

SEASONAL CHANGES OF THE CONDITION INDICES AND HAEMOCYTE COUNTS OF THE *AUSTROPOTAMOBIOUS TORRENTIUM* POPULATION IN KRALJEVEC STREAM, CROATIA

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ABSTRACT

The stone crayfish, *Austropotamobius torrentium* (Schrank, 1803) is one of the four native European crayfish living in Croatian freshwaters. Kraljevec Stream is situated on the southern slopes of Medvednica Mountain (north-west Croatia) at 400 m above-sea level. Our research was carried out for one year, from May 2003 to April 2004. During the study period water temperature varied between 2 to 17.2°C, alkalinity between 100 and 200 mg CaCO₃/L, the mean oxygen concentration and pH was 12.1 mg/L and 8.3, respectively. Crayfish were trapped by baited hand made traps, which were secured in a stream current with the stones and roots of the surrounding trees. We caught 100 crayfishes, 68 males and 32 females. Hemolymph was sampled 10 to 15 min after the catch. EDTA anticoagulant was used to prevent hemolymph coagulation and 10% formalin was used to fix haemocyte for differential counts. Each month five or six specimens of each sex, if possible, were sacrificed and hepatosomatic, gonadosomatic and muscle indices were measured. We found statistically significant differences in hepatosomatic and gonadosomatic indices between months and between sexes. Muscle indices were significantly different between males and females. The average total haemocyte counts were 7.6×10^5 cells/ml and 6.5×10^5 cells/ml hemolymph for males and females, respectively. The average hyalinocyte, granulocyte and semigranulocyte ratio was 77: 14.5: 8.5%. There were no statistically significant differences in total haemocyte counts between months and between sexes.

Key-words: *Austropotamobius torrentium*, condition indices, haemocyte counts, Croatia.

CHANGEMENTS SAISONNIERS DES INDICES DE CONDITION ET DES DÉNOMBREMENTS D'HÉMOCYTES DANS LES POPULATIONS D'*AUSTROPOTAMOBIOUS TORRENTIUM* DU RUISSEAU DE KRALJEVEC, CROATIE

RÉSUMÉ

L'écrevisse pierre (« écrevisse des torrents en français »), *Austropotamobius torrentium* est l'une des quatre écrevisses natives européennes vivant dans les eaux douces croates. Le ruisseau de Kraljevec est situé sur le versant sud de la montagne de Medvednica (Nord-Ouest de la Croatie) à 400 mètres au-dessus du niveau de la mer. Notre travail de recherche s'est déroulé sur un an, de mai 2003 à avril 2004. La température a varié entre 2 °C et 17,3° C, la concentration moyenne d'oxygène était de 12,1 mg/L, le pH

moyen 8,3 et l'alcalinité entre 100 et 200 mg CaCO₃/L. Nous avons capturé les écrevisses au moyen de pièges apâtés faits à la main, protégés du courant au moyen des pierres et des racines des arbres environnants. 100 écrevisses ont été capturées, 68 mâles et 32 femelles. Les échantillons d'hémolymphe ont été prélevés 10 à 15 minutes après la capture. L'anticoagulant EDTA a été utilisé pour empêcher la coagulation de l'hémolymphe et une solution de formaldéhyde à 10 % pour fixer les hématocytes en vue du dénombrement. Chaque mois 5 à 6 animaux ont été sacrifiés pour mesurer les indices hépatosomatiques, gonadosomatiques, et musculaires. Nous avons trouvé des différences statistiquement significatives dans les indices hépatosomatiques et gonadosomatiques des deux sexes et selon les mois. Les indices musculaires étaient considérablement différents entre mâles et femelles. Le nombre total moyen d'hématocytes pour les mâles était de $7,6 \times 10^5$ cellules/ml d'hémolymphe et pour les femelles de $6,5 \times 10^5$ cellules/ml. Le ratio moyen de hyalinocytes, granulocytes et semigranulocytes était de 77 : 14,5 : 8,8 % respectivement. Il n'y a pas de différence statistiquement significative entre les nombres totaux d'hématocytes des différents mois et entre les sexes.

Mots-clés : *Austropotamobius torrentium*, indices de condition, dénombrement d'hématocytes, Croatie.

