Characterisation of the Warm Acclimated Protein gene (wap65) in the Antarctic plunderfish (*Harpagifer antarcticus*)

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Abstract
Physiological adaptation to increased environmental temperatures has been studied experimentally in a number of fish species, with the up-regulation of several genes identified as being associated with the process, such as the warm-acclimated protein (*wap-65*). This article describes the cloning and characterisation of the wap65-2 gene from the Antarctic plunderfish (*Harpagifer antarcticus*). The transcriptional expression of this gene in response to elevated seawater temperatures over a time course series is presented. Initially there is strong down regulation of this gene to a maximum of 40 fold within 4 hours, followed by recovery to almost control levels within 48 hours, indicating that this gene does not play a role in the potential temperature adaptation of *H. antarcticus*.

Introduction

Whilst environmental adaptation and the capacity to cope with change is a species-specific phenomenon, poikilothermic animals are clearly far more vulnerable to environmental temperature changes compared to homeotherms. However many eurythermal aquatic poikilotherms experience and adapt to wide variations in natural water temperatures. Examples of such include a seasonal 30ºC temperature range for goldfish and carp and 13°C weekly variations experienced in ephemeral pond environments inhabited by the annual killifish *Austrofundulus limnaeus*. Therefore within each species there is the capacity for physiological and biochemical reorganisation to enable adaptation to either warmer or cooler seasonal temperatures, a process termed acclimatization (Hazel and Prosser, 1974). In the experimental context this process has been re-named acclimation to delineate laboratory manipulation from conditions found in the natural environment.

Protein 2-D gel electrophoresis experiments identified a 65-kDa protein that accumulated in various tissues in goldfish (*Carassius auratus*) and carp (*Cyprinus carpio*) acclimated to 30ºC for a minimum of 5 weeks (Watabe et al, 1993; Kikuchi et al, 1993). This was subsequently named Warm Acclimated Protein (*wap65*). These fish proteins showed 31% sequence identity to rat hemopexin. This is a protein synthesised in the rat liver and plays an important role scavenging haems from blood. The mRNA for wap65 in *C. auratus* is rapidly amplified in the liver to a maximum of a 40 fold increase on day 3 of temperature acclimation experiments with a subsequent decline to a steady 10 fold increase for long-term acclimated stocks (Kikuchi et al, 1997). This finding is concurrent with the gene product having an acclimation function at least in these fish species. Further analyses on other fish species (*Oncorhynchus mykiss, Ictalurus punctatus, Danio rerio, Oryzias latipes* and *Takifugu rubripes*) have shown that in contrast to the single isoform found in mammals, wap65 is present in duplicate in fish, presumably as a result of the teleost whole genome duplication event (Amores et al, 1998). These fish isoforms display different affinities for haem, tissue distributions and development patterns (Hirayama et al, 2003; 2004; Nakaniwa et al, 2005).

In contrast to the eurythermal examples quoted earlier, some animals exist within a very narrow environmental temperature envelope. For example most Antarctic marine species are highly stenothermal (Somero and DeVries, 1967; Peck et al, 2000) having adapted to life in the Southern Ocean at stable sub zero temperatures. The ability of these animals to adapt/acclimatize to warmer sea temperatures is of prime importance given the current IPCC Third Assessment climate models and predictions of global climate change.

In our laboratory, a number of experiments have been carried out to investigate the effects of elevated water temperatures on the gene expression profile of the Antarctic plunderfish (*Harpagifer antarcticus*). During EST screening a number of clones were identified with high sequence similarity wap65. Here we describe the characterisation of the gene for the *H. antarcticus* wap65 and the effects of an elevated water temperature regime (6ºC) on expression levels over a 48-hour time course.

Materials and Methods

Animal sampling

H. antarcticus used in the experimental work were collected at Rothera Research Station, Adelaide Island, Antarctic Peninsula (67^{o} 34' 07" S, 68^{o} 07' 30" W) by SCUBA divers during the austral summer. The animals were returned to the UK in a refrigerated transport aquarium and maintained in a recirculating aquarium at close to 0 ^{o}C until required for experimental work. Thirty five fish were transferred to the

experimental tank at time zero and maintained at 6.0 ± 0.08°C. Five animals were killed at zero, 2, 4, 8, 12, 24 and 48 hours using Home Office approved procedures.

