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Preliminary multispecies test of a model for non-lethal estimation of metabolic activity in freshwater crayfish

Preliminarni test modela za oceno metabolne aktivnosti pri več vrstah potočnih rakov

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Abstract: We tested the applicability of electron transport system (ETS) derived from a single leg as a tool for non-lethal assessment of metabolic activity in freshwater crayfish. ETS activity of the whole body and of a leg was measured in four crayfish (Arthropoda, Crustacea, Decapoda) species: two European (*Astacus astacus*, *Austropotamobius torrentium)*, and two North American (*Orconectes limosus, Pacifastacus leniusculus)*. Mass scaling of whole body ETS activity (ETS_{whole}) and leg ETS activity (ETS_{leg}) was not significantly different for the European *A. astacus* and the American *O. limosus.* Therefore common models were constructed and tested on the remaining two species. The ratio ETS_{whole}/ETS_{leg} was significantly positively related to body mass. In the first model (model 1) ETS_{whole} was calculated from ETS_{leg} multiplied by the ratio estimated from the known body mass. ETS_{whole} of *A. torrentium* was underestimated by this model, because they mature at smaller body size than the larger species. A direct relation between ETS_{leg} and ETS_{whole} was therefore proposed as a general model (model 2), since they are correlated similarly in the studied species. The results show that model 2 is suitable for estimating the whole body ETS activity from leg ETS activity for the four investigated decapods.

Keywords: electron transport system (ETS) activity, crayfish, size scaling, method

Izvleček: V raziskavi smo testirali uporabnost modela za oceno metabolne aktivnosti pri potočnih rakih z merjenjem aktivnosti elektronskega transportnega sistema (ETS) le na eni nogi. Aktivnost ETS smo merili na celih osebkih in na nogi pri štirih vrstah potočnih rakov: dveh evropskih (*Astacus astacus*, *Austropotamobius torrentium)*, in dveh severnoameriških (*Orconectes limosus, Pacifastacus leniusculus)*. Ker se spreminjanje aktivnosti ETS celega telesa (ETS_{whole}) in aktivnosti ETS noge (ETS_{leg}) ni razlikovalo med jelševcem *A. astacus* in trnavcem *O. limosus,* smo naredili skupni model, ki smo ga testirali na preostalih vrstah. Razmerje ETS_{whole}/ETS_{leg} je bilo v pozitivnem razmerju z maso telesa. Pri prvem modelu (model 1) smo ETS_{whole} izračunali iz ETS_{leg} tako, da smo jo pomnožili z razmerjem, ki smo ga ocenili iz znane mase rakov. Izkazalo se je, da smo s tem modelom podcenili ETS_{whole} pri koščaku *A. torrentium,* verjetno zaradi nastopa zrelosti pri manjši velikosti kot pri večjih vrstah. Tako smo predlagali kot splošni model neposredno povezanost med ETS_{leg} in ETS_{whole}, ki sta pri vseh vrstah rakov v podobnem razmerju. Rezultati so pokazali, da na podlagi modela

2 lahko na osnovi izmerjene aktivnosti ETS pri nogi napovemo aktivnost ETS pri vseh testiranih vrstah rakov.

Ključne besede: aktivnost elektronskega transportnega sistema (ETS), potočni raki, velikost, metoda

Introduction

Freshwater crayfish are the largest freshwater macroinvertebrates. They are becoming recognized increasingly for their importance in the natural elimination of the dead organisms (Covich et al. 1999, Nyström 2002). In Europe, the increasing loss of freshwater habitats, coupled with the spread of non-indigenous North American crayfish species, and by the infection of *Aphanomyces astaci* that selectively kills the European species, populations of European crayfish has dramatically reduced (Holdich et al. 2009). The ecology of the native and introduced species and interactions between them have been studied intensively (e.g. Tamkevičiene 1988, Firkins and Holdich 1993, Holdich et al. 1995, Gil-Sánchez and Alba-Tercedor 2002, Paglianti and Gherardi 2004, Hudina et al. 2011). However, our knowledge about the role of crayfish species in the ecosystem in regard to energy flux and nutrient cycling through their metabolic activity is very limited.

Crayfish are key energy transformers among the different trophic levels since animals show omnivorous feeding character. They make major sources of energy (detrital material, decaying wood, dead organisms and periphyton) available to higher trophic levels at a more rapid rate than any other consumers (Momot et al. 1978). An estimate of crayfish metabolic activity could provide useful information for ecophysiological studies that deal with energy flow through ecosystems with crayfish populations. However, many crayfish species are threatened with population decline or extinction (Taylor 2002), so the number of experimental animals that can be taken from the wild is usually limited. Therefore, an elaboration of a new method that could provide samples of crayfish without killing them, or even without taking them from the wild, would be revolutionary.

