

Potential utility of haemolymph analysis in non-lethal conservation studies on threatened Australasian freshwater crayfish: portability and practicality

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Abstract.—Straightforward and inexpensive analysis of blood constituents can provide quantifiable information on sub-lethal stress in an animal and a measure of their overall physical fitness. Such methods have been widely used on a range of marine and terrestrial species, primarily those of commercial or recreational importance. Freshwater crayfish in many regions of the world face a common suite of threats and threatening processes that include: exotic species (including other freshwater crayfish, associated diseases and parasites), habitat fragmentation and destruction, anthropogenic pollution, overexploitation and increased environmental temperature. Although some studies have investigated the effects of these on freshwater crayfish in-part (i.e., measured by gross symptoms), the subtle, often asymptomatic physiological effects are poorly understood. The analysis of haemolymph provides a simple, inexpensive, high resolution, portable (i.e., suitable for field analysis and assessment in remote areas) and non-lethal method for the evaluation of sub-lethal stress and immunocompetence status in freshwater crayfish. There is considerable scope for application of these existing techniques in conservation initiatives for rare and endangered freshwater crayfish in Australasia, in particular by providing: i) non-lethal stress assessments, ii) quantification of compromised health and iii) increased understanding of the physiological impacts from key threats and threatening processes.

INTRODUCTION

Techniques for evaluating the stress-effects of threats and threatening processes on freshwater crayfish either do not exist, or those that are available only provide relatively coarse resolution data (i.e., usually

simple mortality or survival). Typically, such techniques are laboratory based and also require the sacrifice of substantial numbers of animals (e.g., for competition trials, or dissections) and are therefore unsuitable for use at remote field sites, or on rare and/or endangered species. The resulting lack of understanding is an impediment to conducting and revising freshwater crayfish conservation assessments, such as the IUCN Red List of threatened species (see Coughran & Furse, 2010), and to the implementation of management plans or conservation measures (Furse & Coughran, 2011b).

The analysis of blood constituents has been used to evaluate physiological stress in numerous marine and terrestrial animals including fishes (Meka & McCormick, 2005), crustaceans (Uhlmann *et al.*, 2009), snails (Renwranz & Spielvogel, 2011), deer (Bateson & Bradshaw, 1997) and freshwater crayfish (Hamann, 1975; Malev *et al.*, 2010). For example, in Australia, crustacean haemolymph is often analysed to assess sub-lethal stress or morbidity of commercially and recreationally important decapods. However, the aim of such studies is generally to maximise the post harvest (Evans *et al.*, 1999a; Jussila *et al.*, 1999; Poole *et al.*, 2008) or discard survival of exploited species (Uhlmann *et al.*, 2009; Butcher *et al.*, 2012).

Rapid or chronic changes in extrinsic factors (e.g., environmental temperature or hypoxic conditions) are often inherent in capture and discard processes and can cause deviation from normal physiological function (Jussila *et al.*, 1997, 1999; Poole *et al.*, 2008; Uhlmann *et al.*, 2009). The quantification of changes in haemolymph chemistry can



Fig. 1. Extracting haemolymph through the ventral sinus at the basis of the fifth pereopod, with a small-gauge needle (*Euastacus sulcatus* Riek).

identify periods of elevated stress, particular stressors (Evans *et al.*, 1999b; Malev *et al.*, 2010), reduced immunocompetence (Fotadar *et al.*, 2001), and when homeostasis returns (Pascual *et al.*, 2003).

Commonly used physiological stress indicators include total and differential haemocyte counts (THC and DHC) (Jussila *et al.*, 1997), clotting capacity (Jussila *et al.*, 2001), total protein (typically measured as refractive index (RI)) (Dall, 1975; Leavitt & Bayer, 1977), glucose and lactate (Malev *et al.*, 2010) and ion concentrations (e.g., potassium, magnesium and calcium) (Uhlmann *et al.*, 2009). However, other factors must be considered when analysing haemolymph parameters, including moult-stage, gender (Pascual *et al.*, 2003; Malev *et al.*, 2010) and size (Uhlmann *et al.*, 2009), due to associations with haemolymph changes during the ecdysial cycle (Greenaway, 1993).

