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Analysis of monoamines, adenosine and GABA in tissues of the land snail *Helix lucorum* and lizard *Agama stellio stellio* during hibernation

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Summary

The aim of the present study was to determine the levels of monoamines, GABA and adenosine in the brain, heart and haemolymph of the land snail *Helix lucorum* and in the brain, heart and blood of lizard *Agama stellio stellio* during long-term hibernation. We measured levels of the monoamines serotonin (5-HT) and its main metabolite 5-hydroxyindole-3-acetic acid (5-HIAA), dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanilic acid (HVA), norepinephrine (NE) and epinephrine (E). The most abundant amines detected in the brain and heart of active *H. lucorum* were 5-HT and DA. Of the metabolites examined only 5-HIAA was found in the brain. NE was found at very low levels but only in the brain, while E was not detected in the brain and heart. The levels of 5-HT and 5-HIAA increased in the brain and heart of *H. lucorum* within the first months of hibernation, showing a significant decrease thereafter. The levels of DA did not change during hibernation. The results indicated that 5-HT might be involved in preparing snails for entry into hibernation. GABA was only found in the brain of *H. lucorum*, and the levels were low; these levels remained during hibernation. Adenosine was present in brain and heart of *H. lucorum*, and during hibernation, the level of adenosine decreased significantly in the brain but remained steady in the heart. The monoamines 5-HT, DA and NE were present in the brain of active lizards *A. stellio stellio*, whereas E was found only at very low levels. Moreover, the metabolites 5-HIAA, DOPAC and HVA were detected in the brain of active lizards. The monoamines 5-HT, DA, NE and E were also detected in the heart and blood of active lizards. During hibernation the levels of these four monoamines were decreased significantly in the brain and heart of *A. stellio stellio*. In contrast, the levels of E increased in the heart and blood of hibernating lizards. Adenosine was detected in both heart and brain of active lizards, but hibernation caused a marked decrease in its levels at both tissues. GABA was found at higher levels than monoamines and adenosine in the brain of active lizards, and hibernation caused a significant increase in its levels, indicating an important role of GABA in inhibition of neuronal activity in hibernating lizards.

Key words: land snail, *Helix lucorum*, lizard, *Agama stellio stellio*, neurotransmitter, hibernation, metabolic depression.

Introduction

Metabolic depression is a strategy employed by many invertebrates and vertebrates when faced with environmental stress such as hibernation or anoxia. However, the mechanisms that induce metabolic depression remain elusive. It has been reported that, as regulators of neuronal activity, neurotransmitters might play a key role in regulating metabolic depression in vertebrates and invertebrates. For example, increased levels of the inhibitory neurotransmitter GABA in the brain of animals might contribute to metabolic depression by reducing neuronal activity (Nilsson and Lutz, 1993). However, most evidence supporting such a role for increased GABA levels in metabolic depression comes from studies on anoxia-tolerant animals, where GABA increases in their brain during anoxia and seems to exert an inhibitory effect on the nervous system (Nilsson et al., 1990, 1991; Nilsson and Winberg, 1993). As in anoxia-tolerant animals, GABA increases in some mammals and reptiles during hibernation, indicating that it might play a key role in promoting hypometabolism (Mihailovic et al., 1965; Al-Bardy and Taha, 1982; Lust et al., 1989; Abdel Raheem and El Mosallamy, 1979). As in vertebrates, GABA seems to exert an inhibitory role on the nervous sytem of most invertebrates (Gerschenfeld, 1973; Nistri and Constanti, 1979; Walker, 1986; Walker and Holden-Dye, 1991). However, the role of GABA in promoting metabolic depression in invertebrates during hibernation has been investigated to a lesser extent than in vertebrates.

In addition to GABA, some evidence seems to indicate a contribution of adenosine to metabolic depression in

vertebrates and invertebrates. Adenosine is an inhibitory neuromodulator in the vertebrate brain, which decreases neuronal excitability as well as neurotransmitter release. It has been reported that adenosine is causally involved in the depression of neural activity during entrance into hibernation (Pakhotin et al., 1993; Wang, 1993; Spangenberger et al., 1995). Moreover, adenosine reduces the rate of oxygen consumption in the marine invertebrate *Sipunculus nudus* (Reipschlager et al., 1997) and modulates monoamine release from the nervous system of the marine bivalve *Mytilus edulis* (Barraco and Stefano, 1990). In anoxia-tolerant vertebrates, adenosine is released in their brain during anoxia and might be involved in the metabolic depression and anoxic survival of the brain (Nilsson and Lutz, 1992; Lutz and Kabler, 1997; Pek and Lutz, 1997).

Another group of neurotransmitters that seem to be implicated in metabolic depression are the monoamines, serotonin (5-HT), dopamine (DA) and norepinephrine (NE). The role of these monoamines in promoting metabolic depression has been studied, especially in the anoxic brain of turtles and crucian carp. The levels of 5-HT and NE are maintained in the brains of turtles and crucian carp during extended periods of anoxia and they might contribute to anoxic survival (Nilsson, 1989a,b; Nilsson et al., 1991).

The role that neurotransmitters might play in promoting metabolic depression in several species during hibernation is not fully understood, nor is it well known whether these neurotransmitters could promote metabolic depression in vertebrates and invertebrates during hibernation. This prompted us to determine the levels of monoamines GABA and adenosine in the brain, heart and blood of the hibernating land snail *Helix lucorum* and the lizard *Agama stellio stellio*. These two species were chosen for this work as they both are found in northern Greek habitats facing the same environmental conditions. Also, their responses to harsh environmental conditions of winter are similar as they both hibernate buried into the ground for similar periods of time.

