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The Activity of Cholinesterases in Diapausing and Flying Red Mason Bees *Osmia bicornis* (Megachilidae)

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The red mason bee (*Osmia bicornis*) is a highly effective pollinator that is exposed to various xenobiotics. The organism's potential resistance to the toxic effects of xenobiotics can be determined based on cholinesterase activity. The activity of cholinesterases (ChEs) towards acetylcholine (ACh) and butyrylcholine (BCh) was determined in extracts of diapausing (between October and late March) and flying bees (May). In both males and females, enzyme activity was higher towards ACh than towards BCh. The ratio of ACh/BCh activity was determined in the range of 1.43 to 4.15 in diapausing females and 3.00 to 7.18 in diapausing males. No significant changes in ChE activity towards ACh were observed in females before December and in males before February. Enzyme activity towards ACh increased dynamically in the second half of March. Enzyme activity towards BCh remained stable in both sexes until mid-March, after which it increased significantly. Excluding mid-March, enzyme BCh activity was significantly higher in females than in males. The activity of carboxylesterase towards 4-p-nitrophenyl butyrate was determined in females to assess the involvement of non-specific esterases in the hydrolysis of choline esters. Carboxylesterase activity was low in comparison with cholinesterase activity, and it remained practically unchanged throughout diapause, suggesting that choline esters in female *O. bicornis* extracts were hydrolyzed mainly by acetylcholinesterases.

Key words: *Osmia bicornis*, cholinesterases, carboxylesterases, solitary bee, diapause.

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Contemporary agriculture relies heavily on crop protection, including insecticides that also affect beneficial insects. Chemical compounds present in therapeutic agents, insecticides and fungicides can interact to deliver even more toxic effects (JOHNSON *et al*. 2013). Many of them, including organophosphates and carbamates, affect the nervous system of insects and inhibit cholinesterase activity (ChE) (CASIDA & DURKIN 2013). Acetylcholinesterase (AChE) is a biomarker of environmental contamination with chemical compounds that pose a threat to the health of humans and animals (BADIOU & BELZUNCES 2008; CASIDA & DURKIN 2013). Insect enzymes, mostly AChEs in honey bees, are also used as biomarkers (BADIOU-BÉNÉTEAU *et al*. 2013).

Acetylcholinesterase (EC 3.1.1.7), an enzyme involved in the termination of impulse transmission, is considered an important target for insecticides and acaricides (CASIDA & DURKIN 2013). Adaptive mutations of AChE genes are associated with an increase in insect resistance to insecticides (CASSANELLI *et al*. 2006; SHANG *et al*. 2012). Recent research has demonstrated that besides vertebrates, AChE also induces noncholinergic effects in insects. AChE gene silencing disrupts larval development, locomotor activity and fertility in insects (WILLIAMSON & WRIGHT 2013; WILLIAMSON *et al*. 2013).

In addition to true acetylcholinesterase that participates in neurotransmission, vertebrates also possess a less specific enzyme, butyrylcholinesterase (BChE, EC 3.1.1.8), which is involved mainly in detoxification processes. BChE neutralizes organophosphate and carbamate inhibitors via esterase activity before they reach acetylcholinesterase, thus protecting AChE (AURBEK *et al*. 2009). Unlike other esterases, AChE and BChE rapidly hydrolyze choline esters, but differ in their specificity for various esters. AChE hydrolyzes acetylcholine much faster than other choline esters.

It is also much less active towards butyrylcholine. BChE demonstrates lower activity towards both choline esters than AChE (COKURGRAS 2003).

AChEs in insects are coded by two genes: *ace 1* and *ace 2*. Their products, enzymes AChE1 and AChE2, differ in tissue distribution and molecular and kinetic properties. In many insects, AChE1 is characterized by much higher expression and activity in CNS, and is considered the major cholinesterase responsible for neurotransmission; AChE2 has lower activity and is less sensitive to xenobiotics (LI & HAN 2002; KIM & LEE 2013; XIAO *et al.* 2014). A reverse role for both enzymes was observed in *Apis mellifera* by KIM *et al*. (2012).