We see gaining an understanding of fine-scale haemolymph changes as essential to determine abiotic and biotic variables that impact on rare and endangered freshwater crayfish (e.g., increasing environmental temperature, effects of pollutants and exotic species interactions). These methods might offer substantial advantages over existing techniques, because only small volumes of haemolymph are required (e.g., ~25–300 μ L per test) and the small-gauge needles used are minimally intrusive (e.g., 26G needle, 0.45 mm \varnothing). Repetitive sampling over time is unlikely to be lastingly injurious or lethal to specimens (Hamann, 1975; Paterson *et al.*, 1997), although the possibility for adverse side effects (i.e., infection) cannot be absolutely excluded. Furthermore, as haemolymph constitutes ~30% of a crayfishes biomass (marine species, see Belman, 1975) and total volume can be quickly adjusted through biological functions (Greco *et al.*, 1986), these methods are suitable for use on rare and endangered species.

The objective of this paper is to outline examples of how existing methods of haemolymph constituent analysis might benefit freshwater crayfish conservation. The potential application and utility of such studies are discussed within the context of

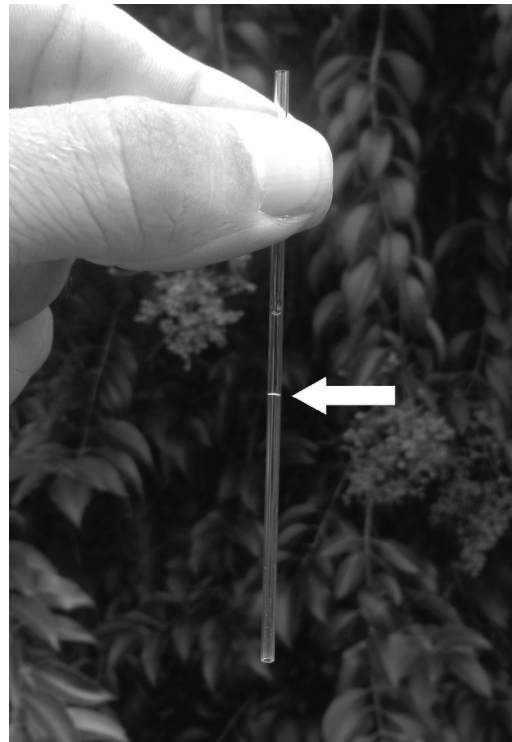


Fig. 2. The endpoint of an *in-situ* haemolymph clotting capacity test (clotted haemolymph indicated by the arrow).

previously identified knowledge gaps for rare and endangered Australasian freshwater crayfish species.

EXAMPLES OF EXISTING FIELD DEPLOYABLE AND INEXPENSIVE METHODS

Haemolymph extraction

Haemolymph samples can be extracted using a 1 mL syringe (cost <US\$1.00 each) from the ventral sinus at the basis of either fifth pereopods (Fig. 1) (Shields *et al.*, 2003; Uhlmann *et al.*, 2009), or from under the abdomen (Stewart *et al.*, 2004). Samples for haemocyte counts are withdrawn into a chilled syringe (on ice) pre-filled with a suitable anti-coagulant (e.g., 1% formalin, at 9 parts formalin to 1 part haemolymph) (Malev *et al.*, 2010). Before storage, the actual volume of haemolymph extracted must be noted, for subsequent volume adjustment

(to account for dilution).

Total and differential haemocyte counts

Typically, THC and DHC are determined using smearing and staining techniques (e.g., May–Grünwald). However, some studies have differentiated between hyaline (agranular) and granular cells (granulocytes and semi-granulocytes) using differences in size, morphology and granular content (LeMoullac *et al.*, 1997; Butcher *et al.*, 2012). Butcher *et al.* (2012) validated the repeatability and accuracy of this technique by comparing counts of stained and unstained cells. While this differentiation technique may reduce fine-scale differences (i.e., between granular cell types) it can be readily implemented to quantify broad-scale shifts in haemocyte proportions.