Most comparative physiological studies on crayfish have been conducted on adult specimens (e.g. Demers et al. 2006, Styrishave et al. 2007). However, the effect of environmental pollution in the early development stages could be very different. For the study of this, the survey of the intra- and inter-specific, and size-dependent physiological reactions is very necessary. Knowledge of the metabolic activity of different sized crayfish would serve as a basis for such other ecophysiological population studies, in which the metabolic activity of entire crayfish populations has to be estimated, taking into account age and size. Most studies on the relationship between metabolic rate and body size in crustaceans have used respiration rate as a measure of metabolic activity (Buikema 1972, Ivleva 1980, Wheatly 1989, Glazier 1991, Marshall et al. 2003), which is impractical when dealing with large and endangered crayfish species. However, if organisms are basically similar in body size, size-related changes in most biochemical and physiological processes should parallel the scaling of metabolism (Peters 1983). Therefore, the use of enzyme activity to assess metabolic rates appears to be a better approach, as demonstrated in studies on several crustacean species which also took into account the body size effects (Berges and Ballantyne 1991, Berges et al. 1990, 1993, Simčič and Brancelj 2003).

The test of the enzymatic respiratory electron transport system (ETS) activity is a useful tool for estimating metabolic potential in aquatic organisms, since the result indicates the amount of oxygen would be consumed if all enzymes functioned maximally (Muskó et al. 1995). The method is simple, rapid and sensitive and has been used extensively on zooplankton (e.g. Bamstedt 1980, 1988, Borgmann 1978, James 1987, G.-Tóth and Drits 1991, G.-Tóth et al. 1995a; Simčič and Brancelj 1997, 2004), various amphipod and isopod species (e.g. Muskó et al. 1995, Simčič and Brancelj 2006, 2007, Simčič et al. 2005, 2010, Mezek et al. 2010) and fish (G.-Tóth et al. 1995b). Nevertheless, it has been rarely used in decapods. Borgmann (1977) studied ETS activities in various tissues of the crayfish *Orconectes pro-*

pinquus (Girard, 1852), while Simčič et al. (2012) used whole animal homogenization to measure the ETS activity of the whole animal (ETS_{whole}) in the noble crayfish *Astacus astacus* (Linnaeus, 1758) in order to relate it to oxygen consumption. To avoid killing animals and to circumvent the impractical whole body homogenization procedure in further crayfish studies, Simčič et al. (2012) proposed a new approach to estimate the metabolic activity of whole crayfish based on measuring ETS activity of a leg (ETS_{leg}). The leg can easily be removed in the field and regenerates afterwards without harmful effects on the animal. The method was however tested only for a single species, while it is essential to know whether a general model could be established and used for different crayfish species.

The four species included in the present study belong to two crayfish families with natural distribution ranges in Europe (EU) and North America (NA): Astacidae (EU: *Astacus astacus* (Linnaeus, 1758), *Austropotamobius torrentium* (Schrank, 1803) NA: *Pacifastacus leniusculus* (Dana, 1852)), and Cambaridae (NA: *Orconectes limosus* (Rafinesque, 1817)). All species are distributed in lentic and lotic freshwater ecosystems in temperate regions (Holdich 2002). The aims of the present study were to determine size scaling of the relationship between ETS_{whole} and ETS_{leg} in two crayfish species, in order to establish a general model that can be used for estimating the metabolic activity of a whole crayfish on the basis of measured ETS_{leg}. The proposed model has been tested on additional two crayfish species.

Material and Methods

Four crayfish species were included in the study. Two species are indigenous to Europe, *Astacus astacus* (AA; n = 35), *Austropotamobius torrentium* (AT; n = 6), and two to North America, *Orconectes limosus* (OL; n = 12) and *Pacifastacus leniusculus* (PL; n = 5). One European (AA) and one North American (OL) species were used for model construction and the other two for validation of the models.

All specimens used in the laboratory tests were sampled in natural water bodies in Slovenia and Italy in 2009 and 2010. Protected and endangered species were collected under special licence No. 35601-135/2010-10 (issued by the Slovenian Environment Agency) in limited numbers. Live crayfish were transported to the laboratory in thermo-isolated bags in order to reduce stress effects. In the laboratory all specimens were maintained, prior to use, in aerated dechlorinated tap water for three weeks at 10 °C and photoperiod light:dark = 16:8 hours. They were fed a commercial food (Sera crabs natural) *ad libitum*. Before measurements, the animals were weighed to the nearest 0.1 mg.