Our knowledge of esterases in solitary bees is still very limited. In *Megachile rotundata*, the expression of esterases varied during development, and the observed changes were sex-related. The presence of ChEs was determined based only on substrate specificity in a zymogram analysis of its extracts (FROHLICH 1990; FROHLICH *et al.* 1990). *Megachile rotundata* overwinters in the prepupae stage. Its development is completed in spring or early summer, and adults are active in summer. This study analyzed ChEs activity in diapausing and flying red mason bees (*Osmia bicornis*) of the family Megachilidae, which overwinter as adults and are active in spring and early summer. The obligate diapause of *O. bicornis* lasts from autumn to spring, and its duration can be somewhat altered by beekeepers through temperature control (WÓJTOWSKI & WILKANIEC 1978; GIEJDASZ & WILKANIEC 2002). Red mason bees are easy to keep, and they are increasingly used as pollinators in orchards and horticulture (BILIŃSKI & TREPER 2004; TREPER & BILIŃSKI 2009). Crop protection products are frequently applied by fruit growers. The results of our study relating to the activity of ChEs can be used to evaluate and compare the sensitivity of red mason bees to xenobiotics with that of other beneficial insects. The results have important implications for the future because the protection of pollinating entomofauna is a key challenge in the face of the global deficit in pollinating insects (NEUMANN & CARRECK 2010; POTTS *et al.* 2011). This study contributes valuable information about i) the activity of ChEs, a family of enzymes that play an important role in neurotransmission and detoxification, and ii) changes in ChEs activity in diapausing and flying bees of both sexes.

Material and Methods

Bees

The material (cocoons) for raising *O. bicornis* was supplied by the apiary of the Poznań University of Life Sciences. The experimental bees were kept in the area of Olsztyn (Poland) (N 53.71383, E 20,55733), and reed (*Phragmites australis*) was used as nesting material. The apiary was kept between April 2010 and May 2011 according to the procedure described by WÓJTOWSKI and WILKANIEC (1978).

The study was conducted on red mason bee (*Osmia bicornis)* imagines. Hibernating females and males were sampled at the beginning of each month between October 2010 and February 2011. In March, samples were collected three times: at the beginning (March 1), in the middle (March 2) and at the end of the month (March 3). Diapausing red mason bees were transported to a laboratory. The cocoons were removed from reed and *O. bicornis* specimens were isolated. On the 5th of May, flying bees of both sexes that emerged from cocoons in the field apiary were caught with an entomological net. All bees were weighed, placed individually in Eppendorf tubes and immediately frozen in liquid nitrogen. The material was freeze stored at -71°C until analysis.

Preparation of *O. bicornis* extracts

Every month (including in mid-March and at the end of March), 15 females and 15 males were randomly selected from the population of red mason bees. Enzymatic extracts were prepared according to the method described by DMOCHOWSKA-ŚLĘZAK *et al.* (2015). Individual bees were homogenized in an ice bath for 2 minutes with 0.6% NaCl, 1:10 (w/v), at 5000 rpm in the Omni TH-2 homogenizer. The homogenates were centrifuged at 15,000 g for 15 minutes at 4°C. The supernatants (n = 15) were used to analyze enzyme activity and protein content.

Determination of enzyme activity

a) Cholinesterase (EC 3.1.1.7) activity in extracts was determined by the method described by ELLMAN *et al*. (1961) by measuring the rate of hydrolysis of choline esters, acetylthiocholine iodide (ACh) and butyrylthiocholine iodide (BCh). The analysis was conducted in 96-well microplates. Each well was filled with 150 µl of 0.15 mM of substrate solution in Ellman's reagent in buffer and 50 µl of 0.05 M-sodium phosphate buffer (pH 7.5). 25 µl of the analyzed extract was added by gently stirring the contents (each extract was analyzed in three replications). Absorbance was measured at a wavelength of 410 nm in an ELISA ASYS340 plate reader (Biogenet with software Mikro Win 2000) immediately after and 3 minutes after incubation at 30°C. The rate (Ach/BCh) of extract activity towards Ach and BCh was calculated.

b) Carboxylesterase (EC 3.1.1.1). The activity of carboxylesterase towards 4-p-nitrophenyl bu-

tyrate (C4) was determined by the method described by WALZ and SCHWACK (2007) to verify the involvement of non-specific esterases in the hydrolysis of choline esters. Due to limited availability of material from males, C4 activity was measured only in extracts of female bees. The assay was performed with p-nitrophenyl butyrate (C4) as the substrate. The reaction mixture contained 50 µl of the analyzed extract, 50 µl of 10 mM solution substrate and 200 µl of 0.1 M phosphate buffer with pH 7.4. After incubation at 30°C for 5 min, absorbance was measured at 415 nm with a microplate reader.