INTRODUCTION

The stone crayfish, *Austropotamobius torrentium* (Schrank, 1803) is one of the four native European crayfishes living in Croatian freshwaters and is protected by law nationally (NARODNE NOVINE 72/94; NARODNE NOVINE 76/98). This species is well adapted to lower water temperature and velocities typical for streams at higher altitudes (BOHL, 1987). *A. torrentium* is not as well investigated as other species of Astacidae family in Europe and recent research of this species in Croatia contributed to the knowledge on its biology and ecology (BOHL, 1987; LAURENT, 1988; MAGUIRE, 2002). Information on organosomatic indices and haemocyte counts in this species are lacking although some Croatian authors measured muscle and hepatopancreas participation in the body weight of *A. torrentium* (OBRADOVIĆ, SEKULIĆ and RAC, 1988).

Changes in the relative size of hepatopancreas (hepatosomatic indices) and its moisture content are used to evaluate nutritional status, as well as crayfish condition and exposition to environmental stress (MANNONEN and HENTTONEN, 1995). The annual cycle of freshwater crayfish can be characterized by changes in hepatopancreas energy content, which serves as the main source for the ovarian development, growth and moulting of the crayfish (HUNER, KÖNÖNEN and LINDQVIST, 1990; MANNONEN and HENTTONEN, 1995; JUSSILA, 1997). KYOMO (1988) studied the relationship between gonad indices and hepatopancreas indices in males and females of *Sesarma intermedia* (De Haan, 1935) while YAMAGUCHI (2001) investigated changes of hepatopancreas and testis indices during the reproductive period of male fiddler crabs, *Uca lactea* (De Haan, 1835). If tail muscle is considered as a storage site of the energy reserves, tail muscle to body weight ratio and tail moisture levels can be considered as a condition index. Muscle indices were used as a measure of condition in dietary studies (FOTEDAR, 1998; TSVETNENKO *et al.*, 1999), as well as in comparative studies of morphology and edible tissues of two commercially important crayfishes (HUNER, LINDQVIST and KÖNÖNEN, 1988).

Changes in total haemocyte counts were used as an indicator of stress caused by injuries (JONES, 1999), transport conditions (JUSSILA *et al.*, 1999) or environmental contamination (LE MOULLAC and HAFFNER, 2000; YILDIZ and BENLI, 2004). Also, JOHANNSON *et al.* (2000) noticed that total haemocyte counts change during the moulting period. Published data on total haemocyte counts in the Astacidae family are lacking while there are some data on haemocyte counts in other species belonging to Decapoda (CORNIC and STEWART, 1978; JUSSILA, 1997; YILDIZ and BENLI, 2004).

It is generally accepted that in Decapoda there are three basic types of haemocytes: hyalinocytes, semigranulocytes and granulocytes (WOOD and VISENTIN, 1967; ANDERSON, 1981; BAUCHAU, 1981; MARTIN and GRAVES, 1985; MARTIN and HOSE, 1992) and VACCA and FINGERMAN (1983) described changes of differential haemocyte counts during moulting period.

As there are almost no data on organosomatic indices of *A. torrentium* the aim of this research was to measure these indices and describe their seasonal changes. Standard condition indices (hepatosomatic, gonadosomatic and muscle indices) were chosen and compared between months and between sexes. Moreover, there are no data on total and differential haemocyte counts in *A. torrentium* at all so these parameters were measured as well and compared between seasons and between sexes. Kraljevec Stream is located in the protected area of the Nature Park Medvednica, which is close to the capital city of Croatia, Zagreb and is very popular destination of the urban population. As many populations of *A. torrentium* in that area were vanished, mainly because of anthropogenic activities, research was made on a small sample size, which was approved by the management of the Nature Park.

MATERIAL AND METHODS

Stone crayfish individuals were trapped from the Kraljevec Stream located on the southern slopes of Medvednica Mountain (north-west Croatia) at 400 m above the sea level in a beech and chestnut-tree forest. Baited hand-made traps were applied for one year from May 2003 to April 2004. Physical and chemical parameters of the stream were measured (temperature, oxygen, alkalinity, pH). Hemolymph of all crayfish caught was sampled 10 to 15 minutes after catch. For total haemocyte counts hemolymph was diluted with EDTA based anticoagulant (0.49 M NaCl, 30 mM trisodium citrate, 10 mM EDTA), the hemolymph and anticoagulant ratio was 1:1. Counts of un-coloured cells were made in Neubauer chamber at 50 × microscope magnification (VÁSQUEZ *et al.*, 1997). Each month, hemolymph from a few randomly chosen crayfish was sampled for differential haemocyte counts. For that purpose, hemolymph was diluted with 10% formaline at 3:1 ratio and haemocytes were fixed 2-3 hours on 6°C. Afterwards, smears were made and stained with the WRIGHT stain, which stained the granule of granulocytes in orange-red and the granule of semigranulocytes in green-blue. On each of two randomly chosen smears, 200 hemocytes were counted and the ratio of three different cell types was calculated (according to MIX and SPARKS, 1980).