Materials and methods

Chemicals

Norepinephrine (NE; norepinephrine hydrochloride), epinephrine (E; L-epinephrine), dopamine (DA; 3-hydroxytyramine hydrochloride), serotonin (5-HT; 5-hydroxytryptamine-creatine sulphate complex), 3,4-dihydroxyphenylacetic acid (DOPAC), 5-hydroxyindole-3-acetic acid (5HIAA) and homovanillic acid (HVA) were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals were of reagent grade.

Animals and induction of hibernation

According to its biological cycle, *Helix lucorum* L. enters hibernation at the end of October and is aroused at the end of March (Staikou et al., 1988). Thus adult specimens of *Helix lucorum* (largest shell diameter ranged between 38 and 42 mm) were collected at the end of September 2000, in the vicinity of Edessa, in northern Greece. The snails were kept in an active state at a temperature of 25±0.5 °C and subjected to a 10.00 h:14.00 h L:D photoperiod in large glass boxes, with a daily supply of lettuce leaves and water. High humidity (85±1 %) was maintained by sprinkling the interior of the boxes with water every day. To induce hibernation, the animals were put into a cool room where the temperature was adjusted to 5 °C and exposed to the 10.00 h:14.00 h L:D photoperiod. The animals kept under these conditions are referred to as hibernating, because these conditions are equivalent to the natural state. Hibernation started at the end of October 2000 and lasted for 4 months. At the end of each month, samples of hibernating animals were used for tissue sampling and analysis of neurotransmitters.

The lizard *Agama stellio stellio* L. begins to enter hibernation between late September and mid-October and emerges at the beginning of March (Loumbourdis, 1984). Thus, active animals were collected in mid-September 2000 and kept in an active state at a temperature of 25±0.5 °C and subjected to a 10.00 h:14.00 h L:D photoperiod in large glass boxes. Hibernation, which commenced in late October 2000, was induced in a similar way to that described for the snails and lasted for 4 months. At the end of each month, samples of hibernating animals were used for tissue sampling and analysis of monoamines, GABA and adenosine.

Tissue sampling and preparation of homogenates

After hibernation periods of 1, 2, 3 or 4 months, snails were decapitated, haemolymph collected and brains (the circumesophageal ganglia) and hearts dissected out, immediately frozen in liquid nitrogen and kept at –80 °C. Similarly, after hibernation periods of 1, 2, 3 or 4 months, lizards were decapitated, blood collected and brains and hearts rapidly removed, frozen in liquid nitrogen and kept at –80 °C. 500 µl of haemolymph and blood were mixed with 200 µl of ice-cold perchloric acid (PCA) containing 0.2 % EDTA and 0.05 % sodium bisulfite and then centrifuged at 20 000 ***g*** for 10 min. The supernatants were collected and used for analysis of monoamines, adenosine and GABA. The brains and hearts from the snails and lizards were homogenized in 4 % (w/v) ice-cold PCA containing 0.2 % EDTA and 0.05 % sodium bisulfite using a Potter–Elvehjem homogenizer (Nilsson, 1990). The homogenates (6 % w/v) were centrifuged (20 000 ***g*** for 10 min) and the supernatants stored at –80 °C for 3 days for monoamine and adenosine analysis and for several weeks for GABA analysis.

Brains, hearts and blood and haemolymph from active snails and lizards were treated as described above and used as controls.

Determination of monoamines

The monoamines tested were serotonin (5-HT) and its main metabolite 5-hydroxyindole-3-acetic acid (5-HIAA), dopamine (DA) and its metabolites dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA), norepinephrine (NE) and epinephrine (E). The amounts of monoamines and monoamine metabolites were quantified using reverse-phase ion-pair