Electron transport system (ETS) activity was measured using the method originally proposed by Packard (1971) and improved by G.-Tóth (1999). The third walking leg or the whole crayfish was homogenized in liquid nitrogen using a mortar. A weighed amount (50–90 mg wet mass) was sonicated in 4 ml of ice-cold homogenization buffer (0.1 M sodium phosphate buffer pH = 8.4; 75 μM $MgSO_4$; 0.15% (w/v) polyvinyl pyrrolidone; 0.2% (v/v) Triton-X-100) for 20 sec (4710; Cole-Parmer) and centrifuged at 8500 x g for 4 min at 0 ºC (Centrifuge Sigma). Three 0.5 ml samples from each homogenate were incubated for 30 min at 10 °C in 1.5 ml substrate solution (0.1 M sodium phosphate buffer pH = 8.4; 1.7 mM NADH; 0.25 mM NADPH; 0.2% (v/v) Triton-X-100) with 0.5 ml 2.5 mM 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) solution. The reaction was ended by addition of 0.5 ml of stopping solution (formalin: H_3PO_4 conc = 1:1 v/v). Blanks (1.5 ml substrate solution and 0.5 ml INT solution) were incubated and treated as for the samples, followed by addition of 0.5 ml of homogenate. Formazan production was determined spectrophotometrically from the absorbance of the sample at 490 nm against the control blank within 10 min of stopping the reaction (WTW PhotoLabSpektral). ETS activity was calculated according to Kenner and Ahmed (1975).

Since body protein content measured in homogenates of whole animals is generally a constant fraction of organism composition and therefore often used in size scaling studies (Berges and Ballantyne 1991), we measured the protein concentration in whole body ($PROT_{whole}$) and legs ($PROT_{leg}$), using a commercial BCA Protein assay kit (Pierce). 0.1 ml of homogenate, from either the leg or the whole body, was pipetted into a test tube

with 2.0 ml of reagent and mixed well. Samples were incubated at 37 °C for 30 min. Absorbance was measured at 562 nm against the blank (distilled water) within 10 min and the protein concentration determined from a calibration curve constructed with bovine albumin as standard.

For model construction we used two species, one indigenous (AA) and one non-indigenous (OL), for each of which we obtained a sufficiently large amount of data to distinguish between general and species-specific patterns. The effect of sex on body mass, ETS_{whole}, ETS_{leg}, $PROT_{whole}$, $PROT_{leg}$, and on the ratio ETS_{whole}/ETS_{leg} was tested with separate paired t-tests for each species. Since there was no significant effect ($P > 0.05$), both sets of data were pooled in further analyses. Least-squares regression analyses were performed to establish the mass scaling of the ETS activity and protein content variables. Regressions were performed with a power model of the form $y = ax^b$, where y is ETS activity or protein content, x is wet body mass of a crayfish and *a* and *b* are regression coefficients. We tested whether a single mass scaling equation can be used for both AA and OL. The improved fit obtained by taking into account species identity was evaluated by multiple regression in which species identity was added with a binary dummy variable A. The variables were *ln* transformed as necessary to fit a linear regression model: $lny = ln(a)+blnx+cA+d(A \times lnx)$. The coefficient *c* tests the significance of the difference in intercepts while *d* tests for the difference in slopes. Separate models were deemed necessary if either slope or intercept, or both, differed significantly between species. If not, a joint equation was calculated from the pooled AA and OL data. The statistically significant equations are shown in the Figures 1–4.

Pearson correlation between ETS activity and protein content was calculated separately for whole bodies and for legs. The relationship between ETS activity and protein content was established, by power regression, separately for whole bodies and for legs. The difference between the regression models for AA and OL was tested as for mass scaling.

We tested two models for predicting ETS_{whole} from ETS_{leg}. The first model involves the use of the ratio ETS_{whole}/ETS_{leg}, estimated from wet mass (WW). ETS_{whole} is thus obtained by multiplying the ETS_{leg} value with the estimated ratio. The final model is:

$$ETS_{whole} = aWW^b\, ETS_{leg} \qquad \text{(model 1)}$$

The alternative model predicts ETS_{whole} directly from ETS_{leg}:

$$ETS_{whole} = aETS_{leg}{}^b \qquad \text{(model 2)}$$

Body mass was excluded from model 2, since it did not contribute significantly to the fit ($P > 0.05$). We tested for the difference between the models for AA and OL by including the dummy variable A. Both models ultimately contained the same number of parameters to be estimated, so they could be compared on the basis of the adjusted r^2 values.

Finally we evaluated the applicability of the joint AA and OL models for two other crayfish species (PL, AT). Values of ETS_{whole} measured in these species were compared with those predicted from the two joint AA and OL models with paired t-tests. The same method was used to test whether mass scaling equations for protein content and ETS activity constructed with AA and OL data function also for the other two crayfish species. All statistical analyses were conducted in SPSS 13.0.

Results

Size-structured samples (based on their body mass) of 58 crayfish specimens were used in laboratory experiments. Models were constructed on the basis of measurements on 47 specimens (AA, OL), and later evaluated on the basis of 11 specimens (AT, PL).

