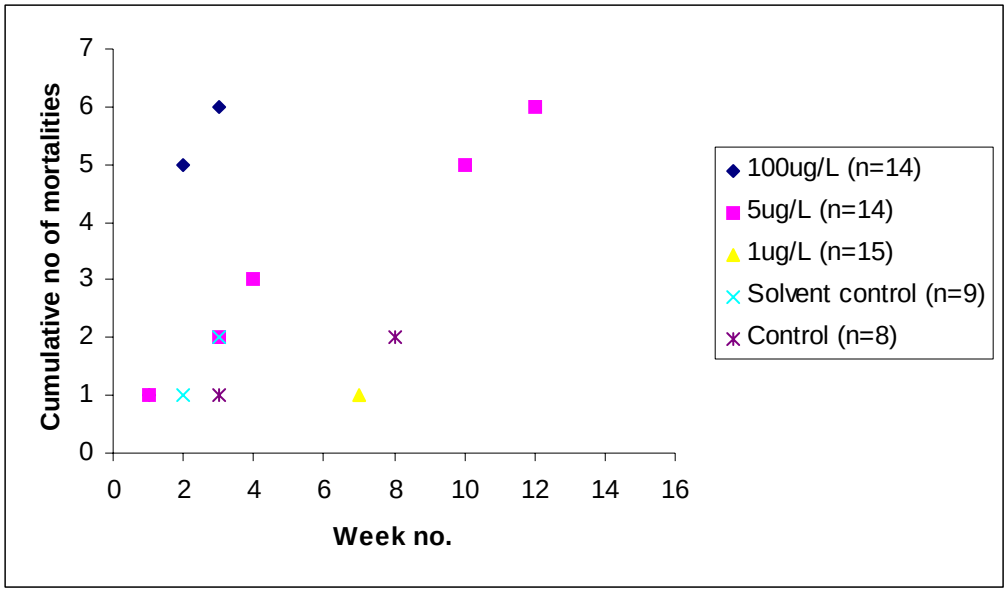


Figure 4 - Cumulative mortality in 20-hydroxyecdysone exposed and control *C.maenas*. (n= number of individuals at beginning of exposure period).

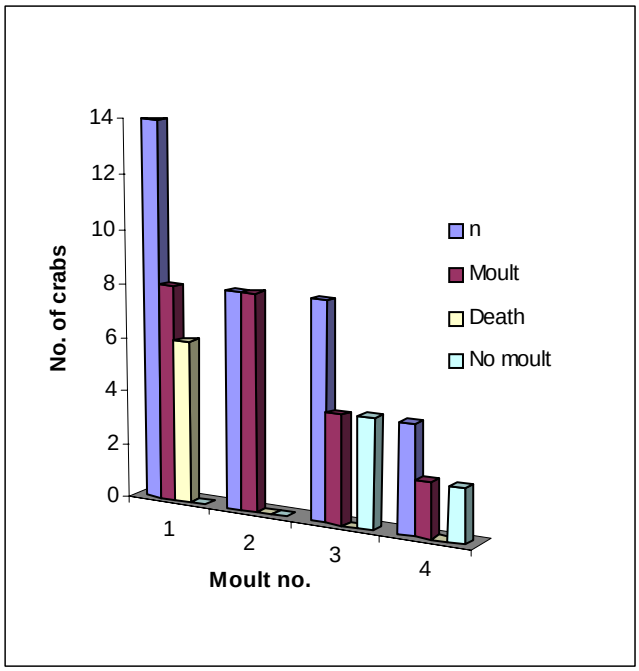


Figure 5- Number of moults, deaths and non moults in the 100µg/L treatment group.

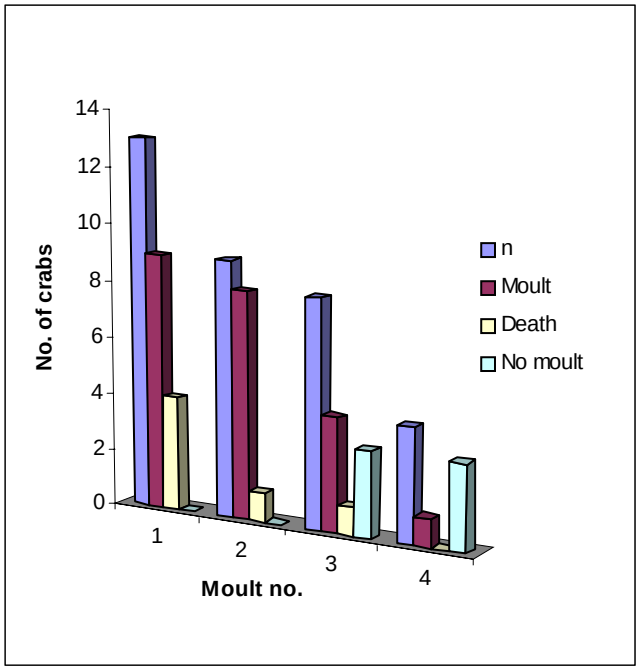


Figure 6- Number of moults, deaths and non moults in the 5µg/L treatment group.

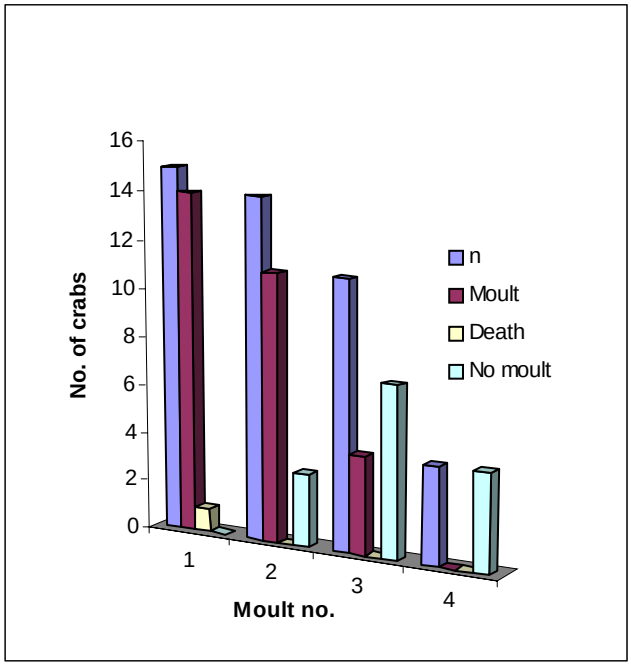


Figure 7- Number of moults, deaths and non-moults in the 1µg/L treatment group.

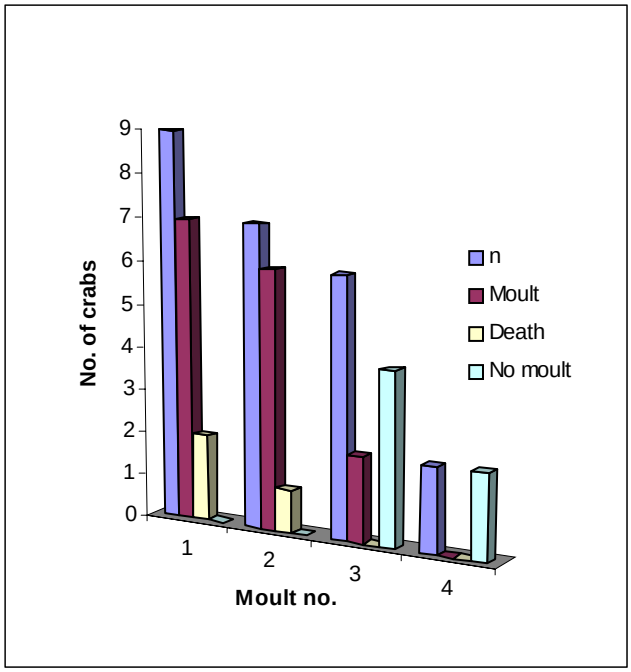


Figure 8- Number of moults, deaths and non-moults in the solvent control group.

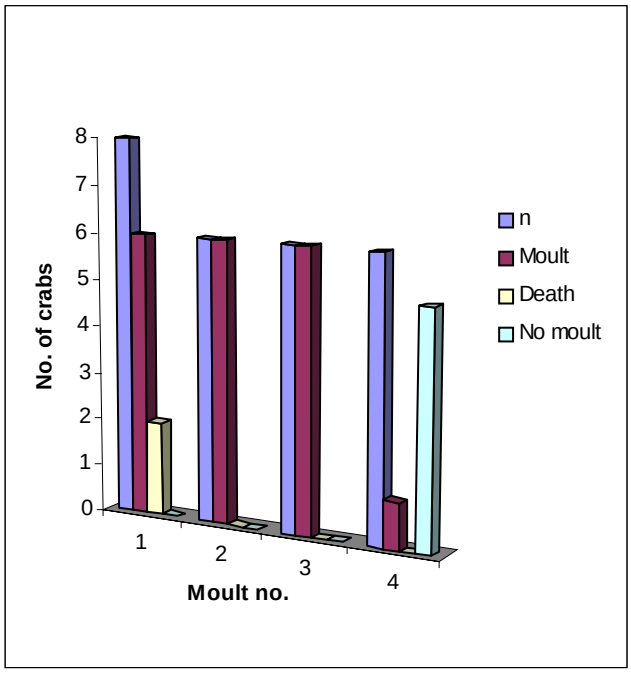


Figure 9- Number of moults, deaths and non-moults in the control group.

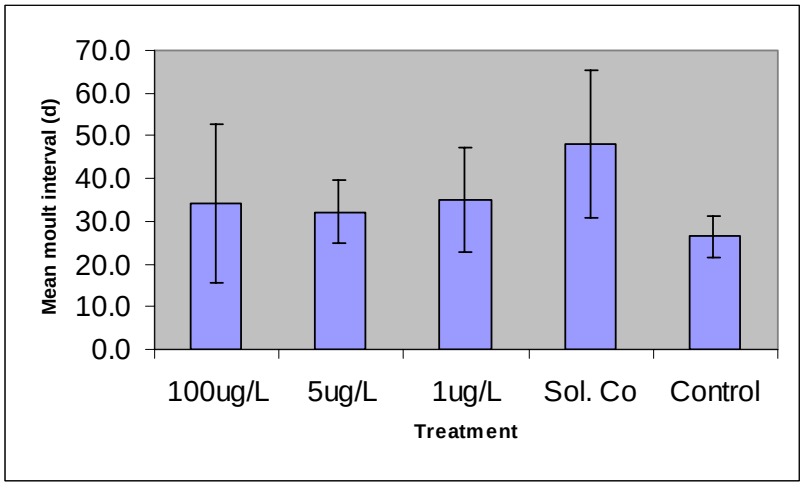


Figure 10- Mean moult interval (1st to 2nd moult) for all treatment groups.

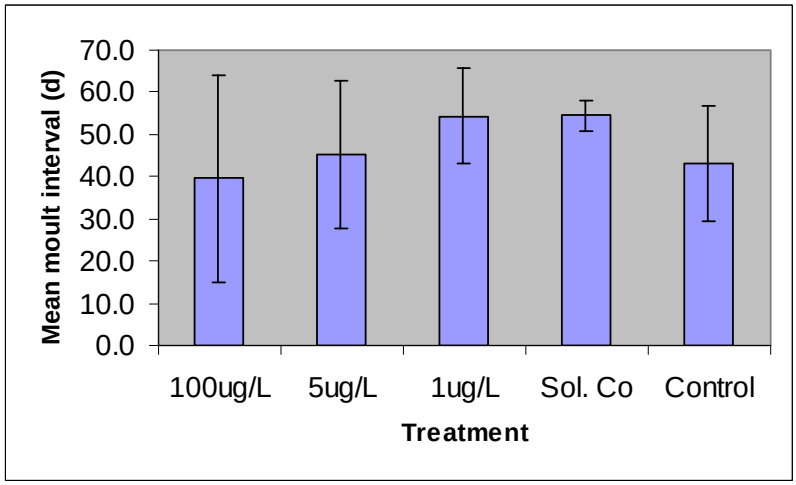


Figure 11- Mean moult interval (2nd to 3rd moult) for all treatment groups.