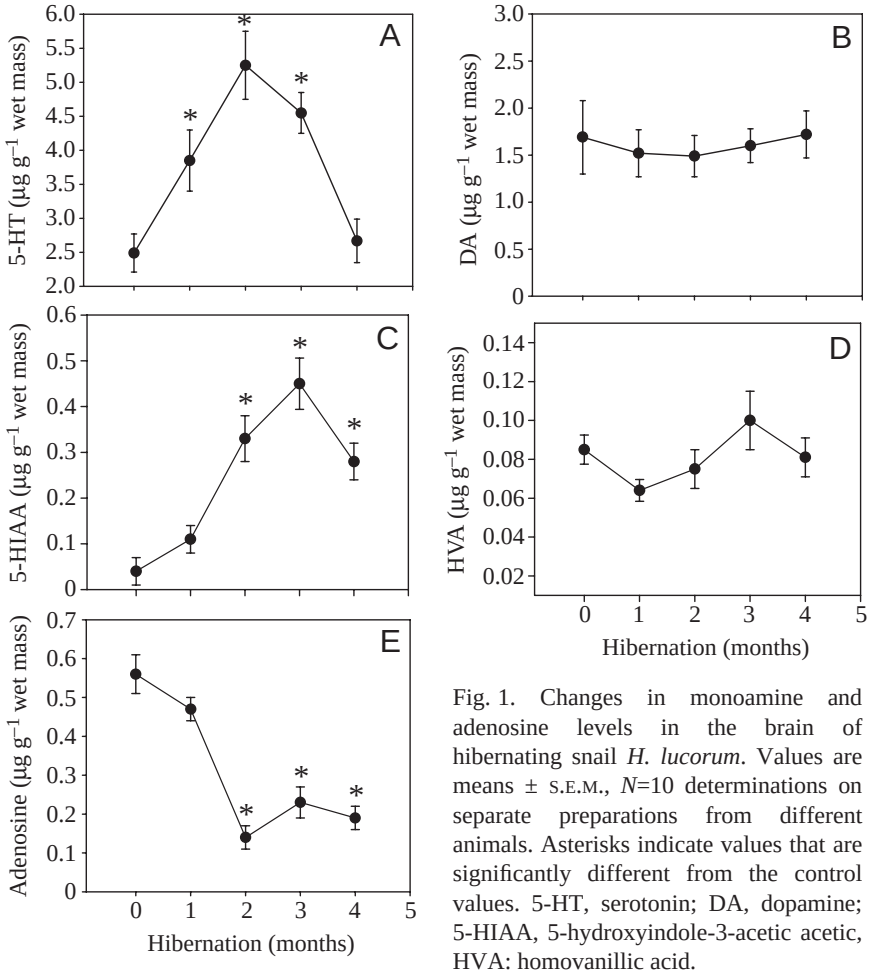

Fig. 1. Changes in monoamine and adenosine levels in the brain of hibernating snail *H. lucorum*. Values are means ± S.E.M., *N*=10 determinations on separate preparations from different animals. Asterisks indicate values that are significantly different from the control values. 5-HT, serotonin; DA, dopamine; 5-HIAA, 5-hydroxyindole-3-acetic acetic, HVA: homovanillic acid.

high-performance liquid chromatography (HPLC), with electrochemical detection similar to the method described by Reipschlager et al. (1997). In brief, the system for HPLC consisted of a Shimadzu solvent delivery unit with a double-plunger reciprocating pump (LC-9A; Kyoto, Japan), a sample-injector fitted with a 20 µl loop (Model 7125; Reodyne, Cotati, CA, USA) a reverse-phase column (C_{18} Nucleosil 120, 4.6×75 mm, 3 µm diameter, Macherey-Nagel, Duren, Germany), thermostated to 40 °C and an electrochemical detector (model 141 Gilson, Middleton, WI, USA). The mobile phase was prepared as described by Reipschlager et al. (1997) and it consisted of 100 mmol l^{-1} NaH_2PO_4, 0.5 mmol l^{-1} EDTA, 30 mg l^{-1} sodium octylsulfate and 6 % methanol, pH 3.7, and the flow rate was 1 ml min^{-1}. The working electrode of the electrochemical detector was a small disc of glassy carbon (3 mm diameter) and the electrode potential was maintained at +750 mV set against an Ag–AgCl reference electrode. The electrochemical detector was linked to a PC Pentium IBM-compatible computer *via* a 14-bit AD-DA card. Standards were dissolved in H_2O and diluted 1:1000 with 4 % (w/v) PCA containing 0.2 % EDTA and 0.05 % sodium bisulfite.

Determination of adenosine

Adenosine was determined in a similar way to that described by Reipschlager et al. (1997) using HPLC (isocratic) with spectrophotometric detection. In brief, a reverse-phase column (C_{18} Nucleosil 120, 4.6 mm × 75 mm, 3 µm diameter, Macherey-Nagel, Duren, Germany) was used and the mobile phase consisted of 10 mmol l^{-1} NaH_2PO_4, 0.25 mmol l^{-1} EDTA and 6 % methanol, pH 6.5, at a flow rate of 1 ml min^{-1}. The concentrations of adenosine in the homogenates were quantified by comparison with standards prepared in the same neutralized PCA solution as the extracts.

Determination of GABA

GABA levels were determined by reverse-phase HPLC (isocratic) with fluorescence detection after derivation with *o*-phthaldialdehyde (OPA), according to the method used by Kamisaki et al. (1990), after modification. A Symmetry C_8 column, 4.6 mm × 250 mm and 5 µm diameter was used. Excitation was set at 340 nm, and emission was measured at 456 nm. The mobile phase consisted of sodium citrate, acetonitrile and methanole (590:320:90 v/v) and the flow rate was set at 1.0 ml min^{-1}. The derivatization reagent was prepared by mixing 100 mg OPA, 2 ml methanol, 8 ml borate buffer (pH 10.45) and 200 µl 2-mercaptoethanol. 60 µl of the supernatant obtained after PCA extraction and centrifugation were mixed with an equal quantity of derivatization reagent. After 2 min, 100 µl of the mixture were injected into the sample loop and the run started at the same time. Concentrations were calculated by comparison with standards prepared in the same PCA solution as the extracts.

Statistical analysis

The results are presented as means ± S.E.M. Significance of difference was tested with Bonferroni's test, which takes into consideration multiple comparisons. The limit of significance was set at $P<0.05$.

Results

Concentrations of monoamines, GABA and adenosine in the brain, heart and haemolymph of Helix lucorum

The main monoamines detected in the brain of active *H. lucorum* were 5-HT and DA, at levels of 2.49±0.28 and 1.69±0.39 µg g^{-1} wet mass, respectively. The metabolites 5-HIAA and HVA were detected at lower levels, 0.04±0.005 and 0.085±0075 µg g^{-1} wet mass, respectively, in the brain of active snails. In contrast, NE was found at very low levels (>0.01 µg g^{-1} wet mass), while E could not be detected at all with this method. The other metabolite of DA, DOPAC, was

not detectable in the brain of active *H. lucorum*. During hibernation, the levels of 5-HT and its metabolite 5-HIAA changed significantly. As shown in Fig. 1A, the levels of 5-HT increased in the brain from 2.49±0.28 μg g^{-1} wet mass to 5.25±0.50 μg g^{-1} wet mass within the first 2 months of hibernation. Thereafter, levels of 5-HT gradually declined during hibernation to 2.67±0.36 μg g^{-1} wet mass. A similar pattern of changes was observed for the main metabolite of 5-HT, 5-HIAA (Fig. 1C). Its level increased from 0.04±0.005 μg g^{-1} wet mass to 0.32±0.04 μg g^{-1} wet mass within the first 2 months of hibernation, reaching 0.28±0.04 μg g^{-1} wet mass by the fourth month. In contrast, no significant changes were observed for DA (Fig. 1B) and its metabolite HVA (Fig. 1D) in the hibernating snails.

