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Migration-related changes in gene expression in leg muscle of the Christmas Island red crab *Gecarcoidea natalis*: seasonal preparation for long-distance walking

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SUMMARY

During their annual breeding migration the Christmas Island land crab *Gecarcoidea natalis* sustains locomotion aerobically for up to 12 h per day compared with just 10 min during the dry season when their muscles quickly become anaerobic. A seasonal transition to an endurance-muscle phenotype would thus seem essential for migrating crabs. The current study employed a gene discovery approach comparing two expressed sequence tag (EST) libraries, one each for leg muscle from dry (non-migrating) and wet season (migrating) crabs. The 14 most abundant transcripts differed in their representation between the two libraries. The abundances of transcripts of genes predicted to code for different proteins forming contractile muscle components, including actin, troponin and tropomyosin, were significantly different between seasons and thus between physiological states. The shift in the isoform composition of the contractile elements provided evidence for a switch from slow phasic (S1) to slow tonic (S2) fatigue-resistant muscle fibres. A tropomyosin (*tm*) transcript aligned with a *tm* isoform of lobster (*tmS2*), and semi-quantitative RT-PCR confirmed this isoform to be more abundant in the migrating crab muscle. Two LIM protein coding genes, a paxillin-like transcript (*pax*) and a muscle LIM protein (*mlp*), were relatively up-regulated in muscle of wet season crabs. These proteins have a fundamental role in muscle development and reconstruction, and their comparative up-regulation is consistent with a remodelling of leg muscle for migration in the wet season. Such a transition would result in an increased representation of aerobic endurance-type fibres concomitant with the greater aerobic exercise capacity of the migrating red crabs.

Key words: land crab, migration, muscle, EST, tropomyosin, LIM protein.

INTRODUCTION

The red land crab *Gecarcoidea natalis* (Pocock 1888) is endemic to Christmas Island, Indian Ocean, where it inhabits the rainforest, often more than 200 m above and 5 km distant from the ocean (e.g. Green, 1997; Adamczewska and Morris, 2001a). In common with other gecarcinid crabs, *G. natalis* undertakes an annual breeding migration but this is a significantly longer trek to the ocean for this crab than for most other gecarcinids. Consequently, *G. natalis* exhibits seasonally dichotomous behaviour and during the dry season these crabs are mostly quiescent within their burrows to avoid desiccation (Green, 1993; Green, 1997). However, within hours of the first monsoonal rain at the start of the wet season the crabs may walk over 1 km day^{-1} on their migration to the coast (e.g. Adamczewska and Morris, 2001a). Furthermore, since these breeding activities are synchronised with the lunar cycle the migrating red crabs must in some years sustain locomotion for up to 12 h day^{-1} during 5–6 consecutive days (Hicks, 1985; Green, 1997; Adamczewska and Morris, 2001a). This apparently instantaneous switch from hypoactivity to hyperactivity must be supported by extensive preparative changes in the physiology of the crab.

During the dry season *G. natalis* crabs are unable to sustain even moderate exercise without recourse to supplemental anaerobiosis (Adamczewska and Morris, 1994a; Adamczewska and Morris, 1994b; Adamczewska and Morris, 2000; Adamczewska and Morris, 2001b; Morris and Adamczewska, 2002). In contrast, the crabs migrating to the ocean showed no reliance on anaerobiosis and no evidence of metabolic acidosis (Adamczewska and Morris, 2001a; Adamczewska and Morris, 2001b). Generally, there is limited information on the mechanisms of, and limitations on, sustained exercise in migratory crabs, most especially in the field. Clearly, the spectacular annual migration of *G. natalis* must be facilitated by the adoption of a phenotype appropriate to long-distance walking.

The oxidative capacity and fatigue resistance of muscle depends on the highly variable metabolic profile and isoform composition of the fibre proteins (for reviews, see Mellon, 1992; Schiffino and Reggiani, 1996; Clark et al., 2002). Sets of myofibrillar proteins can be selectively activated and contractile characteristics of muscle fibre types, including contractility, contraction speed, tension and endurance, are defined by these respective isoform compositions (e.g. Schiffino and Reggiani, 1996). In crustaceans, myofibrillar isoforms allow at least three fibre types to be distinguished; fast, slow phasic (S1) and slow tonic (S2) (Mykles, 1985a; Mykles, 1985b; Silverman et al., 1987; Mykles, 1988; Medler and Mykles, 2003; Medler et al., 2004). These fibre types tend to exhibit a specific suite of muscle protein isoforms, including different myosin heavy chains and isoforms of tropomyosin, troponin T (TnT), troponin I (TnI) and the fast fibre protein P75 (Mykles, 1985a; Mykles, 1985b; Mykles, 1988; Mykles, 1997; Medler and Mykles, 2003; Medler et al., 2004; Perry et al., 2009).

Slow aerobic fibres exhibit a higher endurance suitable for long-distance walking whereas faster anaerobic fibres are useful for sprinting (e.g. Mykles, 1988; Perry et al., 2009). Crayfish have two types of fast fibres that exhibit differences in sarcomere width, TnI isoform composition and activation properties (Koenders et al., 2004), and differences in isoform expression have been matched to functional properties of slow and fast muscle fibres in ghost crabs (Perry et al., 2009). Crustacean muscle fibres can show a high degree of polymorphism (e.g. Medler and Mykles, 2003) such that the

muscles can be a mixture of several different fibre types (e.g. Mellon, 1992; Boyle et al., 2003; Kinsey et al., 2005) as well as mixed phenotype fibres (e.g. Govind et al., 1987; Govind, 1992; Medler and Mykles, 2003; Medler et al., 2004; Perry et al., 2009). Muscle fibre phenotypes can respond to altered functional demands (for a review, see Pette and Staron, 2001), which may be important for the putative seasonal changes in *G. natalis* muscle physiology. Crustacean muscles show a phenotypic plasticity which can persist throughout the life of the animal (e.g. Kent and Govind, 1981; Govind et al., 1987; Mykles, 1997). The underlying mechanisms for this plasticity are not fully understood (Gruhn and Rathmayer, 2002). In mammals, chronic low frequency stimulation (CLFS) induces changes in myosin expression and expression of troponin, tropomyosin, α-actinin and proteins of the sarcoplasmic reticulum such that fast isoforms are exchanged with slower isoforms (for reviews, see Pette and Vrbová, 1992; Pette and Vrbová, 1999; Pette and Staron, 2001). These suites of transitions are considered by Pette and Staron to be consequent on 'orchestrated changes in gene expression' (Pette and Staron, 2001).

Less is known about the plasticity of crustacean muscles and alterations in their properties (Govind and Kent, 1982; Govind et al., 1987; Mykles, 1997; Boyle et al., 2003; Johnson et al., 2004; Kinsey et al., 2005). It is apparent that CLFS can have similar consequences in decapod muscle (Gruhn and Rathmayer, 2002), and in crayfish CLFS transformed phasic muscle fibres to a tonic-like state by altering isoforms of paramyosin, troponin-T, troponin-I and two unidentified proteins (Cooper et al., 1998). Thus, fibre transformation in response to seasonal demand is at least a strong possibility.

In the red crab *G. natalis* the ecological requirement for sustained locomotion is accompanied by increases in walking capacity. A seasonal transition to an endurance muscle phenotype would seem important for crabs about to migrate. Accordingly, the present study investigated putative migration-related changes in the leg muscles of *G. natalis* at the level of gene transcription, by comparing two expressed sequence tag (EST) libraries, one each for dry season (non-migrating) and wet season (migrating) crabs. The difference in representation of ESTs between the libraries prepared from these two sources was used as a measure of the relative transcription of these genes. The overall aims of this study were 2-fold: (i) to discover genes from *G. natalis* and (ii) to quantify changes in the expression of these genes between crabs from distinctly different seasons. Emphasis was given to relating differences in gene expression to derived physiological function and thereby the likely importance of such changes in supporting endurance exercise of migrating red crabs at the onset of the wet season.

