水研センター研報，第20号，53－57，平成19年
Bull. Fish. Res. Agen. No. 20, 53-57, 2007

Novel molecular approach to study moulting in crustaceans

Anna KUBALLA [*1] and Abigail ELIZUR [*2]

Abstract Moulting occurs in all arthropods, from insects to crustaceans, it is essential for growth, reproduction and metamorphosis. Moulting occurs in cycles and involves the shedding of the hard exoskeleton to expose a soft new shell, the uptake of water from the animals' immediate surroundings causing the new exoskeleton to expand, and finally the hardening of the new exoskeleton. Moulting is a complex process that is affected by a range of external factors such as temperature, photoperiod, nutrition and eyestalk ablation. However despite extensive research the moulting process in crustaceans still remains poorly understood. Microarray technology provides a powerful, holistic approach to study gene expression in relation to changing physiological states. It enables not only the ability to profile the expression of genes already known to be involved in moulting, but also facilitates the discovery of new, as yet unknown, genes that may be important in the moulting process.

Understanding, and consequently controlling, the process of moulting, has significant potential for a range of commercial applications in crustaceans, such as the propagation of valuable seafood products. There are three areas within moulting control that have been identified as having potential commercial significance:

1. controlling the timing of the moult
2. manipulating the synchrony of moulting within a population (mass moulting)
3. controlling the process of shell hardening.

Key words: moulting, crustacean, microarray

Introduction into crustacean moulting

Moulting is common to all crustaceans and is essential for growth, metamorphosis and reproduction. Moulting is a complex process, affected by a range of environmental cues and regulated by a cascade of hormonal signals involving changes in gene expression, cellular commitment, mitotic and secretory activity, endocrinology, behaviour and cell death (Loeb, 1993).

The moult cycle refers to the period between two successive moults and has been subdivided into 4 major stages (Drach, 1939). These are known as the moult (E), postmoult (A-B), intermoult (C), and premoult (D) (Table 1). The moult stage, referred to as ecdysis, involves the shedding of the exoskeleton through a rapid uptake of water or air from the environment, causing the exoskeleton to rupture. During postmoult, or metecdysis, further water uptake expands the new, still soft, exoskeleton; this expansion is essential for the growth of the animal. Exoskeleton mineralisation and hardening then occur. The intermoult period, or anecdysis, is the so-called period of non-activity, and by far the longest stage of the moult cycle. During this time, muscle regeneration occurs, and energy reserves such as glycogen and lipids are accumulated in the hemolymph and midgut for the succeeding moult. Premoult, or proecdysis, sees the atrophy of somatic muscle, the resorption of the old exoskeleton, and the formation of a new exoskeleton in preparation for the onset of ecdysis.

Two types of endocrine organs are associated with the moulting process, traditional androgenic

Received: November 25, 2005
[*1] Australian Fresh Research & Development Corporation, c/o Queensland Department of Primary Industries and Fisheries, Bribie Island Aquaculture Research Centre, PO Box 2066 Woorim, Queensland, 4507, Australia Email: anna.kuballa@dpi.qld.gov.au
[*2] Queensland Department of Primary Industries and Fisheries, Bribie Island Aquaculture Research Centre, PO Box 2066 Woorim, Queensland 4507, Australia

