FRDC PROJECT 2000/263

Reducing Rock Lobster Larval Rearing Time through Hormonal Manipulation

Rock Lobster Enhancement and Aquaculture Subprogram

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There is substantial commercial interest in the development of an aquaculture sector for Australian rock lobster species, due to the high value of these species in world markets and the fact that while the Australian wild fisheries are well managed, capacity to increase yield is limited. To be fully independent of wild stock there is the need to develop a closed cycle aquaculture industry which does not rely on the supply of wild broodstock, larvae or juveniles. To do this it is necessary to develop larval-rearing protocols that are reliable on a commercial scale. At present there is no commercial aquaculture sector where the larval phase exceeds 50 days. By contrast, the larval phase of Australian Rock Lobsters is believed to vary from 4-22 months, depending on the species. To date there have been no commercial scale successes in rearing rock lobsters from newly-hatched phyllosoma through to a settled juvenile phase.

There are a number of strategies that can be used to overcome the barriers to commercial-scale larval rearing. Foremost among these are development of suitable diets and microbial control measures. These factors, and other environmental manipulations, are being examined in related projects within the FRDC Rock Lobster Enhancement and Aquaculture Subprogram. However, even if suitable diets are developed and the microbial issues can be addressed, larval rearing will be a costly exercise for rock lobsters due to the length of time required to complete metamorphosis through the different larval stages (11 in total, plus a final molt into the settled puerulus stage). Hence the goal of the present project was to initiate work to determine whether the process of larval development could be speeded up by hormonal manipulation.

Rock lobsters undergo a gradual larval development, with morphological development occurring each time there is a molt to a new larval instar. In addition, there may be multiple molts within an instar, without accompanying metamorphic

change. In insects, larval molting and metamorphosis is controlled by the sequential activation of genes required for these processes, and the timing of this gene activity is orchestrated by titres of the ecdysteroid hormone 20-hydroxyecdysone (ecdysone).

Ecdysone acts by binding to a receptor protein, the ecdysone receptor. The ecdysone receptor is one of a family of proteins found throughout the animal kingdom which are activated by binding to a steroid hormone, and then act directly to regulate gene expression. In insects the ecdysone receptor is the master switch in this system, as it responds with exquisite sensitivity to the circulating levels of ecdysone, being activated when ecdysone levels start to rise, but repressed once ecdysone reaches a maximal level. When activated, the ecdysone receptor triggers a cascade of gene activity, leading to the eventual molt and possible metamorphic transformation. Genes activated by the ecdysone receptor include a number of other nuclear hormone receptors.

At the outset of this work, it was known that ecdysone plays a similar role in regulating molting in crustaceans. However, only nine partial or complete nuclear hormone receptor genes had been identified from crustaceans, and there had been no study of their role in larval development. The overall objective of the present project was "to identify triggers for molting to evaluate a shortening of the larval phase". This was addressed by isolating candidate nuclear hormone receptors from the tropical rock lobster, *Panulirus ornatus*, and studying the role of these nuclear hormone receptors in larval development. The underlying strategy was to determine how similar the molecular mechanisms controlling crustacean larval development are to those controlling insect larval development, as the knowledge base in the scientific literature is almost all on insect rather than crustacean larval development.

Five candidate nuclear hormone receptor genes were identified from *P. ornatus*. These had homology to the following nuclear receptors from the fruit fly, *Drosophila melanogaster*:

1. Ecdysone receptor (EcR): Master regulator of ecdysone response. Binds ecdysone as a heterodimer with USP and activates genes with ecdysone response elements.
2. Ultraspiracle (USP): Functions as a heterodimer with EcR to activate early response genes in *Drosophila*
3. Hormone receptor 3 (Hr3): Induced by EcR. Functions in *Drosophila* metamorphosis by repressing "early genes" induced in response to ecdysone and inducing competence factor for metamorphosis.
4. Hormone receptor 4 (Hr4): Evidence indicates it has a similar role to Hr3.
5. Hormone receptor 78 (Hr78): This receptor is reported to have a critical role in the onset of metamorphosis in *Drosophila*.

Sensitive assays were developed to measure the activity of these genes during *P. ornatus* larval development, using the newly developed technique of quantitative real-time reverse transcription PCR. Larval samples were collected from five independent spawnings, and daily samples were analysed for the activity of four of these five genes (ultraspiracle was omitted from the analysis). This included analysis of samples across larval molts i.e. Phyllosoma stage 1 to stage 2, phyllosoma stage 2 to stage 3 and, in two spawnings, phyllosoma stage 3 to stage 4.

The analysis was complicated by the fact that larval molting is not synchronous. In the spawnings analysed, the transformation from one larval stage to the next took up to 7 days for the entire cohort of larvae to complete a molt. Subsamples of newly molted larvae were collected at the beginning of each molt to ensure that the newly molted larvae were synchronized. However, larvae collected just prior to the onset of molting in a population could comprise larvae that were 1-7 days pre-molt, and this problem of mixed developmental stages compromised the analysis. Nevertheless patterns of gene regulation did appear, and the most significant finding is the strong similarity to patterns of gene expression observed during insect larval development. This is highly significant as it confirms that insects, for which there is an extensive knowledge base, can serve as model systems for the understanding and manipulation of larval development in decapod crustaceans.

The ecdysteroid titre was also measured in two out of the five spawnings. This confirmed that the ecdysone levels varied in the same manner reported in insects and in other crustacean species i.e. a surge in the ecdysone titre was observed late in the molt cycle, with ecdysone levels dropping precipitously immediately before the actual physical process of ecdysis or shedding of the old cuticle.

In summary, this research has provided the initial tools to dissect the molecular control of larval development, and, importantly, has confirmed that control of larval development and metamorphosis closely parallels that in insects. This work leads toward a detailed study of the impacts of hormonal manipulation on larval development. The shortening of the larval cycle in rock lobsters is essential for the commercial success of closed cycle rock lobster aquaculture. A mechanistic understanding of larval molt and metamorphosis will contribute significantly towards this goal.

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1. BACKGROUND

Worldwide demand for rock lobsters

Worldwide (tropical) rock lobster wild fisheries have a value in excess of US$500 million, and demand for this high value seafood product is constantly increasing (FAO Statistics – www.fao.org/fi/default.asp). However, as these fisheries worldwide have reached their sustainable limits, there is no further capacity in the wild fishery to meet increased consumer demand. Hence there is considerable interest in Australia and elsewhere in developing a rock lobster aquaculture sector.

World landings of rock lobsters are dominated by the Astacidea, or clawed lobsters, from the north Atlantic. The Palinuridea, or rock lobsters (also referred to as spiny lobsters), make up approximately 37 % of world landings. Of these, tropical species (including the Caribbean lobster) make up 70% of rock lobster landings (FAO Statistics). As clawed lobsters are not an Australian species, they are not candidates for aquaculture in Australia.

Rock lobsters account for 20% of the total value of fisheries production in Australia, and are the single most valuable fisheries commodity (ABARE, 2004). However, the wild harvest has now plateaued and the only possible means to expand production is by development of an aquaculture industry.

Attempts at rock lobster aquaculture

Attempts at the aquaculture production of tropical rock lobsters (Palinuridea) span over a quarter of a century (Cobb and Phillips 1980; Kittaka 1997). Major research efforts have taken place in Japan, New Zealand and the USA (Moe 1991; Kittaka 1997). None of these efforts have met with commercial success.

A rock lobster aquaculture sector can take two forms. One is to collect newly settled juveniles from the wild, in order to by-pass the larval rearing phase of the production cycle. This is the basis of a successful cottage industry in coastal villages in Vietnam, where such juveniles are on-grown to about 1 kg in cages in coastal bays. However, the unpredictability of supply due to high variation in annual recruitment of juveniles, coupled with questions about sustainability of an industry reliant on collection of juveniles, makes this a difficult prospect for a large scale commercial industry.

The second option is to develop a true closed life cycle aquaculture sector, in which adult broodstock are spawned in hatcheries, larvae are reared through to settlement

and these juveniles are on-grown to marketable size. Advantages of the closed life cycle approach are reduced pressure on wild stocks, an ability to control the disease status of the stocks and the ability to implement selective breeding.

Hence, if rock lobster aquaculture is going to develop into a viable sustainable industry, the closing of the life cycle is necessary. The selection of a particular species as an aquaculture candidate is determined by the possession of favourable characteristics that are amenable to farming conditions. Amongst others, these include suitable life history traits, ability to be reared in high densities, disease resistance, nutritional requirements, and tolerance of variations in water quality.

The challenge of larval rearing in aquaculture

The only commercially viable crustacean aquaculture industries at present are those which farm species with a short larval phase (Lee and Wickens, 1992). The larval rearing period is considered to be the most labour intensive and technically difficult, and is often associated with high mortality which may be due to pathogenic microbial infections. By far the largest crustacean aquaculture sector is that for penaeid prawns. Penaeids have a larval phase of only 15-20 days. Success in larval rearing of some crabs has also been reported and in all cases they also have a short (15-30 day) larval life.

The rock lobster has one of the longest planktonic larval phases of any marine organism. The planktonic larval phase is complex and characterised by delicate, 'spider-like' larvae known as phyllosomas (Figure 1.1). Typically there are 11 morphological phases, with even more molts, that the larvae pass through before metamorphosing into pueruli (a non-feeding stage which has the adult body plan) and then finally settling and becoming juvenile lobsters (Booth and Phillips, 1994; Kittaka and Abrunhosa, 1997). The key to achieving commercial scale larval rearing of rock lobster will be improving larval survival and condensing the larval phase into the shortest time period possible.

A number of rock lobster species occur in Australian waters, and the larval phase is estimated to last anywhere from 4-6 months in *Panulirus ornatus*, the tropical rock lobster, to over 16 months in the Southern Rock Lobster, *Jasus edwardsii*. *P. ornatus* is therefore likely to be more amenable than most rock lobster species to rearing from egg to settled juvenile, but an aquaculture sector would benefit significantly from further reduction in duration of the larval phase.

Growth rate in Crustacea is in part determined by molt frequency

Growth and development in animals with an exoskeleton, including Crustacea, is in part limited by molt frequency: in order to increase in size beyond a certain point, crustaceans must first shed their confining exoskeleton (Chang et al., 1993). The increase in mass which occurs at molting, primarily due to uptake of water driving expansion of the new exoskeleton, is termed the molt increment and may be of the order of 20-30% (Abdu et al., 2001).

The impact of increasing the molt frequency is demonstrated most effectively by studies on decapods that have been bilaterally eyestalk ablated. As discussed in greater detail below, the eyestalks of decapods contain a neuroendocrine gland, named the sinus gland, which contains a suite of neuropeptides that regulate aspects of crustacean physiology. One process that is regulated by one or more of these peptides is molting, with molting being inhibited by the neuropeptides(s) secreted from the eyestalk – hence they are referred to as molt inhibiting hormones or MIH. If both eyestalks are removed from a decapod, then endocrine constraints on the intermolt duration are reduced or removed, molting occurs more frequently and the resulting animals increase in biomass much more rapidly than their siblings with intact eyestalks. Examples include the American lobster (Chang, 1989), several shrimp species (Choy, 1987; Chu and Chow, 1992) and crayfish (Sindhukumari et al., 2005).

Control of molt frequency to regulate growth rate may in fact be a normal component of crustacean physiology – for example oceanic copepods during starvation conditions or in diapause show population level shifts towards earlier stages of the molt cycle (Johnson, 2003; Sindhukumari et al., 2005).

Larval development can be modified by hormonal manipulation

A premise of the present work is that it might ultimately be possible to influence larval development by hormonal manipulation. That it is possible to influence molting and growth by deliberate chemical manipulation is demonstrated by a number of studies, including those referred to above where animals were subjected to bilateral eyestalk ablation. While most reports focus on juveniles or adults, eyestalk ablation has also been carried out on the larvae of some species, notably homarid lobsters, again with the result that molt cycle is decreased in length (Snyder and Chang, 1986).

Manipulation of larval development has also been examined by addition of exogenous hormones, primarily 20-hydroxyecdysone due to its central role in controlling the molt cycle (see below). A number of examples are reviewed by Skinner (Skinner,

1985) and by Chang (Chang, 1989). 20-hydroxyecdysone is generally supplied either by immersion, or, in juvenile or adult animals, by injection. Critical factors in inducing a successful molt without associated developmental abnormalities include the timing of ecdysone administration, and the dosage. In the majority of cases although ecdysis has been successfully induced, there are associated mortalities or developmental abnormalities (Skinner, 1985; Chang, 1989).

The ability to manipulate larval development is exploited in a number of both natural and artificial insect control measures. For example, ecdysone agonists are used to induce precocious molts of insect pests, leading to their death (Sundaram et al., 1998). In fact, hormonal manipulation of larvae has long been exploited by some plant species as a defence against insects (Toong et al., 1988).

Molting is controlled by ecdysteroids

Molting is a complex process. At its simplest, it involves the laying down of a new cuticle or exoskeleton, secreted by the epidermal cell layer. When this new exoskeleton is complete, a series of behaviours is initiated which results in the shedding of the old exoskeleton, in a process called ecdysis (Mesce and Fahrbach, 2002). Following ecdysis, the new exoskeleton must rapidly harden.

The molt cycle is formally divided into a number of different stages based on observed changes in the exoskeleton and underlying epidermis (Skinner, 1985; Chang, 1989). The actual shedding of the old exoskeleton, ecdysis, is the most obvious component of the molt cycle and generally lasts only a few minutes. Stages A and B are collectively termed Metecdysis, and in these stages the new exoskeleton hardens and the animal recovers from the process of ecdysis e.g. the endocuticle (the inner most layer of the exoskeleton) is formed and muscle is repaired either where it has degenerated, or in newly regenerating limbs. This is followed by anecdysis or intermolt (stage C), a period of relative stasis with regard to the molt process, although reserves for the ensuing ecdysis are accumulated during this period. Proecdysis or stage D initiates with apolysis, the separation of the epidermis from the exoskeletion. This is followed by stage D2 when the outer layers of the new exoskeleton are secreted, and the final preparations for actual ecdysis occur in stages D3-D4. The duration of each stage varies considerably, with stage C or the intermolt being by far the longest stage.

Molt cycles may also encompass substantial developmental changes, depending on the life stage of the organism. In holometabolous insects which undergo a complete metamorphosis, including *Drosophila* on which much of our knowledge of the neuroendocrine control of molting in arthropods is based, molts may be larval instar to larval instar, larval instar to pupal, or pupal to adult. In insects this is normally

the final molt. However, in crustaceans, there will be larval instar-larval instar molts, the larval –adult transition and subsequent adult-adult molts throughout the lifetime of the organism. The majority of larval-larval molts, as well as larval-adult molts, involve morphological differentiation as well as the act of molting.

Larval molt and metamorphic transformations in insects are regulated by hormonal cycles, primarily changes in ecdysteroid titres. Likewise, the various transformations through the larval stages and molts are under endocrinological and molecular controls in Crustacea (Chang, 1984) All the major endocrine organs, including the X-organ-sinus gland complex in the eyestalk, the Y-organ and the mandibular organ are involved. The X-organ-sinus gland complex is the source of molt inhibiting hormone (MIH), as discussed earlier for prawns. The Y-organ is the source of ecdysteroids which are the major steroid hormones regulating molt. MIH has been shown to act on the Y-organ to inhibit secretion of ecdysteroids, thus inhibiting progression to the final stages of the molt process (Soumoff and O'Connor, 1982; Watson and Spaziani, 1985; Mattson and Spaziani, 1986; Spaziani et al., 1989). It is not known what triggers the release of the Y-organ from MIH control, although there is some evidence to suggest subsequent feedback from high titres of ecdysone may reduce MIH activity (Mattson and Spaziani, 1986).

A third neuroendocrine organ, the mandibular organ is the source of methyl farnesoate (MF), a juvenile hormone-like hormone. There is evidence for the involvement of methyl farneosoate in reproduction, but only scant evidence for a rold of either MF or the mandibular organ in molting (Charmantier et al., 1997).

Ecdysteroid molecules in particular are known to be critical in controlling molting and metamorphosis in insects and crustaceans. In Crustacea, ecdysone is produced in the Y-organ, and secreted to the haemolymph, where it is converted enzymatically to the active form, 20-OH-ecdysone (Chang, 1993). The Y-organs of some crustaceans may also secrete other ecdysteroids, namely 3-dehydroxyecdysone and 25-deoxyecdysone (Soumoff and Skinner, 1983; Chang, 1993). However, when the ecdysteroid composition of *Homarus americanus* (American lobster) larvae was analysed by thin layer chromatography, it was found to comprise only 20-hydroxyecdysone (Chang and Bruce, 1981).

The pattern of changes in ecdysteroid titres during the molt cycle appears to be conserved across insects and crustaceans. Ecdysteroid titres build up in the haemolymph to high levels and then drop again to basal levels immediately prior to molt (Mesce and Fahrbach, 2002). If this drop does not occur, ecdysis does not proceed (Zitnan et al., 1999). The same pattern of a rise in ecdysteroid titre shortly before ecdysis, followed by a drop in the ecdysteroid titre immediately pre-ecdysis, is observed in crustaceans (Chang and Bruce, 1981; Soumoff and Skinner, 1983).

In insects the final stage of the molt cycle, the actual physical behaviours of ecdysis, is controlled by a number of peptide hormones: ecdysis triggering hormone (ETH); eclosion hormone (EH); and crustacean cardioactive peptide (CCAP). Evidence from studies in both *Drosophila* and the tobacco hornworm, *Manduca sexta*, suggests that ETH, which is synthesised in seven pairs of epitracheal Inka cells, begins to be secreted at low levels at precisely the time in the developmental cycle when ecdysis must be initiated. This then establishes a positive feedback loop between ETH and EH, which mutually reinforces secretion of these two neuropeptides. The most widely held model is that this augmentation of EH secretion triggers a range of events, including release of CCAP which appears to be the most immediate trigger of the motor events that effect ecdysis. However, even in insects the picture is not entirely clear and there is also evidence of redundancy between EH and CCAP (Clark et al., 2004).

The events of ecdysis are also coordinated by ecdysteroid levels. Expression of the ETH gene is induced by rising ecdysteroid levels, but their release from the Inka cells only initiates when ecdysteroid levels fall (Zitnan et al., 1999). Ecdysteroids appears to induce ETH expression by induction of transcription and this correlates with an increase in the level of the Ecdysone receptor (B1 isoform) in the Inka cells (Zitnan et al., 1999).

Ecdysteroid response is mediated by nuclear hormone receptors

Ecdysteroids act by binding to specific receptors in the cell nucleus and inducing conformational changes in those receptors which lead to the activation or inhibition of specific gene expression. Much of the temporal and tissue specificity of ecdysteroid responsiveness is modulated by the presence or absence of specific nuclear hormone receptors (Perera et al., 1998). A large number of nuclear hormone receptors have now been described. In particular, with the completion of the genome sequence of *Drosophila melanogaster*, it has been possible to predict all possible nuclear hormone receptor genes, which total 21 in this organism (Maglich et al., 2001).

The wide variety of genetic and genomic tools available for the study of the model organism *Drosophila melanogaster*, have led to the greatest depth of knowledge about the role of nuclear hormone receptors in molting and larval development being available in this species, although considerable information is also accumulating from studies of the tobacco hornworm, *Manduca Sexta* (Riddiford et al., 2003). It was observed relatively early that ecdysone triggers a cascade of gene expression in *Drosophila*, as demonstrated by the observation of successive waves of "puffing" of the *Drosophila* polytene chromosomes. These chromosomes occur in the salivary glands of late prepupal larvae, and have undergone multiple rounds of DNA replication without concomitant cell division. Hence the banding patterns of

the chromosomes are very clearly visible, and, when transcription of genes occurs, this is visualised as "puffs" associated with specific bands of the *Drosophila* chromosomes. The temporal sequence in which puffing is observed in the different genes is believed to represent a genetic hierarchy, i.e. the genes that are expressed later on are in some way dependent on expression of the early genes. It has also been observed that puffing or expression of the early genes does not require protein synthesis, whereas puffing or expression of later genes is dependent on protein synthesis, indicating that expression of additional proteins, most likely the early genes, is required (Ashburner, 1990).

These early observations of polytene chromosome puffing led to the initial description of the temporal hierarchy of gene expression in response to ecdysone. The first gene to be induced is the ecdysone receptor (EcR). This is rapidly followed by a set of at least five additional "early" genes, the best characterised of which is the E75 nuclear hormone receptor (many genes in *Drosophila* were initially named after the banding location on the salivary polytene chromosomes where puffing associated with gene transcription was observed). Following on from the early genes a set of "delayed early" genes is induced, including the nuclear hormone receptors E78B and DHR3, both of which require prior protein expression. This is followed in turn over a 4-6 hour period by sequential induction of additional nuclear hormone receptor genes, including βFTZ-F1 and, in the later stages, DHR78.

Since these initial descriptions, much has been learned about the functioning of nuclear hormone receptors, and how they interact with each other. Nuclear receptors have a five domain structure. The A/B N-terminal domain is believed to be involved in transcriptional activation. The C domain is the domain which binds to specific DNA sequences and so acts to activate or repress specific gene expression – also referred to as the DNA binding domain or DBD. The C domain also plays a role in determining which other nuclear receptors a given nuclear receptor binds to, as many function as heterodimers. The D domain acts as a hinge between the C and E domains. The E domain is also known as the ligand binding domain, and has additional roles in dimerisation of the receptor and transcriptional activation. There may also be a non-conserved C-terminal F domain (Laudet, 1997; Hu et al., 2003; Escriva et al., 2004). Importantly for this work, nuclear receptors appear to have originated early in evolution, and are sufficiently conserved to allow the ready isolation of nuclear receptor gene sequences from a diversity of species using degenerate primers (Laudet, 1997; Devine et al., 2002; Bertrand et al., 2004).

The ecdysone receptor is encoded by a single gene, but three different isoforms are expressed, EcR-A (849 aa), EcR-B1 (878 aa) and EcR-B2 (669 aa). These isoforms all share the same DNA binding and ligand binding domains, but differ in the N-terminal region due to alternate mRNA splicing (Talbot et al., 1993). The specific isoforms

show tissue specific expression during larval development and metamorphosis, indicating that another level of regulatory specificity may be imposed by differential mRNA splicing (Talbot et al., 1993). EcR appears to function as a heterodimer with another receptor named ultraspiricle (USP), and, in the presence of 20-OH ecdysone, this heterodimer binds to specific DNA target sequences upstream of regulated genes known as ecdysone response elements (EREs) (Jones and Sharp, 1997; Cherbas et al., 1991). Studies have shown that the EcR in *Drosophila* is induced when ecdysone reaches approximately 2 x 10^9 M, and is repressed again when the levels increase by tenfold (Thummel, 1996). This autoregulation is typical of *Drosophila* nuclear hormone receptor genes.

The potential complexities of gene regulation are best illustrated with the ecdysone receptor, but may apply to any of the nuclear hormone receptors involved in molting and metamorphosis. As mentioned above, one level is alternate mRNA splicing to give rise to different isoforms. In addition, it has been shown that nuclear hormone receptors generally require co-regulators which interact with the nuclear receptor to enable activation or repression of genes, and it is believed that this effect may result from changes in chromatin structure induced by the coregulator(s) (Torchia, 1998). This was initially demonstrated for vertebrate nuclear hormone receptors, and recently coregulators have also been demonstrated in *Drosophila* (Tsai et al., 1999; Sedkov et al., 2003).

A second level of complexity is afforded by the formation of heterodimers. For example, the ecdysone receptor can only bind ecdysone and activate transcription when in a heterodimer with Ultraspiracle (USP). However, a third receptor termed DHR38 can bind to USP and sequester it, to prevent activation of USP/EcR dependent genes (Sutherland et al., 1995).

Further complexity is afforded by the interplay of different hormones. A second key hormone in insect development is juvenile hormone (JH). This is produced in *Drosophila* from the late embryonic stages until the last larval instar. The presence of juvenile hormone ensures that ecdysone induces a larva-larva molt. Progression to the final metamorphic molt only occurs when the JH titre drops. This effect appears to be mediated in *Drosophila* by selective repression of a set of regulatory genes that, when expressed, trigger the activity of a series of genes required for metamorphosis – these include genes involved in programmed cell death, and genes involved in cell proliferation and differentiation. The exact mechanism by which JH mediates this effect is unknown, but recent evidence suggests it may be by binding to the USP/EcR dimer and recruiting a co-repressor which prevents activation of genes required for metamorphosis, as opposed to molting (Maki et al., 2004; Dubrovsky, 2005). It is not known whether there is a similar activity in crustaceans. The most likely candidate to play such a role, however, is methyl farnoesoate, a

compound chemically related to JH, which is produced by the mandibular organs as discussed earlier (Chang, 1984; Charmantier et al., 1997).

Despite the extent of information available, and the variety and sophistication of genetic and genomic tools available in *Drosophila*, a complete understanding of the control of molting and metamorphosis is still some way off even in this species. It should also be remembered that development in *Drosophila* is holometabolous, i.e the larvae undergo a complete metamorphic change at pupation, in which larval tissues are destroyed and adult tissues form *de novo* from imaginal discs. By contrast, larval development in Crustacea is more akin to that observed in hemimetabolous insects such as bugs or cockroaches which undergo gradual morphological shifts at each molt, rather than the dramatic complete rearrangement of body plan observed during *Drosophila* metamorphosis.

Few nuclear hormone receptors are known in Crustacea

The partial and complete nuclear receptor genes that have been identified in crustaceans at the time of preparation of this report are listed in Table 1.1. Only nine of these sequences were available at the initiation of this project. The *Artemia* sequences are extremely short, originating from an evolutionary survey of nuclear hormone receptors across all animal phyla (Laudet, 1997) and there is no associated functional data. The lobster HHR3 gene was studied in muscle and eyestalk tissue of juvenile lobsters, and regulation was found to broadly parallel that observed in insects i.e. induction in response to ecdysone administration, although the opposite pattern of regulation was observed in eyestalk (El Haj et al., 1997). The role of crab homologs of the ecdysone receptor and ultraspiracle were studied during limb regeneration, with both genes being induced several days after limb loss when the regeneration process initiates (Chung et al., 1998). A shrimp homolog of the E75 gene was found to be expressed in epidermis, eyestalk and nerve cord of mature *Metapenaeus ensis* (Chan, 1998). The expression of another shrimp nuclear receptor homolog, an FTZ-F1 homolog, was investigated in reproductive tissues and in larvae, and it was found to be most highly expressed in mature oocytes (Chan and Chan, 1999). However its role in molting was not investigated. It should be noted that none of these studies address the role of these nuclear hormone receptor homologs in larval molting and metamorphsis.

NEED

There is substantial commercial interest in the development of an aquaculture sector for Australian rock lobster species, due to the high value of these species in world markets and the fact that the Australian wild fisheries are fully exploited. To develop a closed cycle aquaculture industry, i.e. one which is not reliant on supply

of broodstock, larvae or juveniles from the wild, it is necessary to develop hatchery protocols that are reliable on a commercial scale. The greatest challenge to overcome is larval rearing. At present there is no commercial aquaculture sector where the larval phase exceeds 50 days. By contrast, the larval phase of Australian Rock Lobsters is believed to vary from 4-16 months, depending on the species. To date there have been no commercial scale successes in rearing rock lobsters from newly-hatched phyllosoma through to a settled juvenile phase.

There are a number of strategies that can be used to overcome the barriers to commercial-scale larval rearing. Foremost among these are development of suitable diets and microbial control measures, as mass mortalities of phyllosomas routinely occur due to disease epizootics, with growth rates and ability to resist infection probably compromised by poor nutritional status. These factors, and other environmental manipulations, are being examined in related projects within the FRDC Rock Lobster Enhancement and Aquaculture Subprogram. However, even if suitable diets are developed, and the microbial issues can be addressed, larval rearing will be a costly exercise for rock lobsters due to the length of time required to complete metamorphosis through the different larval stages (11 in total, plus a final molt into the settled puerulus stage). Hence the goal of the present project was to initiate work to determine whether the process of larval development could be speeded up by hormonal manipulation.

OBJECTIVE

The specific objective of this project was to

"identify triggers for molting to evaluate a shortening of the larval phase"

This was addressed by cloning candidate nuclear (steroid-responsive) receptors in *P. ornatus* and tracking changes in expression of these genes over the transition from one phyllosoma stage to the subsequent stage. In parallel with the molecular biology experiments, an enzyme-linked immunoassay was adapted to measure ecdysone in *P. ornatus* phyllosomas be used to examine the temporal changes in hormone titre in groups of phyllosomas. Together, this gave an overall picture of how changes in hormone levels might be translated into changes in gene expression, with consequent implications for the manipulation of larval molt by exogenous addition of hormone.

Figure 1.1: Rock lobster phyllosoma have 11 different instars, with multiple molts within single instars in some cases.

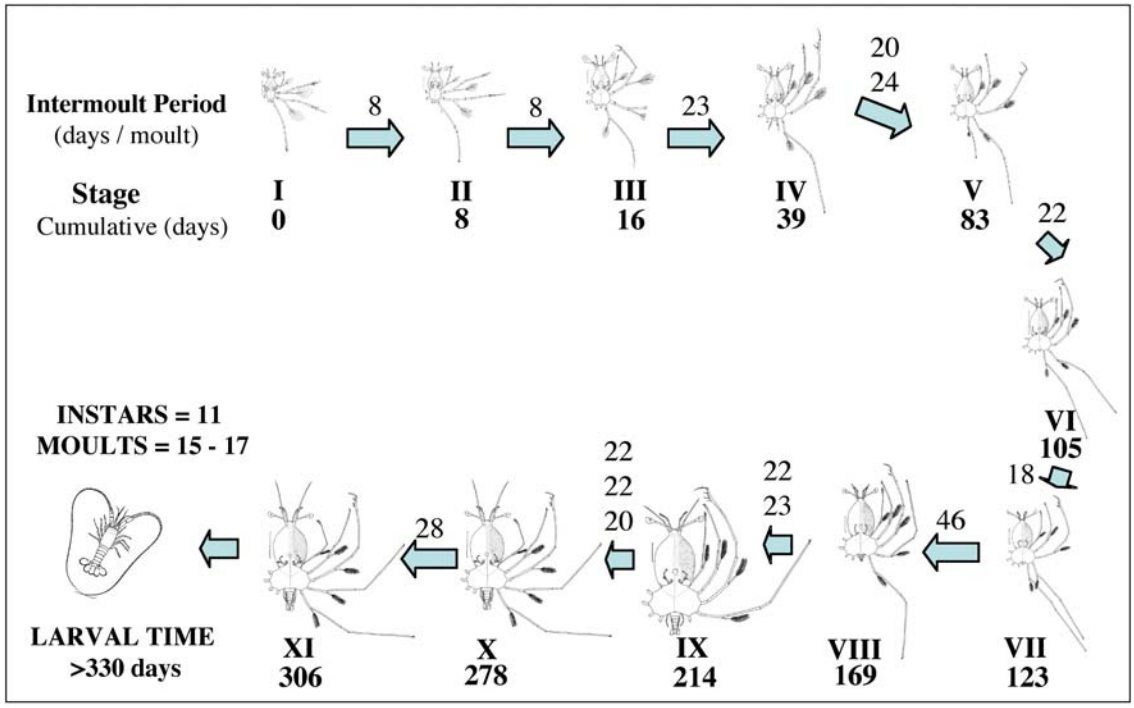

Table 1.1: Partial and complete nuclear receptor genes that have been identified in crustaceans.

ACCESSION NUMBER	DATE ENTERED	GENE	SPECIES	SEQUENCE LENGTH	FUNCTION	REFERENCE
Not in database	-	HHR3	*Homarus americanus*, American lobster			(El Haj et al., 1997)
AF034086	Apr 2001	UpEcR	*Uca pugilator*, fiddler crab	4562	Ecdysteroid receptor	(Chung et al., 1998)
AF032983	Apr 2001	UpRXR	*Uca pugilator*, fiddler crab	1492	Ultraspiricle homolog	(Chung et al., 1998)
AY496927	Jan 2004	EcR	*Carcinus maenas*, green crab	1008 (partial cDNA sequence)	Ecdysteroid receptor	Chung,J.S. and Webster,S.G., unpublished
AY496928	Jan 2004	RXR	*Carcinus maenas*, green crab	3890 (partial cDNA sequence)	Vertebrate Retinoid X receptor / *Drosophila* Ultraspiricle homolog	Chung,J.S. and Webster,S.G>, unpublished
AF092946	Nov 1998	E75	*Metapenaeus ensis*, shrimp	3538		(Chan, 1998)
AF159132	July 1999	FTZ-F1	*Metapenaeus ensis*, shrimp	4700		(Chan and Chan, 1999)
AY346367	Aug 2003	EcR	*Litopenaeus vannamei*	190		Morales-Covarrubias,M.S. and Garcia-Gasca, A. unpublished
AY346368	Aug 2003	RXR	*Litopenaeus vannamei*	174		Morales-Covarrubias,M.S. and Garcia-Gasca, A. unpublished
AF086806	Aug 1998	EcR Homolog	*Uca pugilator*	705	different to above EcR sequence	(Chung et al., 1998)
U93419	Dec 1998	FTZ-F1	*Artemia salina*	109bp		(Laudet, 1997)
U93418	Dec 1998	FTZ-F1	*Artemia salina*	104bp		(Laudet, 1997)
U93417	Dec 1998	EcR	*Artemia salina*	95bp		(Laudet, 1997)

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2. ISOLATION OF *P. ORNATUS* GENES CORRESPONDING TO NUCLEAR HORMONE RECEPTORS

Introduction

A key goal of this project was to isolate a variety of candidate nuclear hormone receptor genes. Nuclear hormone receptors have a modular structure, aspects of which are well conserved, facilitating the isolation of homologous genes in a diversity of metazoan species. There are five domains. The A/B N-terminal domain is believed to be involved in transcriptional activation. The C domain is the domain which binds to specific DNA sequences and so acts to activate or repress specific gene expression – also referred to as the DNA binding domain or DBD. The C domain also plays a role in determining which other nuclear receptors a given nuclear receptor binds to, as many function as heterodimers. The D domain acts as a hinge between the C and E domains. The E domain is also known as the ligand binding domain, and has additional roles in dimerisation of the receptor and transcriptional activation (Laudet, 1997; Hu et al., 2003; Escriva et al., 2004). There may also be a non-conserved C-terminal F domain.

The DNA binding domain is particularly strongly conserved among nuclear receptors, and this has enabled the identification of nuclear receptors in unrelated organisms through use of conserved, degenerate primers. For example, an evolutionary survey of nuclear receptors was undertaken using this approach (Escriva et al., 1997), and a suite of nuclear receptors from ascidians was likewise identified using conserved PCR primers (Devine et al., 2002). In the present work, the conserved primers from the latter publication were used to isolate fragments of candidate nuclear hormone receptor genes from *Panulirus ornatus*. Further work was then undertaken to isolate and characterize full length sequences of the identified genes.

Materials and Methods

Isolation of genomic DNA encoding putative Nuclear Hormone Receptors

Genomic DNA was isolated from *P. ornatus* and from *P. cygnus*. A 2-4 mm^2 sample of leg muscle tissue was ground with a sterile plastic pestle in 500 µl lysis buffer (10 mM Tris pH 7.4, 1 mM EDTA, 0.5% SDS) with 10 µl QIAGEN Protease and incubated at 65°C for 90-120 min. 70 µl 5 M NaCl was then added to bring the overall NaCl concentration to 0.7 M, prior to addition of 65 µl CTAB buffer (10% w/v CTAB, 0.7 M NaCl). This was incubated at 65°C for 1 hr, and then extracted three times with an equal volume of chloroform. An equal volume of isopropanol was added to the supernatant and the resulting white flocculent DNA precipitate washed with 70%

ethanol. The pellet was then air dried, resuspended in 200 µl TE (10 mM Tris pH 7.4, 1 mM EDTA) and treated with RNAse A (100 µg ml^{-1}) at 37°C for 1 hr. RNAse A was removed by phenol-chloroform extraction, and the DNA reprecipitated using 1/10th vol 3M NaOAc + 2.5 vol ethanol. The resulting pellet was washed with 70% ethanol, air dried and resuspended in 80 µl TE.

Candidate nuclear hormone receptor genes were then amplified from the genomic DNA using the degenerate primers RR-1 and RR-2 (Table 2.1) which recognize the conserved protein sequences CEGCKGFF and CQYCRYQKC (Devine et al., 2002). The reaction contained 2-10 ng genomic DNA, 200 nM dNTPs (Invitrogen), 1 µM RR-1, 1 µM RR-2, 5U Taq DNA Polymerase in supplied 1x PCR Buffer (Qiagen) in a 50 µl reaction. Cycling conditions were [94°C for 1 min] x1 cycle; [94°C for 1 min, 47°C for 1 min, 72°C for 1 min 20 s] x 30 cycles; [72°C for 10 min]. This resulted in a very faint band ~ 150 bp, which was gel purified and re-amplified under identical conditions as above, except with primer concentrations used at 1 µM, 5 µM and 10µM. The resulting products were gel purified using a Qiagen Gel Extraction Kit, cloned into the pGemTeasy vector following the manufacturer's instructions (Promega A1360) and transformed into One-Shot Top-10 Cells according to the manufacturer's instructions (Invitrogen C4040).

The resulting colonies were screened by PCR using universal primers (NEB #1224, and NEB #1233, Table 2.1). Individual colonies were transferred to 30 µl sterile MilliQ-H_2O using sterile toothpicks. 2 µl of the resuspended colony was used in a 20 µl PCR reaction with 200 nM dNTPs (Invitrogen), 1 µM NEB1224, 1 µM NEB1233, 1 U Taq DNA Polymerase in supplied 1 x PCR Buffer (Qiagen) as follows: [95°C for 5 min] x1 cycle; [94°C for 30 s, 55°C for 40 s, 72°C for 1 min] x 25 cycles; [72°C for 5 min] x 1 cycle; [Hold at 4°C]. Colonies that showed the expected 150 bp insert were inoculated into overnight cultures in 5 ml LB with ampicillin added to 100 µg ml^{-1} and used for plasmid preparation using the Qiagen Miniprep Kit. Miniprep DNA from 29 clones was sequenced using the Dyenamic ET terminator sequencing kit.

DEPC treatment of water

RNase-Free water was generated by incubating 1000 ml MilliQ-grade H_2O with 1 ml diethylpyrocarbonate (DEPC) overnight at room temp or 37°C. DEPC destroys all RNAse activity. The water was subsequently autoclaved for 20 min to inactivate the DEPC.

3' RACE to obtain further cDNA sequence

Rapid Amplification of cDNA Ends or RACE is a means to obtain full length cDNA sequence without the necessity to construct and screen a cDNA library. mRNA is prepared from an appropriate tissue likely to express the target gene(s) and reverse

transcribed, such that specific primers are added at both 5' and 3' ends. Amplification then proceeds with the 5' or 3' added primers combined with internal gene specific primers.

RNA was isolated from 8 day old *P. ornatus* larvae which were collected fresh and snap frozen in liquid nitrogen. 50-100 mg of each larval sample were ground under liquid nitrogen and RNA was isolated using Trizol LS Reagent according to the manufacturer's instructions (Life Technologies #10296). RNA pellets were resuspended in 50-100 µl DEPC-treated H_2O. The RNA concentrations were determined by OD_{260} and the integrity was analysed by formaldehyde agarose gel electrophoresis.

Reverse Transcription (RT) reactions were performed using 1 µg template RNA and either Omniscript RT (Qiagen #205111) or Superscript II reverse transcriptase (Life Technologies #18064-014) according to the manufacturer's instructions, primed with the UNI-dT primer (Table 2.1). To carry out 5' and 3' RACE, gene specific primers were designed using the 81 bp fragments of sequence that had been obtained from analysis of the cloned DBD regions. PCR was then carried out as follows: one of the gene-specific nuclear hormone receptor primers (Porn056F, Porn060F, Porn062F or Porn063F, Table 2.1) was used as the forward primer and the UNI-Primer, which binds to the 5'-extension of the UNI-dT primer, was used as the reverse primer. All primers were used at a final concentration of 0.2 µM in 50 µl reactions with 200 µM dNTPs, 1.6 U Taq DNA Polymerase in supplied 1 x PCR Buffer and 1 x Q-Solution (Qiagen), 1.6 U Pfx DNA Polymerase (Invitrogen), 100 ng cDNA. The PCR cycling parameters were set according to Qiagen's Long PCR Protocol: [94°C for 1 min] x 1 cycle; [94°C for 10 s; 51°C for 1 min; 68°C for 4 min 30 s] x 10 cycles; followed by an additional 10 s for each extension after the first 10 cycles for a further 20 cycles, [68°C for 10 min] x 1 cycle. 3-4 kb products were obtained in all reactions. These were gel purified, cloned into pGemTEasy and the resultant minipreps sequenced as described above.

5' RACE and suppression step-out PCR for the EcR homolog

To carry out 5' RACE, the suppression PCR technique of Matz et al. was used (Matz et al., 1999). This uses a 3' poly dT oligonucleotide with an additional primer sequence (3' PstI-adapter-oligo-dT, Table 2.1) to prime reverse transcription, and a template switching oligo (TS-NotI, Table 2.1). This latter oligo contains a run of 3 Gs at the 3'end, which is complementary to the 3-5 additional C residues that MMLV reverse transcriptase typically adds at the 5' end of a template. Hence the template switching oligo anneals, and the RT enzyme proceeds to synthesise a complete copy of the original cDNA, with a specific primer added at the 5' end. This process preferentially selects for full length transcripts, as the C residues are preferentially

added only where the cDNA reaches the extreme 5' end of an mRNA template. The defined sequences that have been incorporated at both 5' and 3' ends are then used as templates in subsequent PCR reactions, along with gene specific primers, to amplify the 5' or 3' ends of specific gene targets. The primers used here have been modified slightly from Matz (Matz et al., 1999). A 20 µl reverse transcription reaction was set up using 2.5 µg *P. ornatus* total RNA isolated from a pool of 6-12 day old larvae, 0.5 µM 3'PstI-Adaptor Oligo-d(T) Primer, 0.5 µM TS-NotI-Oligo (Table 2.1), 1 mM each dNTP (Invitrogen), 1 µl PowerScript® RT in supplied 1 x First-Strand Buffer (Clontech) and 2 mM DTT, (Clontech #8460-1). The RT reaction was set up according to the manufacturer's instructions for PowerScript® RT.

Subsequent PCR amplification used the suppression PCR technique described by Matz et al. (Matz et al., 1999). This reduces background amplification resulting from any cDNA products that inadvertently contain the Template-Switching (TS) oligo at both 5'- and 3'- ends of the cDNA molecule, due to the TS oligo also annealing to internal C-rich regions and forming a 3'- priming template for the RT reaction, resulting in the TS oligo at both ends of the cDNA. Prior to use in the RACE reactions, the cDNA was diluted 1 in 10 in nuclease-free water giving a final concentration of ~ 12.5 ng μl^{-1}. The 5' RACE reaction for isolation of the ecdysone receptor cDNA contained the following: 2.5 µl cDNA @ ~12.5 ng μl^{-1} (~31 ng), 0.2 mM each dNTP (Invitrogen), 1U Platinum® Taq DNA Polymerase with supplied 1 x HiFiPCR Buffer and 2 mM $MgSO_4$ (Invitrogen #11304-102), 0.15 µM EcR-long-REV, 0.02 µM Heel-Carrier-NotI, 0.15 µM Heel-Specific primers (Table 2.1). The Heel-carrier-NotI primer is included at low concentration and serves only to incorporate a 22 bp "heel" sequence 5' of the template-switching primer sequence. The majority of the PCR reactions use the Heel-specific primer and the EcR gene specific primer, which are present at higher concentrations. This technique serves to reduce background because there is now a 52 bp long added primer at the 5' end of all cDNAs, and also at the 3' end in cases where there was internal annealing of the TS-switching primer. Products that contain this sequence at both ends will therefore form a hairpin loop structure during the annealing phase of the PCR, and as this is thermodynamically favourable due to the long series of hydrogen bonds, the hairpin loop structures will be unavailable for further PCR. Thus the geometric nature of the PCR reaction can only proceed in cases where there is a heel primer sequence at one end and a gene specific sequence at the other. A touch-down PCR cycling program was used: [94°C for 2 min] x 1 cycle; [94°C for 10 s, 72°C for 30 s, 68°C 3 min 30 s] x 5 cycles; [94°C for 10 s, 70°C for 30 s, 68°C 3 min 30 s] x 5 cycles; [94°C for 10 s, 60°C for 30 s, 68°C 3 min 30 s] x 30 cycles with the extension time increasing by 10 s every cycle for the last 30 cycles; [68°C for 10min] x 1 cycle; [4°C HOLD].

3' RACE and suppression step-out PCR for the EcR homolog

The 3' RACE contained the same reagents as the 5' RACE, except that primers were 0.15 µM EcR-long-FWD, 0.02 µM 3'-PstI-StepOut, 0.15 µM 3'-StepOut-Specific (Table 2.1). The 3' PstI-StepOut primer acts in the same manner as the heel-carrier-NotI primer, to incorporate a long additional sequence in this case at the 3' end of the cDNA, which will form a hairpin loop in cases where there has also been internal priming of the 3' PstI-adapter-oligo-dT primer at A-rich regions. PCR parameters were as for the 5' RACE reaction.

Semi-nested PCR for the EcR homolog

Following the 5' and 3' primary PCRs, products were excised from an agarose gel and subjected to a second round of semi-nested PCR i.e. using the same heel / stepout primer, but a nested EcR primer. Both 5'-RACE and 3'-RACE semi-nested (suppression) 50 µl PCR reactions each contained 2.5 µl of gel-purified RACE fragments (undiluted), 1 U HotStart Taq DNA Polymerase with supplied 1 x PCR Buffer (Qiagen #203205), 200 µM each dNTP (Invitrogen #10297-018). In addition, the semi-nested 5'-RACE contained 0.15 µM EcR-Nested-REV and 0.15 µM Heel-Specific primers, while the semi-nested 3'-RACE contained: 0.15 µM EcR-Nested-FWD and 0.15 µM 3'-StepOut-Specific primers (Table 2.1). PCR reactions were run on a touch-down program as follows: [94°C for 15 min] x 1 cycle; [94°C for 10 s, 72°C 3 min 30 s] x 5 cycles; [94°C for 10 s, 70°C for 3 min 30 s] x 5 cycles; [94°C for 10 s, 68°C for 15 s, 68°C for 3 min 30 s] x 4 cycles with a decrease in annealing temp of 2°C per cycle (68°C →60°C); [94°C for 10 s, 58°C for 15 s, 68°C for 3 min 30 s] x 14 cycles; [68°C for 10 min] x 1 cycle; [4°C, HOLD]. Products were again separated by agarose gel electrophoresis, gel purified, and the resulting fragments cloned into pGemTEasy as described above. A number of independent clones deriving from each gel-purified fragment were sequenced with the Dyenamic ET Terminator kit according to the manufacturer's instructions (Amersham).

5' RACE isolation of other candidate nuclear hormone receptor genes:

Attempts were also made to isolate 5' RACE fragments for Hr4, Hr3, Hr78 and USP genes using the same protocols as described for the EcR gene, except that a different oligo dT primer was used (Oligo-dT-Kpn) for the RT reaction and the following gene specific primers were used in the subsequent PCRs: Hr3 used PoHr3R1 followed by PoHr3R2 for the nested PCR; Hr4 used Hr4R1 followed by Hr4R2 for the nested PCR; Hr78 used Hr78R1 followed by Hr78R2 for the nested PCR; and Usp used Usp-R1 followed by Usp-R2 for the nested PCR.

Sequence analysis

Public databases were searched using the programs BlastX, BlastN and BlastP (Altschul et al., 1990; Altschul et al., 1997). BlastX searches a nucleotide sequence translated in all six frames against protein sequence databases; BlastN searches the nucleotide databases directly with a nucleotide query sequence; BlastP searches the protein databases. These programs and databases were accessed using Biomanager provided by the Australian National Genome Information Service (ANGIS – www.angis.org.au).

Results

Identification of nuclear receptor homologs

Twenty-nine different clones obtained from PCR using the RR1 and RR2 degenerate primers were sequenced, revealing eight unique gene sequences, four of which aligned to known nuclear receptor proteins when analysed using BlastX (nucleotide sequence translated in all six frames against a non-redundant protein sequence database), JS056, JS060, JS062, JS063. Only one of these four sequences (JS056) showed any significant alignment with known DNA sequences when analysed using BlastN (nucleotide sequence against a non-redundant nucleotide sequence database). The remaining four clone sequences (JS054, JS055, JS092, JS096) did not show any alignment with any protein or nucleotide sequences in the database, and also did not show any characteristics of nuclear hormone receptors. These four sequences were therefore not studied further. Table 2.2 presents the nucleotide sequence of the four isolates that aligned with known nuclear receptors and Table 2.3 indicates the results of database searches with these four clones, and the alignments of the predicted protein sequences with the corresponding *Drosophila* receptors.

None of these clones showed homology to the ecdysone receptor. Clearly, analysis of this receptor was integral to any study of the role of ecdysone in regulating molting and metamorphosis. Fortunately, use of the degenerate primers on a related Panulirid species, *Panulirus cygnus*, did isolate a sequence with homology to the ecdysone receptor. This *P. cygnus* sequence was subsequently used to design primers for RACE isolation of the *P. ornatus* ecdysone receptor (see Table 2.2 for details of DNA Binding Domain sequences of the *P. cygnus* DBD).

Isolation of 5' and 3' RACE fragments

5' and 3' suppression step-out PCR was successful in isolating several 1.4 kb 5' RACE clones for EcR and a 1.2 kb 3' RACE clone for EcR. The overlapping sequence between the two clones, which lies in the DNA binding domain, was confirmed by

PCR and sequence analysis of genomic DNA, as the primers used in isolating the *P. ornatus* gene had in fact been designed from *P. cygnus* sequence. The complete deduced sequence of the *P. ornatus* EcR gene is shown in Figure 2.1.

3' RACE products were also isolated for Usp, Hr3, Hr4 but not Hr78 (Figs 2.2-2.4). Conversely, a 5' RACE product was isolated for Hr78, but not for Hr3, Hr4 or Usp (Fig 2.5).

The sequences that were obtained were translated into conceptual protein sequences, as illustrated in Figures 2.2-2.5, and these protein sequences were used to search protein databases. As considerably more protein sequence than the DNA binding domain was being used for searching, this search identified a different set of homologs. Alignments with the proteins identified in these searches are presented in Figures 2.6-2.10.

Discussion

When the degenerate RR1 and RR2 primers were used on the ascidian *Herdmania*, a total of eight different nuclear hormone receptors were identified (Devine et al., 2002). In the present work, one round of PCR and screening by sequencing gave rise to four independent putative nuclear hormone receptor clones from *P. ornatus*. As the goal of the present work was to isolate a set of nuclear hormone receptor candidates for further gene expression analysis, rather than to undertake an evolutionary survey of all nuclear receptor genes in *P. ornatus*, no further effort was made to isolate additional genes using these primers.