MATERIALS AND METHODS

Muscle tissue collection

Adult inter-moult male red crabs, *G. natalis*, were collected on Christmas Island: (a) during their annual breeding migration in December, 2003 (243±10 g, mean ± s.e.m., *N*=10) and (b) in the dry season in July, 2004 (251±15 g, *N*=14). Migrating crabs were collected immediately adjacent to the Parks Australia Research Station (10°29′32″S: 105°38′45″E) while in the dry season crabs were collected from the rainforest (10°30′55″S: 105°38′50″E). Crabs were encouraged to autotomise the penultimate walking legs and muscle tissue from the merus was divided into four sub-samples (each ≤150 mg) for storage in 1.8 ml of RNA-later® in each case (Qiagen GmbH, Hilden, Germany). Samples in RNA-later® were held overnight at 4°C before storage at –20°C and onward airfreight (DEWHA, Australia export and Defra UK import licences) to the UK in dry-ice.

RNA isolation

The muscle tissue samples were blotted free of RNA-later® and portions of 314±6 mg were homogenised using a TM50 homogeniser (OMNI International, Marietta, GA, USA) in 2 ml of RNeasy Lysis Buffer® (Qiagen), to which was then added 2.4 ml TRI Reagent (Sigma-Aldrich Co., Gillingham, Dorset, UK). The RNA was isolated following the manufacturer's protocol with the exception that chloroform and isopropanol were used at volumes of 0.33 ml and 1.0 ml per 1 ml of TRI Reagent. Total RNA from separate isolations from batches of leg muscles of either migrating (*N*=10) or non-migrating (dry season) animals (*N*=14) was pooled to provide two samples. Poly(A^+) RNA was purified using superparamagnetic oligo($dT)_{25}$ polystyrene beads (Dynabeads mRNA purification kit, Dynal Biotech ASA, Oslo, Norway).

cDNA synthesis and library construction and storage

The two cDNA libraries, one corresponding to migrating, wet season crabs (LibW, wet season library) and the other to non-migrating, dry season crabs (LibD, dry season library), were constructed using a pBluescript II XR cDNA library construction kit (Stratagene, La Jolla, CA, USA). Size separation of cDNA was performed by SizeSep 400 spin columns (Amersham Biosciences Corp., Piscataway, NJ, USA). The resulting libraries for 'W' and 'D' crabs were amplified to provide secondary libraries, LibW providing 4.9×10^{10} and LibD with 2.8×10^{11} colony forming units, respectively. Cells containing cloned inserts were identified by standard blue/white screening and were individually selected at random and grown, and preserved as glycerol stocks.

Template preparation, sequencing and data analysis, and handling for the W and D libraries

Insert spanning PCR from T3 and T7 flanking promoter sequences was performed on each glycerol stock in a 96 well format using HotStartTaq DNA Polymerase (Qiagen) with subsequent treatment with shrimp alkaline phosphatase (USB Corporation, Cleveland, OH, USA) and Exonuclease I (USB Corporation) at 37°C (45 min) followed by an enzyme denaturation step at 80°C (15 min). Sequencing was performed *via* capillary electrophoresis (MegaBACE™ 1000, Amersham) using the DYEnamic™ ET Dye Terminator Kit (Amersham) as described previously (Wilson et al., 2004). A quality value was assigned to each base using the phred Q20 algorithm (Ewing et al., 1998) and the high quality base calls were screened for contaminants from either the vector or *E. coli*. Processing and analysis of EST data including clean up, BLAST searching and clustering were performed as described previously (Wilson et al., 2004). This methodology included web-based data mining tools for electronic northern and digital differential displays (these tools are available on request). From the 4208 sequencing attempts, 2118 (50.3%) passed the clean-up filter process and these EST files were prepared for submission to GenBank (http://www.ncbi.nlm.nih.gov/dbEST/). The complete cap3 cluster assembly (Huang and Madan, 1999) is available at http://bio-sanger.bio.bris.ac.uk/crab.htm.

The ESTs were grouped into contigs, in which each contig putatively represents a different gene, isoform or splice variant, as generated using Sequencher (Gene Codes Corporation, Ann Arbor, MI, USA) using the plurality mode. The generated sequences were subsequently used in an NCBI BLASTX query against SwissProt (Table 1; August 2005) and the presence of a sequence within the database was recorded if BLASTX alignment produced a candidate with an E-value <1e–05. All consensus contig sequences (hereafter referred to as contig sequences) that remained unaligned were

Table 1. List of contigs (ranked by number of included ESTs) comprising ≥5 ESTs aligned with the most significant alignment of their contig sequence during BLASTX analysis against GenBank and SwissProt followed by their respective library abundance

Contig[a]	Most significant alignment[b]	Species	GenBank[c]	SwissProt[d]	E[e]	Identity[f]	LibW[g]	LibD[h]
72	Actin-3, muscle specific	*Bactrocera dorsalis*	gi_1168323	sp_P45886	0.0	92%	86 (8.2)	201 (18.9)
105	Arginine kinase (AK)	*Callinectes sapidus*	gi_25453074	sp_Q9NH49	0.0	97%	77 (7.3)	99 (9.3)
131	Muscle LIM protein Mlp84B	*Drosophila melanogaster*	gi_2497676	sp_Q24400	$2e^{-22}$	71%	117 (11.1)	49 (4.6)
52	Troponin I	*Pontastacus leptodactylus*	gi_136223	sp_P05547	$9e^{-49}$	80%	46 (4.4)	37 (3.5)
122	Troponin I	*Pontastacus leptodactylus*	gi_136223	sp_P05547	$9e^{-60}$	86%	33 (3.1)	48 (4.5)
38	Tropomyosin	*Homarus americanus*	gi_14285796	sp_O44119	$1e^{-36}$	93%	54 (5.1)	17 (1.6)
68	Troponin I	*Pontastacus leptodactylus*	gi_136223	sp_P05547	$4e^{-46}$	85%	20 (1.9)	48 (4.5)
32	Tropomyosin	*Homarus americanus*	gi_14285796	sp_O44119	$4e^{-123}$	96%	33 (3.1)	24 (2.3)
97	16S rRNA			No protein			10 (1.0)	25 (2.3)
39	Actin	*Procambarus clarkii*	gi_1168338	sp_P45521	$5e^{-39}$	95%	8 (0.8)	23 (2.2)
27	Alignment only with EST: c4h08up.r1*,†	*Celuca pugilator*	gi_151349207	gb_EV249429.1*	0.0	91%	25 (2.4)	5 (0.5)
59	Actin	*Procambarus clarkii*	gi_1168338	sp_P45521	$8e^{-57}$	93%	19 (1.8)	5 (0.5)
35	Alignment only with EST: c2f07up.f1*,†	*Celuca pugilator*	gi_151348529	gb_EV248753.1*	0.0	90%	15 (1.4)	7 (0.7)
127	Sarcoplasmic calcium-binding protein I	*Pontastacus leptodactylus*	gi_134309	sp_P05946	$1e^{-68}$	69%	4 (0.4)	15 (1.4)
66	Alignment only with EST: c2h19up.r1*,†	*Celluca pugilator*	gi_151348584	gb_EV248808.1*	$2e^{-130}$	94%	7 (0.7)	12 (1.1)
62	Alignment only with EST CS–hyp_66h09*,†	*Celluca pugilator*	gi_151348832	gb_EV249054.1*	$4e^{-111}$	80%	6 (0.6)	11 (1.0)
117	Myosin regulatory light chain 2	*Drosophila melanogaster*	gi_127183	sp_P18432	$1e^{-34}$	57%	8 (0.8)	7 (0.7)
87	Troponin C, isotype gamma	*Pontastactus leptodactylus*	gi_136032	sp_P06708	$2e^{-53}$	76%	9 (0.9)	4 (0.4)
33	Myosin regulatory light chain 2	*Drosophila melanogaster*	gi_127183	sp_P18432	$3e^{-39}$	55%	8 (0.8)	3 (0.3)
63	Cytochrome C oxidase subunit 3	*Locusta migratoria*	gi_117063	sp_P14574	$1e^{-72}$	71%	4 (0.4)	7 (0.7)
58	Muscle-specific protein 20	*Drosophila melanogaster*	gi_22096358	sp_P14318	$3e^{-46}$	50%	5 (0.5)	5 (0.5)
65	60S ribosomal protein L44	*Ochlerotatus triseriatus*	gi_31340370	sp_Q9NB33	$1e^{-39}$	77%	0 (0)	10 (1.0)
18	Glyceraldehyde-3-phosphate dehydrogenase	*Palinurus versicolor*	gi_6016083	sp_P56649	$3e^{-86}$	93%	6 (0.6)	4 (0.4)
129	Myosin light chain alkali	*Drosophila virilis*	gi_2498027	sp_Q24756	$8e^{-25}$	60%	5 (0.5)	4 (0.4)
26	Actin	*Procambarus clarkii*	gi_1168338	sp_P45521	$8e^{-55}$	91%	5(0.5)	4 (0.4)
75	Actin-3, muscle specific	*Bactrocera dorsalis*	gi_1168323	sp_P45886	$5e^{-73}$	94%	2 (0.2)	7 (0.7)
140	Paxillin	*Mus musculus*	gi_81902126	sp_Q8VI36	$9e^{-57}$	71%	8 (0.8)	0 (0)
102	Cytochrome C oxidase subunit 1	*Drosophila yakuba*	gi_116976	sp_P00400	$5e^{-58}$	65%	2 (0.2)	6 (0.6)
41	Alignment only with EST: Cm_mx1_85d01*	*Carcinus maenas*	gi_89501019	gb_DY656815.1*	$5e^{-10}$	90%	2 (0.2)	6 (0.6)
120	Alignment only with EST: c4i17up.r1*,†	*Celuca pugilator*	gi_151349238	gb_EV249460.1*	$4e^{-10}$	70%	4 (0.4)	4 (0.4)
54	Actin	*Procambarus clarkii*	gi_1168338	sp_P45521	$6e^{-27}$	97%	4 (0.4)	3 (0.3)
50	Ubiquitin	*Homo sapiens*	gi_51703339	sp_P62988	$8e^{-37}$	100%	1 (0.1)	6 (0.6)
93	Alignment only with EST: c4i17up.f1*,†	*Celuca pugilator*	gi_151349237	gb_EV249459.1*	$6e^{-59}$	78%	4 (0.4)	3 (0.4)
28	Alignment only with EST: a3m04up.r1*,†	*Celuca pugilator*	gi_84041136	gb_DW175845*	$2e^{-9}$	66%	2 (0.2)	4 (0.4)
45	Ribosomal protein L24	*Plutella xylostella*	gi_52783249	sp_Q6F444	$5e^{-36}$		5 (0.5)	0 (0)
76	60S acidic ribosomal protein P1(EL12′/EL2′-P)	*Artemia salina*	gi_133047	sp_P02402	$2e^{-22}$	73%	3 (0.3)	2 (0.2)
77	40S ribosomal protein S15 (RIG protein)	*Gallus gallus*	gi_51338644	sp_P62846	$3e^{-46}$	85%	0 (0)	5 (0.5)
82	40S ribosomal protein S2	*Urechis caupo*	gi_1350976	sp_P49154	$4e^{-108}$	89%	0 (0)	5 (0.5)
49	Glutamine synthetase	*Panulirus argus*	gi_417059	sp_Q04831	$6e^{-29}$	89%	4 (0.4)	1 (0.1)
104	Cytochrome b	*Drosophila mauritiana*	gi_47115567	sp_Q9MGM4	$6e^{-41}$	86%	1 (0.1)	4 (0.4)
119	Ferritin	*Pacifastacus leniusculus*	gi_26006755	sp_Q26061	$2e^{-29}$	77%	0 (0)	5 (0.5)
42	Alignment only with EST: CS_hyp_40f05*	*Callinectes sapidus*	gi_53704408	gb_CV479632.1*	$1e^{-10}$	87%	3 (0.3)	2 (0.2)