Cloning and sequencing

Approximately 1,000 clones were sequenced from a directionally cloned non-normalised *H. antarcticus* liver cDNA library. All clones were vector and quality clipped before subjecting to BLAST sequence similarity searching. Analysis of BLAST results identified 10 ESTs, which showed high sequence similarity to the warm-temperature-acclimation-related-65 protein (accession number Q4W7I1). These 10 EST clones were concatenated and edited using the phred, Phrap and consed packages (Ewing and Green 1998; Gordon et al, 1998). Sequence analysis was performed using the EMBOSS suite of open source software http://emboss.sourceforge.net. Alignments were exported into Boxshade (http://www.ch.embnet.org/software/BOX_form.html) for annotation. The *H. antarcticus* Wap65 sequence was submitted to the EMBL database with the accession number AM408054.

RNA Isolation and Q-PCR

Total RNA was extracted from the tissue samples using TRI Reagent (Sigma) according to the manufacturer's instructions. 1µg of total RNA was DNase treated and reverse transcribed using a first strand synthesis kit (Promega). Profiling of tissue-specific expression and Q-PCR was carried out using the following primers: Acclim2F (TAGAGCACTACTACTGTTTCCA) and Acclim2Rev (AGGCCGTCACGCTTGGTGT); (ActinF: ACAGACTACCTCATGAAGATCCT; ActinR: GAGGCCAGGATGGAGCCTCC). Actin was used as the housekeeping sequence for both RT and Q-PCR experiments as it had previously been shown not to change under the experimental conditions used (data not shown). For Q-PCR, both primer sets were checked over a four fold 10x dilution series with RSq values and PCR efficiency values (1.00 and 100.8% respectively for Wap65) calculated using the MxPro - MX3000P v 3.00 Build 311 Schema 74 software. Wap65 and actin sequences were amplified from each time point using Brilliant SYBR® Green QPCR Master Mix (Stratagene) and an MX3000P (Stratagene). PCR conditions were as follows: 95°C 10 minutes, 40 cycles of 95°C 30 seconds, 60°C 1 minute and 72°C for 1 minute with a final dissociation curve step. The plate set-up for each Q-PCR experiment consisted of 5 control individuals and 5 experimental individuals (both in triplicate). Analysis was performed using the MxPro - MX3000P v 3.00 Build 311 Schema 74 software and data exported into the Relative Expression Software tool (REST) (http://www.gene-quantification.info/), which incorporates the Pfaffl method of compensating for the PCR efficiency and also uses a Pair Wise Fixed Reallocation Randomisation Test (Pfaffl et al, 2002). The results were also subjected to a 2-sample t-test using MINITAB v14 to determine significance and delineate the 95% confidence range.

Results

Concatenation of EST data (10 clones) produced a 1367nt consensus sequence of high quality reads. BLAST sequence similarity searching of this sequence revealed 72% sequence identity (Expect = 1.9^{e-174}, Score = 1711) with the warm-temperature-acclimation-related-65 protein (accession number Q4W7I1) from the Medaka fish *Oryzias latipes*. Sequence comparisons identified that the full-length wap-65 coding sequence of 431 amino acids was present with 23 nts and 51nts of 5' and 3' UTR respectively. Sequence alignments using Clustal W (Figure 1) indicate that the *H. antarcticus* gene shares greater sequence similarity to the fish wap65-2

isoform (72.2% sequence identity to *O. latipes* wap65-2, but only 49% sequence identity to *O. latipes* wap65-1). No library clones were identified with high sequence similarity to wap65-1. Examination of the *H. antarcticus* gene with regard to hemopexin motifs indicates a similar protein structure with 10 cysteines conserved (out of 12 in mammals) to produce disulphide bridges. This gene also contains 6 (Trp196, Tyr201, Phe208, Tyr222, tyr229 and Phe230) out of the 7 aromatic residues plus Pro 294 that have been defined as important for the structure and stability of the haem pocket and also both histidine (His213 and His266) residues which form the bis-histidyl Fe(III) complex involved in haem axial ligand binding (Paoli et al, 1999). However the *H. antarcticus* gene only contains a single N-glycosylation site and N-glycosylation has been shown to be important in haem binding. Tissue distribution of *H. antarcticus* wap65-2 was examined by RT-PCR over a range of 15 tissues in control animals. There was a very limited distribution with strong expression in the liver and much lower expression in the posterior kidney only (Figure 2). Q-PCR revealed that *H. antarcticus* wap65-2 was not induced in response to an increased environmental temperature of 6ºC. In fact a sigmodal-shape response was produced when the data is plotted as log fold expression change verses time, with considerable initial down regulation (approximately 40 fold) for the first 8 hours followed by re-equilibration to base-line levels around zero (Figure 3). The smallest p value for this dataset is 0.069, which is not significant at the 95% level, but the p values are supportive of the general trend outlined above. In this experiment, variation in gene expression was high due to a limited data set and high inter-individual variation, as would be expected from a wild population study. This wide genetic variation clearly affects significance testing and the resultant p values.