The mass of specimens did not differ significantly between sexes in AA and OL ($P > 0.05$), so differences in investigated parameters between sexes were tested using t-test. Since sex had no effect on values of ETS_{whole}, ETS_{leg}, $PROT_{whole}$, $PROT_{leg}$, or on the ratio ETS_{whole}/ETS_{leg} in AA and OL (t-tests, $P > 0.05$), the data for both sexes were pooled for further analysis.

Values of mass scaling of ETS_{whole} for AA (0.877) and OL (0.907) did not differ significantly (Table 1), so a single equation for pooled data was established (Fig. 1a). Insignificant differences were also observed between the two species in

the regression of ETS_{leg} and wet mass (Table 1; Fig. 1b). Both ETS_{whole} and ETS_{leg} decreased with increasing wet mass.

The regression models for mass scaling of $PROT_{whole}$ for AA and OL differed significantly (Table 1). $PROT_{whole}$ of AA was independent of

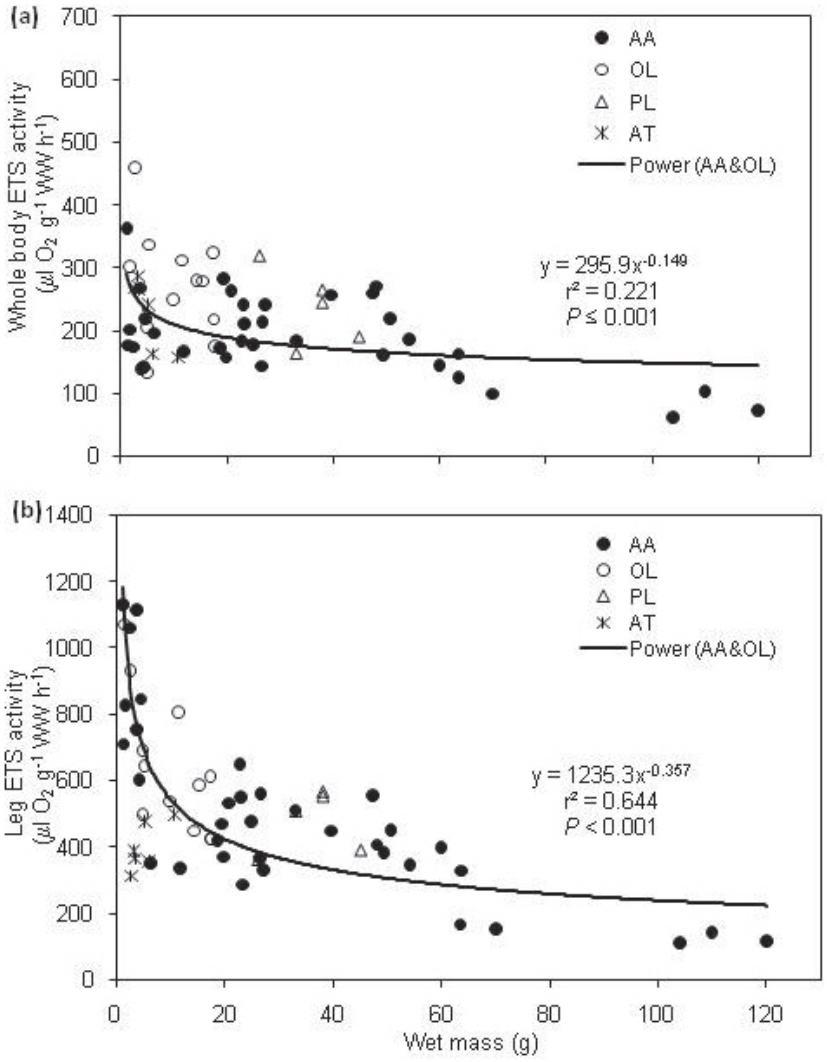

Figure 1: The relationship between wet mass and (a) electron transport system (ETS) activity of whole body and (b) ETS activity of a leg in different crayfish species (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). The function was derived based on AA and OL data only.

Slika 1: Razmerje med svežo maso in (a) aktivnostjo elektronskega transportnega sistema (ETS) celega telesa in (b) aktivnostjo ETS noge za različne vrste potočnih rakov (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). Funkcija je bila narejena le na osnovi podatkov za AA in OL.