The total number of ESTs in any contig is the sum of the values shown for LibW and LibD. Values in parentheses are % of total EST in each of the respective libraries.

[a]Contig number; [b]most significant alignment during BLASTX search against SwissProt (August 2005); [c]GenBank sequence identification number; [d]SwissProt accession number; [e]E-value of the alignment; [f]identity of the alignment; [g]number of ESTs present in the LibW library constructed from leg muscle of migrating crabs; [h]number of ESTs present in the LibD library constructed from leg muscle of crabs collected during dry season.

*No SwissProt identity – EST BLAST.

†Recent BLAST alignment May 2009.

inserted into an NCBI BLASTX search against the NR database (NCBI non-redundant GenBank CDS) and into NCBI BLASTN searches against the NR database and against the EST database (May 2009), and significant alignments (E-value of <1e–05) are included in Table 1. Nucleotide contig sequences were translated into protein sequences with the ExPASy Translate tool (http://us.expasy.org/tools/dna.html). Protein sequence comparisons were generated using the LALIGN program (http://www.ch.embnet.org/software/LALIGN_form.html).

To determine whether there were significant differences in the representation of contigs between the W (LibW) and D (LibD) libraries, EST representation between these two libraries was compared with the null hypothesis of an equal distribution using Fisher's exact test (Siegel, 1956; Agresti, 1996). These analyses were performed for all contigs that contained seven or more ESTs and with the critical *P*-value appropriately reduced through the application of a Bonferroni correction for multiple tests.

Semi-quantitative assessment of selected transcripts – RT-PCR

The transcript abundance of five putative genes, selected on the basis of apparent differential expression in the LibW and LibD libraries, was further compared in the muscle tissue of *G. natalis* crabs. These transcripts were selected based on their perceived importance to muscle restructuring between seasons; contig 131 (*mlp*), contig 38 (*tmS2*), contig 32 (*tmS1*), contig 140 (*pax*) and contig 105 (*ak*). This required the quantification of the specific PCR products and hereafter these will be referred to by the identity of the most likely gene assignment.

For this targeted amplification, pooled total RNA from migrating and non-migrating crabs was prepared as described above, and quantified both by Nanodrop 1000 (Labtech International, Ringmer, East Sussex, UK) and Agilent 2001 bioanalyser (Agilent Technology, Palo Alto, CA, USA). Replicate equal quantities of RNA from wet and dry season crabs were used to generate cDNA using the QuantiTect Reverse Transcriptase Kit (Qiagen), which incorporates a genomic DNA removal process. Two forward (F1 and F2) and two backward (R1 and R2) primers were constructed for each transcript (Table 2) based on the respective nucleotide sequences. This nested primer approach allowed confirmation of primer specificity for four of the five sequences of interest but required a modified approach for *tmS2*. Consequently, to determine the exponential phase of amplification, fragments of each of these genes were amplified from cDNA transcripts using the primer combination F1/R1 for *mlp* (213 bp), *pax* (178 bp) and *ak* (206 bp) but F2/R2 for *tmS1* (192 bp) and *tmS2* (246 bp). The different PCR reactions were sampled every three cycles for cycles 14–35. Subsequently, the PCR products were resolved on 1.2% (w/v) agarose gels containing ethidium bromide and quantified by comparison with a DNA marker (ABgene, Epsom, UK). Replicate PCRs were completed with each of four total RNA samples from migrating and non-migrating *G. natalis*, using the five different primer sets in reactions performed in parallel (26 cycles for *mlp*, *tmS1*, *tmS2* and *ak*, and 32 cycles for *pax*). The concentrations of PCR products were compared using *t*-test or Mann–Whitney rank sum test where appropriate (SigmaStat, Jandel Corp., San Rafael, CA, USA) and plotted as product *vs* cycle number, as previously (Crook et al., 2005).

Since statistical analyses of this comparison revealed no differences for *ak*, the values obtained for *mlp*, *tmS1*, *tmS2* and *pax* were also normalised against the *ak* values gained from the respective sample during parallel PCR. The comparison of the values gained for migrating and non migrating crabs was analysed as described above with $P<0.05$ taken as significant. All means are reported with their standard error.

Table 2. *Gecarcoidea natalis* nucleotide sequences (5′–3′) of specific primers used for conventional and semi-quantitative RT-PCR amplification of specific transcripts of interest

Target gene	Primer name	Primer sequence (5′–3′)
Muscle LIM protein	*mlp* F1	CTCCTTCCACAAGGAATGCT
	mlp F2	ACTTGCACGAGAATGTGCTG
	mlp R1	GAGGCGTAGGAGAGCGATTA
	mlp R2	CGCTTTGAGTTCATGACTGG
Tropomyosin S1	*tmS1* F1	CTCCAGAAGGAGGTCGACAG
	tmS1 F2	TTCAGCGAACTGTCTGGCTA
	tmS1 R1	CCAGCTGGGCATAAGAAGAG
	tmS1 R2	CGAATGATTTGGTGATGTCG
Tropomyosin S2	*tmS2* F1	GCTGAGTTCGCTGAAAGGTC
	tmS2 F2	CTGCGGACTGACTTGACTGA
	tmS2 R1	CCAGCTGTTTGCCACACTTA
	tmS2 R2	CATCTAGACGAGGGCCAAAC
Paxillin	*pax* F1	AACCCACCAAGAGTCCTTCC
	pax F2	GGCGAACCAGCTTGATTCTA
	pax R1	GTAACGCCCTGCTCAGTCAT
	pax R2	GCCGTCACGTTCAAAGAAGT
Arginine kinase	*ak* F1	ATGGCAAGTTCGTGATCTCC
	ak F2	GCCCAGTACAAGGAGATGGA
	ak R1	ATCGTCGATCAGCTTCTGCT
	ak R2	ACCACACCAGGAAGGTCTTG

Designed using Primer3 (http://frodo.wi.mit.edu/primer3).