Discussion

The designation of wap65, isoform 2 via sequence similarity analyses was validated by the tissue distribution. Wap65-2 has a much more restricted tissue distribution (mainly liver), compared to wap65-1, which is present in multiple tissues in *O. latipes* and *Takifugu* (Hirayama et al, 2003; 2004). In spite of the presence of the two conserved histidine residues proposed to be essential for haem binding, other fish orthologues of Wap65-2, which also contain these residues, do not show an affinity for haem (Hirayama et al, 2004). There is sufficient conservation of gene sequence between *H. antarcticus* wap65-2 and *O. latipes* wap65-2 to suggest that the two genes are functional orthologues. Although true acclimation experiments for periods of several weeks were not carried out on *H. antarcticus*, the 6ºC time course assay was carried out for a 48 hour period, during which, based on previously published findings, wap65 should have been significantly induced. In contrast, in *H. antarcticus* expression of wap65-2 was down regulated for a period of between 8-12 hours, after which levels returned to those of the control base line. The consequences of which are that the *H. antarcticus* wap65-2 is not involved in temperature acclimation. The initial drop in expression may well be due to a primary "shock" response. This is mirrored in other genes that we have surveyed from the same animals in this time course assay (unpublished data), with the fish subsequently adjusting, at least in terms of gene transcription processes, to the elevated water temperature. If this is subsequently proved to be the situation, then the speed of the initial drop in expression levels and lag to return to a steady state is probably a function of mRNA stability and gene regulation respectively. Several experiments have been successfully carried out to acclimate Antarctic fish to 4°C (Carpenter and Hofmann, 2000; Lowe et al, 2005; Jin et al, 2006). Therefore, although these fish survive in a stable environment of –1.8ºC to +1.0°C almost year-round, they do potentially have the ability to acclimate to higher water temperatures

than they experience in the natural environment. However, this acclimation process is unlikely to involve wap65-2.

This finding adds further data to the complex regulation of wap65 isoforms in fish. This gene set has also been shown to be induced in response to immunological stimulus using LPS and hypoxia in *C. auratus* (Kikuchi et al, 1997; Gracey et al, 2001). However, there is no elevation in the expression of either of the two wap65 isoforms to induction by LPS in *O. latipes* (Hirayama et al, 2004) or to environmental temperature increases in *O. latipes* and *T. rubripes* (Hirayama et al, 2003). Indeed, so far, the correlation of increased expression levels of wap65 with increased environmental temperature have only been identified in the Cypriniformes, specifically the wap65-1 isoform. *Takifugu*, *H. antarcticus* and *O. latipes* belong to the orders: Perciformes and Atheriniformes, so potentially this acclimation function of wap65 is phylogenetically constrained. The biochemical processes, by which Antarctic fish acclimate to warmer sea temperatures remains unknown. Experiments are on-going in our laboratory using cDNA microarrays and long term acclimation experiments to decipher this process.

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Figure Legends

Figure 1
ClustalW alignment of translated wap-65 genes from a number of fish species. Species ID and accession numbers= Tru: *Takifugu rubripes* (Q75UL8, Q75UL9), Ola: *Oryzias latipes* (Q8JIP8, Q8JIP9), Han: *Harpagifer antarcticus* (AM408054), Tni: *Tetraodon nigroviridis* (Q4STQ5), Xhe: *Xiphophorus helleri* (Q2EF31) and Cca: *Cyprinus carpio* (Q90WF7). Annotation above the sequence: hemopexin-like repeats are shown by lines, conserved cysteine residues are denoted by a "*", N-glycosylation site by a line ended with diamonds, residues important for the structure and stability of the haem pocket are denoted with a "+" and the two histidine residues which form the bis-histidyl Fe(III) complex involved in haem axial ligand binding are indicated by a "#".

