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Parasitic castration by the digenian trematode *Allopodocotyle* sp. alters gene expression in the brain of the host mollusc *Haliotis asinina*

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Abstract **Infection of molluscs by digenean trematode parasites typically results in the repression of reproduction – the so-called parasitic castration. This is known to occur by altering the expression of a range of host neuropeptide genes. Here we analyse the expression levels of 10 members of *POU*, *Pax*, *Sox* and *Hox* transcription factor gene families, along with genes encoding FMRFamide, prohormone convertase and β-tubulin, in the brain ganglia of actively reproducing (summer), non-reproducing (winter) and infected *Haliotis asinina* (a vetigastropod mollusc). A number of the regulatory genes are differentially expressed in parasitised *H. asinina*, but in only a few cases do expression patterns in infected animals match those occurring in animals where reproduction is normally repressed.**

Keywords: Digenean; Hox; Pax; POU; Transcription factor; Sox; *Haliotis*

1. Introduction

Digenean trematodes are platyhelminth parasites with complex lifecycles, typically involving at least three different hosts including a definitive, a first intermediate and a second intermediate host [1]. The first intermediate host is almost always a mollusc, with a given digenean species capable of infecting only one or two mollusc species [1]. These strict associations have allowed specialised interactions to evolve, including parasite-induced alterations in host behaviour, defence function, nutrition, metabolism and reproduction [2–4]. One common outcome of digenean infection in molluscs is castration [5–8], which can result in the incomplete or total disruption of host gamete production and presumably allow for the redeployment of metabolites to the parasite [9].

In most cases castration occurs indirectly, either through the deprivation of essential nutrients [6] or by the interruption and utilisation of the host's natural endocrine system [7,10,11]. In the interaction between the snail, *Lymnaea stagnalis*, and the schistosome trematode, *Trichobilharzia ocellata* [7,10,11], castration appears to be caused by induced changes in gene expression of the neuroendocrine system that controls growth and reproduction [2].

The neurosecretory system of molluscs consists primarily of individual or small clusters of neurons distributed throughout the cerebral, pleural, pedal and visceral ganglia [12]. The antagonism between growth and reproduction appears to be controlled by neurohormones synthesised by cells in these ganglia [13]. Physiological changes occurring in both normal and infected *L. stagnalis* correlate with changes in expression of genes encoding neuropeptides, such as: (i) schistosomin, which inhibits the action of gonadotropic hormones in reproductive organs; (ii) neuropeptide Y, which appears to regulate reproduction and growth; and (iii) caudodorsal cell hormone, which is known to regulate egg-laying and accompanying behaviours [2]. The activities of specific cell clusters in the ganglia with known roles in reproduction and growth are also affected by parasitosis [7,14,15]. Regulatory transcription factors are likely to act as key controllers in the normal physiological transitions operating in molluscan ganglia [16], and coordinate the expression of gene batteries that are required for the mollusc to obtain a reproductive or growing physiological state.

Haliotis asinina (Vetigastropoda) has a distinct and predictable reproductive season that lasts for about six months in the summer [17]. During this season the ovaries are full of mature oocytes, while during the non-reproductive winter period, the female gonad consists of small numbers of immature oocytes and granular debris [26]. Approximately 2% of wild population of *H. asinina* of Heron Island Reef are infected with an opecoelid digenean in the genus *Allopodocotyle* [8]. The condition of the gonads of these parasitised abalone imitates that of the non-gravid winter gonads, with no direct structural damage observed. In infected abalone, the sporocyst and cercarial stages inhabit the haemocoel suggesting that parasitic castration is likely to be because of effects to the physiology of the host and not direct consumption of the gonad by the trematode. Infections of other host molluscs largely appear to follow similar patterns of castration [5–7]. In this study, we have quantitatively assessed the expression of 10 transcription factor genes, along with FMRFamide, prohormone convertase 2 (PC2), and tubulin genes in the cerebral and pleuropedal ganglia of *H. asinina* that are either: (i) actively reproducing; (ii) parasitized and castrated; and (iii) not reproducing. The transcription factor families targeted for quantification – *Hox*, *Pax*, *POU* and *Sox* – are known to play key roles in

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Abbreviations: ANOVA, one-way analysis of variances; PC2, prohormone convertase 2; QPCR, quantitative real-time reverse transcription polymerase chain reaction

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development of a wide range of animals and in some cases physiological processes such as growth [18,19]. These genes are expressed in tissue-restricted patterns in *H. asinina* and in brain ganglia [16,20–23].

2. Materials and methods

2.1. Collection of samples

H. asinina were collected on Heron Island Reef Australia. Parasitised abalone were identified by inspecting the gonad region for obvious infestation of sporocysts as per [8]. Total RNA was extracted from individual dissected cerebral and pleuropedal ganglia from reproducing (summer) abalone, non-reproductive (winter) abalone or parasitised summer abalone as described in [16].

2.2. Quantitative real-time PCR

cDNA was synthesised using 0.2 μg of RNA, 10 μM random hexamer primers (Promega) and MMLV-reverse transcriptase (Promega) according to the manufacturer's specifications. Gradient polymerase chain reactions (PCRs) were performed on all primer sets (oligonucleotide primer sequences available upon request) to ascertain a uniformly suitable annealing temperature, using 0.01 μg cDNA per 20 μl reaction. The cycling conditions for all gradient PCRs were 96 °C for 2 min, 35 cycles of 94 °C for 30 s, gradient annealing from 53 to 68 °C for 1 min, 72 °C for 1 min followed by a final extension of 72 °C for 10 min and a cooling to 25 °C for 10 min.