Table 1. The stages of the moult cycle*

STAGE	DESCRIPTION	DURATION	% DURATION
A	Newly moulted		
A1	Exoskeleton is a soft membrane; Crab is inactive; water absorption continues; exocuticle mineralisation begins	8 – 12 hrs	1
A2	Exoskeleton feels like parchment; crab begins to move about; water content stabilises to ~ 86%; endocuticle deposition and mineralisation begin (secretion of the postecdysial procuticle)	1 – 2 days	2
B	Recently moulted		
B1	Exoskeleton is deformable without breaking	1½ – 3 days	3
B2	Parts of exoskeleton become rigid; feeding may start	2½ – 4 days	5
C	Intermoult		
C1	Carapace almost completely rigid; main period of tissue growth	4½ – 5 days	8
C2	Carapace completely rigid; tissue growth continues	7 – 10 days	14
C3	Continuation of endocuticle mineralisation	7½ - 11 days	15
C4	Inner membranous layer complete – should remain attached to the epidermis; tissue growth complete; accumulation of metabolic reserves; water content 61%	15 – 22 days	30 +
C4T	Terminal anecdysis; animal is fully grown;		
D	Premoult		
D1	Epidermis separates from the membranous layer and secretes a new epicuticle; reserves are mobilised; glycogen builds up in the epidermal tissues; atrophy of somatic muscle; regeneration of autotomised limbs	3½ – 6 days	8
D2	New exocuticle secretion begins; old membranous layer degenerates in to a gelatinous layer; resorption of the old exoskeleton begins; reduced activity; feeding stops as the crabs loses its muscle insertions	3½ – 6 days	8
D3	Main period of old exoskeleton resorption	1½ - 3½ days	4
D4	Resorption of old exoskeleton is complete; old exoskeleton splits along epimeral lines; water uptake begins	12 – 15 hrs	1
E	Ecdysis		
	Animal withdraws from old exoskeleton and takes up water rapidly	Few minutes	

*Moult cycle stages based on (Drach, 1939) for *Cancer pagurus*. Durations based on (Hiatt, 1948)

glands, and neurosecretory cells, which are specialized neurons that form part of the central nervous system (CNS). The Y-organ and mandibular glands are examples of androgenic glands, they synthesize and secrete ecdysteroids and methyl farnesoate (MF) respectively (Claerhout *et al*., 1996). Ecdysteroids are steroidal moulting hormones that are responsible for the growth, development, and reproduction of arthropods (Spaziani *et al*., 1989). MF has also been implicated in the regulation of crustacean morphogenesis, metamorphosis, reproduction and moulting (Laufer *et al*., 1993; Abdu *et al*., 1998; Laufer and Biggers, 2001). Studies into ecdysteroid synthesis have shown that MF directly stimulates the secretion of ecdysteroids in *Cancer magister* Y-organs (Tamone and Chang, 1993; Chang *et al*., 1993). Regulation of MF synthesis by the mandibular organ is negatively controlled via Mandibular Organ Inhibiting Hormone (MOIH), a neurohormone produced in the eyestalk (Liu *et al*., 1997; Chaves, 2001).

The neurosecretory cells of the crustacean eyestalk are collectively termed the Sinus gland/X-organ complex (SG/XO). This is the primary negative regulatory system in crustaceans and is the site of synthesis and storage for the Moult Inhibiting Hormone (MIH). As MIH negatively regulates ecdysteroid production by the Y-organs, precocious moults may be induced by eyestalk ablation. This results in a reduction of circulating MIH and therefore the immediate rise of ecdysteroids in the haemolymph. While eyestalk ablation is effective at inducing moulting, it also leads to lethal ecdysis in some species (Wheatly and Hart, 1995).

Novel molecular approach to study moulting in crustacea

In spite of extensive research investigating the physiological process underlying moulting, there is still no clear understanding of the cascade of events that regulate this process. Classical molecular approaches have focused on genes specifically related to moulting but have failed to comprehensively cover this complex process. New and powerful technologies such as microarrays offer a holistic approach to gene discovery and the study of gene function in relation to changing physiological states (i.e., moulting). In order to explore the full set of genes involved in the moulting process, microarray technology has been implemented to investigate differential gene expression in *Portunus pelagicus* at various stages of the moult cycle. This has enabled both assessment of expression profiles of known genes, and the discovery of new genes that play a role in the moult cycle of crustaceans. *P. pelagicus* (commonly known as the blue swimmer crab) was used as a model species to study moulting due to its similar morphology to *Callinectes sapidus* (a highly valued soft shell seafood product in the United States of America), and due to hatchery technology developed at the Bribie Island Aquaculture Research Centre (BIARC) which allows for its complete culture in captivity, eliminating the need for wild caught animals.

Methods

P. pelagicus individuals were moult staged by examination of pleopod paddles for epidermal retraction and grouped into the following moult stages; moult (shedding of the exoskeleton), postmoult (pliable exoskeleton), intermoult (hard exoskeleton with no evidence of epidermal retraction) early and late stage premoult (based on the extent of epidermal retraction) (Freeman and Perry, 1985).