To obtain THC and DHC transfer 25 μ L of fixed haemolymph into a NEUBAUER™ haemocytometer with a pipette (Jussila *et al.*, 1997). The total number of cells in five (of 25) large squares (dependant on total cell abundance) are counted (at 100 \times magnification) and recorded. The total cell count gives the THC value and the number of granular cells observed comprises the DHC, and both are multiplied by the haemocytometer conversion factor. Ideally haemocyte counts should be performed within 1–5 days (with samples stored at 4°C) (e.g., Uhlmann *et al.*, 2009) to avoid the need for re-suspension.

Refractive index (for total protein)

Place ~25 μ L of fluid haemolymph immediately onto the prism of a VetQuip™ (VQ5600) refractometer with automatic temperature compensation (calibrated with distilled water) and record the RI (scale: 1.340–1.360 nD units) (Dall, 1975; Leavitt & Bayer, 1977). This can be reported simply as refractive index (Poole *et al.* 2008), or converted to total protein (Shields *et al.*, 2003; Butcher *et al.*, 2012). For the latter, take 20–30 haemolymph samples (of known RI) and analyse for total protein (mg/mL). Once the correlation between RI and total protein is known (e.g., Poole *et al.*, 2008), the equation for the line of best fit can be used to convert future RIs for that species (and co-generics) in the field.

Glucose, lactate and ion concentrations

Field measurements of glucose concentrations can be made with a hand-held reflectometer (e.g., One Touch® II Meter, Lifescan, Milpitas, CA, USA, ~US\$100.00) using commercial grade test strips (Malev *et al.* 2010). Similarly, lactate can be quantified using a Accutrend plus™ meter (Roche Diagnostics, Australia, ~US\$250.00) (Wells & Pankhurst, 1999), with a ~25 μ L sample of fluid haemolymph. For samples that are below the minimum detectable limit, 50% of that value can be substituted.

If ion analyses are required (e.g., calcium, magnesium or potassium), haemolymph samples can be placed in Eppendorf™ vials and immediately frozen in liquid nitrogen. Subsequent laboratory analyses of these parameters (for <US\$5.00 per sample) can then be performed concurrently with glucose and lactate concentrations using an OLYMPUS™ AU400 automated clinical analyser.

Clotting time

Extract ~25 μ L of haemolymph and expel into a 1.5 ml vial. Immediately transfer 12–25 μ L of haemolymph into a vertical glass micro-pipettor tube (1.2 mm internal Ø) following Jussila *et al.* (2001). Unclothed haemolymph will move downward and reach the bottom, at this point the tube is inverted 180°. This process is repeated until the clotting ‘endpoint’ occurs; which is defined by the absence of downward movement (Fig. 2). Record the time elapsed from the initial extraction until the endpoint is reached. Generally, clotting times do not exceed 2–3 minutes, and values exceeding this are recorded as “unclothed” (Jussila *et al.*, 2001; Uhlmann *et al.*, 2009).

DISCUSSION

Recent global conservation assessments have: i) identified freshwater crayfish as among the world’s five most endangered animals groups (Dewhurst, 2010 *personal communication*), ii) emphasised the highly endangered status of many freshwater crayfish species, and iii) identified several key threatening processes affecting these

crayfish (Coughran & Furse, 2010; Furse & Coughran, 2011a; IUCN, 2011b). For example, translocated species (i.e., *Cherax quadricarinatus* (von Martens) and *Cherax destructor* Clark) have the capacity to displace native populations (Horwitz, 1990; Coughran & Daly, 2012; Leland *et al.*, 2012). Australia is home to 135 freshwater crayfish species (Coughran & Furse, 2012), of which 47% have been assessed as Critically Endangered, Endangered or Vulnerable versus IUCN Red List criteria (IUCN, 2011b), indicating the highly endangered nature of the fauna, and likely increased conservation needs in the future.