However, as a candidate ecdysone receptor was not among these four genes isolated, further work was carried out to identify the *P. ornatus* ecdysone receptor. Fortuitously, an ecdysone receptor candidate gene had been identified from the related species *Panulirus cygnus* in an independent project using the RR1 and RR2 primers (Nick Wade, University of Queensland, personal communication). This *P. cygnus* sequence was then used to design primers (Table 2.1) that were successfully used to isolate the *P. ornatus* ecdysone receptor homolog.

The DNA binding domain DNA sequences obtained were used to search protein databases. Table 2.3 lists the database sequences with the greatest homology to each DNA binding domain region identified. It also presents the tentative identification of the nuclear hormone receptor, and the DNA binding domain alignments to the corresponding *Drosophila* genes. The alignments to the *Drosophila* genes are presented as in *Drosophila* the complete set of nuclear hormone receptor genes has been identified, and, in addition, more is known about the function of each nuclear hormone receptor in *Drosophila* than in any other species (Maglich et

al., 2001; The Interactive Fly, 2005). Thus most of the interpretation in this and the subsequent chapter on the possible function of these nuclear hormone receptor genes is based on our knowledge of *Drosophila*.

The gene presented in Figures 2.2 and 2.6 shows extremely strong homology with other ecdysone receptor genes. The greatest homology is with the ecdysone receptor from the fiddler crab, *Uca pugilator* (Chung et al., 1998). The ecdysone receptor is probably the most studied of all receptors in *Drosophila*. It is known to exist in three isoforms which differ only in the N-terminal amino acid sequence. These isoforms, which result from differential splicing of the same gene, appear to be differentially expressed in different tissues, leading to the presumption that they play subtly different regulatory roles (Talbot et al., 1993; Mouillet et al., 2001). The ecdysone receptor is induced when the ecdysone titre begins to rise during late intermolt, and appears to be the key trigger for the subsequent cascade of gene expression. It acts as a heterodimer with the nuclear hormone receptor ultraspiricle, a member of the vertebrate retinoid X receptor family. The EcR-Usp heterodimer, when bound to ligand, is an active transcriptional factor activating expression of several genes, including other nuclear hormone receptor genes. There is also an autofeedback loop whereby the EcR-Usp complex actually represses expression of the EcR gene when ecdysone titres become too high (Thummel, 1996; Riddiford et al., 2003). We were also successful in identifying the ultraspiricle gene in *P. ornatus*, as illustrated in Figures 2.6 and 2.10.

Hormone receptor 3 (also named hormone receptor 46) in *Drosophila* is a member of the early-late gene class (Koelle et al., 1992), being induced rapidly in response to ecdysone. This induction, however, requires protein synthesis, apparently through the activity of the EcR-USP heterodimer i.e. DHr3 is induced by EcR-Usp activity (Riddiford et al., 2003). DHr3 acts to repress the activity of the ecdysone receptor, apparently by a direct interaction between the two receptors rather than by acting as a transcriptional repressor (White et al., 1997). This repression of the ecdysone receptor also leads to loss of activity of early genes that are activated by the EcR-Usp complex. DHr3 also serves to induce expression of other genes involved later in the *Drosophila* larval-puparium transition. In *Drosophila* it is transiently expressed during embryonic development, it is again transiently and weakly induced during the first and second larval phases, but its primary role appears to be in puparium formation and metamorphosis (Koelle et al., 1992; Sullivan and Thummel, 2003). DHr3 is an orphan receptor, i.e. its ligand is not known.

The receptor termed *P. ornatus* Hr4 is identified as such due to the overall homology of the deduced protein sequence, although the homology search of the DNA binding domain indicated that it also shows homology to the βFTZ-F1 receptor. However, this may in fact be a result of incorrect identification of the *Artemia* gene, for which

only a short stretch of sequence (109 bp) is available, and for which identification was made prior to the discovery of the *Drosophila* DHr4 gene. At present little data is available on the function of the DHr4 gene, but it is believed to act as a transcriptional repressor regulating genes at puparium formation in *Drosophila*. Analysis of patterns of transcriptional activity reveal that it is expressed in mid-embryonic development, and also during the later stages of the second and third instar molts in *Drosophila*, with expression continuing after DHr3 expression ceases (Sullivan and Thummel, 2003).

The final receptor that was identified in this work was a homolog of the DHr78 receptor. This receptor is expressed at a low level throughout the second larval instar in *Drosophila*, and only induced at higher levels at the onset of puparium formation (Sullivan and Thummel, 2003). This is consistent with its apparent role as a critical regulator at the onset of metamorphosis in *Drosophila* (Fisk and Thummel, 1998).

Figure 2.2: *P.ornatus* Ecdysone Receptor, full cDNA

FEATURE	DNA LOCATION	PROTEIN LOCATION
5'-UTR	1-678	
Start Codon (in red)	678-680	1 aa
Stop Codon (in red)	1989-1991	437 aa
3'-UTR	1992-2153	
A/B Domain: Ligand independent Domain[a]	678-1148	1-157 aa
C Domain: DNA Binding Domain[a]	1149-1349	158-224 aa
D Domain: Hinge Region[a]	1350-1646	225-323 aa
E Domain: Ligand Binding Domain[a]	1647-1788	324-437 aa

[a]Based on homology to *Uca pugilator* (Chung et al., 1998)

```
   1  TCTCCCATATGTCGACCTGCAGGCGGCCGCGAATTCACTAGTGATTGTAATACGACTCAC
  61  TATAGGGCAAGCGGCCGTATCAACGCAGAGTACGCGGGCTGGTTCCCGTTACCGGGTYCC
 121  TGTACAGGCATGTCACGACTGTAGCCGCAAACACAAAAAATGGAGTAACGGCATTCCCGT
 181  GCACGCTGCTGCTGCTGCTGCCAGCACGCCCTCCGCCTCCCTCCACTCACACCCCTC
 241  CAGATCCACATCACTTGCTCCCATTTCGATTATTTTTCTGTTCCTGACTCTTCGCTGAAG
 301  GAAAGCAGAGTGAAAGGAAAAGTTGGCGACAACCTGACCCGGTAACCGGCGTGACCTGAC
 361  CCAAATATTACGCTAACCCATTCACCCAGAGCTAGACTGGTACACACTTGACCCAGAGGA
 421  GCCATTCACAGCGAGAGTGGGAGTGATGCCAGCCTAGCTAGCCCCGCCCGCCGCACCCCT
 481  GAAGATGGCACTCAGAGGACTTAAAGTCGGTCTTTATGACACAAGCAGACGATGTGCTTG
 541  CATGTGACGACCTCGCCTTCTATCGCACATTGACCCACGCCAAGACTTATCCCTCCCGGG
 601  TAGTTATCAAGCTGTGCGCGGAGATTATCGAATTTGGAACACGGGGCCGATAGCGACACT
661           CACTGAAAGTTCCACGCATGGCCAAAGTGCTGGCGACGGCACGTGCCGCTGGA
1                                 M  A  K  V  L  A  T  A  R  A  A  G
714   ATGTTCGTCTTGGGCTCTGGTGTGGCTACTCTCAACCTCTCAGCTATGGGGGACGAAAGC
13    M  F  V  L  G  S  G  V  A  T  L  N  L  S  A  M  G  D  E  S
774   TGCTCCGAGGTGTCTAGCTCCTCACCGCTCACCAGCCCTGGGGCTCTCTCGCCGCCCGCC
33    C  S  E  V  S  S  S  S  P  L  T  S  P  G  A  L  S  P  P  A
834   CTCGTAAGCGTCGGCGTCAGCGTTGGAATGAGTCCGCCCACCTCTCTGGCCTCCTCGGAC
53    L  V  S  V  G  V  S  V  G  M  S  P  P  T  S  L  A  S  S  D
894   ATCGGCGAGGTCGACTTAGATTTCTGGGACCTGGACCTCAACTCGCACAGTCCTCCCCAT
73    I  G  E  V  D  L  D  F  W  D  L  D  L  N  S  H  S  P  P  H
954   GGCATGGCGGCGGCGGCCTCCACCACCACCTCCCTCCTGGTCAACCCAAGGAGCCTCACA
93    G  M  A  A  A  A  S  T  T  T  S  L  L  V  N  P  R  S  L  T
1014  TCGCTCTCTGACGCCTCCTCCATCTCAGGTCGAGATGACATGTCCCCGCCCTCCTCCCTC
113   S  L  S  D  A  S  S  I  S  G  R  D  D  M  S  P  P  S  S  L
1074  ATTAGTTTGAGCACGGATTCCTATGGCGACCTCAAAAAGAAAAAAGGCCCAATACCCCGC
133   I  S  L  S  T  D  S  Y  G  D  L  K  K  K  K  G  P  I  P  R
1134  CAACAGGAGGAATTGTGCCTCGTGTGTGGCGACAGGGCGTCAGGCTACCATTATAATGCG
153   Q  Q  E  E  L  C  L  V  C  G  D  R  A  S  G  Y  H  Y  N  A
1194  CTTACCTGTGAGGGATGCAAAGGTTTCTTTCGGAGATCCATCACCAAGAATGCAGTCTAC
173   L  T  C  E  G  C  K  G  F  F  R  R  S  I  T  K  N  A  V  Y
1254  CAATGTAAATACGGCAACAACTGTGAAATGGACATGTACATGAGACGCAAATGTCAAGAA
193   Q  C  K  Y  G  N  N  C  E  M  D  M  Y  M  R  R  K  C  Q  E
1314  TGTCGCCTAAAGAAATGTCTTAGTGTTGGCATGCGACCGGAATGTGTGGTGCCTGAGTCA
213   C  R  L  K  K  C  L  S  V  G  M  R  P  E  C  V  V  P  E  S
1374  CAATGTCAGGTGAAACGTGAACAAAAGAAAGCTCGGGATAAAGATAAAAAGGATTATCCT
233   Q  C  Q  V  K  R  E  Q  K  K  A  R  D  K  D  K  K  D  Y  P
1434  AGCATGGGTTCCCCAATAGCAGAGGATAATAAGGGCATCCATCTTAGTCCATGTAAACCC
253   S  M  G  S  P  I  A  E  D  N  K  G  I  H  L  S  P  C  K  P
1494  AAAGGACCATCAACTGCATCTGGTATGCAGTTCAAAAATCTTGTGGGAAGCAGTAACATC
273   K  G  P  S  T  A  S  G  M  Q  F  K  N  L  V  G  S  S  N  I
1554  ACTCTAAGTCCTTTGCCTACAACACCAAGGGTCAATATTAAGCCTTTGACTAAGGAGCAA
293   T  L  S  P  L  P  T  T  P  R  V  N  I  K  P  L  T  K  E  Q
1614  GAAGAACTGATCAATACTCTAGTCTACTATCAAGAGGAGTTTGAACAGCCTTCAGAGGAA
313   E  E  L  I  N  T  L  V  Y  Y  Q  E  E  F  E  Q  P  S  E  E
1674  GACCTAAAGAAAATCAAATTTACCTTCGATGGTGAAGATACAAGTGGCATGAGATTCAGG
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333   D  L  K  K  I  K  F  T  F  D  G  E  D  T  S  G  M  R  F  R
1734  CACATAACCGAGATGACGATCCTCACAGTTCAGCTCATAGTGGAATTCTCCAAGCAACTA
353   H  I  T  E  M  T  I  L  T  V  Q  L  I  V  E  F  S  K  Q  L
1794  CCAGGTTTCGGCACTCTTCAACGAGAAGACCAGATTACTCTGCTCAAGGTACACAGTCGA
373   P  G  F  G  T  L  Q  R  E  D  Q  I  T  L  L  K  V  H  S  R
1854  GCACATGACAATGGTGGATTGACGAGCAAATTTCTTACAGGAAATATATTTTATGTCTGT
393   A  H  D  N  G  G  L  T  S  K  F  L  T  G  N  I  F  Y  V  C
1914  TGGGAGACTTCTTGGTTGGAAGAATCTGTGAGCTCTCAGAAATCAAGAATTTCCAGTTAT
413   W  E  T  S  W  L  E  E  S  V  S  S  Q  K  S  R  I  S  S  Y
1974  TGGAATGAAAGAAATTAGTTTTAAATGAACCCAGRAATCTCCATTCCAGGTGAACAAYGR
433   W  N  E  R  N
2034  AACTTTTCGGGACGGCAGCATATTTATGGGGGAAAGTCATTCATAATCTGATGATGGTGG
2094  TCATTGTGACCATGTAACTCCTCTGTACTGCTGTTTCAGTAATAAAAACCTTCATGATMT
2154  AAAAAAAAAAAAAAAAAAAAAAAAAA
```

Figure 2.3: *P.ornatus* Hr3, partial cDNA

FEATURE	DNA LOCATION	PROTEIN LOCATION
5'-UTR	incomplete	
Start Codon	incomplete	
Stop Codon (in red)	1231-1233	
3'-UTR	1234-3182	
A/B Domain: Ligand independent Domain[a]	incomplete	
C Domain: DNA Binding Domain[a]	Partial domain 1-108	1-36 aa
D Domain: Hinge Region[a]	109-498	37-166 aa
E Domain: Ligand Binding Domain[a]	499-1230	167-399
End translated region	1230	410 aa

[a]Based on homology to *Drosophila melanogastes* (Koelle et al., 1992)

```
1      TCGGTGGTGAACTACCAGTGCCCCCGCCAGAAGAACTGCGTCGTGGACCGCGTCAACCGA
1      S  V  V  N  Y  Q  C  P  R  Q  K  N  C  V  V  D  R  V  N  R
61     AACCGCTGCCAGTACTGCCGCCTTCAGAAGTGTCTCGCCTTAGGCATGTCCAGGGACGCG
21     N  R  C  Q  Y  C  R  L  Q  K  C  L  A  L  G  M  S  R  D  A
121    GTGAAGTTCGGGCGCATGAGCAAGAAACAGCGGGAAAAGGTTGAGGATGAGGTGCGGTAC
41     V  K  F  G  R  M  S  K  K  Q  R  E  K  V  E  D  E  V  R  Y
181    CACAAGGCTGCTCAGATGGTGGGCATGACGGGTCAGGAGACCTCGCCGGACTCCTCCATG
61     H  K  A  A  Q  M  V  G  M  T  G  Q  E  T  S  P  D  S  S  M
241    TACGAACCACCAACACCCACCTCCTCGGATATATACACCCCTACGTACTATGGCTCAGAG
81     Y  E  P  P  T  P  T  S  S  D  I  Y  T  P  T  Y  Y  G  S  E
301    ATTGCCTCTTTCACCTCCCCAGGATATACNTACACACCTCAAACCACCACACCCACCATA
101    I  A  S  F  T  S  P  G  Y  T  Y  T  P  Q  T  T  T  P  T  I
361    CCTTTCGAAATCAGCCCAGAGTATGCTGTTGATTCAACAACATTTGATCATCGTCAGACG
121    P  F  E  I  S  P  E  Y  A  V  D  S  T  T  F  D  H  R  Q  T
421    CCGTTAGATCCACTGCCAGACTCTGGAGCCATGTCACCAGTTGTGTCTTCTGATCCCACG
141    P  L  D  P  L  P  D  S  G  A  M  S  P  V  V  S  S  D  P  T
481    CAACTTGATGAGATTGTTGCACGTTGGGTAGCTGATGCTCATTTAAGAACGTGTCTATAC
161    Q  L  D  E  I  V  A  R  W  V  A  D  A  H  L  R  T  C  L  Y
541    TCAAGTGAACACATAGCAGATGTTTTAAGAAAACAAGCTCTTGACATTTCCAAAGTCACC
181    S  S  E  H  I  A  D  V  L  R  K  Q  A  L  D  I  S  K  V  T
601    TACTACAAAAATATGGCACATGAAGAACTTTGGTTTGATTGTGCCCAAAAGTTGACATTA
201    Y  Y  K  N  M  A  H  E  E  L  W  F  D  C  A  Q  K  L  T  L
661    GTAATCCAGCAAATCATTGAATTTGCAAAGGCTGTCCCAGGCTTCAGGAAATTCTCTCAA
221    V  I  Q  Q  I  I  E  F  A  K  A  V  P  G  F  R  K  F  S  Q
721    GATGATCAGATTGTTCTTCTGAAAGCAGGGAGTTTNGAGTTAGCCGTGCTGCGGATGACC
241    D  D  Q  I  V  L  L  K  A  G  S  X  E  L  A  V  L  R  M  T
781    CGGTACTACGATGTGAATCAGAATTGCGTGGTGTATGGAGATNCTCTTCTACCAATGGAG
261    R  Y  Y  D  V  N  Q  N  C  V  V  Y  G  D  X  L  L  P  M  E
841    GCCTTTCTTACCACAGAGACAGTTGAAATGAAACTAGTTAACAACGTCTTTGAATTNGCC
281    A  F  L  T  T  E  T  V  E  M  K  L  V  N  N  V  F  E  X  A
901    AAAACTATCGCTGAACTTAAATTGACAGACACAGAGTTAGGCTTATACTCTGCTCTTGTG
301    K  T  I  A  E  L  K  L  T  D  T  E  L  G  L  Y  S  A  L  V
961    CTGTTACAAGCAGATCGGCCAGGTCTCCGTGGTACTGAGGAGATAGCAAAACTTAATGAA
321    L  L  Q  A  D  R  P  G  L  R  G  T  E  E  I  A  K  L  N  E
1021   GCTGTAGGACGATCATTGTGTCTGGAGTTGGACAAGACACACAGGTATCCAGTCAAAGGG
341    A  V  G  R  S  L  C  L  E  L  D  K  T  H  R  Y  P  V  K  G
1081   GATGTTAGTGTTTACGCCTTCCTTCTGGCTAAGANGCCTGCCCTCAGGGAGCTGAGTATG
361    D  V  S  V  Y  A  F  L  L  A  K  X  P  A  L  R  E  L  S  M
1141   CTACATCAGGAAGCACTGAGCAAGTTCAAGCGGGCTGCACCACACCTCCAGTTCTCTGAT
381    L  H  Q  E  A  L  S  K  F  K  R  A  A  P  H  L  Q  F  S  D
1201   CTCCATAAGGAAATCTTCAATGTAGATTCCTGAGGTGCCTATACAAGATTGCCTGGTTAT
401    L  H  K  E  I  F  N  V  D  S
1261   GATGATAAGTGCAGGGAGCTAYCTGCTCCTCTGGCTCTGTTGCTCCATCACATCCTTTTT
1321   CTACAAGAGAATGAACTGTCTGTACATTACATTTGTGATTTGTTTGTTAGTGKTACTTGT
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1381  TAGTGCAGAGGACTATGGACTCTTTAAAATACCATTATAGATGTGTTGTGAGTTTTACAG
1441  AGTCTAACAAGTGTTCTCTTTAGAGGAGTGGGGTGCTCAAAAACTCCAGTCTCAGATAAA
1501  ATATATTCATATGAAGCCTCCAACAGCCCTTTGTAGATTATAATGTTGATAATGATGCCT
1561  TACAATCATAGTCCAGCAGAGTTGGCCGTTGGCTTCTTAGCTGAGTGTTAGTGAGTCCCC
1621  ACTAAGATAGAGATAGTGAGTCTCTCTTGAAATGTCCAAGGTTGATGATACTATACAGTT
1681  TATTTTTTAAGGAGTCGTATAAACACCCTTGACAATGATCCTTATCTTTGTAAATTGAAA
1741  ATATTGTATACTTAAAAAATATTTAATTACATTATTACATAAATTGTTGTATACTATAAA
1801  TCTTTATTGCCTATAGAAAAAGTTATCATTCTCATACTTATCATTGTCAACAAAAGGTAT
1861  AAAGAAAATTACCATTGATRTCATATCATCATCTCCATCATTATGTTTATAAGTTTTTTA
1921  TAAGTTTAGCTAGCCTATAATTTTGTATAGTTTAATTTATTTTTGTTCAGCTTTTCCCAG
1981  CAAACACATATTGAAATCATATATTGTGCCAGTTGATACTAAYCTGCAAATGATGGGGTC
2041  ATGATTGGCCGTGCTTGCTCGAGTCCTGGCAAAAAGCAAATAACTGAAGGAGGGATGGAA
2101  CAGTGGTACAAAGAGGATGAGAGTCCTGTAGTTGGTCACTAGTACAAGCAGAAAGTTGTA
2161  GAAAACTGCATTTCCACTGGATTTATGTAACAGTGCTTGAGTTTTGAAGAGACTTTAAAA
2221  TGAATTAAAGACTCAGTTGACAAATATATTGACTAAATTGGAATGAAAATCTGTGTTATG
2281  CTCAAGTGGTGAATATGAAATCCAATGTATGATGTACTGAGCGTCCCAGACTCTCARCAT
2341  GGCCTCAGACTCTCTTGGAAGCGCTGTGTTGCGTCCTTTGCCCATTCCAGCCGTGCTAGA
2401  GTACAAATAGGTTAGAGCCGAGGCCATCCTGCCCTGTCTTGTCTCCTGCAGTCCTGCCCT
2461  GGACCCACTGGCCCGTGACCGTGTCCTCTCAAGGTCTTTGGTCTAAAGGTTCCTAAAGCC
2521  ATCTCAGTCAATCCTTGTCTCAATGACCGACATGTGTTCAGGCCTGTGAGCCACCTTTCT
2581  GTTGGCCATTATATTTTGTTTCTCTGGATGGAATCTTWAATTTAAGCTAACATGGTTGGC
2641  TTTAGGTCCATTTGACTGCAGACACAAAGTGTACAGAAAGACACAGCTTCTATTGTAGAT
2701  GATAAGGGGCGGCAGTTGCTGGAGGATCTCTAGTGCTTAGTATCTAGAGTCTAGTGTGCT
2761  TTGGAGAGTTTGGGGCTAGCCTAGTCCTCCCCCAGCCCACCCACAGAAGAGTTACCTATT
2821  TGTGGGTATTGCTGTGGGGTTAGGGAATTGGTCCAGCCCCACTCTGCNGGCATCAGGTCA
2881  CAGCCCTACTGTACAAACTCTCCAGCCGCCGCCATGATGCTTGCTGCCTGCTGCCACCAA
2941  CAAGCTATTTATTTATTTGTGTGATGACCATCCCATTCTGTGCTGTGCATCACAAGATGG
3001  ACTACACAGCAAGCAGCAAGTTACAACTCTGTATATTTTATACAGCTCCTCCTATTGGTT
3061  ATTGTGCATTAAACAACTCTCCCTTTGGTCCTTCCTCTTTGGGGTGCGGGAAATAACCAG
3121  CTAGGACATCTTGTACTCACCACACATAAACACAGGGGTTACAGTAAACTGGTATTTTGG
3181  CT
```

Figure 2.4: *P.ornatus* Hr4, partial cDNA

This *P.ornatus* Hr4 sequence may not be correct at the start as a stop codon is located at 20-22 bp but the reading frame aligns with *T.molitor* from 23 bp onwards.

FEATURE	DNA LOCATION	PROTEIN LOCATION
5'-UTR	incomplete	
Start Codon	incomplete	
Stop Codon (in red)	1571-1573	
3'-UTR	1574-3783	
A/B Domain: Ligand independent Domain[a]	Partial domain 23-260	
C Domain: DNA Binding Domain[a]	260-466	80-148 aa
D Domain: Hinge Region[a]	467-955	149-311 aa
E Domain: Ligand Binding Domain[a]	956-1084	312-354 aa
End of translated region	1570	516 aa
Poly A tail	3784	

[a] Based on homology to *Tenebrio molitor* (Mouillet et al., 1997)

```
1                                                    GTTTATGCCTGAAAGCCTCTAA
23    AGCAGAATCCCGGCTAAAGAAGCAACGATATGTGTACGGCTAGCATTGGGTCAAGGCCAC
1     S  R  I  P  A  K  E  A  T  I  C  V  R  L  A  L  G  Q  G  H
83    GAATATAACGACAAGCTTCCAGGAGACCGAACCCAATTGCCTCAAACTCAACCAGATTCT
21    E  Y  N  D  K  L  P  G  D  R  T  Q  L  P  Q  T  Q  P  D  S
143   CCAGCAGCTCTAAACTTGTCGACAGTGGGAATGTGGGGTGCCCGCACTGCCAGTGCTGAC
41    P  A  A  L  N  L  S  T  V  G  M  W  G  A  R  T  A  S  A  D
203   AGTGTCGTCAGCCCTCCTACCCAACCTACACCTGTTGAAGACGAAGAAGAAGACCAGCCA
61    S  V  V  S  P  P  T  Q  P  T  P  V  E  D  E  E  E  D  Q  P
263   ATGATTTGTATGATCTGTGAGGACCGAGCCACTGGACTTCACTATGGTATCATCACATGT
81    M  I  C  M  I  C  E  D  R  A  T  G  L  H  Y  G  I  I  T  C
323   GAAGGATGCAAAGGCTTTTTCAAGCGAACAGTACAGAATACGCGGGTATACACATGTGTT
101   E  G  C  K  G  F  F  K  R  T  V  Q  N  T  R  V  Y  T  C  V
383   GCTGATGGAGATTGTGAAATCACAAAAGCACAGCGTAACCGTTGCCAGTATTGTCGATTT
121   A  D  G  D  C  E  I  T  K  A  Q  R  N  R  C  Q  Y  C  R  F
443   CAGAAGTGCCTACGTCAGGGAATGGTGCTTGCTGCTGTGAGAGAAGACCGGATGCCTGGT
141   Q  K  C  L  R  Q  G  M  V  L  A  A  V  R  E  D  R  M  P  G
503   GGTCGAAATTCAGGAGCAGTTTATAACCTTTACAAGGTAAAGTACAAAAAGAAGAACAAA
161   G  R  N  S  G  A  V  Y  N  L  Y  K  V  K  Y  K  K  K  N  K
563   AAGAATGGTCAAATAAAGCAAGGCGGTACTTCTCCAGAAAAGAAAATGAGTCTGGATCCC
181   K  N  G  Q  I  K  Q  G  G  T  S  P  E  K  K  M  S  L  D  P
623   CTAATGATGGCTGGAACAACACCCCTTACAAGCACATCGTTACCCATGCCCATCTCAAGC
201   L  M  M  A  G  T  T  P  L  T  S  T  S  L  P  M  P  I  S  S
683   CCTCTCACTTCTCCACCTGTTACTTCTCCCATCCCCAGTGGCTTAAATAGTGGACACATC
221   P  L  T  S  P  P  V  T  S  P  I  P  S  G  L  N  S  G  H  I
743   CTAAAAGCTGCTCTCACAAATCCATCTGAGGTGGCCCACTTCCGGCAAAGGTTGGACACG
241   L  K  A  A  L  T  N  P  S  E  V  A  H  F  R  Q  R  L  D  T
803   ACAGTGACTTCTTCAAAAGAGCGTTTCATGCCCTACCCTGTTGCCCAGGCCATGATTAGG
261   T  V  T  S  S  K  E  R  F  M  P  Y  P  V  A  Q  A  M  I  R
863   ATGCTAATAGAATGTGATGATTTTGAAGACATTGCCACATTGAAGAATCTAGATGATTTG
281   M  L  I  E  C  D  D  F  E  D  I  A  T  L  K  N  L  D  D  L
923   TTGGACCACAATAGTGATTTGTCAACCAAATTATGCCAGTTGGGAGACTCTATTTCCATA
301   L  D  H  N  S  D  L  S  T  K  L  C  Q  L  G  D  S  I  S  I
983   AAGCTAGTGCAGTGGACCAAACGCCTCCCCTTCTACCAAGAACTACCAGTGGAGGTTCAC
321   K  L  V  Q  W  T  K  R  L  P  F  Y  Q  E  L  P  V  E  V  H
1043  ACACGGTTACTTACACATAAGTGGCATGAGCTTCTGGTGCTCACTACTTCTGCTTATCAA
341   T  R  L  L  T  H  K  W  H  E  L  L  V  L  T  T  S  A  Y  Q
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```
1103  GCTATTTATGGCCTACAAAAGTTGGGTTCTAGATCTTCAGATGGTACTGAAGCAGAGTTC
361   A  I  Y  G  L  Q  K  L  G  S  R  S  S  D  G  T  E  A  E  F
1163  CATCAAGAGGTATCAAACAACTTGTGCACTTTGCAAACCTGCCTAAATTCCATGATGGGG
381   H  Q  E  V  S  N  N  L  C  T  L  Q  T  C  L  N  S  M  M  G
1223  CAACCTATCACTATGGACCAACTGCGGCACGAGGTTGGTGTAATGGTTGAGAAGATAACC
401   Q  P  I  T  M  D  Q  L  R  H  E  V  G  V  M  V  E  K  I  T
1283  CATGTCACGCTTGCGCTACGAAAGATAAAAATACAGATTGAGGAGTATGTCTGCCTTAAA
421   H  V  T  L  A  L  R  K  I  K  I  Q  I  E  E  Y  V  C  L  K
1343  GTCATCGCCATGTTAAATCAGTCACGAATCGGCCACAAGGAACTGGAAGTAATCCAGGAA
441   V  I  A  M  L  N  Q  S  R  I  G  H  K  E  L  E  V  I  Q  E
1403  CGATACATGGGCTGTCTTCGGTCGTTCTGTGAAACCCATTATCCTTCCCAACCCAACAGA
461   R  Y  M  G  C  L  R  S  F  C  E  T  H  Y  P  S  Q  P  N  R
1463  TACCAGGACCTGCTAGTCAGACTCCCAGATATCCAAGCAGCTGCAGCTAAACTGTTAGAA
481   Y  Q  D  L  L  V  R  L  P  D  I  Q  A  A  A  A  K  L  L  E
1523  ACAAAGATGCTGTATGTTCCCTTCCTGTTGAACTCCACCATCAACAGATAG
501   T  K  M  L  Y  V  P  F  L  L  N  S  T  I  N  R
1574                CAATAAAGAGATGGTTGGTTCTTAGAGCAAGTCAGCTGCAAGGCGTC
1621  TTGTAGTGAACCACGTGCCGTCCTATGTCTCTGCTGTAGCCAGCAGCAGTGGGGGTGCTG
1681  CARCCACTCTCATCATCATGCCTAGCAAGCCACTCACATCATGACTGACTCATTGAGTAG
1741  TCAGTCCATGAACGTTAACAATGTCTGCAAGAAGCATGATGTATATGAAGTGACAATCTA
1801  GCCAGAGCATTTATTTGGCTAAAGGAAATCAACAGCTGCTTTGCCCAGTGGTTGTGCAAC
1861  CAATTTACCTGTGATGGGATGTGTACCAGTTTCCGAGCAATTTAAATATAATATCTTGTG
1921  AASATTTTATAWAWAACTTCCTNCAGACAGTTTACCAAAGGCTTTCTTNAATAGACTGTN
1981  CATGGGTAAGTGTTAGGATTACANAAATCCTNAGACCCAATACAACAGATACTTAACGGN
2041  TGTGTGACTGACAAGTNGTGCAATTATTATTGGTNCAACTTCCCTTCCACTGCTATTTTT
2101  CACCACCAACTCTAAAACTTCCTACTAGTCGCTGAATGCTGAGTCTACTTAAAAGAAGAA
2161  TTCTAGTTTYATGGTGTTCTCAAGAGTACGACCGAGCACAAGACAATGATGGCAGTTTTT
2221  CATGGTAGCCGCAAGTGGGGTGTGGGGAGGCAGTATGCTGGTGCAGGAAAGTTGTTGGCT
2281  GGTAATGTGATCCTCAGCTCCCTCTAGCAGGGTCACATGACTGTGTTCTCCTGTGCTGTG
2341  TGAGGGAGTACTTACAATGGTACCTGCAAGGATGGGCTATCAAACTCGTTCACTACATGA
2401  ACAGTATTGTATGAACGAATCCACTCTTTTAGTATTACCAACAGTAACAAGGAAACAGTT
2461  CCTAGAAAGATCCTACTAACAGTTTTCACTGCACTGATCTCGAAAGTGCAAAGAGTTATT
2521  CTATGTCTTGTAAAACACATCACTTTTAAATGTTAAGGTTTATACAGATGATTTGTATCT
2581  CAGAGGCACAAGTCTCATTATTGTCCTGTGCTGCCATATGACAGTGTACCATTTATACCT
2641  TTATTAAGGATGGATAATTCTTAGAAATAATATATGGGAAAATCTTTTATTAAGAGTACA
2701  TTTTTGTAAATACACTTTTGCAGATGAACCTTTTGTTTGTACAGTTTTCGGTACTAGAAC
2761  TGGTGTATGTTGTGAAGGTGTGAGTGTGCAGTCTGTGCCTGCCAATGTAGGGCACAGGCG
2821  GGAGACAGTTTACAGAGTACTCATCAGAAATGGCATTTTTCCTTCACGTCTACTTTCTCT
2881  AATGTCTTACAAAATCAGATACATTTTTTTAGACAAACTTAAAAGGTGCAGTTGATACAA
2941  AGTGATTCTAAAATAACAAGTCTGAAATATTGATTTAAGAGTCTATTGTTGTAAGTTACT
3001  TTTAACCTATTCAGTCATTTTTCTTATTCATTTATTTATTTTATTTATTTATATAATCTT
3061  AGCTAAGACACTACATGTTTTCCTATGAATGTGGTGAAAACCTCCTAGGAAAGGGGTATA
3121  TCATTCTAAAGATACAGGATGACCCTGTGCTTTTCATGCCTAATTACTCCTGTGTAACAG
3181  CTTAGCACCCAGGACTGAACCTCAATGCTTGAGTTGTGAATGTTACAGTATATCGTGATT
3241  TTAAAGAAAATATTCATTAAAAATGAATGTACCTATCAGTTTGTACATTAATGATTTCCA
3301  CTAAACTTTAATCCATGATGAAGCATCTTTTCAGACGTATGAGTTTGCAATAGAGTATCA
3361  GTGTTGCAGTGTTGTCTTCAGACTGTTAAGTTACCAGTGGCTGCAGAGTAAATCTGATGT
3421  CAGATGACCTATGCTGCAATAGGGTGCAGCATCACTACCAATGTTTTTCATGCATTTTAC
3481  AAGACTGGAAATGGAAATTACTTGGGAAGTGATTGGTATTATGGGGTAGTGGCATTGCAG
3541  TGGCCAACACTAGTCTCTGATCTCATTCTTATCTTTAAATTTATTGTCTTAATGGCTGAA
3601  AATTTGAAGCTCAATACTATTTGAGGTGTGATAGTAATACTCTTATAAAATGAGTGGTAA
3661  TGGTGAGGCAGCAGCAGCCTAAGTCATAAGCAGCCATGAGTACACTGTAAGCTGACTCCT
3721  TAATGTAGTCAAGCAATCAAATTTGTTGCTTCTATATTTAATGAAATAAACAAGTGAACT
3781  TTGAAAAAAAAAAAAAAA
```

Figure 2.5: *P.ornatus* Hr78 homolog, partial cDNA

Two 5'-RACE clone sequences were isolated for Hr78. The clones vary in length and sequence up until ~65 bases upstream of the start codon from which point (indicated by the arrow) they are identical. This would possibly indicate there are multiple copies of this gene in the genome. The two clones are presented below.

FEATURE	DNA LOCATION	PROTEIN LOCATION
5'-UTR (long clone)	Possibly 1-457	
5'UTR point where clones are identical	458	
5'UTR (short clone alternate sequence)	*165*	
Start Codon (in red)	Possibly 523-525	1 aa
Stop Codon	incomplete	
3'-UTR	incomplete	
A/B Domain: Ligand independent Domain[a]	Possibly 523-774	1-84 aa
C Domain: DNA Binding Domain[a]	775-930	85-136aa
D Domain: Hinge Region[a]	incomplete	
E Domain: Ligand Binding Domain[a]	incomplete	
End of translated region	incomplete	
Poly A tail	incomplete	

[a]Based on homology to *Tenebrio molitor* (Mouillet et al., 1999)

```
   1                                                                AGAGG
   6  CATCGCGTCAGAGCTCTCCGGATGGATTTCTTGTTTGTGTTTTTTTGTACTACATGCTGA
  66  GTTTATTATATGGTCTTCATCGTAGGGATGTGCAATTGTGGTAGTGTTGTGGAATTGACG
 126  TGCCTTAGGAGAGGATAACAGTGATAAGTTGTTGGTTGTAGAGTGTTGATAAGAGAGGGC
 186  GGCAGGACATTCGACCTCTGTCTGACACTACGTACGGTGCCAACGCTTCGACACCCACTG
 246  CAATCTTGGCTTATTCTACCCCCCATGGCTTGCTACTCCGACCTTGAGGTGTGGTGACGG
 306  CGGAAGCTTGTGAACCTCAGGTCAAACCTCTTGGTCTTCACTAGTCATCCCCAGGGGCCG
 366  TGACACTCTCCCTCCGCCACCACCATCACCATCACCATCACCACCATCACCACCATCACC
                                          ↓
 426  ATCGCCAGCCGCAGCCGGGACACGCTACTAAGGTGATCTAGTGGACAGCACAAGTGCCCT
 486  ATCCAACCCCCTGTTAGTGACCAGTGCAGGTGGCTTAATGGTGACCAACCCTGCCCTCTT
                                          M  V  T  N  P  A  L  F
 546  CACCCACACCATGCTCAGTGCCGCCCCCACGCTCCTCCTCGGAAATTCCCCTGTCCTTGG
       T  H  T  M  L  S  A  A  P  T  L  L  L  G  N  S  P  V  L  G
 606  GAGTGACTGGGTGGGACGTCTAAAAGAACTGCAGGAGAGAGGAGGTCTGGCAGCAGCAGC
       S  D  W  V  G  R  L  K  E  L  Q  E  R  G  G  L  A  A  A  A
 666  AGCTGCTGCAGCTGCTGCCAGCTCTGGCAGTGACATTGACAGCAGAGACTTAGATCATTC
       A  A  A  A  A  A  S  S  G  S  D  I  D  S  R  D  L  D  H  S
 726  CCACCAGTACACATCCCGTAACACAGGCCAGCCTGTTACCATGGACCTGTGTGTCGTGTG
       H  Q  Y  T  S  R  N  T  G  Q  P  V  T  M  D  L  C  V  V  C
 786  CGGTGACCGAGCCTCAGGACGTCACTATGGAGCCATCAGTTGTGAGGGCTGTAAAGGGTT
       G  D  R  A  S  G  R  H  Y  G  A  I  S  C  E  G  C  K  G  F
 846  CTTCAAAAGGTCAATCCGTAAGCAGCTCGGCTACACTTGCCGGGGGAACAAGACGTGCGA
       F  K  R  S  I  R  K  Q  L  G  Y  T  C  R  G  N  K  T  C  E
 906  GGTCACTAAACACCACCGCAATAGG
       V  T  K  H  H  R  N  R

  Alternate sequence of short clone:
                                                          ATTTATTGATAAT
      GGTGGACTTCCTTCTTTTTCTTGTCGATCTCCAGCACCTAAAAAGCTATTAGGTTTTCGT
      TCTGATTGGACTGTTTTGTACAAATCCTTAGTATCGTTCTTACAGTCGCTGCGCATGCCC
      ACCGCCTTCCCCAGGAGACGATAGTAAAGGAA (sequences identical from here)
```

Figure 2.6: *P.ornatus* Ultra spiracle Homolog partial cDNA

Feature	DNA Location	Protein Location
5'-UTR	incomplete	
Start Codon	incomplete	
Stop Codon (in red)	917-919	
3'-UTR	920-1366	
A/B Domain: Ligand independent Domain[a]	incomplete	
C Domain: DNA Binding Domain[a]	Partial domain 1-115	1-38 aa
D Domain: Hinge Region[a]	116-220	39-73 aa
E Domain: Ligand Binding Domain[a]	221-916	74-305 aa

[a]Based on homology to *Uca pugilator* (Chung et al., 1998)

```
   1 CAAGCGAACAGTTCGTAAAGACCTCACATATGCATGTCGAGAAGATAAAT  50
   1  K  R  T  V  R  K  D  L  T  Y  A  C  R  E  D  K  S  17
  51 CGTGTACCATTGACAAGAGACAAAGAAATCGATGTCAGTATCGCCGATAC 100
  18   C  T  I  D  K  R  Q  R  N  R  C  Q  Y  R  R  Y   33
 101 CATGAGGTGTTTATCCATGGGCATGAAGAGAGAAGCGGGTCCAATGTAGG 150
  34 H  E  V  F  I  H  G  H  E  E  R  S  G  S  N  V  G  50
 151 GGGAGTAGAGGAAGAGCGTCAGCGTACAAGAGGAGACAAAGGTGATGGTG 200
  51  G  V  E  E  E  R  Q  R  T  R  G  D  K  G  D  G  D  67
 201 ACACAGAGTCATCATGTGGAGCTATCTCAGACATGCCTATAGCAAGCATT 250
  68   T  E  S  S  C  G  A  I  S  D  M  P  I  A  S  I   83
 251 CGTGAGGCAGAGCTCTGTGTTGAACAGACAGATGAACAGCCTCTTGACCA 300
  84 R  E  A  E  L  C  V  E  Q  T  D  E  Q  P  L  D  Q  100
 301 AGGGGATGCTGTAACCAACATTTGCCAGGCTGCAGATAGACACTTAGTAC 350
 101  G  D  A  V  T  N  I  C  Q  A  A  D  R  H  L  V  Q 117
 351 AGTTGGTGGAATGGGCTAAACATATCCCACACTTTACTGATCTTCCAGTT 400
 118   L  V  E  W  A  K  H  I  P  H  F  T  D  L  P  V   133
 401 GAAGACCAAGTTGTGTTACTTAAGGCTGGTTGGAATGAACTACTAATTGC 450
 134 E  D  Q  V  V  L  L  K  A  G  W  N  E  L  L  I  A  150
 451 TTCTTTCTCACACCGGAGCATGGGAGTTGAAGATGGCATAGTTTTAGCTA 500
 151  S  F  S  H  R  S  M  G  V  E  D  G  I  V  L  A  T 167
 501 CAGGACTTGTGGTACACAGAAGCAGTGCTCATCAGGCAGGTGTTGGAACC 550
 168   G  L  V  V  H  R  S  S  A  H  Q  A  G  V  G  T   183
 551 ATTATTGATCGAGTATTATCAGAGTTGGTTGCTAAGATGAAGGAAATGAA 600
 184 I  I  D  R  V  L  S  E  L  V  A  K  M  K  E  M  K  200
 601 GATGGACAAGACTGAGCTAGGCTGCTTACGCTCTGTGGTGCTATTCAACC 650
 201  M  D  K  T  E  L  G  C  L  R  S  V  V  L  F  N  P 217
 651 CTGATGCCAAGGGATTGACTTGCTGTAATGATGTAGAGATATTACGTGAG 700
 218   D  A  K  G  L  T  C  C  N  D  V  E  I  L  R  E   233
 701 AAGGTTTATGCAGCTCTAGAGGAGTACACACGCACTAGCTACCCTGATGA 750
 234 K  V  Y  A  A  L  E  E  Y  T  R  T  S  Y  P  D  E  250
 751 GCCTGGCCGATTTGCAAAACTTCTCCTACGACTACCAGCGTTGAGATCCA 800
 251  P  G  R  F  A  K  L  L  L  R  L  P  A  L  R  S  I 267
 801 TAGGCCTAAAGTGCCTTGAATATCTCTTCTTGTTCAAGCTGATTGGAGAC 850
 268   G  L  K  C  L  E  Y  L  F  L  F  K  L  I  G  D   283
 851 ACACCCTTGGACAACTACCTGATGAAAATGCTCGTTGACAATCCCAACAA 900
 284 T  P  L  D  N  Y  L  M  K  M  L  V  D  N  P  N  N  300
 901 TAATTCCCCATCAAGTTAAGATGTGGTATACATGTGTGATAAAATGGCAG 950
 301  N  S  P  S  S
 951 TCACGACCTTTAGTGTTTCTCTAATACTCATATTAATCGCATGCTTTAAG 1000
1001 ATGAGCCTTATTGAAGCACAGTGATGAATTGATTTTACATTGTCCAAATG 1050
1051 TTATTGCTTAACATAGAGGAGACTTTATAGTTTTGGGCGTATCTCTCTTG 1100
1101 TGAGTATAATGAAATATAGACAGCTATTTTAAAAAAAAAATGTGAATGCA 1150
1151 GTAAATGAAATTGAGTGTTGCTTCAAAACTGTGGGTGATAATTGATACAT 1200
1201 TATGAAGTGAAGGAAATATAGAAAAATAAGAATGAGAGGTTTTACTGATA 1250
1251 GTTTGATGATCCCAGAACTTGAATTACCTTCCAGTAAATGAACTGGACAA 1300
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1301 GCTGAAGGGATGTTTATGATGACAGTCTTCATGAACAGTAAAGTGATGAG 1350
1351 ACTGAAAATTATTAGTAGGTTCAAGTGATAACACTGAAGAAAAAACATTG 1400
1401 TTTCTGTTGTAAATAGTCTAGCAGGTTTATATTTGTGATGAATGTTTATA 1450
1451 GTTATATTTCTTCAAATGATTTGCTCAGGGAAATTTTAAATTTCTAAATT 1500
1501 TTCCTCTAGGAAATTTAAAATTTCCAGACAAACATGATTAACAGTTATTT 1550
1551 TTCCGATTACATTTGCCTTGAAAGGAATTTATAAGGAACAAAAAAATTGA 1600
1601 AGCAGTCTTGTCATAAAACTTTCTCTTTTGTAAAATTCAAAGCATTCTGA 1650
1651 TTGTTCAATTTTTAATCAATATACAATTCCTCTGTGATCCGTATAGTGGA 1700
1701 GGTCATTCAAGTGTTAAAAGTGGTATATACGTATATATATGATTCATTTG 1750
1751 A
```

Figure 2.6: Sequence Alignment between the *P. ornatus* ecdysone receptor and the *Uca pugilator* ecdysone receptor

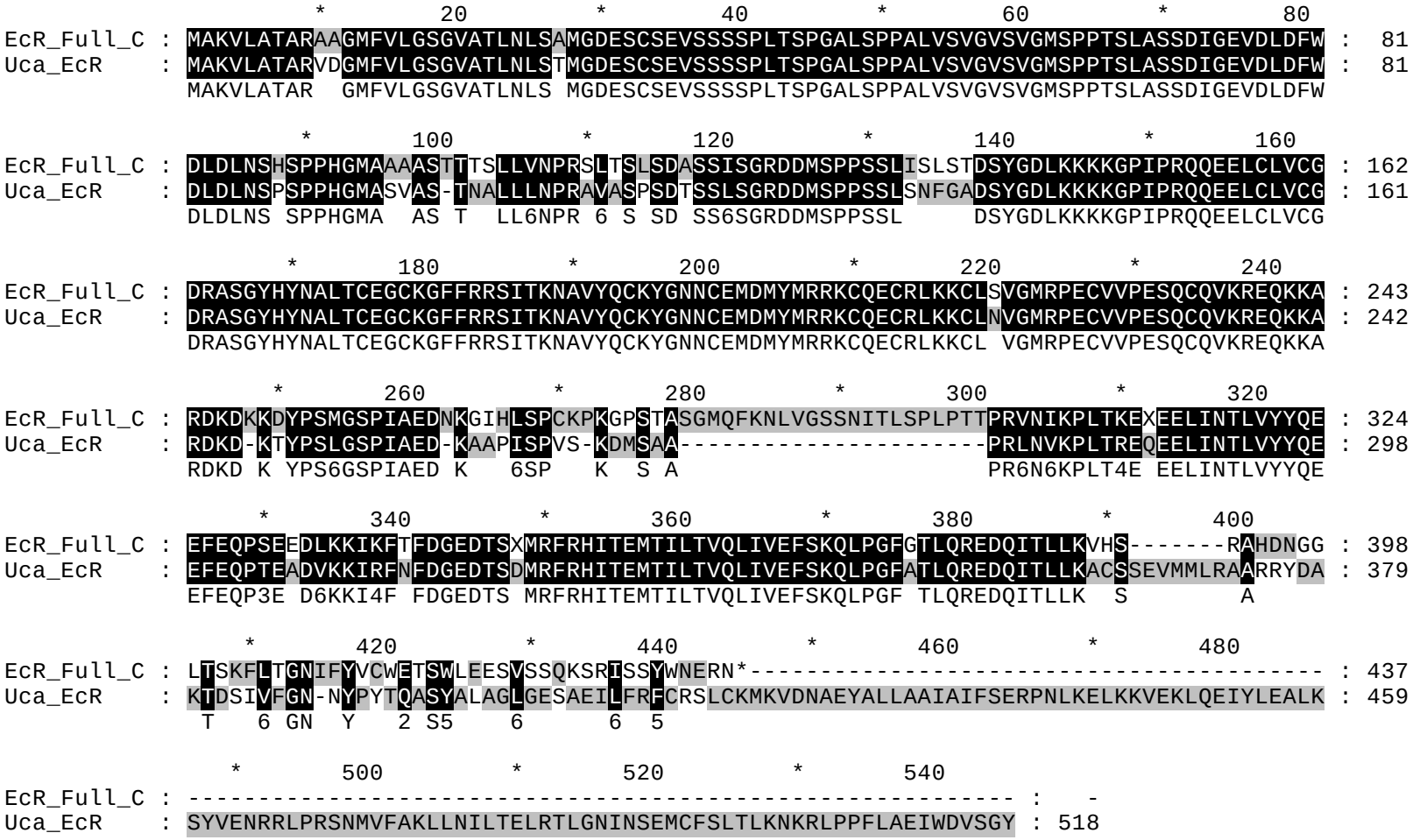

Figure 2.7: Sequence alignment between the *P. ornatus* Hr3 receptor and the *Drosophila* Hr3 receptor