Protein analysis

Protein concentrations in the extracts were determined by the method proposed by BRADFORD (1976). Enzyme activities were expressed in international units (U) in terms of 1 mg of protein in the extract. All samples were run in triplicate, and the appropriate controls were made. All chemicals were supplied by Sigma.

Statistical analysis

Statistical analyses were conducted using the Statistica (Statsoft, V. 9.0) software package. Multi-factor ANOVA was used to measure the effect of month, sex and substrate on the analyzed parameters. Statistically significant results of ANOVA were analyzed by the Bonferroni post-hoc test. The results were considered statistically significant at $\alpha<0.05$.

Results

In every analyzed month, enzyme activity was higher towards ACh than towards BCh in both male and female bees (Fig. 1). The ratio of enzyme activity towards Ach and BCh (ACh/BCh ratio) is a true indicator of AChE function. In diapausing bees, Ach/BCh varied more extensively in males (3.00-7.18) than in females (1.43-4.15), which could be attributed to generally lower activity towards BCh in males than in females (Fig. 2). Sex-related differences in enzyme activity towards both substrates were statistically significant (Fig. 2).

The differences between the mean of enzyme activity towards ACh and BCh were statistically significantly in females, excluding those noted on March 3 (Fig. 2A), and in males (Fig. 2B). Three stages of AChE activity were identified in females: higher activity in the three first months of diapause, lower activity between January and mid-March, and a rapid increase in activity in the final period (Fig. 2A). In males, AChE activity remained fairly stable between October and February, and it began to increase in early March (Fig. 2B). The highest AChE activity was observed in May in flying bees of both sexes.

The direction of changes in enzyme activity towards BCh was similar in both sexes. Enzyme activity remained fairly stable until mid-March, after which it increased significantly (Fig. 2).

In extracts of female bees, carboxylesterase activity remained almost unchanged throughout diapause. Carboxylesterase activity increased only in flying bees (Fig. 3).