We had no catch in traps during January and February and moreover, females were caught only from May until September. Therefore, the statistical analysis was made only for that period when crayfish were active. Each month, 5 or 6 males and females were sacrificed and dissected within 20 to 24 hours of their collection. Hepatopacrea, gonads and tail muscle were removed, placed in a foil cups and weighed. Organs were then dried at 80°C for 24 hours and weighed again. Organosomatic indices were expressed as wet organosomatic index (Oi_{wet} ; equation 1) and organ moisture content (OM; equation 2) (JUSSILA, 1997).

$Oi_{wet} = W_{wo} \times 100/W_t$ and (2) $OM = (W_{wo} - W_{do}) \times 100/W_{wh}$, where: W_{wh} = weight of wet organ (g); W_t = total weight of crayfish (g); and W_{do} = weight of dry organ (g)(1)

Results were expressed as mean values. The normality of data distribution is tested by Shapiro-Wilks'W test and afterwards differences in condition indices between sexes and between months were analysed using nonparametric analysis of variance (KRUSKAL-WALLIS test). SPEARMAN correlation test was used for correlations between the weights of the organs and the whole animal. Parametric one-way analysis of variance (ANOVA) was used to test difference in differential haemocyte counts between sexes and between

months. We were using Statistica 5.0 for statistical analysis. Results were considered statistically significant at $p < 0.05$.

RESULTS

We caught altogether 100 crayfish, 68 males and 32 females. The sex ratio (males to females) was 2.1:1 ($S_0 = 0.36$). The average male weight was 18.8 ± 1.3 g, it varied between 2.9 and 4.6 g while the average female weight was 12.9 ± 1 g and it varied between 5 and 26 g. Total lengths (TL) of males range between 4.1 and 9.2 cm, the mean was 7.0 ± 0.1 cm and the total length of the females ranged between 2.7 and 8.8 cm with its mean value at 6.7 ± 0.2 cm. Ten males less than 5 cm TL and three females less than 6 cm TL were excluded from the analysis. Analysis was made for males caught from March until December (ten months analysed) and for females caught from May until September (five months analysed).

The average annual temperature of the stream was $9.4 \pm 1.6^\circ\text{C}$, its oxygen concentration $12.1 \pm 0.8^\circ\text{C}$, pH 8.3 ± 0.1 and its alkalinity varied between 100 and 200 mg CaCO_3/L .

Stone crayfish males had a higher relative hepatopancreas weight (Hi_{wet} 6.7-7.2%) and lower moisture content (HM 45-64%) at the beginning of the year (March, April) and during the mating period in October when Hi_{wet} was 6.6% and HM was 61% (Figures 1 and 2). During moulting (from June until September) the Hi_{wet} of males varied between 2.4 and 5.7% and HM varied between 67.3 and 83% (Figures 1 and 2). Females had lower Hi_{wet} in September (4.6%) than during moulting when it was around 5.6%. The moisture content of hepatopancreas in females decreased from June (83%) to September when 72% of moisture was determined.