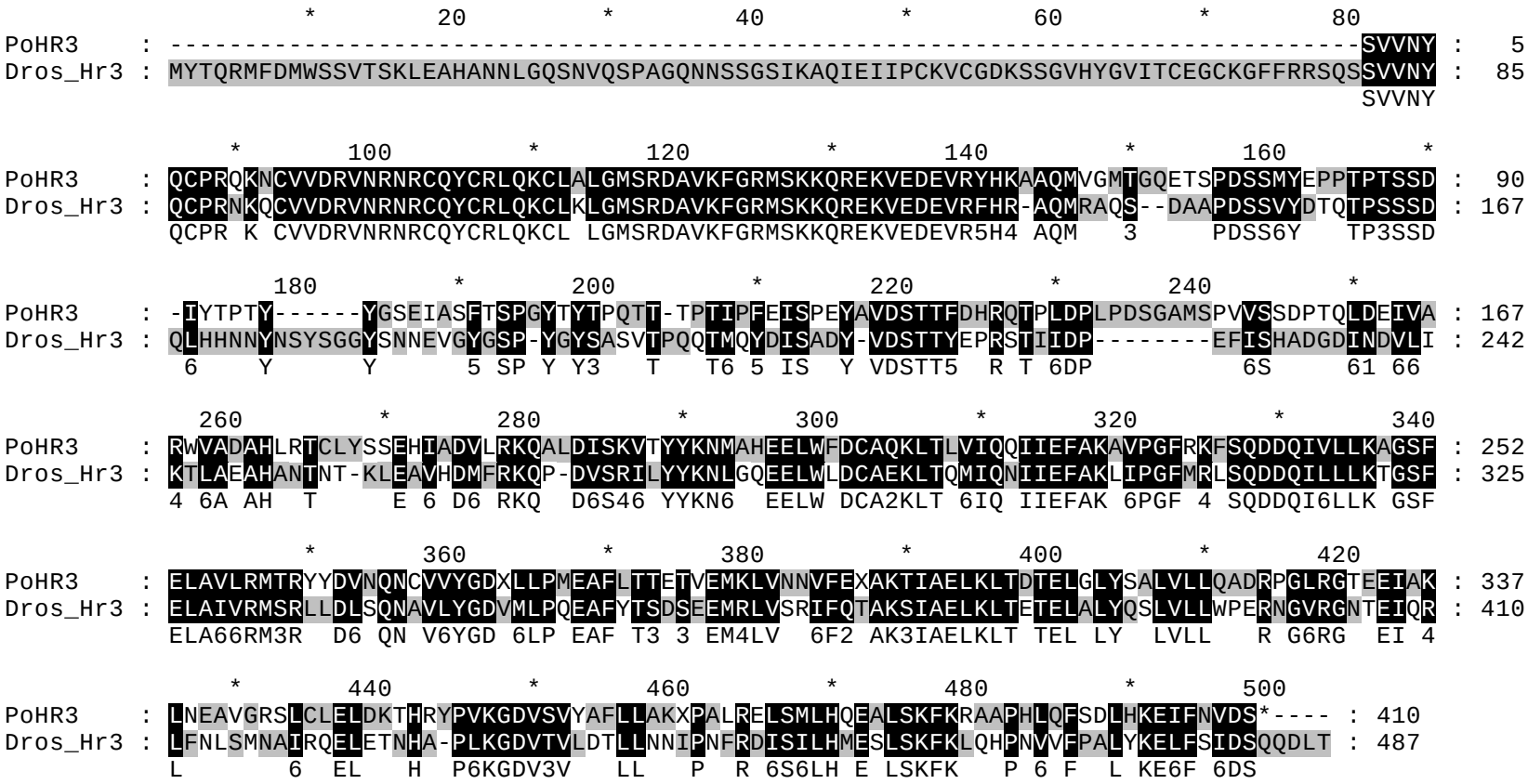

Figure 2.8: Sequence alignment between the *P. ornatus* Hr4 receptor and the *Tenebrio molitor* Hr4 receptor

```
            260         *       280         *       300         *       320         *       340
PoHR4   : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~SR-IPAKEATICVRLALGQGHEYNDKL- :  26
Tm_Hr4  : RTMIWSQPTLGASPTSPVESPHHATTSWASGANMSNTEESAAQMLLNLGQDRLRSPVSRTLVSPQSPSTARFTTTPLN--MERLW : 338
                                                                   SR 6   2     R           4L

                   *        360        *        380        *        400        *        420
PoHR4   : PGDRTQLPQTQPDSPAALNLSTVGMWGARTASADSV-VSPPTQPTPVED----EEEDQPMICMICEDRATGLHYGIITCEGCKGF : 106
Tm_Hr4  : AGDLRQLPVNQQTQALNLSSPTPGPPGVYCGSVSDVKISIMNESSTSESQEATEEEEQPMICMICEDKATGLHYGIITCEGCKGF : 423
           GD  QLP  Q      L   T G  G    S   V 6S   2 3  E     EEE QPMICMICED4ATGLHYGIITCEGCKGF

              *        440        *        460        *        480        *        500        *
PoHR4   : FKRTVQNTRVYTCVADGDCEITKAQRNRCQYCRFQKCLRQGMVLAAVREDRMPGGRNSGAVYNLYKVKYKKKNKKNGQIKQGGTS : 191
Tm_Hr4  : FKRTVQNRRVYTCVADGNCEITKAQRKRCPYCRFKKCIEQGMVLQAVREDRMPGGRNSGAVYNLYKVKYKKHKKP--ACKQPQKA : 506
          FKRTVQN RVYTCVADG1CEITKAQR RC YCRF KC6 QGMVL AVREDRMPGGRNSGAVYNLYKVKYKK  K     KQ

                  520        *        540        *        560        *        580        *
PoHR4   : PEKKMSLDPLMMAGTTPLTSTSLPMPISSPLTSPPVTSPIPSGLNSGHILKAALTNPSEVAHFRQRLDTTVTSSKERFMPYPVAQ : 276
Tm_Hr4  : AEKNI---------------LSQQFKVEQP-------SSIPANLVNGTILKTALTNPSEVVRLRQRLDSAVSSSRDRNFSIEYSL : 569
           EK 6                S    6  P       S IP  L  G ILK ALTNPSEV   RQRLD3 V3SS4 R

            600         *       620         *       640         *       660         *       680
PoHR4   : AMIRMLIECDDFEDIATLKNLDDLLDHNSDLSTKLCQLGDSISIKLVQWTKRLPFYQELPVEVHTRLLTHKWHELLVLTTSAYQA : 361
Tm_Hr4  : SMIKTLIDCDEFQDIATLQNLDDLLDHNTDLSEKLCHIGDSIVYKLVQWTKRLPFYLELPVEVHTRLLTHKWHELLVLTTSAYQA : 654
           MI4 LI CD F2DIATL NLDDLLDHN3DLS KLC 6GDSI  KLVQWTKRLPFY ELPVEVHTRLLTHKWHELLVLTTSAYQA

                   *        700        *        720        *        740        *        760
PoHR4   : IYGLQKLGSRSSDGTEAEFHQEVSNNLCTLQTCLNSMMGQPITMDQLRHEVGVMVEKITHVTLALRKIKIQIEEYVCLKVIAMLN : 446
Tm_Hr4  : IH---KAGDQLTTVIKTDFNHEVETNLCTLQSCLTSMMGREITIEQLRQDVGLMIEKITHVTLMFRQIKLTMEEYVCLKVITMLN : 736
          I    K G   3      F  EV  NLCTLQ3CL SMMG  IT6 QLR  VG6M6EKITHVTL  R IK6 6EEYVCLKVI MLN

              *        780        *        800        *        820        *        840
PoHR4   : Q----SRIGHKELEVIQERYMGCLRSFCETHYPSQPNRYQDLLVRLPDIQAAAKLLETKMLYVPFLLNSTINR* : 516
Tm_Hr4  : QAKPASSSGNSELESIHERYMTCLRVYTQHMYPQQTTRFQDLLGRLPEIQSAAFLLESKMFYVPFLLNSAIQR~ : 810
          Q    S  G  ELE I ERYM CLR 5 2  YP Q  R5QDLL RLP IQ AA LLE3KM YVPFLLNS I R
```

Figure 2.9: Sequence alignment between the *P. ornatus* Hr78 receptor and the Tenebrio molitor Hr78 receptor

```
                        *        20         *        40         *        60         *        80
Tm_HR78 : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~MDIEHSIKSEILSDKIRNNSNN :  22
PoHR78  : MVTNPALFTHTMLSAAPTLLLGNSPVLGSDWVGRLKELQERGGLAAAAAAAAASSGSDIDSRDLDHS--HQYTS---RNTGQP :  79
                                                                        D6 HS   2  S   RN

                 *       100         *       120         *       140         *       160
Tm_HR78 : LCLTVELCVVCGDRASGRHYGAISCEGCKGFFKRSIRKQLGYQCRGSKNCEVTKHHRNRCQYCRLQKCLACGMRSDSVQHERKP : 106
PoHR78  : --VTMDLCVVCGDRASGRHYGAISCEGCKGFFKRSIRKQLGYTCRGNKTCEVTKHHRNR~~~~~~~~~~~~~~~~~~~~~~~~~ : 136
            6T6 LCVVCGDRASGRHYGAISCEGCKGFFKRSIRKQLGY CRG K CEVTKHHRNR
```

Figure 2.10: Sequence alignment between the *P. ornatus* Usp receptor and the *Uca pugilator* Usp receptor

```
                   *        20         *        40         *        60         *        80
USP     : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ :   -
Uca_RXR : MIMIKKEKPVMSVSSIIHGSQQRAWTPGLDIGMSGSLDRQSPLSVAPDTVSLLSPAPSFSTANGGPASPSISTPPFTIGSSNTTGL :  86

             *       100         *       120         *       140         *       160         *
USP     : ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~KRTVRKDLTYACREDKSCTIDKRQRNRCQYRR :  32
Uca_RXR : STSPSQYPPSHPLSGSKHLCHPLSGSKHLCSICGDRASGKHYGVYSCEGCKGFFKRTVRKDLTYACREERSCTIDKRQRNRCQYCR : 172
                                                                KRTVRKDLTYACRE 4SCTIDKRQRNRCQY R

               180         *       200         *       220         *       240         *       2
USP     : YHEVFIHGHEERSGSNVGGVEEERQRTRGDKGDGDTESSCGAISDMPIASIREAELCVEQTDEQPLDQG----------------- : 101
Uca_RXR : YQKCLTMGMKREA------VQEERQRTKGDKGDGDTESSCGAISDMPIASIREAELSVDPIDEQPLDQGVRLQVPLAPPDSEKCSF : 252
          Y      G           V2EERQRT4GDKGDGDTESSCGAISDMPIASIREAEL V   DEQPLDQG

          60         *       280         *       300         *       320         *       340
USP     : -----------------DAVTNICQAADRHLVQLVEWAKHIPHFTDLPVEDQVVLLKAGWNELLIASFSHRSMGVEDGIVLATGLV : 170
Uca_RXR : TLPFHPVSEVSCANPLQDVVSNICQAADRHLVQLVEWAKHIPHFTDLPIEDQVVLLKAGWNELLIASFSHRSMGVEDGIVLATGLV : 338
                           D V3NICQAADRHLVQLVEWAKHIPHFTDLP6EDQVVLLKAGWNELLIASFSHRSMGVEDGIVLATGLV

               *       360         *       380         *       400         *       420         *
USP     : VHRSSAHQAGVGTIIDRVLSELVAKMKEMKMDKTELGCLRSVVLFNPDAKGLTCCNDVEILREKVYAALEEYTRTSYPDEPGRFAK : 256
Uca_RXR : IHRSSAHQAGVGAIFDRVLSELVAKMKEMKIDKTELGCLRSIVLFNPDAKGLNCVNDVEILREKVYAALEEYTRTTYPDEPGRFAK : 424
          6HRSSAHQAGVG I DRVLSELVAKMKEMK6DKTELGCLRS6VLFNPDAKGL C NDVEILREKVYAALEEYTRT3YPDEPGRFAK

                 440         *       460         *       480
USP     : LLLRLPALRSIGLKCLEYLFLFKLIGDTPLDNYLMKMLVDNPNNN--SPSS* : 305
Uca_RXR : LLLRLPALRSIGLKCLEYLFLFKLIGDTPLDSYLMKMLVDNPNTSVTPPTS~ : 475
          LLLRLPALRSIGLKCLEYLFLFKLIGDTPLD YLMKMLVDNPN     P3S
```

Table 2.1: Primer sequences used in this analysis

PRIMER NAME	PRIMER SEQUENCE	PRIMER USE
RR1	5' TAYWSNTGYGARGGNTGYAARGGNTTYTT 3'	Initial amplification of nuclear hormone receptor homologs
RR2	5' RCAYTTYTGRTANCGRCARTAYTGRCA 3'	Initial amplification of nuclear hormone receptor homologs
NEB 1224	5' CGCCAGGGTTTTCCCAGTCACGAC 3'	Screening of colonies for inserts in plasmids
NEB 1233	5' AGCGGATAACAATTTCACACAGGA 3'	Screening of colonies for inserts in plasmids
UNI-dT		Reverse transcription of mRNA for 5' and 3' RACE
UNI		PCR for 3' RACE reactions
Porn056F	5'TCGGTGGTGAACTACCAGTG 3'	Gene specific primer for 3' RACE of Hr3 homolog
Porn060F	5'TAAACACCACCGCAATAG 3'	Gene specific primer for 3' RACE of Hr78 homolog
Porn062F	5'ACCATTGACAAGAGACAAAG 3'	Gene specific primer for 3' RACE of USP homolog
Porn063F	5'ATCACAAAAGCACAGCGTAACC 3'	Gene specific primer for 3' RACE of Hr4 homolog
3' PstI-adapter-oligo-dT	5'AAG CAG TGG TAA CAA CGC AGA GCT GCA GT_{30}VN 3'	Reverse transcription of mRNA for 5' and 3' RACE
TS-NotI,	5'AAG CGG CCG TAT CAA CGC AGA GTA CGC GGG 3'	Template switching during cDNA synthesis
EcR-long-REV	5' TTT GCG TCT CAT GTA CAT GTC CAT TTCACA GT 3'	Gene specific primer for 5' RACE of EcR homolog
Heel-Carrier-NotI	5' GTA ATA CGA CTC ACT ATA GGG CAA GCG GCC GTA TCA ACG CAG AGT 3'	Incorporates heel sequence into DNA 5' of original TS-NotI primer sequence
Heel-Specific	5' GTA ATA CGA CTC ACT ATA GGG C 3'	Amplification during 5' RACE
EcR-long-FWD	5' CCG GAG ATC CAT CAC CAA GCC TGC AGT CT 3'	Gene specific primer for 3' RACE of EcR homolog
3'-PstI-StepOut	5' AAT TAA CCC TCA CTA AAG GGA AGC AGT GGT AAC AAC GCA GAG C 3'	Incorporates step out sequence into DNA 5' of original 3' PstI adapter oligo dTprimer sequence
StepOut-Specific	5' AAT TAA CCC TCA CTA AAG GG 3'	Amplification during 3' RACE
EcR-Nested-REV	5'CAC AGT TTT TGC CGT ATT TAC ATT GGT AGA CTG C 3'	5' semi-nested PCR
EcR-Nested-FWD	5' GCA GTC TAC CAA TGT AAA TAC GGC AAA AAC TGT G 3'	3' semi-nested PCR
PoHR3R1	5' CCG TCA TGC CCA CCA TCT GAG CAG 3'	Primary PCR for 5' RACE for Hr3
PoHR3R1	5' CAG CCT TGT GGT ACC GCA CCT CAT CCT 3'	Semi-nested PCR for 5' RACE for Hr3
HR4-R1	5' CCT AAT CAT GGC CTG GGC AAC AGG GT 3'	Primary PCR for 5' RACE for Hr4
HR4-R2	5' CGG AAG TGG GCC ACC TCA GAT GGA TT 3'	Semi-nested PCR for 5' RACE for Hr4
HR78R1	5' CGC ACG TCT TGT TCC CCC GGC 3'	Primary PCR for 5' RACE for Hr78
HR78R2	5' GGC AAG TGT AGC CGA GCT GCT TAC GGA T 3'	Semi-nested PCR for 5' RACE for Hr78
USP-R1	5' GGG ATA TGT TTA GCC CAT TCC ACC AAC TG 3'	Primary PCR for 5' RACE for Usp
USP-R2	5' CAG CCT GGC AAA TGT TGG TTA CAG CA 3'	Semi-nested PCR for 5' RACE for Usp
Oligo-dT-Kpn1	5' GTA AGT ACA CAA ACA GGT ACC TTT TTT TTT TTT TTT TTV 3'	Reverse transcription of mRNA for 5' and 3' RACE
Kpn1-primer	5' GTA AGT ACA CAA ACA CCT ACC 3'	PCR primer for 3' RACE

Table 2.2: Nucleotide sequence of *P. ornatus* and *P. cygnus* DNA binding domains isolated using degenerate RR-1/RR-2 primers

NAME	**NUCLEOTIDE SEQUENCE**	*DROSOPHILA* **HOMOLOG**
JS056 (AIMSPorn1)	1 CCGCTGGTCGCAGTCCTCGGTGGTGAACTACCAGTGCCCCCGCCAGAAGAACTGCGTCGT 61 GGACCGCGTCAACCGAAACCGC	Hr3
JS060 (AIMSPorn2)	1 CAAAAGGTCAATCCGTAAGCAGCTCGGCTACACTTGCCGGGGGAACAAGACGTGCGAGGT 61 CACTAAACACCACCGCAATAGG	Hr78
JS062 (AIMSPorn3)	1 CAAGCGAACAGTTCGTAAAGACCTCACATATGCATGTCGAGAAGATAAATCGTGTACCAT 61 TGACAAGAGACAAAGAAATCGA	Usp
JS063 (AIMSPorn4)	1 CAAGCGAACAGTACAGAATAAGCGGGTATACACATGTGTTGCTGATGGAGATTGTGAAAT 61 CACAAAAGCACAGCGTAACCGT	Hr4
P. cygnus EcR	1 CCGGAGATCCATCACCAAGAATGCAGTCTACCAATGTAAATACGGCAAAAACTGTGAAAT 61 GGACATGTACATGAGACGCAA	EcR

Table 2.3: Database homologs and sequence alignments of the DNA binding domain sequences isolated from *P. ornatus* and *P. cygnus*

NAME	STRONGEST DATABASE HOMOLOG	*DROSOPHILA* HOMOLOG	ALIGNMENT TO *DROSOPHILA* HOMOLOG	FUNCTION OF *DROSOPHILA* HOMOLOG
P. c. EcR	Ecdysteroid receptor *Carcinus maenas* (green crab) GenBank AY496927	EcR – ecdysone receptor	P.c.EcR: RRSITKNAVYQCKYGKNCEMDMYMRRK RRS+TK+AVY CK+G+ CEMDMYMRRK EcR: RRSVTKSAVYCCKFGRACEMDMYMRRK	Functions as master regulator of ecdysone response. Binds ecdysone as a heterodimer with USP and activates genes with ecdysone response elements (EREs)
P.o. EcR	Ecdysteroid receptor *Uca pugilator* (Atlantic sand fiddler crab) GenBank AF034086	EcR – ecdysone receptor	P.o. EcR: RRSITKNAVYQCKYGNNCEMDMYMRRK RRS+TK+AVY CK+G CEMDMYMRRK EcR: RRSVTKSAVYCCKFGRACEMDMYMRRK	
P.o. Usp	Ultraspiracle *Aedes aegypti* (Yellowfever mosquito) GenBank AF305214	Usp - ultraspiracle	P.o. Usp: KRTVRKDLTYACREDKSCTIDKRQRNR KRTVRKDLTYACRE+++C IDKRQRNR USP: KRTVRKDLTYACRENRNCIIDKRQRNR	Functions as a heterodimer with EcR to induce transcription of early response genes in *Drosophila*
P.o. Hr3	NHR23_CAEEL (nuclear hormone receptor family chr-3) *Caenorhabditis elegans* (nematode) SWISS-PROT P41828	Hr3 Hormone receptor 3	P.o. Hr3: RWSQSSVVNYQCPRQKNCVVDRVNRNR R SQSSVVNYQCPR K CVVDRVNRNR Hr46: RRSQSSVVNYQCPRNKQCVVDRVNRNR	Functions in *Drosophila* metamorphosis by repressing "early genes" induced in response to ecdysone in larvae and inducing βFTZ-F1 competence factor for puparium formation. Can function as a monomer.
P.o. Hr4	FTZ-F1 *Artemia salina* (Brine shrimp) GenBank U93419	Hr4 Hormone receptor 4	P.o. Hr4: KRTVQNKRVYTCVADGDCEITKAQRNR KRTVQN+RVYTCVADG CEITKAQRNR Hr4: KRTVQNRRVYTCVADGTCEITKAQRNR	Hr4 is expressed in the epidermis as ecdysteroid levels begin to decline, and prior to βFTZ-F1
P.o. Hr78	THR6 *Tenebrio molitor* (Yellow mealworm) GenBank AJ005765	DHr78 Hormone receptor 78	P.o. Hr78: KRSIRKQLGYTCRGNKTCEVTKHHRNR KRSIRKQLGY CRG CEVTKHHRNR Hr78: KRSIRKQLGYQCRGAMNCEVTKHHRNR	Orphan receptor, expressed as ecdysone titre rises in third instar larvae prior to puparium formation.

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3. DEVELOPMENT OF REAL-TIME QUANTITATIVE PCR TESTS FOR *PANULIRUS ORNATUS* NUCLEAR HORMONE RECEPTOR HOMOLOGS

Introduction

A key goal of this project was to determine how specific nuclear receptors might be involved in larval molting and metamorphosis. It is known from work with *Drosophila* and other insects that there are precisely timed cascades of gene expression associated with larval development. Hence the approach taken in the present project was to examine changes in the expression of *Panulirus ornatus* nuclear hormone receptor genes across several larval molt cycles.

The technique used for this was real-time PCR. Real-time PCR can use a variety of specific methodologies, but in each case amplification of template molecules is detected in real-time i.e. while the PCR reaction is proceeding, and the detection method, usually fluorescence based, is such that the detection is directly proportional to the amount of product that has been formed.

TaqMan® Real time PCR technology was used in the present work. The assay consists of a forward and reverse primer as would be found in a traditional PCR, together with a dual-labelled probe. The probe is an oligo-nucleotide that contains a fluorescent dye at the 5'-base and a quenching dye on the 3'-base (Figure 3.1, (1)). Due to the close proximity of the quencher to the reporter, the fluorescence of the reporter is transferred to the quenching dye which also fluoresces, but at a different wavelength which is not detected in the assay. The probe is designed to hybridise to the target gene sequence between the two primer sites, but before the primers during the annealing or hybridization phase of the PCR reaction, ensuring it is in place before amplification begins (Figure 3.1 (2)). During the extension phase of the PCR, the probe is degraded by the 5'-exonuclease activity of the Taq DNA polymerase, separating the reporter dye from the quencher, enabling detection of its fluorescent signal as shown (Figure 3.1 (3)). For each product amplified a fluorescent signal is released from the labeled probe and detected by the real-time machine. Hence fluorescence increases in direct proportion to the number of target genes amplified during the log-linear phase of PCR amplification.

Furthermore, as PCR is a geometric reaction with the number of molecules doubling in every cycle, the number of cycles taken for a given amount of product to be synthesized is proportional to the amount of starting template. Thus for an abundantly expressed gene, for which there are a large number of templates in a

given cDNA preparation, a "critical threshold" of fluorescence will be reached after only a few cycles; by contrast, a gene which is only expressed at a low level will only have a very few starting template molecules in the initial cDNA preparation, and so the "critical threshold" of fluorescence may only be reached after a significantly higher number of cycles (Figure 3.2). While the associated mathematics are complex, the concept of the critical threshold lies at the heart of using real-time PCR to quantify levels of gene expression, with a low critical threshold implying high abundance and, conversely, a high critical threshold implying that a gene is expressed only at low levels in the tissues being studied.

Development of the real-time PCR assays for four of the nuclear hormone receptor homologs, Hr3, Hr4, Hr78 and EcR, and for a "house-keeping gene", the 18S rRNA gene, is discussed in this chapter, along with the considerable difficulties that were encountered.

Methods

DEPC treatment of water

RNase-Free water was generated by incubating 1000 ml MilliQ-grade H_2O with 1 ml diethylpyrocarbonate (DEPC) overnight at room temp or 37°C. DEPC destroys all RNAse activity. The water was subsequently autoclaved for 20 min to inactivate the DEPC.

RNA extraction

RNA EXTRACTION METHOD-1

RNA was extracted using Trizol LS Reagent according to the manufacturer's instructions (Invitrogen #10296-010) with pellets being resuspended in 50 µl DEPC-treated H_2O. When RNA was pelleted, it was dark brown and resuspension of pellets maintained the pigmented colour. As pigments have been shown to interfere with downstream processes (Giambernardi et al., 1999), the RNA sample was spun at 2000 r.p.m for 2 min through a 0.5 ml spin-column packed with Sepharose CL6B (Amersham) equilibrated in DEPC-treated water to remove most of the pigment. Each RNA sample was then treated with DNaseI (Ambion #1906) according to the manufacturer's instructions with incubation at 37°C for 60-120 min. RNA was again passed through a CL6B-DEPC-H_2O column to remove any degraded DNA, prior to quantification of the RNA sample by OD_{260}.

RNA EXTRACTION METHOD-2

Approximately 50-100 mg snap frozen larvae were ground in NucleoSpin II grinding buffer using a micro-pestle and microfuge tube. Samples were then homogenized

through a 21G-needle and spun through the shredding filters provided. RNA extraction was continued as outlined in the NucleoSpin II instructions (Machery-Nagel #740955).

Synthesis of cDNA by Reverse Transcription

Reverse transcription was carried out using 500 ng template RNA primed with 2.5 µM random hexamers, 4 µM each dNTP, 50 U StrataScript Reverse Transcriptase in supplied 1x StrataScript Buffer (Stratagene #600085), 40 U RNaseOut (Invitrogen #10777-019), and made up to 50 µl with nuclease-free water. Two 50 µl negative RT control reactions were set up in the same way except the StrataScript RT was replaced with nuclease-free water. The RT reactions and controls were incubated at 42°C for 90 min prior to inactivating the StrataScript at 90°C for 5 min.

Primer design and synthesis

Primers and Taqman probes were designed using the ABI software Primer Express, which designs primer / probe combinations which fit very robust, standardized PCR parameters. The primers and probes for *P. ornatus* EcR, Hr3, Hr4 and Hr78 were designed based on the 81 bp sequences obtained as described in Chapter 2 and are shown in Table 3.1. The primers and probe for the 18S gene were designed by aligning sequences available in the database for the Caribbean spiny lobster *Panulirus argus* (PAU19182), the shrimp *Litopenaeus vannamei* (AF186250) and for *Penaeus monodon* (AIMS unpublished data) to identify a region that is highly conserved among decapod 18S rRNA genes (positions 964-1047 on the *P. argus* complete 18S rRNA sequence). The *L. vannamei* sequence, which only differed from the *P. argus* sequence at two positions in this region, was then used in Primer Express to design the primer and probe sequences shown in Table 3.1. All TaqMan® probes were synthesized by Applied Biosystems. The location of the primers and probes on the target sequences is shown in Table 3.2.

Primer concentration optimisation

Optimal primer concentrations for each assay were determined by the Primer Limiting Assay as outlined in ABI's User Bulletin-2 (Livak, 1997). A series of primer dilutions was prepared at 9 µM, 3 µM, 0.5 µM for both the forward and reverse primers for each target gene, and all nine possible primer concentration combinations were set up in triplicate 50 µl reactions containing 2 x TaqMan® Universal reaction mix and 10 ng cDNA. Universal Master Mix is supplied in a 2 x concentration and contains buffer, dNTPs and Taq Polymerase, all optimized for real-time quantitative PCR. The cDNA was assumed to be 10 ng μl^{-1} based on the input RNA amount used in the RT reaction. Reactions were run on the ABI Prism® 7700 Sequence Detection System as follows: [50°C for 2min] x 1 cycle; [95°C for

10min] x 1 cycle; [95°C for 15s, 60°C for 1 min] x 40 cycles. SDS software (Applied Biosystems) was used to compute the Ct and ΔRn values for each primer combination.

Confirming specificity of nuclear hormone receptor primers

Four plasmid DNA templates containing the DNA binding domain region of each nuclear hormone receptor (described in Chapter 2) were screened by PCR with each of the four different TaqMan® primer combinations (EcR-Fwd and-Rev, Hr3-Fwd and Rev, Hr4-Fwd and Rev and Hr78-Fwd and Rev). Each PCR was set up using 10 ng plasmid DNA, 1 µM forward and 1 µM reverse primers, 200 nM dNTPs, 0.2 U HotStart-Taq DNA Polymerase in supplied 1x PCR Buffer. Cycling was performed on a Perkin-Elmer GeneAmp9700 PCR machine under the TaqMan® cycling conditions. PCR products were analysed by agarose gel electrophoresis.

Linear dynamic range assays

All reactions were set up in a final volume of 50 µl. TaqMan® reaction mix was prepared to give 1 x TaqMan® Universal Master Mix, optimized concentrations of forward and reverse primers, 250 nM probe and nuclease-free water to 45 µl per reaction. A series of dilutions of cDNA were prepared, and 5 µl of each dilution was added to triplicate wells, giving a range of cDNA amounts from 80 pg to 50 ng per well. Negative RT control reactions were also included.

PCR efficiency determinations for larval samples

Two dilution ranges were used for determining PCR efficiency due to the high abundance of the 18S house-keeping gene (HKG) in relation to each of the four genes of interest (GOI). In order to be within the linear dynamic range of the HKG, 10-fold serial dilutions were prepared in nuclease-free water (Ambion) from 2.5 ng μl^{-1} down to 2.5 x 10^{-4} ng μl^{-1}. However, to be within the linear dynamic range of the GOIs, a mixture of 2-fold and 10-fold dilutions were used to prepare triplicate serial dilutions covering 10, 5, 0.5, 0.25, 0.025 ng μl^{-1}. Two µl of each triplicate dilution were assayed as described in a 25 µl PCR reaction for all four nuclear hormone receptor genes and for the 18S rRNA gene on the ABI7700 and results were analysed using the SDS-Software. Resulting Ct values were averaged for triplicate wells and plotted against log input cDNA (based on the original RNA concentration determined by OD, assuming 100% reverse transcription efficiency). The slope of the line for each assay, calculated using the Linear Regression Analysis function in Excel (Microsoft), was then used to calculate the PCR Efficiency (E) according to the equation: E=10(-1/slope) (Livak, 1997; Rasmussen, 2001; Bustin, 2002; Pfaffl et al., 2002).

Preparation of 18S plasmid for standard curve

P. ornatus cDNA was used as a template to amplify the 18S gene by standard PCR using 1 µM 18S-FWD and 1 µM 18S-REV Q-PCR primers, 200 nM dNTPs, 1 x Q-Solution, 1 U Taq DNA Polymerase in supplied 1 x PCR Buffer. Cycling was performed on a Perkin-Elmer GeneAmp® PCR System 9700 as follows: [94°C for 1 min] x 1 cycle; [94°C for 15 s, 60°C for 1 min] x 40 cycles; [60°C for 5 min] x 1 cycle.

The ~86bp 18S fragment was purified using a Qiaquick PCR Purification Kit (Qiagen), cloned into pGemTeasy (Promega) according to the manufacturer's instructions, and transformed into DH10α chemically competent cells. Large-scale plasmid stocks were prepared using a Qiagen Maxi-Prep Kit. Plasmid DNA was quantified by A_{260}, and the copy number per ng plasmid DNA calculated. The 18S plasmid standard was then used to prepare a standard curve in 10 mM Tris, 0.1 mM EDTA pH 8.0 ranging from 10^9-10^2 copies μl^{-1}.

The cloned 18S plasmid DNA was also sequenced using the Dyenamic ET terminator sequencing kit (Amersham).

Constitutive expression of 18S gene

cDNA was prepared as outlined above from a range of larval samples of different ages. 200 ng of each cDNA sample was subsequently treated with RNase in a 10 µl reaction according to the SuperScript® protocol using 1 U *E. coli* RNaseH (Invitrogen) for 20 min at 37°C. RNase-treated cDNA samples were cleaned by Phenol/Ether extraction and ammonium acetate precipitation. Pellets were resuspended in 10 µl nuclease-free water and quantified using A_{260}.

Master mix containing 1 x TaqMan® Universal Master Mix, 600 nM 18S-FWD and 600 nM 18S-REV primers and 250 nM 18S-TAMRA-VIC probe was prepared for all larval samples and standard curve samples to be analysed in the constitutive expression experiment. Sample reactions were set up in duplicate each with a total of 24 µl reaction mix with 1 µl sample containing either 10 ng or 1 ng cDNA. Standard curve samples were set up in triplicate each with 24 µl reaction mix and 1 µl plasmid standard ranging from 10^9-10^2 copies per well. Larval samples from each age group were treated as replicates. Statistical analysis was then performed between each age group to determine if there were any significant differences between the Ct values recorded using the SDS-Software "Stat".

Genomic DNA contamination determination: selection of suitable sample

Negative RT reactions i.e. reverse transcriptase reactions with all components added except for reverse transcriptase itself, were used to determine genomic DNA contamination in RNA samples according to equation 1 (Livak, 1997).

Equation-1: Determination of Genomic DNA Contamination

$$\text{Genomic DNA Contamination (\%)} = E^{[Ct(+RT) - Ct(-RT)]} \times 100$$

E = PCR efficiency factor.
Ct(+RT) = Ct value of the target for the cDNA sample.
Ct(-RT) = Ct value of the target when using a –RT sample.

Analysis of results

The mean normalised expression was calculated using Q-Gene Equation-3:

Formula for the Calculation of the mean Normalised Gene Expression by Averaging the Three Concomitant Ct Values

$$MNE = \frac{E_{HKG}^{(Ct1+Ct2+Ct3/3)}}{E_{GOI}^{(Ct1+Ct2+Ct3/3)}} = \frac{E_{HKG}^{(Ct\text{-}mean)}}{E_{GOI}^{(Ct\text{-}mean)}}$$

MNE = mean normalised expression.
E_{HKG} = PCR efficiency value of house-keeping gene (18S).
E_{GOI} = PCR efficiency value of gene of interest (nuclear hormone receptor genes).
Ct-mean = Mean critical threshold for each sample.

Results

Optimal primer concentrations

Primer concentrations were optimized to select those that give the highest ΔRn and lowest Ct. ΔRn is the fluorescence signal detected after normalisation to a standard fluorophore (ROX), which is incorporated in all reactions, and subtraction of background fluorescence i.e. that which is observed in reactions without added template. Ct is the cycle number at which a particular ΔRn value, referred to as the critical threshold, is crossed for any given sample. The optimal primer concentrations were found to vary for each target gene (Table 3.1).

Specificity of nuclear hormone receptor primers

Due to the highly conserved nature of the DBD region in which the TaqMan® assays were designed, each set of TaqMan® NHR primers were tested against plasmid DNA

containing the DBD domain of each other receptor being assayed in order to confirm that there was no cross-amplification of any of the other nuclear hormone receptors being analysed. This analysis confirmed the specificity of the assays, as each set of nuclear hormone TaqMan primers only amplified product for their respective nuclear hormone plasmids with no primer pair amplifying product from any other NHR tested.

Linear dynamic range

The linear dynamic range assay determines the range of template amounts, i.e. input cDNA amounts, suitable for analysing the gene of interest (GOI). It is determined by assaying a serial dilution of an appropriate template. The Ct is plotted against the log(ng input cDNA) and the linear range of this assay determines the appropriate range of starting template. As the expression level of the nuclear hormone receptor genes was unknown, a large dilution range was tested with the template cDNA varying from 80 pg to 50 ng per well. As different genes may be expressed at quite different levels in the tissue of interest, the linear dynamic range has to be tested for each GOI, as it may vary for different genes with different expression levels. The relationship between Ct and initial cDNA amount was initially tested for Hr3 and was found to be linear across the entire range tested with a correlation coefficient r^2 >0.99 (Figure 3.3). The testing of the linear dynamic range for the other nuclear hormone receptor genes was incorporated into the PCR efficiency tests (see chapter 4).

It was anticipated at the outset of this analysis that the 18S rRNA gene would be expressed at much higher levels than the four nuclear hormone receptor genes, as the 18SrRNA gene is normally present in thousands of copies per genome (Wyngaard et al., 1995). Initial Linear Dynamic Range analysis of 18S (Figure 3.3) confirmed significant differences in expression levels between the 18SrRNA gene and the nuclear hormone receptor gene Hr3. For example, Figure 3.3 shows that 5 ng cDNA (log = 0.7) assayed for 18S rRNA resulted in a Ct value of ~14 while Hr3 gave Ct values of ~26. While these Ct values are acceptable, if the GOIs are significantly down-regulated during larval development, then the Ct values will be >30. As this represents extremely low levels of gene expression, it inevitably introduces greater error due to inter-sample variability. Hence it was decided to use 10 ng cDNA per reaction for analysis of the genes of interest, but to perform a further 1/10 dilution for the 18S rRNA assay to 1 ng per reaction. This meant that the housekeeping gene analysis had to be performed in separate wells from analysis of the gene of interest, which had implications for calculation of the results as discussed below.

Genomic contamination

No matter what RNA preparation method is used, there is always some genomic DNA contamination. This genomic DNA therefore also contaminates the cDNA preparation used in the real-time PCR reactions, and hence will generate signal in the Q-PCR reaction. The only way to completely avoid this is to design primers / probes to span messenger RNA intron-exon splice junctions, so that they can amplify cDNA which has been generated from correctly spliced mRNA, but cannot amplify the original genomic DNA sequences. This was not possible for the nuclear hormone receptor genes as we have no knowledge of the location of any introns in the genes.

It is generally acceptable to ignore genomic contamination if the signal contribution is less than 1% of the total signal from the positive RT-PCR reaction, but it was necessary to test this by analysing the signal generated from negative reverse transcription reactions i.e. RT reactions without added enzyme. The four nuclear hormone receptor genes were first tested for the degree of genomic contamination for 24 different larval cDNA samples from a single spawning run. All assays indicated <1% genomic DNA contamination, with the maximum recorded value being 0.58% contamination for the Hr3 gene. As it is too expensive to run negative RT reactions for every individual assay, it was decided to use the Hr3 assay to monitor % genomic DNA contamination in all subsequent spawnings – see chapter 4.

Choice of quantification method

There are several different methods of quantification available for real-time PCR. The choice of quantification method is determined both by the information required, and also by the parameters of the specific assay.

Quantification can be grouped into two different broad methods. Absolute quantification uses a standard curve which enables the absolute amounts of a target gene in a sample to be calculated. This is most useful in cases where there is intrinsic value in knowing the absolute amounts of a target gene e.g. when real-time PCR is used to quantify copies of a viral template. For example, real-time PCR has been used to quantify the numbers of viral genomic templates for the gill associated virus, a virus known to cause disease outbreaks in the black tiger prawn, *Penaeus monodon*, in order to investigate the effect of different treatments on the viral infection status of the prawns (de la Vega et al., 2004). The results are normalised to some factor such as input RNA amount, and are expressed as DNA or RNA copy numbers per unit used for normalisation e.g. copy numbers per ng total RNA.

The alternative method is relative quantification. In this case the absolute amounts of a target gene are unimportant. Rather it is how expression of a target gene changes under different circumstances that are important. The relative method

generally has two steps. In the first step, measured expression of the gene of interest is normalised relative to a housekeeping gene which is expressed at a constant level. This corrects for variations in sample input, pipetting accuracy etc. In the second step, the normalised expression of the gene of interest is compared to that of a calibrator sample. This might be the expression level of the gene in a particular tissue, or at day one in a time series – the choice depends on the experimental design and objectives. The results are then expressed relative to those of the calibrator, and therefore do not have any units associated with them. This method was selected for the present work, and hence a range of experiments were undertaken to test the assumptions underlying this method, and to modify it accordingly if certain key assumptions were not met.

Design of an assay for a housekeeping gene

The 18S rRNA gene was selected for use as the house-keeping gene for normalisation of the assays because sequences for other potential house-keeping genes (β-actin, GAPDH) were not available for *P. ornatus* or close relatives. While 18S rRNA sequence was also not available for *P. ornatus,* 18S rRNA sequence was available in Genbank for two other decapods, the Caribbean spiny lobster *Panulirus argus* and the Pacific white shrimp *Litopenaeus vannamei,* and AIMS also possessed 18S rRNA sequence for the black tiger shrimp *Penaeus monodon*. As the 18S rRNA gene is extremely highly conserved across species, it was possible to use these sequences to design the real-time quantitative PCR primers for the *P. ornatus* 18S rRNA gene.

To confirm the suitability of the designed primers (18S-Fwd and 18S-Rev) for use as Q-PCR primers for *P. ornatus*, these primers were used to amplify the 18S rRNA gene from *P. ornatus*. The expected size PCR product (84 bp) was obtained and this was cloned and sequenced to check the internal region for specificity with the TaqMan probe designed on the *L. vannamei* sequence. The *P. ornatus* sequence in the region of the TaqMan probe (a 29 bp sequence) was found to be identical to that of *L. vannamei*, and hence the primers and probe designed on the *L. vannamei* sequence were suitable for analysis in *P. ornatus*.

Constitutive expression of the housekeeping gene

In order to use a house-keeping gene for normalisation, it is necessary first to determine with considerable accuracy that expression of the house-keeping gene is indeed constant in the tissues / samples being analysed. As we were comparing gene expression levels throughout larval development, 18S expression levels were analysed in several larval samples representing a range of developmental time points. RNA-free cDNA was prepared to allow accurate quantification of each cDNA sample so that identical template amounts could be used in each Q-PCR assay. 18S expression per ng cDNA was then determined for a range of samples, and quantified

using a standard curve generated with a cloned plasmid containing the target 18S sequences i.e. this experiment used the absolute quantification approach. The standard curve and the measurements of 18S rRNA expression in different larval samples are shown in Figure 3.4. Testing for significant differences to 99% confidence limit revealed that there was no significant differences between any of the Ct values recorded for the different samples. This indicated that the 18S rRNA copy number per ng cDNA was constant in all samples. Hence the 18S rRNA expression could be considered constitutive and the 18S rRNA gene is suitable for normalisation of gene expression in *P. ornatus* larval samples.

PCR efficiency

The PCR amplification efficiency is a measure of the efficiency with which all available template copies of a gene of interest are copied with each round of amplification, leading to the geometric nature of PCR amplification. Ideally the PCR efficiency will be 100%, but frequently efficiencies below 100% are observed. The PCR efficiency is particularly important when using standard housekeeping genes to normalise expression, as normalisation will only be accurate if the efficiency of amplification of the housekeeping gene is virtually identical to that of the gene of interest. Constant amplification efficiency between individual samples, e.g. larval samples harvested on different days, is also important if the relative expression of the gene of interest is to be compared across a series of samples e.g. a time series. For example, a difference in PCR efficiency of 3% can lead to falsely calculated differences in expression ratio of 40-200% after 25 cycles of amplification (Pfaffl, 2004).

PCR efficiency can be calculated from the data generated by the linear dynamic range experiments. Ct values are plotted against log input cDNA (based on the original RNA concentration determined by OD, assuming 100% reverse transcription efficiency) and the slope of the line is used to calculate the PCR Efficiency (E) according to the equation: $E=10^{(-1/slope)}$ (Bustin, 2002;Pfaffl et al., 2002).

PCR efficiencies were determined for the nuclear hormone receptor genes plus the 18S rRNA in a set of independent cDNA samples generated from larval samples obtained from two independent spawnings and three different days after hatching. Table 3.3 clearly illustrates that there were differences in PCR efficiency between the nuclear hormone receptor homologs and the housekeeping gene in the same sample, and between samples for a particular gene.

Relative efficiency calculations

These data were then used to determine whether the relative PCR efficiencies met ABI's criteria for analysing samples using the Comparative Ct Relative Expression

Technique. ΔΔCt (ΔCt18S - ΔCtGOI) was plotted for each nuclear hormone receptor gene against log (ng RNA) for a range of template dilutions and a regression analysis was performed. The slope of this trend line must be < ± 0.1 for the PCR efficiencies to be considered close enough for the ΔΔCt method to be used directly for analysis (Livak, 1997). A list of the slopes from the Relative Expression Plots for a set of five independent larval samples is given in Table 3.4. The slope of all but three of the relative efficiency plots exceeds ± 0.1 indicating that the 18S rRNA gene cannot be used for normalisation of nuclear hormone receptor gene expression in most of these samples without correction for the differing PCR efficiencies.

As the real-time quantitative PCR technique developed, it became apparent that many quantitative PCR assays fail to meet the strict criteria of near-identical PCR efficiencies between the gene of interest and the housekeeping gene in all samples. Therefore equations have been developed which take into account differing PCR efficiencies (Pfaffl, 2001), using the best available estimate of PCR efficiency for each gene being amplified. This initial analysis determined that it would be necessary to use equations with a PCR efficiency correction factor in all subsequent relative expression analysis of the nuclear hormone receptor genes.

Analysis of results using PCR efficiency correction factor

Calculation of relative gene expression using the PCR efficiency correction factor has been reported by both Pfaffl et al. and Muller et al. (Pfaffl et al., 2002; Muller et al., 2002). Both approaches are very similar, but comparison of the available software for each method revealed that the Q-Gene program by Muller et al (Muller et al., 2002) was more suitable for analysing the spawning experiments than the REST-Software by Pfaffl et al. (Pfaffl et al., 2002) (both programs are available from http://www.wzw.tum.de/gene-quantification/index.shtml).

The Q-Gene Software offers two options for calculating the mean normalised expression values i.e. expression values after using the HKG to normalise all samples for variations in input cDNA. As each sample is analysed in triplicate, the mean normalised expression can be calculated by either averaging three independently calculated normalised values of the triplicate (Q-Gene Equation-2) or by averaging the Ct values of the GOI and the HKG and then calculating the normalised value (Q-Gene Equation-3). If gene analysis is to be performed in a multiplex reaction then Q-Gene's Equation-2 would be the most suitable as the HKG and GOI can be used as paired values. However, when analysis of the GOI and HKG are performed in separate reactions, as was the case for the spawning experiments, then Q-Gene's Equation-3 is the most appropriate equation. This approach was therefore adopted for all subsequent calculation of Mean Normalised Expression for the *P. ornatus* nuclear hormone receptor genes.

Conclusions

Development of the real-time PCR assays for the nuclear hormone receptor genes involved a number of steps. The initial steps were the design of suitable primers and probes based on the DNA sequences reported in chapter 2, followed by optimization of primer concentration. It was then necessary to establish the range of starting material, i.e. the amount of larval RNA that could be used to generate cDNA for use in a quantitative PCR reaction to generate reliable signal. In this case, the range of input RNA or the linear dynamic range was from at least 80 pg to 50 ng RNA translated into cDNA per well for the Hr3 gene, and results presented in chapter 4 indicate a similar linear dynamic range for the other nuclear hormone receptor genes. As the typical yield from a stage 1 phyllosoma was appropriately 250 ng RNA, this indicates that in theory it would be possible to analyse gene expression in individual larvae, although in practice for technical reasons larval RNA was generated from pooled samples of 5-10 larvae.

The relative expression method was selected for the quantitative PCR analysis of the nuclear hormone receptor homologs. This method requires use of a housekeeping gene which has been demonstrated to be constitutively expressed in the samples being analysed. The house-keeping gene is analysed in all samples along with the gene(s) of interest and used to normalise between samples for variations in input template amounts in each reaction. In the case of *P. ornatus*, the 18S rRNA gene was selected, primarily because suitable sequence for designing primers was not available for any other candidate housekeeping genes. However, this proved to be a suitable selection as quantitative analysis of a range of samples indicated that the 18S rRNA gene is indeed expressed constitutively in *P. ornatus* larvae. As ongoing research is continuing to cast doubt on the suitability of other candidate housekeeping genes e.g. β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Goidin et al., 2001; Moe et al., 2001; Bustin, 2002), due to variations in the level of expression of these genes in different samples, it is likely that the 18S rRNA gene would have been the most suitable choice even if other candidate housekeeping genes had been available. The only drawback with the use of the 18S rRNA gene for this purpose is its extremely high level of expression making it necessary to carry out further dilutions of the sample for the quantitative PCR analysis, and therefore prohibiting the use of multiplexed reactions (carrying out PCR for the housekeeping gene and the gene of interest in the same reaction tube).

The major problem encountered in establishing the assays related to the varying PCR efficiencies. As described earlier, differences in PCR efficiencies as small as 3%, either between one of the genes of interest and the housekeeping gene, or between individual samples for a specific gene, can lead to very large inaccuracies in

calculating the relative expression, due to the geometric nature of the PCR reaction. It became clear from analysis presented in this chapter that there are indeed differences of this magnitude between different genes within a sample, and between samples for a given gene. Therefore, it was determined that it would be necessary to use PCR correction factors to calculate the relative expression of the four nuclear hormone receptor genes across a range of different rock lobster larval samples as described in the following chapter.

Table 3.1: Primers and Taqman probes used in quantitative PCR assays

PROBE OR PRIMER NAME/ TARGET GENE	SEQUENCE	LABEL	CODING STRAND OR COMPLEMENTARY STRAND	OPTIMISED CONCENTRATION IN REACTION	COMMENTS
EcR probe	6FAM-TCC CTC ACA GGT AAG C-MGB	FAM	Complementary		Taqman MGB probe for EcR
EcR-DBD-Fwd	GGG CGT CAG GCT ACC ATT ATA A			100 nM	Forward primer for EcR Q-PCR
EcR-DBD-Rev	GAT GGA TCT CCG AAA GAA ACC TT			100 nM	Reverse primer for EcR Q-PCR
HR3 probe	6FAM-ATC ACC AGT GCC CCC GCC AGA AG-TAMRA	FAM	Coding		Taqman TAMRA probe for HR3
PoHR3-FWD	CGC AGT CCT CGG TGG TG			300 nM	Forward primer for HR3 Q-PCR
PoHR3-REV	CGG TCC ACG ACG CAG TT			300 nM	Reverse primer for HR3 Q-PCR
HR4 probe	6FAM-ACA ATC TCC ATC AGC AAC A-MGB	FAM	Complementary		Taqman MGB probe for HR4
PoHR4-FWD	CGA ACA GTA CAG AAT AAG CGG GTA T			300 nM	Forward primer for HR4 Q-PCR
PoHR4-REV	GTT ACG CTG TGC TTT TGT GAT TTC			300 nM	Reverse primer for HR4 Q-PCR
HR78 probe	6FAM-CCT CGC ACG TCT TGT T-MGB	FAM	Complementary		Taqman MGB probe for HR78
PoHR78-FWD	CGT AAG CAG CTC GGC TAC ACT T			100 nM	Forward primer for HR78 Q-PCR
PoHR78-REV	CCT ATT GCG GTG GTG TTT AGT GA			100 nM	Reverse primer for HR78 Q-PCR
18S probe	VIC-CTT CGA ACC TCT AAC TTT-MBG	VIC	Complementary		Taqman TAMRA probe for 18S
18S-Fwd	AAA GCA TCT GCC AAG GAT GTT T			900nM	Forward primer for 18S Q-PCR
18S-Rev	GGT TAG AAC TAG GGC GGT ATC TGA			900nM	Reverse primer for 18S Q-PCR

6FAM = 6-carboxyflurocein, absorption wavelength maximum 495 nm, emission wavelength maximum 521 nm.
VIC = Proprietary to Applied Biosystems, absorption wavelength maximum 538 nm, emission wavelength maximum 552 nm.
TAMARA = N,N,N^1,N^1-tetramethyl-6-carboxyrhodamine, absorption wavelength maximum 555 nm, emission wavelength maximum 580 nm
MGB = Minor Groove Binder, a 'dark quencher' which inserts into the minor groove of double helical DNA and quenches fluorescence from the 5' fluorescent label by absorbing emitted fluorescence and re-emitting it as heat-energy not fluorescence.

