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RESEARCH PAPER

The Size Driven Variations in Physiological Responses of the Bearded Horse Mussel *Modiolus barbatus* and the Noah's Ark Shell *Arca noae*

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Abstract

Bearded horse mussel and Noah's ark shell are sometimes observed to live together in groups. Oxygen consumption, ammonia excretion and clearance rate were investigated on these species under laboratory conditions considering different size of individuals divided into three size groups (less than 25 mm; 25 to 50 mm and larger than 50 mm). Body size of both species significantly influenced oxygen consumption, ammonia excretion and clearance rate of the Noah's ark shell. In both species, respiration and clearance rate decreased with body size while excretion rate increased with body size. For Noah's ark shell groups of 25 to 50 mm and smaller than 25 mm, the atomic ratio between oxygen consumption and ammonium excretion i.e. O:N ratio was detected to be greater than 30. The same applies to a group of the bearded horse mussels smaller than 25 mm. Animals of both species larger than 50 mm have O:N ratio smaller than 30 indicating use of proteins as metabolic substrate, compared to use of lipids and carbohydrates in younger animals.

Keywords: Oxygen consumption, ammonia excretion, clearance rate, O:N ratio, size, bearded horse mussel, Noah's ark shell.

Introduction

Arca noae Linnaeus, 1758 and *Modiolus barbatus* (Linnaeus, 1758) are sometimes observed to live together in groups. In addition to farmed bivalves, occasionally on the local markets fisherman bring bearded horse mussel and the Noah's ark shell caught from natural habitats. This however does not meet market demand, therefore there is need for farming of these species. Knowledge about the species that are potential aquaculture candidates needs to be expanded. Growth potential and potential of the bearded horse mussel for aquaculture were investigated by Lok, Acarli, Serdar, Kose, and Goulletquer (2006), Peharda *et al.* (2007), Peharda, Ezgeta-Balić, Davenport, and Vrgoč (2013). Possibility of experimental farming of the Noah's ark shell was investigated by Peharda *et al.* (2013), Župan, Rogošić, Šarić, and Kanski (2013), Župan *et al.* (2014). Sustainable management of bivalve molluscs requires an interdisciplinary approach involving knowledge of the physiology of farmed species. Physiological responses of the bearded horse mussels have been investigated with regard to stress caused by temperature changes (Katsikatsou, Pörtner, & Michaelidis, 2009; Katsikatsou, Anestis, Pörtner, & Michaelidis, 2010; Ezgeta-Balić *et al.*, 2011). The influence of the size of animals on the metabolism was also investigated (Widdows, 1978a; Yukihira, Klumpp, & Lucas, 1998; Jadhav & Bawane, 2012; Taware & Muley, 2014). However, there is no available data on the impact of the size of the Noah's ark shell and the bearded horse mussel on physiological responses. In this paper, the influence of the size of the Noah's ark shell and the bearded horse mussel on metabolic responses was investigated, including oxygen consumption (OC), ammonia excretion (AE), the atomic ratio between oxygen consumption and ammonia excretion, i.e. O:N ratio and clearance rate (CR). Comparison of physiological responses of these two species due to the size of the individuals was also made. When assessing the suitability of certain species for culture, it is important to know how physiological responses are influenced by the size of an animal. The results of this study brings us to better understanding of the physiology of the Noah's ark shell and the bearded horse mussel and thus provide the contribution to the increase of knowledge about these species for the purpose of possible commercial use.

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Materials and Methods

Sampling and Design of Experiments

The samples of bearded horse mussel and Noah's ark shell were collected by autonomous diving in May 2014 and May 2015, respectively in Bistrina Bay (42° 52'11.41"N, 17° 42'06.73"E) within the Mali Ston Bay. On sampled individuals measurements of oxygen consumption and ammonia excretion were performed. The animals of both species, for clearance rate measurements, were collected at the same place, in May 2016. Living bivalves were transferred to the laboratory in Dubrovnik. The acclimation period of shellfish to the aquarium conditions lasted for seven days. Shellfish were fed daily with the flagellate *Tetraselmis suecica* in a density of $3x10^3$ cell ml^{-1} according to Widdows and Staff (2006).