Figure 2
RT- defined tissue distribution of wap65-2 in *H. antarcticus* control (non-treated) animals. Actin RT-PCR was used as a quantification control for the different tissues.

Figure 3
A: Q-PCR results using liver tissue for *H. antarcticus* wap65-2 gene over a 48 hour time course series with a 6ºC temperature heat shock.
B: Graphical representation of log expression fold changes (with error bars) in the wap65-2 gene in *H. antarcticus*.

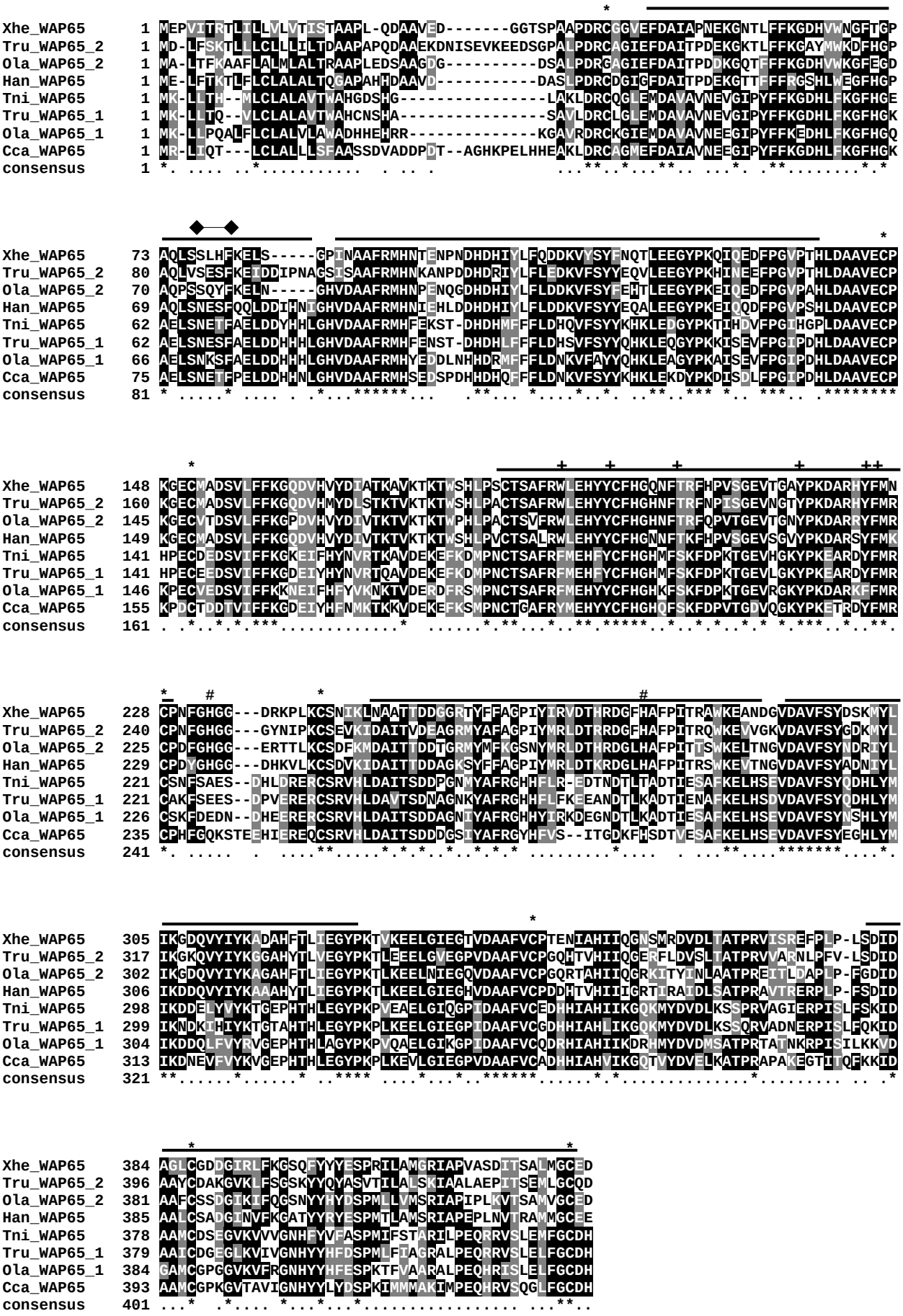
Xhe_WAP65
Tru_WAP65_2
Ola_WAP65_2
Han_WAP65
Tni_WAP65
Tru_WAP65_1
Ola_WAP65_1
Cca_WAP65
consensus