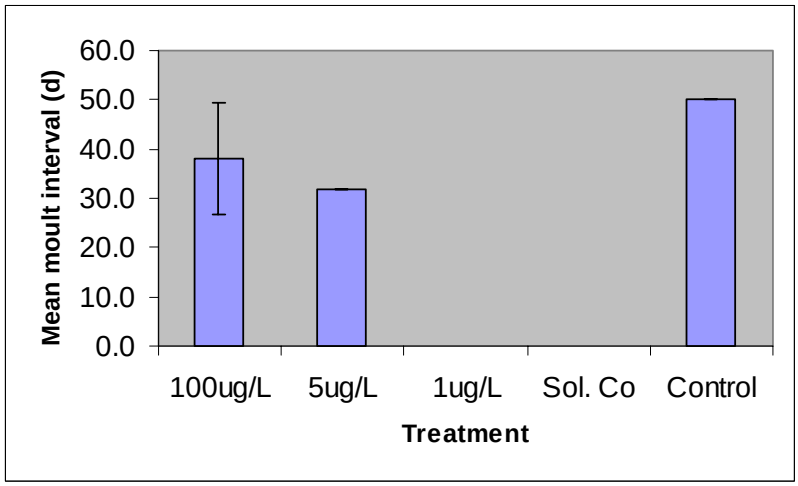


Figure 12- Mean moult interval (3rd to 4th moult) for all treatment groups.

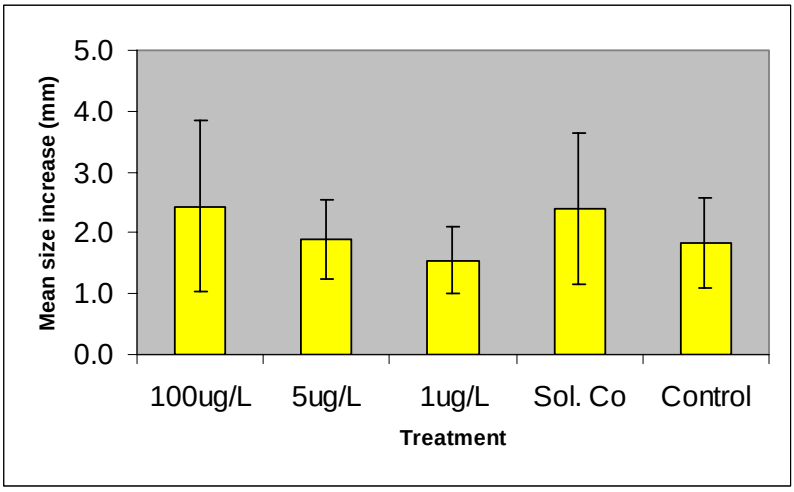


Figure 13- Mean size increase following moult (1st – 2nd moult) in all treatment groups.

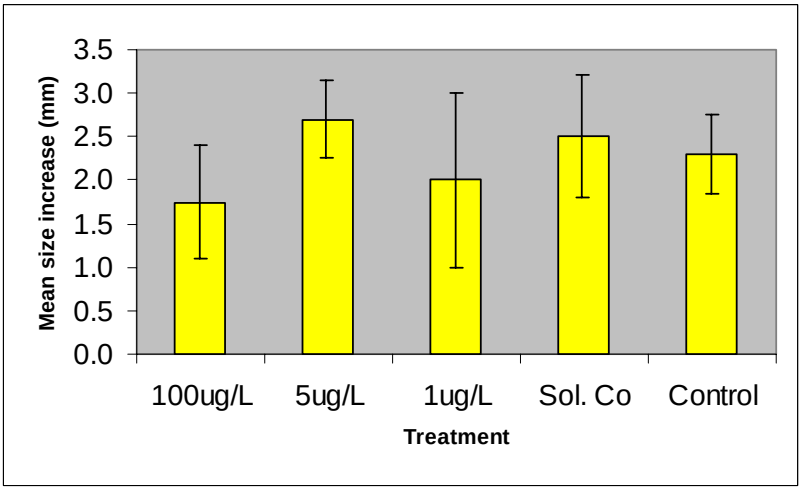


Figure 14 - Mean size increase following moult (2nd – 3rd moult) in all treatment groups.

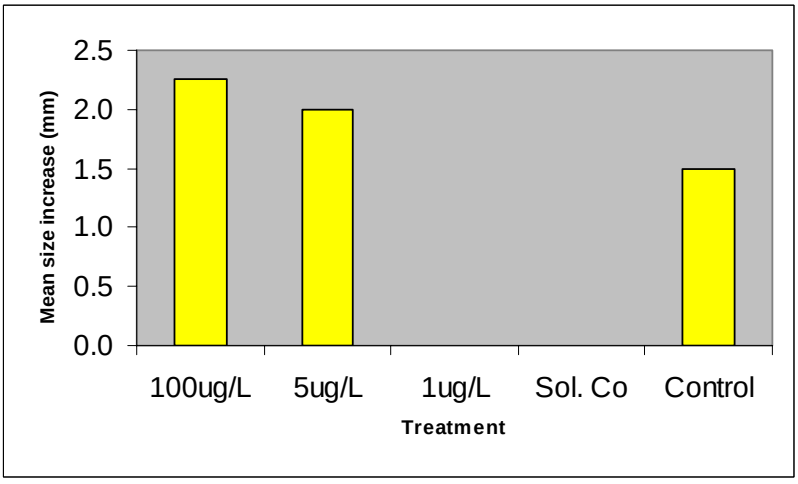


Figure 15 - Mean size increase following moult (3rd - 4th moult) in all treatments

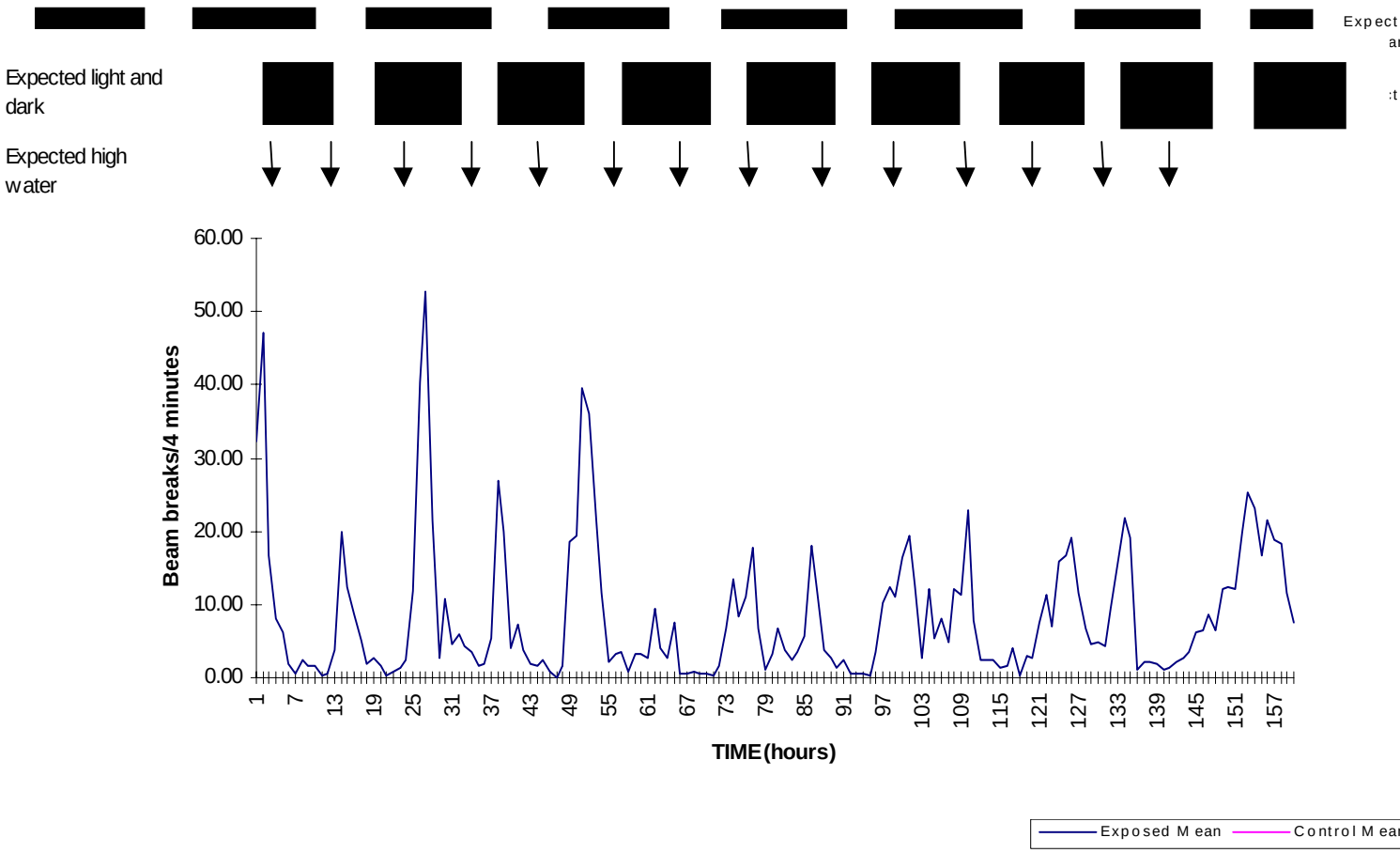


Figure 16- Mean locomotor activity for 8 crabs kept under control conditions.

Figure 17- Mean locomotor activity for 4 20-hydroxyecdysone (100µg/L) exposed crabs and 4 control crabs.

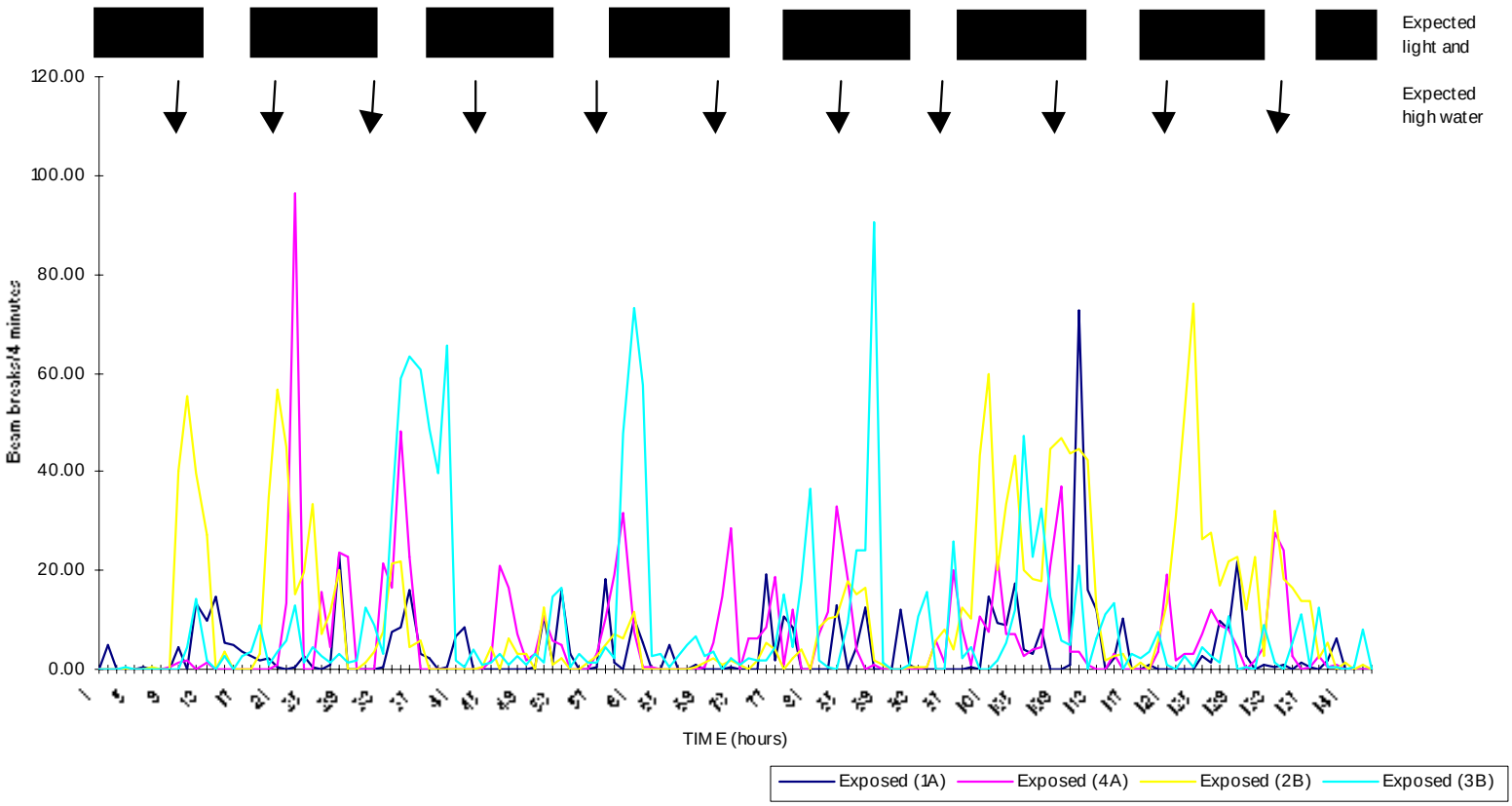


Figure 18- Individual locomotor activity for 4 20-hydroxyecdyson (100µg/L) exposed crabs (trial 1).