5-HT and DA were the only monoamines detected in the heart of active *H. lucorum*. The concentration of 5-HT was found to be at the same level (2.95±0.34 μg g^{-1} wet mass) as that measured in the brain, while the level of DA was much lower (0.27±0.032 μg g^{-1} wet mass) than that measured in the brain of active snails. During hibernation, changes in the levels of 5-HT were similar to those observed for the brain. Specifically, the concentration of 5-HT increased to 4.32±0.30 μg g^{-1} wet mass within the first 2 months, followed by a decrease thereafter (Fig. 2A). In contrast, a significant decrease in the level of DA was observed by the fourth month after the onset of hibernation (Fig. 2B).

The concentration of adenosine and the changes in its levels in the brain and heart of hibernating *H. lucorum* are shown in Fig. 1E and Fig. 2C, respectively. As indicated, levels of adenosine decreased from 0.57±0.05 μg g^{-1} wet mass to 0.19±0.04 μg g^{-1} wet mass in the brain by the fourth month of hibernation (Fig. 1E), while the levels of adenosine did not change statistically in the heart of hibernating snails (Fig. 2C).

GABA was detectable only in the brain of active *H. lucorum* and its concentration was found to be 0.35±0.054 μg g^{-1} wet mass. Hibernation did not cause any significant change in its level. However, none of the neurotransmitters examined was detected in the haemolymph of active and hibernating snails.

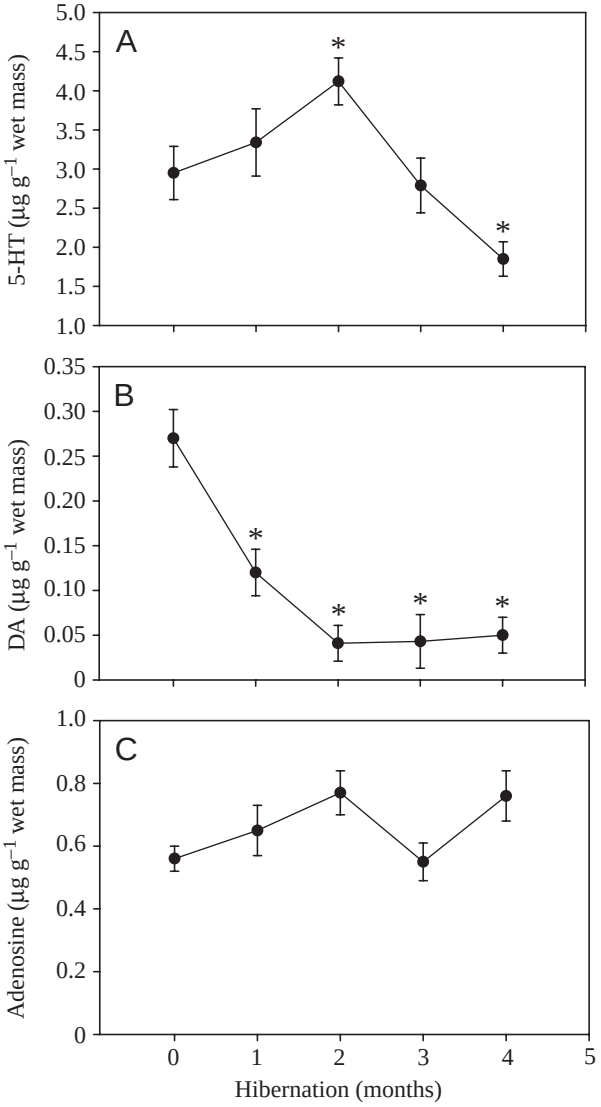

Fig. 2. Changes in monoamine and adenosine levels in the heart of hibernating snail *H. lucorum*. Values are means ± S.E.M., *N*=10 determinations on separate preparations from different animals. Asterisks indicate values that are significantly different from the control values. 5-HT, serotonin; DA, dopamine.

Concentrations of monoamines, GABA and adenosine in the brain, heart and blood of A. stellio stellio

As in *H. lucorum*, 5-HT and DA and their metabolites 5-HIAA and HVA were detected in the brain of active lizard *A. stellio stellio*. In contrast to *H. lucorum*, however, both NE and E and DOPAC, the metabolite of DA, were found in the brain of *A. stellio stellio*. Hibernation caused significant decreases in the levels of 5-HT and 5-HIAA. The concentration of 5-HT decreased from 1.67±0.21 μg g^{-1} wet mass to 0.25±0.032 μg g^{-1} wet mass and that of 5-HIAA from 0.14±0.024 μg g^{-1} wet mass to 0.028±0.005 μg g^{-1} wet mass by the fourth month (Fig. 3A, B, respectively). As with 5-HT, the levels of DA decreased from 0.29±0.03 μg g^{-1} wet mass to 0.042±0.006 μg g^{-1} wet mass by the fourth month of hibernation (Fig. 3C), while the principal metabolites of DA, HVA and DOPAC, were not detectable in the brain of hibernating lizards. The concentration of NE measured in the brain of active lizards was higher (0.67±0.064 μg g^{-1} wet mass) than that of DA (0.29±0.03 μg g^{-1} wet mass). Within the first 2 months of hibernation, the levels of NE decreased significantly (0.27±0.032 μg g^{-1} wet mass), then increased within the next 2 months, reaching a value of 0.40±0.052 by the fourth month (Fig. 3D). In contrast, the concentration of E (0.11±0.015 μg g^{-1} wet mass) remained at the same levels in the brain of lizards during hibernation (Fig. 3E).