RESULTS

Library and contig analysis

A total of 2118 ESTs with an average sequence read length of 282 bp were obtained, 1052 (49.7%) from the LibW and 1066 (50.3%) from the LibD library. Overall, 1100 of the individual ESTs (51.9%) had significant individual BLAST alignments. The contig analysis grouped 1666 ESTs into 157 contigs consisting of two or more ESTs; 813 and 853 ESTs from LibW and LibD, respectively. The remaining 452 ESTs were singleton contigs, i.e. single EST contigs. Therefore, the 2118 ESTs represented 609 contigs, which are putative genes or isoforms. All contigs containing five or more ESTs are listed in Table 1 where their most significant BLAST alignment is shown. The BLASTX analysis failed to allocate 426 gene transcripts (Fig. 1). Singleton contigs were less likely than larger contigs to have a significant BLASTX alignment.

Transcription profiles

The abundance of ESTs representing any single gene is at least in part related to the level of gene transcription, and thus contigs with large numbers of ESTs are likely to be genes whose transcription was greatest in the sampled tissues (Table 1). The largest contig by far was contig 72 (287 ESTs; Table 1) which was composed of ESTs with high similarity to actin genes. The seven next largest contigs (>50 ESTs each) were allocated to further genes for muscle function-related proteins; i.e. arginine kinase (contig 105; 176 ESTs), muscle LIM protein (contig 131; 166 ESTs), troponin I (contigs 52, 122 and 68 with 83, 81 and 68 ESTs, respectively), and tropomyosin (contigs 38, 32 with 71 and 57 ESTs, respectively). In total, 1198 ESTs (56.6% of total ESTs) could be assigned to genes for muscle function-related proteins, as would be expected given the tissue from which the RNA was isolated, either directly or following their identification by assignment to a contig (Fig. 1).

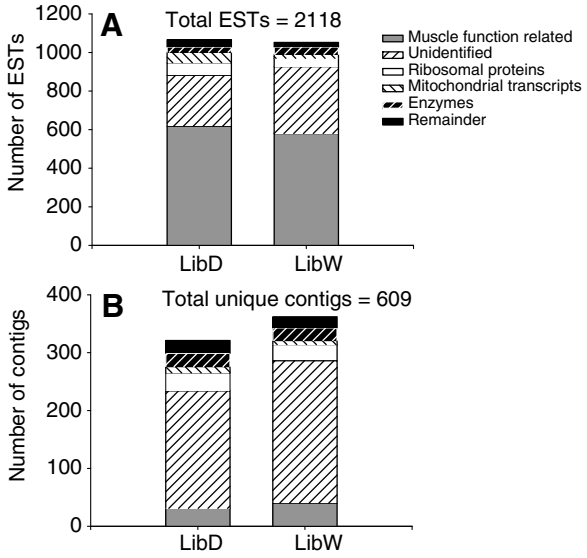

Fig. 1. (A) Functional distribution of expressed sequence tags (ESTs) within and between the two cDNA libraries (LibD and LibW); and (B) comparison of the functional distribution of distinct transcripts distinguished by contig analyses. The cDNA library LibW was constructed from leg muscle of migrating (wet season) *Gecarcoidea natalis* and library LibD from leg muscle of non-migrating *G. natalis* in the dry season.

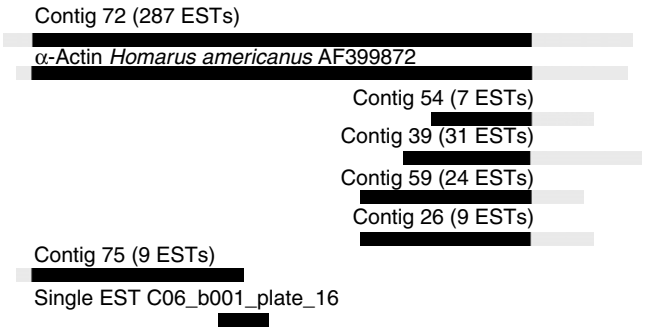

Fig. 2. The relative alignment of the α-actin sequence of *Homarus americanus* (AF399872) with the seven actin sequences (post-contig analysis) from the cDNA libraries of *G. natalis* (contigs 72, 54, 39, 59, 26 and 75 and one singleton; below). The black bars mark the area of the predicted open reading frame (ORF); the grey bars represent the 5′-untranslated region (UTR; left) and 3′UTR (right). Note this figure does not represent sequence identity.

The next highest represented group of contigs, as grouped by general function of the probable expressed protein, were ribosomal protein-coding genes (5.1%), followed by those aligned to genes of mitochondria (3.6%), and those with high similarity to genes coding for enzymes (3.1%; Fig. 1). The remaining 125 identified ESTs (5.9% of all ESTs) aligned most significantly with a range of genes including those coding for structural proteins (e.g. tubulins and histones) and for regulatory proteins (e.g. elongation factors, ubiquitin and Hsp89) (Fig. 1). Of the 2118 ESTs, 611 (28.8%) could not be positively identified (Fig. 1) and may represent genes novel to this species.

Comparison of the abundance of transcripts within the two libraries

The number of ESTs belonging to each contig in the LibW (1052 ESTs) and LibD libraries (1066 ESTs) was compared (Fisher's exact test), and this showed that ESTs of six contigs had a highly significant difference in abundance between the libraries (Table 3). Three of these, contigs 72, 68 and 65, were more abundant in LibD while the other three, contigs 131, 38 and 27, were more abundant in LibW. A further eight contigs revealed at least some differences in abundance between the libraries (Table 3), including contig 140, showing putative differential expression of paxillin (Table 3). Data for some of the transcripts (post-BLASTX analysis) whose differential expression was significant and which were of putative importance to muscle restructuring were considered in more detail.

Actin

There were seven contigs with significant similarity to actin. The most significant alignment of contig 72 differed depending on whether it was searched against Swiss Prot or the NCBI NR database: for the former the most significant alignment was to Actin-3 of *Bactrocera dorsalis* (Table 1); for the latter the most significant alignments were to skeletal muscle actin 2 (Ha-ActinSK2; ACI23565.1) and α-actin (Ha-ActinSK1; AF399872) of *Homarus americanus*. The deduced amino acid sequences of the remaining five contigs (Table 1), and one singleton contig with significant alignment to actin, also had a greater similarity to at least one of the Ha-ActinSK1 and/or Ha-Actin-SK2 isoforms compared with the 10 other isoforms from *H. americanus*. Importantly, while the sequence of contig 72 spanned the entire length of the *H. americanus* cDNA of α-actin, the additional six *G. natalis* contigs aligned with only sections of the *H. americanus* sequence (Fig. 2). ESTs from contig 72 were considerably more frequent in the muscle tissue of non-migrating crabs (201 in LibD) than in that of migrating crabs (86 in LibW). Of the six additional contigs coding for actin-like genes, the EST distribution ratio for contig 59 was LibW/LibD=4 and that for contig 39 was LibW/LibD=0.34 (Table 3). The sequences of contigs 54, 39, 59 and 26 aligned with the 3′-region of the *H. americanus* sequence (Fig. 2) while fewer than 19% of the ESTs of contig 72 aligned with this same 3′-region. The deduced amino acid sequence of this 3′-region of contig 72 is identical to that of the C-terminus of α-actin of *H. americanus*. The consensus sequences of contig 59, 54 and 26 differed from that of contig 72 within the predicted open reading frame (ORF) in a few nucleotides, but this only alters the amino acid sequence for contig 26. The sequence of contig 39 differed from that of contig 72 in the 3′-untranslated region (UTR). Within contig 72, ~81% of the ESTs were concentrated in the 5′-region of the *H. americanus* actin sequence, a section to which the contig sequence of contig 75 and a singleton contig (C06_b001_plate_16) also aligned.