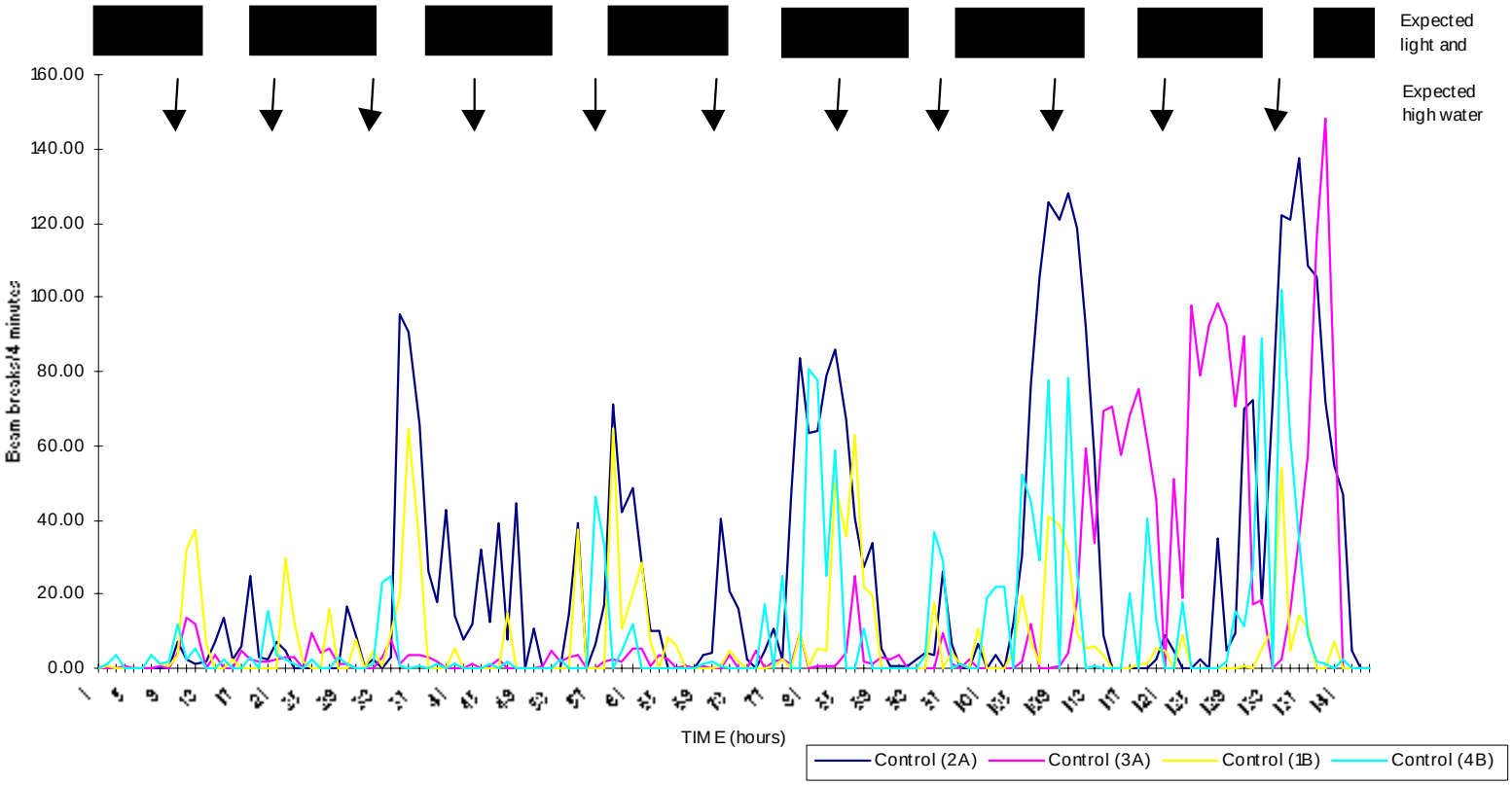


Figure 19- Individual locomotor activity for 4 control crabs (trial 1).

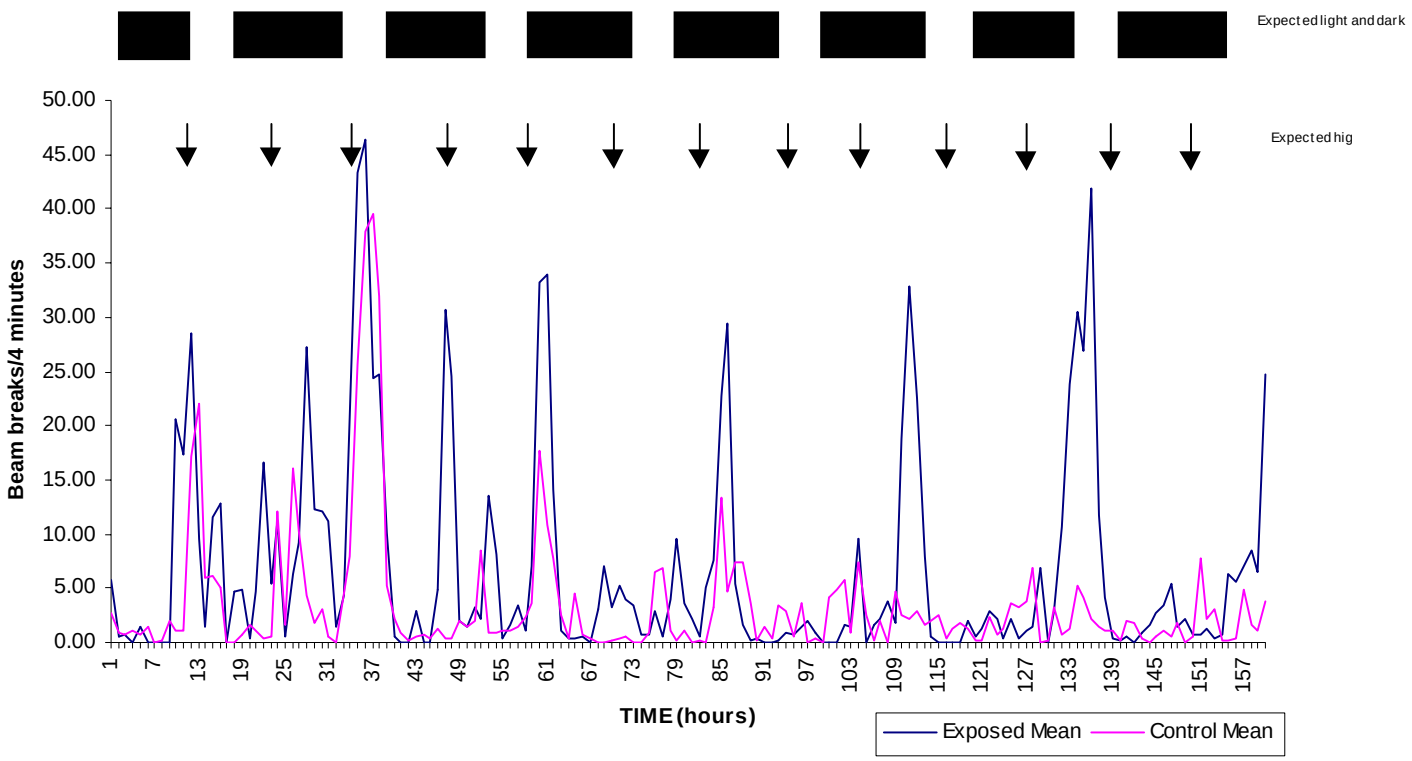


Figure 20- Mean locomotor activity for 4 20-hydroxyecdysone (100µg/L) exposed crabs and 4 control crabs (trial 2).

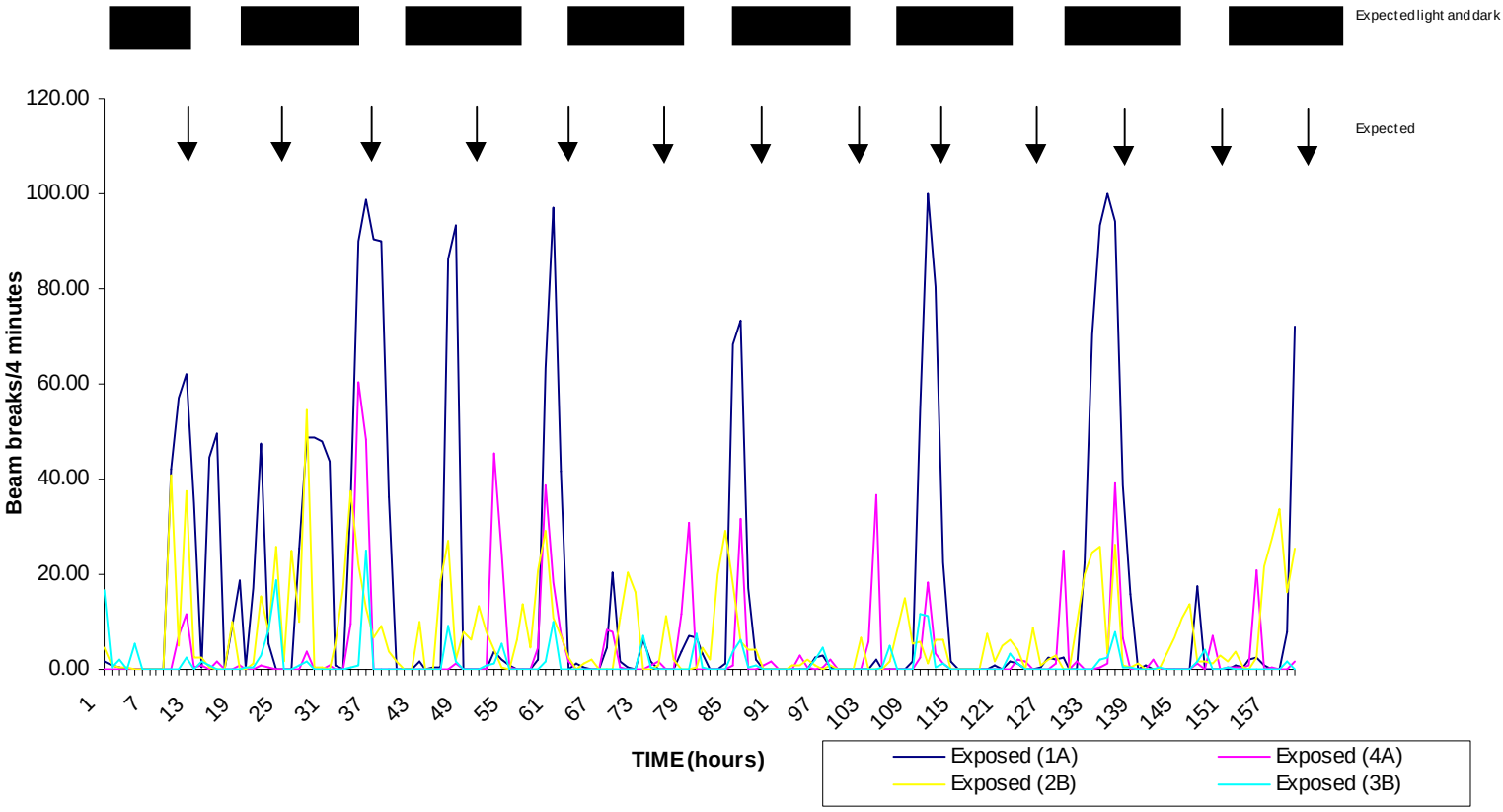


Figure 21- Individual locomotor activity for 4 20-hydroxyecdysone (100µg/L) exposed crabs (trial 2).

