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Genes induced during the early developmental stages of the Cane Toad, *Bufo* (Chaunus) *marinus*

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ABSTRACT

Metamorphosis, a critical stage in the development of toads and frogs, involves rapid levels of morphological change. In the current study, we have used microarray analysis to identify shifts in gene expression between tadpole and toadlet stages of the cane toad, *Bufo* (Chaunus) *marinus*. Here, we report on nine genes that show the greatest induction during metamorphosis; the gut-associated gastrokine and trefoil factor, blood components haemoglobins α/β, apolipoprotein and serum albumin, a nasal gene olfactomedin, a lens gene γ-crystallin, and a novel gene with low homology to frog harderin. We present both temporal and spatial expression patterns of these genes identified in developing and adult cane toads. This study extends our knowledge of the molecular basis of toad metamorphosis, and not only offers insights to the genes induced during the general remodelling that occurs but also reveals possible targets for control and manipulation of amphibian pest species, for example, the cane toad in Australia.

1. Results and discussion

Frogs and toads (Order Anura) undergo major morphological changes in the transition from an aquatic to a semi-terrestrial existence. This process, known as metamorphosis, takes place in a remarkably short period considering the degree of transformation that occurs. During metamorphosis, many tissues and cellular systems in the anuran are affected by remodelling; changing from larval- to adult-type cells (Yoshizato, 1992). Gross morphological changes occur in a coordinated fashion, such that the development of legs is accompanied by reabsorption of the tail, lungs replace gills and a shift in diet requires alterations to the mouth and gut (Nieuwkoop and Faber, 1967). Cellular and tissue modifications include alterations to blood composition (Katherine et al., 1997; Benbassat, 1974), skin structure (Suzuki et al., 2002) and intestinal length (Schreiber et al., 2005).

At the genetic level, it is clear that metamorphosis is modulated by significant alterations in gene expression, primarily driven by hormonal mediators such as thyroid hormones and corticosteriods (Buchholz et al., 2003; Tata, 1998; Das et al., 2006). Cataloguing genetic events, such as those seen in the tail, limbs, liver and gut, has provided valuable insights to organ development and tissue regression in the anurans *Xenopus* and *Rana* (Shi and Brown, 1993; Buckbinder and Brown, 1992; Brown et al., 1996; Lyman and White, 1987).

Abbreviations: ORF, open-reading frame; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; TFF, trefoil factor; HDL,high-density lipoprotein; vs, versus; dpf, day(s) post-fertilisation; aa, amino acid(s).

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Bufo (Chaunus) marinus (cane toad or marine toad) is a large anuran species native to the America's and introduced to a number of East Asian, Pacific and Caribbean nations (Easteal, 1985; Slade and Moritz, 1998). These toads are of interest as they produce cardiotonic toxins that impart a substantial ecological advantage over non-toxic native species. Cane toads have become a major invasive pest in countries like Australia, where they have reached densities at least 10 times greater than in their native Venezuelan and Brazilian habitats (Lampo and De Leo, 1998).

The genetic data for the cane toad, particularly with regards to metamorphosis is currently lacking and would greatly aid our understanding of this animal's development and may provide insights into targets for control strategies. In this study, we have used microarray analysis systems to identify shifts in gene expression between pre-metamorphic (tadpole) and post-metamorphic (toadlet) stages of cane toads. In particular, our focus has been the isolation of genes and gene fragments that are switched on during or late in metamorphosis. Such genes offer a unique opportunity for manipulation since early tadpole stages are potentially naive to these later expressed proteins, and premature exposure may compromise development (Maniatis et al., 1969). Here, we report on a number of metamorphosis-induced genes including haemoglobin α/β chains, apolipoprotein A1, γ-crystallin, serum albumin, trefoil factor 1, olfactomedin, gastrokine and a harderin-like gene. In addition, we present both temporal and spatial expression patterns of these genes in developing and adult toads, respectively. As the cane toad continues to be a major invasive pest in its introduced ranges, we highlight these metamorphosis-induced genes with an aim to test and explore tailored and specific control strategies.

1.1. Microarray analysis

Patterns of differential gene expression during development were assessed in this study, and hybridisations were organised with different aged pre-metamorphic samples (9, 18 and 28 dpf) compared to post-metamorphic samples (30 and 53 dpf), see Fig. 1. Across the six comparisons the total number of genes that passed the statistical cutoff ($p < 0.005$) was 870, with 301 up-regulated genes and 576 down-regulated genes. Several genes were considered complex, being up- and down-regulated in different comparisons. As our interest was only in those genes significantly induced during metamorphosis, a final selection from the 301 up-regulated genes was based on the strength of signal (>2-fold) and frequency of occurrence across multiple contrasts.