Muscle LIM protein

The deduced protein sequence for contig 131 showed a significant BLAST alignment to muscle LIM proteins (MLP) of *Drosophila* (Table 1, Fig. 3), which belong within the family of cysteine-rich proteins (CRPs). The ESTs of this contig 131 were more than twice as abundant in LibW (migrating) than in LibD (non-migrating) (Tables 1 and 3).

The ORF of the *Drosophila* MLP-coding gene lay towards the 5′-region of the contig 131 sequence. Consistent with highly similar described LIM proteins, the initiation codon of the ORF is postulated to be the ATG at nucleotide position 102–104. Thus, the predicted open reading frame has 282 nucleotides corresponding to a 93 amino acid protein (Fig. 3). The deduced protein product of contig 131 is

Table 3. Contigs that exhibited a significant difference in abundance between LibW and LibD either after Bonferroni correction (bold) or only prior to such correction (Fisher's exact test)

Contig[a]	BLASTX search of all SwissProt entries[†]: most significant alignment[b]	Library representation		*P*-value[e]	Corrected *P*-value[f]
		LibW[c]	LibD[d]		
72	Actin-3, muscle specific, *Bactrocera dorsalis*[†]	86	201*	$6.05e^{-13}$	$1.82e^{-11}$
131	Muscle LIM protein Mlp84B, *Drosophila melanogaster*	117*	49	$1.39e^{-9}$	$4.17e^{-8}$
38	Tropomyosin *Homarus americanus*	54*	17	$1.13e^{-6}$	$3.39e^{-5}$
27	No significant alignment to SwissProt (EST alignment in Table 1)	25*	5	$7.21e^{-5}$	$2.16e^{-3}$
68	Troponin I, *Pontastacus leptodactylus*	20	48*	$4.39e^{-4}$	$1.32e^{-2}$
65	60S ribosomal protein L44, *Ochlerotatus triseriatus*	0	10*	$1.21e^{-3}$	$3.63e^{-2}$
59	Actin, *Procambarus clarkii*	19*	5	$1.70e^{-3}$	n.s.
140	Paxillin *Mus musculus*	8*	0	$3.16e^{-3}$	n.s.
39	Actin, *Procambarus clarkii*	8	23*	$4.95e^{-3}$	n.s.
97	16S rRNA	10	25*	$7.21e^{-3}$	n.s.
127	SCP I; Sarcoplasmic calcium–binding protein I *Pontastacus leptodactylus*	4	15*	$9.31e^{-3}$	n.s.
105	Arginine kinase (AK), *Callinectes sapidus*	77	99*	$2.34e^{-3}$	n.s.
122	Troponin I, *Pontastacus leptodactylus*	33	48*	$3.03e^{-2}$	n.s.
35	No significant alignment to SwissProt (EST alignment in Table 1)	15*	7	$3.28e^{-2}$	n.s.
32	Tropomyosin *Homarus americanus*	33*	24	$4.07e^{-2}$	n.s.
52	Troponin I, *Pontastacus leptodactylus*	46*	37	$4.18e^{-2}$	n.s.

The total number of ESTs in any contig is the sum of the values shown for LibW and LibD.
[a]Contig number; [b]most significant alignment during BLASTX search against SwissProt; [c]number of ESTs in the LibW library (migrating crabs); [d]number of ESTs in the LibD library (non-migrating crabs); [e]*P*-value of the Fisher's exact test; [f]*P*-value after Bonferroni correction.
*Significant difference in abundance between LibW and LibD.
[†]SwissProt identity (see text for BLASTX search against the NCBI non-redundant GenBank CDS).

composed of a single LIM domain which, similar to other CRPs of vertebrates, exhibits the sequence C-X_2-C-X_{17}-H-X_2-C-X_2-C-X_2-C-X_{17}-C-X_2-C (Fig. 3; key amino acids underlined) and is linked to a glycine-rich region. Examining the abundance of only those ESTs in contig 131 which lay within the ORF of the *Drosophila mlp* gene still provided twice as many ESTs in LibW (74 ESTs) than in LibD (36 ESTs).

Tropomyosin and troponin I

Contigs 32 and 38 from the leg muscle of *G. natalis* aligned closely with the same sequence coding for slow muscle tropomyosin (*tm*) in *H. americanus* (Fig. 4). While the sequence of contig 32 encompassed the entire ORF of *H. americanus tm*, that of contig 38 was limited to just the 3′-region. However, there are two different *tm* isoforms from *H. americanus* which are similar in their 5′-region and it is therefore possible that the construction of contigs 32 and 38 allocated 5′-region ESTs from both isoform sequences to just contig 32 – a potential bias in the abundance analysis. Further analyses of these *G. natalis* sequences showed the 3′-regions of contigs 32 and 38 were even more closely and specifically aligned to the *tmS1* or *tmS2* isoform sequences from *H. americanus* (Fig. 4).

ESTs of contig 38 (putatively *tmS2*) were 3.2-fold more abundant in LibW than in LibD, and those of contig 32 were possibly also more abundant (1.4-fold) in LibW (Table 3).

Four contigs from *G. natalis* showed significant alignment to the sequence for troponin I (contig 52, 68, 122 and 145) (Table 1, contig 145 not listed as it comprised two ESTs). The sequences of the four different contigs all commenced before the start codon of the ORF (*Pontastacus leptodactylus*; Table 1) and encompassed parts of the 5′-region but only contig 52 spanned the entire ORF. While four putative genes may be implied by the four contigs in *G. natalis*, the region between nucleotides 250 and 380 of their predicted ORFs revealed only two different nucleotide sequences, one of which occurred in contigs 52 and 145, and the second in contigs 68 and 122. The ESTs of contig 68 were more than twice as abundant in LibD than in LibW and contig 122 showed a similar but less significant relative abundance. However, contig 52 showed evidence of the opposite relative abundance (Table 3).

Semi-quantitative analysis of specific transcripts

The abundance of mRNA corresponding to five contigs of interest, 105 (*ak*), 131 (*mlp*), 140 (*pax*), 32 (*tmS1*) and 38 (*tmS2*), was tested

```
Contig 131      MP.FKAPPTP KCPKCGKSVY HAEARVVGDI SFHKECYKCN  39
DROMEMlp84B     **S*QPIEA* ***R****** A**E*LA*GY V***N*F**G  40
DROMEMlp60A     **.*VPVE** ***A****** A**E**A*GY K***T*F**S  39
HUMANCSRP3      **NWGGGA.. **GA*E*T** ***EIQCNGR ****T*FH*M  38
HUMANCSRP2      **VWGGGN.. **GA**RT*A YH*EVQCDGR ***RC*FL*M  38
HUMANCSRP1      **NWGGGK.. **GV*Q*T** F**EVQCEGN ****S*FL*M  38

Contig 131      MCNKMLDSTN CNLHENVLYC KTCHGRKFSP KGYGFGGGAA  79
DROMEMlp84B     ****S***** *TE**RE*** ********** ******T**G  80
DROMEMlp60A     ****A***** *TE**KE*F* *N*****Y** *********G  79
HUMANCSRP3      A*R*A****T VAA**SEI** *V*Y**RY** **I*Y*Q**G  78
HUMANCSRP2      V*R*N****T VAI*DEEI** *S*Y*K*Y** ****Y*Q**G  78
HUMANCSRP1      V*R*N****T VAV*GEEI** *S*Y*K*Y** ****Y*Q**G  78

Contig 131      GLSMDTGSQF EEKQ       Stop                   93
DROMEMlp84B     T****N**** LRENGDVPSV .. .. .. .            495
DROMEMlp60A     C**T***AHL NRE        Stop                   92
HUMANCSRP3      c**T***EHL GLQFQQSPKP .. .. .. .            194
HUMANCSRP2      T*N**R*ERL GI*PESVQPH .. .. .. .            192
HUMANCSRP1      T**T*K*ESL GI*HEEAPGH .. .. .. .            192
```

Fig. 3. Amino acid sequence deduced from the sequence of contig 131 of *G. natalis* aligned with the two most similar sequences identified by BLASTX analysis: DROME Mlp84B *Drosophila melanogaster* muscle LIM protein (gi|2497676; sp|Q24400), DROME Mlp60A *D. melanogaster* muscle LIM protein (gi| 1709057; sp| P53777); and three most closely related human proteins: HUMAN MLP_{CSRP3} human muscle LIM protein (gi|1705933; sp| P50461.1), HUMAN CSRP2 cysteine- and glycine-rich protein-2 (gi| 2497674; sp| Q16527) and HUMAN CSRP1 cysteine- and glycine-rich protein-1 (gi| 118161; sp| P21291). CCHC and CCCC zinc-binding sites of the LIM domain are italicised and underlined, the glycine residues that comprise the glycine-rich region following the LIM domain are shaded in black and the potential nuclear targeting signal is bold and underlined.