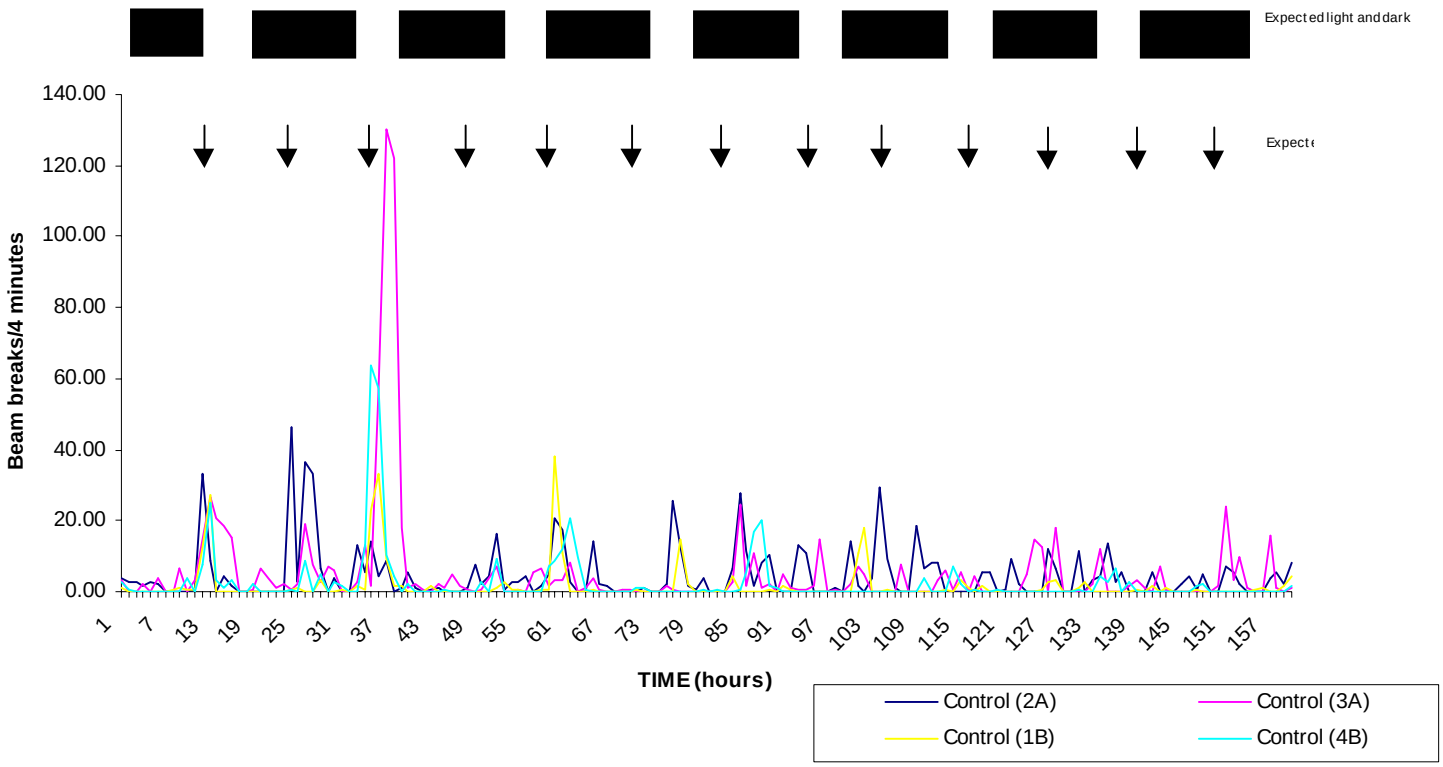


Figure 22- Individual locomotor activity for 4 control crabs (trial 2).

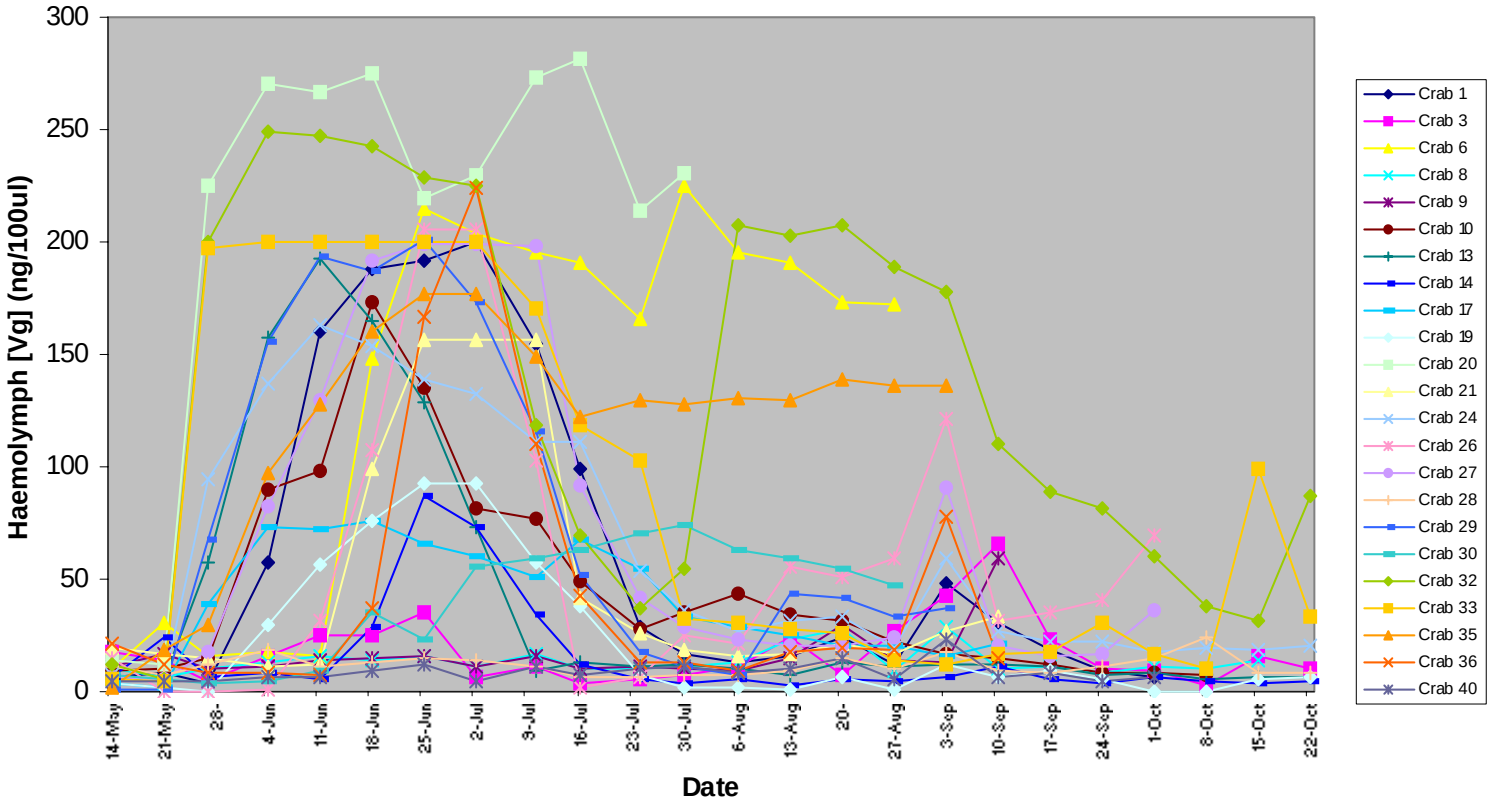


Figure 23- Haemolymph vitellogenin levels (ng/100µl) of selected baseline individuals.

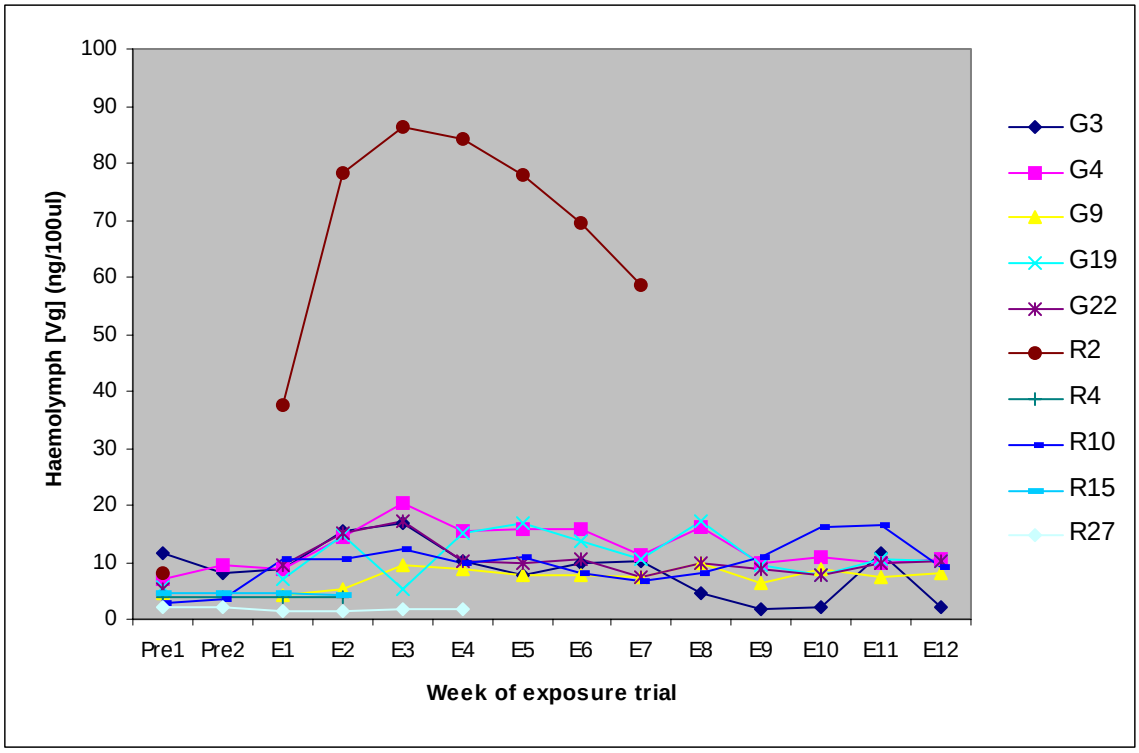


Figure 24- Haemolymph vitellogenin levels of 20-hydroxyecdysone exposed (100µg/L) individuals.

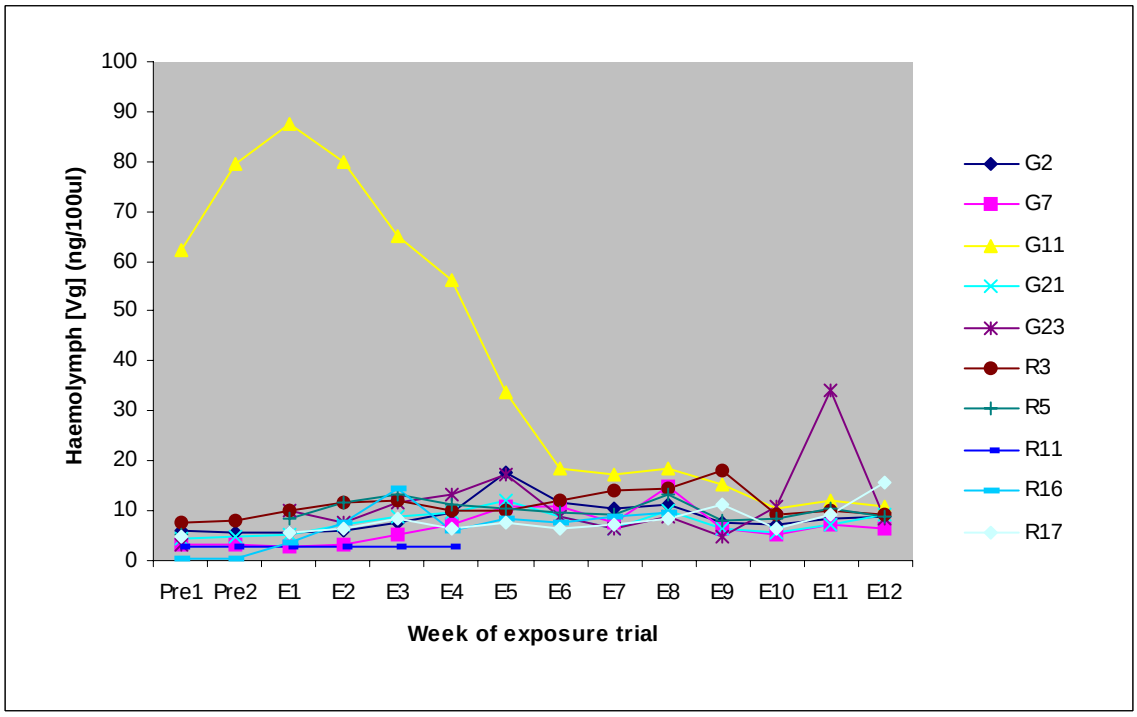


Figure 25- Haemolymph vitellogenin levels of 20-hydroxyecdysone exposed (5µg/L) individuals.