As in the brain, NE was found at higher levels (0.81±0.064 μg g^{-1} wet mass) than DA (0.039±0.004 μg g^{-1} wet mass) and 5-HT (0.13±0.02 μg g^{-1} wet mass) in the heart of active *A. stellio stellio*. During hibernation, all three

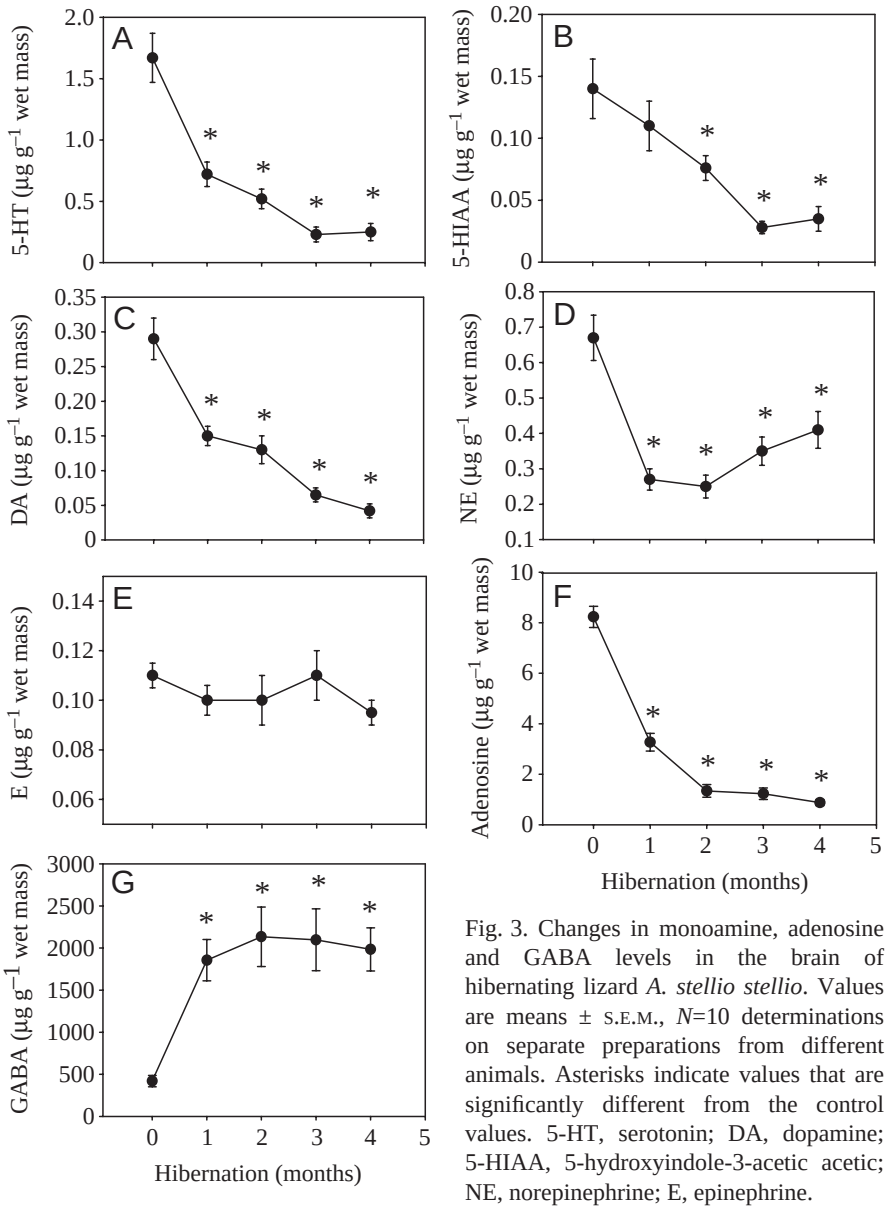

Fig. 3. Changes in monoamine, adenosine and GABA levels in the brain of hibernating lizard *A. stellio stellio*. Values are means ± S.E.M., *N*=10 determinations on separate preparations from different animals. Asterisks indicate values that are significantly different from the control values. 5-HT, serotonin; DA, dopamine; 5-HIAA, 5-hydroxyindole-3-acetic acetic; NE, norepinephrine; E, epinephrine.

monoamines showed approximately the same pattern of changes in the heart as those in the hibernating brain (Fig. 4). E was below the detectable limits in the heart of active *A. stellio stellio*, although it could be determined in the heart of hibernating lizards (Fig. 4D).

Adenosine was detected in the brain and heart of active *A. stellio stellio*, but the level of adenosine was about twofold higher in the brain (8.23±0.42 μg g^{-1} wet mass) (Fig. 3F) than in the heart (3.56±041 μg g^{-1} wet mass) of active lizards (Fig. 4E). Hibernation caused a marked decrease in the levels of adenosine in both brain and heart of lizards.

GABA was detected only in the brain of active *A. stellio stellio* at 420±22 μg g^{-1} wet mass, which is higher than those of the monoamines examined. In contrast to hibernating snails, however, GABA levels increased markedly in the brain of hibernating lizards. As shown in Fig. 3G, the levels of GABA increased fivefold in the brain within the first 2 months of hibernation (from 420±52 μg g^{-1} wet mass to 2.198±302 μg g^{-1} wet mass) and they remained high, reaching levels of 1.984±256 μg g^{-1} wet mass of brain in the fourth month.

