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Thyroid hormone modifies the metabolic response of air-breathing perch (*Anabas testudineus* Bloch) to nimbecidine exposure

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SUMMARY

The action of thyroid hormones on metabolite regulation of nimbecidine-exposed climbing perch was studied to understand the role of these hormones in tolerance mechanisms of fish to nimbecidine exposure. *Anabas testudineus*, the climbing perch, was treated with nimbecidine, a neem-based biopesticide, at nominated concentrations (60 and 600 μgL^{-1}) for 48 h, with or without THs, and the levels of triiodothyronine (T_3) and thyroxine (T_4), metabolite and nucleic acid were quantified. The treatment of THs along with nimbecidine produced a different metabolite pattern. A significant rise in serum T_3 occurred in the nimbecidine-treated fish but the serum T_4 level remained unaffected. Nimbecidine exposure increased the serum triglycerides and the serum urea but the serum glucose and liver total protein, RNA and DNA remained unchanged. Significant reduction in the aspartate aminotransferase and alkaline phosphatase activities occurred in the serum and liver of nimbecidine-treated fish but the lactate dehydrogenase and alanine aminotransferase activities remained unaffected. The data indicate that TH modifies the metabolite pattern of climbing perch during its exposure to nimbecidine and, thus, suggest that TH is involved in the mechanism of stress tolerance in fish.

Key words: *Anabas testudineus*, climbing perch, fish, metabolism, neem, nimbecidine, thyroid

Introduction

Complex neuro-endocrine responses, that release catecholamines (Perry and Reid, 1993) and corticosteroids (Sumpter, 1997; Wendelaar Bonga, 1997; Iwama et al., 2006), occur in fish during environmental stress. Stressors which impose challenges, in turn, influence the rate of energy utilization and, consequently, disturb metabolism and growth of fishes (Barton, 1997; Peter and Peter, 1997; Leji et al., 2007). Evidences to the effect that thyroxine (T_4) and triiodothyronine (T_3), the principal thyroid hormones (THs), play pivotal roles in the regulation of metabolic machinery of a number of fish species have been presented (Matty, 1985; Leatherland, 1988, 1994; Oommen and Matty, 1997; Peter, 2007; Peter and Peter, 2007). For instance, exogenous T_3 and T_4 have been shown to play important roles in intermediary metabolism of the air-breathing perch *Anabas testudineus* (Nair and Oommen, 1998; Varghese et al., 2001). Furthermore, stimulatory roles of THs in mitochondrial oxidative metabolism (Peter and Oommen, 1989, 1993) and lipid metabolism (Varghese and Oommen, 1999) have been reported in this species. In addition, exogenous THs have been shown to regulate oxidative metabolism of this fish treated with neem extract (Peter and Oommen, 1991). Besides these studies, little is known about thyroid responses to environmental toxicants in fishes (Brown, 1993; Peter, 1996; Wendelaar Bonga, 1997; Peter, 2007).

Fishes encounter pollutants in their natural environment and these toxicants reach their body through branchial and oral surfaces, which could later impair the metabolic and ionic equilibria (Perry and Laurent, 1993; Wendelaar Bonga, 1997; Peter et al., 2004; Lock and Wendelaar Bonga, 2008). It is known that toxicants of various origins disturb physiological processes in fishes (Barton and Iwama, 1991; Wendelaar Bonga, 1997) including metabolic regulation (Barton, 1997; Lawrence et al., 2003). These stressors affect the energy balance of fishes (Wendelaar Bonga, 1997) by exerting compensatory changes in the biochemical and physiologic processes (Barton and Iwama, 1991; Lawrence et al., 2003; Peter, 2007). It is likely that the altered hormonal status due to disturbed endocrine axis might modulate the

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tolerance mechanism that operates in fishes during exposure to stressors.

Synthetic pesticides, the most prominent chemical pollutants, contaminate the environment and cause a wide range of toxic effects on non-target species including fishes. Efforts to minimize the pollution impact of chemical pesticides led to the development of many biodegradable insecticides which are believed to have a little impact on the biota of an ecosystem. Nimbecidine, an insecticidal formulation extracted from the Indian neem tree *Azadirachta indica* (Family Meliaceae) is widely used in India. Nimbecidine has been claimed to be biodegradable, less precipitatable and eco-friendly. For this reason, very little study has been carried out to assess its impact on fish fauna (Peter, 1996; Peter and Peter, 1997). Azadirachtin, the active ingredient in nimbecidene, has been proved for its insecticidal properties (Purohit et al., 1990), though its effect on vertebrate physiology is far from clear. Use of neem products as effective alternatives to chemical pesticides in agriculture has evoked some interest among fish physiologists in view of pollution hazards, if any, posed by these products. Neem-induced alterations in the turnover of biochemical constituents and enzyme activities may, therefore, have significance in the evaluation of adverse health effects of these products. Studies on the endocrine control of toxicant–induced metabolic regulation thus appear to be vital to understand the mechanism of tolerance in fishes to stressors. We, therefore, examined the effects of nimbecidine exposure on thyroid activity and metabolic machinery and addressed whether THs modulate the metabolite regulation of climbing perch during its exposure to nimbecidine.