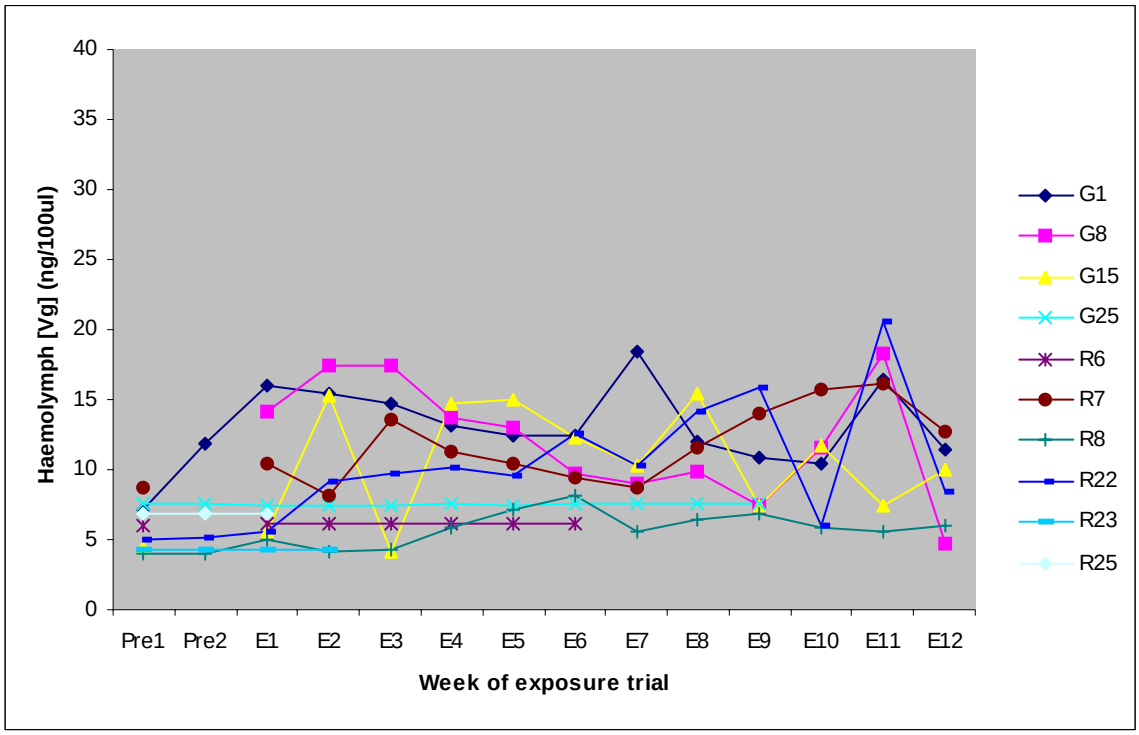


Figure 26- Haemolymph vitellogenin levels of 20-hydroxyecdysone exposed (1µg/L) individuals.

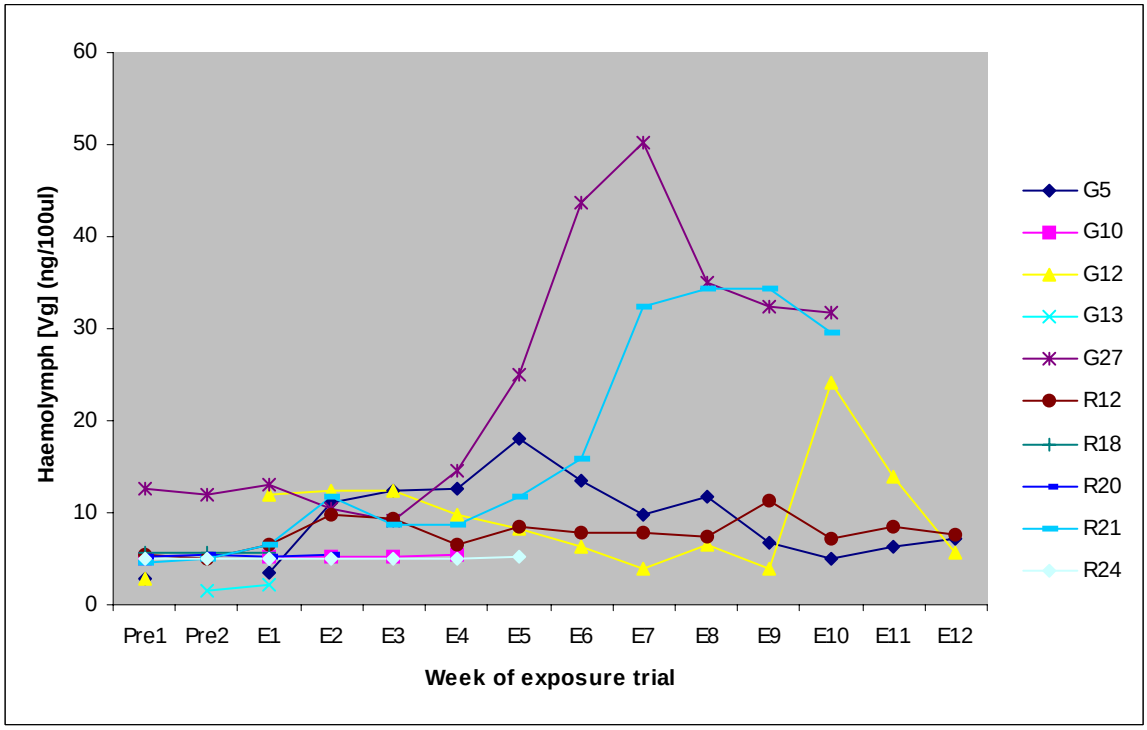


Figure 27- Haemolymph vitellogenin levels of 20-hydroxyecdysone exposed (100ng/L) individuals.

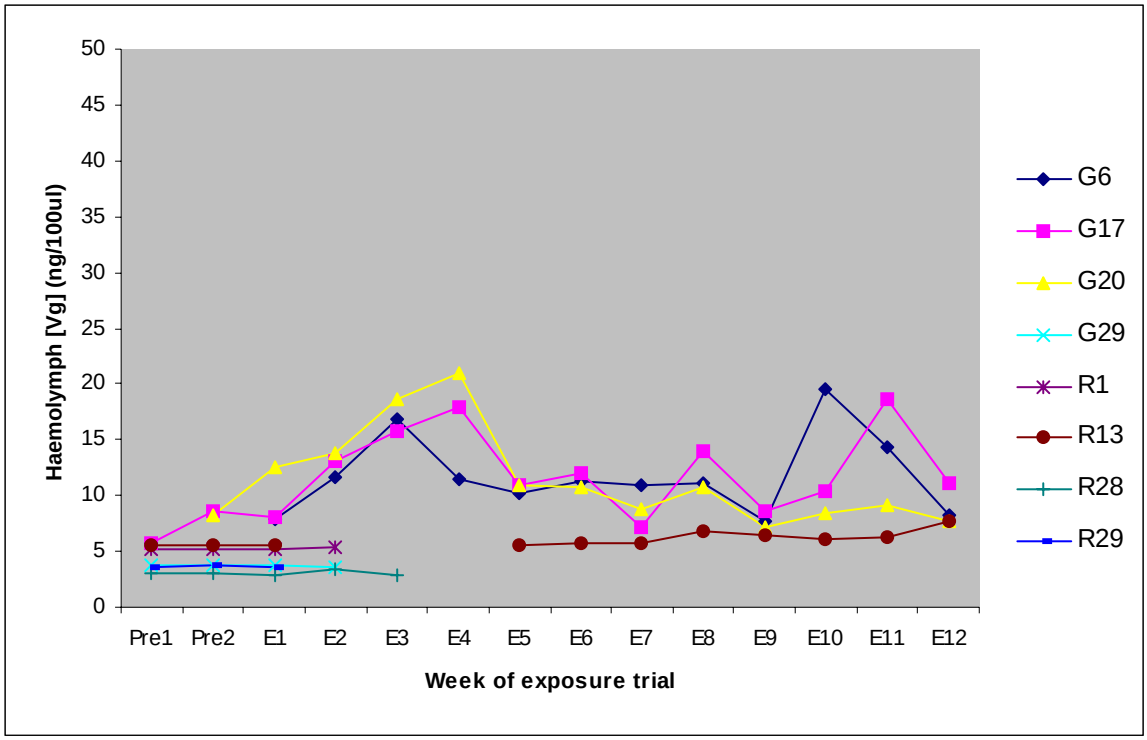


Figure 28- Haemolymph vitellogenin levels of solvent control individuals.

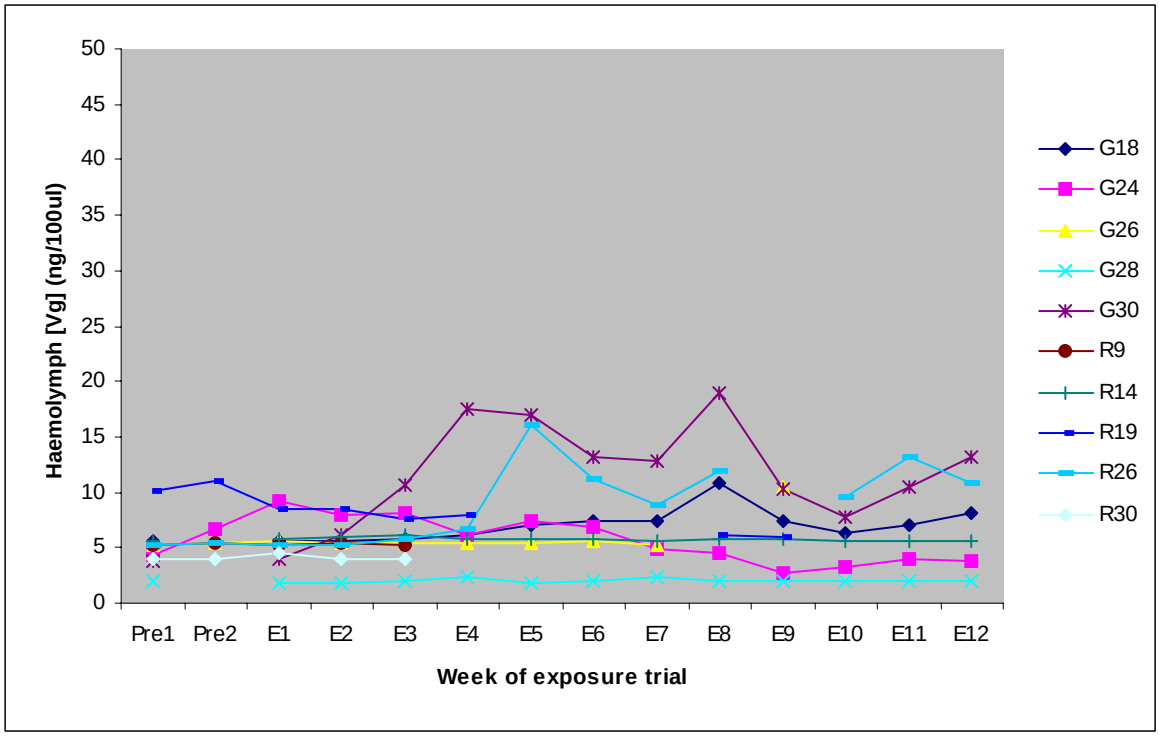


Figure 29- Haemolymph vitellogenin levels of control individuals.

DISCUSSION

Moulting

The moulting trial produced little conclusive evidence of disruption to this process caused by exogenous 20-hydroxyecdysone. Increased mortality in the two highest exposure groups (100 and 5µg/l) was the only result to indicate any disruption due to exposure. The majority of these mortalities were amongst the smallest size class of crabs (5-6mm carapace width) which is to be expected, since moult frequency is highest in crabs of this size (Crothers, 1967). Therefore, the risk from moulted related complications is increased. However, exposure to 20-hydroxyecdysone at the concentrations used has had no discernible effect on moult frequency or on growth following moult. A recent study produced a similar result in *Daphnia magna* (Baldwin *et al* 2001).

