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Identification of differentially expressed reproductive and metabolic proteins in the female abalone (*Haliotis laevigata*) gonad following artificial induction of spawning

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ABSTRACT

Inefficient control of temperate abalone spawning prevents pair-wise breeding and production of abalone with highly marketable traits. Traditionally, abalone farmers have used a combination of UV irradiation and application of temperature gradients to the tank water to artificially induce spawning. Proteins are known to regulate crucial processes such as respiration, muscle contraction, feeding, growth and reproduction. Spawning as a pre-requisite of abalone reproduction is likely to be regulated, in part, by endogenous proteins. A first step in elucidating the mechanisms that regulate spawning is to identify which proteins are directly involved during spawning. The present study examined protein expression following traditional spawning induction in the *Haliotis laevigata* female. Gonads were collected from abalone in the following physiological states: (1) spawning; (2) post-spawning; and (3) failed-to-spawn. Differential protein abundance was initially assessed using two-dimensional difference in-gel electrophoresis coupled with mass spectrometry for protein identification. A number of reproductive proteins such as vitellogenin, vitelline envelope zona pellucida domain 29 and prohibitin, and metabolic proteins such as thioredoxin peroxidase, superoxide dismutase and heat shock proteins were identified. Differences in protein abundance levels between physiological states were further assessed using scheduled multiple reaction monitoring mass spectrometry. Positive associations were observed between the abundance of specific proteins, such as heat shock cognate 70 and peroxiredoxin 6, and the propensity or failure to spawn in abalone. These findings have contributed to better understand both the effects of oxidative and heat stress over abalone physiology and their influence on abalone spawning.

1. Introduction

Abalone are edible marine snails highly appreciated for their palatability. Abalone aquaculture has emerged as a strategy to fulfill the market demands and to avoid depletion of wild stocks. At the current time the world production of aquacultured abalone has exceeded the fisheries production, mainly due to extensive farming in China and Korea (Gordon and Cook, 2013). In Australia, the production value of fisheries and farmed abalone was estimated at AUD 190 million for the year 2012–13 (Stephan and Hobsbawn, 2014). In addition to meeting market demand, abalone aquaculture also aims to implement selective breeding programs (SBPs), for example, between specific individuals with desirable traits in order to produce offspring with highly marketable characteristics. Despite the commercial importance, abalone reproduction is a poorly understood process and lack of full control over spawning is a current issue in the Australian abalone industry.

In the wild, successful reproduction of broadcast spawning molluscs, such as abalone, relies on a myriad of integrated environmental factors including tides, moon cycle, food availability, atmospheric pressure, photoperiod, temperature and salinity (Kikuchi and Uki, 1974; Counihan et al., 2001; Grubert and Ritar, 2005). Farmed abalone rely on many, if not all, of these factors and inability to artificially reproduce these cues prevents successful spawning on demand. Research has been undertaken with an aim to gain control over abalone spawning. Manipulation of physical and chemical parameters in seawater has been shown to induce gonad conditioning (maturation of gametes) and spawning in certain abalone species (Kikuchi and Uki, 1974, 1977; Morse et al., 1977; Grubert and Ritar, 2005). However, in the greenlip abalone, *Haliotis laevigata*, full control over abalone spawning remains unsolved.

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In molluscs, there have been many attempts to elucidate the mechanisms underpinning reproduction, especially in model organisms such as the pond snail *Lymnaea stagnalis* and the sea hare *Aplysia californica* (Hermann et al., 1997; Cummins et al., 2008). Much of the research effort has been aimed toward identifying the peptide hormones. Gonadotropin-releasing hormone (GnRH) is a hypothalamic peptide hormone that initiates reproduction in vertebrates. To date several molluscan analogues of GnRH have been identified (Iwakoshi et al., 2002; Cummins and Hanna, 2004; Zhang et al., 2008; Cummins et al., 2010). Invertebrate and vertebrate GnRHs are thought to evolve from a common ancestor, but the molecular mechanisms underlying GnRH function in molluscs are yet to be fully elucidated (Iwakoshi et al., 2002; Zhang et al., 2008; Tsai et al., 2010). The ovulation hormone caudodorsal cell hormone (CDCH) known to stimulate sexual maturation, mating and spawning has been identified in *L. stagnalis* (Hermann et al., 1997). An orthologue of CDCH, the egg-laying hormone (ELH) has been identified in *A. californica* (Cummins and Hanna, 2004; Cummins et al., 2010). The role of abalone ELH (aELH) isolated from blacklip abalone *Haliotis rubra* (Cummins and Hanna, 2004) in reproduction was evidenced by the induction of sexual maturation and spawning in *Haliotis asinina* and in the giant prawn *Macrobrachium rosenbergii* (Ngernsoungnern et al., 2009; Nuurai et al., 2010) after injection.

Neuropeptide hormones and neurotransmitters with the ability to stimulate sexual maturation and/or spawning have attracted attention as a potential means to manipulate reproductive processes on farm. In *H. asinina*, injections of the tetrapeptide APGWamide into both sexes was undertaken and was noted to stimulate spawning within 2–4 h exclusively in males (Chansela et al., 2008). An attractin-like pheromone predicted to play a role in mating behaviour was identified in both sexes of *H. asinina* (Kuanpradit et al., 2010). The exogenous administration (in tank water) of the neurotransmitter serotonin stimulated contractions in gonadic tissue of *H. rubra* that resembled those occurring naturally during abalone spawning (Panasophonkul et al., 2009). Protein enzymes such as prohormone convertases are responsible for cleaving neuropeptide precursors yielding mature bioactive neuropeptides that act to regulate reproductive processes. Prohormone convertase 1 was identified in *H. diversicolor supertexta* and was postulated to play a critical role in abalone reproductive processes as judged by its high mRNA expression in pre-breeding and during-breeding stages which declined significantly in the post-breeding stage (Zhou and Cai, 2010). While many of the signalling molecules have been identified, little is known about their partners, the receptors. In the gonads of the marine snail *Thais clavigera*, high levels of estrogen receptor 1 mRNA were expressed in spring in preparation for seasonal gonad development (Kajiwara et al., 2006).