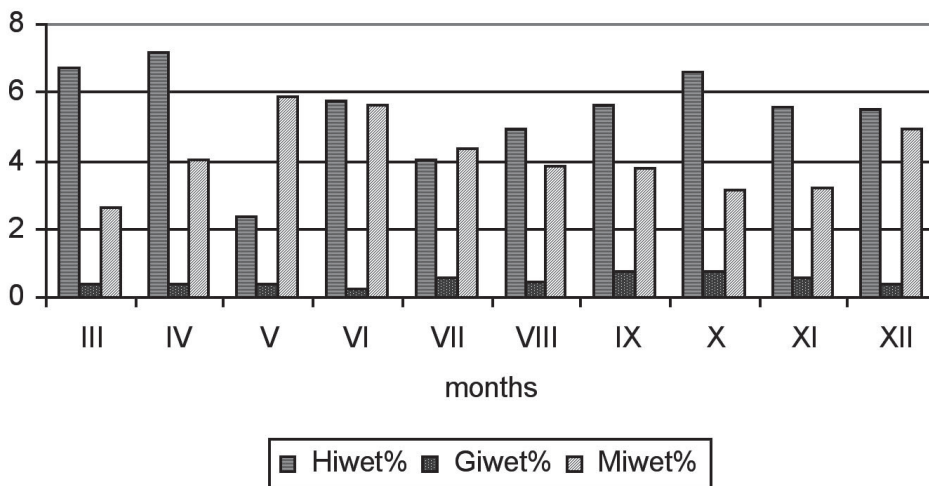


Figure 1
 Mean values of relative weight of hepatopancreas (Hi_{wet} %), gonads (Gi_{wet} %) and tail muscle (Mi_{wet} %) of *Austropotamobius torrentium* males from March to December, in Kraljevec Stream, Croatia.

Figure 1
 Valeurs moyennes du poids relatif de l'hépatopancreas (Hi_{poids} %), des gonades (Gi_{poids} %) et du muscle de la queue (Mi_{poids} %) d'*Austropotamobius torrentium* mâle de mars à décembre, dans le ruisseau Kraljevec, Croatie.

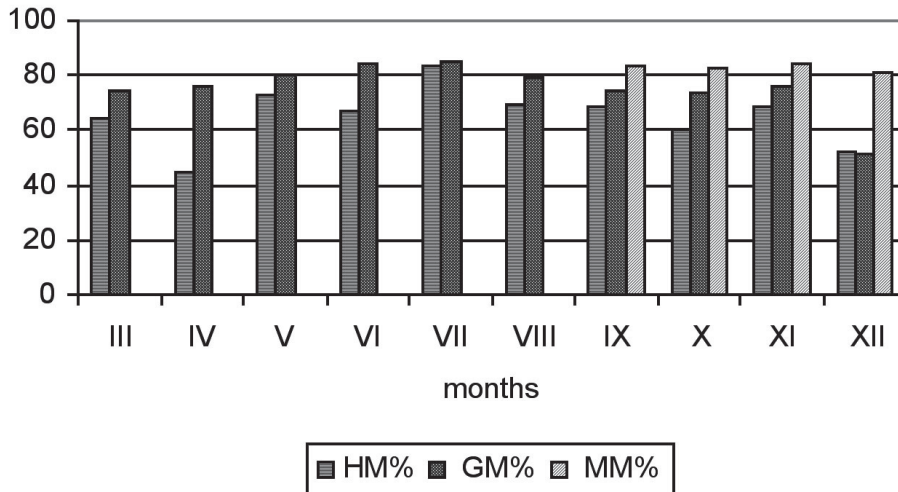


Figure 2

Average moisture content of hepatopancreas (HM%), gonads (GM%) and tail muscle (MM%) of *Austropotamobius torrentium* males from March to December, in Kraljevec stream, Croatia.

Figure 2

Humidité moyenne du contenu de l'hépatopancreas (HM%), des gonades (GM%) et du muscle de la queue (MM%) d'*Austropotamobius torrentium* mâles de mars à décembre, dans le ruisseau Kraljevec, Croatie.

The relative weight of the ovaries gradually increased from May ($G_{i_{wet}}$ 0.7%) until September ($G_{i_{wet}}$ 6.4%) while the weight of testis increased from 0.4 to only 0.8% during the same period (Figure 1). The relative testis weight decreased from 0.8 to 0.4% from October to April (Figure 1). The moisture contents of the ovaries were the lowest in August and September; they varied between 43.7-58.5% while from May to July moisture content ranged between 80 and 90%. The moisture content of the testis was the lowest in December (around 50%) while from March to November varied between 73 and 85% (Figure 2). While preparing to mate and during mating (from August to November) the moisture content of testis was lower than during moulting, it ranged between 75 and 80% (Figure 2).

The KRUSKAL-WALLIS test indicated statistically significant differences in all hepatosomatic and gonadosomatic indices between months ($p < 0.05$) (Table I). Males and females differed significantly in the hepatopancreas moisture content and wet gonad index (Table I).