It was anticipated that an expression profile for some of the differentially expressed genes could be established which would map their expression during metamorphosis. While strong changes in gene expression were observed between pre- and post-metamorphosis, the low expression of differentially expressed genes during all pre-metamorphic time points meant that a statistically significant time course analysis was not possible. There was an interesting difference in the number of differentially expressed genes between the 30 and 53 dpf toadlet samples contrasted with 28 dpf pre-metamorphs. In particular the contrast at metamorphic climax, illustrated by the 30 vs 28 dpf comparison, showed strong induction of gene expression (67 genes) with little down-regulation (3 genes) (Fig. 2a). The 53 vs 28 dpf comparison on the other hand revealed a more even regulation ratio (Fig. 2b; 20 up, 17 down), with these older toadlets (53d) showing stable gene regulation levels when compared to the newly metamorphosed 30-day-old toadlets.

1.2. Differential genes induced during B. marinus metamorphosis

Top ranking up-regulated clones, as depicted in Fig. 2 (blue asterisks), were sequenced and analysed for homology to known genes. Of the top 32 clones examined, 10 revealed no significant homology with published sequences (i.e., unknowns). For the remaining clones, after duplicate spots/clones had been combined, 11 different genes were assigned putative identities based on closest sequence homology (Supplementary Table 3). In this paper, we present data on nine of these differentially expressed genes, each summarised briefly below with reference to putative identities (see Table 2 and Fig. 3 for additional details, including accession numbers).

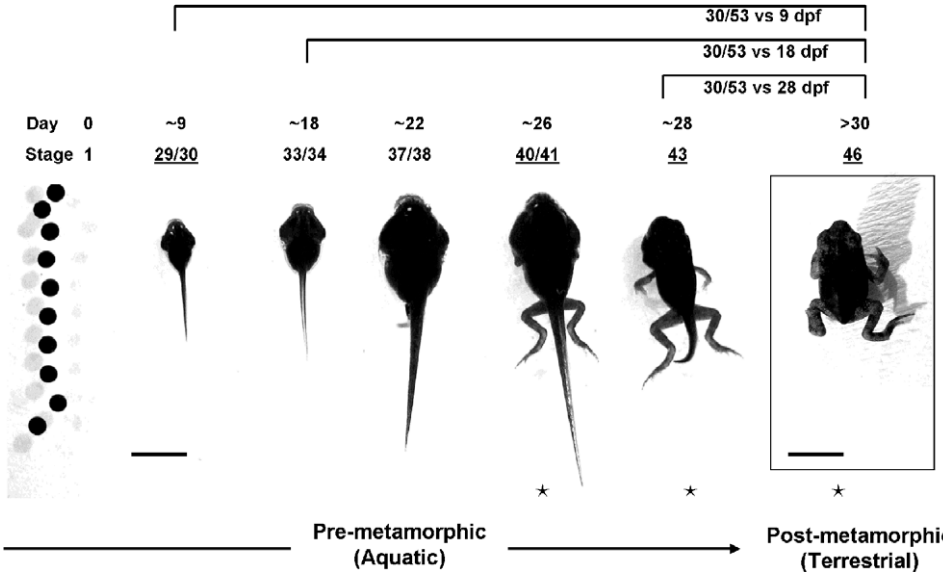

Fig. 1. Selected metamorphic stages of *B. marinus* showing the progression from eggs and tadpoles through to terrestrial toadlet. Aquatic pre-metamorphic (Stage 1–43, 0–28 days post-fertilisation) and terrestrial post-metamorphic (Stage 46, $\geqslant$30 dpf) stages are shown with the period of extensive morphological change, known as the metamorphic climax, indicated by stars ($*$). Contrasting developmental time points used for microarray analysis are shown by brackets, with our six comparisons being 30 vs 9 dpf, 53 vs 9 dpf (top bracket), 30 vs 18 dpf, 53 vs 18 dpf (middle bracket), 30 vs 28 dpf and 53 vs 28 dpf (bottom bracket). Stages that are representative of the animals used for the temporal analysis of gene expression are underlined. Approximate age in days post-fertilisation (dpf) is designated, however, note that the time taken to metamorphosis can vary substantially based on temperature, food and density. Animals were staged according to Limbaugh and Volpe (1957). Scale bars equal 5 mm.