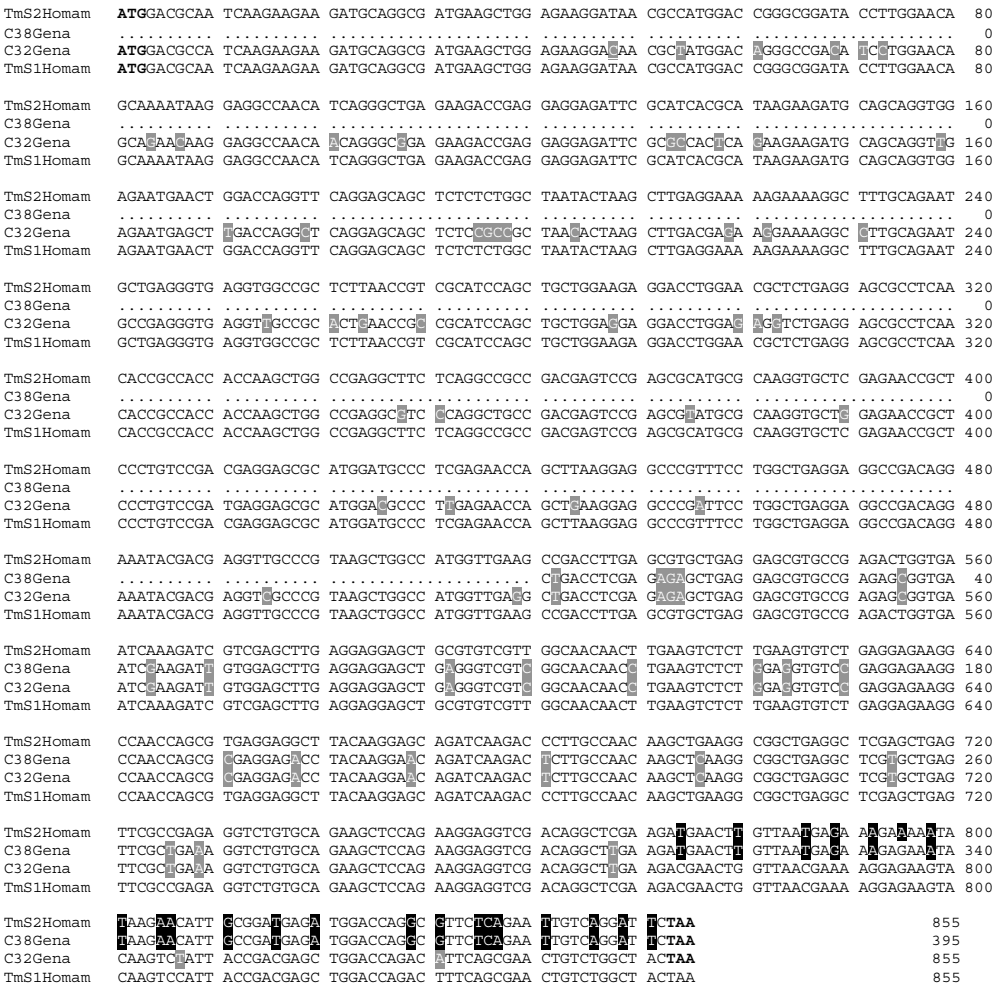

Fig. 4. Alignment of the nucleotide sequence of the ORFs of the tropomyosin isoforms *tmS1* (TmS1 Homam; AF034953) and *tmS2* (TmS2 Homam; AY521627) of *H. americanus* with the respective matching regions of contig 38 (C38 Gena) and contig 32 (C32 Gena). Differences between either of the two *G. natalis* contig sequences and those of *H. americanus* are shaded in grey while differences between the tropomyosin isoforms are shaded in black. Start and stop codons are shown in bold text.

between the wet season and dry season muscle tissues of *G. natalis* by semi-quantitative RT-PCR. Transcripts allocated to arginine kinase *ak* in muscle from each of the two seasons showed no difference in their abundance (dry season 3.07±1.12 ng DNA μl^{-1} and wet season 2.48±0.91 ng DNA μl^{-1}). The wet/dry season ratio was 0.81 which is extremely similar to that of the library comparison (LibW/LibD=0.78). However, *ak* expression provided an additional mechanism for normalising the transcription of the other selected genes.

LIM proteins

Consistent with the library comparison, the gene transcript coding for paxillin (*pax*), or a closely related protein, was twice as abundant in the muscle tissue of wet season crabs (wet/dry ratio=2.01; Fig. 5A). This difference persisted when *pax* transcription was normalised against values for *ak* (Fig. 5B), as the *pax*/*ak* ratio in dry season muscle was less than half that in the muscle tissue of migrating animals.

In the case of the *mlp*, neither the direct comparison of the concentrations of the fragments (Fig. 5A; wet/dry ratio=0.94) nor the comparison normalised against the respective *ak* values (Fig. 5B, wet/dry ratio=1.18) showed any significant difference in the transcript levels of *mlp* within the muscle tissue of wet and dry season crabs.

Tropomyosin

The 'pattern' of frequency of expression of genes related to *H. americanus tmS1* and *tmS2* isoforms revealed in the EST libraries (LibW/LibD=1.4 and 3.2, respectively) was confirmed by the more quantitative PCR method. For *tmS2* transcripts, both the direct comparison of the concentrations of the amplified *tmS2* fragments (Fig. 5C; wet/dry ratio=1.24) and the comparison relative to *ak* (Fig. 5D; wet/dry ratio=1.52) indicated a significantly higher abundance of the *tmS2* transcript in the muscle tissue of wet season crabs. For *tmS1* transcripts, the wet/dry ratio=0.96 (Fig. 5C) and corresponding values for *ak*-normalised amounts (wet/dry ratio=1.19; Fig. 5D) indicated no significant difference between transcript abundance in crabs from the different seasons, consistent with the marginal difference between LibW and LibD (Table 3).

DISCUSSION

The ESTs provided sequence information for 609 putatively unique transcripts from the muscle tissue of the terrestrial Christmas Island red crab *G. natalis*; 426 ESTs showed no significant alignment and potentially represent novel genes. This study has added significantly to the database resource for decapod crustaceans, most especially for land crabs. Importantly, comparison of EST abundances within the two libraries from *G. natalis* leg muscle provided new data indicating changes associated with the seasonal preparation for endurance exercise in crustaceans. All 14 of the most abundant transcripts showed some difference in their abundance, which is consistent at the transcriptional level with extensive regulation of protein composition of muscle in preparation for the demands of the breeding migration. The changes in gene transcript abundance for some of the more important proteins implied deserves detailed consideration.