The oxygen consumption (OC), ammonia excretion (AE) and clearance rate (CR) were measured with respect to the different size categories of shellfish. The animals were divided into three groups: smaller than 25 mm; 25 to 50 mm and larger than 50 mm. Each group consisted of 30 individuals. The measurements were performed on five individuals in each of three replicas. OC was measured at the end of the period between two feeding to avoid the effect of the feeding (Widdows & Staff, 2006). Both OC and AE were measured individually for each animal in the closed respiratory chamber with volume of 429.39 ml for larger animals and 74.35 ml for small animals. Prior to measurement the shellfish were cleaned off from fouling. For the first 30 minutess animals were kept in the chamber with flow through of seawater in order to adapt to chamber conditions, opening of the shell and start of breathing. After closing the flow through of water, for the next 40 min the values of dissolved oxygen were taken at intervals of five minutes. The oxygen concentration drop was measured by the Oxyscan graphic probe (UMS Gmbh, Germany). No significant drop in oxygen was observed in the control chamber without shellfish during the experimental period. After physiological measurements the volume of all individuals was measured and soft tissue was extracted and dried in tissue drier (TMA, Croatia) on 60°C for 24 hrs to constant mass The oxygen consumption rate i.e. VO_2 (mg O_2 h^{-1}) was calculated according to Widdows and Johnson (1988) using formula:

$$VO_2 = 60 \times \left[C\left(t_0\right) - C\left(t_1\right) \right] \times \left(Vr\right) / \left(t_1 - t_0\right)$$

where: t_0 and t_1 are the initial (min) and the final time (min) of the measurement period, C (t) is the oxygen concentration in water at time t, Vr is the volume of respirometry reduced by volume of shellfish. After oxygen measuring period, 50 ml of the sea water sample was taken from the closed chamber and fixated with 2 ml of phenol-alcohol solution for AE measurement. Prior to each measurement AE in the sea water was carried out and the obtained values were subtracted from the values measured in the experimental chamber. The concentration of ammonium was determined by the indolfenol blue method (Solorzano, 1969; Strickland & Parsons, 1972) with modification to Ivančić and Degobbis (1984). The ammonia excretion (AE) rate was calculated according to Sobral and Widdows (1997) using formula:

$$U = \left(T - C\right) \times \left(V/1000\right) / t$$

where: U is the ammonium excretion rate (μM NH_4 h^{-1}), T is the concentration of ammonium (μM) in the sample, C is the concentration of ammonium in the control sample, V is the volume (ml) of the seawater in which the animal is immersed, t is the time (h).

To standardize values all physiological rates were converted to a specific physiological rate per gram of dry mass of the animal. After the experiment, the soft tissue of the bivalve was dried in a drying chamber at 60°C for 24 h to a constant mass and dry soft tissue weight was obtained. Dry shellfish soft tissue weights were taken as the basis for calculating the specific physiological rates according Bayne and Newell (1983) using the following equation:

$$Ys = \left(Ws/We\right)^b \times Ye$$

where: Ys is the physiological rate for the animal of standard mass (1 g), Ws is the standard mass (1 g), We is the observed mass of the individual in grams, Ye is the ucorrected (measured) physiological rate, b is the mass exponent for the physiological rate. The mean employed b exponent of mass is 0.67 for CR and 0.75 for OC of bivalve (Savina & Pouvreau, 2004), and a value of 0.78 was used for AE according to Hawkins *et al.* (2000).

The atomic ratio of oxygen and nitrogen was determined on the basis of oxygen consumption and ammonia excretion, expressed in atomic equivalents according to Hawkins *et al.* (2002) using formula:

$$O/N = \left(mg\, O_2/16\right) / \left(mg\, NH_4/14\right)$$

Energetic expenditures are respiratory energy expenditure (R) and energy lost in excreta (U). Calculation of R and U (all in $Jg^{-1}h^{-1}$) is according to Widdows and Johnson (1988) as follows:

$$R = VO_2\left(ml\ O_2 g^{-1} h^{-1}\right) \times 20.33\ J\ ml^{-1} O_2$$

$$U = mg\ NH_4 g^{-1} h^{-1} \times 19.4\ J\ mg^{-1} NH_4$$

The clearance rate (CR) is the volume of water cleared from suspended particles in the unit of time. A closed system was used in which the clearance rate (CR) was calculated from exponential drop in algal cell concentration in the water tank over a period of 1.5-2 hours (Widdows & Staff, 2006). The algal cell density was measured in the tank half an hour after the addition of 20×10^{3} cell ml^{-1}, which was repeated at 60, 90 and 120 min. Bivalves were cleaned from fouling, placed in a container, left for 20 minutes to open the shells and then the algal cells (*T. suecica*) were added. Mixing of water was achieved by aeration. No significant drop in the cell abundance was observed during the experimental period in the control tanks without shellfish. CR measurements were carried out in containers of varying volume with respect to the size of the shellfish (Riisgård, 1988),volume of 5 l was used for larger and 2 l for smaller animals. CR of each bivalve was calculated using the following formula (Coughlan, 1969):

$$CR\left(l\,h^{-1}\right)=\mathrm{Vol}(l)\times\left(\log_e C1-\log_e C2\right)/time\ interval\ \left(h\right)$$

where: Vol is the volume of water, C_1 and C_2 are the cell concentrations at the beginning and the end of time interval respectively. Measurement of algal culture density was performed by measuring absorbance of chlorophyl at λ=750 nm in the medium using Hach DR2500 spectrophotometer. Regression analysis of the dependence of absorbance on known population density yielded the calibration curve, and subsequently, polynomial equation was used to calculate population density from absorbance data (Rodrigues, Raya-Rodriguez, & Fontoura, 2011).

Statistical Analysis

The data collected were tested for the homogenity of variance using Levene's test using Statistica package 12.0 (Statsoft Inc.). Normality of data was estimated by Kolmogorov-Smirnov and Liliefors test. Upon confirmation of normal distribution of values within groups, further analysis was carried out using parametric ANOVA and Tukey post-Hoc analysis. For the analysis of OC, AE, O:N ratio and CR with regard to different size of bivalves, one way ANOVA (P<0.01) was used. To compare the metabolic rates between species *Arca noae* and *Modiolus barbatus*, a t-test was used.

Results

Mean oxygen consumption of both *A. noae* (ANOVA, P=0.000195, F=10.543) and *M. barbatus* (ANOVA 0.000006, F=16.63) depended significantly on body size (Figure 1). In both investigated species, there was a trend of higher respiration rate in smaller animals. Post hoc Tukey test for *A. noae* revealed significant difference between groups <25 and 25-50 (Tukey, P<0.01) mm and between groups <25 mm and >50 mm (Tukey, P<0.001). For *M. barbatus* the post hoc Tukey test showed that there was a significant difference between the group <25 mm and 25-50 mm (Tukey, P<0.001) and between the group <25 and >50 mm (Tukey, P<0.001). Mean values of respiration energy loss of *A. Noae* ranged from 1.68±1.10 to 5.63±4.20 J $g^{-1}h^{-1}$, and for *M. Barbatus* from 2.44±0.90 to 11.63±7.48 J $g^{-1}h^{-1}$. T-test showed statistically significant difference for respiration rate between these two species for groups smaller than 25 mm (t=-2.610; P<0.001). The smallest and the largest groups of *M. barbatus* showed two times higher metabolic cost than same groups of *A. noae.*

Excretion rate (AE) of *A. noae* depended strongly (ANOVA, P<0.000676, F=8,899) on body size (Figure 2) and significant difference was observed for largest animals compared to the other size groups (Tukey, P<0.01). Ammonia excretion of *M. barbatus* was not influenced by body size. Mean

