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Biochemical investigations into the absence of *rigor mortis* in the Norway lobster *Nephrops norvegicus*

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ABSTRACT

It was found that the striated muscle of the Norway lobster (*Nephrops norvegicus*) does not exhibit the *rigor mortis* state otherwise typical for this type of muscle. This absence of *rigor* was investigated, concentrating on changes in the structure, ultrastructure and post-mortem biochemistry of the muscle. Samples were initially fixed for light and electron microscopy at the time of death and at different times post-mortem (3, 6, 12 and 24 h). Protein extracts were obtained in parallel to compare the banding patterns of the myofibrillar proteins using SDS-PAGE. A Western blot was applied to elucidate if myosin – a representative major myofibrillar protein – was degraded post-mortem. And finally, ATP levels in the muscle were analyzed using HPLC. Using TEM imaging it was found that between 12 and 24 h post-mortem at a storage temperature of 10 °C, when *rigor mortis* should set in (according to the muscular ATP concentrations), an extensive, but rather specific breakdown of myofibrillar proteins occurred. The Z-disks were degraded and the myofibrillar structure was lost. SDS-PAGE and Western blot clearly demonstrated the post-mortem breakdown of myosin. The nature of the observed protein breakdown seems to impede *rigor mortis* in some way by the activation of at least one of the several proteolytic systems (cathepsins, calpains and others) found in vertebrates and invertebrates. It is speculated that the proteolysis simply overtakes the *rigor*-inducing post-mortem changes.

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1. Introduction

The phenomenon of *rigor mortis* in muscle occurs when ATP levels drop below 1.0–2.0 μmol g^{-1} (depending on species) (Sikorski et al., 1990) and is due to the arrest of cross-bridge cycling and the permanent linking of myosin heads to actin filaments (Reedy et al., 1965). In fish and other vertebrates, *rigor mortis* usually begins between 1 and 6 h after death (species, post-mortem temperature and ante-mortem stress dependent), and is resolved after 1–2 days, when stored at low temperatures (0–4 °C) (e.g. Sikorski et al., 1990) or after several hours when stored at higher temperatures.

Although the fast striated muscles of invertebrates, such as insect flight muscle or those in the crayfish abdomen, are known to be structurally, functionally and physiologically very similar to vertebrate twitch muscle (Yagi and Matsubara, 1977) and the processes of calcium-activation, contraction and relaxation are equivalent (Millman, 1998), the literature on *rigor mortis* in invertebrates, is sparse. A few reports of the development of *rigor mortis* exist (Flick and Lovell, 1972)), but have been based on mainly subjective methods of assessment, and are therefore not conclusive. For example Flick and Lovell (1972) used only a simple visual assessment of rigor in the tail of the gulf shrimp *Penaeus aztecus*, and tail rigidity itself was not monitored.

An alternative interpretation, based on the observation that *rigor mortis* does not seem to occur during the early post-mortem storage of Norway lobsters (*Nephrops norvegicus*) (Gornik et al., 2008), is that *rigor mortis* does not in fact occur in such crustacean muscles. Several causes for an absence of the *rigor* state can be suggested, such as differences in muscle ultrastructure or abnormal post-mortem biochemistry. Either of these, or a combination of the two, could cause a change in the standard course of post-mortem events, leading to the failure of the musculature to stiffen post-mortem. In order to test these possibilities, the absence of *rigor mortis* in the Norway lobster (*N. norvegicus*) has been investigated by examining changes in the structure, ultrastructure and post-mortem biochemistry of the muscle.

2. Materials and methods

2.1. Capture and transport of N. norvegicus

N. norvegicus were caught in the Clyde Sea Area (55.41°N, 4.56°W), Scotland (UK) using baited traps (creels). Only male *N. norvegicus* in the intermoult stage (as determined following the method of Aiken (1980)) within a size range of 35 mm to 55 mm of carapace length (distance from the posterior margin of the eye-socket to the midline of

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the posterior carapace edge) were used. On board the fishing vessel the animals were kept in flow-through seawater holding tanks. Once landed, the animals were transferred to tube-sets (vertically sub-divided containers), immersed in fresh seawater (without aeration) and transported to the University of Glasgow (transport duration: 1 to 1.5 h).

2.2. Aquarium maintenance

Animals were kept in separation at a temperature of 8 °C and a salinity of 33–35 pss in holding tanks (W 40 cm × H 40 cm × L 100 cm), which were fed by a close circuit flow-through seawater system, equipped with a coarse sand filter, an active charcoal filter and a biofilter unit. Only animals that presented themselves healthy over a period of 4 weeks were selected for the actual experiment. The lobsters were fed twice a week with a fish paste, which was obtained by homogenizing frozen round-fish (from by-catch).

2.3. Experimental design

Rested, unstressed animals were sacrificed by separating the carapace from the abdomen using a sharp pair of scissors. Great care was taken not to stress lobsters prior to the culling. The samples were incubated at 10 °C in a tabletop incubator (ECHOtherm® IN30, Torrey Pines Scientific) in a humid chamber (to avoid dehydration of the samples). An initial sample was taken at 0 h and afterwards samples were taken at 3, 6, 12 and 24 h post-mortem, fixed for microscopy or frozen in liquid nitrogen and stored at −80 °C until extraction and analysis.