P. pelagicus microarray chips were custom printed by AgGenomics (Melbourne, Victoria, Australia), using cDNA libraries created from whole crablets (juvenile crabs with a 3-5 cm carapace width) in each of the five moult stages discussed above, and from the following crab organs brain, eyestalk, mandibular gland, Y-organ dissected from adult crabs in each of the five moult stages.

Experiments on the microarray chips were carried out to assess moult cycle related differential gene expression. This was done by hybridising cDNA labelled with Cy 3 (green) and Cy 5 (red) flurophores to the chip. The cDNA used to probe the slides was isolated from whole crabs in the following moult stages: Moult, Postmoult, Intermoult, Early Premoult and Late Premoult. An Arrayworx scanner was used to scan the slides and data analysed via the

Biodiscovery software package, which includes Imagene for detecting signal intensity and spot morphology and Genesight for expression pattern analysis.

Results and Discussion

Previous studies describe the stimulatory effects of MF on ecdysteroid secretion (Tamone and Chang, 1993; Chang *et al*., 1993). Farnesoic acid O-methyltransferase (FaMeT) is the enzyme responsible for the conversion of farnesoic acid (FA) to MF in the final step of MF synthesis. In this study FaMeT was found to be highly up-regulated in the intermoult stage of the moult cycle. This up-regulation implicates the direct involvement of FaMeT and indirectly MF in initiating the moulting process in crustaceans.

Synthesis and hardening of a new exoskeleton are essential to the arthropod moulting process. Cuticle proteins previously identified in calcified regions of the exoskeleton and those from flexible arthrodial membranes (soft cuticle at the joints) (Andersen, 1999; Wynn and Shafer, 2005) were found to be highly up-regulated in the moult and postmoult stages of the moult cycle indicating that these proteins are necessary for the formation and hardening of the exoskeleton. Cuticle proteins are suggested to be involved in the calcification process (Andersen, 1999; Kragh *et al*., 1997) and in chitin binding (Wynn and Shafer, 2005). Another gene associated with exoskeleton formation is cryptocyanin, the expression of this gene was found to be highly up-regulated in the premoult stage of the moult cycle. Crustacean cryptocyanin belongs to a suit of hemolymph proteins, and is thought to transport hormones, phenols and/or some cuticular proteins to the arthropod hypodermis during premoult (Terwilliger, 1999), where they may become directly incorporated into the new exoskeleton (Terwilliger *et al*., 1999; Haunerland, 1996). Metallothionein, also a hemolymph protein, was found be up-regulated in the premoult and postmoult stages. Metallothionein is involved in copper homeostasis associated with the degradation and synthesis of hemocyanin, prior to and post moult, respectively (Syring *et al*., 2000).

Many interesting expression profiles of "new" as yet unidentified genes were also determined. This was done by comparing the expression profiles of genes already known to be involved in moulting, with those profiles of as yet uncharacterised genes. If unknown genes display expression profiles which are similar (or opposite) to the profiles expressed by genes known to be important to the moulting process, they are selected for further analysis.

Molecular studies aimed at investigating the hormonal regulation of moulting in crustaceans through new and powerful technologies such as microarrays may provide a path into methods of controlling the crustacean moult cycle. Such control would have a direct impact on the large scale / commercial production of a variety of soft shell high value crustacean species.

Acknowledgements

This work was funded by the Queensland Government through the Aquaculture Industry Development Initiative of the Department of Primary Industries and Fisheries. Special thanks go to the supervisors of this project Abigail Elizur, Tim Holton and David Merritt. I would also like to extend my gratitude to the workshop organizers including Dr Sakai, Dr Yokoyama, Dr Aono and Dr Awaji of the National Research Institute of Aquaculture and H. Matsuda of Mie Prefectural Science and Technology Promotion Centre.

References

Abdu, U., Takac, P., Laufer, H., and Sagi, A., 1998: Effect of methyl farnesoate on late larval development and metamorphosis in the prawn *Macrobrachium rosenbergii* (Decapoda, Palaemonidae): A juvenoid-like effect? *Biol. Bull*., **195**, 112–119.