In contrast to *H. lucorum*, NE, DA, E and 5-HT were detected in the blood of active *A. stellio stellio* and their levels were 0.15±0.023, 0.33±0.045, 0.018±0.0013 and 0.92±0.12 μg ml^{-1}, respectively. During hibernation, the levels of NE, DA and 5-HT decreased significantly in the blood, while that of E increased (Fig. 5).

Discussion

The present work proves DA and 5-HT to be the dominant monoamines in the brain of *H. lucorum*. In addition, the results show that 5-HT and DA are the main biogenic amines involved in cardioregulation in *H. lucorum*. In contrast, NE was found at low levels in the ganglia of *H. lucorum*, as has been found in the nervous system of other molluscs (Osborne and Cotrell, 1970; Guthrie et al., 1975; Osborne, 1984; Ottoviani et al., 1988; Hetherington et al., 1994). These results agree with those from previous studies, which have shown that DA and 5-HT, in particular, are abundant in the nervous system of gastropods (Gerschenfeld, 1973; Walker, 1986; Sloley et al., 1990). The distribution of serotonergic neurons in the CNS of land snails has been studied and there is strong evidence for a central transmitter role for 5-HT at both central and peripheral synapses (Walker, 1986; Hernadi et al., 1989).

From our results presented here, DA does not seem to play any key role in regulating hibernation in *H. lucorum*. The levels of DA remained stable in the brain (Fig. 1B), and no significant changes were observed in the levels of the DA metabolite HVA (Fig. 1D). In contrast, the levels of 5-HT increased by the second month, followed by a significant reduction thereafter (Fig. 1A). Moreover, the pattern of changes in 5-HIAA levels is similar to that of 5-HT (Fig. 1C). It should be pointed out, however, that these data do not necessarily imply an increase in extracellular levels of 5-HT. The modulatory effects of 5-HT at peripheral and central synapses during hibernation remain undetermined. Nevertheless, the changes in the levels of 5-HT in the brain of hibernating *H. lucorum* agree well with other data indicating an activation of serotonergic neurons in the land snails during the first months of hibernation and a reduction in the activity of these neurons thereafter. Specifically, biochemical

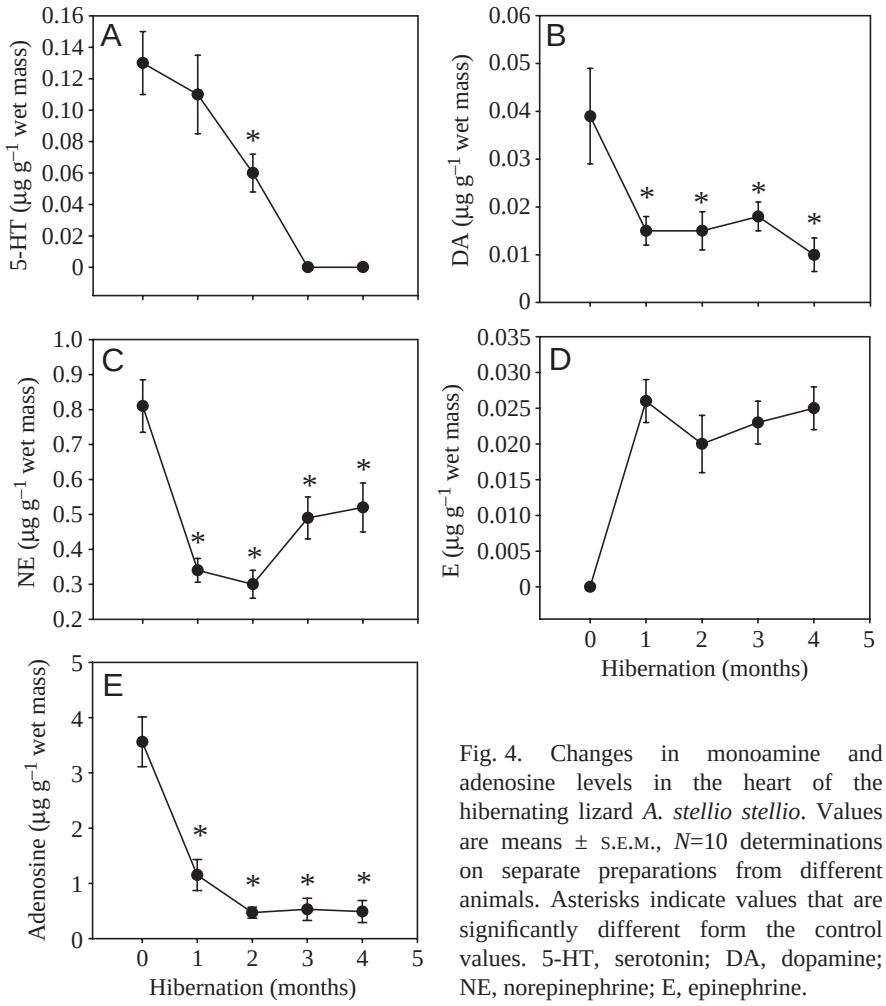

Fig. 4. Changes in monoamine and adenosine levels in the heart of the hibernating lizard *A. stellio stellio*. Values are means ± S.E.M., *N*=10 determinations on separate preparations from different animals. Asterisks indicate values that are significantly different form the control values. 5-HT, serotonin; DA, dopamine; NE, norepinephrine; E, epinephrine.