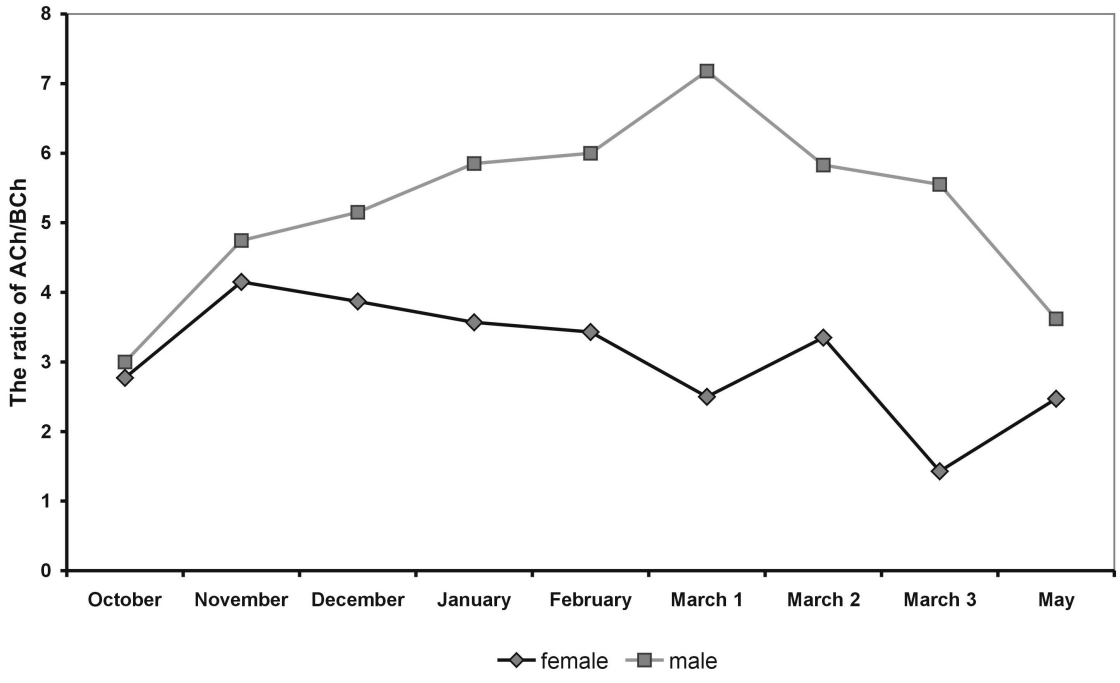

Fig. 1. The ratio of enzyme activity towards ACh and BCh as substrates (ACh/BCh) in female and male extracts of *Osmia bicornis*.