Figure 1

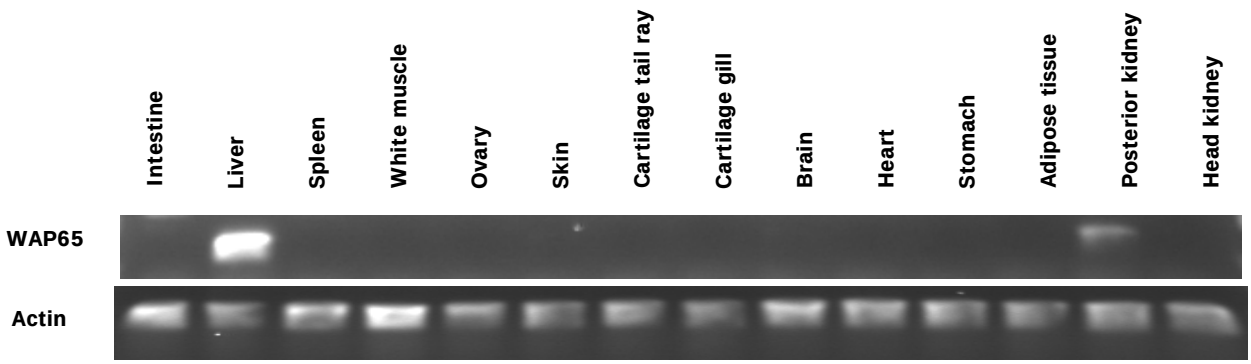
Intestine
Liver
Spleen
White muscle
Ovary
Skin
Cartilage tail ray
Cartilage gill
Brain
Heart
Stomach
Adipose tissue
Posterior kidney
Head kidney
WAP65
Actin

Figure 2

A

Time (hours)	REST P-value	Fold change	Range	Gene Regulation
2	0.277	0.046	0.017-0.122	-21.64 down regulated
4	0.243	0.025	0.006-0.106	-39.33 down regulated
8	0.069	0.045	0.010-0.144	-22.20 down regulated
12	0.996	0.690	0.310-1.530	-1.43 down regulated
24	0.768	0.460	0.143-1.530	-2.13 down regulated
48	0.695	0.670	0.020-1.970	-1.49 down regulated

B

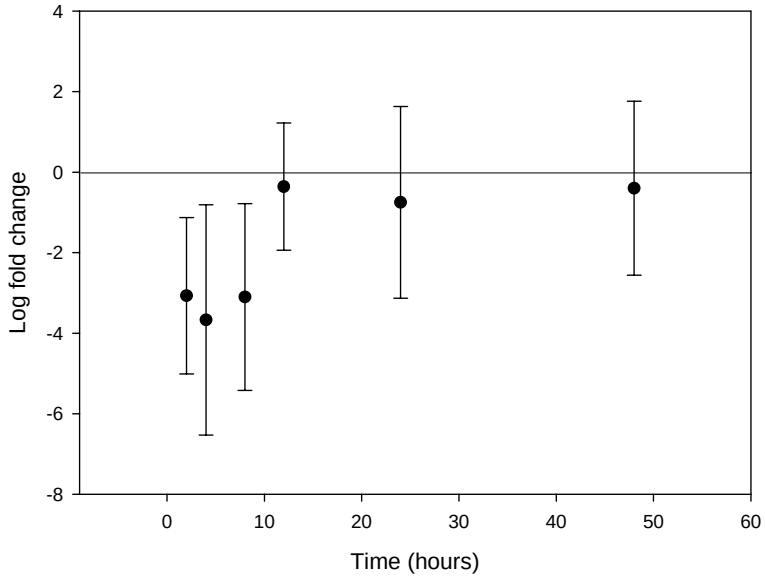

Figure 3