Differences in the relative weight of the tail muscle between the months were close to statistical significance (KRUSKAL-WALLIS test; $p = 0.0559$). In males, lower relative weights of the tail muscle were detected during the moulting and mating period (from July to November) (Figure 1). Tail muscle moisture content varied in males from 80 to 87% between September and December (Figure 2). In females, the tail muscle/body weight ratio gradually decreased from 8.9% measured in June to 7.5% measured in September. Females had a significantly higher tail muscle/body weight ratio than males (KRUSKAL-WALLIS test; $p = 0.008^*$). The tail muscle/body weight ratio varied between 6.3 and 8.9% in females while it was between 2.7 and 5.9% in males.

Statistically significant positive correlation was found between the weight of the hepatopancreas, gonads, tail muscle and total body weight (SPEARMAN correlation test;

Table I

Statistical analysis (Kruskal-Wallis test) of hepatosomatic and gonadosomatic indices of *Austropotamobius torrentium* compared between months and between sexes, in Kraljevec Stream, Croatia.

Tableau I

Analyse statistique (test de Kruskal-Wallis) des indices hépatosomatiques et gonadosomatiques d'*Austropotamobius torrentium* sur plusieurs mois et entre sexes.

| Kruskal-Wallis test | Hepatosomatic indices | p | Gonadosomatic indices | p | F |
|-------------------------|-----------------------|---------|-----------------------|---------|---|
| Compared between sexes | $H_{i_{wet}}$ | 0.6398 | $G_{i_{wet}}$ | 0.0003* | 1 |
| | HM | 0.0101* | GM | 0.0645 | 1 |
| Compared between months | $H_{i_{wet}}$ | 0.0303* | $G_{i_{wet}}$ | 0.0436* | 8 |
| | HM | 0.0219* | GM | 0.0088* | 8 |

* Statistically significant difference.

* Différence statistiquement significative.

$r = 0.605^*$, $r = 0.3812^*$, $r = 0.672^*$, respectively). Correlation between hepatopancreas, gonads and tail muscle relative weight and total body weight were negative and statistically significant (SPEARMAN correlation test; $r = -0.387^*$; $r = -0.3812^*$; $r = -0.389^*$, respectively).

Seasonal changes of the total haemocyte counts (THC) are presented in Figure 3. There were no significant differences in the THC between the months (KRUSKAL-WALLIS test; $p = 0.3279$) or the sexes (KRUSKAL-WALLIS test; $p = 0,4179$) although the mean THC was higher during the moulting period compared to the rest of the year (Figure 3). The average total haemocyte count was 7.6×10^5 cells/ml and 6.5×10^5 cells/ml hemolymph for males and females, respectively.

Differential haemocyte counts (DHC) were not significantly different between males and females (ANOVA; hyalinocytes $p = 0.6556$; granulocytes $p = 0.519$; semigranulocytes $p = 0.9854$). The average ratio of hyalinocytes, granulocytes and semigranulocytes was 77.6: 13.9: 8.5% in males, while a slightly different ratio, 76.5: 15:8.5%, was determined in females. The ratio of all three-cell types was significantly different between months (ANOVA; hyalinocytes $p = 0.021295^*$; granulocytes $p = 0.001570^*$; semigranulocytes $p = 0.033814^*$).

DISCUSSION

Kraljevec Stream is, by its characteristics, an ideal habitat for the stone crayfish if compared with the description given by BOHL (1987). Physical and chemical characteristics are in accordance with the data previously reported as being optimal for this species (MAGUIRE, 2002). Crayfish population of the stream was estimated on 1 218 individuals (between 1 039 and 1 472) by mark-recapture method at the year 2002 (MAGUIRE, 2002). The absence of crayfish in traps during January and February is probably the result of very low water temperature (between 2 and 2.8°C) while the absence of females from October to May indicated the period when they bear eggs. Increase in the water temperature in March (4.4°C) induced the activity of crayfish as it was previously described by other authors (BOHL, 1987; MAGUIRE, 2002). Thus, the unequal sex ratio as a result of seasonal