measurements have shown higher 5-HT content in the CNS of *H. pomatia* during the first months of hibernations (Hiripi and Salanki, 1973). Moreover, immunocytochemical studies have shown that the number of serotonergic neurons changes in a seasonal manner in the land snails, a smaller number being found during winter (Hernadi et al., 1989; Bernocchi et al., 1998). As shown in previous studies, 5-HT is an excitatory agent (Jones, 1983) and enhances the glycolytic rate in some tissues of land snails (Michaelidis and Vasiliou, 1997). Thus changes in 5-HT levels, depending on seasonal conditions, might influence the rate of several physiological processes, in this way contribute to metabolic depression in the hibernating land snails. However, at peripheral sites in the snail, 5-HT is not always excitatory and acts as a relaxing agent, e.g. in the penis retractor muscle of *H. pomatia* (Wabnitz and Von Wachtendonk, 1976).

In some hibernating vertebrates 5-HT seems to play a pivotal role in the central neural control of hibernation. Brain 5-HT levels increased during hibernation in a number of mammalian species, including the hedgehog (Uuspaa, 1963), the ground squirrel (Kudryavtseva and Popova, 1973) and the Syrian hamster (Novotna et al., 1975). In addition, the level of 5-HIAA was higher in some hibernating animals in winter, suggesting an increase in the activity of their serotonergic system (Duncan and Tricklebank, 1978; Novotna et al., 1975).

GABA was found to be at low levels in the brain of *H. lucorum*, and these results agree with those reported for *H. pomatia* (Osborne et al., 1972). Previous studies reported the existence of GABA-like immunoreactivity in the CNS of several gastropods, including *Helix* species (Hernadi, 1994; Richmond et al., 1991; Hatakeyama and Ito, 2000). However, the role of GABA as a transmitter in snails is poorly understood, since the enzyme glutamic acid decarboxylase, involved in the synthesis of GABA from glutamate, is detected in the nervous system (Bradford et al., 1969). Moreover, pharmacological studies of GABA action on snail neurons have shown that GABA may produce inhibitory or excitatory effects, depending on the concentrations applied (Gerschenfeld and Lasansky, 1964; Walker, 1986; Takeuchi, 1992; Zhang et al., 1997). During hibernation, the concentration of GABA remained at the same levels in the brain of *H. lucorum*, indicating that GABA may have a minor role as an inhibitory neurotransmitter in hibernating land snails.

To our knowledge, there are have been no previous reports of the levels of adenosine in land snails. The levels of adenosine determined in the brain and the heart of *H. lucorum* are comparable to those found in the invertebrate *Sipunculus nudus* (Reipschlager et al., 1997). It has been reported that adenosine is causally involved in the depression of neural activity during entry into hibernation (Pakhotin et al., 1993; Wang, 1993; Spangenberger et al., 1995). Also, a recent investigation showed that the levels of adenosine increase in *S. nudus* during anoxia, so it might be involved in metabolic depression since it reduces the oxygen consumption of this worm (Reipschlager et al., 1997). Several lines of evidence, including the existence of all requisite adenylate-metabolizing enzymes (Lazou, 1989) and adenosine receptors on the neurons of land snails (Cox and Walker, 1987), and the inhibition of 5-HT and DA release by adenosine from the neurons of marine bivalve *Mytilus edulis* (Barraco and Stefano, 1990), indicate a possible modulation of the nervous system of invertebrates by adenosine. From the results presented, it is difficult to speculate on the mobilization of monoamine stores by adenosine in the brain of hibernating *H. lucorum*. It has been suggested that the initial increase in the levels of adenosine in the brain of anoxia-tolerant animals during anoxia is responsible for the decrease in energy utilization (Nilsson, 1993). Thereafter, GABA seems to become responsible for the maintenance of depressed energy consumption at a later stage

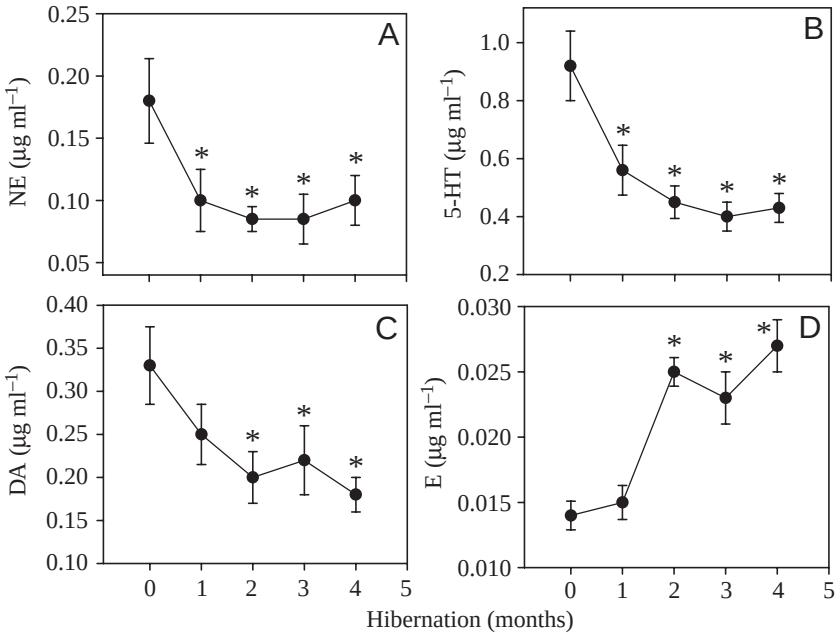

Fig. 5. Changes in monoamine levels in blood of the hibernating lizard *A. stellio stellio*. Values are means ± S.E.M., *N*=10 determinations on separate preparations from different animals. Asterisks indicate values that are significantly different from the control values. 5-HT, serotonin; DA, dopamine; NE, norepinephrine; E, epinephrine.

of anoxia. Unfortunately, it was impossible to determine the levels of adenosine in the brain of snails during the initial stages of hibernation, because the animals responded quickly to handling during the first stages of imposed hibernation, so it remains unclear whether this type of initial increase in the level of adenosine takes place in the brain of *H. lucorum* in response to hibernation.