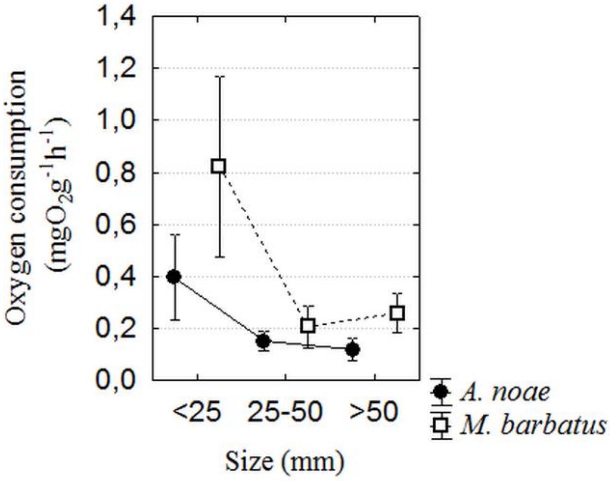

Figure 1. Relationship between oxygen consumption (mg $O_2g^{-1}h^{-1}$) and shell length (mm) of *A. noae* and *M. barbatus.* Values are given as mean±sd.

values of excretion energy loss of *A. Noae* were from 0.08±0.06 to 0.89±0.94 J g^{-1} h^{-1}, and for *M. Barbatus* from 0.22±0.30 to 0.38±0.22 J g^{-1} h^{-1}. AE rates are higher for larger animals. Metabolic cost of excretion is higher for small *M. barbatus* than *A. noae* of the same size group, but it is conversely for animals >50 mm. T-test showed a statistically significant difference for AE rate between *A. noae* and *M. barbatus* for organisms smaller than 25 mm (t=2.173; P<0.05).

O:N ratio of Noah's ark did not depend significantly on body size, but was signficant for bearded horse mussel (ANOVA P<0.001, F=25.6436) (Figure 3), and there was a trend of higher O:N ratio in both smaller animals. The post hoc Tukey test showed a significant difference between the groups <25 mm and >50 mm (Tukey, P<0.05) for *A. noae*, and between the groups <25 mm and 25-50 mm (Tukey, P<0.05) and the groups <25 mm and > 50 mm (Tukey, P<0.05) for *M. barbatus*. T-test did not show statistically significant difference of O:N ratio between two species .

Clearance rate of Noah's ark was significantly affected by the body size (ANOVA p=0,000027, F=28,7397) (Figure 4), and for bearded horse mussel the relation was not found to be significant. In both studied species, there was a trend of higher clearance rate in smaller animals. Post hoc Tukey test showed significant difference between the largest animals compared to the other size groups for *A. noae* (Tukey, P<0.01), and for *M. barbatus* between the groups <25 mm and> 50 mm (Tukey, P<0.01). T-test showed statistically significant differences between the investigated species for the size group from 25 to 50 mm (t-test, t=2.330; P<0.05) and for the organisms smaller than 25 mm (t-test, t = 2.794; P<0.05).

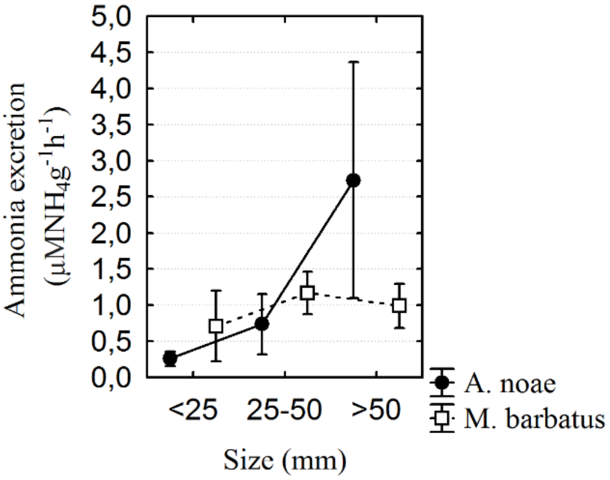

Figure 2. Relationship between ammonia excretion (µM NH4$g^{-1}h^{-1}$) and shell length (mm) of *A. noae* and *M. barbatus*. Values are given as mean±sd.