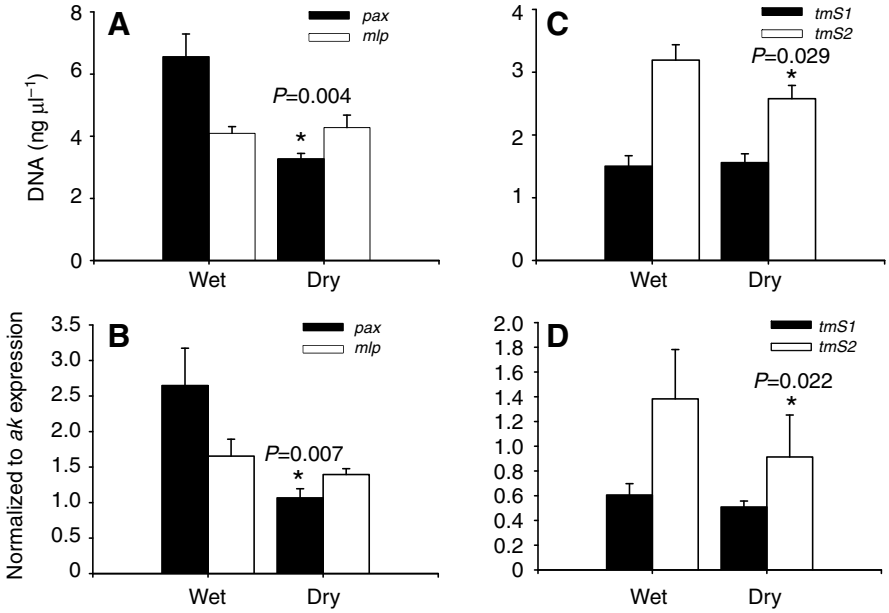

Fig. 5. Semi-quantitative real time RT-PCR analysis of transcript abundance of four transcripts selected from library comparisons for samples of the muscle tissue of migrating (wet) and non-migrating (dry) *G. natalis*. Data are means + s.e.m. (A) Total paxillin (*pax*; 32 cycles) and muscle LIM protein (*mlp*; 26 cycles) normalised against total RNA. (B) Total *pax* (32 cycles) and *mlp* (26 cycles) transcript normalised against arginine kinase (*ak*) transcript amount (26 cycles). (C) Total tropomyosin S2 (*tmS2*) and tropomyosin S1 (*tmS1*) (both after 26 cycles) normalised against total RNA. (D) Total *tmS2* and *tmS1* (both after 26 cycles) normalised against *ak* transcript amount. *Significant difference between dry and wet season transcript amount. *N*=7 amplifications in all cases.

Actin

The variation in the abundance of actin-coding gene(s) ESTs was consistent with the putative migration-related changes of the muscle structure of the leg of *G. natalis*. Actin is abundant and highly conserved in eukaryotes (Sheterline et al., 1999) and besides comprising part of the contractile apparatus, actin is also part of the cytoskeleton and thus involved in a variety of cellular processes. The mouse, human and fly genomes contain six actin loci (Röper et al., 2005). However, while these actin proteins vary by just a few amino acids, temporal and spatial differences in their expression are consistent with distinctly different functions. Thus, the cytoplasmic actin isoform of *Drosophila* is ubiquitous in cells (e.g. Fyrberg et al., 1983) while four other actin isoforms seem to be restricted to muscle cells. Furthermore, while actin 87E is expressed throughout life in *Drosophila*, actin 79B and 88F transcripts are most abundant in pupae and adults, while actin 57 transcripts occur mainly in embryonic and larval muscle (Fyrberg et al., 1983; Röper et al., 2005). Actin isoforms are often co-expressed within the same cells, and in *Drosophila*, for example, each of the six actin isoforms is expressed at some stage in muscle cells (Fyrberg et al., 1983; Nongthomba et al., 2001; Röper et al., 2005).

Consequently, it seems likely that the different actin contigs from *G. natalis* muscles represent different, but co-expressed, actin isoforms. However, only one of the identified actin contigs encompassed the entire ORF of the actin gene and it remains possible that the currently unknown regions of the other actin contigs may be very similar to each other. Thus, while it is reasonable to conclude that different actin isoforms are co-expressed in *G. natalis* leg muscle it is not yet clear exactly how many there are and consequently what the relative abundances of the several distinguished actin transcripts might ultimately be. Recently 12 actin isoforms and their tissue-specific expression patterns were described for *H. americanus*, including eight skeletal muscle isoforms (Ha-ActinSK1 to SK8) with muscle fibre type-specific distribution (Kim et al., 2009). Two 'slow-type' actins, Ha-ActinSK1 (100% identical to α-actin AF399872) and Ha-ActinSK2, were co-expressed in slow-twitch fibres (S1), while the remaining six skeletal muscle isoforms were expressed in different compositions in the fast muscle fibres of the cutter claw closer and deep abdominal muscles (Kim et al., 2009). All *G. natalis* contigs with significant alignment to actin showed highest similarity to the two 'slow type' actins of *H. americanus*. Although complete sequence information of the *G. natalis* actin genes is not yet available, these data are consistent with the idea that the leg muscle of *G. natalis* is predominantly composed of slow muscle fibres. Variations in the 3′-UTR such as between contigs 72 and 39 could separate expression of otherwise identical actin isoforms and promote migration-related changes in the transcriptional regulation of actin isoforms. Similar migration-related differences in the level of actin expression were also observed in the migratory locust (*Locusta migratoria*) (Kang et al., 2004). Up-regulation of transcription of seven distinct actin-related transcripts in the hind leg of the solitary form (non-migrating) of the locust was associated with the relatively longer limbs and stronger leaping ability of the legs. Increased protein turnover is characteristic of muscle undergoing structural remodelling (e.g. Termin and Pette, 1992; Mykles, 1997) with a concomitant rise in poly(A^+) RNA (for reviews, see Swynghedauw, 1986; Pette and Vrbova, 1992). Thus, the lower relative number of actin-coding ESTs apparent in LibW (migrating crabs) may be due to a background of generally increased transcription.

Troponin I

The thin-filament complex troponin (*tn*) composed of three subunits (TnC, TnI and TnT) is the main regulator of muscle contraction (Herranz et al., 2005). In crustaceans, up to seven isoforms of TnI can be differentially expressed in the various fibre types (Mykles, 1988; Miegel et al., 1992; Neil et al., 1993; Sohn et al., 2000; Medler and Mykles, 2003; Perry et al., 2009). Several contigs allocated to TnI were distinguished in *G. natalis*, which is not surprising since multiple isoforms of TnI are co-expressed in muscle tissue. In lobster and ghost crab muscles, different isoforms of troponin T and troponin I provide convenient markers of fibre type, albeit with low consensus between the two decapod species (Perry et al., 2009), most especially with regards to TnI. In the lobster, five TnI isoforms exist and are expressed in different proportions in different fibre types. In the ghost crab muscles only two isoforms were identified which were variably co-expressed in the different fibre types.

Perhaps the most notable finding with regards to TnI was that the sequence between nucleotides 250 and 380 of the predicted ORFs was similar for contigs 52 and 145 but different from a second sequence common to the ORF of contigs 68 and 122. This

association of contigs prompts the suggestion that TnI coded in contigs 68 and 122 is down-regulated in migrating crabs while that of contig 52 and 145 is in contrast up-regulated. In the crayfish *Cherax destructor* variations in the TnI isoform composition are correlated with changes in muscle functional properties (Koenders et al., 2004). In a different crayfish, *Procambarus clarkii*, CLFS altered TnI characteristics from those of fast to those more like slow muscle fibres (Cooper et al., 1998), akin to a similar increase in TnI transcription during a CLFS-induced transition from fast to slow muscle fibres in mammals (Härtner and Pette, 1990). The LibW/LibD values for the *tn*-aligned contigs varied between 0.4 and 1.2 and were thus not readily amenable to semi-quantitative RT-PCR. However, the differential library abundance of at least one of the TnI transcripts (contig 68) is consistent with a probable migration-related shift in the muscle properties.

Tropomyosin

Three different but co-expressed tropomyosin (*tm*) isoforms have been identified to date in *H. americanus* (Mykles et al., 1998; Medler et al., 2004). These isoforms are differentially expressed within the three main muscle fibre-type categories – fast, slow phasic (S1) and slow tonic (S2) – but their expression changes gradually along a continuum of hybrids from pure S1 to pure S2 fibres (Medler et al., 2004). The striking similarity of the two Tm transcripts in *G. natalis* to either *tmS1* or *tmS2* indicates these two isoforms or close variants are co-expressed in the leg muscle of *G. natalis*.

Regarding the comparison of *tm*-coding EST abundance, only the sequence of contig 32 covered the entire ORF of tropomyosin, which is near identical for the *tmS1* and *tmS2* isoforms in *H. americanus* except for the 3′-end of the ORF (Medler et al., 2004). In such a case some of the ESTs clustered towards the 5′-region of contig 32 (*tmS1*) might possibly be 5′ sequence for *tmS2* leading to some overestimate of the relative abundance of *tmS1* within each library with a concomitant under-estimation of *tmS2*. However, contig 38 (highly transcribed in LibW) was composed of ESTs that represent only *tmS2* and were thus a true reflection of the relative frequency of *tmS2* in the two libraries.