Andersen, S.O., 1999: Exoskeletal proteins from the crab, Cancer pagurus. *Comp. Biochem. Physiol*., **A123**, 203-211.

Chang, E.S., Bruce, M., and Tamone, S., 1993: Regulation of crustacean moulting. *Am. Zool*., **33**, 324–329.

Chaves, A.R., 2001: Effects of sinus gland extract on

mandibular organ size and methyl farnesoate synthesis in the crawfish. *Comp. Biochem. Physiol.*, **A128**, 327-333.

Claerhout, T., Bendena, W., Tobe, S.S., and Borst, D.W., 1996: Characterization of methyl transferase activity in the mandibular organ of the American lobster, *Homarus americanus*. *Biol. Bull.*, **191**, 304.

Drach, P., 1939: Mue et cycle d'intermue chez les Crustaces Decapodes. *Annales De L Institut Oceanocraphique*, **19**, 103-391.

Freeman, J.A. and Perry, H.M., 1985: The crustacean molt cycle and hormonal regulation: its importance in soft shell blue crab production. *National Symposium on the Soft-Shelled Blue Crab Fishery*, 23-30.

Haunerland, N.H., 1996: Insect Storage Proteins: Gene Families and Receptors. *Insect Biochem. Mol. Biol.*, **26**, 755-765.

Hiatt, R.W., 1948: The biology of the lined shore crab, *Pachygrapsus crassipes*. *Pacific Science*, **2**, 135-213.

Kragh, M., Molbak, L., and Andersen, S.O., 1997: Cuticular proteins from the lobster, *Homarus americanus*. *Comp. Biochem. Physiol.*, **B118**, 147-154.

Laufer, H. and Biggers, W.J., 2001: Unifying concepts learned from methyl farnesoate for invertebrate reproduction and post-embryonic development. *Am. Zool.*, **41**, 442-457.

Laufer, H., Ahl, J., and Sagi, A., 1993: The role of juvenile hormones in crustacean reproduction. *Am. Zool.*, **33**, 365-374.

Liu, L., Laufer, H., Wang, Y., and Hayes, T., 1997: A neurohormone regulating both methyl farnesoate synthesis and glucose metabolism in a crustacean. *Biochem. Biophys. Res. Comm.*, **237**, 694-701.

Loeb, M., J., 1993: Hormonal Control of Growth and Reproduction in the Arthropods: Introduction to the Symposium. *Am. Zool.*, **33**, 303-307.

Spaziani, E., Watson, R.D., Mattson, M.P., and Chen, Z.F., 1989: Ecdysteroid biosynthesis in the crustacean Y-organ and control by an eyestalk neuropeptide. *J. Exp. Zool.*, **252**, 271-282.

Syring, R.A., Hoexum Brouwer, T., and Brouwer, M., 2000: Cloning and sequencing of cDNAs encoding for a novel copper-specific metallothionein and two cadmium-inducible metallothioneins from the blue crab *Callinectes sapidus*. *Comp. Biochem. Physiol.*, **C125**, 325-332.

Tamone, S.L. and Chang, E.S., 1993: Methyl farnesoate stimulates ecdysteroid secretion from crab Y-organs *in vitro*. *Gen. Comp. Endocrinol.*, **89**, 425-432.

Terwilliger, N.B., 1999: Hemolymph proteins and molting in crustaceans and insects. *Am. Zool.*, **39**, 589-599.

Terwilliger, N.B., Dangott, L., and Ryan, M., 1999: Cryptocyanin, a crustacean molting protein: evolutionary link with arthropod hemocyanins and insect hexamerins. *Proc. Natl. Acad. Sci. U.S.A.*, **96**, 2013-2018.

Wheatly, M.G., Hart, M.K., 1995: Hemolymph ecdysone and electrolytes during the molting cycle of crayfish: A comparison of natural molts with those induced by eyestalk removal of multiple limb autotomy. *Physiol. Zool.*, **68**, 583-607.

Wynn, A. and Shafer, T.H., 2005: Four differentially expressed cDNAs in *Callinectes sapidus* containing the Rebers-Riddiford consensus sequence. *Comp. Biochem. Physiol.*, **B141**, 294-306.