Despite the lack of gross perturbations to moult processes in this preliminary study, potential disruption due to a moult mimic such as tebufenozide cannot be ruled out. Without appropriate chemical analyses, the persistence of 20-hydroxyecdysone in the exposure system is not known. It is possible that it is rapidly degraded and therefore is only active and able to exert an effect for a relatively short period of time. In contrast, moult inhibiting insecticides such as tebufenozide are by their nature more persistent, in order to maximise the chance of exerting a toxic effect. Further work is presently underway at the University of Plymouth to compare the effect of tebufenozide with that of 20-hydroxyecdysone to fully determine its potential for disruption to moult processes in non-target invertebrates such as *C.maenas*.

Future work should also include the use of other species of crustacean and also other life stages, particularly larval forms which are invariably more sensitive to chemical stress. Seasonal differences in sensitivity are also worth investigating.

Actograph

The locomotor activity experiments were tentatively included in the experimental program, due to the lack of available information on the hormonal control of rhythmicity. No obvious link with 20-HE exposure was found, although other behaviours could have indirectly been influenced by disruption elsewhere (eg: Bolingbroke & Kass-Simon, 2001). Investigation into such behaviours was beyond the scope of the present study, due to the simplicity of the actograph system. Only a crude level of “activity” over time could be measured using this system. Research into more complex behavioural patterns could be the focus of future work, utilising new high-tech behavioural observation systems recently acquired by the University of Plymouth.

Preliminary exposure studies suggested the abolition of activity by 20-HE, but the present study does not support this. The preliminary study was undertaken during the summer (Bamber 2000, unpublished results) when large proportions of the population are actively migrating with the tide. During winter however, the number of migrants declines (McGaw & Naylor, 1992). As a result, it is possible that the present study tested crabs with more persistent locomotor activity not so easily overridden by exogenous influences.

Hunter and Naylor (1993) report differences in locomotor activity between “colour-forms”, with green (early intermoult) male crabs being more active and

making more extensive intertidal migrations than red (late intermoult) crabs. Green intermoult males were chosen for the present exposure trial and their characteristically active locomotory habits seemed to have prevailed despite 20-hydroxyecdysone exposure. The hormone could potentially have a more profound effect on different "colour forms" whose locomotory activity patterns are not quite as robust. This could form the basis for further research.

Vitellogenesis

As mentioned previously, there is some evidence reported in the literature for the involvement of ecdysone in vitellogenic events although its role is still poorly understood. It has been suggested that ecdysteroids may stimulate vitellogenesis (Fingerman 1997) and ovarian maturation and protein synthesis (Chan 1995, Oberdorster and Cheek 2001). Conversely, in some species, administration of ecdysteroids has inhibited vitellogenesis (Chang 1989). It appears that the ability of ecdysteroids to promote vitellogenesis in the hepatopancreas is species dependent (Loeb 1993) as is the need for ecdysteroids for the completion of vitellogenesis (Pinder *et al* 1999). Correlations between haemolymph ecdysteroid titres and vitellogenesis have been reported (Chang 1993, Okumura *et al* 1992, Young *et al* 1993a,b). However, it is still unclear as to whether ecdysteroids directly influence vitellogenesis, or that their levels during vitellogenesis are simply indicative of the corresponding stage of the moult cycle.

The results of the present study have unfortunately provided little furthur clarification of this situation. No obvious changes in vitellogenic events were observed in exposed individuals and consequently no real conclusive evidence of disruption by exposure to 20-HE could be found. The present study has however successfully led to the optimisation of a quantitative immunosorbent assay for *C.maenas* vitellogenin and highlighted the areas in which our knowledge of the temporal progression and associated physiological processes of vitellogenesis is lacking.

It is clear from the findings that the fundamental baseline data on vitellogenesis needs to be more comprehensive. A more thorough knowledge of the biology of vitellogenesis is needed before it can be categorically concluded that ecdysone exposure has had no effect.

A year round picture of the vitellogenin profile correlated with a clear measure of reproductive status (ovarian development) and moult/colour stage is required. In this way, a vitellogenic continuum could be combined with a moult/reproductive component so that deviations from the norm might be identified. For example, females could be collected regularly throughout the year and moult staged by a specific measurement of shell thickness or colour. The reproductive status could also be ascertained by investigation of ovarian development. These parameters could then be compared with an ELISA derived haemolymph Vg titre for each individual. A clearer picture could then be composed of the interplay between these processes over an entire year or perhaps more. Mean data on haemolymph Vg level and ovarian development would then exist for females of a certain size and colour/moult stage in any month, or even week of the year. Females sampled from a population could then be compared to the established baseline data set. If the Vg level does not correlate with the expected level of an individual sharing the same physiological characteristics at the same time of year, then there could be a case for a level of "disruption" to vitellogenic events caused by exogenous influences. It is important to note here that,

due to regional differences in the timing and duration of various physiological events in crustacean populations, allowances would need to be made to account for variations between these populations.

On a similar note, the present study has illustrated that a simple measure of Vg level at the time of collection tells us little about the true stage of vitellogenesis an individual has reached. The pre-screen provides us with limited information since it does not tell us which side of the large vitellogenic baseline peak the individual is. Additionally, crude classification of crabs into "green" or "red" is no guarantee that their respective positions in the vitellogenic continuum are before or after the large peak seen in the baseline.

The absence of high vitellogenin titres in exposed crabs may be a simple issue of the timing of the trail. The exposure trial was started later in the summer and after the time period encompassing the vitellogenic peak in the majority of the baseline samples (from mid May to late July). A possible explanation for the lack of increased Vg titres could be that the period of increased vitellogenin synthesis had already occurred in the exposure trial individuals. This increase in synthesis and circulation of Vg is clearly the crucial stage for our investigation and alterations in Vg level would be most easily detected during this time.

During the initial planning of experiments, however, the above scenario was considered unlikely, as it is reported that vitellogenesis can occur over a wide time period and well into late summer/early autumn (Crothers 1967). The results of the present work now cast doubt on this assumption, at least in the population studied. It is possible that the population sampled at Bantham is characterised by a compressed temporal period for the events of vitellogenesis. For example, where vitellogenesis might occur throughout some populations from early May to October, with a maximum in June, crabs from Bantham may be characterised by vitellogenesis occurring from late May to early August only. Later in August and through September into October, the proportion of the population exhibiting the increased Vg titres would therefore be smaller. This would explain the small number of crabs in the exposure trial which showed any kind of vitellogenic peak. The need to avoid repetition of this situation further illustrates the need for a fully comprehensive baseline data set.

The majority of crabs in the exposure trial and a small number in the baseline group were characterised by a Vg titre which stayed at basal or "background" levels (<10ng/100µl) throughout the exposure period. If still in the intermoult or early premoult stages such individuals are most probably non-vitellogenic (Vafopoulou & Steel, 1995) and may lie to the left of the peak on the vitellogenic continuum. Without an accurate measure of moult stage however, this remains conjectural. Alternatively females may have previously undergone vitellogenesis ie: are now situated to the right of the vitellogenic peak on the continuum. These are individuals whose ovaries have sequestered the vitellogenin previously circulating in their haemolymph where it is converted to the ovarian form vitellin (Lee *et al* 1996, Chen and Chen 1994). Recirculation into the haemolymph and/or basal levels of protein synthesis/turnover might account for the small amounts found in samples throughout the exposure period (Vafopoulou & Steel, 1995). A measure of ovarian development might confirm this, but this was not undertaken in the present study, due to time constraints. Again, comparison to a well-established and comprehensive baseline would clarify the situation.

The disparity between the amplitude of the vitellogenic peaks of the haemolymph of baseline and exposure crabs may be partially explained by the activity of the Vt standard used to run the ELISA. The baseline haemolymph samples were analysed using aliquots of a concentrated stock of Vt. These aliquots were stored at -20°C for several months and thawed individually when required. Inevitably, all may have undergone partial thawing and re-freezing when individual aliquots were removed for use in the ELISA. As a result, the "binding affinity" of the standard may have decreased over time. In contrast, the Vt standard used in the analysis of the exposure samples was aliquotted from the concentrated stock (kept at -80°C) just prior to analysis and kept at -20°C for only a few weeks whilst running the ELISA. The absorbance values produced by the "fresher" standards were therefore higher despite their concentrations being identical. As a result, interpolation from the steeper curve produced lower values. This effect can be minimised by regular "normalisation" of standards against an initial "fresh" curve and is important now that the Vg ELISA is fully quantitative.

As mentioned briefly above in reference to the moulting experiments, some form of chemical analysis would be a valuable addition to any future work. It is not presently known whether waterborne exposure to 20-hydroxysone is effective in elevating endogenous ecdysone levels in *C.maenas*. An unfortunate disadvantage of the semi-static renewal system of dosing experiments is the potential for degradation and/or loss of the test chemical from the exposure system. Monitoring of waterborne levels over time would offer insight into the degree of these processes and provide information on the required frequency of redosing. Nevertheless, the present study minimised potential degradation and loss by a regime of regular water changes and redosing.

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

Moulting

- No gross effects on moulting processes besides increased mortality in the two highest exposure groups.
- No discernible effect on moult frequency or on growth following moult.
- Future work- Investigate the effect of potentially more persistent "mimics" eg:tebufenozide to assess risk of disruption to moult processes in non-target invertebrates such as *C.maenas*. Use of other species of crustacean and life stages (larval forms). Seasonal differences in sensitivity of juveniles.

Locomotor activity

- No obvious effects of 20-HE exposure.
- Future work- Research into more complex behavioural patterns, utilising modern behavioural observation systems. Exposure of different "colour-forms" in actograph system

Vitellogenesis

- No obvious changes in vitellogenic events observed in exposed individuals.
- Successful optimisation of a quantitative immunosorbent assay for vitellogenin.
- Identified need for normalisation of standards against an initial "fresh" curve. Important for quantitative Vg ELISA.
- Highlighted gaps in knowledge of vitellogenesis and need for comprehensive baseline data set.

Future work (vitellogenesis)

- Development of a long term baseline data set for a more thorough understanding of the nature, extent and timing of vitellogenic events in *C.maenas*. eg: A year long haemolymph vitellogenin profile correlated with a clear measure of reproductive status (ovarian development) and moult/colour stage.
- Future research should be timed to ensure major vitellogenic events are included.
- Chemical analyses to determine rates of potential degradation and/or loss of the test chemical from the exposure system.

APPENDIX

DEVELOPMENT OF QUANTITATIVE ELISA FOR VITELLOGENIN

In order to measure any changes in haemolymph vitellogenin (Vg) titres due to exogenous ecdysteroids, it was necessary to develop a quantitative assay for Vg. The results of preliminary experiments show that Vg preferentially binds to the plate wells. This conclusion was reached following several experiments investigating the binding characteristics of vitellin (Vt) standards. Vt is immunologically identical to Vg, the haemolymphatic form (Lee *et al* 1996). The results of each experiment led to the subsequent step in the development of the quantitative ELISA and are described in turn below.

1- Microplate well capacity for vitellin. Vitellin standards from 10ng/100µl to 1µg/100µl were run using a standard ELISA to determine the capacity of the plate wells for vitellin only. When absorbance is plotted against Vt concentration an absorbance response plateau at approximately 400ng/100µl can be seen. ie: above 400ng/100µl all binding sites are occupied and concentrations in excess of this amount will therefore produce the same absorbance response. This experiment was repeated several times and found to be reproducible. (See appendix fig 1).

2- Male haemolymph proteins vs vitellin. Having determined the well capacity for vitellin to be approximately 400ng/100µl, it was necessary to determine whether this behaviour was affected by the presence of other proteins competing for space on the walls of the well. To do this, vitellogenin-free haemolymph from male crabs was sampled and first assayed to determine it's total protein concentration. Solutions of male haemolymph in coating buffer were then spiked with known quantities of vitellin standard to yield a standard series of vitellin from 10ng/100µl to 400ng/100µl with an overall concentration of 400ng/100µl. For example 10ng Vt + 390ng male protein in 100µl, 20ng Vt + 380ng male protein in 100µl and so on, up to a final standard of 400ng Vt + zero male protein in 100µl. The presence of male haemolymph had no effect on the response seen, suggesting preferential binding of Vt to the well (See appendix fig 2).

To further validate this finding, four additional experiments were carried out.