The abundance of *tmS1* and *tmS2* in crustacean muscle (i.e. *H. americanus*) is positively correlated with that of the myosin heavy chain (MHC) isoforms MHC S1 and MHC S2, respectively (Medler et al., 2004), and thereby with the main feature which distinguishes fibre type S1 (slow phasic) from S2 (slow tonic). Since the up-regulation of slow tonic *tmS2* (contig 38) in the LibW was corroborated by quantification of the specific transcript, the strong implication is that the relative abundance of slow tonic muscle fibres increased in migrating red crabs. A similar increase in the mRNA levels for proteins specific to slow fibres in rabbit muscle was seen during fast to slow fibre transition stimulated by CLFS (for a review, see Pette and Vrbova, 1992). Among the decapod Crustacea, crayfish have been shown to respond to CLFS by changing MHC to the slow fibre form (Cooper et al., 1998). In crabs, tonic fibres predominate in muscles that must sustain tension for a long period without tiring (Mykles, 1988; Govind et al., 1986). The S2 (tonic) fibres have a higher oxidative capacity than S1 (phasic) fibres (e.g. Neil et al., 1993; Perry et al., 2009) consistent with S2 fibres specialised for long-term force generation and S1 fibres adapted to slow movement (Galler and Neil, 1994). Thus, a shift toward a higher S2 fibre content would signify the switch towards a muscle of higher endurance suitable for long-distance migration. Such physiological modifications must underpin the migration in *G. natalis* which can thus be accomplished aerobically and without recourse to anaerobic glycolysis (Adamczewska and Morris, 2001b).

Regulatory LIM proteins

The LIM domain is a conserved CRP sequence that forms a double zinc finger motif, which functions as a mediator of protein–protein interactions (for reviews, see Sánches-García and Rabbitts, 1994; Dawid et al., 1998; Bach, 2000). This motif is found in a wide range of proteins and may occur as multiple domains. LIM proteins are involved in a variety of fundamental biological processes including cytoskeleton organisation, cell lineage specification and organ development (for reviews, see Bach, 2000; Weiskirchen and Guenther, 2003). The muscle LIM proteins (MLP) are found in the cytoplasm and nucleus of developing muscles (Arber et al., 1994; Stronach et al., 1996). The cytosolic form is associated with actin filaments and the Z-line of sarcomeres, suggesting a role in muscle fibre architecture (Kempler and Brenig, 1999). Furthermore, MLP has been identified as an essential factor in myogenesis and myogenic differentiation (Arber et al., 1994).

The LIM proteins are classified into three groups: containing LIM domain A and B (group 1), mainly LIM domain class C (group 2), or primarily class D LIM domains (group 3) (Dawid et al., 1998; Bach, 2000). In *G. natalis* two LIM proteins have been identified from contig sequences which show differences in their library abundance between LibW (migrating) and LibD (non-migrating crabs). One transcript (contig 131) showed highest similarity to members of LIM group 2, while the second (contig 140) was allocated to paxillin, which belongs to the more heterogeneous group 3 of the LIM proteins.

Muscle LIM protein-related contig 131

The amino acid sequence deduced from the sequence of contig 131 exhibited high similarity to the muscle LIM protein Mlp84B of *Drosophila melanogaster* (Stronach et al., 1996; Stronach et al., 1999). In vertebrate tissues these proteins are characterised by two copies of the LIM domain, each followed by a short glycine-rich region (reviewed by Weiskirchen and Guenther, 2003). In contrast, the two closely related CRPs found in the muscle of *D. melanogaster* (Mlp60A and Mlp84B) exhibit single and five tandem LIM–glycine modules, respectively (Stronach et al., 1996).

Induction by environmental cues of both *mlp* gene transcription and expression of MLP was observed in the goldenrod gall moth *Epiblema scudderiana* (Bilgen et al., 2001). The transcription of EsMlp, closely related to Mlp60A, was up-regulated in the larvae by cold exposure with a consequent rise of the EsMlp protein concentration in the early spring (Bilgen et al., 2001), consistent with a role for EsMlp in the preparation for development and metamorphosis needed in spring. Furthermore, the up-regulation of *mlp* during CLFS promoted fast-to-slow fibre transformation which implies that MLP has an essential role in transformation processes of adult muscle fibres (Schneider et al., 1999). In *Drosophila*, Mlp84B cooperates with D-titin to maintain muscle structural integrity (Clark et al., 2007). Together, these findings support a suggestion that MLPs play a crucial role in the remodelling of muscle structures in response to external cues. Additionally, the higher abundance of two MLPs in the EST catalogue of the leg muscle from migrating phase locusts (Kang et al., 2004) is evidence that MLP is necessary for developing migration season muscle properties. The elevated abundance in *G. natalis* of the MLP-related transcript (contig 131) in LibW was thus indicative of a migration-related restructuring of the muscle tissue of the walking crabs, in which the encoded MLP might play a role in the regulation of this process and/or be required as a structural element.

It should be noted that the quantification of transcripts corresponding to contig 131 did not confirm the different abundance

seen between LibW and LibD. This outcome may arise from the discriminatory sensitivity of the RT-PCR method but may be inherent in the clustering of ESTs into almost two discrete groups within the derived sequence of contig 131. It seems apparent that contig 131 was chimaeric and composed in part of the *mlp* ORF but conjoined with a further and very likely separate transcript, all of which may have confounded the semi-quantitative RT-PCR.

Paxillin-related contig 140

The second transcript assigned as coding for LIM proteins (contig 140) showed highest similarity to paxillin. The up-regulation of the gene for this protein is a further strong indication for a structural transformation of the muscle fibres in response to the demands of migration. Paxillin is a multi-domain adaptor protein that plays a role in the integration and dissemination of signals from integrin and growth factor receptors to effect efficient cell migration. Paxillin family members contribute to the transactivation of steroid receptors and a common feature of the disparate extracellular cues mediated by paxillin signal transduction is their promotion of significant changes in the organisation of the cytoskeleton and/or the state of cellular proliferation (for a review, see Brown and Turner, 2004).

In *G. natalis*, the semi-quantitative RT-PCR of the paxillin-related transcript confirmed the significantly greater transcript abundance seen in the library (LibW) from migrating crabs. The assembled evidence underscores a role for paxillin in preparing these land crabs for their seasonal migration, perhaps through varying the signal transduction properties of neuronal impulses or more likely through enhanced muscle proliferation/transformation processes. The role of the paxillin-related transcript in the latter could be manifold since paxillin and members of the paxillin family are involved in cell motility and adhesion during development, but also serve as integrative elements in the coordination of multiple signal transduction pathways regulating diverse cellular responses such as gene expression and reorganisation of the cytoskeleton.

Physiological implications for migrating red crabs

The comparison of LibW and LibD revealed changes in the abundance of transcripts with respect to the seasonal physiology of *G. natalis* and generally important in preparation for the annual migration. The shift in the isoform composition of the contractile elements provided evidence for a switch from slow phasic (S1) to the most slow, fatigue-resistant, muscle fibres (S2). Such a transition would result in an increased representation of aerobic fibres which is also consistent with the concomitant improvement in aerobic exercise capacity of the red crabs at the onset of the annual migration (Adamczewska and Morris, 2001b). Additionally, up-regulation of paxillin and probably a MLP is likely to be fundamental in the regulation of muscle development and reconstruction, and provides the first clues to regulatory mechanisms in the adaptation of the muscle to the migration-related demands. The transcript for the crustacean fast muscle protein P75 (Costello and Govind, 1984; Medler and Mykles, 2003; Mykles, 1985b; Mykles et al., 2002; Perry et al., 2009) was absent from both libraries and thus it may be that leg muscles of *G. natalis* generally have a low population of genuinely fast fibres of the type found, for example, in ghost crabs (Perry et al., 2009).

The EST-based gene discovery approach adopted here would always be unlikely to reveal differences in the expression of the least abundant transcripts, for example those for the enzymes of the glycolytic pathway. The important pieces of information reported here now need to be complemented by further investigations. These might include a proteomic probe of the muscle tissues together with examination of subtractive cDNA libraries (for less abundant transcripts), or perhaps comprehensive EST studies employing very high throughput quantitative gene discovery such as 454 DNA sequencing (Margulies et al., 2005; Goldberg et al., 2006). The seasonal dichotomy in the ecological physiology of red crabs provides a near unique model for this work. It is now crucial to integrate these molecular approaches into a detailed accounting of the concomitant changes in functional morphology of the muscles of *G. natalis*; comparing not just quiescent dry season crabs with those undergoing the annual migration but crabs throughout their seasonal cycle such that the preparative changes in functionality can be properly documented.

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