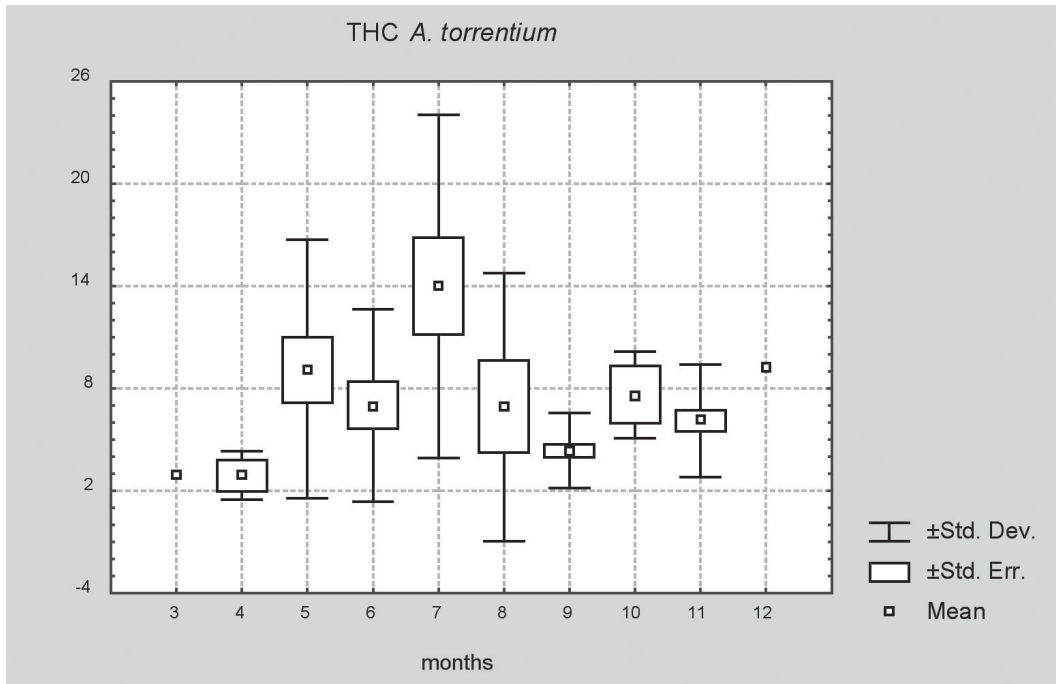


Figure 3

Standard deviations, mean and standard errors of total haemocyte counts (THC) in *Austropotamobius torrentium* from March to December, in Kraljevec Stream, Croatia.

Figure 3

Déviations standards, erreurs moyennes et standards des dépouillements d'hémocytes totaux (THC) d'*Austropotamobius torrentium* de mars à décembre, dans le ruisseau Kraljevec, Croatie.

inactivity of females during bearing eggs was also recorded in other species of Astacidae family (ACKEFORS, 1999; ALEKNOVICH, KULLESH and ABLOV, 1999).

Changes in the relative size of the hepatopancreas (hepatosomatic indices), gonads (gonadosomatic indices) and tail muscle (muscle indices) were detected in farmed as well as in wild crayfish populations (HUNER, LINDQVIST and KÖNÖNEN, 1988; HUNER, KÖNÖNEN and LINDQVIST, 1990; JUSSILA, 1997; YAMAGUCHI, 2001; CARMONA-OSALDE *et al.*, 2004). The higher hepatopancreas moisture content and at the same time lower relative hepatopancreas weight during the moulting period of males (from July to September) is the result of spending their energy reserves in the postmoult period as it was previously described by some authors (DAUPHIN-VILLEMANT, BÖCKING and SEDLMEIR, 1995; YAMAGUCHI, 2001). Moreover, most of the males caught during that period were already moulted and thereby consumed nutrients stored in hepatopancreas during the intermoult period. With approaching the mating period (October) wet hepatopancreas weight increased and moisture content decreased which is the opposite than what YAMAGUCHI (2001) and VIKINKOSKI *et al.* (1995) found during the mating period. One possible explanation of this difference might be the small sample size used in our research. On the other hand, it is also possible that males accumulated energy in the hepatopancreas and testis at the same time because the energy demand of the spermatogenesis is very low (GHERARDI, 2002). Similarly to our findings, no clear relationship between the hepatopancreas and gonad indices was found in *Sesarma intermedia* males by KYOMO

(1988). The energy accumulation in the testis is indicated by the slight increase of $G_{i_{wet}}$ and the decrease of GM while approaching to mating period (Figures 1 and 2). KYOMO (1988) detected that the gonad index of males fluctuated all year round within a very small range, i.e. between 0.5 and 0.9% while in our study it fluctuated between 0.4 and 0.8%. In the females $H_{i_{wet}}$ and HM decreased by approaching the mating season in October while continuous and significant increase of $G_{i_{wet}}$ and decrease of GM was detected during the period from May to October. These findings indicated that the relative hepatopancreas weight decreased with the increase of relative ovaries weight while energy accumulated in both organs although the energy accumulation is more intensive in ovaries (decrease of GM from 82.7 to 72%). Many other authors also found an increase in the relative gonad weight with approaching to mating period (PALACIOS, IBARRA and RACOTTA, 2000; ZAPATA *et al.*, 2003; CARMONA-OSALDE *et al.*, 2004).