Table 3.2: Location of Q-PCR primers and probes on *P. ornatus* nuclear hormone receptor DNA binding domain sequences, and on 18S rRNA gene

Name	Nucleotide Sequence
P. ornatus Hr3	1 CCGCTGGTCGCAGTCCTCGGTGGTGAACTACCAGTGCCCCCGCCAGAAGAACTGCGTCGTGGACCGCGTCAACCGAAACCGC
P. ornatus Hr78	1 CAAAAGGTCAATCCGTAAGCAGCTCGGCTACACTTGCCGGGGGAACAAGACGTGCGAGGTCACTAAACACCACCGCAATAGG
P. ornatus Hr4	1 CAAGCGAACAGTACAGAATAAGCGGGTATACACATGTGTTGCTGATGGAGATTGTGAAATCACAAAAGCACAGCGTAACCGT
P. ornatus EcR	1 GACAGGGCGTCAGGCTACCATTATAATGCGCTTACCTGTGAGGGATGCAAAGGTTTCTTTCGGAGATCCATCACCAAGAATG
P. ornatus 18S rRNA gene	1 AAAGCATCTGCCAAGGATGTTTTCATTGATCAAGAACGAAAGTTAGAGGTTCGAAGGCGATCAGATACCGCCCTAGTTCTAACC

Single underline = forward and reverse real-time primers
Double Underline = Real-time probe (designed on positive strand)
Double Underline = Real-time probe (designed on negative strand)

Table 3.3: PCR efficiencies for PoHr3, PoHr4, PoHr78, PoEcR and 18S assays for 5 larval samples

Sample	Age (days)	PCR Efficiency (%)					
		PoHR3	PoHr4	PoHR78	PoEcR	18S rRNA	Range of Efficiency between Assays
RLLR-14	7	92.0	94.6	83.8	87.0	86.9	83.8-94.6
RLLR-24	7	101.7	84.3	83.9	87.7	96.6	83.9-101.7
RLLR-16	8	104.2	91.2	98.1	94.2	86.0	86.0-104.2
RLLR-25	8	94.9	90.5	81.5	85.6	92.7	81.5-94.9
RLLR-18	9	102.4	94.5	76.7	100.0	93.9	76.7-102.4
Range of PCR Efficiency between samples		92.0-104.2	84.3-94.6	76.7-98.1	85.6-100	86.9-96.6	

Table 3.4: Slope from relative expression analysis to compare PCR efficiencies between NHR assays and 18S

	Sample				
Assay	RLLR-14	RLLR-24	RLLR-16	RLLR-18	RLLR-25
PoHr4	0.224	-0.3599	0.1575	0.0167	-0.0611
PoHR78	-0.1172	-0.3753	0.341	-0.568	-0.3527
PoEcR	0.0056	-0.2522	0.2398	0.1626	-0.212

Only the values highlighted meet the criterion that the slope of the plot of the relative efficiencies of the gene of interest compared to the housekeeeping gene should be < ±0.1.

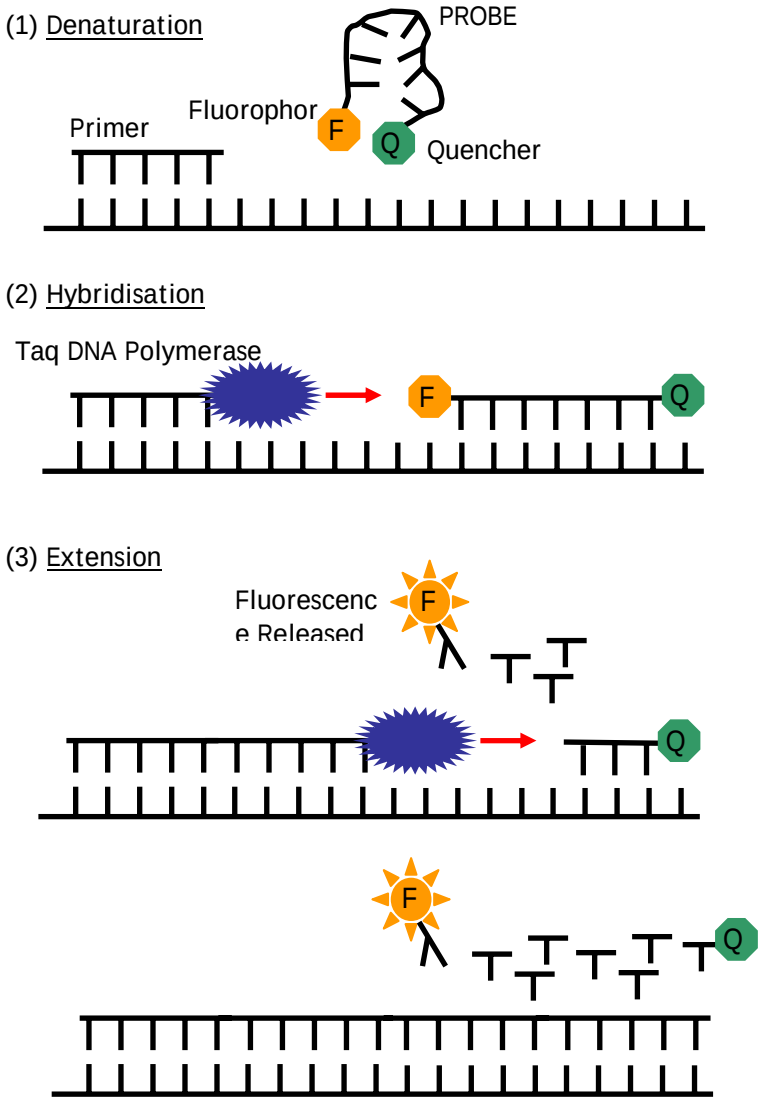

Figure 3.1: TaqMan Probe System
(Figure 3.1 is taken from http://biology.queensu.ca/~tyshenko/settingup.htm)

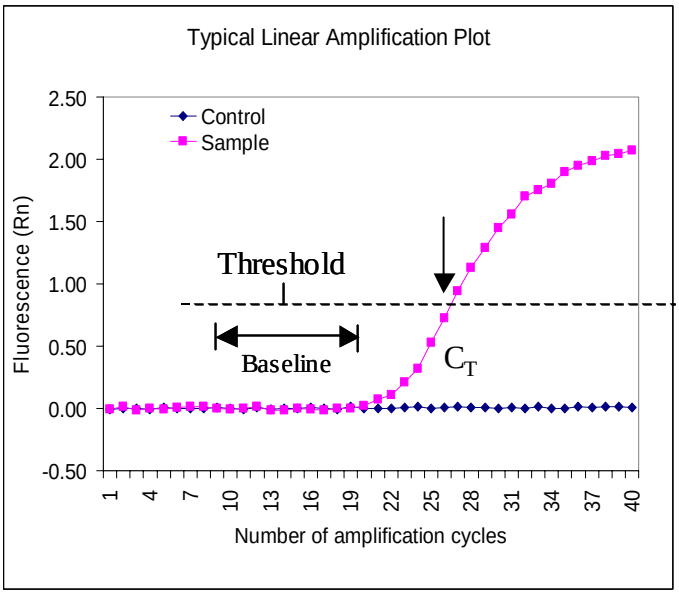

Figure 3.2: Illustration of a typical Taqman assay for a highly expressed gene and a less abundant gene.

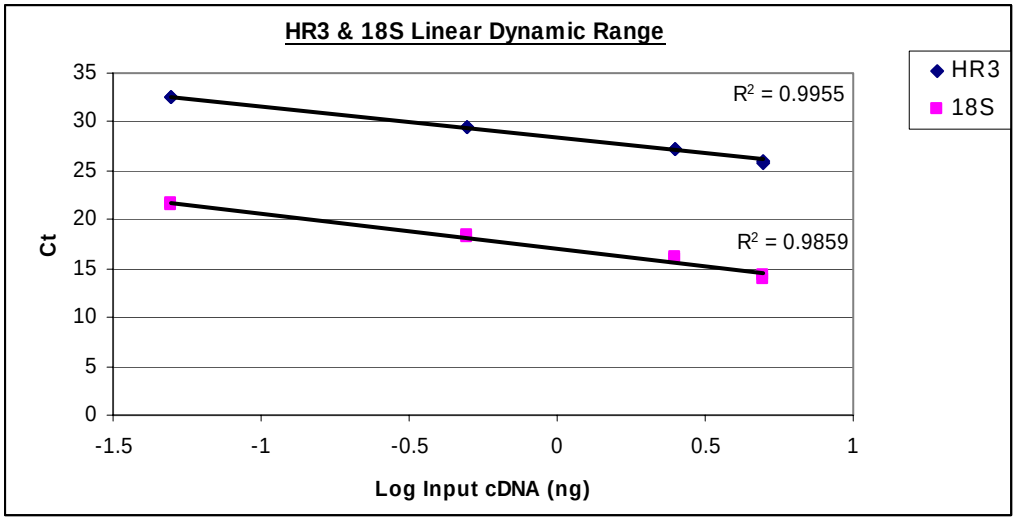

Figure 3.3: Hr3 & 18S linear dynamic range experiment.

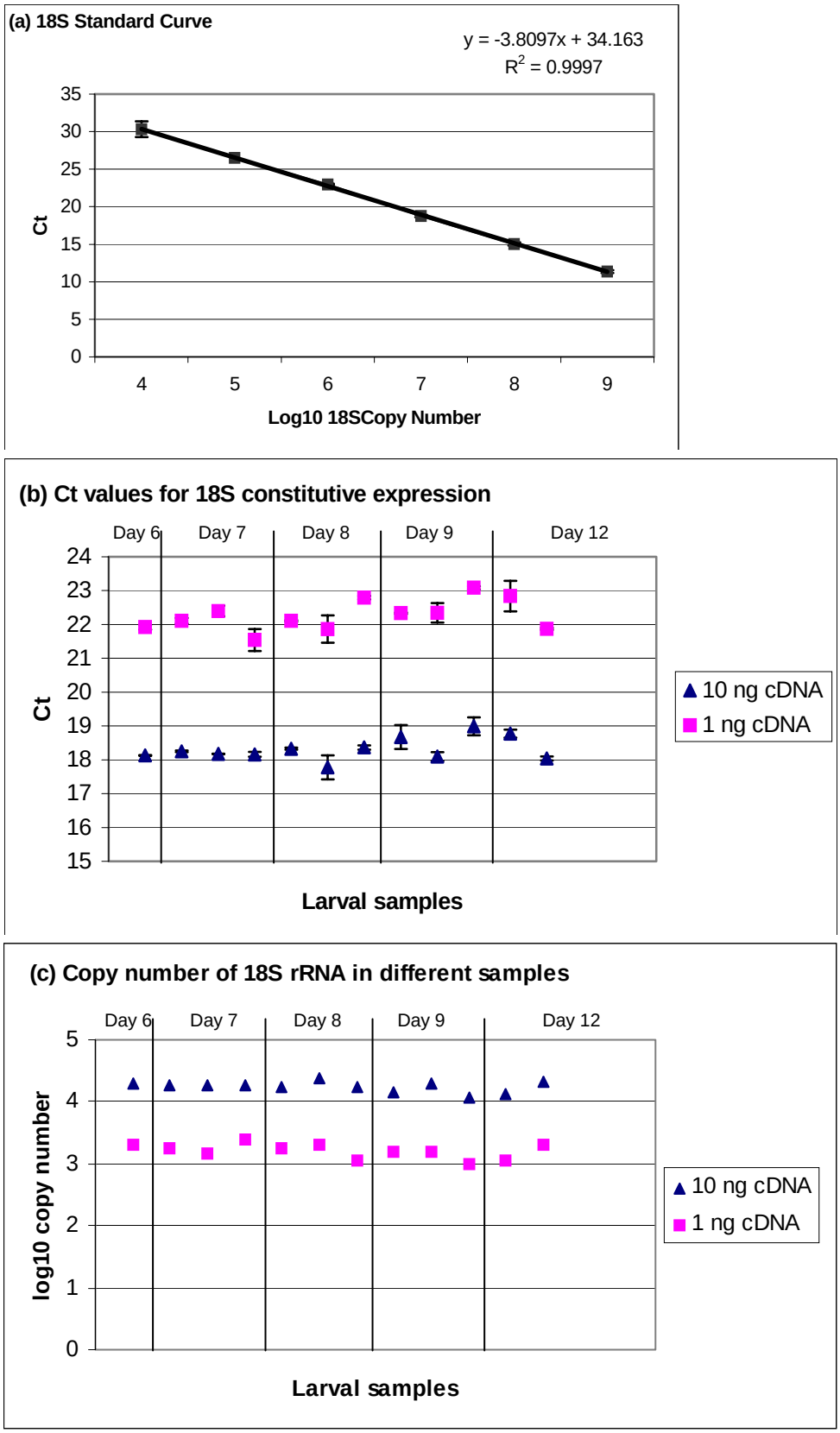

Figure 3.4: 18S constitutive expression. (a) Standard curve generated using cloned plasmid DNA containing the target 18S gene sequence. (b)Variation in critical threshold measured for the 18S rRNA gene using either 1 ng or 10 ng of each cDNA sample. cDNAs derive from replicate larval samples taken on different days of development. Results showed no significant differences between samples. (c) Data as in b) expressed as log10 copy number per sample.

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4. ANALYSIS OF NUCLEAR HORMONE GENE EXPRESSION THROUGH EARLY *P. ORNATUS* LARVAL DEVELOPMENT

Introduction

A central goal of this project was to analyse changes in the abundance of nuclear hormone receptors during larval development. Chapter 2 described isolation of the candidate nuclear hormone receptors from *P. ornatus*, EcR, Hr3, Hr4 and Hr78. Chapter 3 described the development of real-time quantitative PCR assays for these genes, and for a control housekeeping gene the 18S rRNA gene. In this chapter we describe the application of these assays to study changes in the expression of these genes through successive larval molts in *P. ornatus* phyllosomas.

Materials and Methods

Broodstock

P. ornatus broodstock were obtained from M.G, Kailis sourced from the Torres Strait.

Captive broodstock were maintained in 4.5 m^2 diameter outdoor tanks with a water depth of 0.85 m. Incoming water was obtained from the near-shore waters off Cape Ferguson, settled for 24 hours and passed through a 5 micron filter before flowing into the holding tank. There was 100% water exchange per day. Annual variation in water temperature was 20-29.5°C. During the breeding season (Nov-Mar) water temperature ranged between 26-29.5°C, salinity between 29-35 ppt and pH between 8.1 and 8.5. The broodstock were fed daily *ad libitum* with a combination of squid, mussels and pippies. Males and females were maintained at a ratio of 5 females/2 males in the same tanks.

Hatching

Berried females were removed from holding tanks several days prior to hatching and treated with antibiotics (0.4 g/100 L oxytetracycline, 0.6 g/100 L erythromycin and 1.0 g/100 L streptomycin) and 25 ppm formalin for 2 hrs prior to placement in a 5000 L hatching tank (refer to Appendix IV). Quality and development of the eggs were checked every second day and if filamentous bacteria were observed on the eggs, the hatching tank received an extra treatment of 1 ppm Virkon S and a 40% water exchange. After the phyllosoma hatched they were transferred into a larval rearing tank. The female was then returned to its holding tank.

Larval rearing

Larvae hatched into 5000 L tanks typical of the commercial mass culture tanks used in penaeid prawn hatcheries. Upwelling was created with a submerged inlet pipe along the length of the tank bottom, with an average flow rate of 600 L/hr. Larval rearing systems were set up as a recirculatory system and given a 50% daily water exchange with 3 ppm of Virkon S.

All phyllosomas were fed on *Artemia* (Prime Brine Shrimp, A Grade). Cysts were decapsulated in 50 gram batches per 50 L hatching tank. Eighteen hours after *Artemia* hatched they were supplemented with 0.6 g/L of DC Super Selco and enriched for 17-24 hrs. Phyllosomas were fed twice daily at 09:00 and 16:00 hours. *Artemia* were added to obtain a density of 1-4 per ml. If *Artemia* remained in the tank by the next feed, the tank was only topped up to reach the 4 per ml target.

For the first 7 days, phyllosomas were fed newly-hatched *Artemia*. Subsequently they were fed with a 50:50 mixture of newly hatched and enriched *Artemia.* After day 21 they were only fed enriched *Artemia*.

The larval rearing tanks were treated with a variety of antibiotic and chemical treatments depending on the appearance and quality of the tank and water. Details of additives to the rearing tanks can be found in Appendix IV (Table 22).

Larvae that were sub sampled for molt synchronization were transferred to 50 L tanks. Other details of water treatment and feeding were as for the 5000 L tanks.

Larval sampling

For each spawning run, larvae were collected daily from the first day after hatching through as many molt stages as larval survival allowed. Due to the fact that transition from one stage to the next occurs over several days, larvae were collected daily from a given stage up until the appearance of a new molt. At this time collection of the previous stage ceased and sufficient numbers of larvae (~1000 larvae) that had reached the next molt stage were transferred to separate larval rearing tanks to synchronise the molt cycle as much as possible.

For each of five spawnings, up to twenty larvae were collected where possible from tanks at each time point, placed directly into RNALater (Ambion, Sigma, Qiagen) and stored at 4°C until larval collection was completed. The number of larvae required for RNA extraction decreased as larvae increased in size. While a minimum of 20 larvae were required at stage P1, only 5 larvae were required by the P4 stage.

RNA preparation

RNALater was first removed and then RNA was extracted from pooled larvae from each day of sampling using NucleoSpin® RNAII (Machery-Nagel #740955) following the manufacturer's instructions. Larvae were ground using a Teflon pestle in a 1.5 ml microfuge tube, then sheared by 10-20 passages through a 21 G needle. RNA was DNase-treated using the on-column digestion as outlined in the NucleoSpin® protocol, quantitated by $OD_{260/280}$ and stored at –80°C.

Reverse transcription reactions

All RT reactions for a given spawn were set up at the same time using 200 U SuperScriptIII (Invitrogen #18080-044) according to the manufacturer's instructions with 400 ng total RNA, 40 U RNAseOUT Recombinant Ribonuclease Inhibitor (Invitrogen #10777-019) and 250 nM random hexamers in a 20 µl reaction. Both positive and negative (i.e. with nuclease free water added in place of reverse transcriptase) RT reactions were set up for each larval sample. Reactions were incubated at 45°C for 30 min and inactivated at 70°C for 15 min at completion of transcription.

TaqMan assays

SAMPLE DILUTION DETAILS

Due to the extremely high expression of the 18S rRNA gene in relation to the nuclear hormone receptor genes (refer to template optimisation assays – Chapter 3), analysis of samples was performed using 1 ng cDNA for 18S and 10 ng cDNA for ECR, Hr3, Hr4, Hr78. The concentration of larval cDNA samples resulting from reverse transcription was assumed to be equivalent to the amount of input RNA. Samples were diluted to provide sufficient template for analysis of all four nuclear hormone receptor genes, together with sufficient template for subsequent dilution for the 18S rRNA assay. Each cDNA sample (and negative RT sample) was diluted to 5 ng μl^{-1}, 1 µl was removed and diluted further to 0.5 ng μl^{-1}. The cDNA dilution at 5 ng μl^{-1} was used for analysing the nuclear hormone receptor genes (2 µl = 10 ng in triplicate wells for four genes), and the cDNA dilution at 0.5 ng μl^{-1} was used for analysing the 18S rRNA (2 µl = 1 ng in triplicate wells).

PCR EFFICIENCY MEASUREMENTS FOR INDIVIDUAL SPAWNINGS

To determine the PCR efficiency for PoHr3, PoEcR, PoHr4, PoHr78 and 18S rRNA for each spawning experiment, a pooled cDNA sample was prepared by combining equal volumes (2 µl) of every larval RT reaction within a spawning. The PCR efficiencies were calculated by preparing a series of cDNA dilutions in triplicate, and then analysing these triplicate samples for each gene by Q-PCR as described in Chapter 3.

Q-PCR MASTER MIX

To reduce variability between samples and consecutive Q-PCR runs, sufficient stocks of primer/probe reaction master mix were prepared for each assay to allow all samples from five spawnings to be analysed. Primer/probe reaction mixes consisted of 0.595 µM probe and forward and reverse primer concentrations of 0.238 µM for EcR, 0.238 µM for Hr78, 0.714 µM for Hr3, 0.714 µM for Hr4 and 2.14 µM for 18S (primer sequences are in Chapter 3, Table 3.1). Sufficient volumes were prepared to subsequently allow 10.5 µl of each primer/probe reaction mix to be mixed with 12.5 µl 2x TaqMan Universal Mix and 2 µl template cDNA (or negative RT reaction) for a final 25 µl PCR reaction. Stocks were frozen in aliquots at -20°C.

Q-PCR REACTIONS

All assays were performed in ABI 96-well bar-coded plates (ABI 4346906) and heat-sealed using an optically clear adhesive cover (ABI 4313663). One tube of Primer/Probe Reaction Mix was thawed for each target gene being assayed. 178.5 µl of primer/probe mix and 212.5 µl of 2 x TaqMan Universal Mix was then mixed per 96-well plate to make the Final Master Mix. Sufficient 2 x Taqman Universal Master Mix was purchased from a single batch to ensure that the same batch number was used for all assays from a single spawning. 23 µl of each Final Master Mix were aliquotted using a positive displacement pipette into triplicate wells for each sample. All Master Mix stocks were aliquotted in a "clean" PCR workstation area and then plates transferred to a standard lab bench for addition of 2 µl template cDNA or negative RT reaction. Final concentrations were 10 ng cDNA per well for the nuclear hormone receptor genes or 1ng cDNA per well for the 18S rRNA gene, 250 nM probe, 100 nM EcR or Hr78 primers, 300 nM Hr3 or Hr4 primers, or 900 nM 18S primers. Each 96-well plate contained triplicate analyses for four larval samples for all five genes, plus a negative RT reaction analysed for Hr3, plus single wells of no template control for each gene.

All Q-PCR reactions were performed on the ABI Prism® 7700 Sequence Detection System using the standard non-UNG TaqMan cycling parameters as described in Chapter 3 (see also Livak, 1997).

The Mean Normalised Expression for each sample, taking into account the measured PCR efficiencies, was calculated as described in Chapter 3. The Relative Expression was then calculated by dividing the MNE for each sample by the MNE for the calibrator. In this work, the MNE for Day-1 larvae for each spawn was used as the calibrator. Hence Relative Expression was calculated as:

$$\text{Relative Expression} = \frac{MNE_{sample}}{MNE_{calibrator}} = \frac{E_{sample\text{-}HKG}^{(Ct\text{-}mean)} / E_{sample\text{-}GOI}^{(Ct\text{-}mean)}}{E_{calibrator\text{-}HKG}^{(Ct\text{-}mean)} / E_{calibrator\text{-}GOI}^{(Ct\text{-}mean)}}$$

Where E = PCR efficiency and Ct means critical threshold.

The Q-gene software was used for these calculations (Muller et al., 2002), available at http://www.wzw.tum.de/gene-quantification/index.shtml.

Genomic DNA contamination controls

During optimisation (Chapter 3) the Hr3 nuclear receptor assay was selected for use as an indicator of genomic DNA contamination due to the limitation of the number of wells on each assay plate. The Hr3 assay was chosen as it gave the highest genomic DNA signal of the assays tested during optimisation (although still below 1% - see Chapter 3). Hence negative RT reactions were run for Hr3 for each of spawns 5, 6, 8 and 11. Due to limited stocks of the Hr3 probe for the final analysis of Spawn-14, the genomic DNA contamination assay for these samples was performed with Hr4 – the assay that gave the next highest DNA contamination result during optimisation.

Results

Spawnings used for sample collection

Spawnings that were suitable for Q-PCR analysis of nuclear hormone receptor gene expression had to provide sufficient numbers of larvae for sampling through to at least the phyllosoma P3 stage (third instar), and ideally to the P4 stage. Due to early problems in large scale larval rearing, with mass mortalities at the P2 stage (Bourne et al., 2004), not all spawnings were suitable. Of fourteen spawning runs, only five were suitable for analysis: Spawn-5, 6, 8, 11 and 14.

Larval sampling strategy

A larval molt ideally is completed for all larvae in a cohort over a 2-3 day period. However, depending on the health of the larvae this can extend out to 10 days or more – see Table 4.1 for the duration of the molts in the larval samples used in the present work. In order to obtain sufficient RNA to analyse all the target genes, larval samples collected on each day had to be pooled. The fact that the larvae do not all molt on the same day presented serious difficulties for the sampling regime, as there is no morphological way of distinguishing which larvae are just about to molt from ones that may not be ready to molt for several days. This means that there is a high likelihood that a combined sample is in fact a sample of larvae at quite different stages of the molt cycle. This in turn could cause a dilution effect, wholly or

partially masking any spikes in gene expression that may occur at specific points during the molt cycle.

To try to minimize this effect, larvae were resynchronised at each molt. This was achieved by removing a subset of larvae that molted at the onset of the molt period, rearing them in a separate tanks, and taking subsequent daily samples from this tank until the next molt. Thus for example in spawn 6, phyllosoma began to molt to stage P2 on the 8th day after hatching, but all phyllosoma only completed this transition on the 11th day after hatching. Thus the larval sample collected on day 7 (20 larvae) probably comprised a mixture of larvae that were between 1-4 days pre-molt. Those that had molted to stage P2 on day 8 were collected and transferred to a separate tank, thus resynchronising the next molt period. Nevertheless, the next molt from P2-P3 was again spread out over 5 days, and the molt had to be resynchronised by collecting newly molted P3s on day 15 and transferring them to a separate tank for daily sampling. Table 4.1 briefly outlines the sampling regime for each of these spawns. Full details of sampling and collection can be found in Appendix IV: Tables 16-22.

PCR efficiency

As PCR efficiency may vary between individual samples, Pfaffl et al. (2002) recommend using a pool of all available cDNAs being assayed to determine a common PCR efficiency value for each gene which can then be applied to all samples during subsequent calculations of gene expression. Different template ranges were used to calculate PCR efficiencies for the nuclear hormone receptor genes compared to the 18S gene, due to the fact that the 18S assay was not linear above 5 ng cDNA and the nuclear hormone receptor genes could not be detected much below 0.05 ng cDNA template.

Figure 4.1 illustrates a typical PCR efficiency experiment, and actual PCR efficiencies for the five genes analysed, across all five spawnings, are presented in Table 4.2.

Genomic DNA contamination controls

Generally genomic DNA contamination was not a problem, as it represented <1% of the total signal. There were a few samples from Spawn-5 and Spawn-14 where the negative reverse transcription control for genomic contamination indicated possible genomic contamination in excess of 1%. A list of the individual samples and the percentage of genomic DNA contamination observed is detailed in Table 4.3 and asterisked in Figures 4.2-4.5. While the contamination was still very low, this should be borne in mind as a possible source of error for those particular samples.

Nuclear hormone expression analysis of P. ornatus larvae

The Relative Expression models of Pfaffl (2002) and Muller (2002) were used, taking into account variations in PCR efficiencies. Relative expression measures gene expression relative to a given calibrator, which might be a gene expression at a particular time point, in a particular tissue, or from samples prior to some specific treatment. In the present work, the levels of gene expression in day 1 larvae were used as the calibrator (Figs 4.2-4.5 and 4.6a-4.10a). The relative gene expression is thus the level of gene expression relative to day 1 larvae. As it is calculated by dividing the mean normalised gene expression on day X by that on day 1, it does not have units. Relative expression was also calculated using the day on which the lowest mean normalised expression was observed for each gene in each sample (Figs 4.6b-4.10b).

In the present work, differences in PCR efficiencies were detected between individual samples within a spawn (Chapter 3). However, since the difference in PCR efficiency between individual samples could not be overcome, an averaged value for the PCR efficiency correction factor was determined for each assay using a pooled cDNA sample consisting of equal amounts of cDNA from each sample within a Spawn as recommended in the literature (Pfaffl et al., 2001; Rasmussen, 2001). Using this approach, it is assumed that the average correction factor will be close enough to that for individual samples to allow detection of when certain genes are up or down regulated during the molt cycle. However, the fact that these samples did not fully meet the current accepted requirements for the mathematical model for Comparative Relative Expression means that some degree of uncertainty may lie in the exact values calculated for the changes in gene expression.

Relative gene expression for each nuclear hormone receptor analysed is presented as a set of graphs. Figures 4.2-4.5 comprise the respective nuclear hormone receptor results for each spawn analysed, presented separately with standard errors. Note that the scales differ between individual graphs. Figures 4.6-4.10 present the combined data for each nuclear receptor for individual spawns. The data are expressed both as gene expression relative to that observed on day 1 (a), and as gene expression relative to the lowest detected value of gene expression (b). The raw data for each graph can be found in the Appendix II.

Discussion

Figures 2-10 illustrate the inherent variability with these data, as there is clear variation in results both between different spawns – e.g. spawns 8, 11 and 14 seem to show a higher fold induction of individual genes than spawns 5 and 6 – and

between different larval instars e.g. compare the variation of expression in receptor genes in spawn 14 P1 with the lack of variation in spawn 14 P2.

This variability could be attributed to a number of factors. One is the lack of synchronisation among sampled larvae. For example, in spawn 14 both the P1-P2 transition and the P2-P3 transition spread over 6 days before the entire cohort of larvae had completed their molt, and hence larvae sampled just prior to the transition could have been at any stage from 1-6 days pre molt. Unfortunately there is no way of determining which larvae are about to molt from their morphology.

A second issue is the extreme sensitivity of the real-time PCR assay, which may in fact lead to significant errors. For example, the issue of variations in PCR efficiency between samples was addressed here, as in other published work, by calculating a PCR efficiency for each gene for pooled larval samples from a given spawn. This was done because the logistics and expense make it impractical to calculate PCR efficiencies for every individual sample and gene. However, as discussed in chapter 3, even small discrepancies in PCR efficiency can be substantially magnified due to the geometric nature of the PCR reaction, leading to major errors when comparing two samples.

Another level of error may be introduced in variations in the efficiency of the reverse transcriptase reaction. Here the assumption was made, as in the published literature, that reverse transcriptase converts RNA into cDNA with 100% efficiency. In fact, our unpublished data and published work (Stahlberg et al.,2004) suggest that this efficiency can vary from well below 100% to substantially over 100% - the latter presumably due to reverse transcriptase transcribing the same template more than once. Although every attempt was made to standardise conditions in the present work, by always using identical reaction conditions and by buying in a large amount of a single batch of reverse transcriptase to ensure that all larval samples from a given spawn were processed with the same batch of enzyme, this cannot be ruled out as another source of error.

The only way to control for the errors that may be inherent in the reaction is to analyse replicate samples from the same day. However, the additional expense involved would be considerable, as it would require the entire processing, from larval RNA extraction through reverse transcription to quantitative PCR. At the outset of this project we made a decision to invest in replicating the analysis across as many spawns as possible, rather than replicating large numbers of samples within a single spawn.

Despite these reservations, and the obvious variability in the data, some general trends do emerge. The Hr4 receptor is clearly the receptor that shows the greatest variation in the measured level of expression across molts, with a 45 fold variation in the level of expression being apparent in spawn 8. It also appears to show peak

expression just prior to a molt, with the level of expression declining to basal levels on molting. The Hr3 gene shows a similar pattern of expression, with substantial induction prior to molt. Its pattern of expression appears to closely mirror that of Hr4, except that Hr4 expression may persist slightly after that of Hr3 declines. This exactly parallels the pattern of expression observed for these genes in the second larval instar of *Drosophila*, where DHr3 and DHr4 were both induced pre-molt, but DHr3 expression declined immediately pre-molt whereas DHr4 expression declined exactly at molt (Sullivan and Thummel, 2003).

The *P. ornatus* Hr78 receptor gene showed relatively little variation in the level of expression. What variation was detected indicated a possible increase in expression just post-molt (see Figure 4.5 and Figures 4.9 and 4.10 in particular). In *Drosophila*, DHr78 is expressed at a fairly constant level throughout the second and third larval instars, but shows an increase in expression just after puparium formation, indicating a commitment to metamorphosis. While it is hard to draw parallels with the gradual metamorphic shifts in *P. ornatus*, it is conceivable that the *P. ornatus* Hr78 is playing a role in directing new morphological development at each larval stage.

The ecdysone receptor is a key regulator of the transcriptional hierarchies associated with molting and metamorphosis. The overall variation in the level of expression was quite small across all spawnings (Figures 4.6-4.10). However, it is apparent that expression does peak mid-molt. This is consistent with it being induced early in the regulatory cascade, and before induction of other nuclear hormone receptors such as Hr3 and Hr4. It should be noted that in *Drosophila* and other species there are several isoforms of the ecdysone receptor, expressed from the same gene but created by differential mRNA splicing (Talbot et al., 1993) and the pattern of expression of individual isoforms differs (Sullivan and Thummel, 2003). While we have not investigated the possible existence of isoforms in *P. ornatus*, the quantitative PCR assay used targeted the DNA binding domain, which is common to all isoforms, and therefore it would not have distinguished between the different isoforms.

In summary, we have been able to measure the pattern of expression of four candidate nuclear hormone receptor genes through early larval development in four *P. ornatus* spawnings. While the data are variable, it is possible to recognise patterns of gene expression which suggest that patterns of hierarchical gene expression occur in *P. ornatus* in a similar manner to that observed in *Drosophila* and other insects. Moreover, the hierarchical pattern parallels that observed in *Drosophila*, suggesting that mechanisms controlling molting and metamorphosis may well be conserved between insects and crustaceans. This is the first analysis of its kind to demonstrate hierarchies of gene expression controlling molting in a crustacean.

Table 4.1: Details of spawns and sampling regimes

SPAWN NO.	DATE OF SPAWN	P1 SAMPLES COLLECTED	P1-P2 TRANSITION	P2 SAMPLES COLLECTED	P2-P3 TRANSITION	P3 SAMPLES COLLECTED	P3-P4 TRANSITION	P4 SAMPLES COLLECTED	COMMENTS
5	22/01/2003	10 days (Day 1-10)	7 days (Day 11-17)	6 days (Day 14[a]-19)	3 days (Day 19-22)	8 days (Day 20-27)	5 days (Day 27-31)	5 days (Day 27-31)	Insufficient numbers of larvae to resynchronise P4s.
6	04/02/2003	7 days (Day 1-7)	4 days (Day 8-11)	7 days (Day 8-14)	5 days (Day 15-19)	8 days (Day 15-22)	none	none	
8	09/04/2003	9 days (Day 1-9)	5 days (Day 8-12)	9 days (Day 8-16)	6 days (Day 16-21)	5 days (Day 16-20)	none	none	
11	22/01/2004	9 days (Day 1-9)	4 days (Day 8-11)	9 days (Day 8-16)	7 days (Day 20-26)	11 days (Day 19-29)	none	none	
14	04/04/2004	9 days (Day 1-9)	6 days (Day 9-14)	7 days (Day 9-15)	6 days (Day 16-21)	11 days (Day 16-26)	3 days (Day 24-26)	3 days (Day 24-26)	Insufficient numbers of larvae available to resynchronise P4 samples.

[a] *Spawn-5*: Rather than selecting P2s at first day of molt, P1 larvae were collected on Day 13 that were thought to be close to molting and transferred to a separate tank. On Day 15 all P1s that had not molted to P2s were removed, leaving a mixture of P2s at stage P2 day 1-2 in the tank for sampling.

Table 4.2: PCR efficiency values for each gene and each larval spawning run.

		PCR EFFICIENCY (%)				
ASSAY	TEMPLATE RANGE	SPAWN-5	SPAWN-6	SPAWN-8	SPAWN-11	SPAWN-14
18S	0.5 pg-0.5 ng	87	85	86	82	82
EcR	50 pg-20 ng	101	93	95	106*	96
HR3	50 pg-20 ng	91	98	102	93	91
HR4	50 pg-20 ng	93	95	92	87	85
HR78	50 pg-20 ng	101	90	95	91	77

*Spawn-11 EcR Assay template range: 500 pg-20 ng

Table 4.3: Samples in which Genomic DNA contamination was potentially >1%.

SPAWN	SAMPLE	%GENOMIC DNA CONTAMINATION
5	P1-d1	3.12
5	P1-d2	1.23
5	P1-d3	1.02
5	P1-d8	1.30
14	P1-d1	1.35
14	P1-d5	3.47
14	P2-d4	1.45
14	P2-d5	1.11
14	P2-d6	2.50
14	P3-d2	1.79
14	P4-d2	1.57

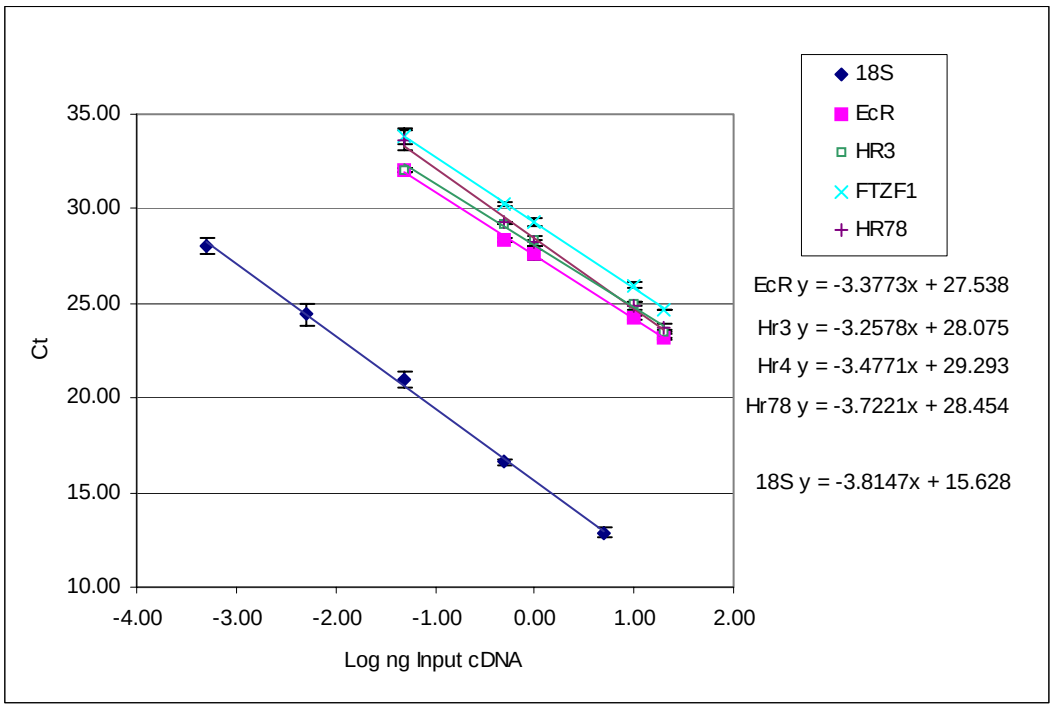
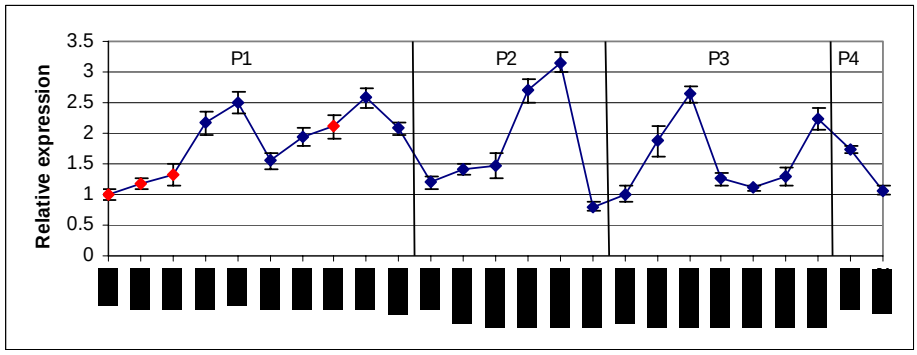


Figure 4.1: Example of Linear Dynamic Range (PCR Efficiency) plots for each Q-PCR assay from a single spawning (pooled larval samples).

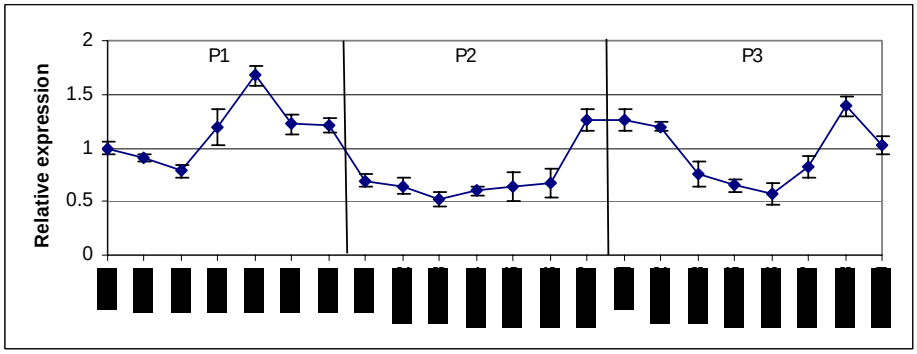
Figures 4.2-4.5: Expression of EcR (Fig. 4.2), Hr3 (Fig. 4.3), Hr4 (Fig. 4.4) and Hr78 (Fig. 4.5) across all five spawns. The standard errors are the errors of three replicate Q-PCR analyses but do not represent independent larval samples. Data are based on pooled larval samples as described. Where a range of days is indicated for a particular stage, this is because the larvae were not resynchronised until more than one day after the molt began (see Appendix IV, for full details of sampling regimes). Hence it is not possible to specify with certainty whether the larvae were, for example, 1 or 2 days old when separated into a new tank. Red data points represent samples were genomic DNA contamination was detected at >1%. Note that because the values represent relative gene expression rather than absolute values, and because larval sampling was carried out over a different number of days for each spawn, the graphs for each spawn have individual scales on the x and y axes.

Figure 4.2: EcR Expression

Spawn 5



Spawn 6



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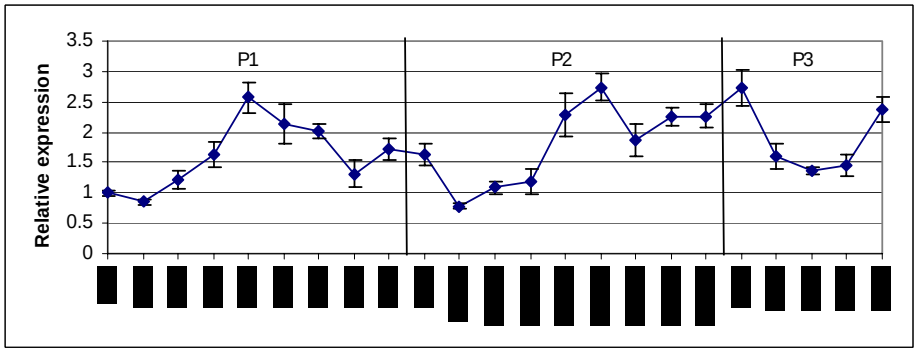
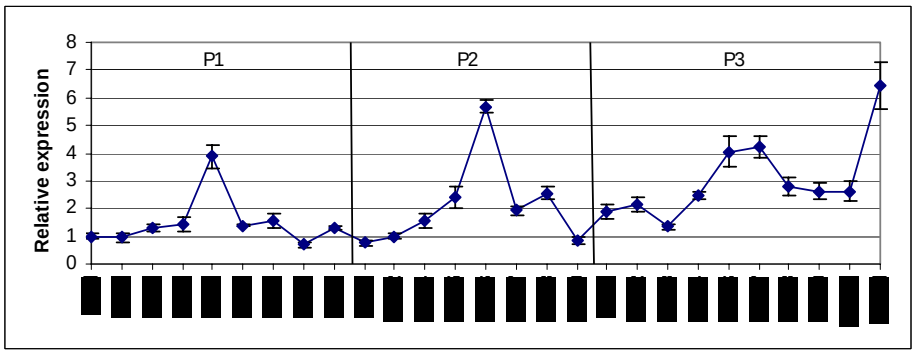


Spawn 11



Spawn 14

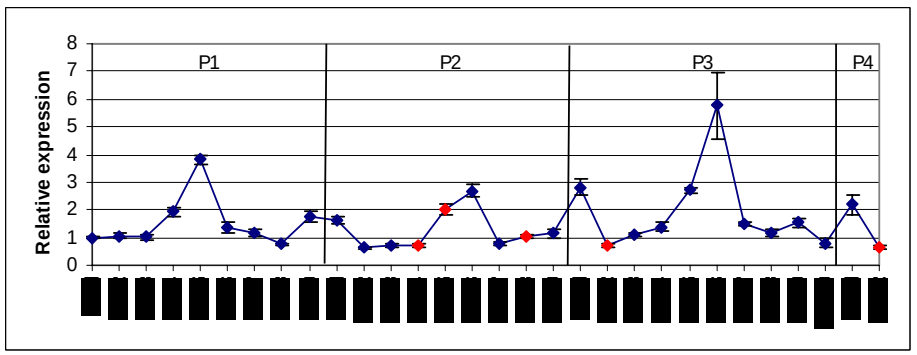
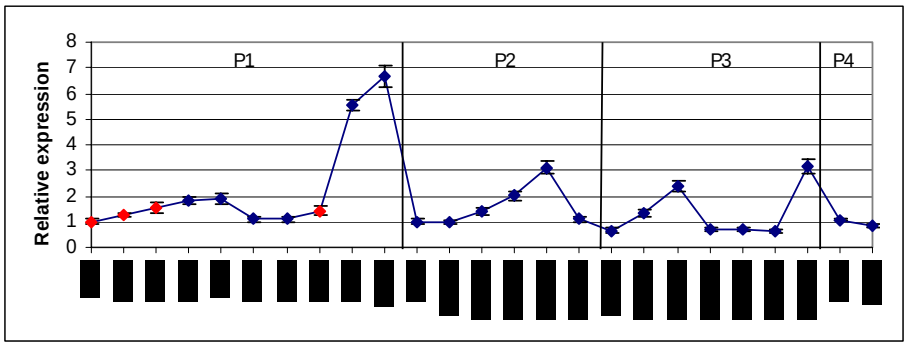


Figure 4.3: Hr3 Expression

Spawn 5



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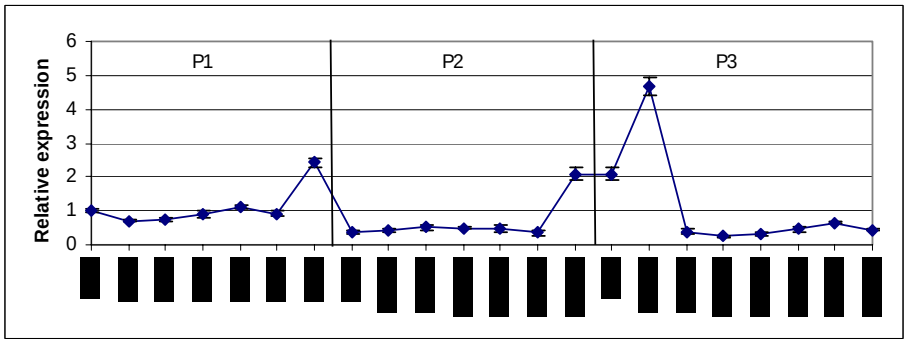
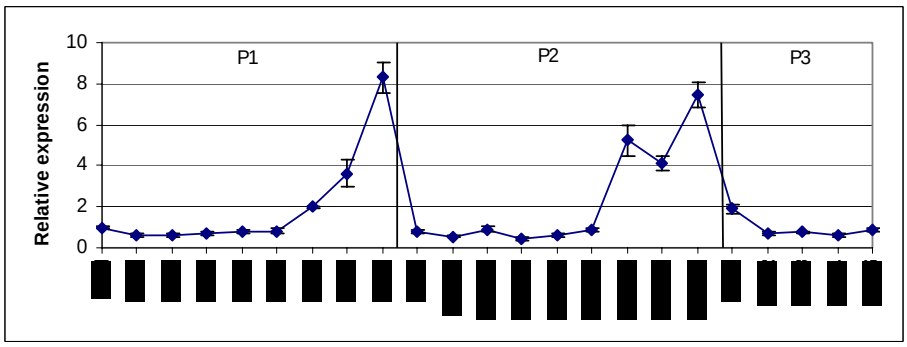


Spawn 8



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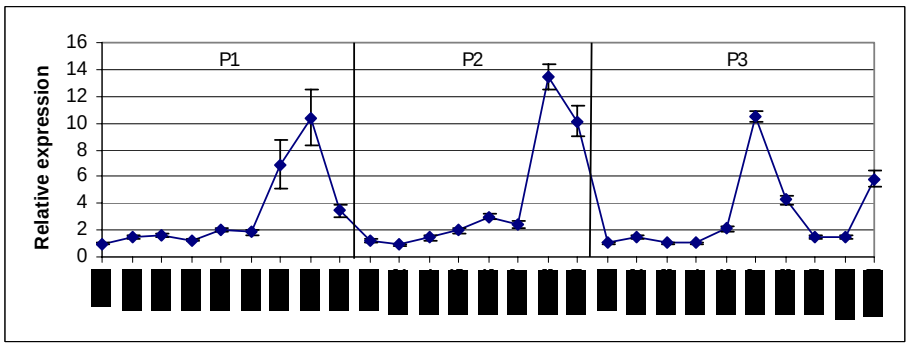

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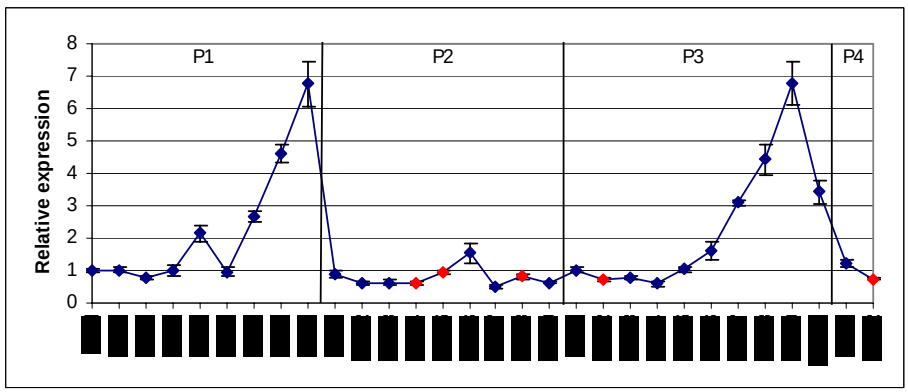
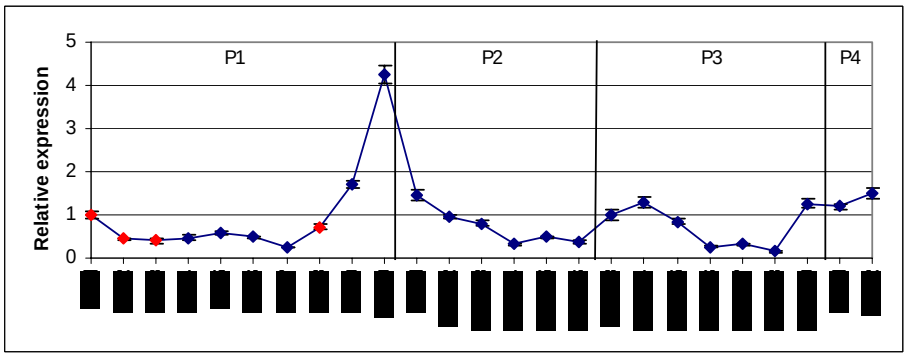
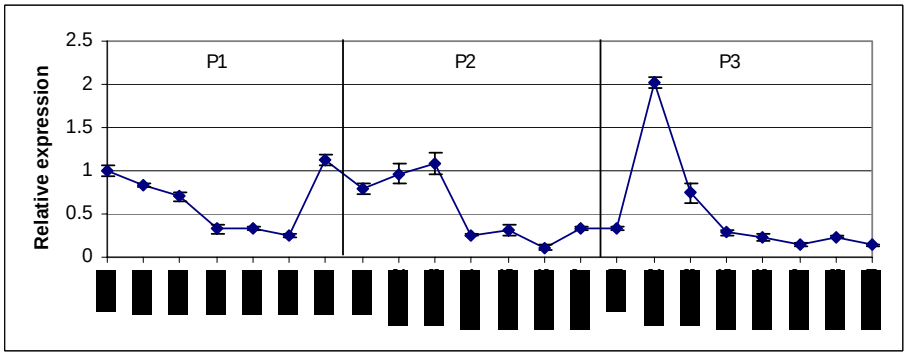