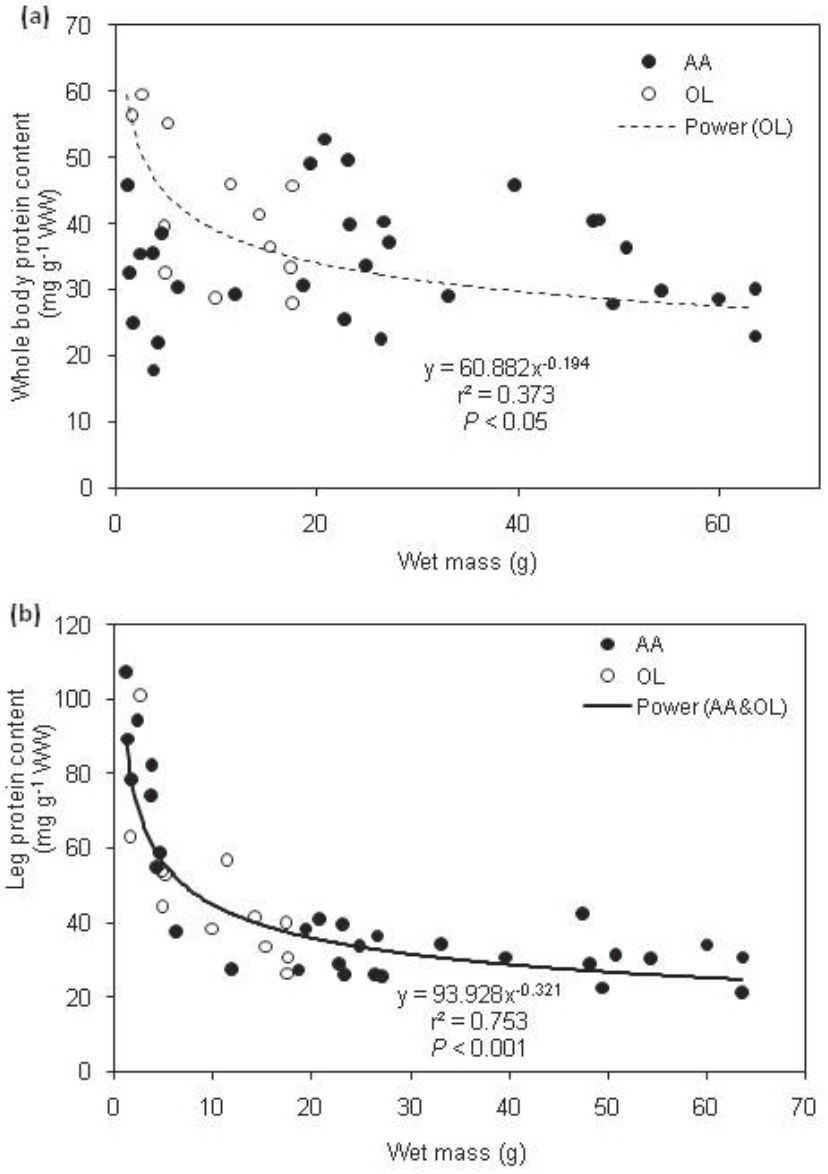

Figure 2: The relationship between wet mass and (a) protein content of whole body and (b) protein content of a leg in *Orconectes limosus* (OL) and *Astacus astacus* (AA).

Slika 2: Razmerje med svežo maso in (a) vsebnostjo proteinov v celem telesu in (b) vsebnostjo proteinov v celem telesu pri *Orconectes limosus* (OL) in *Astacus astacus* (AA).

body mass ($r^2 = 0.005$, df = 28, $P > 0.05$), whereas that of OL decreased with body mass (Fig. 2a). On the other hand, $PROT_{leg}$ in AA and in OL decreased similarly with wet mass according to a power law (Fig. 2b). ETS activity correlated significantly with $PROT_{whole}$ ($r = 0.788$, $n = 42$, $P < 0.001$) and in $PROT_{leg}$ ($r = 0.870$, $n = 42$, $P < 0.001$). Similar scaling exponents *b* were observed for AA and

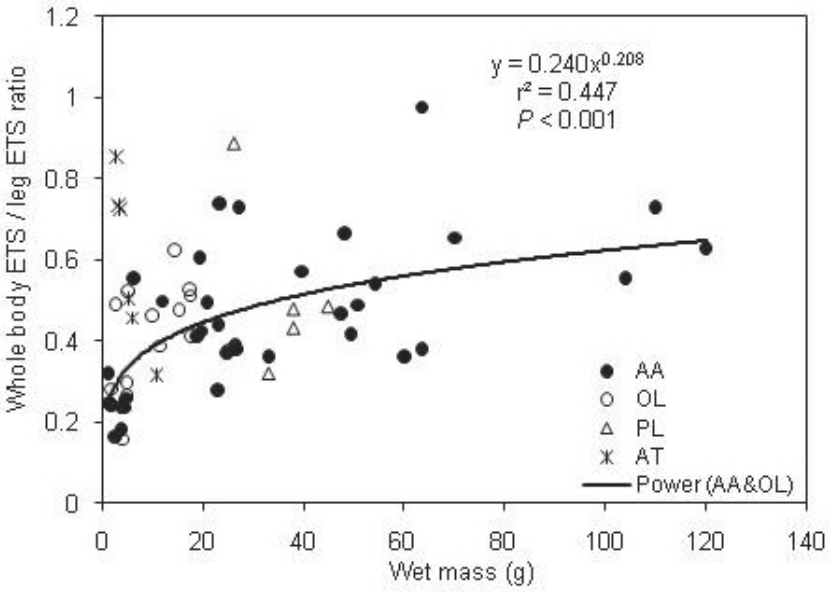

Figure 3: The relationship between wet mass and the ratio of electron transport system (ETS) activity of whole body to that of a leg, in different crayfish species (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). The function was derived based on AA and OL data only.