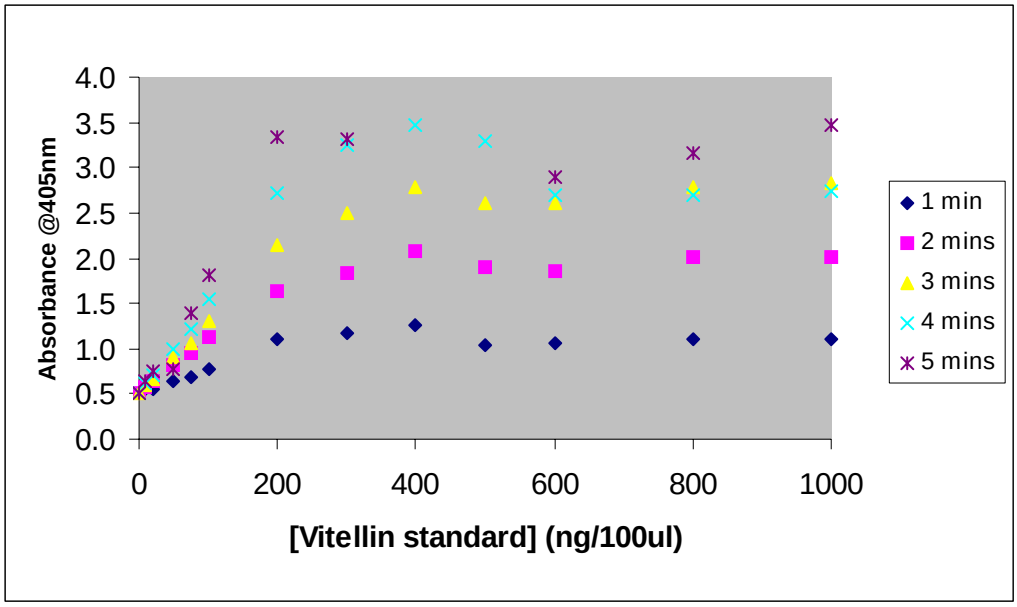
3- Increasing male haemolymph proteins vs fixed concentration of Vt. First, a series of standards, containing a fixed amount of vitellin (200ng/100µl) but increasing amounts of male protein (from zero up to 900ng/100µl) were added to wells in triplicate. Despite the presence of additional protein, the response produced was approximately the same in all standards (See appendix fig 3).

4- 50% vitellin and 50% male protein. Secondly, a series of standards, containing a fixed ratio of 50% vitellin and 50% male protein (from 10ng/100µl to 800 ng/100µl) were added to the wells in triplicate. Again, these standards behaved in the same way as pure vitellin standards, with a plateau seen at 400ng/100µl vitellin. ie: Additional haemolymph proteins, even when their total concentration was equal to that of vitellin, did not compete with Vt for binding sites (See appendix fig 4).

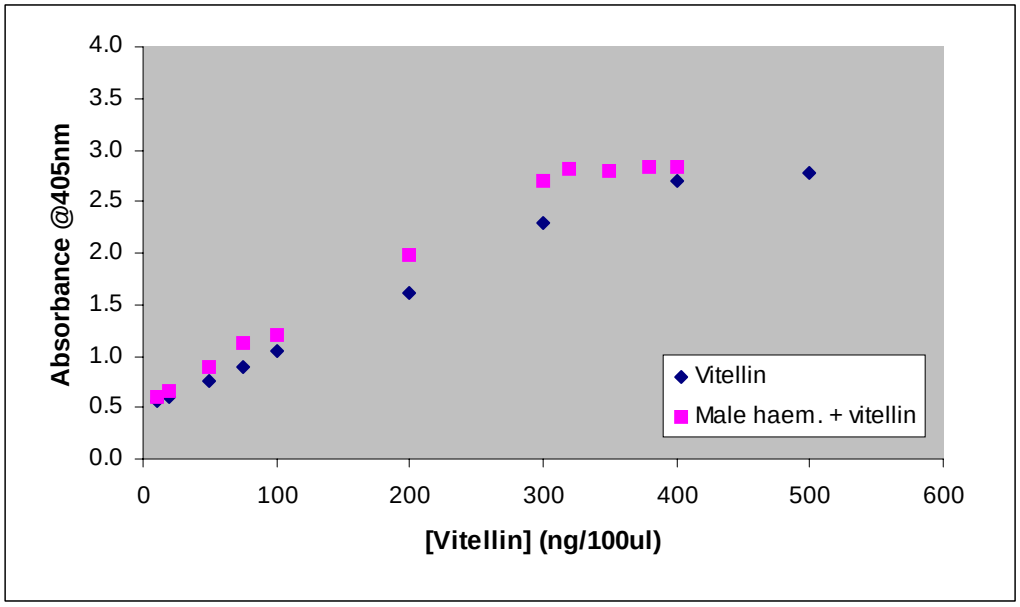
5- Increasing vitellin vs fixed concentration of male protein. A third series of standards, containing a fixed amount of male protein (400ng/100µl) and an increasing amount of vitellin (10 to 800 ng/100µl) were added to the wells in triplicate. The response was again the same as the pure vitellin standard series, with a plateau seen at 400ng/100µl of vitellin (See appendix fig 5).

6- Non-vitellogenic *female* haemolymph proteins vs vitellin. Lastly, the preferential binding theory was tested using non-vitellogenic *female* haemolymph proteins. This was to investigate whether female specific proteins influence vitellin binding. A series of solutions of haemolymph protein from non-vitellogenic green and red females were spiked with known quantities of Vt (in the same way as in the initial experiment with male haemolymph). It was found that non-vitellogenic female haemolymph proteins do not affect vitellin binding (See appendix fig 6).

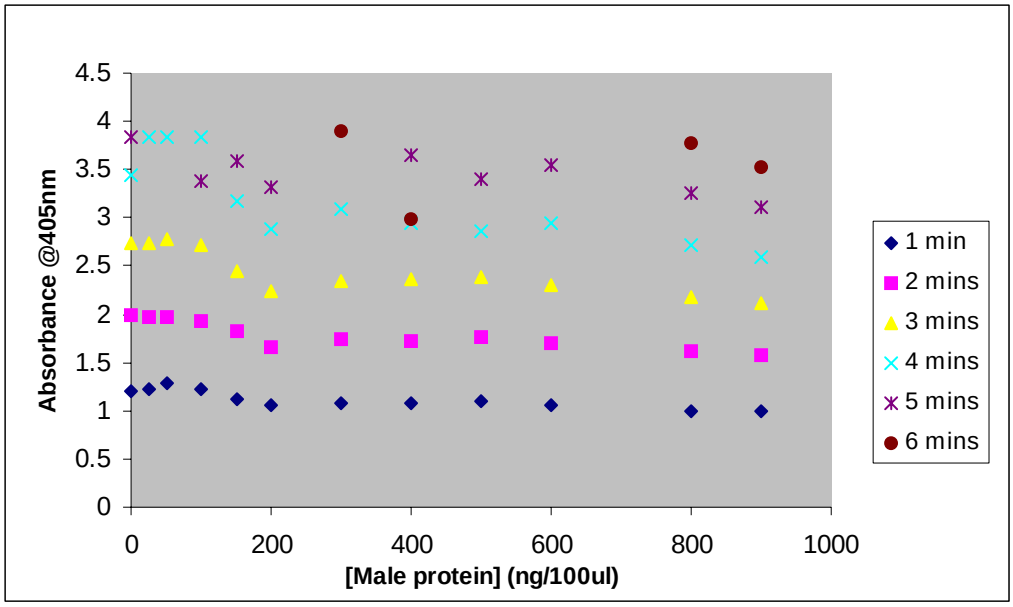
These experiments suggest that vitellin is preferentially binding to the well and that other haemolymph proteins (male or female) do not compete for binding sites. As a result, it can be assumed that all the vitellogenin in any given sample will bind when added to a microplate well, provided its concentration is not greater than 400ng/100µl. Any change in total vitellogenin concentration (per 100µl of sample) can be therefore be measured using the present ELISA protocol. The ELISA can then be applied to confidently quantitate Vg in haemolymph samples, allowing the measurement of any fluctuations in Vg level associated with exposure to exogenous ecdysteroids (see methods and results in main report).



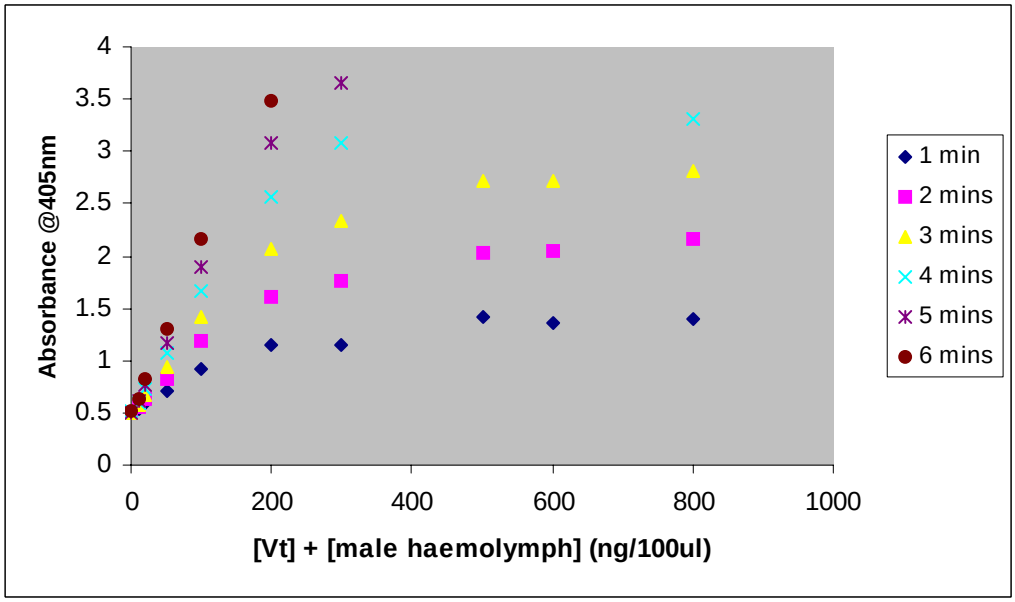
Appendix figure 1- Absorbance measurement at minute intervals of vitellin standard series (10ng/100µl to 1µg/100µl).



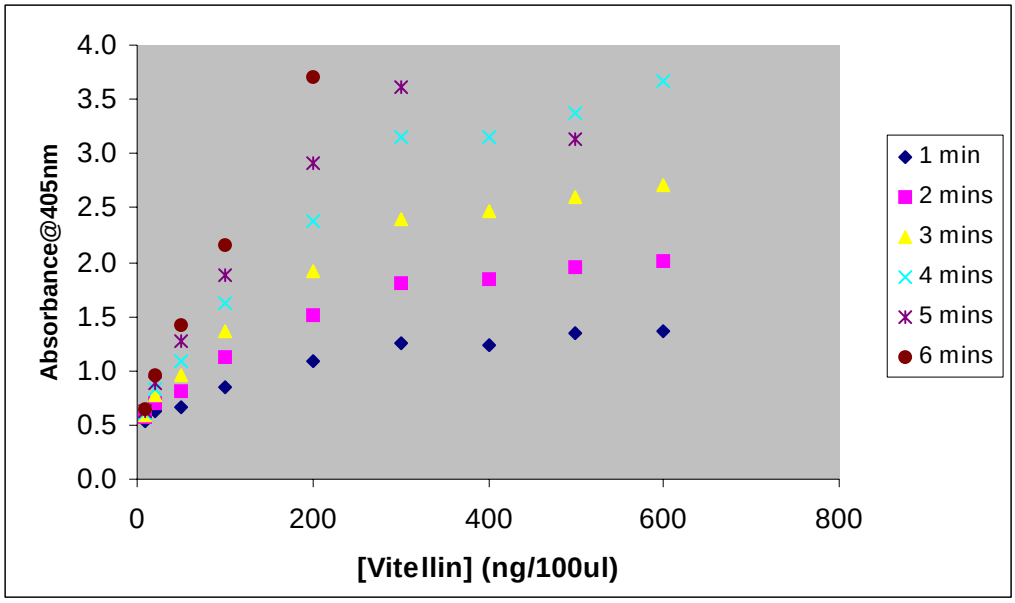
Appendix figure 2- Absorbance of male haemolymph spiked with vitellin standard (10ng/100µl to 400ng/100µl).