Figure 4.4: Hr4 Expression

Spawn 5



Spawn 6



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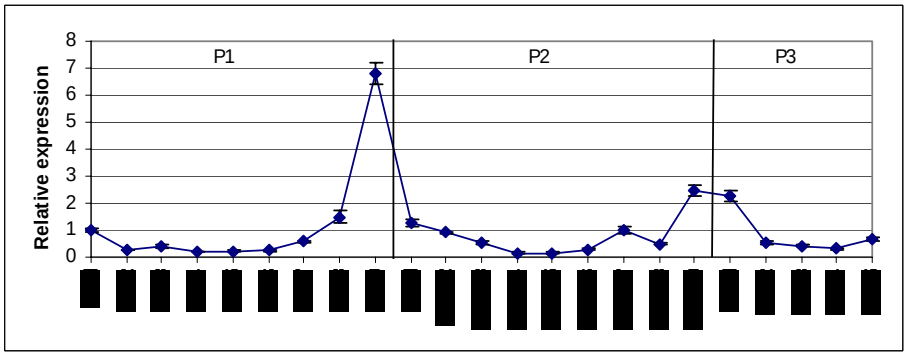
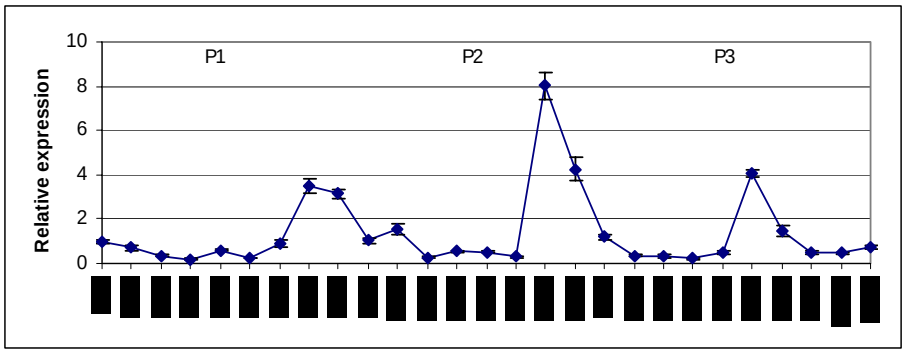


Spawn 11



Spawn 14

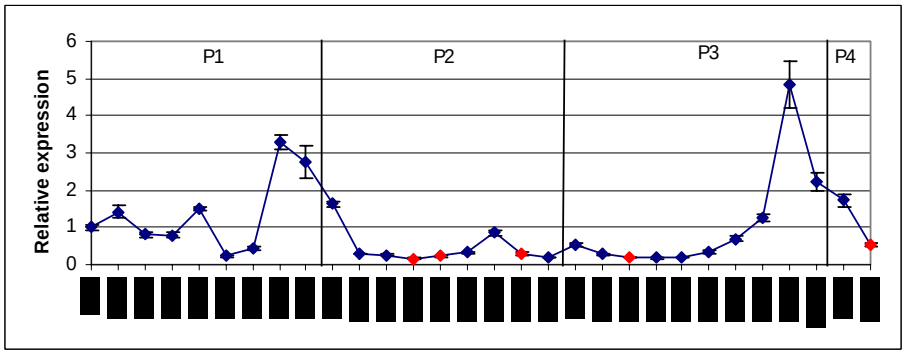
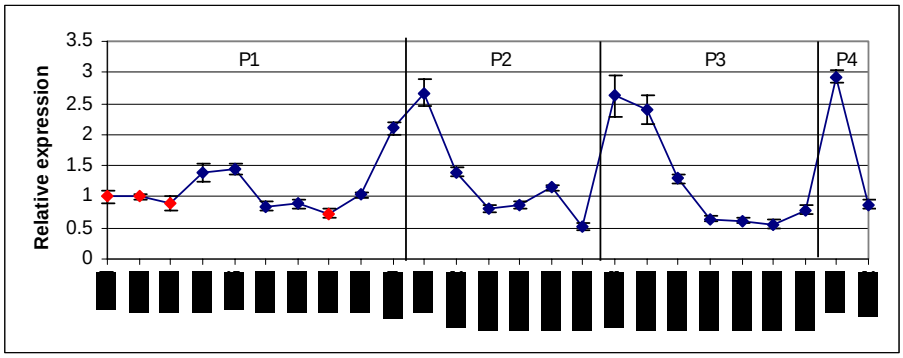
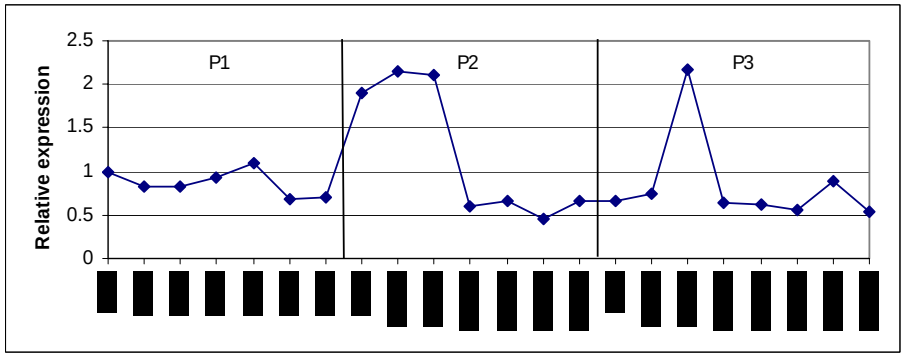


Figure 4.5: Hr78 Expression

Spawn 5



Spawn 6



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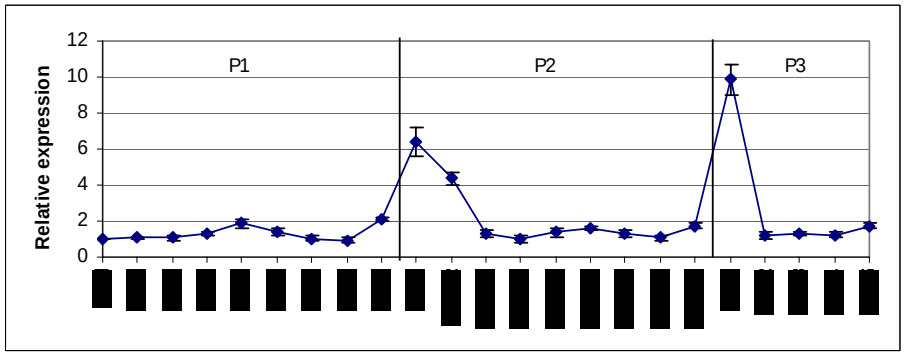
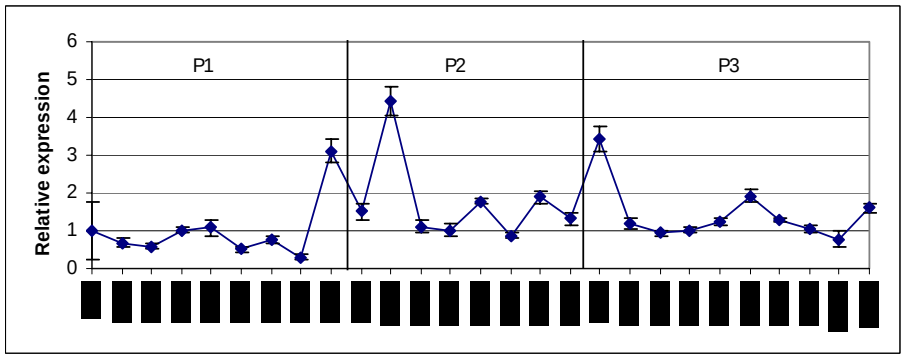


Spawn 11



Spawn 14

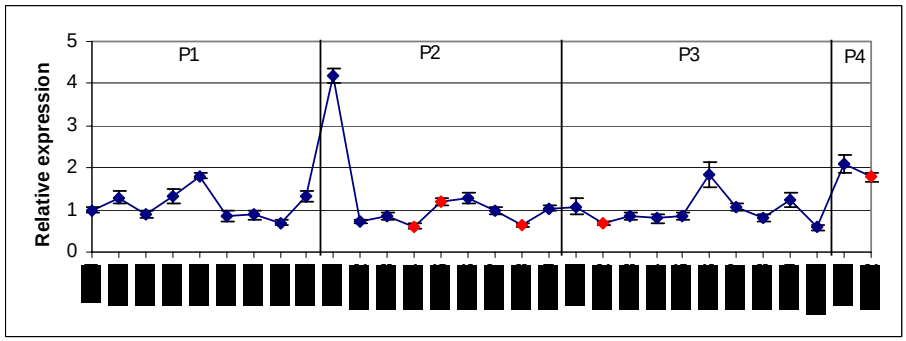


Figure 4.6: Spawn5

a) Expression of all four nuclear hormone receptor genes relative to the expression level measured on day 1

b) Expression of all four nuclear hormone receptor genes relative to the lowest measured expression for each gene. This was as follows: EcR P2 days 5-6; Hr3 P3 days 6-8; Hr4 P3 days 6-8; Hr78 P2 days 5-6 (arrowed on the graph).

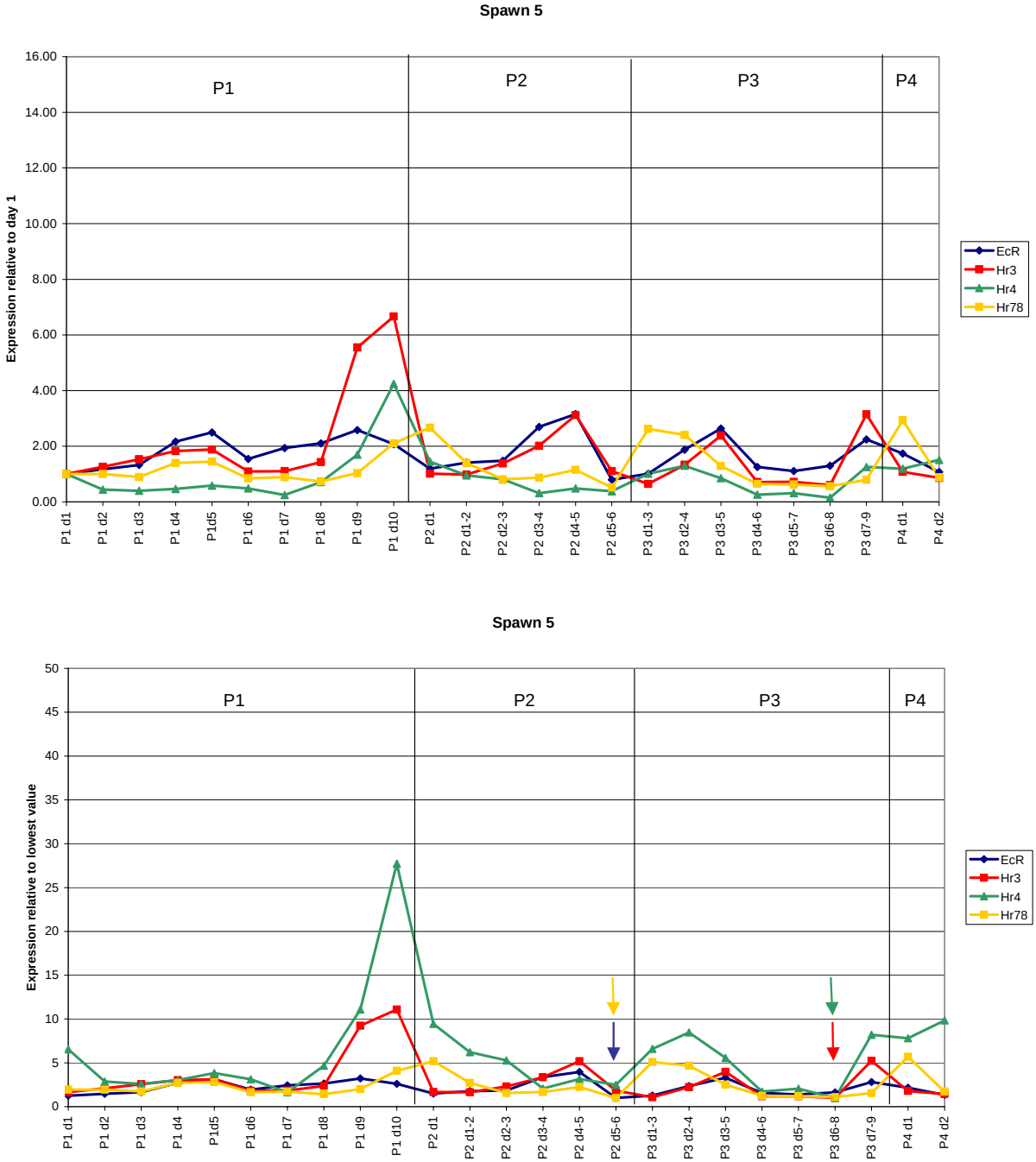
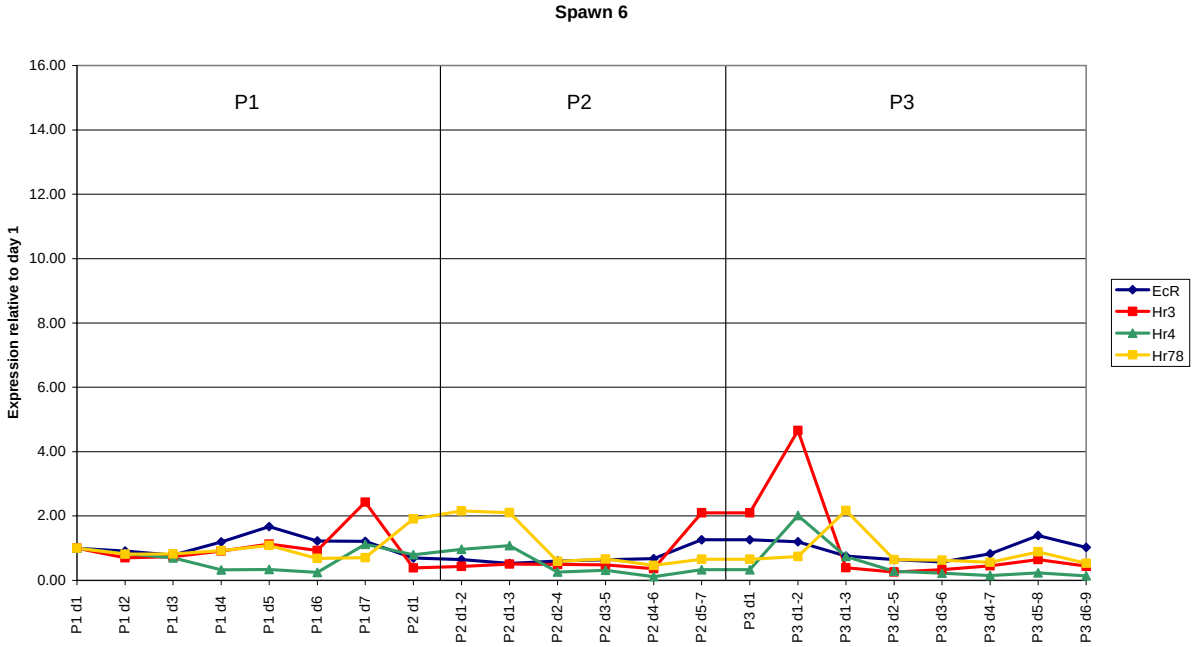


Figure 4.7: Spawn6

a) Expression of all four nuclear hormone receptor genes relative to the expression level measured on day 1

b) Expression of all four nuclear hormone receptor genes relative to the lowest measured expression for each gene. This was as follows: EcR P2 days 1-3; Hr3 P3 days 2-5; Hr4 P2 days 4-6; Hr78 P2 days 4-6 (arrowed on the graph).



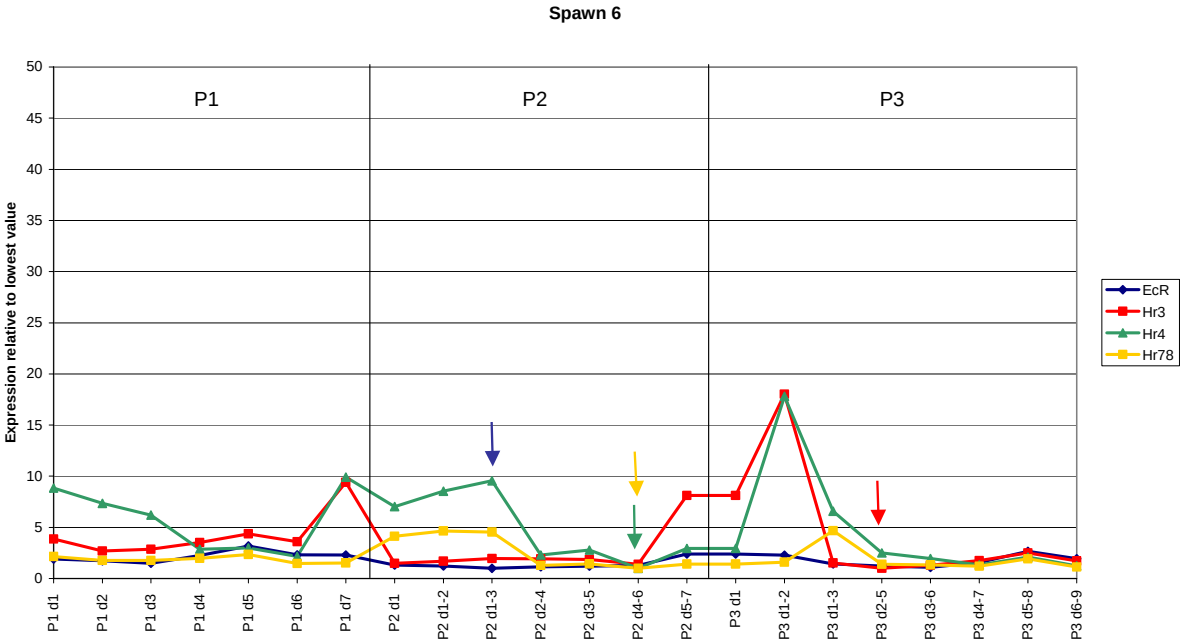
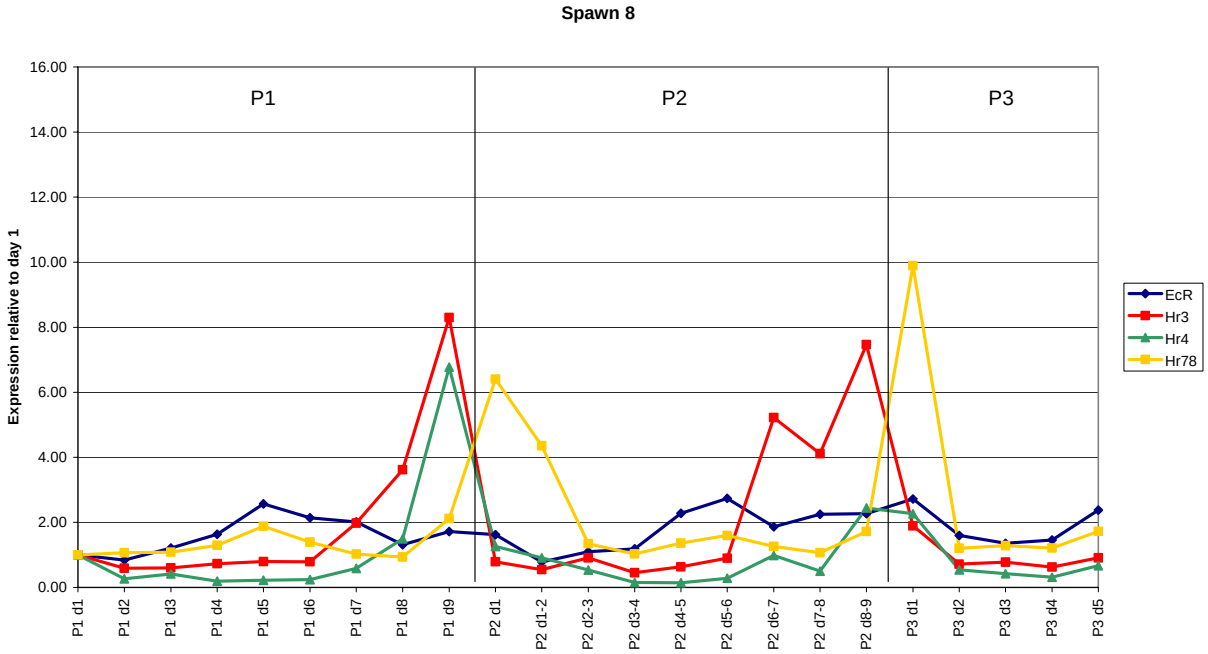


Figure 4.8: Spawn8

a) Expression of all four nuclear hormone receptor genes relative to the expression level measured on day 1

b) Expression of all four nuclear hormone receptor genes relative to the lowest measured expression for each gene. This was as follows: EcR P2 days 1-2; Hr3 P2 days 3-4; Hr4 P2 days 3-4; Hr78 P1 day 8 (arrowed on the graph).



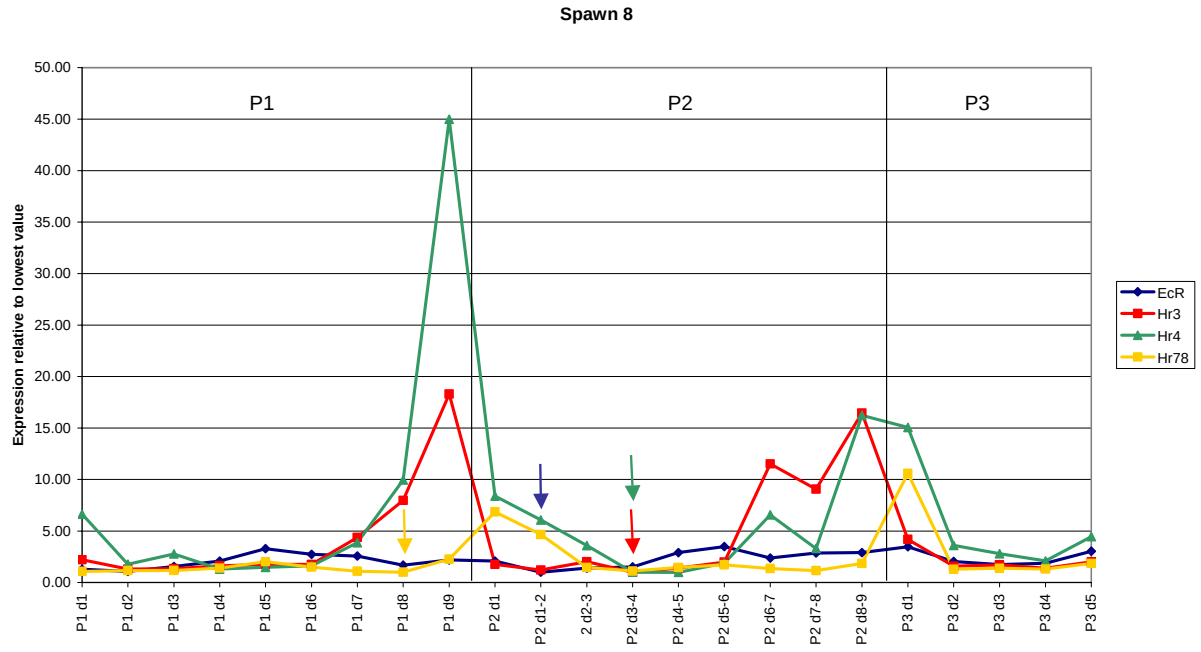
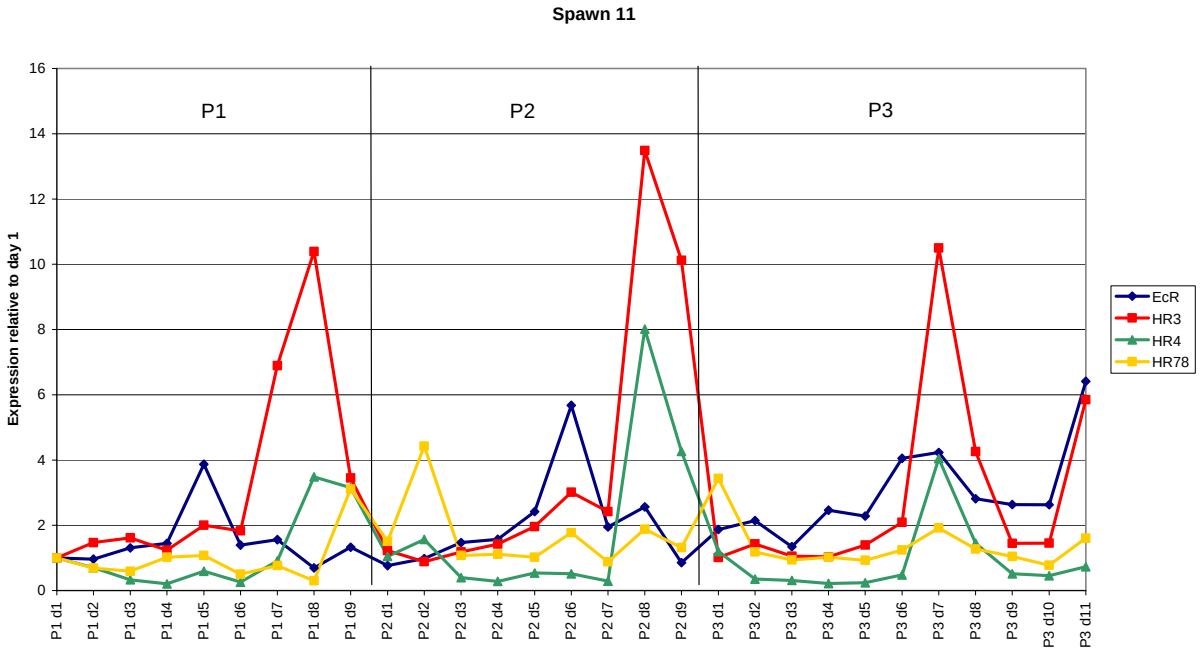


Figure 4.9: Spawn11

a) Expression of all four nuclear hormone receptor genes relative to the expression level measured on day 1

b) Expression of all four nuclear hormone receptor genes relative to the lowest measured expression for each gene. This was as follows: EcR P1 day 8; Hr3 P2 day 2; Hr4 P1 day 4; Hr78 P1 day 8 (arrowed on the graph).



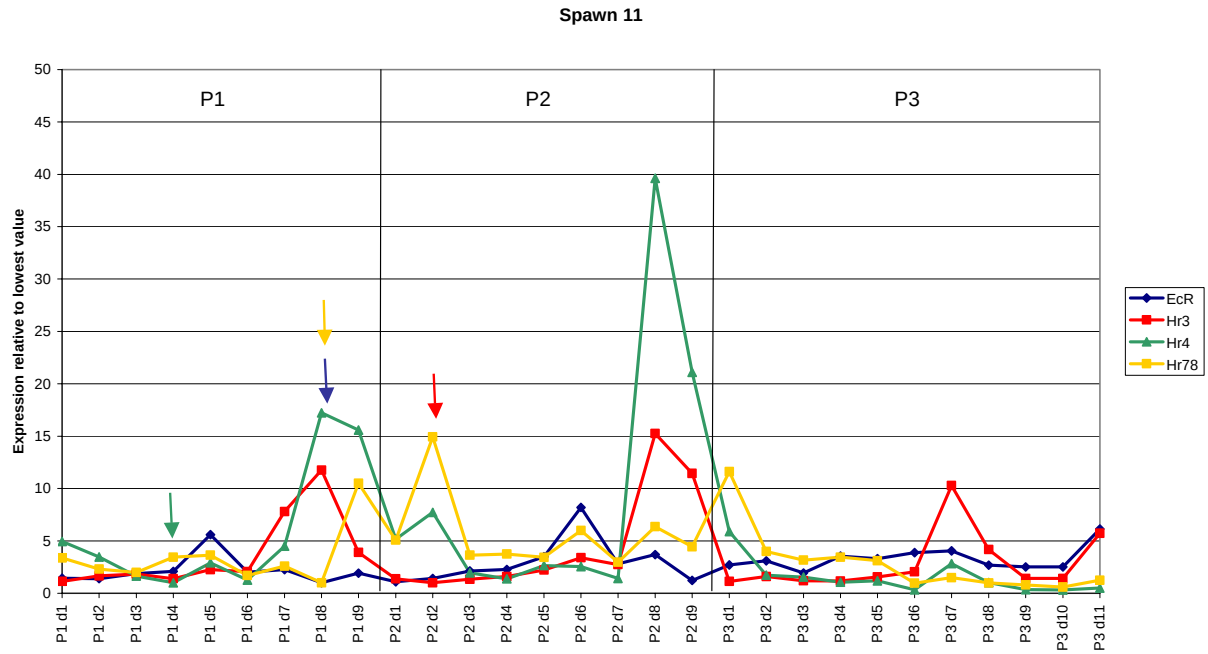
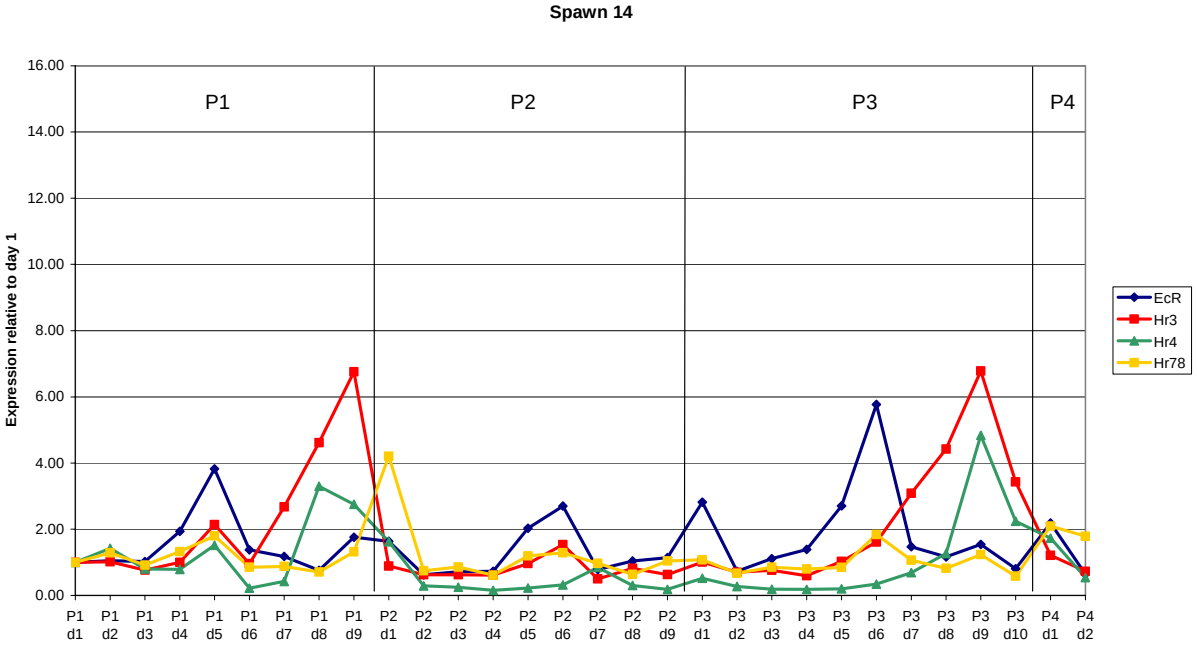
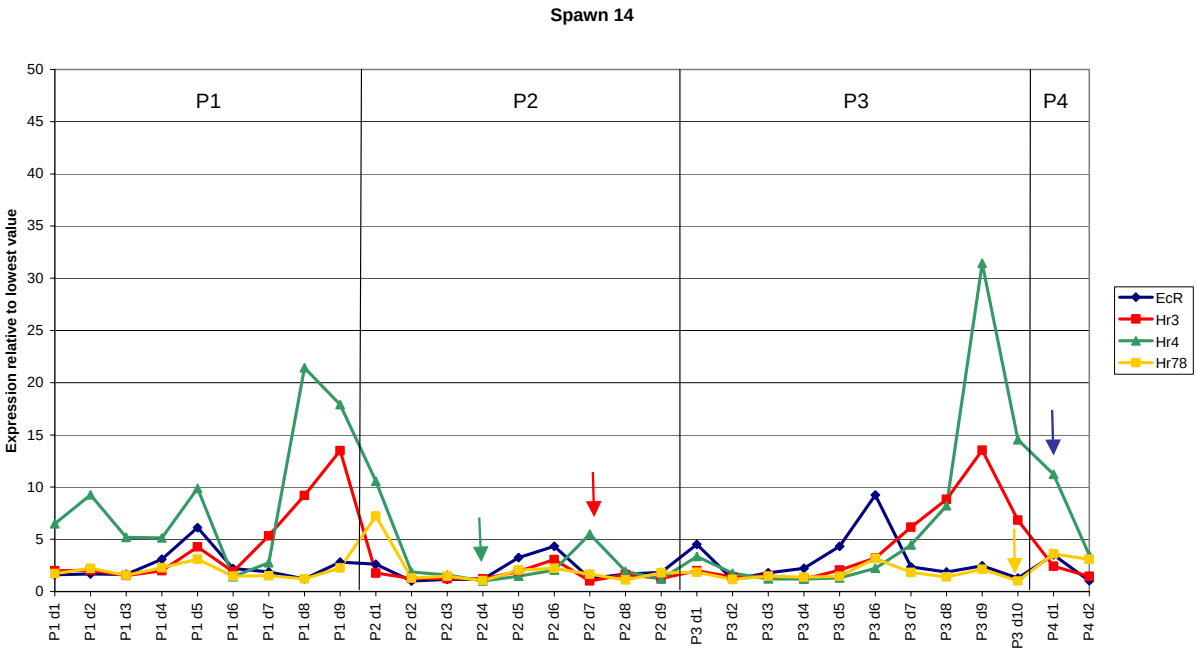


Figure 4.10: Spawn14

a) Expression of all four nuclear hormone receptor genes relative to the expression level measured on day 1

b) Expression of all four nuclear hormone receptor genes relative to the lowest measured expression for each gene. This was as follows: EcR P2 day 2; Hr3 P2 day 7; Hr4 P2 day 4; Hr78 P3 day 10 (arrowed on the graph)





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5. ECDYSONE LEVELS THROUGHOUT *P. ORNATUS* LARVAL DEVELOPMENT TO STAGE P4

Introduction

Ecdysone Enzyme ImmunoAssay (EIA)

The ecdysone Enzyme ImmunoAssay (EIA) is an assay that estimates ecdysone levels using a competitive Enzyme-Linked Immunosorbent Assay (ELISA) method (Figure 5.1). An anti-IgG antibody is first immobilised on a 96-well microtitre plate (Figure 5.1a).

An horseradish peroxidase (HRPO)-labelled ecdysone molecule is then added as a tracer, together with the unknown sample, followed by an anti-ecdysone antibody. The ecdysone in the unknown sample competes with the labelled ecdysone tracer for the anti-ecdysone antibody and the anti-ecdysone antibody becomes bound to the plate by the coating anti-IgG. The plates are then washed and an HPRO substrate is added to enable colour development for a set time. If the unknown sample has only a small amount of ecdysone, then a large amount of labelled ecdysone tracer binds to the antibody and hence results in a strong colour development. If there is a high level of ecdysone in the unknown, then less labelled ecdysone tracer will bind to the antibody and this results in a weak colour development. Colour development is measured quantitatively using a spectrophotometer and calibrated against a standard curve generated with different amounts of ecdysone tracer.

This method has been used to assay ecdysone levels in numerous arthropod species over the years, including crayfish (Marco et al., 2001), prawn (Petit et al., 1997), *Drosophila* (Chavez et al., 2000) and mealworm (Mouillet et al., 1997).

In this chapter we describe its use to assay ecdysone titres in early stage *P. ornatus* larvae.

Methods

Ecdysone EIA protocol

For all buffer preparation, coating of ELISA plates, ecdysone standard curve, tracer dilutions and substrate preparation for EIA, refer to Appendix V. All plates were washed using a 12-well Nunc-Immuno Washer (Nunc #400175) and blotted dry on paper towels prior to subsequent use.

The EIA protocol of Porcheron et al. (1989) was followed with minor modifications as outlined by Von Gliscynski et al. (1995). Three α-ecdysone antibodies, RUD-1, RUD-

2, RUD-3, were kindly provided by Dr D. Sedlmeier, Institut für Zoophysiologie, Universität Bönn. The ecdysone tracer, 20-hydroxyecdysone conjugated to horseradish peroxidase, was kindly provided by Dr J.P Delbeque, Laboratoire de Neurobologie des Reseaux, UMP CNRS, Universite Bordeaux I, France.

Briefly, goat anti-rabbit IgG monoclonal antibodies (Zymed Laboratories #81-6100) were immobilised on 96-well microtitre plates by coating wells with 200 µl of a 125 µg ml^{-1} solution of monoclonal antibody in 50 mM phosphate buffer (pH 7.4). After overnight incubation at room temperature, 100 µl of 3 mg ml^{-1} bovine serum albumin in 50 mM phosphate buffer (pH 7.4) were added to each well for blocking purposes. Plates were incubated overnight at room temperature prior to sealing with plastic wrap and storing for up to one month at 4°C prior to use. Before use, plates were extensively washed with EIA Wash Buffer (50 mM phosphate buffer pH 7.4, 0.05% w/v Tween-20) in order to remove any unbound antibody. The EIA assay was performed in a total volume of 150 µl composed of 50 µl diluted ecdysone tracer, 50 µl of sample ecdysone standard or biological extract and 50 µl of α-ecdysone antibody (RUD-2 @ 1:8000) which were added successively to each well in the order listed. B_o controls were also prepared in which the 50 µl sample was replaced with EIA sample buffer (i.e. no ecdysone other than tracer). At least 6 wells were reserved for B_o determination on each plate. Non-specific binding (NSB) controls were run at least in triplicate on each plate and received 50 µl ecdysone tracer and 100 µl EIA sample buffer only (ie. no sample or anti-ecdysone antibody). All dilutions of tracer, standard, samples and antibody were prepared in EIA Sample Buffer (0.1 M phosphate buffer pH 7.4, 0.4 mM NaCl, 0.1 mM EDTA, 1% w/v BSA).

The 150 µl reagent mix was incubated for 3 hr at room temperature and gently mixed on an orbital shaker (or overnight at 4°C followed by 1 hr at room temperature on the shaker). Plates were washed with 4-6 well volumes of EIA Wash Buffer. Each well then received 100 µl 3,3',5,5'-Tetramethylbenzidine dihydrochloride (TMB Sigma #T-3405) prepared according to the manufacturer's instructions and was incubated in the dark for 10-20 min to allow colour development. Colour development was stopped by the addition of 25 µl 2 M H_2SO_4 and the absorbance read at 450 nm within 30 min.

Optimising α-ecdysone antibodies for EIA

Three α-ecdysone antibodies, RUD-1, RUD-2, RUD-3, were compared. An antibody titration assay was performed using 2-fold serial dilutions in EIA sample buffer from 1:500 to 1:64000 to determine the optimal binding capacity of each antibody. The Ecdysone EIA assay was performed as outlined above and in Appendix V using a constant amount of tracer.

Testing tracer stocks

Three different stocks of tracer for the Ecdysone EIA were kindly provided by Dr JP Delbeque. To compare the relative sensitivity of each tracer stock, a series of standard curves was prepared as described (Appendix V) using the optimised dilution of RUD-2 anti-ecdysone (1:8000) and dilutions of tracer ranging from 10^{-6} M to 10^{-11} M. Each tracer stock was used at 13 µl per 6 ml EIA Sample Buffer.

Sample collection

Details of larval rearing and collection were as outlined for Q-PCR larval sampling (Chapter 4). Details of larval rearing conditions can be found in Appendix IV. Briefly, 60 x P1 larvae, 30 x P2 and 30 x P3 larvae were collected to enable triplicate samples to be analysed with the minimum number of larvae per well for the EIA assay. All larvae collected were carefully separated into developmental stages (i.e. P1s, P2s or P3s) using a dissecting microscope, blotted on filter paper to remove excess water and stored at –20°C in 2 ml tubes with O-ring seals (Quantum Scientific #520-GRD). Sufficient numbers of larvae were stored in individual tubes to allow one replicate of analysis (see Table 5.1). Ecdysone extractions were performed as outlined below.

Ecdysone extraction procedure

Ecdysone extraction from whole *P. ornatus* phyllosoma was performed according to the protocol of Block et al. (2003). Briefly, frozen phyllosoma samples were removed from the freezer and 250 µl ice-cold methanol-water 4:1 v/v (80% MeOH) were added. Two 3 mm and one 2 mm glass beads were added to each sample which were then placed in a chilled aluminium 96-well bead beater block and homogenised for 3 min (Daintree Industries, Bead-Beater). Samples were then sonicated in a iced-water bath for 3 min prior to overnight incubation at –20°C (~16 hr). Samples were then centrifuged 10 min at 16000 rpm in an Eppendorf microfuge and supernatants transferred to a fresh microfuge tube. The pellet was re-extracted with the addition of an extra 100-200 µl 80% MeOH, vortexed 30 s, sonicated again in an iced water bath for 3 min and re-pelleted for 10 min at 16000 rpm. The re-extracted supernatant was added to the original extract for each sample. All samples dried in a vacuum centrifuge and stored at –20°C for subsequent analysis.

Ecdysone ImmunoAssay analysis of P. ornatus larvae

TRIAL ECDYSONE ANALYSIS THROUGH TWO MOLT STAGES

To determine the levels of ecdysone in *P. ornatus* larvae, a trial experiment was performed in which *P. ornatus* larvae were collected daily throughout two molt cycles and stored as described. Twenty P1, 10 P2 and 10 P3 larvae were collected

per tube (in triplicate). One set of the triplicate samples collected was initially measured in the EIA assay, i.e. 20 P1s and 10 each of P2s and P3s were extracted for ecdysone as described and each sample resuspended in 200 µl EIA sample buffer for triplicate well analysis (50 µl per well) in the EIA assay. Therefore each well had the equivalent of 5 P1 larvae or 2.5 P2/P3 larvae for ecdysone analysis. Subsequently, rather than resuspending each sample in 200 µl EIA sample buffer and splitting this between triplicate wells in the EIA, each sample was resuspended in just 50 µl EIA sample buffer and assayed in a single well. This resulted in the equivalent of 20 P1 larvae and 10 P2/P3 larvae assayed per well in the EIA.

ECDYSONE ANALYSIS: SPAWN-11 & SPAWN-14

For each spawn, two EIA plates were run with a standard curve on each plate and samples were analysed against the standard curve from the plate on which they were assayed. The average B_o values for each plate were determined by averaging the six replicate B_o wells. Raw OD values for standard curves from each plate were converted to B/B_o (%) using the averaged B_o value calculated for each plate. Standard curves were plotted with log fmol ecdysone/50 µl on the X-axis and a 4-order polynomial regression line was fitted using Microsoft Excel. From the polynomial regression line, the points of the standard curve that fell within the linear range were selected and used to create a linear plot for each standard curve. Individual well B/B_o (%) values were converted to log fmol ecdysone/50 µl using the appropriate linear equation from their respective plates. Log fmol ecdysone/50 µl were converted to fmol ecdysone/50 µl and then to fmol ecdysone *per larva* by dividing by the number of larvae in each well. Average values and standard deviations were then determined from the triplicate values for each sample.

Results

EIA standard curve

EIA results are expressed as $B/B_o \times 100$ as a function of the log dose where B represents the absorbance measured of the labelled ecdysone tracer in the presence of competitor (unknown) and B_o represents the absorbance of the tracer in the absence of any competitor. Therefore, B_o is the maximum signal of the assay in the absence of any sample, whereas B is the reduced signal strength due to the displacement of tracer by the ecdysone in the unknown sample. Hence $B/B_o \times 100$ represents the signal strength expressed as a percentage of the maximum signal for the assay. The EIA plate also requires a non-specific binding (NSB) control to be run using an incubation mixture in which the anti-ecdysone antibody and sample are replaced by buffer (i.e. only tracer is present in the coated well). At least quadruplicate B_o controls and triplicate NSB controls are always run on each plate.

To determine ecdysone levels in unknown samples, a standard curve is run on each plate using a known concentration of ecdysone (commercial standard) titrated over at least four log dilutions. According to Porcheron et al. (1989) B/B_o x 100 is plotted as a function of the log dose and a four-order polynomial fitted to the curve (Figure 5.2a). The linear region of the curve is determined (usually lying between 20-80% B/B_o) and a linear plot is then prepared using only those values that fall within the linear region (Figure 5.2b). Once a linear plot is prepared, the resulting regression line is used to determine the concentration of the unknown samples (whose values must also fall within the linear range of the standard curve). Figure 5.2 is an example of a standard curve generated in the course of the present work.

A typical EIA assay has a detection limit for ecdysone between 150-5000 fmol per 50 µl, as illustrated in Figure 5.2.

Optimisation of Ecdysone ImmunoAssay

Prior to use, the ecdysone immunoassay required optimisation to determine the optimal antibody preparation and concentration to use, and to determine the optimal tracer stock to use, as these two reagents are critical to the success of the assay.

The three different antibody stocks provided were compared. The optimal working dilutions for RUD-1 and RUD-2 were found to be 1:2000 and 1:8000 respectively (Figure 5.3). RUD-3 produced a very weak signal (max OD ~0.2), hence was not suitable for subsequent assays. The RUD-2 antibody was selected for use in ecdysone EIA analysis as it showed the strongest ecdysone reaction. Von Gliscynski et al. (1995) used RUD-1 and RUD-2 at 1:20000 whereas RUD-3 was used at 1:2000.

The three tracers stocks provided were also tested. Two were found to work well while the third gave very low signals and was not suitable for use (data not shown). Of the two suitable tracer stocks, tracer batch: 21-06-2003 ("New") was selected for use in all EIA assays.

Ecdysone ImmunoAssay of P. ornatus larvae

TRIAL ECDSYONE EIA ASSAY

This trial experiment provided valuable information about the expected ecdysone levels in *P. ornatus* larvae and provided data on the number of larvae required to obtain ecdysone levels within the linear region of the EIA standard curve (200-5000 fmol/50 µl). Figure 5.4(a) shows that many of the samples analysed fell below the limit of detection of the EIA when 5 P1 and 2.5 P2/P3 larvae were analysed per 50 µl. Subsequent analysis using 20 P1 and 10 P2/P3 larvae per 50 µl provided much more reliable data with all values falling within the detectable range of the EIA

assay, as shown in Figure 5(b). Table 5.1 indicates the expected range of ecdysone levels from each *P. ornatus* phyllosoma from stage P1 to P3, based on this initial trial.

Chang and Bruce (1981) reported ecdysone levels for *Homarus americanus* larvae of 2000-10000 fmol ecdysone per larva, depending on the stage of the molt cycle. However, these early stage larvae were approximately 10 mg wet weight, increasing to about 20 mg by the third instar. When the figures were expressed per 10 mg wet weight across all three larval instars, the range was approximately 1000-6500 fmol ecdysone per 10 mg wet weight. The wet weight of bulk *P. ornatus* phyllosoma samples was determined as follows: 20 P1s weighed 5.28 mg $\pm$ 0.63 (n=35); 10 P2s weighed 4.31 $\pm$ 0.34 (n=42); 10 P3s weighed 5.89 $\pm$ 0.79 (n=18). This translates to average weights of 0.26 mg for P1 larvae, 0.43 mg for P2 larvae and 0.59 mg for P3 larvae. Based on the data obtained in this trial assay, this enables calculation of the approximate ecdysone titres on a wet weight basis (Table 5.1). Using the figures in Table 5.1, the range of ecdysone concentrations per 10 mg wet weight for *P. ornatus* larvae would be approximately 400-4,500 fmol ecdysone per 10 mg, which is highly comparable to the figures of 1000-6500 fmol ecdysone per 10 mg wet weight for *H. americanus*.

P. ornatus *ecdysone levels through two molts (Spawn-11 & Spawn-14)*

The results presented in Figure 5.4 are derived from a spawning where larvae were collected "en masse" from the large larval rearing tank, and no attempt was made to resynchronise phyllosomas at the onset of each new molt phase (see chapter 4 for further discussion of this effect). Variations in ecdysone titre were subsequently measured in two molts for which quantitative PCR data were also available, spawns 11 and 14 (see Chapter 4). For these spawnings, phyllosoma were separated into a different tank on the first day of each new molt, to resynchronise larvae within that specific molt stage.

Figures 5.5 and 5.6 present the measured variation in ecdysone titre on a per larva or per mg basis across two molt cycles, and most of the way through the third molt cycle for two spawnings. These figures illustrate clearly the pattern of an increase in ecdysone titre pre-molt, and a precipitous drop just prior to molt.
Sampling constraints in the present work restricted the level of resolution of analysis that was possible. First, sampling occurred only on a daily basis. Secondly, and perhaps most importantly, the temporal spread of each molt transition for a cohort of larvae meant that, particularly towards the end of a molt cycle, we were actually sampling larvae in a variety of pre-molt stages. For example, for molt 14, the transition from P1-P2 actually took place over four days. Hence, larvae sampled as P1s on day 9 actually comprised a mixture of P1s that were between 1-4 days pre

molt. Hence, it is not possible to determine with precision how long pre ecdysis the ecdysone titre peaks.