Seasonal changes in hepatosomatic and gonadosomatic indices were statistically confirmed in all tested parameters. Males and females differed significantly in hepatopancreas moisture content and relative gonad weight and this finding confirms the different energy demands of oogenesis and spermatogenesis (GHERARDI, 2002). Similarly to our results, HUNER, LINDQVIST and KÖNÖNEN (1988) also found no significant differences in the relative hepatopancreas weight between males and females. These findings probably reflect many important functions of this gland.

Female stone crayfish had significantly higher weight of abdominal muscle than males. This data is in agreement with the findings of HUNER, LINDQVIST and KÖNÖNEN (1988) for species *Astacus astacus* and *Procambarus clarkii*. The bigger tail muscle in females is connected with its role in bearing eggs. Decrease in the relative tail muscle weight of both males and females during the moulting and the mating periods (Figures 1 and 2) may reflect the higher level of activity during that period.

The positive correlation between organ weight and total body weight and negative between relative organ weight and total body weight also confirmed the findings of OBRADOVIĆ, SEKULIĆ and RAC (1988) on the same species. These results indicated that hepatopancreas, gonads and tail muscle relative weights do not increase proportionally with the total body weight. Similarly, HUNER, LINDQVIST and KÖNÖNEN (1988) found that the proportions of tail muscle and hepatopancreas do not increase geometrically with the increase of total body lengths.

We did not find significant differences in total haemocyte counts (THC) between males and females of the stone crayfish and as there are no data on sexual differences in this respect in other articles we assume that there are no sex differences in other species, either (JUSSILA, 1997; EVANS *et al.*, 1999; JUSSILA *et al.*, 1999; LE MOULLAC and HAFNER, 2000; YILDIZ and BENLI, 2004). Although THC is often used as an indicator of stress (JONES, 1999; YILDIZ and BENLI, 2004; EVANS *et al.*, 1999) we agree with the opinion of JOHANSSON *et al.* (2000) that this parameter is not a reliable condition index, mainly because of great individual differences noticed during the counting of haemocytes. Differences in THC between months were not significant although mean THC-s were higher during the moulting period (Figure 3) which may be connected with the role of hemocytes in the tanning and mineralization of the exoskeleton after moulting. VACCA and FINGERMAN (1983) and HOSE *et al.* (1992) established that hyalinocyte number increased during moulting while granulocyte number grew during intermoult period. Significant changes in the ratio of different cell types during the year found in our research are in agreement with the findings of VACCA and FINGERMAN (1983) and HOSE *et al.* (1992). No significant differences in differential haemocyte counts of males and females found in our research confirmed the findings of CORNICK and STEWART (1978) in *Homarus americanus*. Haemocyte ratio similar to what we found was published by VASQUEZ *et al.* (1997) for freshwater shrimp *Macrobrachium rosenbergii* (70% hyalinocytes, 20% granulocytes and 10% undifferentiated cells) while HARDY (1892) mentioned that *Astacus*

fluviatilis has three times more hyalinocytes than granulocytes which is also in agreement with our findings for *A. torrentium*. On the other hand, marine Decapods have different haemocyte ratio (CORNICK and STEWART, 1978; HOSE, MARTIN and GERARD, 1990).

CONCLUSIONS

The annual cycle of the stone crayfish is characterized by changes in hepatosomatic and gonadosomatic indices. Gonadosomatic indices increased gradually with the approaching reproductive season while the changes in the hepatosomatic indices were not so clear. Hepatosomatic and gonadosomatic indices of males and females were significantly different because of different energy demands of oogenesis and spermatogenesis. Relative tail muscle weight is higher in females than in males. The weight of hepatopancreas, gonads and tail muscle is directly proportional to the total body weight but their ratio to the body weight is indirectly proportional to it. Total haemocyte counts did not change significantly during the year while the ratio of different cell types did. As the total and differential haemocyte counts show great individual differences they should not be used as reliable condition parameters. As the studied species is protected by law in Croatia and the population is located in a protected area of Nature Park Medvednica the number of specimens approved for sacrifice and analysis was small and the trends identified here need to be confirmed during a second year of the study.

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