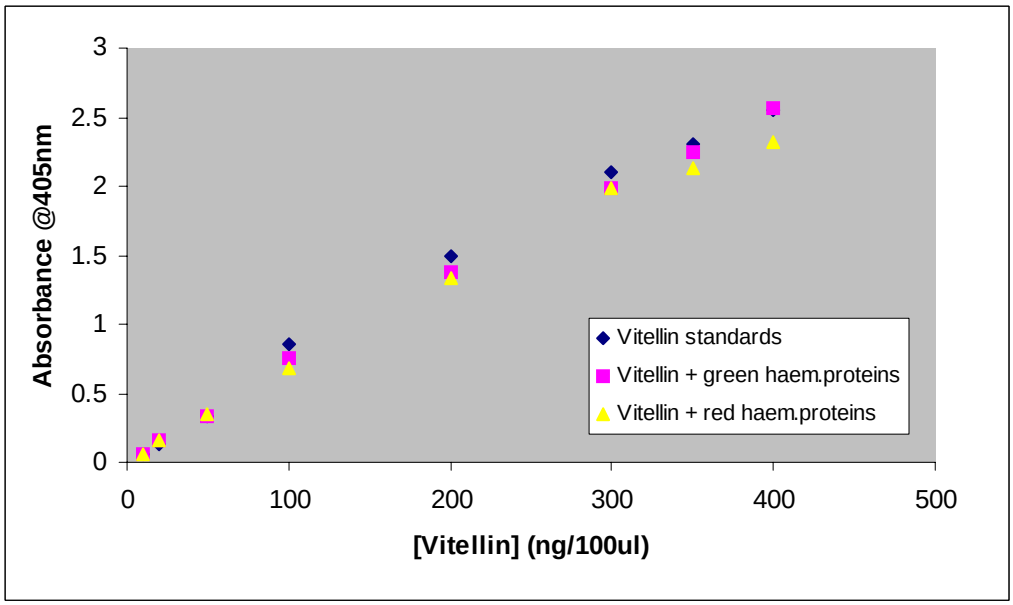
Appendix figure 3. Absorbance measurements at minute intervals of vitellin standard (200ng/100µl) spiked with increasing amounts of male protein (from 0-900ng/100µl).



Appendix figure 4. Absorbance measurements at minute intervals of a 50:50 vitellin/male haemolymph standard series from 10-800ng/100µl.



Appendix figure 5. Absorbance measurements at minute intervals of standard series consisting of a fixed concentration of male protein (400ng/100µl) spiked with increasing amounts of vitellin (from 10-600ng/100µl).



Appendix figure 6. Absorbance values of a series of protein standards from non-vitellogenic green and red females spiked with increasing quantities of vitellin (from 10-400ng/100µl).

REFERENCES

Adiyodi, R.G (1985). "Reproduction and its control" Chapter 3, pp147-215, in The Biology of Crustacea, Vol 9- Integument, Pigments and hormonal processes. Eds Dorothy E. Bliss and Linda H. Mantel, Acadmeic Press, Inc.

Arechiga, H., Huberman, A. & Naylor, E. (1974). Hormonal modulation of circadian neural activity in *Carcinus maenas* (L.). Proc.R.Soc. Lond. B, **187**, pp299-313.

Arechiga, H., Williams, J., Pullin, R.S.V. & Naylor, E. (1979). Cross sensitivity to neuro-depressing hormone and its effect on locomotor rhythmicity in two different groups of crustaceans. Gen. Comp. Endocrinol., **37**, pp350-357.

Baldwin, W.S., Bailey, R., Long, K.E. & Klaine, S. (2001). Incomplete ecdysis is an indication of ecdysteroid exposure in *Daphnia magna*. Env. Toxicol. Chem., **20**, No7, pp1564-1569.

Bolingbroke, M. & Kass-Simon, G. (2001). 20-hydroxyecdysone causes increased aggressiveness in female American lobsters, *Homarus americanus*. Hormones and Behaviour, 39, No2, pp144-156.

Bolt, S.R.L & Naylor, E. (1986). Entrainability by salinity cycles of rhythmic locomotor activity in normal and eyestalk ablated *Carcinus maenas* (L). Mar.Behav.Physiol., **12**, pp257-267.

Chaix, J.C. & de Reggi, M. (1982). Ecdysteroid levels during ovarian development and embryogenesis in the spider crab *Acanthonyx lunulatus*. Gen. Comp. Endocrinol., **47**, pp7-14.

Chan, S.M. (1995). Possible roles of 20-hydroxyecdysone in the control of ovary maturation in the white shrimp *Penaeus-vannamei* (Crustacea, Decapoda). Comp.Biochem.Physiol C- Pharmacology Toxicology and Endocrinology, **112**, No1, pp51-59.

Chang, E.S (1989). Endocrine regulation of molting in Crustacea. Reviews in Aquatic Sciences, **1**, No1, pp131-157.

Chang, E.S (1993). Comparative endocrinology of molting and reproduction: Insects and Crustaceans. Ann.Rev.Entomol, **38**, pp161-180.

Chang, E.S (1995). Physiological and biochemical changes during the molt cycle in decapod crustaceans: an overview. Jnl. Exp. Mar. Biol. Ecol., **193**, pp1-14.

Chen, C-C. & Chen S-N. (1994). Vitellogenesis in the giant tiger prawn, *Penaeus monodon* Fabricius, 1789. Comp.Biochem.Physiol B- Biochemistry & molecular biology, **107**, No3, pp453-460.

Colborn, T., Peterson Myers, J. and Dumanoski, D. (1996). Our Stolen Future, Little Brown and Co, Boston, p 306.

Crothers, J.H. (1967). The biology of the shore crab (*Carcinus maenas* (L)).1- The background- anatomy, growth and life history. Field studies, **2**, No4, pp407-434.

deFur, P.L., Crane, M., Ingersoll, C. & Tattersfield, L.J. (Eds) (1999). Proceedings from the International SETAC workshop on Endocrine Disruption in Invertebrates: Endocrinology, Testing and Assessment, 12-15 December, 1998, Noordwijkerhout, The Netherlands. SETAC Technical Publications Series, SETAC press.

Depledge, M.H. & Billinghurst, Z. (1999). Ecological significance of endocrine disruption in marine invertebrates. Mar. Poll. Bull, **39**, No1-2, pp32-38.

Fingerman, M. (1987). The endocrine mechanisms of crustaceans. Jnl. Crustacean Biology, **7**, No1, pp1-24.

Fingerman, M. (1995). Endocrine mechanisms in crayfish with emphasis on reproduction and neurotransmitter regulation of hormone release. Am. Zool., **35**, pp68-78.

Fingerman, M. (1997). Crustacean endocrinology: A retrospective, prospective and introspective analysis. Physiological Zoology, **70**, No3, pp257-269.

Guillette, L.J., Gross, T.S., Masson, G.R., (1994). Developmental abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. Env. Health. Perspectives, **102**, pp680-688.

Hunter, E. & Naylor, E. (1993). Intertidal migration in the shore crab *Carcinus maenas*. Mar.Ecol.Progr.Ser, **101**, No1-2, pp131-138.

Lachaise, F., Goudeau, M., Hetru, C., Kappler, C. & Hoffmann, J.A. (1981). Ecdysteroids and ovarian development in the shore crab, *Carcinus maenas*. Hoppe-Seyler Z.Physiol. Chem., **362**, pp521-529.

Lachaise, F., Le Roux, A., Hubert, M. & Lafgont, R. (1993). The molting gland of crustaceans: localization, activity and endocrine control (A review). Jnl. Crustacean Biology, **13**, No2, pp198-234.

Lee, C-Y., Umphrey, H.R. & Watson, R.D. (1996). Developmental changes in the level of vitellin-immunoreactive proteins in hemolymph and tissues of the blue crab *Callinectes sapidus*: relation to vitellogenesis. Jnl. Crustacean Biology, **16**, No1, pp1-9.

Loeb, M.J. (1993). Hormonal control of growth and reproduction in the arthropods: Introduction to the symposium. Amer. Zool, **33**, pp303-307.

Matthiessen, P. and Gibbs, P.E. (1998). Critical appraisal of the evidence for tributyltin-mediated endocrine disruption in molluscs. Env. Toxicol. Chem. **17**, pp37-43.

McGaw, I.J. & Naylor, E. (1992). Distribution and rhythmic locomotor patterns of estuarine and open nshore populations of *Carcinus maenas*. J. Mar. Biol. Ass. U.K., **72**, No3, pp599-609.

Naylor, E. (1985). Tidally rhythmic behaviour of marine animals. Symposia of the society for experimental biology, **39**, pp63-93.

Oberdorster, E. & Cheek, A.O. (2001). Gender benders at the beach: Endocrine disruption in marine and estuarine organisms. Env. Toxicol. Chem, **20**, No1, pp23-36.

Okumura, T. & Aida, K. (2000). Hemolymph vitellogenin levels and ovarian development during the reproductive and non-reproductive molt cycles in the giant fresh-water prawn *Macrobrachium-rosenbergii.* Fisheries Science, 66, No4, pp678-685.

Okumura, T., Han, C.H., Suzuki, Y., Aida, K. & Hanyu, I. (1992). Changes in haemolymph vitellogenin and ecdysteroid levels during the reproductive and non-reproductive molt cycles in the fresh-water prawn *Macrobrachium-nipponense*. Zool. Sci., **9**, No1, pp37-45.

Pinder, L.C.V., Pottinger, T.G., Billinghurst, Z. & Depledge, M.H. (1999). Endocrine function in aquatic invertebrates and evidence for disruption by environmental pollutants. R&D Technical Report E67, Environment Agency, Bristol.

Reid, D.G. & Naylor, E. (1985). Free-running endogenous semilunar rhythmicity in a marine isopod crustacean. J. Mar. Biol. Ass. U.K., **65**, No1, pp85-91.

Sharpe, R.M and Skakkebaek, N.E (1993). Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet, **341**, pp1392-1395.

Skinner, D.M. (1985). Molting and regeneration. Chapter 2, pp43-146, in The Biology of Crustacea, Vol 9- Integument, Pigments and hormonal processes. Eds Dorothy E. Bliss and Linda H. Mantel, Acadmeic Press, Inc.

Subramoniam, T. (1999). Endocrine regulation of egg production in econmically important crustaceans. Current Science, **76**, No3, pp350-360

Subramoniam, T. (2000). Crustacean ecdysteroids in reproduction and embryogenesis. Comp.Biochem.Physiol C- Pharmacology Toxicology and Endocrinology, **125**, No2, pp135-156.

Sumpter, J.P (1995). Feminized responses in fish to environmental estrogens. Toxicology Letters, **82/83**, pp7373-7742.

Tamone, S.L. & Chang, E.S. (1993). Methyl farnesoate stimulates ecdysteroid secretion from crab Y-organs *in vitro*. Gen. Comp. Endocrinol., **89**, pp425-432.

Tom, M., Fingerman, M., Hayes, T.K, Johnson, V., Kerner, B., Lubzens, E. (1992). A comparative-study of the ovarian proteins from 2 penaeid shrimps, *Penaeus-semisulcatus dehaan* and *Penaeus-vannamei* (Boone). Comp. Biochem. Physiol B-

Biochemistry and molecular biology, **102**, No3, pp483-490.

Vafopoulou, X. & Steel, C.G.H.(1995). Vitellogenesis in the terrestrial isopod, *Oniscus asellus* (L.): Characterization of vitellins and vitellogenins and changes in their synthesis throughout the intermoult cycle. Invertebrate Reproduction and Development, **28**, No2, pp87-95.

Webb, H.M. (1983). "Persistent rhythms in decapod crustaceans", Chapter 8, pp197-216, in Studies in Adaptation: The Behaviour of Higher Crustacea, Eds. S. Rebach & D.W.Dunham, John Wiley and Sons.

Webster, S.G. (1993). High-affinity binding of putative molt-inhibiting hormone (MIH) and crustacean hyperglycemic hormone (CHH) to membrane-bound receptors on the Y-organ of the shore crab, *Carcinus maenas*. Proc.R.Soc. Lond. B, **251**, No1330, pp53-59.

Williams, J.A. (1985). Evaluation of optic tract section on the locomotor activity rhythm of the shore crab *Carcinus* maenas. Comp.Biochem.Physiol A- Physiology, **82**, No2, pp447-453.

Young, N.J., Webster, S.G. & Rees, H.H. (1993a). Ovarian and hemolymph ecdysteroid titers during vitellogenesis in *Macrobrachium rosenbergii*. Gen. Comp. Endocrinol., **90**, pp183-191.

Young, N.J., Webster, S.G. & Rees, H.H. (1993b). Ecdysteroid profiles and vitellogenesis in *Penaeus monodon* (Crustacea, Decapoda). Invertebrate Reproduction and Development, **24**, No2, pp107-118.