Figure 5.7 graphs the combined changes in ecdysone titre and ecdysone receptor expression for both spawns 11 and 14. This graph illustrates that induction of the ecdysone receptor occurs soon after ecdysone levels begin to rise, and that it declines again when ecdysone levels peak.

Discussion

The results presented in this chapter confirm that the molt cycle in *P. ornatus* phyllosoma is regulated by ecdysone in the same manner that molting is regulated in both insects (Riddiford et al. 2003) and in other crustaceans (Chang, 1989). In other words, the circulating levels of ecdysone rise and peak in the lead-up period prior to ecdysis, but then drop precipitously just prior to ecdysis. In insects this drop often occurs just hours before the final molt (Mesce and Fahrbach, 2002) and it has been demonstrated that, without this drop in the ecdysone titre, the molt will not proceed to actual ecdysis (Zitnan et al., 1999).

The pattern of expression of the *P. ornatus* ecdysone receptor, and its relation to variations in ecdysone titre, also appears to be consistent with observations in insects. It has been demonstrated in *Drosophila* that the ecdysone receptor is induced when ecdysone levels reach approximately 2×10^{-9} M, but that it is repressed when the ecdysone concentration increases tenfold (Andres et al., 1993; Thummel, 1996). The data presented here indicated that the *P. ornatus* ecdysone receptor is induced when ecdysone levels reach a low titre and starts to decline again when ecdysone levels increase, although the range of ecdysone concentrations apparently inducing this change is less than tenfold. For example, in spawn 11, the ecdysone receptor appears to be induced as ecdysone levels rise from 15-32 fmol per larva, and then to decline again when levels reach 42 fmol per larva. In spawn 14 they rose when ecdysone levels rose form 12-19 fmol per larva, and declined again as ecdysone rose to approximately 36 fmol per larva. It should be noted that the assay used in the present work for the ecdysone receptor would not distinguish between different isoforms of the ecdysone receptor in *P. ornatus*, and there is evidence that the different isoforms in *Drosophila* show different temporal as well as spatial patterns of expression (Sullivan and Thomas, 2003). Nevertheless, the overall picture of ecdysone receptor regulation, and its relation to changes in ecdysone titres, is very similar in *P. ornatus* larvae and to more widely-studies insects.

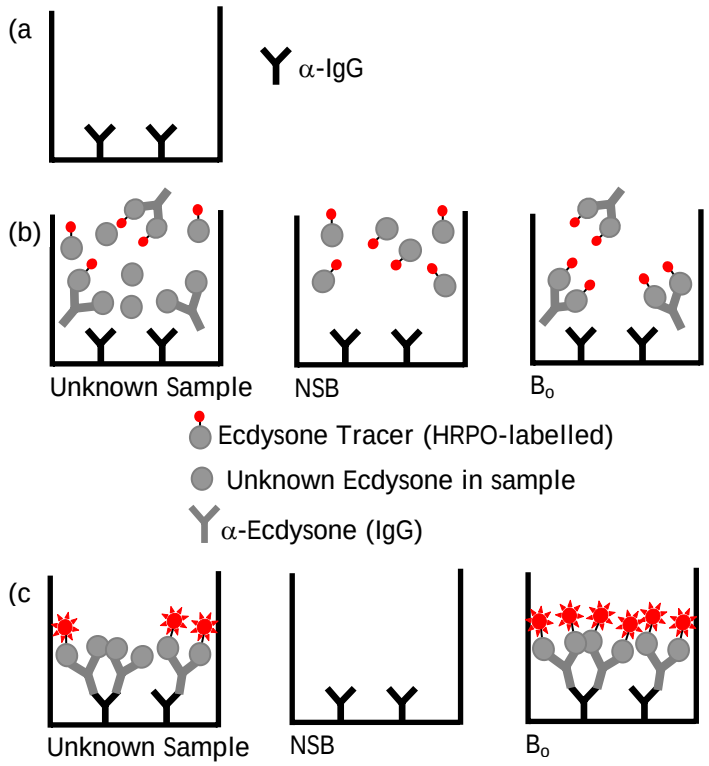


Figure 5.1: Ecdysone ImmunoAssay.

(a) Wells are coated with α-IgG antibody. (b) To analyse unknown samples Ecdysone Tracer, Unknown Sample and α-Ecdysone IgG are added to sample wells. The ecdysone from the Unknown Sample competes with the Tracer for binding to the α-Ecdysone IgG. Non-specific background signal (NSB) is determined by measuring absorbance in the presence of Ecdysone Tracer only. The maximum signal strength of the assay (B_o) is measured by incubating Ecdysone Tracer and α-Ecdysone antibody in the absence of any competitor. (c) Following washing and colour development using an HPRO substrate, signal strength of the Unknown Samples (B) is compared to the signal strength of the B_o control ecdysone levels are determined from an ecdysone standard curve.

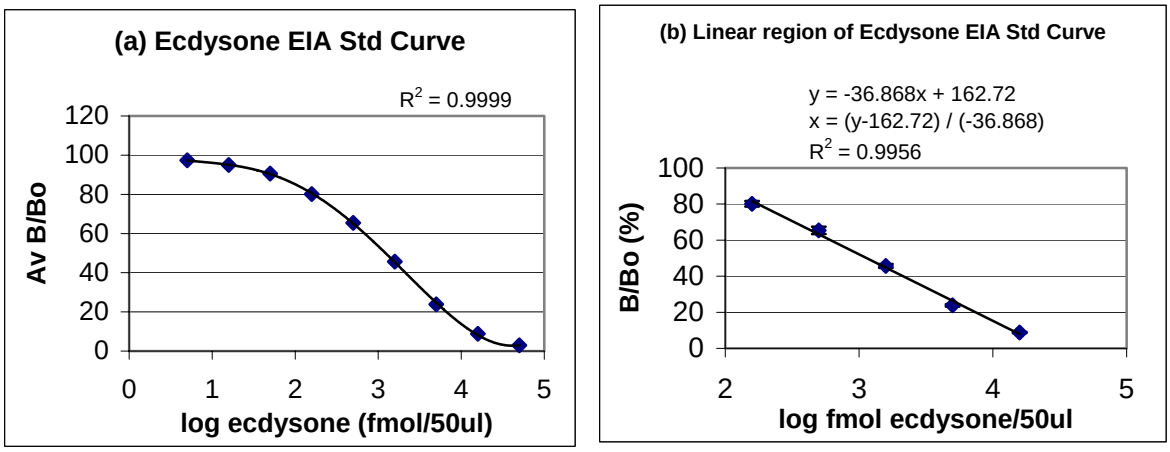


Figure 5.2: Typical Standard Curve for the Ecdysone ImmunoAssay.

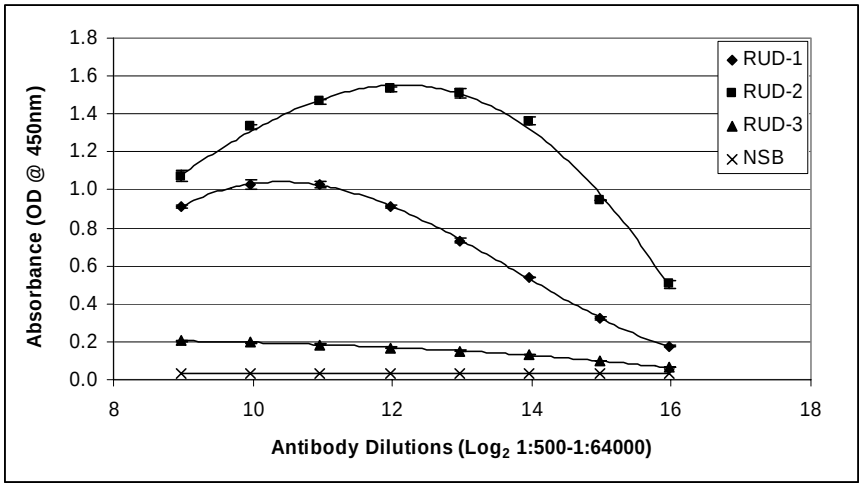


Figure 5.3: α-Ecdysone Antibody Optimisation.

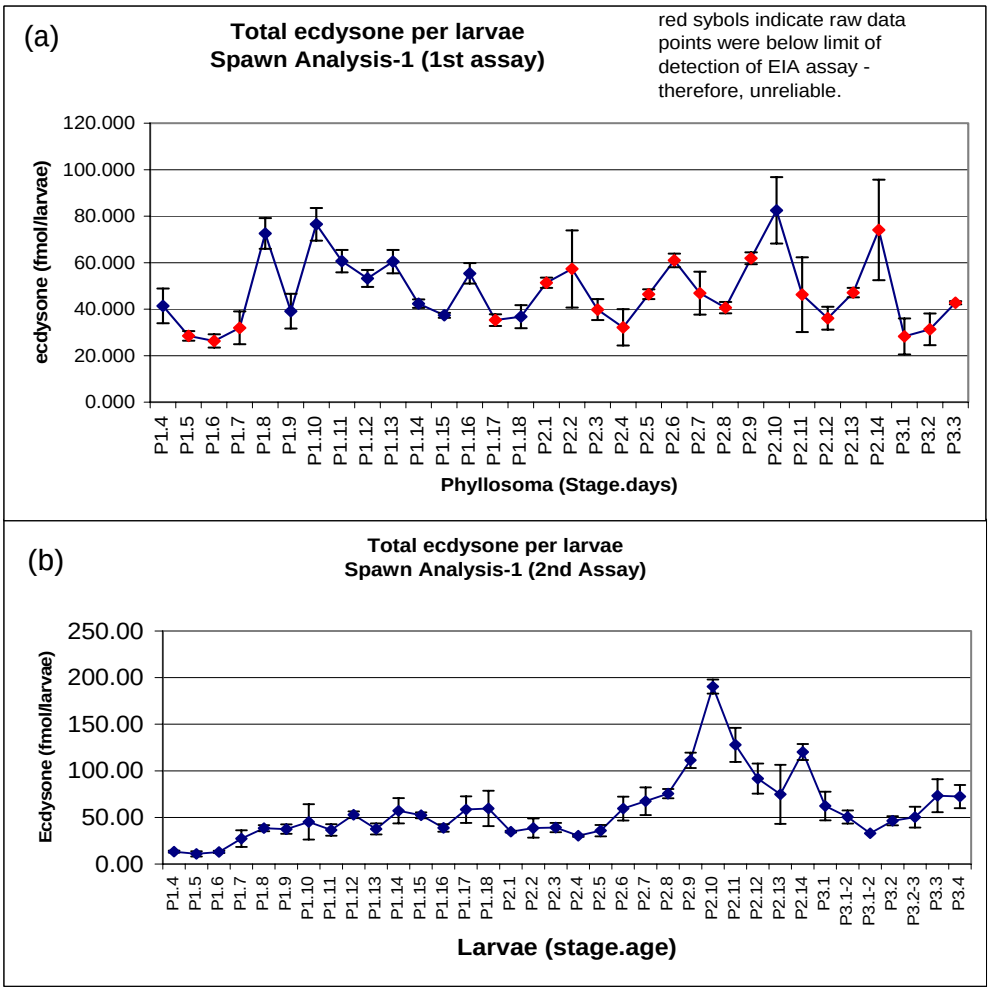
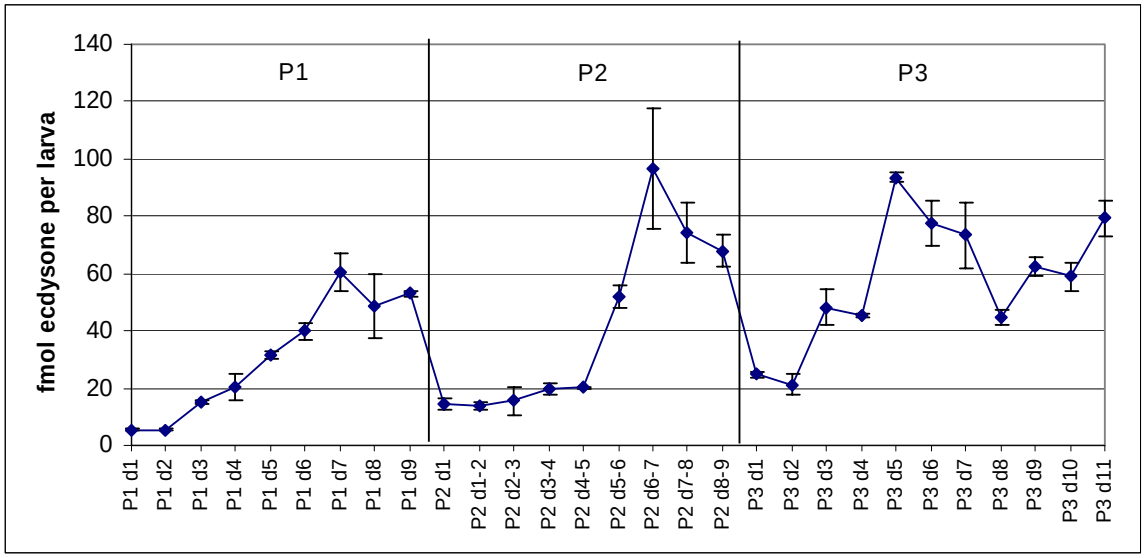


Figure 5.4: Trial Ecdsyone ImmunoAssay.

(a) First trial assay used 5 P1 larvae/ well in triplicate and 2.5 P2 & 2.5 P3 larvae/well in triplicate. Due to the lower number of larvae assayed per well in the first assay, the B/B_o levels for many samples fell outside of the linear region of the B/B_o standard curve (all P2 and P3 samples fell outside the linear region of the standard curve).
(b) Second assay used 20 P1 larvae/well in duplicate and 10 P2 & 10 P3 larvae/well in duplicate. All sample levels fell within the linear region of the B/B_o standard curve.

(a)



(b)

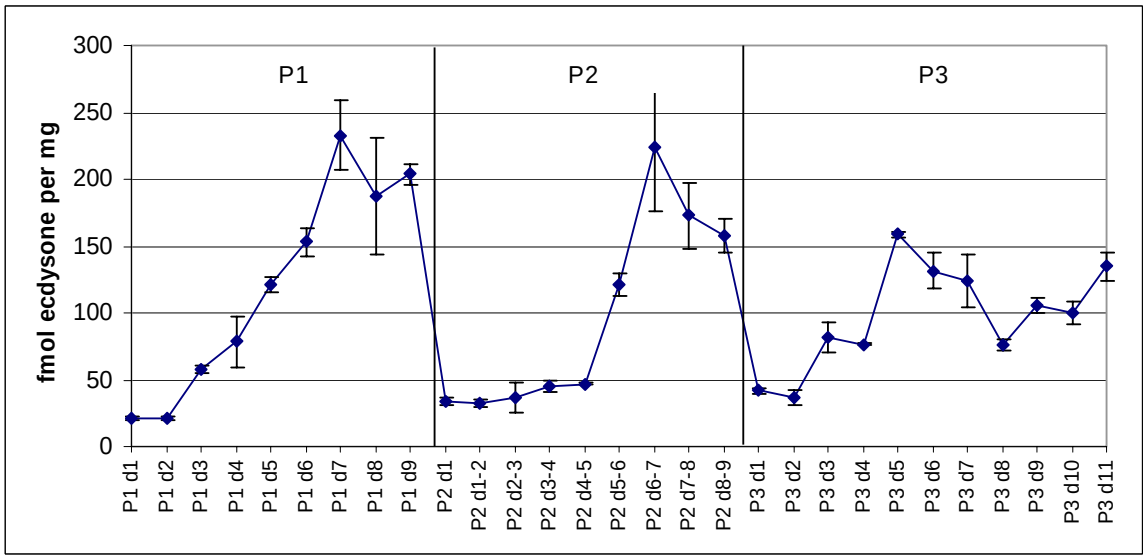
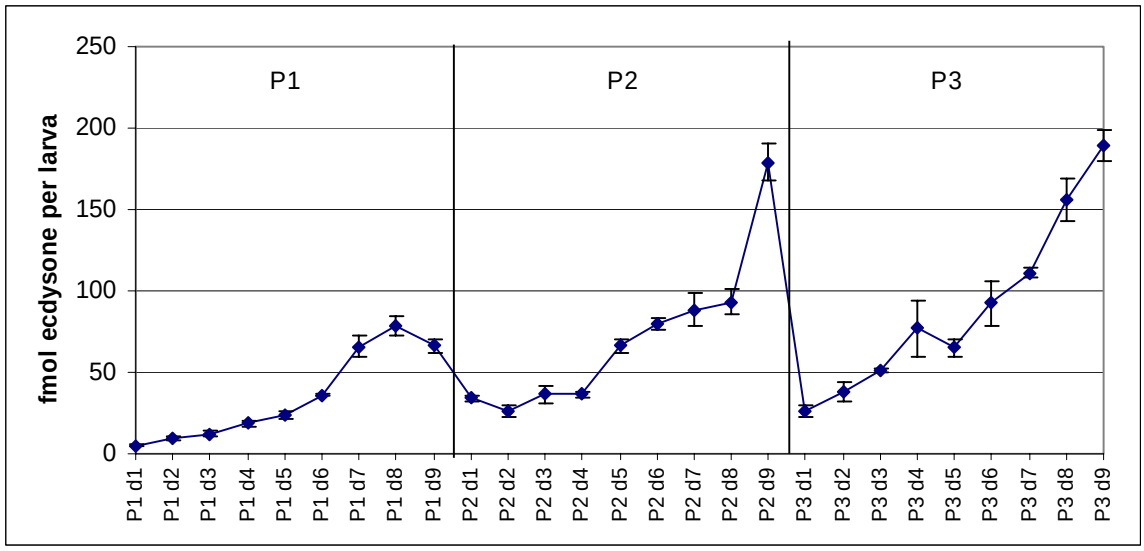


Figure 5.5: *P. ornatus* Ecdysone levels through two molts: Spawn 11.
(a) Ecdysone titre expressed on a per larva basis. (b) Ecdysone titre expressed on a per mg larval tissue basis.

The x-axis represents the days in a given phyllosoma stage. The P2 phyllosoma had a two-day spread for estimated age as P2s were not subsampled for synchronisation until the second day after P2s started to appear in the main tank (see Chapter 4 and Data Appendix IV Table 19). The period for all larvae in this cohort to complete a molt was 2-days for P1-P2 and 4 days for P2-P3.

(a)



(b)

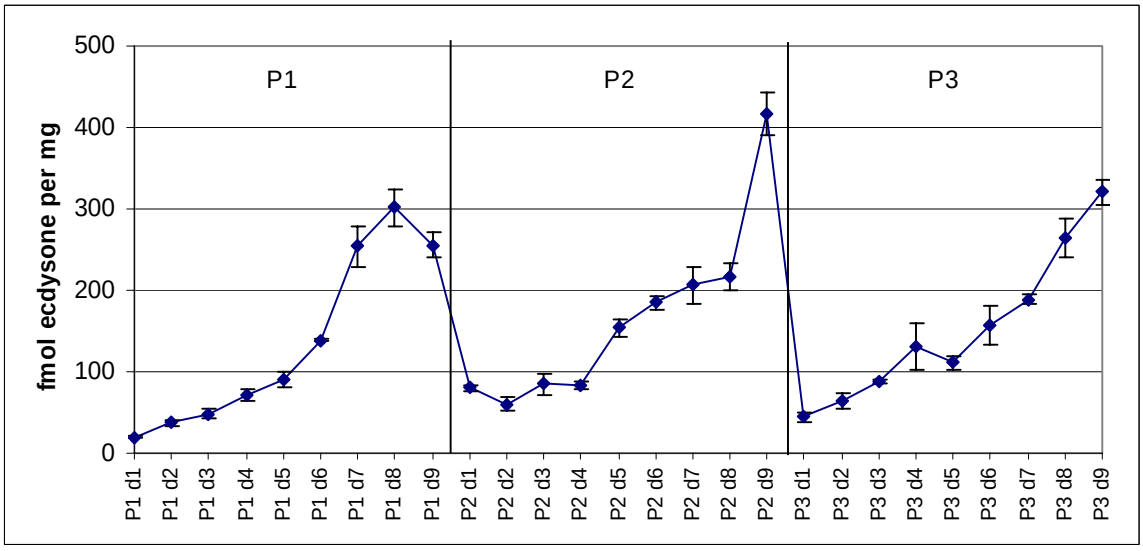


Figure 5.6: *P. ornatus* Ecdysone Levels Through Two Molts: Spawn 14.
(a) Ecdysone titre expressed on a per larva basis. (b) Ecdysone titre expressed on a per mg larval tissue basis.
The period for all larvae in this cohort to complete a molt was 4-days for P1-P2 and 3 days for P2-P3 for this spawn.

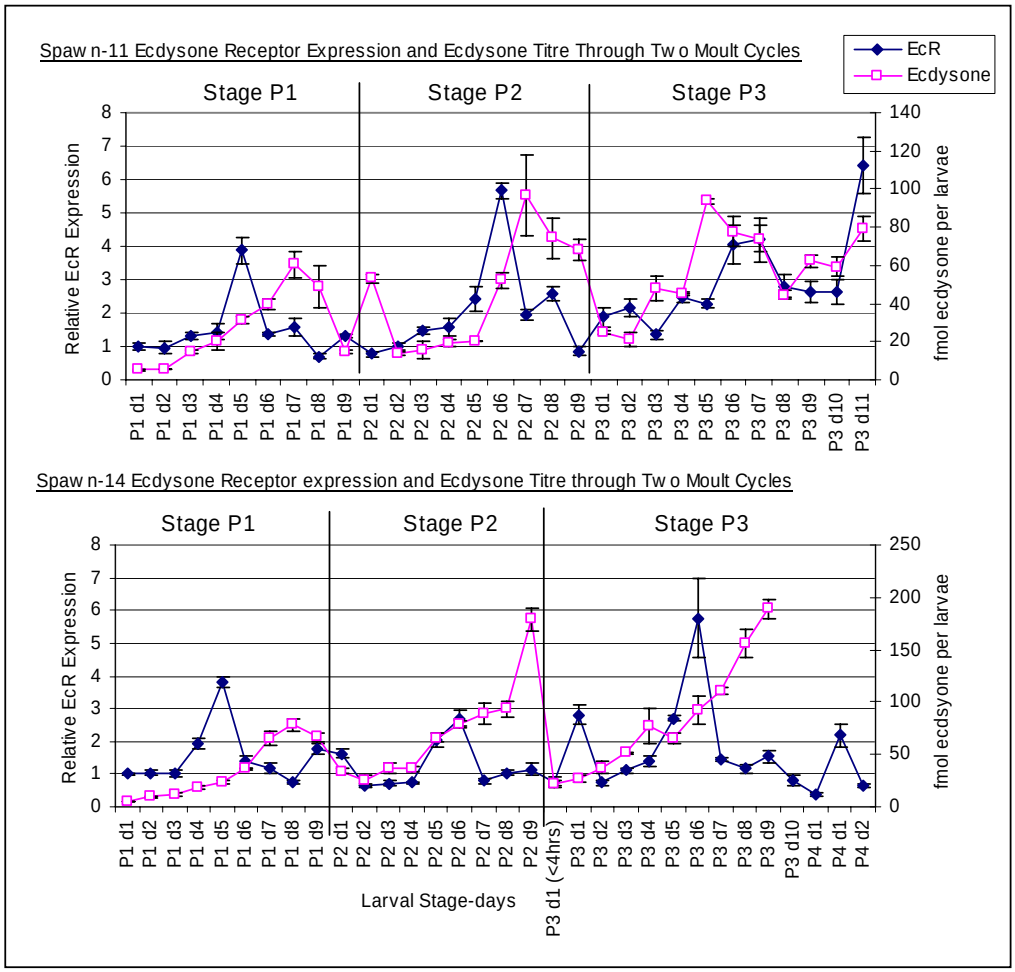


Figure 5.7: Ecdysone levels and ecdysone receptor (EcR) expression through two molts, for two separate spawns.

Table 5.1: Number of larvae required for Ecdysone EIA analysis

Range of Standard Curve: 150-5000 fmol/50μl Desired Sample Ecdysone Levels: ~1500 fmol/50μl				
Stage	Range of ecdysone concentrations (fmol per larva)	Minimum number of larvae required for EIA	Average weight per larva (mg)	Range of ecdysone concentrations (fmol per mg wet weight)
P1	10-60	20	0.26	38-227
P2	30-190	10	0.43	70-441
P3	30-70	10	0.59	51-119

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APPENDICES

Appendix 1. Intellectual Property

Recommendation:

The information generated in this project can be considered public domain. The sequences will be deposited in public DNA databases, and the results will be published in the scientific literature. It is considered that this is the best use of these data, to ensure that high quality Australian scientific work is widely disseminated and acknowledged.

The amount of investment required to translate the current results into a practical application is still substantial. If further investment does focus on testing hormonal manipulation using the results described here, then the results of such a future project may require intellectual property protection.

This suggestion is based on the following analysis:

a) Protection of IP
The only way to protect IP from this project currently is to maintain secrecy i.e. not to deposit sequences in the database, and not to publish it. This is because there is still no clear use for the data produced so far, and hence a patent application is not feasible. Unless substantial further funding is provided towards this work, practical application is unlikely to ensue in the foreseeable future. Hence the overall result of protecting the IP in this manner will be that the information obtained, which is a piece of high quality, innovative scientific research, will not be published and neither the scientists involved nor the FRDC will receive the recognition due for this investment.

b) Decision not to protect IP
This will enable the information to be published. Sequences will be deposited in the public databases, and at least one high quality peer-reviewed publication will result. As much of this work is a first for crustaceans, this will position AIMS and FRDC as intellectual leaders in this field.

Appendix 2. Staff engaged in the project

Name	Role	Institution
Mike Hall	PI	AIMS
Kate Wilson	Molecular Biology Direction	AIMS
Jennifer Swan	Molecular Biology Lab Work	AIMS
Matt Kenway	Tropical Aquaculture Facilities Manager	AIMS
Matt Salmon	Larval Rearing	AIMS
Don Booth	Larval Rearing	AIMS
Lisa Kennedy	Larval Rearing	AIMS
Grant Milton	Larval Rearing	AIMS
Jane Gioffre	Larval Rearing	AIMS
David McKinnon	Live Feeds	AIMS
Samantha Duggan	Phyllosoma Descriptions	AIMS
Liz Howlett	Report Editing and Production	AIMS
Tim Simmonds	Report Production	AIMS
Nicolas Wade	Molecular Biology	UQ / AIMS

DATA APPENDICES

APPENDIX I. Raw Data from Q-PCR Assays for all Spawnings

Ct values were exported from the SDS format from the ABI 7700 into Excel and rearranged into the following tables in an Excel spreadsheet for subsequent export into the Q-Gene Software. All values are raw Ct values recorded directly by the ABI7700. The PCR Efficiency slope and E-values were calculated from the PCR Efficiency analysis – see Tables 11-15 in Appendix III

Table 1: Spawn-5 Ct Values

PCR Effic. Slope:	-3.6897	-3.284	-3.5638	-3.5067	-3.2972
E-value:	1.866486	2.0160751	1.9080922	1.9282724	2.0104239
Triplicate Samples	Reference	Target-1	Target-2	Target-3	Target-4
	Ct-18S	Ct-EcR	Ct-HR3	Ct-FTZF1	Ct-HR78
RNA-26	16.35	26.00	27.04	27.11	26.13
	16.43	25.90	27.06	27.17	25.91
	15.98	25.88	26.91	27.25	26.03
RNA-27	15.96	25.73	26.39	28.15	25.89
	16.06	25.44	26.66	28.19	25.90
	16.14	25.41	26.44	28.17	25.73
RNA-28	15.59	25.32	26.04	28.26	25.78
	15.90	25.09	26.23	28.27	25.96
	16.25	25.25	26.00	28.05	25.91
RNA-29	16.48	25.17	26.36	28.71	25.94
	16.33	25.10	26.29	28.56	25.82
	16.76	24.93	26.50	28.26	25.60
RNA-30	17.05	25.15	26.77	28.50	26.03
	16.86	25.19	26.41	28.43	26.04
	16.68	25.15	26.75	28.52	26.04
RNA-31	16.09	25.05	26.73	27.95	26.04
	15.97	24.94	26.63	27.88	26.02
	15.68	25.02	26.37	27.93	25.83
RNA-32	15.64	24.73	26.32	28.71	25.84
	15.93	24.60	26.46	28.76	25.94
	15.89	24.47	26.66	28.83	25.65
RNA-33	15.69	24.59	25.89	27.30	26.14
	15.73	24.45	26.24	27.34	25.99
	16.14	24.49	26.28	27.26	26.20
RNA-34	16.20	24.57	24.47	26.41	25.87
	16.06	24.58	24.52	26.37	25.89
	16.29	24.39	24.50	26.30	25.98
RNA-35	16.68	25.23	24.45	25.35	25.21
	16.44	25.11	24.54	25.35	25.20
	16.51	25.09	24.68	25.42	25.25
RNA-36	15.78	25.52	25.27	25.76	25.00
	15.97	25.53	25.22	25.73	24.96
	15.60	25.72	25.35	25.41	24.84
RNA-37	15.58	25.33	27.21	27.12	24.63
	15.76	25.28	27.10	27.46	24.46
	15.82	25.33	27.27	27.38	24.98
RNA-38	15.50	25.64	27.52	27.45	25.59
	15.60	25.44	27.44	27.37	25.49
	16.08	25.41	27.58	27.30	25.38
RNA-39	16.08	25.32	27.15	28.60	27.01
	16.04	25.35	27.12	28.68	26.56
	16.40	25.03	26.87	28.71	26.82
RNA-40	16.59	25.28	23.95	25.18	26.54
	16.43	25.16	23.68	25.12	26.57
	16.42	25.37	23.82	25.09	26.53

RNA-41	16.08	25.38	26.64	26.51	24.39
	16.12	25.35	26.68	26.30	24.30
	15.75	25.56	26.92	26.42	24.46
RNA-42	16.01	25.44	26.93	27.18	25.43
	16.26	25.25	26.81	27.13	25.44
	16.13	25.30	27.03	27.11	25.44
RNA-43	15.87	25.44	26.16	27.28	26.08
	15.97	24.84	26.33	27.22	26.19
	16.25	25.23	26.55	27.35	26.10
RNA-44	16.23	24.55	25.76	28.72	26.12
	15.97	24.37	25.96	28.75	26.12
	16.28	24.37	26.04	28.74	26.18
RNA-45	16.80	24.65	25.79	28.86	26.22
	16.80	24.82	26.09	28.58	26.35
	16.85	24.90	25.77	28.72	26.39
RNA-46	14.59	24.58	25.06	26.90	25.31
	14.54	24.60	25.36	26.91	25.24
	14.11	24.65	25.26	26.94	25.44
RNA-47	14.85	25.67	24.35	25.44	25.95
	15.15	25.51	24.46	25.61	25.75
	15.13	25.60	24.51	25.55	26.09
RNA-48	14.52	25.49	25.93	26.47	25.17
	14.82	25.29	26.02	26.59	25.29
	15.19	25.44	25.97	26.54	25.19
RNA-49	15.26	25.18	25.28	27.07	25.63
	15.03	24.99	25.50	27.31	25.58
	15.40	25.00	25.52	27.27	25.62
RNA-50	15.75	24.54	24.85	26.27	25.92
	15.49	24.75	24.86	26.25	26.12
	15.64	24.79	24.85	26.24	26.10
RNA-51	15.89	24.69	23.68	25.87	25.97
	15.84	24.88	24.11	25.85	25.98
	15.81	24.76	24.09	25.88	26.02
RNA-52	15.32	25.28	25.26	26.50	25.77
	15.66	25.28	25.44	26.59	25.61
	15.61	25.18	25.25	26.52	25.70
RNA-53	15.40	25.31	27.01	26.45	23.92
	15.22	25.21	26.67	26.51	24.00
	15.90	25.21	27.20	26.53	24.03
RNA-54	16.03	24.74	26.31	26.56	24.57
	15.67	25.01	26.36	26.57	24.51
	16.18	24.55	26.32	26.51	24.45
RNA-55	17.04	25.19	26.61	27.99	26.23
	17.13	25.26	26.33	28.12	26.35
	16.88	25.22	26.39	28.15	26.43
RNA-56	18.95	25.09	26.84	28.42	26.17
	15.87	25.06	26.98	28.68	26.05
	15.45	25.08	27.05	28.59	26.14
RNA-57	15.42	25.15	26.63	28.26	26.19
	15.62	25.17	27.08	28.24	26.00
	15.64	25.16	26.88	28.12	26.08
RNA-58	15.20	24.73	26.91	29.09	26.09
	15.27	24.91	26.92	29.13	26.05
	15.75	24.77	27.10	29.08	26.14
RNA-59	15.71	24.42	25.12	26.53	25.98
	15.76	24.47	25.05	26.46	26.05
	16.11	24.38	24.88	26.52	25.98
RNA-60	16.79	24.56	25.40	26.24	26.31
	16.42	24.66	25.28	26.31	26.25
	16.66	24.86	25.55	26.34	26.31
RNA-61	16.29	25.35	25.10	26.63	26.92
	16.52	25.61	25.05	26.71	26.96
	16.12	25.63	25.04	26.73	27.02
RNA-62	15.60	25.14	24.41	25.24	26.45
	15.91	25.15	24.39	25.31	26.29
	16.01	25.21	24.49	25.32	26.55

RNA-63	15.82	24.43	27.01	28.54	25.71
	15.57	24.52	27.01	28.51	25.82
	15.58	24.45	26.95	28.49	25.88
RNA-64	16.08	25.02	26.84	26.83	24.37
	16.11	25.10	26.78	26.76	24.45
	16.24	25.01	26.77	26.89	24.35
RNA-65	17.06	25.37	26.87	26.34	25.32
	16.76	25.68	26.91	26.35	25.29
	16.76	25.64	26.91	26.57	25.32
RNA-66	15.88	25.62	25.78	26.28	25.23
	15.93	25.59	25.99	26.29	25.14
	15.52	25.79	25.97	26.25	25.14
RNA-67	15.23	25.14	26.47	25.85	25.51
	15.54	25.15	26.50	25.81	25.46
	15.62	25.08	26.57	26.01	25.53

Table 2: Spawn-6 Ct Values

Slope:	-3.75133	-3.49188	-3.379842	-3.460664	-3.581312
PCR Eff (E)	1.847447	1.9336535	1.9763863	1.9451892	1.9020734
Triplicate Samples	Reference	Target-1	Target-2	Target-3	Target-4
	Ct-18S	Ct-EcR	Ct-HR3	Ct-FTZF1	Ct-HR78
RNA-71	16.18	25.01	26.58	27.03	25.70
	16.43	25.07	26.60	26.90	25.75
	16.21	25.10	26.60	26.96	25.72
RNA-72	16.07	25.02	26.89	27.07	26.00
	16.17	25.12	27.05	27.17	25.80
	16.19	25.11	27.07	27.12	25.94
RNA-73	16.00	25.31	26.83	27.31	25.99
	16.19	25.41	27.02	27.47	26.01
	16.38	25.32	27.04	27.48	25.84
RNA-74	16.19	25.00	26.84	28.90	26.06
	16.33	25.05	26.89	28.69	26.07
	16.89	24.88	26.99	28.92	25.99
RNA-75	16.27	24.03	26.37	28.37	25.42
	16.12	24.28	26.19	28.57	25.46
	16.13	24.25	26.41	28.56	25.61
RNA-76	15.58	24.00	26.05	28.41	25.59
	15.74	24.08	26.00	28.45	25.71
	15.36	24.19	26.13	28.40	25.61
RNA-77	15.62	24.31	24.79	26.31	25.85
	15.79	24.30	24.89	26.36	25.72
	15.91	24.30	24.84	26.32	25.80
RNA-78	15.20	24.55	24.02	24.90	25.80
	15.34	24.59	24.08	24.91	25.72
	15.78	24.52	23.98	24.97	25.66
RNA-79	16.00	25.28	24.75	24.37	24.26
	16.06	25.28	24.81	24.38	24.32
	16.70	25.23	24.61	24.37	24.26
RNA-80	16.77	25.47	26.42	25.09	23.54
	16.78	25.72	26.42	25.14	23.55
	16.75	25.70	26.45	25.21	23.59
RNA-81	15.88	25.14	27.47	26.97	24.27
	16.03	25.19	27.47	26.90	24.31
	15.61	25.29	27.86	26.85	24.33
RNA-82	15.48	25.23	27.21	26.57	24.14
	16.12	25.41	27.71	26.63	24.03
	15.87	25.29	27.30	26.60	24.12
RNA-83	15.53	25.60	27.10	26.40	24.14
	15.70	25.68	27.30	26.46	24.09
	16.19	25.52	27.11	26.39	24.13
RNA-84	16.20	25.07	23.92	24.52	26.08
	16.03	25.13	23.85	24.53	25.94
	16.44	25.08	23.74	24.54	26.00
RNA-85	16.82	25.19	27.45	29.58	26.22
	17.13	25.37	27.59	29.61	26.28
	16.96	25.36	27.45	29.55	26.32
RNA-86	16.10	25.50	27.29	28.73	26.22
	16.03	25.50	27.21	28.68	26.25
	15.75	25.61	27.51	28.68	26.25
RNA-87	15.17	25.28	27.14	28.34	25.97
	16.06	25.39	27.41	28.33	25.85
	16.31	25.36	27.29	28.25	26.04
RNA-88	15.15	24.94	27.38	29.31	26.16
	15.25	25.00	27.32	29.60	26.20
	16.16	24.91	27.53	29.72	26.24
RNA-89	16.15	24.66	25.55	28.50	26.46
	16.16	24.78	25.41	28.59	26.35
	16.51	24.68	25.56	28.76	26.33

	16.49	24.87	24.36	26.05	26.28
RNA-90	16.37	24.85	24.60	26.06	26.35
	16.35	24.99	24.40	25.98	26.30
	16.10	25.50	27.29	28.73	26.22
RNA-91	16.03	25.50	27.21	28.68	26.25
	15.75	25.61	27.51	28.68	26.25
	15.17	25.28	27.14	28.34	25.97
RNA-92	16.06	25.39	27.41	28.33	25.85
	16.31	25.36	27.29	28.25	26.04
	15.15	24.94	27.38	29.31	26.16
RNA-93	15.25	25.00	27.32	29.60	26.20
	16.16	24.91	27.53	29.72	26.24
	16.15	24.66	25.55	28.50	26.46
RNA-94	16.16	24.78	25.41	28.59	26.35
	16.51	24.68	25.56	28.76	26.33
	16.49	24.87	24.36	26.05	26.28
RNA-95	16.37	24.85	24.60	26.06	26.35
	16.35	24.99	24.40	25.98	26.30
	15.33	24.96	27.40	26.93	24.06
RNA-96	16.13	25.09	27.54	26.94	24.07
	15.93	25.09	27.67	27.03	24.06
	15.37	25.11	27.91	28.15	25.82
RNA-97	15.66	25.13	28.10	28.34	25.79
	15.88	25.14	28.01	28.31	25.78
	15.29	25.18	27.61	28.54	25.73
RNA-98	15.18	25.28	27.53	28.57	25.72
	16.11	25.17	27.45	28.49	25.78
	15.46	24.75	27.35	29.19	26.02
RNA-99	15.35	24.75	27.05	29.12	25.98
	15.97	24.65	27.01	29.20	25.92
	16.40	24.42	27.16	29.09	25.81
RNA-100	16.27	24.73	27.40	29.20	26.02
	16.30	24.66	27.27	29.22	26.03
	15.61	24.32	27.10	29.25	26.06
RNA-101	15.77	24.32	27.32	29.27	26.08
	15.39	24.52	27.12	29.31	26.05
	14.99	24.10	25.14	26.03	25.44
RNA-68	15.19	24.26	25.17	26.01	25.56
	15.63	24.16	25.19	26.04	25.52
	16.05	24.08	25.74	27.18	25.37
RNA-69	16.25	24.15	25.67	27.27	25.50
	16.73	24.09	25.75	27.22	25.48
	16.84	24.23	25.98	27.04	25.49
RNA-70	16.70	24.43	25.95	27.04	25.61
	16.41	24.44	26.00	27.11	25.61

Table 3: Spawn-8 Ct Values

PCR Efficiency (E)	1.855994	1.951592	2.021956	1.921804	1.948322
Triplicate Samples	Reference	Target-1	Target-2	Target-3	Target-4
	Ct-18S	Ct-EcR	Ct-HR3	Ct-FTZF1	Ct-HR78
RNA-102	14.26	24.24	25.18	24.88	25.05
	14.36	24.39	25.50	25.09	25.20
	14.26	24.48	25.38	25.00	25.09
RNA-103	14.15	24.49	25.70	27.06	25.03
	14.33	24.64	26.14	26.82	24.91
	14.12	24.47	26.21	26.89	25.06
RNA-104	14.21	23.98	25.97	26.04	24.80
	13.91	24.07	26.07	26.34	24.89
	14.56	24.02	25.98	26.42	24.72
RNA-105	14.65	24.07	26.17	27.77	25.16
	14.45	24.15	26.23	27.86	25.17
	15.03	23.85	26.09	28.03	25.12
RNA-106	15.32	23.65	26.54	28.14	25.03
	14.89	23.68	26.39	28.15	25.09
	15.21	23.90	26.34	28.01	25.08
RNA-107	14.92	23.53	25.74	27.30	24.66
	14.66	23.37	25.99	27.41	24.86
	14.08	23.52	26.01	27.48	24.81
RNA-108	14.30	23.54	24.44	25.85	24.88
	14.43	23.38	24.53	26.01	24.88
	14.40	23.28	24.40	25.83	25.05
RNA-109	14.12	24.02	23.85	24.64	25.51
	14.10	24.08	23.47	24.33	25.42
	14.91	24.02	23.48	24.38	25.47
RNA-111	15.53	24.61	23.58	23.18	25.12
	15.66	24.93	23.41	23.26	25.06
	15.34	24.52	23.26	23.20	25.15
RNA-110	14.75	24.24	26.21	25.22	22.79
	14.76	24.30	26.43	25.24	22.75
	15.25	24.13	26.07	25.22	22.87
RNA-112	14.41	24.93	26.64	25.32	22.97
	14.65	24.91	26.45	25.42	23.26
	14.53	25.02	26.14	25.32	23.13
RNA-113	14.58	24.78	25.71	26.53	25.12
	14.74	24.62	26.10	26.45	24.79
	15.08	24.71	25.97	26.27	25.26
RNA-114	14.30	24.27	26.82	28.14	25.34
	14.05	24.39	26.52	28.03	25.33
	15.01	24.13	26.51	27.95	25.11
RNA-115	14.24	23.51	26.35	28.20	25.18
	14.58	23.51	26.17	28.25	24.91
	15.11	23.36	26.42	28.32	24.91
RNA-116	15.28	23.52	26.14	27.68	25.08
	14.98	23.82	26.46	27.71	25.23
	15.17	23.61	26.15	27.77	25.10
RNA-117	15.12	24.00	23.46	25.58	25.24
	15.10	24.02	23.50	25.56	25.27
	14.44	23.94	23.62	25.58	25.10
RNA-118	14.83	23.90	23.81	26.75	25.55
	15.10	23.73	23.98	26.69	25.26
	15.10	23.83	24.13	26.77	25.44
RNA-119	14.45	24.32	23.07	24.53	25.56
	14.58	24.37	23.08	24.57	25.55
	15.16	24.34	22.88	24.46	25.42
RNA-120	15.11	23.92	23.33	24.50	25.23
	15.05	24.08	23.33	24.48	25.06
	15.47	23.98	23.25	24.49	25.14
RNA-121	16.00	24.11	25.60	25.24	23.04
	15.55	24.47	25.72	25.13	23.09
	15.91	24.28	26.04	25.18	23.13

RNA-122	15.17	24.27	26.32	26.56	25.23
	15.10	24.20	26.41	26.46	25.34
	14.48	24.26	26.40	26.54	25.39
RNA-123	14.99	24.59	26.37	27.09	25.49
	15.17	24.65	26.36	27.13	25.56
	15.13	24.72	26.51	27.01	25.63
RNA-124	14.47	24.32	26.65	27.27	25.24
	14.82	24.27	26.40	27.16	25.34
	15.14	24.26	26.36	27.32	25.33
RNA-125	15.34	24.13	26.46	26.50	25.23
	15.14	24.11	26.32	26.68	25.23
	15.58	23.93	26.48	26.65	25.26

Table 4: Spawn-11 Ct Values

PCR Efficiency Slope	-3.85633	-3.183955	-3.498607	-3.676581	-3.558751
E-value:	1.816827	2.060985	1.9312035	1.8706471	1.9098419
Triplicate Samples	Reference	Target-1	Target-2	Target-3	Target-4
	Ct-18S	Ct-EcR	Ct-HR3	Ct-HR4	Ct-HR78
RNA-146	15.24	26.23	28.78	27.99	26.56
	15.55	26.60	28.93	28.07	26.55
	15.48	26.39	28.80	27.71	26.86
RNA-147	15.65	26.50	28.73	29.07	27.57
	16.13	27.23	28.84	29.12	28.06
	15.88	26.79	28.45	28.61	27.37
RNA-148	16.00	26.39	28.75	30.00	28.28
	16.12	26.77	28.96	30.67	28.13
	16.14	26.58	28.42	30.30	27.81
RNA-149	16.09	26.16	29.01	31.23	27.42
	15.99	26.90	29.28	31.06	27.28
	16.27	26.35	29.13	31.12	27.09
RNA-150	15.80	25.10	28.17	29.38	26.48
	16.19	25.10	28.36	29.08	27.36
	15.92	24.76	28.30	29.41	27.30
RNA-151	14.44	25.18	27.12	29.14	27.08
	14.44	25.23	27.25	29.10	26.37
	14.59	25.13	26.85	29.34	27.13
RNA-152	14.44	25.18	24.66	27.65	26.34
	15.04	25.13	25.75	27.80	26.73
	15.07	25.64	25.74	27.13	26.55
RNA-153	14.82	26.54	24.50	25.66	28.88
	15.19	26.66	25.15	25.67	27.66
	15.39	26.83	25.40	25.63	28.27
RNA-154	15.28	26.92	28.50	28.15	25.78
	15.67	27.09	29.00	28.04	26.31
	15.74	26.67	28.47	27.76	26.37
RNA-155	15.61	26.05	27.27	26.22	24.84
	15.45	26.23	26.71	26.39	25.32
	15.63	26.12	27.27	26.06	24.93
RNA-156	14.98	26.47	28.69	27.47	23.95
	15.32	26.11	28.80	26.69	24.24
	15.24	26.11	28.92	26.77	24.21
RNA-157	15.00	25.71	28.55	29.22	26.49
	15.13	25.52	28.32	29.07	26.20
	15.27	25.67	28.09	29.10	26.13
RNA-158	15.40	26.01	28.47	30.10	26.81
	15.39	26.17	28.43	30.04	26.83
	16.05	25.63	28.52	30.27	26.37
RNA-159	16.19	25.84	28.66	29.75	27.67
	15.91	26.10	28.56	29.74	27.40
	16.55	25.58	28.38	29.54	27.01
RNA-160	16.03	24.42	27.87	29.69	26.21
	16.00	24.49	27.64	29.40	26.41
	16.06	24.61	27.63	29.59	26.37
RNA-161	15.29	25.45	27.65	30.38	27.02
	15.68	25.65	27.55	29.73	26.78
	15.41	25.45	27.38	29.76	26.88
RNA-162	17.31	26.79	26.88	26.35	27.66
	17.45	26.59	26.62	26.67	27.64
	17.56	26.93	26.64	26.55	27.32
RNA-163	15.40	27.10	25.50	26.12	26.25
	15.70	26.59	25.56	25.79	26.63
	16.02	26.89	25.67	25.72	26.60
RNA-164	15.22	25.65	28.58	27.42	24.58
	15.21	25.65	29.06	27.56	24.66
	15.63	25.15	28.63	27.74	24.81

RNA-165	15.74	25.55	28.60	30.02	26.58
	15.96	25.71	28.90	30.16	27.05
	16.10	26.06	28.78	30.08	26.98
RNA-166	15.19	25.66	28.44	29.94	26.32
	15.08	25.72	28.62	29.67	26.75
	15.25	26.00	28.53	29.02	26.50
RNA-167	15.38	25.29	29.10	30.51	26.51
	15.68	25.25	28.77	30.50	26.80
	15.53	25.21	28.78	30.45	26.85
RNA-168	15.14	25.25	28.17	30.07	27.04
	15.26	25.12	28.17	30.06	26.36
	15.41	25.05	28.24	30.03	26.51
RNA-169	15.61	24.61	27.94	29.52	26.73
	15.67	25.08	28.15	29.18	26.63
	16.00	24.56	27.97	29.54	26.54
RNA-170	16.29	25.10	26.15	26.67	26.77
	16.43	25.11	26.10	26.66	26.32
	16.50	25.46	26.22	26.56	26.58
RNA-171	15.41	24.98	26.70	27.81	26.31
	15.44	24.79	26.87	27.39	26.39
	15.60	25.31	26.50	26.96	26.31
RNA-172	16.37	26.10	29.20	29.60	27.38
	16.57	25.65	29.46	30.33	27.79
	16.57	26.13	29.11	30.13	27.59
RNA-173	16.17	26.08	29.22	30.30	28.76
	16.72	26.11	29.43	30.12	28.05
	16.65	25.72	29.13	30.25	27.36
RNA-174	15.91	24.56	26.41	28.89	26.38
	15.80	24.22	26.91	29.07	26.56
	16.06	23.97	26.50	28.74	26.22

Table 5: Spawn-14 Ct Values

PCR Efficiency Slope	-3.753	-3.44188	-3.583157	-3.798441	-3.939056
E-value:	1.846943	1.9522657	1.9014439	1.8334365	1.7941879
Triplicate Samples	Reference Ct-18S	Target-1 Ct-EcR	Target-2 Ct-HR3	Target-3 Ct-HR4	Target-4 Ct-HR78
RNA-175	17.10	27.40	29.27	**29.23**	28.19
	16.88	**27.47**	29.25	29.10	28.33
	16.97	27.53	29.23	29.36	28.20
RNA-176	16.64	27.41	29.06	28.57	27.63
	16.74	27.16	29.11	28.60	27.87
	17.07	27.17	29.01	28.26	27.40
RNA-177	16.37	26.70	29.15	29.19	28.13
	16.53	27.22	29.03	28.87	27.71
	16.46	26.92	29.30	29.13	27.67
RNA-178	16.28	26.02	28.72	29.14	27.01
	16.60	26.15	28.60	29.15	27.55
	16.76	26.06	29.17	29.25	27.34
RNA-179	16.35	24.83	27.32	27.91	**26.58**
	16.46	24.90	27.31	**27.91**	26.57
	16.27	24.94	27.78	**27.91**	26.59
RNA-180	15.88	25.65	28.25	**30.46**	27.28
	15.95	25.76	27.85	30.44	27.26
	15.35	26.09	28.25	30.48	27.03
RNA-181	16.09	26.33	26.96	30.01	27.78
	16.24	26.89	27.04	30.04	27.90
	16.42	26.43	27.06	29.65	27.40
RNA-182	16.27	27.26	26.17	26.53	28.26
	16.11	27.19	26.15	26.55	27.95
	16.41	27.19	26.23	26.52	28.04
RNA-183	16.89	26.75	26.34	27.94	27.90
	16.93	26.65	26.29	27.28	27.85
	17.38	26.69	26.44	27.71	27.82
RNA-184	17.11	26.61	29.19	28.51	25.88
	17.05	26.91	29.72	28.70	25.85
	17.18	27.03	29.78	28.47	26.03
RNA-185	16.12	27.49	29.46	30.56	27.94
	16.28	27.48	29.21	30.49	27.84
	16.31	0.00	29.13	0.00	28.09
RNA-186	16.06	26.90	29.15	30.44	27.72
	15.79	26.98	29.28	30.80	27.30
	16.19	27.33	28.77	30.48	27.42
RNA-187	16.15	27.39	29.26	31.30	28.67
	16.48	27.37	29.22	31.68	28.25
	16.33	27.23	29.67	31.96	28.25
RNA-188	17.01	26.80	29.55	32.28	27.92
	17.21	26.53	29.33	31.89	28.32
	17.21	26.33	29.49	31.37	28.07
RNA-189	17.25	26.40	28.56	31.30	28.42
	17.46	26.47	29.11	31.61	28.15
	17.72	26.43	29.50	31.98	28.40
RNA-190	15.24	26.23	28.78	27.99	26.56
	15.55	26.60	28.93	28.07	26.55
	15.48	26.39	28.80	27.71	26.86
RNA-191	15.65	26.50	28.73	29.07	27.57
	16.13	27.23	28.84	29.12	28.06
	15.88	26.79	28.45	28.61	27.37
RNA-192	16.00	26.39	28.75	30.00	28.28
	16.12	26.77	28.96	30.67	28.13
	16.14	26.58	28.42	30.30	27.81
RNA-193	16.09	26.16	29.01	31.23	27.42
	15.99	26.90	29.28	31.06	27.28
	16.27	26.35	29.13	31.12	27.09

RNA-194	15.80	25.10	28.17	29.38	26.48
	16.19	25.10	28.36	29.08	27.36
	15.92	24.76	28.30	29.41	27.30
RNA-195	16.57	27.48	29.56	30.84	28.29
	0.00	27.84	29.48	30.74	28.70
	16.55	27.34	29.15	31.28	28.47
RNA-196	0.00	26.59	29.06	31.45	28.04
	16.42	26.80	28.94	31.63	28.10
	16.38	26.92	29.36	31.14	27.55
RNA-197	16.22	26.67	29.58	31.57	28.41
	16.82	26.38	29.89	31.50	28.01
	0.00	26.60	29.38	31.56	27.97
RNA-198	17.05	26.09	29.33	32.10	28.85
	17.12	26.16	29.11	31.98	28.36
	17.16	26.03	29.54	31.95	28.75
RNA-199	17.63	25.36	29.00	31.79	28.02
	17.96	25.03	28.81	31.31	27.72
	17.15	25.79	29.43	31.70	27.73
RNA-200	16.29	26.17	26.87	28.81	27.18
	16.35	26.34	26.77	29.19	27.46
	16.24	26.25	26.87	29.45	27.60
RNA-201	16.16	26.38	26.39	28.21	27.92
	16.11	26.61	26.33	28.16	27.97
	16.51	26.73	26.02	27.96	27.60
RNA-202	16.83	29.18	29.43	30.53	29.14
	17.05	28.67	29.55	30.84	28.70
	16.99	28.72	29.73	31.08	29.42
RNA-203	17.81	27.43	27.07	27.63	28.61
	17.55	27.67	27.10	27.21	29.05
	18.12	27.68	27.06	27.61	28.64
RNA-204	18.50	27.19	30.23	29.49	28.15
	18.04	27.27	30.06	29.73	28.22
	18.14	27.84	30.12	29.54	28.47
RNA-205	17.44	28.34	30.10	30.58	27.64
	17.26	28.37	30.04	30.44	27.50
	17.36	28.68	30.16	30.86	27.77
RNA-206	17.75	28.52	28.40	29.11	30.28
	18.07	28.51	28.45	29.01	30.21
	18.26	29.22	28.14	28.75	30.30

BOLD: reactions that didn't work, values are averages of successful reactions

APPENDIX II. Q-Gene Data for Nuclear Hormone Receptor Expression Plots

The raw Ct-values from Table 1-Table 5 were exported directly into the Q-Gene Software and used for calculation of Mean Normalised Expression (MNE) applying Muller's Equation-3 as described in Chapter 3. The MNE values together with the Standard Error of the Mean (SEM), as a percentage value, were exported back into Excel for calculations of Relative Gene Expression. The Relative Gene Expression was calculated by simply dividing the MNE of each sample by the MNE of the calibrator sample (P1 d1 sample from each spawn). The SEM from the MNE from Q-Gene was used to calculate the error values for the subsequent Relative Gene Expression value.