In contrast to *H. lucorum*, in *A. stellio stellio* NE was present in the brain, heart and blood, and the pattern of monoamine changes was different from that of hibernating snails. In addition, DA and E were found to be at lower levels than that of NE in the brain. These results seem to agree with the distribution of catacholamine-containing neurons in the brain of lizards. In particular, results from microspectrofluorometric and pharmaco-histochemical analysis showed that the fluorescent substance in the brain of lizards is mainly NE (Baumgarten and Braak, 1968). The levels of NE in the brain of active lizards *A. stellio stellio* are comparable to those found in other lizards such as *Anolis sagrei* and *Uromastix aegyptius* (Nilsson et al., 1991; Okasha et al., 1995). During hibernation, the levels of DA and 5-HT decreased significantly in the brain, heart and blood of *A. stellio stellio* and remained at low levels until the fourth month. In contrast, the levels of NE decreased within the first 3 months, followed by an increase thereafter (Figs 3, 4 and 5). In agreement with our results, decreases in the levels of monoamines NE, DA and 5-HT have been reported for brain and blood of the hibernating lizard *U. aegyptius* (Okasha et al., 1995). In the latter work, it was also reported that levels of NE increased significantly in the brain and blood of the aroused *U. aegyptius*.

The above data clearly indicate a lower rate of catecholamine synthesis in the brain of lizards during hibernation. The decreases in catecholamine levels could be the result of reduced temperature and may contribute to the low neuronal activity and reduction of metabolic rate that characterize hibernating lizards. For example, the reduction in NE levels in the heart of hibernating lizards (Fig. 4C) indicates lower heart activity and a consequent decrease in the energy used by the contracting myocytes. Moreover, NE as a peripheral transmitter of sympathetic nervous system controls several mechanisms of cold defense. On the other hand, the slight increase in NE levels in the brain of *A. stellio stellio* after the second month of hibernation might be attributable to the peripheral and central functions of NE in thermoregulation, which are very important physiologically for the preparation of animals that are arousing from hibernation. This suggestion is also based on our results, showing an increase in NE levels on the heart (Fig. 4C).

In contrast to active lizards, E was detected in the blood (Fig. 5D) and heart (Fig. 4D) of hibernating *A. stellio stellio*. The present results seem to coincide well with those of earlier studies, which show that the adrenal gland is inactive in the early stages of hibernation and becomes more active as hibernation proceeds (Agid et al., 1961; Duguy, 1963). Adrenaline (epinephrine) may be implicated in the reduction of glycogen content in the liver and muscles of lizards during hibernation, through inducing glycogenolysis *via* activation of phosphorylase. It is known that oxidation of glycogen contributes to energy maintenance in hibernating lizards and adrenaline exerts a hyperglycemic effect on reptiles (Akbar et al., 1978; Coulson and Hernadez, 1983). According to recent findings, glucose seems to play a cryoprotectant role in reptiles that are facing a cold winter (Constanzo et al., 1995; Grenot et al., 2000). It is also interesting that adrenaline, in parallel with its hyperglycemic effect, reduces oxygen consumption in some reptiles (Coulson and Hernandez, 1986). However, it is not known whether adrenaline contributes in this way to metabolic depression in hibernating *A. stellio stellio* or in other lizards.

Based on the present results it is difficult to make any assumptions about the physiological role that adenosine might play in metabolic depression in the hibernating *A. stellio stellio*. Adenosine levels were maintained in the brain during the first 5 days (data not shown), but decreased markedly during prolonged hibernation. As in *H. lucorum*, *A. stellio stellio* responded to handling during the first hours of imposed hibernation. Thus, it remains unclear whether adenosine acts as an initial inhibitory neurotransmitter in the hibernating lizards as it does in anoxia-tolerant turtles (Nilsson and Lutz, 1992; Lutz and Kabler, 1997).

The levels of GABA determined in the brain of active *A. stellio stellio* are comparable to those measured in the brain of *A. sagrei* and *Varagunus griseus* (Abdel Raheem and El Mosallamy, 1979; Nilsson et al., 1991). GABA levels increased markedly in the brain of hibernating *A. stellio stellio* and remained elevated during hibernation (Fig. 3G). Similarly, levels of GABA increased in the brain of hibernating *V. griseus*, while those of the excitatory neurotransmitter glutamate decreased (Abdel Raheem and El Mosallamy, 1979; Abdel Raheem and Hanke, 1980). As reported elsewhere, GABA is the major inhibitory neurotransmitter in all vertebrates and it is suggested that increases in the levels of GABA could mediate metabolic depression and, thus, anoxic survival in ectothermic as well as endothermic vertebrates (Nilsson, 1992; Nilsson and Lutz, 1993). The results presented indicate that GABA might be involved in reducing neuronal activity and metabolic rate in hibernating lizards. We do not know, however, whether an increase in the levels of brain GABA necessarily means an increase in the percentage of bound GABA molecules on the membrane receptors at low temperatures. Moreover, further experimentation is needed to illustrate the mechanism by which a block or activation of GABA receptors modulates hibernation in lizards.

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