2.4. HPLC analyses

An amount of approximately 0.5–1.0 g was taken from previously frozen abdominal muscle samples using clean dissecting tools and the exact weight was recorded. The tissue was homogenized on ice with a 5× volume (w/v) of 0.6 M perchloric acid (PCA) using an Ultra Turrax® T25 (IKA) tabletop homogenizer. The homogenate was centrifuged for 10 min at 4 °C and 16000 ×g in a tabletop centrifuge (Heraeus, Biofuge fresco). A fraction (3 ml) of the supernatant was transferred to a glass vial and the pH was adjusted to 6.5–6.8 with 1.0 M KOH. The sample was then incubated for 30 min on ice and filtered with a 0.22 µm syringe filter (Sartorius, 16534k) into a new tube. An equal volume of 0.1 M phosphate buffer (0.06 M K_2HPO_4, 0.04 M KH_2PO_4, pH 7.0) was added to make up a 6 ml dilution. A sub-sample was finally carried over into a 0.2 ml light-proof HPLC vial and stored at −20 °C until HPLC analysis.

ATP, ADP, AMP, IMP, inosine and hypoxanthine were analyzed by HPLC following a protocol modified from Ryder (1985). A SP8800 ternary HPLC pump was used and coupled to a PDA detector (Thermo Finnigan) set to monitor at 254 nm. The system was operated and controlled using the Xcalibur® systems software (Thermo Finnigan). Peaks were analyzed using the same software package. Separations were carried out using a reverse-phase C18 SYNERGY MAX-RP 80 A column (250 × 4.60 mm, with an internal particle diameter of 4 mm, Phenomenex, Torrance, CA, USA) fitted with an C18 SecurityGuard cartridge (4 × 3.0 mm, I.D., Phenomenex) at 40 °C. The mobile phase was composed of: solvent A (0.04 M KH_2PO_4, 0.06 M K_2HPO_4, pH 7.0) and solvent B (methanol (MeOH)). Conditions used for the analysis are summarized in Table 1. Standard curves were prepared from adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), inosine 5′-monophosphate (IMP), inosine (INO) and hypoxanthine (HX) in concentrations ranging from 0.1 to 1.0 mM (all nucleotides were obtained from Sigma). For the determination of ATP, ADP, AMP, IMP, INO and HX in the samples 10 µl of extract was injected per run. The concentrations of different products of ATP breakdown were calculated by extrapolation from the ΔA of a standard.

Table 1
HPLC conditions used for the analysis of nucleotides in *N. norvegicus* abdominal muscle extracts.

Time interval [min]	Solvent A [%]	Solvent B [%]	Flow rate [ml min^{-1}]
0	100	0	1.0
8	84	16	
8.5	60	40	
13.5	40	60	
14	100	0	
24	100	0	

2.5. Rigor mortis assessment

Rigor mortis was assessed using the method described by Matsumoto and Yamanaka (1991). The stiffness of the muscle (*rigor*) was assessed measuring the curvature angle of the abdomen, after the head, carapace and shell were carefully removed from the abdomen. *Rigor* was assessed just after death, and 3, 6, 12 and 24 h later. In theory, just after death the curvature of the abdominal muscle should equal 0°, when the abdominal muscle begins to stiffen the angle of the curvature will change and become larger. When the angle is largest *rigor* will be fully developed.

2.6. SDS-PAGE of muscle proteins

N. norvegicus muscle proteins were extracted following the method of D'Albis et al. (1986) and Martinez et al. (2001). The procedure was carried out on ice. Approximately 100 mg of the abdominal muscle was dissected and minced in a 1.5 ml tube. 500 ml of a high salt extraction buffer (5 mM EGTA, 15 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 200 µM phenyl-methyl-sulphonyl fluoride (PMSF) and 100 mM $Na_4P_2O_7$, pH 8.5) was added and the mix was homogenized for 1 min using a small hand-held homogenizing tool. The homogenate was vortexed for 10 min and centrifuged for 5 min at 4 °C and 16000 ×g using a tabletop centrifuge (Heraeus, Biofuge fresco). The supernatant (~250 µl) was transferred to a fresh 1.5 ml tube, an equal volume of glycerol was added, mixed by vortex and frozen at −20 °C for storage, or was immediately analyzed for protein content.

The protein content in high salt extraction protein samples was determined using the Bradford assay (Bradford, 1976). Due to high protein content, the muscle homogenate was diluted 1:100 with double-distilled water (H_2O dd.). The diluted protein solution (100 ml) was mixed with 900 ml of 1× Bradford reagent (5× Bradford reagent: 0.5 mg ml^{-1} Coomassie Blue R, 25% MeOH, and 42.5% H_3PO_4) and was incubated at room temperature (RT) for 10 min. The absorbance of a (diluted) protein solution was measured at 595 nm and the protein concentration was determined with reference to a standard curve of known concentrations (10–100 µg BSA ml^{-1}). Samples of 6 animals of the same origin and treatment were pooled after extraction and protein content determination. Amounts of 100 µg of protein from each individual sample were amalgamated in a fresh tube, and after mixing the protein content was determined again for confirmation.

Proteins were separated by polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970). The NuPAGE electrophoresis system (Invitrogen) was used with 4–12% Novex Bis/Tris pre-cast gradient gels. In accordance to Martinez et al. (2001) desalting of samples was not necessary prior to electrophoresis. If not otherwise stated 20 mg of the high salt soluble protein was mixed with 5× Laemmli-loading buffer (0.3 M Tris–HCl pH 6.8, 50% (v/v) glycerol 10% (w/v) SDS, 0.5% (w/v) bromophenol blue and 0.035% β-mercaptoethanol) and was denatured at 100 °C for 5 min. The samples were

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