Tables 6-10 show the raw MNE values exported from Excel together with the SEM and subsequent Relative Gene Expression Values and calculated Standard Error.

Table 6: Gene Expression Data for Spawn-5

RNA Sample	Stage/days	EcR				HR4				HR3				HR78			
		MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM
RNA-26	P1 d1	3.238E-04	9.033	1.000	0.090	1.348E-04	9.105	1.000	0.091	1.522E-04	9.257	1.000	0.093	3.025E-04	9.731	1.000	0.097
RNA-27	P1 d2	3.783E-04	7.858	1.168	0.092	5.928E-05	3.349	0.440	0.015	1.916E-04	6.661	1.259	0.084	3.037E-04	5.047	1.004	0.051
RNA-28	P1 d3	4.298E-04	12.819	1.328	0.170	5.344E-05	12.916	0.397	0.051	2.335E-04	12.895	1.535	0.198	2.699E-04	12.478	0.892	0.111
RNA-29	P1 d4	7.003E-04	9.317	2.163	0.202	6.262E-05	12.160	0.465	0.057	2.782E-04	8.976	1.828	0.164	4.227E-04	10.515	1.397	0.147
RNA-30	P1d5	8.091E-04	6.732	2.499	0.168	7.889E-05	6.935	0.585	0.041	2.866E-04	10.560	1.883	0.199	4.386E-04	6.670	1.450	0.097
RNA-31	P1 d6	5.003E-04	7.936	1.545	0.123	6.473E-05	7.734	0.480	0.037	1.660E-04	10.690	1.091	0.117	2.552E-04	8.927	0.844	0.075
RNA-32	P1 d7	6.262E-04	7.730	1.934	0.150	3.373E-05	6.166	0.250	0.015	1.676E-04	8.940	1.101	0.098	2.681E-04	8.223	0.886	0.073
RNA-33	P1 d8	6.811E-04	9.437	2.104	0.199	9.630E-05	9.119	0.715	0.065	2.177E-04	12.489	1.431	0.179	2.218E-04	9.985	0.733	0.073
RNA-34	P1 d9	8.349E-04	6.015	2.579	0.155	2.287E-04	4.745	1.697	0.081	8.447E-04	4.298	5.550	0.239	3.128E-04	4.803	1.034	0.050
RNA-35	P1 d10	6.720E-04	5.401	2.076	0.112	5.719E-04	4.738	4.244	0.201	1.014E-03	6.464	6.662	0.431	6.368E-04	4.574	2.105	0.096
RNA-41	P2 d1	3.875E-04	8.641	1.197	0.103	1.949E-04	8.469	1.446	0.122	1.539E-04	9.545	1.012	0.097	8.072E-04	8.004	2.668	0.214
RNA-42	P2 d1-2	4.564E-04	6.016	1.410	0.085	1.283E-04	4.735	0.952	0.045	1.493E-04	6.339	0.981	0.062	4.235E-04	4.511	1.400	0.063
RNA-43	P2 d2-3	4.787E-04	14.222	1.479	0.210	1.088E-04	7.570	0.807	0.061	2.098E-04	10.631	1.379	0.147	2.454E-04	7.483	0.811	0.061
RNA-44	P2 d3-4	8.723E-04	7.325	2.694	0.197	4.258E-05	6.028	0.316	0.019	3.069E-04	8.369	2.016	0.169	2.630E-04	6.158	0.869	0.054
RNA-45	P2 d4-5	1.021E-03	5.272	3.154	0.166	6.491E-05	5.762	0.482	0.028	4.743E-04	7.331	3.117	0.228	3.492E-04	3.745	1.154	0.043
RNA-46	P2 d5-6	2.585E-04	9.619	0.798	0.077	5.129E-05	9.545	0.381	0.036	1.678E-04	11.342	1.102	0.125	1.560E-04	10.357	0.516	0.053
RNA-47	P2 d6-8	1.922E-04	6.860	0.594	0.041	2.005E-04	6.979	1.488	0.104	4.315E-04	6.892	2.835	0.195	1.518E-04	9.185	0.502	0.046
RNA-53	P3 d1-3	3.280E-04	12.908	1.013	0.131	1.362E-04	12.806	1.011	0.129	9.844E-05	16.712	0.647	0.108	7.936E-04	12.901	2.623	0.338
RNA-54	P3 d2-4	6.080E-04	13.295	1.878	0.250	1.745E-04	9.533	1.295	0.123	2.032E-04	9.504	1.335	0.127	7.279E-04	9.751	2.406	0.235
RNA-55	P3 d3-5	8.536E-04	4.779	2.637	0.126	1.146E-04	5.716	0.851	0.049	3.629E-04	7.512	2.385	0.179	3.911E-04	6.117	1.293	0.079
RNA-56	P3 d4-6	4.057E-04	7.592	1.253	0.095	3.520E-05	9.264	0.261	0.024	1.086E-04	8.717	0.714	0.062	1.952E-04	7.977	0.645	0.051
RNA-57	P3 d5-7	3.595E-04	4.402	1.111	0.049	4.246E-05	5.349	0.315	0.017	1.089E-04	10.125	0.716	0.072	1.873E-04	5.842	0.619	0.036
RNA-58	P3 d6-8	4.196E-04	11.445	1.296	0.148	2.063E-05	10.840	0.153	0.017	9.141E-05	11.623	0.601	0.070	1.698E-04	10.940	0.561	0.061
RNA-59	P3 d7-9	7.267E-04	8.062	2.245	0.181	1.690E-04	8.001	1.254	0.100	4.794E-04	9.307	3.150	0.293	2.400E-04	8.021	0.793	0.064
RNA-64	P4 d1	5.615E-04	3.658	1.734	0.063	1.608E-04	4.041	1.193	0.048	1.642E-04	3.426	1.079	0.037	8.878E-04	3.739	2.935	0.110
RNA-65	P4 d1	6.099E-04	9.249	1.884	0.174	3.345E-04	8.163	2.482	0.203	2.395E-04	6.310	1.574	0.099	7.285E-04	6.280	2.408	0.151
RNA-66	P4 d1	2.885E-04	9.166	0.891	0.082	1.886E-04	8.103	1.399	0.113	2.427E-04	9.325	1.595	0.149	4.087E-04	8.329	1.351	0.113
RNA-67	P4 d2	3.473E-04	7.579	1.073	0.081	2.029E-04	8.570	1.506	0.129	1.311E-04	7.707	0.861	0.066	2.667E-04	7.564	0.882	0.067
Samples analysed but not included in Expression plots (larval samples extending past appearance of molt to next stage or mixed stage samples																	
RNA-36	d1	3.057E-04	8.078			2.966E-04	10.301			3.800E-04	7.175			4.845E-04	7.470		
RNA-37	d1-2	3.568E-04	4.649			8.737E-05	8.487			9.549E-05	5.695			5.524E-04	11.638		
RNA-38	d1-3	3.151E-04	12.264			8.452E-05	11.577			7.662E-05	11.527			3.173E-04	11.953		
RNA-39	?	5.008E-04	10.086			4.521E-05	7.473			1.404E-04	9.448			1.673E-04	11.583		
RNA-40	d13	5.910E-04	5.477			6.520E-04	3.906			1.637E-03	6.457			2.415E-04	3.539		
RNA-52	d4-8	3.321E-04	7.015			1.344E-04	6.885			3.162E-04	7.905			2.428E-04	7.368		

Table 7: Gene Expression Data for Spawn-6

RNA Sample	Stage/days	EcR				HR4				HR3				HR78			
		MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM
RNA-71	1d1	1.450E-03	5.142	1.000	0.051	3.521E-04	5.445	1.000	0.054	2.950E-04	4.859	1.000	0.049	1.430E-03	4.927	1.000	0.049
RNA-72	1d2	1.318E-03	3.096	0.909	0.028	2.929E-04	2.980	0.832	0.025	2.060E-04	4.500	0.698	0.031	1.169E-03	4.439	0.817	0.036
RNA-73	1d3	1.140E-03	7.052	0.786	0.055	2.469E-04	7.666	0.701	0.054	2.178E-04	8.131	0.738	0.060	1.177E-03	7.565	0.823	0.062
RNA-74	1d4	1.728E-03	13.541	1.192	0.161	1.142E-04	14.009	0.324	0.045	2.689E-04	13.466	0.911	0.123	1.316E-03	13.226	0.920	0.122
RNA-75	1d5	2.426E-03	5.987	1.673	0.100	1.191E-04	5.251	0.338	0.018	3.334E-04	5.484	1.130	0.062	1.556E-03	4.760	1.088	0.052
RNA-76	1d6	1.774E-03	7.675	1.224	0.094	8.621E-05	6.837	0.245	0.017	2.738E-04	7.236	0.928	0.067	9.759E-04	7.170	0.682	0.049
RNA-77	1d7	1.757E-03	5.169	1.212	0.063	3.948E-04	5.263	1.121	0.059	7.166E-04	5.526	2.429	0.134	1.008E-03	5.709	0.705	0.040
RNA-81	2 d1	1.009E-03	8.084	0.696	0.056	2.802E-04	7.890	0.796	0.063	1.139E-04	11.633	0.386	0.045	2.731E-03	7.627	1.910	0.146
RNA-82	2 d1-2	9.329E-04	11.951	0.643	0.077	3.401E-04	11.488	0.966	0.111	1.286E-04	15.510	0.436	0.068	3.088E-03	11.635	2.159	0.251
RNA-83	2 d1-3	7.626E-04	12.520	0.526	0.066	3.804E-04	12.231	1.080	0.132	1.495E-04	12.928	0.507	0.066	3.011E-03	12.184	2.105	0.256
RNA-86	2 d2-4	8.736E-04	6.994	0.603	0.042	9.167E-05	6.656	0.260	0.017	1.467E-04	8.967	0.497	0.045	8.464E-04	6.595	0.592	0.039
RNA-87	2 d3-5	9.257E-04	21.344	0.638	0.136	1.108E-04	21.319	0.315	0.067	1.422E-04	21.891	0.482	0.106	9.493E-04	21.532	0.664	0.143
RNA-88	2 4-6	9.819E-04	19.799	0.677	0.134	3.984E-05	21.319	0.113	0.024	1.065E-04	20.175	0.361	0.073	6.629E-04	19.777	0.464	0.092
RNA-89	2 d5-7	1.830E-03	7.667	1.262	0.097	1.172E-04	8.861	0.333	0.029	6.185E-04	7.979	2.097	0.167	9.376E-04	7.716	0.656	0.051
RNA-94	3 d1	1.830E-03	7.667	1.262	0.097	1.172E-04	8.861	0.333	0.029	6.185E-04	7.979	2.097	0.167	9.376E-04	7.716	0.656	0.051
RNA-95	3 d1-2	1.741E-03	3.938	1.201	0.047	7.096E-04	3.163	2.015	0.064	1.373E-03	5.725	4.654	0.266	1.062E-03	2.999	0.743	0.022
RNA-96	3 d1-3	1.092E-03	15.028	0.753	0.113	2.622E-04	14.905	0.745	0.111	1.158E-04	15.681	0.392	0.062	3.103E-03	14.756	2.170	0.320
RNA-97	3 d2-5	9.388E-04	9.084	0.647	0.059	1.001E-04	9.878	0.284	0.028	7.619E-05	9.806	0.258	0.025	9.229E-04	9.098	0.645	0.059
RNA-98	3 d3-6	8.306E-04	18.157	0.573	0.104	7.833E-05	18.075	0.222	0.040	9.854E-05	18.281	0.334	0.061	8.928E-04	18.048	0.624	0.113
RNA-99	3 d4-7	1.198E-03	11.927	0.826	0.099	5.342E-05	11.842	0.152	0.018	1.342E-04	13.815	0.455	0.063	8.022E-04	11.871	0.561	0.067
RNA-100	3 d5-8	2.021E-03	6.643	1.394	0.093	8.362E-05	3.612	0.238	0.009	1.910E-04	5.306	0.647	0.034	1.272E-03	5.204	0.889	0.046
RNA-101	3 d6-9	1.486E-03	8.065	1.025	0.083	4.966E-05	6.862	0.141	0.010	1.300E-04	8.283	0.441	0.037	7.555E-04	6.785	0.528	0.036
Samples analysed but not included in Expression plots (larval samples extending past appearance of molt to next stage or mixed stage samples																	
RNA-78	1d8	1.214E-03	10.809			8.185E-04	10.824			1.016E-03	10.907			8.556E-04	11.038		
RNA-79	1d9	1.253E-03	13.793			1.949E-03	13.751			1.042E-03	14.330			3.573E-03	13.809		
RNA-80	1d10	1.348E-03	5.317			1.596E-03	2.378			4.463E-04	0.870			7.779E-03	1.121		
RNA-84	1d11	1.376E-03	7.402			1.724E-03	7.310			1.871E-03	8.126			1.156E-03	7.752		
RNA-85	P1d1-5	1.890E-03	6.716			9.467E-05	5.621			2.445E-04	6.354			1.540E-03	5.810		
RNA-90	2 d6-8	1.741E-03	3.938			7.096E-04	3.163			1.373E-03	5.725			1.062E-03	2.999		
RNA-91	2 d7-10	8.736E-04	6.994			9.167E-05	6.656			1.467E-04	8.967			8.464E-04	6.595		
RNA-92	2 d8-10	9.257E-04	21.344			1.108E-04	21.319			1.422E-04	21.891			9.493E-04	21.532		
RNA-93	2 d9-12	9.819E-04	19.799			3.984E-05	21.319			1.065E-04	20.175			6.629E-04	19.777		

Table 8: Gene Expression Data for Spawn-8

RNA Sample	Stage/ days	EcR				HR4				HR3				HR78			
		MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM
RNA-102	1 d1	5.782E-04	5.114	1.000	0.051	5.610E-04	4.477	1.000	0.045	1.220E-04	6.887	1.000	0.069	3.685E-04	3.908	1.000	0.03907902
RNA-103	1 d2	4.893E-04	5.414	0.846	0.046	1.498E-04	6.174	0.267	0.016	7.221E-05	11.947	0.592	0.071	3.931E-04	6.458	1.067	0.068884014
RNA-104	1 d3	6.996E-04	11.745	1.210	0.142	2.338E-04	13.857	0.417	0.058	7.393E-05	11.829	0.606	0.072	3.996E-04	14.420	1.084	0.156368621
RNA-105	1 d4	9.433E-04	12.109	1.631	0.198	1.094E-04	11.638	0.195	0.023	8.928E-05	10.900	0.732	0.080	4.768E-04	10.868	1.294	0.140625341
RNA-106	1 d5	1.484E-03	9.559	2.567	0.245	1.242E-04	8.502	0.221	0.019	9.699E-05	9.028	0.795	0.072	6.937E-04	12.366	1.882	0.232782523
RNA-107	1 d6	1.237E-03	15.739	2.139	0.337	1.368E-04	15.731	0.244	0.038	9.663E-05	16.527	0.792	0.131	5.136E-04	15.417	1.394	0.21486972
RNA-108	1 d7	1.164E-03	5.616	2.014	0.113	3.267E-04	4.444	0.582	0.026	2.416E-04	3.638	1.980	0.072	3.778E-04	12.784	1.025	0.131057965
RNA-109	1 d8	7.591E-04	16.549	1.313	0.217	8.405E-04	17.649	1.498	0.264	4.416E-04	18.697	3.618	0.677	3.441E-04	19.610	0.934	0.183109274
RNA-111	1 d9	9.928E-04	10.110	1.717	0.174	3.800E-03	5.957	6.774	0.403	1.013E-03	8.682	8.297	0.720	7.803E-04	6.011	2.117	0.127275856
RNA-110	2 d1	9.397E-04	10.735	1.625	0.174	7.082E-04	10.215	1.262	0.129	9.655E-05	12.592	0.791	0.100	2.360E-03	12.175	6.405	0.779851838
RNA-112	2 d1-2	4.531E-04	4.845	0.784	0.038	5.122E-04	4.806	0.913	0.044	6.714E-05	11.118	0.550	0.061	1.605E-03	7.046	4.355	0.306873784
RNA-113	2 d2-3	6.329E-04	9.628	1.095	0.105	3.022E-04	10.409	0.539	0.056	1.115E-04	12.177	0.914	0.111	4.963E-04	14.630	1.347	0.19705106
RNA-114	2 d3-4	6.855E-04	18.478	1.186	0.219	8.445E-05	18.142	0.151	0.027	5.536E-05	19.170	0.454	0.087	3.806E-04	18.301	1.033	0.189009023
RNA-115	2 d4-5	1.319E-03	16.007	2.282	0.365	8.245E-05	15.819	0.147	0.023	7.709E-05	16.509	0.632	0.104	5.023E-04	16.104	1.363	0.219530094
RNA-116	2 d5-6	1.583E-03	8.043	2.738	0.220	1.595E-04	5.688	0.284	0.016	1.098E-04	9.168	0.900	0.082	5.896E-04	6.197	1.600	0.099166458
RNA-117	2 d6-7	1.078E-03	13.909	1.865	0.259	5.531E-04	13.823	0.986	0.136	6.374E-04	14.225	5.223	0.743	4.654E-04	16.354	1.263	0.206557297
RNA-118	2 d7-8	1.301E-03	6.470	2.250	0.146	2.792E-04	5.783	0.498	0.029	5.023E-04	8.563	4.116	0.352	4.658E-04	8.049	1.264	0.101732835
RNA-119	2 d8-9	7.711E-04	13.532	1.334	0.180	9.990E-04	13.659	1.781	0.243	8.324E-04	14.253	6.820	0.972	3.934E-04	14.536	1.068	0.155196484
RNA-120	2 d8-9	1.311E-03	8.690	2.268	0.197	1.371E-03	8.119	2.444	0.198	9.111E-04	8.325	7.465	0.621	6.339E-04	8.747	1.720	0.150460413
RNA-121	3 d1	1.571E-03	10.983	2.718	0.298	1.271E-03	8.752	2.266	0.198	2.312E-04	12.560	1.895	0.238	3.644E-03	8.677	9.889	0.858085023
RNA-122	3 d2	9.253E-04	13.638	1.600	0.218	3.036E-04	13.706	0.541	0.074	8.730E-05	13.707	0.715	0.098	4.436E-04	13.875	1.204	0.167013928
RNA-123	3 d3	7.862E-04	4.207	1.360	0.057	2.359E-04	4.086	0.420	0.017	9.510E-05	4.797	0.779	0.037	4.732E-04	8.689	1.284	0.111568918
RNA-124	3 d4	8.433E-04	12.029	1.459	0.175	1.764E-04	12.357	0.314	0.039	7.653E-05	13.564	0.627	0.085	4.459E-04	12.192	1.210	0.147517258
RNA-125	3 d5	1.373E-03	8.942	2.375	0.212	3.750E-04	8.666	0.668	0.058	1.109E-04	8.627	0.909	0.078	6.351E-04	8.110	1.724	0.139780622

Table 9: Gene Expression Data for Spawn-11

RNA Sample	Stage/days	EcR				HR4				HR3				HR78			
		MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM
RNA-146	P1 d1	5.079E-05	9.563	1.000	0.096	2.538E-04	8.839	1.000	0.088	5.717E-05	6.402	1.000	0.064	3.229E-04	8.644	1.000	0.086
RNA-147	P1 d2	4.896E-05	17.436	0.964	0.168	1.778E-04	13.108	0.701	0.092	8.394E-05	11.264	1.468	0.165	2.215E-04	15.632	0.686	0.107
RNA-148	P1 d3	6.659E-05	8.351	1.311	0.109	8.391E-05	12.413	0.331	0.041	9.234E-05	10.668	1.615	0.172	1.918E-04	9.340	0.594	0.055
RNA-149	P1 d4	7.340E-05	16.775	1.445	0.242	5.133E-05	5.800	0.202	0.012	7.083E-05	7.096	1.239	0.088	3.299E-04	7.887	1.022	0.081
RNA-150	P1 d5	1.966E-04	10.705	3.870	0.414	1.495E-04	9.537	0.589	0.056	1.145E-04	7.813	2.004	0.157	3.477E-04	19.615	1.077	0.211
RNA-151	P1 d6	7.064E-05	3.643	1.391	0.051	6.563E-05	5.525	0.259	0.014	1.045E-04	8.308	1.828	0.152	1.621E-04	16.158	0.502	0.081
RNA-152	P1 d7	7.934E-05	16.967	1.562	0.265	2.311E-04	17.656	0.910	0.161	3.940E-04	26.771	6.893	1.845	2.473E-04	14.257	0.766	0.109
RNA-153	P1 d8	3.514E-05	11.679	0.692	0.081	8.847E-04	9.998	3.485	0.348	5.940E-04	20.274	10.391	2.107	9.561E-05	24.872	0.296	0.074
RNA-155	P1 d9	3.884E-05	12.281	0.765	0.094	2.658E-04	11.219	1.047	0.117	6.997E-05	14.177	1.224	0.174	4.861E-04	14.837	1.506	0.223
RNA-154	P2 d1	6.729E-05	5.091	1.325	0.067	8.003E-04	6.868	3.153	0.217	1.971E-04	12.747	3.447	0.439	1.006E-03	10.120	3.115	0.315
RNA-156	P2 d2	4.991E-05	10.624	0.983	0.104	3.971E-04	16.682	1.565	0.261	5.053E-05	7.527	0.884	0.067	1.429E-03	8.547	4.425	0.378
RNA-157	P2 d3	7.473E-05	6.258	1.471	0.092	1.003E-04	5.469	0.395	0.022	6.755E-05	9.902	1.182	0.117	3.480E-04	8.515	1.078	0.092
RNA-158	P2 d4	7.993E-05	17.438	1.574	0.274	7.109E-05	13.733	0.280	0.038	8.133E-05	13.150	1.423	0.187	3.585E-04	16.258	1.111	0.181
RNA-159	P2 d5	1.229E-04	15.498	2.419	0.375	1.360E-04	11.861	0.536	0.064	1.121E-04	12.304	1.961	0.241	3.289E-04	16.612	1.019	0.169
RNA-160	P2 d6	2.883E-04	4.143	5.676	0.235	1.308E-04	5.426	0.515	0.028	1.720E-04	5.262	3.009	0.158	5.730E-04	4.086	1.775	0.073
RNA-161	P2 d7	9.882E-05	8.406	1.946	0.164	7.261E-05	14.948	0.286	0.043	1.384E-04	8.621	2.421	0.209	2.832E-04	8.228	0.877	0.072
RNA-162	P2 d8	1.302E-04	8.340	2.564	0.214	2.034E-03	7.268	8.012	0.582	7.709E-04	6.992	13.485	0.943	6.078E-04	8.334	1.882	0.157
RNA-163	P2 d9	4.334E-05	15.125	0.853	0.129	1.083E-03	13.187	4.267	0.563	5.787E-04	11.179	10.122	1.132	4.250E-04	13.286	1.316	0.175
RNA-164	P3 d1	9.499E-05	14.613	1.870	0.273	3.031E-04	10.094	1.194	0.121	5.779E-05	12.992	1.011	0.131	1.110E-03	9.342	3.438	0.321
RNA-165	P3 d2	1.089E-04	12.560	2.144	0.269	8.880E-05	6.752	0.350	0.024	8.153E-05	8.489	1.426	0.121	3.814E-04	11.352	1.181	0.134
RNA-166	P3 d3	6.817E-05	8.139	1.342	0.109	7.927E-05	17.356	0.312	0.054	6.025E-05	4.531	1.054	0.048	3.032E-04	8.597	0.939	0.081
RNA-167	P3 d4	1.250E-04	5.434	2.460	0.134	5.433E-05	5.300	0.214	0.011	5.908E-05	8.810	1.034	0.091	3.303E-04	8.589	1.023	0.088
RNA-168	P3 d5	1.158E-04	6.301	2.281	0.144	6.102E-05	4.724	0.240	0.011	7.967E-05	4.910	1.394	0.068	2.985E-04	14.137	0.924	0.131
RNA-169	P3 d6	2.058E-04	13.996	4.052	0.567	1.221E-04	10.292	0.481	0.049	1.196E-04	8.428	2.093	0.176	4.008E-04	8.063	1.241	0.100
RNA-170	P3 d7	2.150E-04	9.320	4.233	0.395	1.026E-03	4.292	4.044	0.174	6.000E-04	4.340	10.496	0.455	6.196E-04	9.209	1.919	0.177
RNA-171	P3 d8	1.428E-04	11.537	2.812	0.324	3.682E-04	15.766	1.451	0.229	2.434E-04	7.869	4.257	0.335	4.116E-04	3.921	1.275	0.050
RNA-172	P3 d9	1.337E-04	11.912	2.632	0.314	1.301E-04	14.209	0.513	0.073	8.264E-05	7.971	1.445	0.115	3.371E-04	8.631	1.044	0.090
RNA-173	P3 d10	1.335E-04	13.734	2.629	0.361	1.152E-04	10.854	0.454	0.049	8.295E-05	11.863	1.451	0.172	2.502E-04	28.113	0.775	0.218
RNA-174	P3 d11	3.257E-04	13.158	6.412	0.844	1.856E-04	7.479	0.731	0.055	3.344E-04	11.082	5.849	0.648	5.183E-04	7.786	1.605	0.125

Table 10: Gene Expression Data for Spawn-14

RNA Sample	Stage/days	EcR				HR4				HR3				HR78			
		MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM	MNE	SEM (%)	Relative Expression	SEM
RNA-175	P1 d1	3.513E-04	4.653	1.000	0.047	6.761E-04	6.004	1.000	0.060	2.302E-04	3.988	1.000	0.040	2.270E-03	4.722	1.000	0.047
RNA-176	P1 d2	3.670E-04	9.665	1.045	0.101	9.637E-04	10.341	1.425	0.147	2.348E-04	8.184	1.020	0.083	2.922E-03	11.245	1.287	0.145
RNA-177	P1 d3	3.589E-04	10.475	1.022	0.107	5.403E-04	6.596	0.799	0.053	1.762E-04	5.767	0.765	0.044	2.076E-03	9.057	0.914	0.083
RNA-178	P1 d4	6.802E-04	9.031	1.936	0.175	5.331E-04	8.915	0.789	0.070	2.306E-04	14.116	1.002	0.141	3.008E-03	12.623	1.325	0.167
RNA-179	P1 d5	1.342E-03	4.005	3.820	0.153	1.027E-03	3.379	1.519	0.051	4.929E-04	10.520	2.141	0.225	4.087E-03	3.396	1.800	0.061
RNA-180	P1 d6	4.840E-04	14.604	1.378	0.201	1.484E-04	11.642	0.219	0.026	2.206E-04	14.438	0.958	0.138	1.940E-03	12.531	0.855	0.107
RNA-181	P1 d7	4.131E-04	12.935	1.176	0.152	2.872E-04	9.589	0.425	0.041	6.152E-04	6.173	2.673	0.165	1.993E-03	10.576	0.878	0.093
RNA-182	P1 d8	2.672E-04	5.542	0.761	0.042	2.229E-03	5.344	3.297	0.176	1.062E-03	5.537	4.613	0.255	1.599E-03	7.566	0.705	0.053
RNA-183	P1 d9	6.181E-04	9.832	1.760	0.173	1.862E-03	15.178	2.754	0.418	1.555E-03	10.046	6.756	0.679	2.989E-03	9.734	1.317	0.128
RNA-184	P2 d1	5.740E-04	8.668	1.634	0.142	1.099E-03	4.879	1.626	0.079	2.038E-04	12.265	0.886	0.109	9.542E-03	3.988	4.204	0.168
RNA-185	P2 d2	2.192E-04	3.623	0.624	0.023	1.950E-04	3.820	0.288	0.011	1.440E-04	7.340	0.626	0.046	1.694E-03	5.579	0.746	0.042
RNA-186	P2 d3	2.523E-04	11.413	0.718	0.082	1.652E-04	9.996	0.244	0.024	1.428E-04	12.203	0.620	0.076	1.952E-03	10.273	0.860	0.088
RNA-187	P2 d4	2.559E-04	6.752	0.729	0.049	1.040E-04	12.987	0.154	0.020	1.406E-04	10.938	0.611	0.067	1.384E-03	10.061	0.610	0.061
RNA-188	P2 d5	7.131E-04	9.986	2.030	0.203	1.527E-04	16.494	0.226	0.037	2.223E-04	5.876	0.966	0.057	2.712E-03	7.952	1.195	0.095
RNA-189	P2 d6	9.480E-04	8.450	2.699	0.228	2.136E-04	14.544	0.316	0.046	3.528E-04	19.405	1.533	0.297	2.926E-03	9.764	1.289	0.126
RNA-190	P2 d7	2.738E-04	9.194	0.780	0.072	5.732E-04	8.771	0.848	0.074	1.153E-04	6.504	0.501	0.033	2.200E-03	8.277	0.969	0.080
RNA-192	P2 d8	3.663E-04	7.813	1.043	0.081	2.010E-04	12.048	0.297	0.036	1.879E-04	10.450	0.816	0.085	1.444E-03	8.535	0.636	0.054
RNA-193	P2 d9	4.016E-04	15.672	1.143	0.179	1.251E-04	5.862	0.185	0.011	1.452E-04	7.103	0.631	0.045	2.361E-03	7.517	1.040	0.078
RNA-191	P3 d1 (<4hrs)	2.723E-04	16.549	0.775	0.128	4.130E-04	13.005	0.611	0.079	1.701E-04	11.312	0.739	0.084	1.619E-03	14.692	0.713	0.105
RNA-194	P3 d1 (<24hrs)	9.901E-04	10.371	2.819	0.292	3.501E-04	9.532	0.518	0.049	2.311E-04	7.940	1.004	0.080	2.449E-03	18.039	1.079	0.195
RNA-195	P3 d2	2.554E-04	9.969	0.727	0.072	1.834E-04	10.061	0.271	0.027	1.616E-04	8.071	0.702	0.057	1.516E-03	6.945	0.668	0.046
RNA-196	P3 d3	3.909E-04	6.490	1.113	0.072	1.263E-04	8.704	0.187	0.016	1.750E-04	8.057	0.760	0.061	1.940E-03	10.207	0.855	0.087
RNA-197	P3 d4	4.875E-04	12.128	1.388	0.168	1.252E-04	10.709	0.185	0.020	1.369E-04	14.277	0.595	0.085	1.822E-03	13.430	0.803	0.108
RNA-198	P3 d5	9.504E-04	3.195	2.706	0.086	1.355E-04	3.407	0.200	0.007	2.368E-04	8.218	1.029	0.085	1.927E-03	8.958	0.849	0.076
RNA-199	P3 d6	2.025E-03	20.611	5.766	1.188	2.318E-04	16.968	0.343	0.058	3.703E-04	18.629	1.609	0.300	4.176E-03	15.532	1.840	0.286
RNA-200	P3 d7	5.174E-04	3.821	1.473	0.056	4.647E-04	11.433	0.687	0.079	7.108E-04	2.897	3.088	0.089	2.410E-03	7.476	1.062	0.079
RNA-201	P3 d8	4.092E-04	10.334	1.165	0.120	8.553E-04	9.002	1.265	0.114	1.017E-03	10.671	4.420	0.472	1.851E-03	10.271	0.815	0.084
RNA-203	P3 d9	5.408E-04	11.492	1.540	0.177	3.270E-03	13.074	4.837	0.632	1.561E-03	10.138	6.780	0.687	2.799E-03	13.077	1.233	0.161
RNA-206	P3 d10	2.820E-04	18.181	0.803	0.146	1.513E-03	11.210	2.239	0.251	7.886E-04	11.022	3.426	0.378	1.319E-03	9.268	0.581	0.054
RNA-202	P4 d1	1.362E-04	11.582	0.388	0.045	2.542E-04	10.458	0.376	0.039	1.844E-04	6.900	0.801	0.055	1.361E-03	12.895	0.600	0.077
RNA-204	P4 d1	7.693E-04	16.151	2.190	0.354	1.168E-03	9.648	1.727	0.167	2.792E-04	9.147	1.213	0.111	4.755E-03	10.280	2.095	0.215
RNA-205	P4 d2	2.260E-04	7.941	0.643	0.051	3.638E-04	8.138	0.538	0.044	1.673E-04	3.894	0.727	0.028	4.053E-03	5.565	1.785	0.099

APPENDIX III. PCR Efficiency Determination

Ct-values from triplicate dilution series for 18S, EcR, HR3, HR4 and HR78 for each Spawning were averaged in Excel and the values used to determine the slope. The slope values were then used to calculate the E-value according to the equation $E=10^{(-1/slope)}$

Table 11: Spawn-5 PCR Efficiency

Assay	ng cDNA	log ng cDNA	Avt Ct	Std Dev	Slope	R^2	E
18S	5	0.699	13.567	0.163	-3.690	1.000	1.866
	0.5	-0.301	17.540	0.257			
	0.05	-1.301	21.120	0.070			
	0.005	-2.301	24.877	0.051			
	0.0005	-3.301	28.347	0.136			
EcR	20	1.301	24.093	0.084	-3.284	0.991	2.016
	10	1.000	24.973	0.127			
	1	0.000	27.830	0.265			
	0.5	-0.301	28.773	0.152			
	0.05	-1.301	32.803	0.601			
HR3	20	1.301	24.380	0.092	-3.564	0.999	1.908
	10	1.000	25.567	0.067			
	1	0.000	28.907	0.101			
	0.5	-0.301	29.987	0.129			
	0.05	-1.301	33.763	0.552			
HR4	20	1.301	25.333	0.025	-3.507	0.998	1.928
	10	1.000	26.573	0.112			
	1	0.000	29.673	0.242			
	0.5	-0.301	30.863	0.254			
	0.05	-1.301	34.617	0.632			
HR78	20	1.301	24.210	0.082	-3.297	0.999	2.010
	10	1.000	25.427	0.133			
	1	0.000	28.467	0.158			
	0.5	-0.301	29.590	0.181			
	0.05	-1.301	32.897	0.186			

Table 12: Spawn-6 PCR Efficiency

Assay	ng cDNA	log ng cDNA	Avt Ct	Std Dev	Slope	R^2	E
18S	5	0.699	13.813	0.180	-3.751	0.999	1.847
	0.5	-0.301	17.847	0.136			
	0.05	-1.301	21.760	0.263			
	0.005	-2.301	25.380	0.249			
	0.0005	-3.301	28.803	0.414			
EcR	20	1.301	24.120	0.070	-3.492	0.994	1.934
	10	1.000	25.213	0.042			
	1	0.000	28.313	0.153			
	0.5	-0.301	29.230	0.261			
	0.05	-1.301	33.413	0.251			
HR3	20	1.301	24.527	0.064	-3.380	0.996	1.976
	10	1.000	26.057	0.057			
	1	0.000	29.027	0.365			
	0.5	-0.301	29.943	0.060			
	0.05	-1.301	33.617	0.730			
FTZF1	20	1.301	25.347	0.100	-3.461	0.999	1.945
	10	1.000	26.547	0.097			
	1	0.000	29.773	0.238			
	0.5	-0.301	30.880	0.346			
	0.05	-1.301	34.457	0.545			
HR78	20	1.301	24.383	0.070	-3.581	0.997	1.902
	10	1.000	25.567	0.059			
	1	0.000	28.700	0.241			
	0.5	-0.301	29.973	0.204			
	0.05	-1.301	33.843	0.249			

Table 13: Spawn-8 PCR Efficiency

Assay	ng cDNA	log ng cDNA	Avt Ct	Std Dev	Slope	R^2	E
18S	5	0.699	12.910	0.260	-3.723	1.000	1.856
	0.5	-0.301	16.620	0.250			
	0.05	-1.301	20.603	0.240			
	0.005	-2.301	24.107	0.232			
	0.0005	-3.301	27.783	0.388			
EcR	20	1.301	23.070	0.046	-3.444	0.999	1.952
	10	1.000	24.110	0.118			
	1	0.000	27.397	0.075			
	0.5	-0.301	28.350	0.130			
	0.05	-1.301	32.120	0.204			
HR3	20	1.301	23.000	0.217	-3.270	0.981	2.022
	10	1.000	24.210	0.123			
	1	0.000	27.763	0.188			
	0.5	-0.301	29.237	0.091			
	0.05	-1.301	31.290	1.335			
HR4	20	1.301	24.267	0.102	-3.525	0.999	1.922
	10	1.000	25.437	0.049			
	1	0.000	29.067	0.068			
	0.5	-0.301	30.063	0.076			
	0.05	-1.301	33.437	0.601			
HR78	20	1.301	23.547	0.075	-3.452	0.999	1.948
	10	1.000	24.670	0.020			
	1	0.000	28.073	0.067			
	0.5	-0.301	29.273	0.075			
	0.05	-1.301	32.517	0.273			

Table 14: Spawn-11 PCR Efficiency

Assay	ng cDNA	log ng cDNA	Avt Ct	Std Dev	Slope	R^2	E
18S	5	0.699	12.950	0.166	-3.856	1.000	1.817
	0.5	-0.301	16.743	0.170			
	0.05	-1.301	20.520	0.285			
	0.005	-2.301	24.527	0.433			
	0.0005	-3.301	28.340	0.905			
EcR	20	1.301	25.047	0.047	-3.184	1.000	2.061
	10	1.000	25.977	0.162			
	1	0.000	29.130	0.164			
	0.5	-0.301	30.167	0.111			
	~~0.05~~	~~-1.301~~	~~32.410~~	~~0.300~~			
HR3	20	1.301	26.520	0.044	-3.499	1.000	1.931
	10	1.000	27.697	0.064			
	1	0.000	31.237	0.091			
	0.5	-0.301	32.260	0.082			
	0.05	-1.301	35.640	0.820			
HR4	20	1.301	26.215	0.700	-3.677	0.995	1.871
	10	1.000	28.033	0.038			
	1	0.000	31.220	0.140			
	0.5	-0.301	32.307	0.176			
	0.05	-1.301	36.123	0.295			
HR78	20	1.301	25.093	0.042	-3.559	1.000	1.910
	10	1.000	26.173	0.186			
	1	0.000	29.793	0.132			
	0.5	-0.301	30.833	0.140			
	0.05	-1.301	34.340	0.877			

Table 15: Spawn-14 PCR Efficiency

Assay	ng cDNA	log ng cDNA	Avt Ct	Std Dev	Slope	R^2	E
18S	5	0.699	14.923	0.060	-3.753	0.9993	1.847
	0.5	-0.301	18.673	0.150			
	0.05	-1.301	22.280	0.070			
	0.005	-2.301	26.437	0.319			
	0.0005	-3.301	29.807	0.679			
EcR	20	1.301	25.917	0.192	-3.44188	0.9972	1.952
	10	1.000	26.803	0.193			
	1	0.000	30.113	0.117			
	0.5	-0.301	31.030	0.236			
	0.05	-1.301	34.933	0.272			
HR3	20	1.301	27.020	0.062	-3.58316	0.9977	1.901
	10	1.000	28.163	0.055			
	1	0.000	31.863	0.252			
	0.5	-0.301	33.135	0.092			
	0.05	-1.301	36.250	0.297			
HR4	20	1.301	28.080	0.127	-3.79844	0.9989	1.833
	10	1.000	29.493	0.064			
	1	0.000	32.997	0.186			
	0.5	-0.301	34.157	0.212			
	0.05	-1.301	38.130	0.127			
HR78	20	1.301	26.600	0.135	-3.93906	0.9996	1.794
	10	1.000	27.830	0.391			
	1	0.000	31.605	0.092			
	0.5	-0.301	32.805	0.488			
	0.05	-1.301	36.920	0.269			

APPENDIX IV. Phyllosoma Sample Collection Records

Table 16: Sample Collection for Spawn-5

Tank	% at stage	Date	Days P.H.[a]	Stage	Days P.M.[b]	Sample	NoLarvae/ sample	Notes
LRT4		22/01/2003	1	P1	d1	RNA-26	20	
LRT4		23/01/2003	2	P1	d2	RNA-27	20	
LRT4		24/01/2003	3	P1	d3	RNA-28	20	
LRT4		25/01/2003	4	P1	d4	RNA-29	20	
LRT4		26/01/2003	5	P1	d5	RNA-30	20	
LRT4		27/01/2003	6	P1	d6	RNA-31	20	
LRT4		28/01/2003	7	P1	d7	RNA-32	20	
LRT4		29/01/2003	8	P1	d8	RNA-33	20	
LRT4		30/01/2003	9	P1	d9	RNA-34	20	
LRT4		31/01/2003	10	P1	d10	RNA-35	20	
LRT4		1/02/2003	11	P2	d1	RNA-36	20	P2's first recorded. P1's still present in tank, not sampled after Day-10 RNA-36 not included in data analysis as duplicate sample was collected from tank H31 RNA36=RNA-41
LRT4		2/02/2003	12	P2	d1-2	RNA-37	20	RNA-37 ot included in data analysis as a tighter sample from tank H31 was collected RNA-37 (d1-2) = RNA-42 (d1) from H31 tank
LRT4	80% P2's	3/02/2003	13	P2	d1-3	RNA-38	20	RNA-38 not included in data analysis as duplicate sample was collected from H31 which had a smaller range of P3 ages RNA38(d1-3) = RNA43(d2-3) from H31 tank
LRT4		4/02/2003	14	P2	d2-4			
LRT4	>80% P2's	5/02/2003	15	P2	d3-5			
LRT4		6/02/2003	16	P2	d4-6			
LRT4	100% P2's	7/02/2003	17	P2	d5-7			Spawn-5 took 6 days for all larvae to molt from P1 to P2-quite long
LRT4	95% P2's <5% P3's	8/02/2003	18	P2 P3	d6-8 d1			
LRT4	90% P2's 10% P3's	9/02/2003	19	P2 P3	d7-9 d1-2			
LRT4	80% P2's 20% P3's	10/02/2003	20	P2 P3	d8-10 d1-3	RNA-47 (P2)	20	Due to larvae die-off in H31 tank, had to return to main tank to continue sampling P2 larvae. This sample is not really a consecutive sample to RNA-46 hence was not included in data analysis. Subsequent samles were collected but consisted of a mixture of ages from d6-8 rather than the two-day spread from the H31 tank (ie RNA-46 d5-6) hence were also not included in the analysis. P3's d1-3 transferred from LRT4 to H33
LRT3	50% P2's 50% P3's	10/02/2003	20	P2 P3	d7-10 d1-3	RNA-53 (P3)	20	P3's d1-3 transferred from LRT3 to H33
LRT3	35% P2's 65% P3's	11/02/2003	21	P2 P3	d8-11 d1-4	RNA-48 (P2)	20	Due to larvae die-off in H31 tank, had to return to main tank to continue sampling P2 larvae. RNA-48 to RNA-52 not included in data analysis

LRT3	20% P2's 80% P3's	12/02/2003	22	P2 P3	d9-12 d1-5	RNA-49 (P2)	20	
LRT3	10% P2's 90% P3's	13/02/2003	23	P2 P3	d7-13 d1-6	RNA-50 (P2)	20	
LRT3	5% P2's 95% P3's	14/02/2003	24	P2 P3	d8-14 d1-7	RNA-51 (P2)	20	
LRT4	100% P3's	15/02/2003	25	P3	d1-8	RNA-52 (P3)	20	odd sample - don't include in Q-PCR results
LRT3	100% P3's	15/02/2003	25	P3	d1-8	-	-	Spawn-5 took 7 days to molt P2-P3
LRT4		22/02/2003	32	P3 P4	-	-	-	P3's began to molt to P4's in main tank on Day-32 post-hatch (P4's began to appear Day-27 in segregated larval stocks - see Tank H33 records)
H31		3/02/2003	13		-	-	-	400 P1's from LRT3 transferred to H31 Tank - late stage P1's close to molt
H31		3/02/2003	13	P1	d13	RNA-40	20	RNA-41 not included in data analysis as P1 samples already collected from LRT3 on 1st day of transfer
H31		4/02/2003	14	P1 P2	d14 d1	RNA-41 (P2)	20	
H31		5/02/2003	15	P1 P2	d15 d1-2	RNA-42 (P2)	20	All remaining P1's removed from H31
H31		6/02/2003	16	P2	d2-3	RNA-43	20	
H31		7/02/2003	17	P2	d3-4	RNA-44	20	
H31		8/02/2003	18	P2	d4-5	RNA-45	20	
H31		9/02/2003	19	P2 P3	d5-6 d1	RNA-46 (P2)	20	only 2 x P3's seen
H31	50% P2's 50% P3's	10/02/2003	20	P2 P3	d6-7 d1-2	-	-	Larvae began dying off in this tank and insufficient numbers were available for sampling. Therefore, sampling of P2's was continued from large tanks (LRT4 & LRT3) - although due to different age P2's from LRT's these subsequent samples were not included in analysis (samples were analysed, but expression levels recorded indicated these larvae were slightly behind the segregated samples -with receptor expression peaks occuring about 2-day later than those in segregated samples)
H31	20% P2's 80% P3's	11/02/2003	21	P2 P3	d7-8 d1-3	-	-	
H33		10/02/2003	20	P3	d1-3	-	-	P3's d1-3 transferred from LRT3 & LRT4
H33		11/02/2003	21	P3	d2-4	RNA-54	10	
H33		12/02/2003	22	P3	d3-5	RNA-55	10	
H33		13/02/2003	23	P3	d4-6	RNA-56	10	
H33		14/02/2003	24	P3	d5-7	RNA-57	10	
H33		15/02/2003	25	P3	d6-8	RNA-58	10	
H33		16/02/2003	26	P3	d7-9	RNA-59	10	
H33	95% P3's 5% P4's	17/02/2003	27	P3	d8-10	RNA-60 (P3)	10	
				P4	d1	RNA-64 (P4)	13	P4 from 17/2 & 18/2 pooled for RNA-64 (P4 d1-2) - RNA-64 has a total of 13 larvae
H33	90% P3's 10% P4's	18/02/2003	28	P3	d9-11	RNA-61 (P3)	10	RNA-61 not included in data analysis