Materials and Methods

Fish and maintenance

Adult perch of both sexes weighing 45-50g were collected in large tanks and fed once a day with 1% body weight commercial fish feed. Feeding was stopped for 24 h prior to sacrifice. The fish used were in the post-spawning phase (August-September) and were kept in a 12L : 12D cycle at a water temperature of 28 °C. Two weeks before the start of the experiment, fish were kept in 100 L aquaria.

Protocol

The dose-dependent and the combined effects of nimbecidene with or without T_3 or T_4 were tested. Forty-two laboratory-acclimated fish were divided into seven groups of six each. Fish in group 1 received saline injection and served as control. Fish in groups 2 and 3 were given saline injection and exposed to 60 and 600 mg L^{-1} nimbecidine (T. Stanes, Coimbatore, India) for 48 h. Fish in group 4 received 2 µg of T_3 (Sigma Chemical Co., MO, USA) and those in group 5 received 5 µg of T_4 (Sigma) for 48 h. Fish in group 6 were first given 2 µg of T_3 and those in group 7 were injected with 5 µg of T_4 and these fish were then exposed to 600 mg L^{-1} nimbecidine for 48 h. All batch treatments were done simultaneously to avoid interaction of environmental variables. Strict care was taken to minimize stress as a result of handling and injection. All treatments were done between 8.30 - 9.00 A.M. The hormone or drug that was administered was dissolved in 100 µl per fish of the respective vehicle and was given i.p. The doses of TH were selected on the basis of earlier studies (Peter, 1996).

Sampling

Forty-eight hour after nimbecidine exposure, blood was drawn by caudal puncture and the fish were then killed by decapitation. Serum was collected after centrifuging the blood at 3000 xg for 10 min. A lower lobe of liver tissue was quickly removed and kept in glycerol buffer (pH.7.2) and stored at –20 °C.

Analyses

The levels of serum glucose, triglycerides and urea, liver total protein and the activities of enzymes in serum and liver were determined photometrically at 28° C on a Vital Lab auto analayser (Vital Scientific, The Netherlands), adopting the standard procedures. The supplier's instructions with regard to the pH, incubation time and temperature, specified for individual enzymes, were strictly followed during enzyme assays (E. Merck-India Ltd, Mumbai). The activities of alkaline phosphatase (AlP, orthophosphoric-monoester phosphohydroxylase, alkaline optimum EC 3.1.3.1), aspartate aminotransferase (AST, L-aspartate 2-oxyglutarate aminotransferase EC 2.6.1.1) alanine aminotransferase (ALT, L-alanine

2-oxyglutarate aminotransferase EC 2.6.1.2) and lactate dehydrogenase (EC 1.1.1.27) were measured. Part of the liver was homogenized in 5 vol (w/v) $HClO_4$, and total protein (Folin et al., 1969), RNA (Mejbaum, 1959) and DNA levels (Burton, 1956) were determined.

Serum T_3 and T_4 levels were measured by enzyme immunoassay (EIA) technique based on the magnetic solid phase separation (Serozyme, Guidonia Montecelio, Italy). The sensitivity of this method was checked by comparing to the results of RIA based on competitive binding of ^{125}I-labelled T_3 or T_4 (Peter et al., 2000) with the EIA results. The basal levels of T_3 and T_4 obtained in the present study by EIA are consistent with the hormone levels reported earlier (Leji et al., 2007; Peter and Peter, 2007).

Statistics

Data were obtained from all fish groups and statistically analyzed for one-way analysis of variance supplemented by SNK test using software (Graphpad Instat-3, San Diego, USA). The values are depicted as mean ± SEM for six fish. Statistical significance between treatments was accepted if $P < 0.05$.

Results

Effects of nimbecidine in TH untreated fish

Exposure of perch to varied concentrations (60 and 600 µg L^{-1}) of nimbecidine altered the T_3 level and the pattern of metabolites. There was a significant increase in the serum T_3 level but serum T_4 level remained unchanged (Fig. 1). Serum glucose and liver total protein remained unaffected (Fig. 2). Serum triglycerides and serum urea levels increased (Fig. 3) but there was no effect on LDH activity in serum and liver (Table 1). Significant reduction in the serum and liver AlP and liver AST activities were found (Tables 1, 2). The serum and liver ALT activity, and liver DNA and RNA content remained unaffected (Table 2).