Despite the importance of these contributions to understanding molluscan reproduction, a depth of knowledge similar to that achieved in vertebrate reproduction has yet to be achieved in abalone. A comprehensive analysis of the effects of the current spawning methodology on the proteins of abalone is needed to understand the biological basis for spawning. In this study two-dimensional difference in-gel electrophoresis (2D-DIGE) and scheduled multiple reaction monitoring mass spectrometry (MRM-MS) were employed to examine differential protein expression in abalone that had successfully spawned compared to those that failed-to-spawn following artificial spawning induction.

2. Materials and methods

2.1. Abalone spawning induction and gonad dissection

Sexual maturation assessment of female *H. laevigata* (100–130 mm; 36 months old) was performed using the visual gonad index (VGI). The VGI is a non-invasive technique that evaluates gonad shape and size addressed in four categories (0–3), wherein VGI 3 denotes a swollen-round conical appendage (Kikuchi and Uki, 1974). Sexually mature abalone, with a VGI of 3, were collected from production tanks at Kangaroo Island Abalone (KIAB) (35°35′42.2″ S; 137°25′52.5″ E). On the day of spawning induction, 50 female abalone with VGI of 3 were divided into groups of 10 and placed in resting tanks for ~7 h before artificial spawning induction was performed as previously described (Kikuchi and Uki, 1977) with modifications. In brief, filtered seawater was irradiated with ultraviolet (UV) light (1 × 300 W, Australian Ultra-violet Products, Seven Hills, NSW) and simultaneously heated from 18 to 22 °C over the course of 1 h (Fig. 1). The circulation of the UV irradiated water supply was stopped in the holding tanks as the abalone started to spawn to allow gamete recovery for farm restocking purposes. Abalone were allowed 12 h to spawn from the start of the experiment. Sampled gonads were classified according to the response to the spawning induction. Abalone started spawning within 4 h of induction. Some abalone releasing gametes were immediately taken during gamete release for dissection and classified as spawning (SP), other spawning abalone were left to spawn *ad libitum* and sampled approximately at 7–9 h of initiation of spawning to comprise the post-spawning (PSP) group. Abalone that failed to spawn over the duration of the experiment were classified as failed-to-spawn (FSP) and sampled at the conclusion of the experiment.

The inefficiency in artificial spawning induction on-farm is exemplified by the fact that only 18% of the abalone in this experiment successfully spawned. Five gonads were classified as SP, four PSP and nine were selected to comprise the FSP group. For dissection, the selected abalone were anaesthetized by injecting 500 μL of magnesium chloride (360 mM) into the cephalic arterial sinus using a 30 gauge and 8 mm ultrafine syringe. Gonadic tissue (possibly containing eggs) was snap-frozen in liquid nitrogen and stored at −80 °C prior to analysis.

2.2. Two-dimensional difference in-gel electrophoresis (2D-DIGE)

2.2.1. Protein preparation, IEF and PAGE

Proteins were extracted from gonad tissues using standard protocols recommended by GE Healthcare for 2D-DIGE utilising the Ettan–Dalt system with slight variations. In brief, 100 mg of gonad tissue was homogenized (Ultra-turrax T8, IKA) using 1 mL of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 100 mM dithiothreitol (DTT)) followed by sonication at 30% (amplitude) on ice. Preparations were then incubated at room temperature for 3 h. A clear lysate was obtained by centrifugation at 14,000 × *g* at 4 °C for 30 min. Protein extracts were prepared for isoelectric focusing (IEF) using the 2D-Clean-up kit and quantified using the 2D-Quant kit (GE Healthcare).

Prior to IEF, 50 μg of protein of each sample (representing one animal per physiological state) to be evaluated was labeled with either fluorescent Cy3 or Cy5 dyes according to the manufacturer's directions (Amersham). The fluor Cy2 was used as an internal standard constituted by pooling half of each gonad under analysis to obtain a ratio of differential expression between them. Overall, three 2D-DIGE experiments were performed in which each physiological state was compared in a pair-wise manner as follows: experiment 1: FSP versus SP; experiment 2: PSP versus SP and experiment 3: FSP versus PSP. Proteins were cup loaded into 24 cm strips (3–11 NL, GE Healthcare) and separated (IEF) for 40,850 Vh at 20 °C followed by reduction, alkylation and PAGE separation. Gel images were acquired in a Typhoon scanner variable mode imager (GE Healthcare) and subsequently silver stained in preparation for in-gel digestion (Rabilloud et al., 1988) followed by protein MS identification based on tryptic peptides. Supplemental File 1 provides a detailed description of the 2D-DIGE methodology.

2.2.2. Assessment of differentially expressed proteins

The module Differential In-Gel Analysis (DIA) embedded in the software DeCyder 2D version 6.5 (GE Healthcare) was used for detection of differentially expressed protein-spots. The DIA module utilises algorithms to quantify abundance, dependent on fluorescent intensity, between two corresponding protein-spots in a CyDye linked single gel. The abundance values are then converted to ratios allowing visualisation

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