In contrast, Japan has a single native freshwater crayfish species (*Cambaroides japonicus* (De Haan)), which is considered an Endangered species by the Japanese Fisheries and Environmental Agencies. *Cambaroides japonicus* is also listed by the IUCN as Data Deficient (DD), a classification that does not indicate the species is not threatened, but rather that there is insufficient data to evaluate and place the taxon into any category (IUCN, 2011a). Such listing highlights there are specific knowledge gaps for the species that need addressing. *Cambaroides japonicus* is threatened by two exotic and highly invasive species (the American signal crayfish *Pacifastacus leniusculus* (Dana) and red swamp crayfish *Procambarus clarkii* (Girard)) (Nakata & Goshima, 2003; Kawai & Machino, 2010). These species have dispersed widely and pose serious threats to *C. japonicus* due to competitive advantages, and by acting as disease vectors of the crayfish plague (*Aphanomyces astaci* Schikora) (Yamanaka *et al.*, 1997; Evans & Edgerton, 2002; Nakata *et al.*, 2002; Nakata & Goshima, 2003; Nakata *et al.*, 2004; Nakata *et al.*, 2006; IUCN, 2011b). While threats to the native Australian and Japanese freshwater crayfish have been identified and investigated in part, further research into the sub-lethal effects of these stressors is required.

We feel there is substantial scope to address previously identified knowledge gaps for rare and endangered freshwater crayfish species, refine the resolution at which sub-lethal stress is measured (e.g.,

compromised immunocompetence before physical exhaustion) and reduce the necessity for highly-invasive or lethal methods (e.g., LD/LC50 tests). For example, thermal tolerance studies generally use observation and evaluation of gross symptoms that (i.e., righting response, Nakata *et al.*, 2002), while unambiguous, do not evaluate sub-lethal effects (Bone *et al.*, unpublished).

The use of gross symptoms (i.e., righting response) in thermal tolerance studies indicates the point at which physiological stress renders a freshwater crayfish unable to right itself (see Nakata *et al.*, 2002). Such tests are not benign, and are not suitable for studies on Critically Endangered freshwater crayfish species. However, the methods outlined in this paper should be suitable for use on rare and Critically Endangered species and confer appreciable benefits over some existing and often injurious or lethal methods. In a field of research which is chronically short of funding (Furse & Coughran, 2011b), these inexpensive methods offer an opportunity to address knowledge gaps that would otherwise remain unanswered. Such studies would facilitate conservation assessments and improve understanding of the requirements necessary to manage and conserve threatened species. We believe the Endangered *C. japonicus* and some Critically Endangered Australian *Euastacus* (e.g., *Euastacus bindal* Morgan, and *Euastacus dharawalus* Morgan) are ideal candidates for novel applications of haemolymph constituent analysis.

From an experimental design, execution and cost point of view, these methods are superior to some existing techniques, because of their capacity for large sample sizes, are easily replicated and provide almost immediately quantified information. Furthermore, the equipment for haemolymph extraction is standard and easily sourced throughout the world, most equipment can be obtained from local non-specialist suppliers (i.e., pharmacies), and is inexpensive (<US\$1.00 per sample collection). The cost of equipment for haemolymph analysis is generally not prohibitive, for example the haemocytometer required for THC and DHC range from US\$35.00–250.00 and will last

for many years.

Most haemolymph analysis is performed on standard laboratory equipment (e.g., microscopes), or can be done with an inexpensive portable field microscope. Samples can be easily collected (and preserved) in the field and stored for laboratory analysis. Haemolymph extraction and most analyses are uncomplicated and reasonably easy to perform; any competent person can be trained in basic analysis techniques in a few hours. The straightforward nature of the methods and minimal equipment requirements can enable inexpensive analysis, and therefore remove common problems including lack of specialist expertise, expensive equipment, high per-sample processing costs and remote field sites: all of which are often impediments to critical research on rare and endangered Australasian freshwater crayfish. Application of the existing methods outlined in this paper should prove useful in crayfish conservation studies in other regions of the world.

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