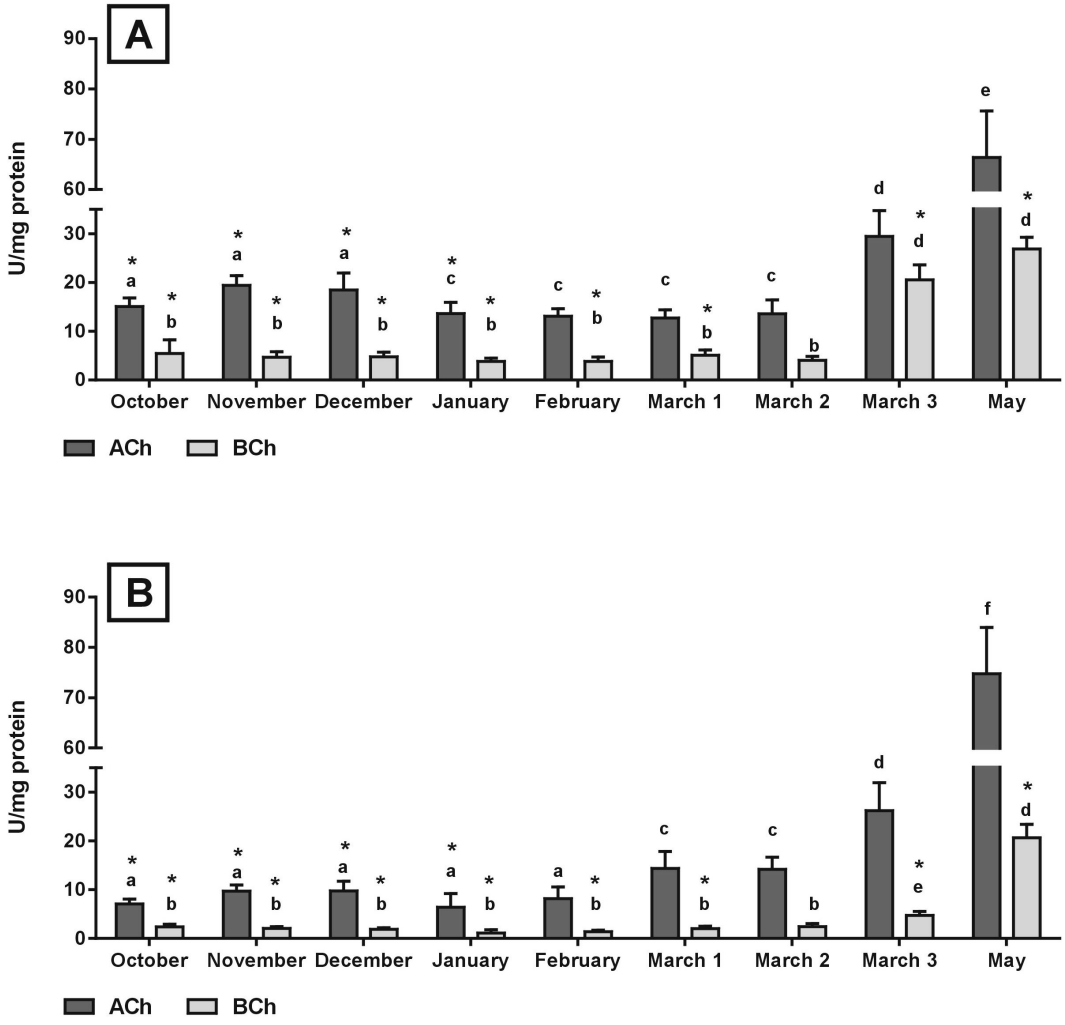

Fig. 2. Cholinesterase activity in extracts of female (A) and male (B) *Osmia bicornis* (U/mg protein). Data are expressed as the mean ± SD. Different letters above the bars in panel A or B represent significant differences between the means of activity towards Ach and BCh ($\alpha<0.05$). Asterisks above the bars indicate significant sex-related differences ($\alpha<0.05$) between the means of activity towards ACh or BCh in the given month.

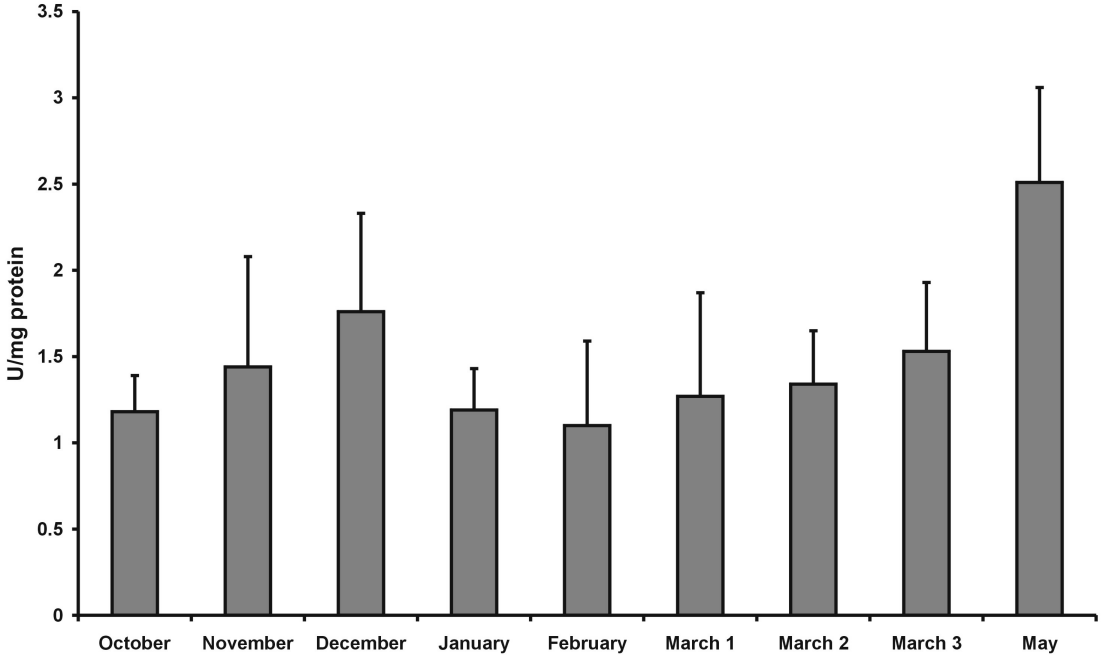

Fig. 3. Carboxylesterase activity in extracts of female *Osmia bicornis* (U/mg protein). Data are expressed as the mean ± SD.

Discussion

The specific activity of AChEs in red mason bees is difficult to compare with that of other insects due to differences in the sources of extracts and activity expression, therefore, our findings can only be related to the results of studies that relied on a similar methodology. In extracts of flying red mason bees, the activity of AChE was several dozen times higher than in extracts of *Aphis gossypii, Sitobion avennae* and *Rhopalpsiphum padi* (SHANG *et al*. 2012; LU & GAO 2013). It was also higher than in crude extracts of the heads of honey bees and *Drosophila melanogaster* from which acetylcholinesterases were isolated (GNAGEY *et al*. 1987; BELZUNCES *et al.* 1988). Cholinesterase activity in *O. bicornis* is indicative of the enzyme's high efficiency, which could be associated with cholinesterase's reduced sensitivity to insecticides (CHARPENTIER *et al*. 2000).