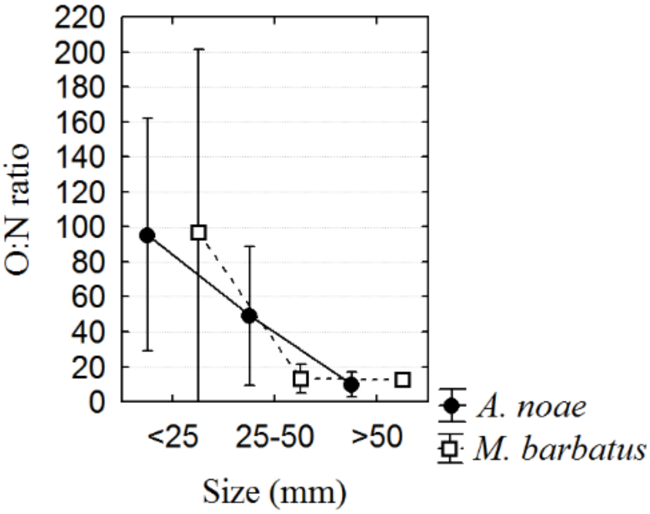

Figure 3. Relationship between O:N ratio and shell length (mm) of *A. noae* and *M. barbatus*. Values are given as

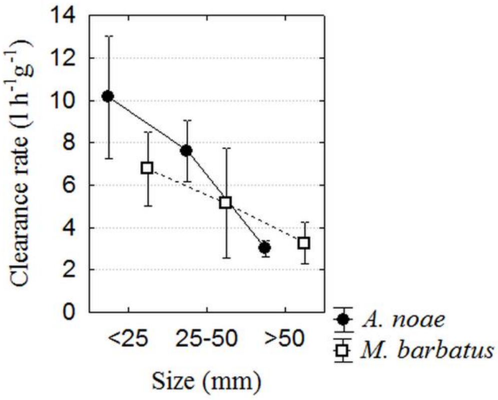

Figure 4. Relationship between clearance rate ($lg^{-1}h^{-1}$) and shell length (mm) of *A. noae* and *M. Barbatus*. Values are given as mean±sd.

Discussion

Metabolic rate is a fundamental biological rate. Brown, Gillooly, Allen, Savage, and West (2004) have developed the theory of varying metabolic rates with regard to body size and temperature. The body size is one of the main endogenous factors influencing the energy costs of organisms (Sukhotin & Pörtner, 2001). Large organisms need more resources but their energy flow is slower than in smaller organisms (Brown *et al*., 2004). The metabolic rate is often estimated as the rate of oxygen consumption (Gosling, 2003; Brey, 2010). In this study, in both *A. noae* and *M. barbatus* the respiration rate i.e. OC rate decreased with body size. Similar trend of higher OC of small individuals compared to larger was found by Mondal (2006), Resgalla, Brasil, and Salomão (2006),Taware and Muley (2014). Bayne and Newell (1983) suggested that the mass-specific metabolic rate was greater in small individuals. The results of the OC in our study are consistent with the values observed by Savina and Pouvreau (2004), Tang, Liu, Yang, and Xiang (2005), Taware and Muley (2014). Contrary to the results of this study, higher OC in larger organisms was shown by Navarro and Winter (1982), Winter, Acevedo, and Navarro (1984), Yukihira *et al*. (1998), they however did not convert measured values of physiological rates into a specific rates. The same authors also observed a higher clearance rate for larger organisms probably because of the same reason. Ezgeta-Balić *et al*. (2011) observed a respiration rate of 9.83 to 20.89 μmol O_2 $h^{-1}g^{-1}$ when studying the energy budget of bearded horse mussels. That study reports somewhat lower values for large animals of *M. barbatus*, which can be caused by performing measurements during different season and different phase of the gametogenetic cycle (Newell & Bayne, 1980) .

The group of bivalves larger than 50 mm, and probably a part of the group of 25-50 mm are aproximately 5-6 years old (Peharda *et al*., 2003; 2007). Some studies reported that the OC rate decreased with the aging of bivalves (Sukhotin & Pörtner, 2001; Zotin & Ozernyuk, 2004). Therefore, the metabolic rate of the largest size group of explored bivalves in our study might has been influenced by the age. In a long-living species the age doesn't affect the respiration rate for the first hundred years (Begum *et al*., 2009). The Noah's ark shell can live 25 years (Puljas, Peharda, Župan, & Bukša, 2015) and the bearded horse mussels about 20 years (Peharda *et al*., 2007) and since they are not long-living species the OC might be affected by their aging.