				P4	d1-2	RNA-64 (P4)	(see above)	
H33		19/02/2003	29	P3	d10-12	RNA-62	10	RNA-62 not included in data analysis
				P4	d1	RNA-65	10	RNA-65 also P4-d1 sampl
H33	50% P3's 50% P4's	20/02/2003	30	P3	d11-13	RNA-63 (P3)	10	RNA-63 not included in data analysis
				P4	d1	RNA-66 (P4)	10	RNA-66 also P4-d1 sample
H33	30% P3's 70% P4's	21/02/2003	31	P3 P4	d12-14 d2	RNA-67 (P4)	10 10	RNA-67, only P4-d2 sample

RNA-## Samples not included in data analysis

Table 17: Sample Collections for Spawn-6

Tank	% at stage	Date	Days P.H[a]	Stage	Days P.M[b]	Sample	NoLarvae/ sample	Notes
LRT1		4/02/2003	1	P1	d1	RNA-71	20	
LRT1		5/02/2003	2	P1	d2	RNA-72	20	
LRT1		6/02/2003	3	P1	d3	RNA-73	20	4ppm Eryth & Oxytet - antibiotic treatment due to growth on larvae, possibly leucothrix
LRT1		7/02/2003	4	P1	d4	RNA-74	20	
LRT1		8/02/2003	5	P1	d5	RNA-75	20	
LRT1		9/02/2003	6	P1	d6	RNA-76	20	
LRT1		10/02/2003	7	P1	d7	RNA-77	20	4ppm Eryth & Oxytet - antibiotic treatment due to growth on larvae, possibly leucothrix
LRT1	10% P2	11/02/2003	8	P1 P2	d8	RNA-78 (P1)	20	
					d1	RNA-81 (P2)	20	First P2' appeared, not enough to transferr 1000 P2's to small tank
LRT1	20% P2	12/02/2003	9	P1 P2	d9	RNA-79 (P1)	20	
					d1-2	RNA-82 (P2)	20	
LRT1	54% P2	13/02/2003	10	P1 P2	d10	RNA-80 (P1)	20	
					d1-3	RNA-83 (P2)	20	Enough P2's (d1-3) to transfer 1000 x P2's to small tank H31
LRT1	95% P2	14/02/2003	11	P1 P2	d11 d1-4	RNA-84 (P1)	20	
LRT1		15/02/2003	12	P2	P2 d2-5	RNA-85	20	Not included in data analysis as this sample was better represented by RNA-86 (d2-4) and RNA-87 (d3-5)
LRT1		16/02/2003	13	P2	P2 d3-6			
LRT1		17/02/2003	14	P2 P3	P2 d4-7 P3 d1			4ppm Eryth & Oxytet - antibiotic treatment due to growth on larvae, possibly leucothrix
LRT-1		17/02/2003			P2- pre molt	RNA-68	20	larvae that looked like they would molt within 12hrs (ie would have molted that night had they not been harvested)
LRT-1		17/02/2003			P2- mid molt	RNA-69	20	larvae that looked like they would molt within 24-36 hrs (ie would have molted within 1-2days night had they not been harvested)
LRT-1		17/02/2003			P2-early molt	RNA-70	20	larvae that looked like they would take 36-48hrs before they molted.
LRT1	<5% P3	18/02/2003	15	P2 P3	P2 d5-8/ P3 d1-2			
LRT1		18/02/2003						Ery + Oxy added Skimmer venturi off. UV light off 11:30pm-4:00pm (at 4:00pm 25ppm Formalin)
LRT1	10% P3	19/02/2003	16	P2 P3	P2 d6-9 P3 d1-3			
LRT1	30% P3	20/02/2003	17	P2 P3	P2 d7-10 P3 d1-4			
LRT1	80% P3	21/02/2003	18	P2 P3	P2 d8-11 P3 d1-5			
LRT1		22/02/2003	19	P3	P3 d1-6			
LRT1		23/02/2003	20	P3	P3 d2-7			
LRT1		24/02/2003	21		P3 d3-8			Die-off in main tank: 99% dead
H31		13/02/2003	10	P2	d1-3			Stocked 500 x P2's (d1-3) from tank LRT1
H31		14/02/2003	11	P2	d2-4	RNA-86		

H31		15/02/2003	12	P2	d3-5	RNA-87		
H31		16/02/2003	13	P2	d4-6	RNA-88		
H31		17/02/2003	14	P2	d5-7	RNA-89		
H31	5% P3	18/02/2003	15	P2	d6-8	RNA-90 (P2)	20	
				P3	d1	RNA-94 (P3)	8	First P3's appeared, 8 x P3's taken as Day-1 P3 sample (RNA-94)
H31		19/02/2003	16	P2	d7-9	RNA-91 (P2)	20	
				P3	d1-2	RNA-95 (P3)	20	
H31	60% P3	20/02/2003	17	P2	d8-10	RNA-92 (P2)	20	
				P3	d1-3	RNA-96 (P3)	10	RNA-96 (10 larvae only)
H31	70% P3	21/02/2003	18	P2 P3	d9-11 d1-4	RNA-93 (P2)	18	
H31		Dumped - no longer needed						
H35		20/02/2003	17	P3	d1-4			Stocked 450 x P3s from LRT1 (P3 range from d1-4)
H35		21/02/2003	18	P3	d2-5	RNA-97	15	
H35		22/02/2003	19	P3	d3-6	RNA-98	10	
H35		23/02/2003	20	P3	d4-7	RNA-99	10	
H35		24/02/2003	21	P3	d5-8	RNA-100	10	
H35		25/02/2003	22	P3	d6-9	RNA-101	9	

RNA-## Samples not included in data analysis

Table 18: Sample Collection for Spawn-8

Tank	% at stage	Date	Days P.H[a]	Stage	Days P.M[b]	Sample	NoLarvae/ sample	Notes
LRT1		9/04/2003	1	P1	d1	RNA-102	20	
LRT1		10/04/2003	2	P1	d2	RNA-103	20	
LRT1		11/04/2003	3	P1	d3	RNA-104	20	
LRT1		12/04/2003	4	P1	d4	RNA-105	20	
LRT1		13/04/2003	5	P1	d5	RNA-106	20	
LRT1		14/04/2003	6	P1	d6	RNA-107	20	
LRT1		15/04/2003	7	P1	d7	RNA-108	20	
LRT3	90% P1	16/04/2003	8	P1	d8	RNA-109	20	
LRT3	10% P2	16/04/2003	8	P2	d1	RNA-110	20	first appearance of P2's - sample collected but insufficient numbers to transfer 1000 x P2's to separate tank.
LRT3	70% P1	17/04/2003	9	P1	d9	RNA-111	20	
LRT3	30% P2	17/04/2003	9	P2	d1-2	RNA-112	20	565 x P2's (day 1-2) were collected and transferred to separate tank, HR28. P2 sample for Day-9 collected during larval separation.
H28	56% P2 (44% P1)	18/04/2003	10	P2	d2-3	RNA-113	20	
H28	90% P2 (10% P1)	19/04/2003	11	P2	d3-4	RNA-114	20	
H28	100% P2	20/04/2003	12	P2	d4-5	RNA-115	20	
H28		21/04/2003	13	P2	d5-6	RNA-116	20	
H28		22/04/2003	14	P2	d6-7	RNA-117	20	
H28		23/04/2003	15	P2	d7-8	RNA-118	10	
H28	90%	24/04/2003	16	P2	d8-9	RNA-119	11	sample not included in data analysis as RNA-119 = RNA-120 some stage 3 present, but only stage-2 collected for this sample P2-d7-9
LTR4		24/04/2003	16	P2	d8-9	RNA-120	20	P2 – selected based on morphology as pre-molt sample
LTR4	10% P3*	24/04/2003	16	P3	d1	RNA-121	10	first appearance of P3's on Day-16 . P3's separated from LRT4 and transferred to bowls (20 phyllosoma/bowl x x-bowls)
LTR4	53% P3* (47% P2)	25/04/2003	17	P3	d2	RNA-122	20	P3 numbers were very low - die off overnight. Larvae transferred from tanks to bowls
LTR4	76% P3* (24% P2)	26/04/2003	18	P3	d3	RNA-123	20	
LTR4	90% P3* (10% P2)	27/04/2003	19	P3	d4	RNA-124	20	
LTR4	98% P3* (2% P2)	28/04/2003	20	P3	d5	RNA-125	20	Due to insufficient larval numbers, no larvae survived out to stage P4

RNA-## Samples not included in data analysis

Table 19: Sample Collection for Spawn-11

Tank	% at stage	Date	Days P.H[a]	Stage	Days P.M[b]	Sample	NoLarvae/ sample for EIA	NoLarvae/ sample for Q-PCR	Notes
LRT3		22/01/2004	1	P1	d1	RNA-146	60	10	
LRT3		23/01/2004	2	P1	d2	RNA-147	60	10	
LRT3		24/01/2004	3	P1	d3	RNA-148	60	10	
LRT3		25/01/2004	4	P1	d4	RNA-149	60	10	
LRT3		26/01/2004	5	P1	d5	RNA-150	60	10	
LRT3		27/01/2004	6	P1	d6	RNA-151	60	10	
LRT3		28/01/2004	7	P1	d7	RNA-152	60	10	
LRT3	97% P1 3% P2	29/01/2004	8	P1	d8	RNA-153	60	10	
LRT3	44% P1 56% P2	30/01/2004	9	P1	d9	RNA-155	60	10	P1 samples not collected after first appearance of molt to P2's
LRT3		29/01/2004	8	P2	d1	RNA-154	30	10	
LRT3	10% P1	30/01/2004	9	P2	d2	RNA-156	30	10	
H1	90% P2	31/01/2004	10	P2	d3	RNA-157	30	10	
H2	100% P2	1/02/2004	11	P2	d4	RNA-158	30	10	
H1-5		2/02/2004	12	P2	d5	RNA-159	30	10	
H1-5		3/02/2004	13	P2	d6	RNA-160	30	10	
H3		4/02/2004	14	P2	d7	RNA-161	30	10	
H3		5/02/2004	15	P2	d8	RNA-162	30	10	
H1,2,4		6/02/2004	16	P2	d9	RNA-163	30	10	P2 samples not collected after first appearance of molt to P2's
H1,2,4		6/02/2004	17	P3	d1	RNA-164	30	10	
H3	90% P2 10% P3	7/02/2004	18	P3	d2	RNA-165	30	5	
H3	70% P1 30% P2	8/02/2004	19	P3	d3	RNA-166	30	5	
H3		9/02/2004	20	P3	d4	RNA-167	30	5	
H3	30% P2 70% P3	10/02/2004	21	P3	d5	RNA-168	30	5	
H5		11/02/2004	22	P3	d6	RNA-169	30	5	These samples not continued on from previous sample collection These samples were collected from the main LRT tank due to an insufficient number of P3's to continue sampling exact age of larvae unknown
H5	100% P3	12/02/2004	23	P3	d7	RNA-170	30	5	
H5		13/02/2004	24	P3	d8	RNA-171	30	5	
LRT3		14/02/2004	25	P3	d9	RNA-172	30	5	
LRT3		15/02/2004	26	P3	d10	RNA-173	30	5	
LRT3		16/02/2004	27	P3	d11	RNA-174	30	5	

Table 20: Sample Collection for Spawn-14

Tank	% at stage	Date	Days P.H.[a]	Stage	Days P.M.[b]	Sample	NoLarvae/ sample for EIA	NoLarvae/ sample for Q-PCR	Notes
LRT1		4/04/2004	1	P1	d1	RNA-175	60	10	
LRT1		5/04/2004	2	P1	d2	RNA-176	60	10	
LRT1		6/04/2004	3	P1	d3	RNA-177	60	10	
LRT1		7/04/2004	4	P1	d4	RNA-178	60	10	
LRT1		8/04/2004	5	P1	d5	RNA-179	60	10	
LRT1		9/04/2004	6	P1	d6	RNA-180	60	10	
LRT1		10/04/2004	7	P1	d7	RNA-181	60	10	
LRT1		11/04/2004	8	P1	d8	RNA-182	60	10	
LRT1		12/04/2004	9	P1	d9	RNA-183	60	10	
LRT1		12/04/2004	9	P2	d1	RNA-184	30	10	2000 x P2's transferred from LRT1 to 5 x 50L tanks each containing ~400 larvae (tanks H1-H5) Tanks H1-H2: no antibiotic treatment Tanks H3-H5: Daily oxytetr @ 4ppm 0.2g per tank - monitor survival
H1	56% P1 44% P2	13/04/2004	10	P2	d2	RNA-185	30	10	<5 dead/tank
H2	23% P1 77% P2	14/04/2004	11	P2	d3	RNA-186	30	10	5 dead/tank
H3	10% P1 90% P2	15/04/2004	12	P2	d4	RNA-187	30	10	H3: 6 dead; H4: 8 dead; H5: 11 dead
H4	4% P1 96% P2	16/04/2004	13	P2	d5	RNA-188	30	10	H3: 3 dead; H4: 8 dead; H5: 7 dead
H5	100% P2	17/04/2004	14	P2	d6	RNA-189	30	10	H3: 6 dead; H4: 5 dead; H5: 6 dead
H1		18/04/2004	15	P2	d7	RNA-190	30	10	H3: 3 dead - no record for other tanks - none dead ?
H2		19/04/2004	16	P3	d1 (<4hr)	RNA-191	30	5	First P3's found late in afternoon, very low % (probably <5-10%) had molted to P3 - not possible to collect enough P3's to stock new tank. Therefore, sampled enough P3's for first day-1 sample (<4hrs old), will collect more P3's tomorrow on day of separation as second P3 day-1 sample (<24 hrs old)
H1-H5	10% P3 90% P2	19/04/2004	16	P2	d8	RNA-192	30	10	H3: 0/1? Dead; H4: 2 dead; H5: 5 dead
H1	30-40% P3 60-70% P2	20/04/2004	17	P2	d9	RNA-193	30	10	All tanks H1-H5 sorted to separate P3's to new tank. ~ 500 P2 from H1-H5 transferred to H4 to monitor length of time for remaining larvae to molt to P3
H1		20/04/2004	17	P3	d1 (<24hr)	RNA-194	30	5	P3 larvae collected <9am to ensure still "day-1" (<24hrs old) All remaining larvae sorted to separate P3's. Newly molted P3's transferred to H6
H6	60% P3 40% P2	21/04/2004	18	P3	d2	RNA-195	30	5	Although not continued to be sampled, remaining P2's were monitored for molt % from tank H4: H4: 58% P3; 42% P2
H6	96% P3 4% P2	22/04/2004	19	P3	d3	RNA-196	30	5	Although not continued to be sampled, remaining P2's were monitored for molt % from tank H4. H4: 96% P3; 4% P2 - length of molt: 2-3 days - fairly tight molt - good! Transfer ~300 P3's from H4 to H6 - leftover P2 & P3 in H4 ~120
H6		23/04/2004	20	P3	d4	RNA-197	30	5	

H6		24/04/2004	21	P3	d5	RNA-198	30	5	
H6		25/04/2004	22	P3	d6	RNA-199	30	5	
H6		26/04/2004	23	P3	d7	RNA-200	30	5	~100 larvae remaining, close to molting to P4
H6		27/04/2004	24	P3	d8	RNA-201	30	5	Possibly some have molted - at least one molt visible during sampling. Only ~ 50 larvae left.
H6		27/04/2004	24	P4	d1	RNA-202	-	3	possibly P4 - difficult to distinguish
H6		28/04/2004	25	P3	d9	RNA-203	-	5	
H6		28/04/2004	25	P4	d1	RNA-204	-	4	
H6		29/04/2004	26	P4	d2	RNA-205	-	3	
H6		29/04/2004	26	P3	d10	RNA-206	-	2	

Table 21: Treatment of Hatching Female for Each Spawn

Spawn	Hatching Female	2hr in 100L with antibiotics & formalin treatment prior to transfer to 5000L Hatching tank				5000L Hatching Tank treatment
		Oxytertacycline[a]	Erythromycin[b]	Streptomycin[c]	Formalin[d]	
Spawn-5	PI/R H1	0.4	0.6	1	50ppm	One treatment: 1ppm Virkon S[e]. 40% water exchange due to filamentous bacteria observed on eggs
Spawn-6	LG/R H2	0.4	0.6	1	25ppm	no additional treatment
Spawn-8	PI/R H2	0.4	0.6	1	25ppm	no additional treatment
Spawn-11	YE/R F1	0.4	0.6	1	25ppm	no additional treatment
Spawn-14	YE/R F1	0.4	0.6	1	25ppm	no additional treatment

[a] Oxytetracycline Hydrochloride - CCD Animal Health - N.R.A. No. 52863/0603
[b] Erythromycin (as the thiocyanate) - CCD Animal Health - N.R.A. No. 50742/0600
[c] Streptomycin Sulphate - SIGMA - S6501-100G
[d] Formalin - UN No. 2209
[e] Virkon S - Janos Hoey

Table 22: Water Treatment of Larval Tanks for Each Spawn

Spawn	Stocking Density in 5000L tank	Sump for 5000L Tank	5000L Tank for P1 Phyllosoma (and backup P2 & P3 Stocks when required)	50L Tank/s for P2 & P3 Phyllosoma	
Spawn-5	Phyllosoma split between two tanks Each tank received ~160 000 phyllosoma	1/4 Tablet Ecomarine, daily	1/12 tablet Ecomarine daily 3ppm Virkon S every other day	**H31** (400 x P2 phyllosoma) no antibiotics used 25ppm formalin on 5/2/03, 8/2/03, 10/2/03.	**H33** (400 x P3 phyllosoma) 4ppm oxytetracycline/erythromycin + 5ppm Virkon S on 12/2/03, 17/2/03 & 18/2/03
Spawn-6	~300 000 phyllosoma	1/4 Tablet Ecomarine, daily	1/12 tablet Eco daily 3ppm Virkon S every 2nd day 4pp oxytetracycline/erythromycin on 6/2/03, 10/2/03 & 17/2/03	**H31** (500 xP2 phyllosoma) 4ppm oxytetracycline/erythromycin + 5ppm Virkon S on 17/2/03 & 18/2/03	**H35** (450 xP3 phyllosoma) no further treatments needed - phyllosoma healthy
Spawn-8	~800 000 phyllosoma	no treatment	5ppm Virkon S every 2nd day Phyllosoma moved to a new tank every 7 days	**H28** (565 x P2 phyllosoma) 5ppm Virkon S every 2nd day	**BOWLS** (20 xP3 phyllosoma per bowl) 250ml/bowl 100% water exchange daily 4ppm oxytet/eryth
Spawn-11	~200 000 phyllosoma	no treatment	1/2, 9/2 Tank treated with 25ppm Formalin on 1/2/04 and 9/2/04 Tank treated with 5ppm Virkon S on 4/2/04, 6/2/04 & 8/2/04	**H1-H5** (400 xP2 phyllosoma) no antibiotics used From 4/2/04, all 50L tanks received 5ppm Viron S daily due to leucothrix on phyllosoma	
Spawn-14	~250 000 phyllosoma	no treatment	4ppm oxytetracycline daily for duration	**H1, H2** (400 xP2 phyllosoma each) no antibiotics used no other treatment **H3,H4,H5** (400 xP2 phyllosoma each) 4ppm oxytetracycline daily for duration	**H6** (300 xP3 phyllosoma) 4ppm oxytetracycline daily for duration

- Unless otherwise noted, all tanks (5000L and 50L) received 50% water exchange daily with chlorinated/dechlorinated water.
- Antibiotic treatment usually given in response to pinkish bacterial growth in tanks
- Virkon S treatment usually given in response to leucothrix growth on phyllosoma to try and improve survival rates
- Formalin treatment usually given in response to appearance of ciliates on phyllosoma to try and improve survival rates. Tanks were treated for 4hrs with formalin (biofilter switched off during treatment), then tanks received the daily 50% water exchange

APPENDIX V. Ecdysone EIA Protocol

The protocol is based on the following two references:
Porcheron, P., Moriniere, M., Grassi, J., Pradelles, P. (1989) Development of an enzyme immunoassay for Ecdysteroids using acetylcholinesterase as label. Insect Biochemistry 19: 117-122.
Von Gliscynski, U., Delbecque, J.P., Böcking, D., Sedlmeier, D., Dircksen, H., Lafont, R. (1995) Three new antisera with high sensitivity to ecdysone, 3-dehydroecdysone and other A-ring derivatives: Production and characterization. European Journal of Entomology 92: 75-79)

EIA Buffers

EIA CONCENTRATE:

1M K-Phosphate pH 7.4 + 0.1% thimerosal
(20 x Stock for coating & blocking plates; 100 x Stock for Wash Buffer)

35 g K_2PO_4 (Mwt: 174) in 200 ml RO-H_2O (1M solution)
8 g KH_2PO_4 (Mwt: 136) in 60 ml RO-H_2O (1M solution)

Added ~ 50 ml 1M KH_2PO_4 slowly to 200 ml K_2PO_4 until pH reached 7.4
Weighed ~0.25 g Thimerosal into 15 ml falcon tube in hood, dissolved using K-Phosphate pH 7.4, then added to remaining ~250ml K-Phosphate.
Aliquotted 40 ml complete buffer to each of 4 Falcon tubes and stored @ -20°C, remaining ~90ml kept at 4°C

EIA SAMPLE BUFFER:

0.1 M phosphate buffer, 0.4 mM NaCl, 0.1 mM EDTA, 1% BSA
Suitable for use for up to 1month when stored at 4°C

50 ml EIA 1 M Concentrate (Bk-145 p53)	→	Final 0.1 M K-Phosphate pH 7.4
11.7 g NaCl	→	Final 0.4 mM
100 µl 0.5M EDTA	→	Final 0.1 mM
0.5 g BSA	→	Final 1%

Bring to 500 ml mQ-H_2O

EIA WASHING BUFFER:

0.01 M K-Phosphate Buffer, 0.05% Tween-20
Prepare as required, use at room temp.

10 ml EIA 1M Concentrate (Bk-145 p53)	→	Final 0.01 M
500 µl Tween-20	→	Final 0.05%

Bring to 1 L mQ-H_2O

Detection Buffers

TMB SUBSTRATE BUFFER (0.05M PHOSPHATE-CITRATE BUFFER)

0.2 M dibasic sodium phosphate:
2.84 g Na_2HPO_4 (SIGMA S-7907)
to 100 ml with mQ-H_2O

0.1 M citric acid:
2.10 g Citric Acid monohydrate - AnalR (BDH-1008)
to 100 ml mQ-H_2O

0.05 M Phosphate-citrate buffer:
25.7 ml 0.2M dibasic sodium phosphate
24.3 ml 0.1M citric acid
50 ml mQ-H_2O
Adjust pH to 5.0 if necessary (usually not necessary) – can be stored at 4°C

Dissolve one TMB tablet (SIGMA T-3405) in 10 ml 0.05M phosphate-citrate buffer pH 5.0. Add 2 µl fresh 30% hydrogen peroxide immediately prior to use.

TMB SUBSTRATE STOP SOLUTION (2 M H_2SO_4)

Sulfuric acid (CPMB A.2.1 Table: 98% solution ~ 18.3 M)
To make 50 ml @ 2 M:
5.46 ml 98% Sulfuric acid
44.54 ml mQ-H_2O

Coating Plates

PLATES:

C96 Maxisorp (Nunc #430341/batch 049452)

ANTIBODY:

Goat α-Rabbit IgG (H&L): Zymax Grade (Zymed Laboratories Inc #81-6100/Lot 21073977R)
Added 1 ml mQ-H_2O to lyophilised antibody according to manufacturer's instructions to produce 1 ml @ 2 mg/ml
Alliquotted ~140 µl/tube x 5 tubes and stored @ -20°C

ANTIBODY BUFFER TO COAT 10 PLATES (125µG/ML IN 50MM PHOSPHATE BUFFER PH 7.4):

0.25 mg GoatαRabbit IgG (125 µl @ 2 mg/ml)
10 ml EIA Concentrate
190 ml mQ-H_2O
Used 200 µl to fill each well of 10 plates
Plates stacked and wrapped with aluminium foil at room temp 24 hrs

Blocking Plates

Prepare 30% BSA blocking solution (3 g/L)
For 10 plates, need to prepare at least 100 ml, prepare 110 ml:

0.33 g BSA
5.5 ml EIA Concentrate
104.5 ml mQ-H_2O
110 ml Total

Add 100 µl BSA solution to 200 µl coating antibody solution. Incubate overnight at room temperature and then seal plates with plastic wrap and store for up to one month at 4°C.

Reagent and Ecdysone-Standard Preparation for EIA

PREPARATION OF ECDYSONE TRACER:

Dilute 10-15 µl Tracer (batch: 21-06-2003) in 6 ml EIA Sample Buffer (keep left-over tracer for testing colour reaction with TMB substrate solution)

Tracer a gift from: Dr. J.P Delbeque 28/7/03, Laboratoire de Neurobologie des Reseaux, UMP CNRS, Universite Bordeaux I, France (provided 1 ml enzymatic tracer: 20-hydroxyecdysone conjugated to peroxidase)

PREPARATION OF α-ECDYSONE ANTIBODY:

Anti-ecdysone antibodies trailed: RUD-1, RUD-2, RUD-3 were kindly supplied by Dr D.Sedlmeier 2 Nov 2000

Most suitable antibody for ecdysone assay found to be RUD-2
Optimised dilution of RUD-2 found to be 1:8000. Prepare sufficient antibody for 50 µl/well in EIA Sample Buffer

PREPARATION OF ECDYSONE STANDARD:

20-Hydroxyecdysone standard (Sigma H-5142/lot 84H3446)
1 mg 20-Hydroxyecdysone was dissolved in 0.5ml Ethanol to give ~4.16 M

The stock solution of 4.16 mM was subsequently diluted to $2x10^5$ M in ethanol and used as the working stock for ecdysone standard curve assays

PREPARATION OF ECDYSONE STANDARD CURVE:

The standard curve was performed as recommended by Dr J.P. Delbeque (verbal communication), except our curve started slightly more concentrated than he recommended. Basically, successive 10-fold serial dilution of ecdysone were prepared from 10^{-6} M to 10^{-11} M in EIA Sample Buffer. Each of these serial dilution was then diluted by a factor of 3.16 in order to provide an intermediate dilution between the 10-fold serial dilutions. Once prepared, 50 µl of standards were used per well for each EIA performed as outlined in the methods section.

10-Fold serial dilutions:

FINAL:	10^{-6M}	10^{-7}M	10^{-8}M	10^{-9}M	10^{-10}M	10^{-11}M
Vol @ χM:	30µl @ $2x10^{-5}$M	60µl @ 10^{-6}M	60µl @ 10^{-7}M	60µl @ 10^{-8}M	60µl @ 10^{-9}M	60µl @ 10^{-10}M
Vol EIA Sample Buffer:	570µl	540µl	540µl	540µl	540µl	540µl

Intermediate 3.16-fold dilutions of each 10-fold serial dilutions:

FINAL:	$3.16x10^{-7}$M	$3.16x10^{-8}$M	$3.16x10^{-9}$M	$3.16x10^{-10}$M	$3.16x10^{-11}$M
Vol @ χM:	126µl @ 10^{-6}M	126µl @ 10^{-7}M	126µl @ 10^{-8}M	126µl @ 10^{-9}M	126µl @ 10^{-10}M
Vol EIA Sample Buffer:	275µl	275µl	275µl	275µl	275µl

Final Std curve dilution series:

Standard[a]	Molarity (M)	log10	fmol /50µl
1	$1x\ 10^{-6}$	-6.00	50000.000
	$3.16x10^{-7}$	-6.50	15822.785
2	$1x\ 10^{-7}$	-7.00	5000.0000
3	$3.16x10^{-8}$	-7.50	1582.2785
4	$1x10^{-8}$	-8.00	500.0000
5	$3.16x10^{-9}$	-8.50	158.2278
6	$1x10^{-9}$	-9.00	50.0000
7	$3.16x10^{-10}$	-9.50	15.8228
8	$1x10^{-10}$	-10.00	5.0000
9	$3.16x10^{-11}$	-10.50	1.5823
10	$1x10^{-11}$	-11.00	0.5000

[a]Standard $3.16x10^{-7}$M was omitted from the standard curve samples due to limited well availability on the plate. The standards above $1x10^{7}$M fell outside the linear region which was used for sample analysis, so omitting this one standard did not impact on data analysis.

APPENDIX VI. Standard Curve Raw Data

Table 23: EIA Standard Curve Data: Spawn-11 & Spawn-14

Spawn	Plate[a]	Ecdysone (fmol/50ul)	Ecdysone (log fmol/50ul)	B (Raw OD - NSB) Std-1	Std-2	Std-3	B/Bo (%) Std-1	Std-2	Std-3	Average B/Bo(%)	B/Bo (SD)
Spawn-11	Plate-1	15822.79	4.20	0.104	0.097	0.096	8.197	7.629	7.532	7.786	0.359
	Bo = 1.268	5000.00	3.70	0.291	0.267	0.259	22.923	21.022	20.411	21.452	1.310
	NSB = 0.040	1582.28	3.20	0.562	0.526	0.554	44.300	41.499	43.683	43.161	1.472
		500.00	2.70	0.822	0.838	0.817	64.785	66.088	64.374	65.083	0.895
		158.23	2.20	0.936	0.978	0.983	73.758	77.108	77.506	76.124	2.059
		50.00	1.70	1.055	1.092	1.099	83.142	86.066	86.622	85.277	1.869
		15.82	1.20	1.189	1.198	1.166	93.763	94.466	91.943	93.391	1.302
		5.00	0.70	1.187	1.174	1.209	93.581	92.587	95.352	93.840	1.401
		1.58	0.20	1.271	1.290	1.266	100.213	101.715	99.820	100.583	1.000
Spawn-11	Plate-2	15822.79	4.20	0.118	0.115	0.120	8.947	8.693	9.082	8.907	0.197
	Bo = 1.323	5000.00	3.70	0.305	0.316	0.310	23.045	23.895	23.445	23.462	0.425
	NSB = 0.040	1582.28	3.20	0.611	0.618	0.633	46.179	46.728	47.822	46.910	0.836
		500.00	2.70	0.884	0.877	0.864	66.842	66.330	65.321	66.164	0.774
		158.23	2.20	1.082	1.101	1.085	81.769	83.269	82.024	82.354	0.803
		50.00	1.70	1.215	1.212	1.210	91.860	91.658	91.479	91.666	0.190
		15.82	1.20	1.239	1.256	1.265	93.653	94.988	95.658	94.767	1.021
		5.00	0.70	1.300	1.334	1.310	98.283	100.874	99.063	99.406	1.329
		1.58	0.20	1.307	1.299	1.325	98.800	98.185	100.206	99.064	1.036
Sapwn-14	Plate-1	15822.79	4.2	0.088	0.090	0.091	7.899	8.105	8.249	8.084	0.176
	Bo = 1.109	5000.00	3.7	0.251	0.254	0.253	22.645	22.936	22.848	22.809	0.149
	NSB = 0.038	1582.28	3.2	0.497	0.504	0.500	44.821	45.449	45.086	45.119	0.315
		500.00	2.7	0.732	0.756	0.770	65.970	68.184	69.482	67.879	1.776
		158.23	2.2	0.902	0.916	0.955	81.325	82.625	86.118	83.356	2.478
		50.00	1.7	0.986	1.033	1.019	88.954	93.178	91.909	91.347	2.167
		15.82	1.2	1.074	1.094	1.099	96.818	98.698	99.124	98.214	1.227
		5.00	0.7	1.109	1.040	1.058	99.991	93.787	95.432	96.403	3.214
		1.58	0.2	1.112	1.106	1.132	100.247	99.787	102.100	100.711	1.224
Spawn-14	Plate-2	15822.79	4.2	0.088	0.089	0.092	7.382	7.452	7.667	7.500	0.149
	Bo = 1.195	5000.00	3.7	0.268	0.262	0.266	22.450	21.954	22.248	22.217	0.249
	NSB = 0.040	1582.28	3.2	0.538	0.540	0.535	45.002	45.181	44.767	44.983	0.207
		500.00	2.7	0.802	0.838	0.804	67.100	70.129	67.247	68.159	1.708
		158.23	2.2	1.004	0.991	0.972	83.973	82.926	81.320	82.740	1.336
		50.00	1.7	1.132	1.110	1.096	94.749	92.864	91.713	93.108	1.533
		15.82	1.2	1.186	1.163	1.215	99.231	97.334	101.663	99.409	2.170
		5.00	0.7	1.215	1.204	1.150	101.657	100.768	96.196	99.540	2.930
		1.58	0.2	1.134	1.198	1.182	94.891	100.241	98.885	98.006	2.782

[a] Bo values represent average of six replicates & NSB represents average of three replicates

Figure 1: EIA Standard Curves Spawn-11

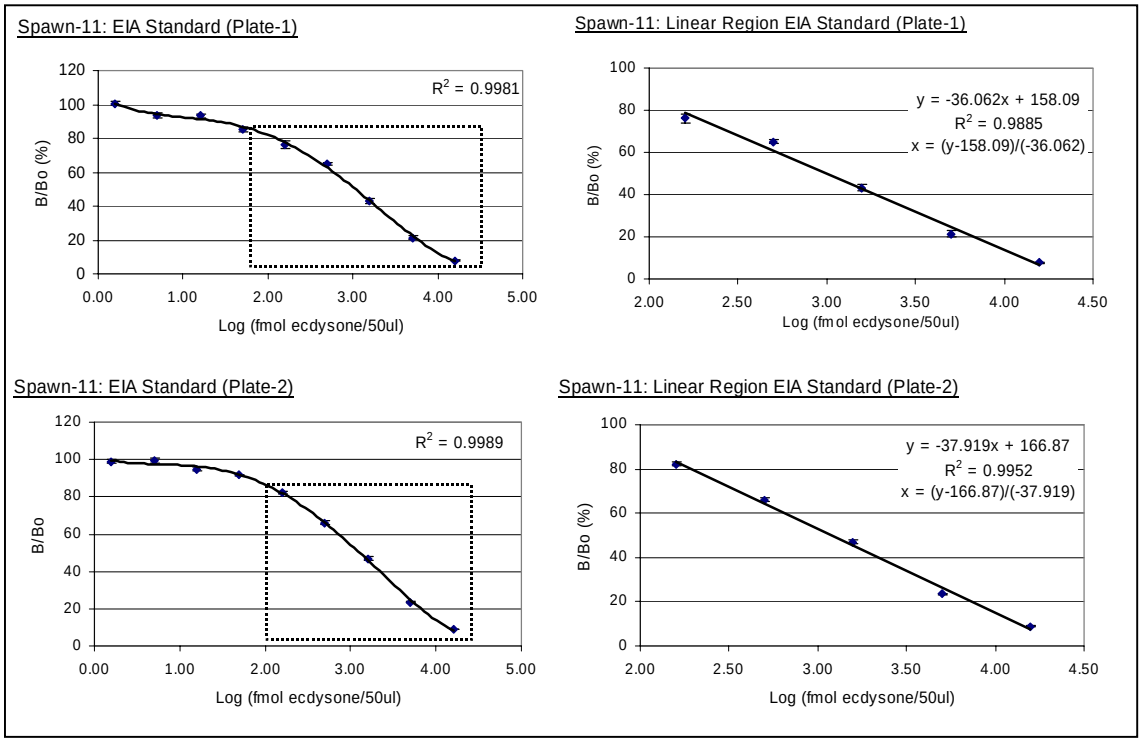
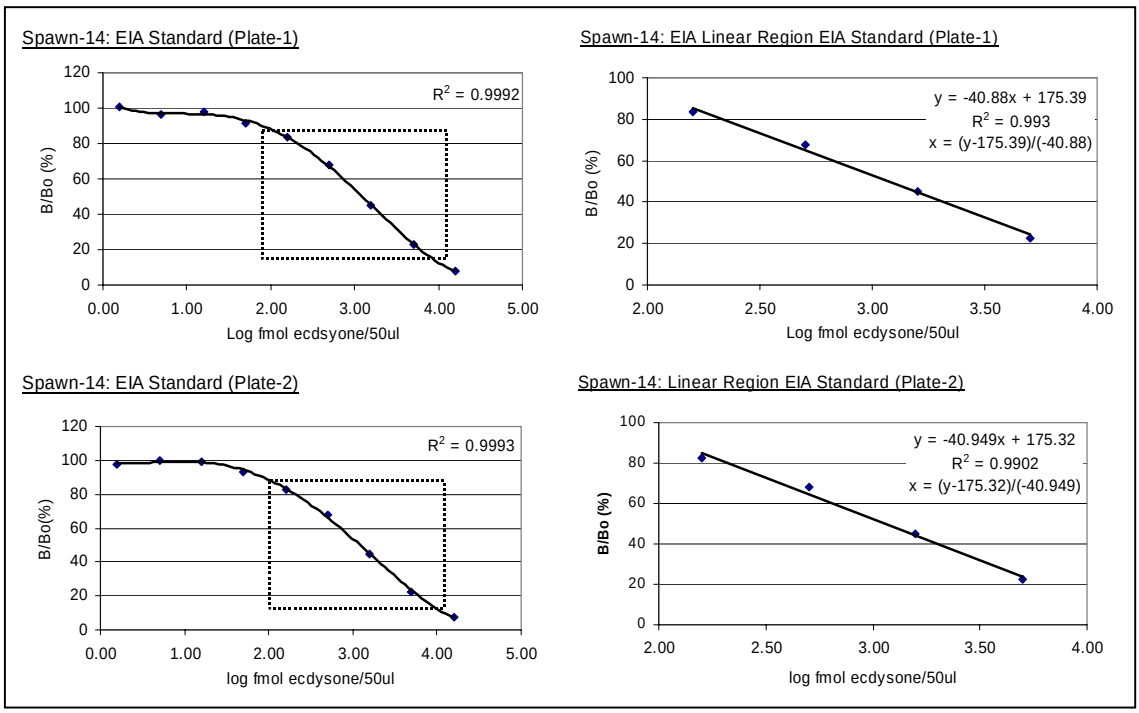


Figure 2: EIA Standard Curves Spawn-14



Spawn-11 & Spawn-14 Raw Data

Table 24: EIA Spawn-11 Raw Data

EIA Plate	Larval Stage-day	EIA #	B (Raw OD-NSB) 1	2	3	B/Bo (%) 1	2	3	log fmol ecdysone/50ul[a] 1	2	3	fmol ecdysone/50ul 1	2	3	#larvae/ well	fmol/larvae 1	2	3	Average Ecdysone fmol / larvae	SD
1	P1-d1	EIA-1	1.073	1.069	1.097	84.576	84.270	86.478	2.039	2.047	1.986	109.282	111.438	96.782	19.6	5.573	5.683	4.936	5.398	0.404
1	P1-d2	EIA-2	1.089	1.070	1.064	85.884	84.396	83.907	2.002	2.044	2.057	100.522	110.543	114.051	19.6	5.127	5.638	5.817	5.527	0.358
1	P1-d3	EIA-3	0.886	0.866	0.879	69.815	68.298	69.317	2.448	2.490	2.462	280.453	308.987	289.528	19.6	14.303	15.758	14.766	14.942	0.743
1	P1-d4	EIA-4	0.780	0.800	0.877	61.486	63.106	69.130	2.679	2.634	2.467	477.357	430.429	292.996	19.6	24.345	21.952	14.943	20.413	4.886
1	P1-d5	EIA-5	0.740	0.721	0.725	58.355	56.852	57.188	2.766	2.807	2.798	582.974	641.700	628.074	19.6	29.732	32.727	32.032	31.497	1.568
1	P1-d6	EIA-6	0.685	0.695	0.668	53.971	54.802	52.641	2.887	2.864	2.924	771.282	731.439	839.646	19.6	39.335	37.303	42.822	39.820	2.791
1	P1-d7	EIA-7	0.604	0.576	0.619	47.629	45.431	48.810	3.063	3.124	3.030	1156.318	1330.581	1072.349	19.6	58.972	67.860	54.690	60.507	6.718
1	P1-d8	EIA-8	0.602	0.641	0.696	47.443	50.505	54.849	3.068	2.983	2.863	1170.137	962.340	729.263	19.6	59.677	49.079	37.192	48.650	11.248
1	P2-d1	EIA-9	1.038	1.018	1.006	81.816	80.232	79.332	2.115	2.159	2.184	130.340	144.212	152.740	9.8	13.295	14.710	15.579	14.528	1.153
1	P1-d9	EIA-10	0.618	0.624	0.634	48.742	49.203	49.970	3.032	3.019	2.998	1077.034	1045.803	995.768	19.6	54.929	53.336	50.784	53.016	2.091
1	P2-d1-2	EIA-11	1.049	1.032	1.015	82.702	81.392	79.984	2.091	2.127	2.166	123.173	133.920	146.515	9.8	12.564	13.660	14.945	13.723	1.192
1	P2-d2-3	EIA-12	0.967	1.055	1.118	76.205	83.158		2.271	2.078	4.384	186.501	119.636		9.8	19.023	12.203		15.613	4.823
1	P2-d3-4	EIA-13	0.983	0.959	0.944	77.476	75.577	74.434	2.235	2.288	2.320	171.959	194.133	208.822	9.8	17.540	19.802	21.300	19.547	1.893
1	P2-d4-5	EIA-14	0.956	0.956	0.950	75.383	75.347	74.900	2.293	2.294	2.307	196.548	197.003	202.697	9.8	20.048	20.094	20.675	20.272	0.350
1	P2-d5-6	EIA-15	0.763	0.783	0.754	60.179	61.747	59.425	2.715	2.672	2.736	518.898	469.452	544.498	9.8	52.928	47.884	55.539	52.117	3.891
1	P2-d6-7	EIA-16	0.601	0.658	0.683	47.347	51.871	53.815	3.071	2.945	2.892	1177.337	881.976	779.029	9.8	120.088	89.962	79.461	96.504	21.089
1	P2-d7-8	EIA-17	0.684	0.677	0.731	53.946	53.370	57.628	2.888	2.904	2.786	772.520	801.450	610.662	9.8	78.797	81.748	62.287	74.277	10.488
1	P2-d8-9	EIA-18	0.722	0.724	0.696	56.941	57.043	54.872	2.805	2.802	2.862	638.064	633.915	728.194	9.8	65.083	64.659	74.276	68.006	5.434
1	P3-d1	EIA-19	0.907	0.923	0.915	71.525	72.792	72.151	2.400	2.365	2.383	251.453	231.910	241.591	9.8	25.648	23.655	24.642	24.648	0.997
1	P3-d2	EIA-20	0.911	0.954	0.972	71.822	75.177	76.615	2.392	2.299	2.259	246.719	199.146	181.681	9.8	25.165	20.313	18.531	21.337	3.433
2	P3-d3	EIA-21	0.841	0.860	**0.899**	63.616	65.053	67.973	2.723	2.685	2.608	528.466	484.308	405.622	9.8	53.904	49.399	41.373	48.225	6.347
2	P3-d4	EIA-22	0.881	0.882	0.875	66.595	66.694	66.171	2.644	2.642	2.656	441.007	438.367	452.527	9.8	44.983	44.713	46.158	45.285	0.768
2	P3-d5	EIA-23	0.718	0.726	0.721	54.298	54.854	54.496	2.969	2.954	2.964	930.575	899.677	919.439	9.8	94.919	91.767	93.783	93.490	1.596
2	P3-d6	EIA-24	0.740	0.767	0.782	55.909	57.966	59.087	2.926	2.872	2.842	843.859	744.759	695.742	9.8	86.074	75.965	70.966	77.668	7.697
2	P3-d7	EIA-25	0.750	0.761	0.817	56.718	57.564	61.792	2.905	2.883	2.771	803.397	763.180	590.345	9.8	81.946	77.844	60.215	73.335	11.546
2	P3-d8	EIA-26	0.873	0.878	0.897	65.981	66.404	67.806	2.661	2.649	2.613	457.765	446.168	409.742	9.8	46.692	45.509	41.794	44.665	2.556
2	P3-d9	EIA-27	0.801	0.824	0.806	60.522	62.267	60.950	2.805	2.759	2.793	637.691	573.585	621.314	9.8	65.044	58.506	63.374	62.308	3.397
2	P3-d10	EIA-28	0.801	0.833	0.832	60.578	62.969	62.928	2.803	2.740	2.741	635.538	549.624	551.004	9.8	64.825	56.062	56.202	59.030	5.019
2	P3-d11	EIA-29	0.740	0.759	0.774	55.915	57.383	58.533	2.926	2.887	2.857	843.565	771.604	719.538	9.8	86.044	78.704	73.393	79.380	6.352

Shaded numbers indicate values that fell outside the linear region of std curve

[a] Log fmol ecdysone/50µl calculated using linear equation from appropriate std curve (Figure 1)

Plate-1: x = (y-158.09)/(-36.062) Plate-2: x = (y-166.87)/(-37.919)

Table 25: EIA Spawn-14 Raw Data

EIA Plate	Larval Stage	EIA #	B (Raw OD-NSB) 1	2	3	B/Bo (%) 1	2	3	log fmol ecdysone / 50ul[a] 1	2	3	fmol / 50ul 1	2	3	# larvae / well	fmol ecdysone / larvae 1	2	3	Average Ecdysone fmol / larvae	SD
1	P1 d1	EIA-67	1.028	1.058	1.031	92.703	95.458	92.978	2.023	1.955	2.016	105.358	90.219	103.742	19.6	5.373	4.601	5.291	5.088	0.424
1	P1 d2	EIA-68	0.919	0.926	0.896	82.910	83.524	80.768	2.262	2.247	2.315	182.903	176.687	206.363	19.6	9.328	9.011	10.524	9.621	0.798
1	P1 d3	EIA-69	0.883	0.836	0.870	79.647	75.364	78.478	2.342	2.447	2.371	219.817	279.777	234.773	19.6	11.211	14.269	11.973	12.484	1.592
1	P1 d4	EIA-70	0.803	0.779	0.767	72.428	70.229	69.137	2.519	2.572	2.599	330.104	373.616	397.327	19.6	16.835	19.054	20.264	18.718	1.739
1	P1 d5	EIA-71	0.760	0.734	0.720	68.550	66.237	64.914	2.613	2.670	2.702	410.676	467.824	504.021	19.6	20.944	23.859	25.705	23.503	2.400
1	P1 d6	EIA-72	0.651	0.656	0.651	58.716	59.128	58.706	2.854	2.844	2.854	714.581	698.219	715.007	19.6	36.444	35.609	36.465	36.173	0.488
1	P1 d7	EIA-73	0.557	0.516	0.531	50.202	46.576	47.926	3.062	3.151	3.118	1154.352	1415.851	1312.208	19.6	58.872	72.208	66.923	66.001	6.716
1	P1 d8	EIA-74	0.494	0.489	0.517	44.592	44.089	46.655	3.200	3.212	3.149	1583.301	1628.787	1409.605	19.6	80.748	83.068	71.890	78.569	5.899
1	P1 d9	EIA-75	0.546	0.521	0.531	49.277	47.016	47.874	3.085	3.140	3.119	1216.074	1381.212	1316.087	19.6	62.020	70.442	67.120	66.527	4.242
1	P2 d1	EIA-76	0.798	0.810	0.789	71.979	73.067	71.177	2.530	2.503	2.549	338.541	318.425	354.204	9.8	34.531	32.479	36.129	34.380	1.829
1	P2 d2	EIA-77	0.885	0.831	0.850	79.775	74.972	76.633	2.339	2.456	2.416	218.237	286.021	260.475	9.8	22.260	29.174	26.568	26.001	3.492
1	P2 d3	EIA-78	0.824	0.773	0.770	74.313	69.709	69.481	2.473	2.585	2.591	296.848	384.726	389.694	9.8	30.279	39.242	39.749	36.423	5.327
1	P2 d4	EIA-79	0.799	0.777	0.790	72.019	70.050	71.217	2.529	2.577	2.548	337.791	377.407	353.406	9.8	34.455	38.496	36.047	36.333	2.035
1	P2 d5	EIA-80	0.673	0.683	0.656	60.673	61.606	59.154	2.806	2.783	2.843	640.006	607.254	697.178	9.8	65.281	61.940	71.112	66.111	4.642
1	P2 d6	EIA-81	0.644	0.630	0.629	58.071	56.794	56.744	2.870	2.901	2.902	741.035	796.280	798.564	9.8	75.586	81.221	81.454	79.420	3.323
1	P2 d7	EIA-82	0.630	0.590	0.620	56.844	53.195	55.874	2.900	2.989	2.924	794.055	975.220	838.657	9.8	80.994	99.472	85.543	88.670	9.628
1	P2 d8	EIA-84	0.604	0.617	0.587	54.470	55.620	52.925	2.958	2.930	2.996	907.667	850.749	990.180	9.8	92.582	86.776	100.998	93.452	7.151
1	P2 d9	EIA-85	0.489	0.465	0.469	44.069	41.950	42.336	3.212	3.264	3.255	1630.609	1837.327	1797.812	9.8	166.322	187.407	183.377	179.035	11.193
1	P3 d1 (<4hrs)	EIA-83	0.918	0.886	0.854	82.798	79.933	76.979	2.265	2.335	2.407	184.063	216.304	255.459	9.8	18.774	22.063	26.057	22.298	3.647
1	P3 d1 (<24hrs)	EIA-86	0.879	0.829	0.847	79.228	74.748	76.427	2.352	2.462	2.421	225.067	289.662	263.521	9.8	22.957	29.546	26.879	26.461	3.314
2	P3 d2	EIA-87	0.878	0.834	0.811	73.480	69.781	67.825	2.487	2.577	2.625	306.899	377.860	421.787	9.8	31.304	38.542	43.022	37.623	5.913
2	P3 d3	EIA-88	0.774	0.778	0.767	64.776	65.076	64.168	2.700	2.692	2.714	500.676	492.310	518.095	9.8	51.069	50.216	52.846	51.377	1.342
2	P3 d4	EIA-89	0.711	0.722	0.638	59.463	60.377	53.337	2.829	2.807	2.979	674.995	641.162	952.598	9.8	68.849	65.398	97.165	77.138	17.430
2	P3 d5	EIA-90	0.738	0.725	0.703	61.719	60.687	58.857	2.774	2.799	2.844	594.588	630.088	698.379	9.8	60.648	64.269	71.235	65.384	5.381
2	P3 d6	EIA-91	0.689	0.632	0.629	57.629	52.858	52.594	2.874	2.991	2.997	748.322	978.572	993.208	9.8	76.329	99.814	101.307	92.483	14.010
2	P3 d7	EIA-92	0.607	0.604	0.616	50.783	50.494	51.529	3.041	3.048	3.023	1099.681	1117.696	1054.507	9.8	112.167	114.005	107.560	111.244	3.320
2	P3 d8	EIA-93	0.559	0.531	0.523	46.747	44.396	43.766	3.140	3.197	3.213	1379.856	1574.877	1631.642	9.8	140.745	160.637	166.427	155.937	13.471
2	P3 d9	EIA-94	0.617	0.596	0.601	51.601	49.903	50.320	3.021	3.063	3.053	1050.253	1155.460	1128.691	5.9	178.543	196.428	191.877	188.950	9.295

[a] Log fmol ecdysone/50µl calculated using linear equation from appropriate std curve (Figure 2)
Plate-1: x = (y-175.39)/-40.88 Plate-2: x = (y-175.32)/-40.949