Research concerning AChE and BChE in insects often deliver confusing and contradictory data. Some authors have ruled out the presence of BChE in insects (see KIM *et al*. 2012), whereas others measured its activity (DE VILLAR *et al*. 1980; KULIEVA *et al*. 1995). Recent research has validated the hypothesis proposed by GNAGEY *et al.* (1987) who argued that insects possess AChE with substrate specificity that occupies the mid range between vertebrate AChE and BChE. The above could be attributed to the fact that AChEs in insects are coded by two genes, *ace 1* and *ace 2*, whose products, enzymes AChE1 and AChE2, have different functions (LI & HaN 2002; KIM & LEE 2013; XIAO *et al*. 2014). KIM *et al*. (2012) demonstrated that AChE2 is the key neurotransmission enzyme in honey bees. AChE1 probably has non-neuronal functions and protects bees against the harmful effects of xenobiotics, therefore, it plays a similar role to vertebrate BChE. In whole extracts of honey bees and fruit flies, cholinesterase activity towards ACh was only two-fold higher than towards BCh (GANGEY *et al*. 1987; KIM *et al*. 2012). These findings were attributed to the presence of carboxylesterases in whole extracts which also hydrolyze choline esters. In our study, enzymes also originated from whole extracts of *O. bicornis*, therefore, the ratio of enzyme activity towards ACh to enzyme activity towards BCh (ACh/BCh) was expected in the range of 0.5-0.6, similarly to that reported in *A. mellifera* and *D. melanogaster* (GANGEY *et al*. 1987; KIM *et al*. 2012). However, the ACh/BCh ratio measured in both substrates was significantly higher in red mason bees (Fig. 1). BCh was far less effectively hydrolyzed by *O. bicornis* extracts, suggesting that red mason bees possess mostly the highly active true AChE which plays a significant role in the hydrolysis of choline esters. The low activity of carboxylesterase (Fig. 3) confirmed this observation at least in the case of females.

The activity of esterases may have an important role in mitigating the adverse effects of xenobiotics, in particular in males whose enzyme activity towards BCh was considerably lower in comparison to females (Fig. 2). Lower activity of male enzymes could be related to the active life span of males which is half that of females (WILKANIEC 1991). Also the question whether it is associated with lower activity of enzymatic protein expressed in a haploid male genome remains open.

It remains unknown whether the estimated two-fold increase in cholinesterase activity in the second half of March, i.e. towards the end of diapause, in both males and females could be induced by environmental regulators of diapause termination and hormonal changes which influence gene expression, as observed in other insects (HUYBRECHTS *et al*. 2004; YOCUM *et al*. 2005; RAGLAND *et al*. 2011) In honey bees, changes in the expression of AChE genes are associated with division of labor (SHAPIRA *et al*. 2001). These enzymes are responsible for the ability to learn and memorize (WILLIAMSON & WRIGHT 2013; WILLIAMSON *et al.* 2013), therefore, they mediate functions that are very important for adult insects. The increase in cholinesterase activity at the end of diapause and high cholinesterase activity in flying red mason bees suggest that these enzymes play similar roles in the evaluated insects. Changes in cholinesterase activity in *O. bicornis* are positively correlated with oxygen consumption in *Osmia lignaria*, a related species that also diapauses in the adult stage (KEMP *et al*. 2004). Higher oxygen consumption towards the end of diapause, in particular in emerged bees, results from a higher metabolic rate, which is accompanied by an increase in enzyme activity. The above observations corroborate our previous findings (DMOCHOWSKA *et al*. 2013; ZAOBIDNA *et al*. 2014; DMOCHOWSKA *et al*. 2015).

This study paves the way for more advanced research into cholinesterases in red mason bees. Our results can be used to monitor periodic changes in the cholinergic system of red mason bees in response to environmental factors.

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