For both studied species increase in AE rate is observed with an increase in the body size, consistent with finding that small animals had a relatively low rate of ammonia excretion during spring (Bayne & Scullard, 1977a). Obtained values of the excreted ammonia are consistent with the results reported by Widdows and Johnson (1988), Navarro and Gonzalez (1998) and James, Weatherhead, and Ross (2001). It should be mentioned that Bayne and Scullard (1977b) investigating specific dynamic action of the mussel *M. edulis* in experiments in which shellfish were fed with flagellate *T. suecica* found an increase in AE 24 to 30 hours after the feeding in almost a half of the experiments. These data suggest that the release of ammonia is likely affected by the type of food used in the experiment. Emerson (1969) found the difference in the excretion of ammonia in two repeated experiments of the same design, and the possible reason was variation from the time of animal collection to experimentation. AE increases to the highest values in the spring and summer when the animal retains mature gametes in the mantle (Widdows, 1978a). Small individuals in the spring have relatively reduced AE, probably due to the use of carbohydrates as energy sources, while larger individuals rely more on proteins in this part of the year, and increased protein metabolism results in increased AE (Bayne & Scullard, 1977a). For both studied species, increase in size causes decrease of the

O:N ratio. The measure of the atomic ratio of oxygen consumption and nitrogen excretion gives the data on the substrate for the maintenance of metabolism (Corner & Cowey, 1968). Values of O:N ratio higher than 30 indicate the degradation of carbohydrates and lipides, while the values lower than 30 indicate the degradation of proteins and stressed condition (Bayne & Thompson, 1970; Widdows, 1978b). For Noah's ark shell size-groups of 25 to 50 mm and less than 25 mm the value of O:N ratio is greater than 30 indicating that they use carbohydrates and lipides as a metabolic substrate. The same applies to a group of the beardeed horse mussels smaller than 25 mm.

In bivalve molluscs, due to a disproportionate reliance on protein catabolism for energy production by smaller individuals the relationship between ammonia excretion rates and body size can be very variable (Bayne & Newell, 1983). This very variable metabolic trait may account for greater variability of oxygen consumption and ammonia excretion and resulting O:N ratio in our group of animals smaller than 25mm.

For energy gains, the clearance rate was investigated. In both *A. noae* and *M. barbatus* the clearance rate decreased with body size. A similar trend of higher clearance rates of small individuals compared to larger ones was found by Sylvester, Dorado, Boltovskoy, Juárez, and Cataldo (2005) and Resgalla *et al.* (2006). Small animals have a higher clearance rate than large ones (Widdows, 1978a) probably due to higher metabolic demands of younger specimens and due to reducing of gill surface to body size ratio with age (Kryger & Riisgard, 1988). Our results for individuals larger than 50 mm are consistent with the values given by Widdows and Johnson (1988), James *et al.* (2001), Widdows, Burns, Menon, Page, and Soria (1990). Ezgeta-Balić *et al.* (2011) documented for the mussel *M. barbatus* a much lower clearance rate (0.17 to 0.23 l h^{-1} g^{-1}). Shellfish in that experiment were fed with larger food quantity and another type of algal food (*Isochrysis galbana*), animals were collected in November, while for this study in May when bivalves are in a different phase of the gametogenetic cycle. Since this species is growing faster from May to August (Peharda *et al.*, 2007), higher energy consumption is required in that part of year.

Comparison of the metabolic rates (OC, AE and CR) between the Noah's ark shell and bearded horse mussel showed statistically significant difference for groups of individuals smaller than 25 mm. Large populations of small animals would filtrate huge amount of seawater during feeding and possibly reduce food quantity. The density of farmed bivalves should not overmatch the available energy in the ecosystem. Understanding energy gains is important for assessing the ecological carrying capacity of the farming area (Gibbs, 2007). Small and middle sized *A. noae* need more food than *M. barbatus* and for that reason *M. barbatus* is favorable species for aquaculture. On the other hand *M. barbatus* needs more oxygen in all size categories. Water replacement condition of chosen site should be taken in account. The present experiment provides a valuable information on physiological responses that can be useful for selecting aquaculture sites. Data on values of metabolic rates with respect to the size categories can help in ecological modelling to achieve more accurate results. The period of the year should also be taken into account and therefore it would be important to investigate the effect of shellfish size on their metabolic responses in different seasons.

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