Slika 3: Odnos med svežo maso in razmerjem med aktivnostjo elektronskega transportnega sistema (ETS) celega raka in noge pri različnih vrstah potočnih rakov (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). Funkcija je bila narejena le na osnovi podatkov za AA in OL.

OL (0.973 and 1.117) when the ETS_{whole} was expressed in relation to protein mass of crayfish with a common scaling exponent of 0.961.

The ratio ETS_{whole}/ETS_{leg} scaled similarly in AA and OL (Table 1) and showed a significant positive correlation with wet mass (Fig. 3). The common model (model 1), predicting ETS_{whole} from ETS_{leg} with the help of this estimated mass ratio, explained 45% of the variation in whole body ETS activity of AA and OL:

$$ETS_{whole} = 0.240\ WW^{0.208}\ ETS_{leg} \quad \text{(model 1)}$$

ETS_{whole} was estimated directly from ETS_{leg} using a power equation (Fig. 4). The regression coefficients for AA and OL did not differ significantly, so a common model (model 2) was constructed:

$$ETS_{whole} = 8.498\ ETS_{leg}^{0.511} \quad \text{(model 2)}$$

This model explained 51% of the variance in ETS_{whole} of OL and AA.

Mass scaling of ETS_{whole} was similar in all the crayfish species in this study. The observed values of ETS_{whole} of PL (t-test, t = 2.388, df = 4, $P > 0.05$), and AT (t-test, t = 0.305, df = 5, $P > 0.05$) did not differ significantly from those predicted with the mass scaling equation established from the AA and OL data. Similar results were obtained for ETS_{leg}, except for the ETS_{leg} of AT, where the observed and predicted values differed significantly (t-test, t = 4.270, df = 5, $P < 0.01$), indicating different mass scaling of ETS_{leg} in AT from those in OL and AA.

The observed values of ETS_{whole} of PL did not differ significantly from the predicted values obtained from model 1 (t-test, t = 0.062, df = 4, $P > 0.05$). However, the predicted values of ETS_{whole} of AT (t-test, t = 2.768, df = 5, $P < 0.05$) differed significantly from the observed values when model 1 was used.

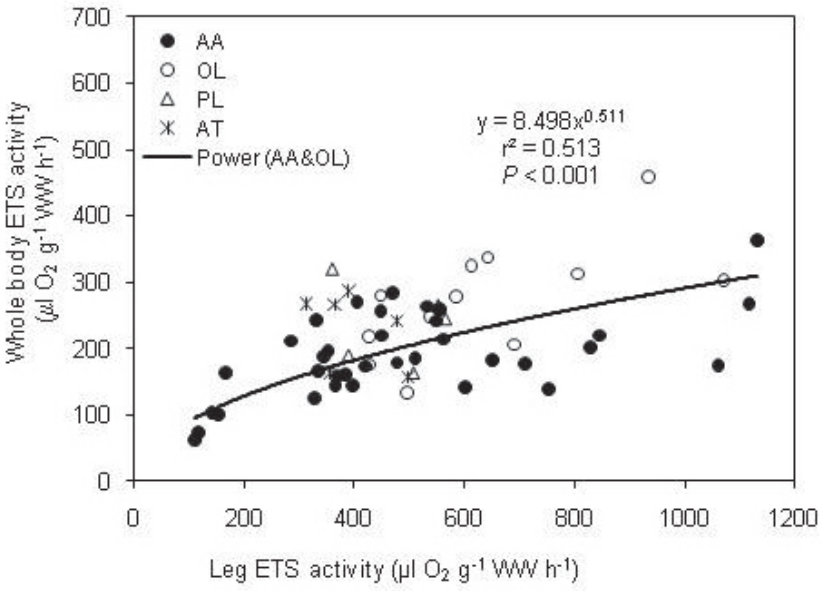

Figure 4: The relationship between electron transport system (ETS) activity in a leg and that of whole body in different crayfish species (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). The function was derived based on AA and OL data only.