Quantitative real-time reverse transcription polymerase chain reaction (QPCR) was conducted on a LightCycler (Roche) using Quantitech SYBR Green PCR Mix (Qiagen). The master mix was optimised to contain 0.75× SYBR Green Mix, 1 μM per primer, and 0.01 μg cDNA per 20 μl reaction. cDNA samples made from differing amounts of initial RNA (0.2 μg, 0.4 μg, 0.6 μg and 1 μg) were run as standards in each run. Standards were run in triplicate and each sample in triplicate for the control gene and the gene of interest. All runs included a negative control. For all analyses the cycling conditions were 95 °C for 15 min, 32 cycles of 94 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min with a read temperature of 80 °C each cycle. Melting curve analysis was used to establish that only one product was amplified per reaction and that the product was the expected amplicon.

2.3. Data analysis

Analysis of fluorescence data was conducted on LightCycler Software version 3.3P9. Once the data had been collected from the LightCycler Microsoft Excel was used to normalise the target genes' expression relative to *HasSox-C* expression. Normalisation was carried out using the average *HasSox-C* expression per each specific run so as to account for variability between runs. All analyses were conducted within a ganglion type. Once normalised the values were then corrected for the uneven expression of the *HasSox-C* gene by multiplying by a correction value derived from the fold difference between the average *HasSox-C* values per treatment over all runs. SPSS Student Version 11.0 was then used to perform either one-way analysis of variances (ANOVA) or the non-parametric equivalent the Kruskal–Wallis test where Levene's test showed variances to be unequal. Post hoc comparisons between treatments were carried out using either parametric or non-parametric tests (Tukey's test or Nemenyi's test, respectively) where applicable. For post hoc comparisons the critical rejection value was adjusted using Bonferoni's method from 0.05 to 0.016. If no homogenous subsets were created at $\alpha = 0.016$ despite a significant difference being found using ANOVA or Kruskal–Wallis post hoc comparisons were re-run at $\alpha = 0.05$. See Reverter et al. [24,25] for a detailed rationale behind the normalisation procedure and statistical approach described here.

3. Results

Using QPCR, we determined the relative abundance of gene transcripts in cerebral and pleuopedal ganglia dissected from *H. asinina* either in the middle of their reproductive cycle (summer abalone), when not reproducing (winter abalone) or when infected by the digenean trematode (parasitised). The infected animals were all obtained in the summer, when they normally would be reproducing.

3.1. Expression of reference gene HasSox-C

*HasSox-C*was chosen as the reference gene as it is widely expressed during development and across adult tissues [16,20,21]. However, QPCR revealed that the *HasSox*-C expression levels in the cerebral ganglia were 1.7-fold different between winter and summer samples (Fig. 1). Expression levels in the pleuropedal ganglia were higher and more variable, with winter samples having the highest level of expression and parasitised samples showing a significantly lower transcript levels than either of the uninfected treatments. *HasSox-C* was employed as a reference despite the uneven expression across seasons and between normal and parasitised animals. The effect of these differences in *HasSox-C* transcript abundance, however, was mitigated by defining a set of correction factors for each ganglia. Expression in each of the parasitised ganglia was set to 1 and the fold difference between parasitised and the other samples was calculated. The correction factors were as follows: cerebral ganglia summer = 0.78 and winter = 1.34; and pleuropedal ganglia summer = 2.79 and winter = 4.25 (Fig. 1; Table 1). These correction factors were applied to the normalised abundance values to produce the standardised abundance values.

3.2. Structural and neuropeptide gene expression profiles

Overall, *HasTub1*, *HasPC2*, and *HasFMRFa* transcripts were more abundant in both ganglia compared to transcripts encoding transcription factors (Fig. 2). *HasTub1* and *HasPC2* were expressed at higher levels in the ganglia of non-reproducing animals in the winter, with 2.4-fold higher expression of *HasTub1* in the cerebral ganglion and approximately 20-fold higher expression of *HasPC2* in both ganglia (Table 1). Altered expression in parasitised abalone for both genes tended to match the changes that existed between reproducing and non-reproducing *H. asinina*. *HasTub1* and *HasPC2* transcript abundance increased in parasitised animals relative to summer

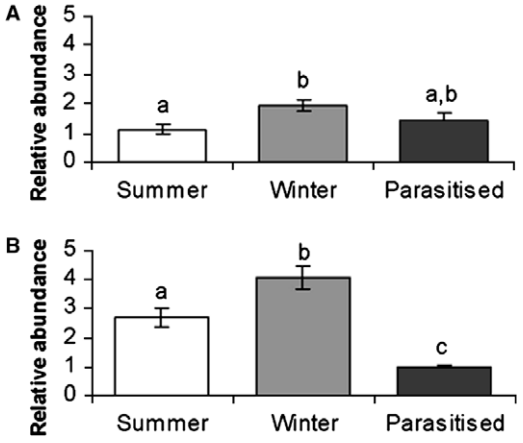

Fig. 1. Mean relative abundance for *HasSoxC* transcripts in *H. asinina* ganglia ± 1 S.E. of the mean. (A) cerebral ganglia; (B) pleuropedal ganglia. Average abundances estimated from 10 QPCR analyses of the cerebral ganglia and 12 analyses of the pleuropedal ganglia. Homogeneous groupings are significant at confidence level of $\alpha = 0.016$.