Slika 4: Razmerje med aktivnostjo elektronskega transportnega sistema (ETS) noge in celega raka pri različnih vrstah potočnih rakov (OL – *Orconectes limosus*, AA – *Astacus astacus*, PL – *Pacifastacus leniusculus,* AT – *Austropotamobius torrentium*). Funkcija je bila narejena le na osnovi podatkov za AA in OL.

Table 1: Results of statistical testing (for details see methods) of the differences in intercept and slope between *Orconectes limosus* and *Astacus astacus* for the specified relationships (ETS_{whole} – whole body ETS activity, ETS_{leg} – leg ETS activity, $PROT_{whole}$ – whole body protein content, $PROT_{leg}$ – protein content of a leg).

Tabela 1: Rezultati statističnega testiranja (za podrobnosti glej metode) razlik v odseku in naklonu med vrstama *Orconectes limosus* in *Astacus astacus* za podana razmerja (ETS_{whole} – aktivnost ETS celega telesa, ETS_{leg} – aktivnost ETS noge, $PROT_{whole}$ – vsebnost proteinov v celem telesu, $PROT_{leg}$ – vsebnost proteinov v nogi).

	Intercept		Slope
Relationship	t	*P*	t
ETS_{whole} – wet mass	0.655	0.516	0.218
ETS_{leg} – wet mass	0.339	0.736	0.574
$PROT_{whole}$ – wet mass	2.758	0.009	2.081
$PROT_{leg}$ – wet mass	0.068	0.946	0.366
ETS_{whole}/ETS_{leg} – wet mass	1.200	0.237	0.426
$ETS_{whole} – ETS_{leg}$	0.720	0.476	0.835

Predicted values of ETS_{whole} obtained with model 2 for PL (t-test, t = 1.248, df = 4, $P > 0.05$), and AT (t-test, t = 1.840, df = 5, $P > 0.05$) did not differ significantly from the measured ETS_{whole}.

Discussion

It seems that estimating the metabolic activity of crayfish from a single leg only is an adjustable

method and that the model for the relationship between ETS_{whole} and ETS_{leg} can be applied to different crayfish species. The relation between ETS activity and wet mass is in agreement with the findings in previous investigations that ETS activity varies with body mass according to a power law (Cammen et al. 1990, Muskó et al. 1995, Simčič and Brancelj 1997, 2003; Simčič et al. 2012). In two crustaceans, *Chirocephalus croaticus* (Steuer, 1899) and *Gammarus fossarum* Koch, 1835, it was shown that ETS activity is related to body size in a manner typical of a metabolic function with the scaling exponent *b* being 0.787 and 0.651, respectively (Simčič and Brancelj 2000, 2003). Since mass scaling of ETS activity did not differ significantly between AA and OL, a common equation for pooled data was proposed. The established *b*-value was in the range reported for the metabolic rate of crustaceans in general (Wolvekamp and Waterman 1960, Ivleva 1980). Comparison of intra- versus inter-specific *b* exponents for oxygen consumption in aquatic crustaceans showed that the intraspecific slope was approximately 0.1 less than the slope of the overall collected data (Wheatly 1989). In crayfish we have found even smaller differences between intra- and inter-specific *b* exponents for ETS activity. Berges and Ballantyne (1991) reported that intra- and inter-specific exponents for whole body maximal enzyme activities in aquatic crustaceans, i.e. *Macrobrachium rosenbergii* (De Man, 1879), *Artemia franciscana* (Kellogg, 1906) and *Daphnia magna* (Straus, 1820), were similar for enzymes such as citrate synthase, but significant differences between species were found for enzymes associated with pathways other than aerobic metabolism. However, several authors have assigned the decrease in mass-specific metabolic activity to an increasing proportion of metabolically inert mass as the animals grow (Glazier 1991, Simčič and Brancelj 2003). Thus, the proportion of metabolically inactive tissue differs with species and varies with size and developmental stage of the same species.

The protein content per gram of whole crayfish mass did not change with increasing wet mass in AA, but decreased significantly in OL (Fig. 2a). However, when the ETS activity was expressed in relation to protein mass, similar exponents were observed for AA and OL. The common scaling exponent for ETS activity of the two species was 0.961, similar to the exponents reported for decapod species *M. rosenbergii* for a variety of enzymes, where whole animals were homogenized and the enzyme activity was expressed in relation to protein mass (Berges and Ballantyne 1991). A closely similar *b*-value (0.955) was found for the relationship between the amount of protoplasm and ETS activity per individual in *G. fossarum* (Simčič and Brancelj 2003). Berges and Ballantyne (1991) reported that an exponent close to 1.0 is characteristic of enzymes capable of functioning catabolically or anabolically. Moreover, for larger crustaceans, such as *M. rosenbergii*, that rely increasingly on anaerobic metabolism as body size increases, scaling exponents closer to 1.0 are expected for the enzymes that function in both anaerobic and aerobic processes. The exponent close to 1.0 obtained for ETS_{whole} in the present study was in accord with the findings of Berges and Ballantyne (1991), since ETS activity measures both aerobic and anaerobic metabolism (Packard 1985).

ETS_{whole} and ETS_{leg} exhibited different mass scaling exponents (Fig. 1). This was expected, because different tissues contribute to the sample for ETS measurements in the two cases. Borgmann (1977) found that ETS activities differed in the various tissues of the crayfish *Orconectes propinquus*. Thus, ETS_{whole} reflects the activity of the mixture of the large number of different metabolically active tissues, body storage materials and exoskeleton, while in a leg sample muscle tissue and exoskeleton material predominate. Moreover, the proportion of protein material in a leg decreased up to 10 g of body mass, while in larger animals it was relatively constant (Fig. 2b). The decrease of $PROT_{leg}$ with similar exponents in the two species could mean a lower variability in $PROT_{leg}$ than in $PROT_{whole}$. The reason for higher variability in the relation between protein content and body mass probably lies in variable amounts of different storage materials and other metabolically inert tissues in the crayfish during their inter-annual life history, as well as in their age. However, the ETS_{whole} and ETS_{leg} correlated well with their protein content, indicating that the latter plays a key role in ETS activity.

Since ETS_{whole} and ETS_{leg} scaled differently with body mass (Fig. 1), the ratio ETS_{whole}/ETS_{leg}

showed a significant, positive power relationship with body mass (Fig. 3). Similar scaling of the ETS_{whole}/ETS_{leg} ratio in AA and in OL indicates a greater influence of body size than of species-specific properties on the relationship between whole body and leg metabolic potential. Therefore estimation of the whole crayfish metabolic potential can be estimated from ETS_{leg} using a common ratio for both species, but this ratio depends on body mass. Thus, calculation of the ETS_{whole}/ETS_{leg} ratio for a crayfish of a given body mass on the basis of the equation in Fig. 3 provides a factor that can be used for the estimation of ETS_{whole} from ETS_{leg} (model 1). The utility of this model for estimating ETS_{whole} in different crayfish species was tested by comparing observed and predicted values in two crayfish species not used in the model construction. For PL, observed did not differ from predicted values, but the ETS_{whole} of AT was underestimated by this model (Fig. 3). The reason probably lies in the different developmental stage of 2–5 g AT from that of other crayfish of this size. Wheatly (1989) reported that the comparison of organisms at different developmental stages is problematic due to the different physiology of immature and adult individuals. Small-sized crayfish species such as AT attain their maturity at a mass of 2 to 5 g, while large-sized species become mature at a mass of more than 5 g (Souty-Grosset et al. 2006). It means that the specimens of AT were actually in a mature developmental stage, but that the same sized individuals of other species were still immature, and therefore possessed different physiological characteristics. Berges et al. (1990) found that the activity per unit mass of the primary anabolic enzyme nucleoside diphosphate kinase (NDPK) decreases with size and that enzyme scaling is affected by differences in growth rate. Thus, different development stages probably contribute to the relatively low ETS_{leg} in AT.

To minimize the potential effect of different development stages on estimated ETS activity, we explored a model relating ETS_{whole} directly to ETS_{leg}. A common model (model 2) was constructed for both species, since the species-specific models did not differ significantly (Fig. 4). High variability in physiological and biochemical variables, especially due to different moulting stages, reproduction cycle and fitness, resulted in scattered data and, consequently, low coefficients of determination. Nevertheless, the results confirmed our expectation that the metabolic potentials of a whole crayfish and of a leg scale similarly in different crayfish species, since the whole body ETS activity estimated from a leg did not differ significantly from the observed value in all species investigated.

The results of this study suggested that the metabolic potential of whole crayfish could be estimated on the basis of the ETS activity measurement in a single leg, using a general model. Due to the non-lethal approach, the new method could allow larger sample sizes to be incorporated into metabolic activity studies on crayfish, which is essential in conducting studies on multi-population and interspecific levels. Direct measurement of respiration is time-consuming and impractical and may subject the animal to stress before and during measurement. The slow response of ETS activity to short-term variations in environmental factors or stress makes the method superior to direct respiratory measurements on incubated animals (Bamstedt 1980). Moreover, the results of the present study also revealed that the two species-specific models did not provide significantly better estimates of whole crayfish metabolic activity than the joint ones. Model 2, by which ETS_{whole} was related directly to ETS_{leg}, seems the most appropriate for an approximate estimation of whole body metabolic activity in all tested species. Further studies taking into account more species and larger samples could contribute to a clearer picture of the generality of model use. The proposed model, in which only population density, size structure (as body mass) and ETS_{leg} are needed for estimating whole population metabolic activity, could be applied, in particular, for the estimation of metabolic activity in endangered and rare crayfish species.

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