Effects of nimbecidine in TH-treated fish

Exposure of perch to nimbecidine after pretreatment with either T_3 or T_4 produced a pattern of response that was different from that found in perch exposed to nimbecidine alone. Nimbecidine exposure to either T_3- or T_4-treated perch further increased the serum T_3 level (Fig. 1). On the other hand, nimbecidine exposure to T_4-treated fish resulted in decrease in serum T_4 (Fig. 1). Serum glucose, liver total protein (Fig. 2) and serum triglyceride (Fig 3) levels remained unaffected after nimbecidine exposure to TH-treated perch. A decrease in serum urea was found in the T_4-treated fish after nimbecidine exposure (Fig. 3). Liver RNA and DNA content decreased in the TH-pretreated perch after nimbecidine exposure (Fig. 4). LDH and AlP activities in both serum and liver remained unaffected in perch pre-treated TH and exposed to nimbecidine (Table 1). Similarly, serum AST and ALT did not respond to nimbecidine in the perch pretreated with TH but liver AST activity decreased (Table 2).

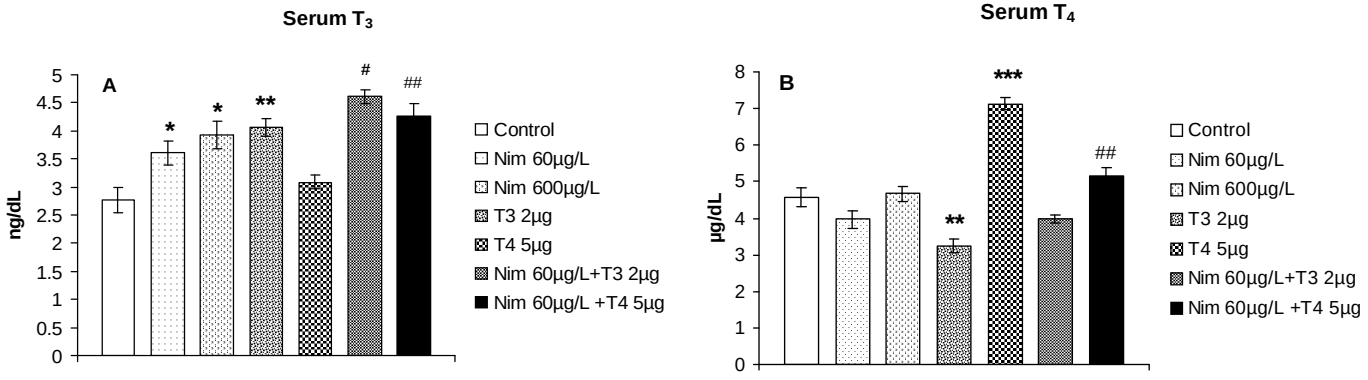

Fig. 1 Levels of serum T_3 (A) and serum T_4 (B) in *A. testudineus* after 48 h nimbecidine (Nim) exposure alone or with thyroid hormones (T_3 or T_4) treatment. Each column is mean ± SEM for six fish.

* ($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$) denote significant differences from control fish

($P< 0.05$) and ## ($P< 0.01$) denote significant differences from thyroid hormones (T_3 or T_4)-treated fish.

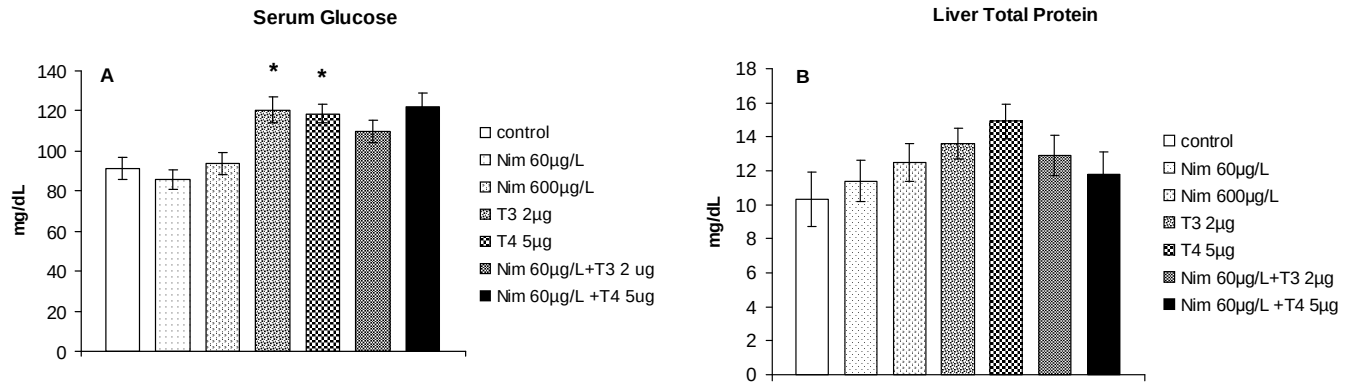

Fig. 2. Levels of serum glucose (A) and liver total protein (B) in *A. testudineus* after 48 h nimbecidine (Nim) exposure alone or with thyroid hormones (T_3 or T_4) treatment. Each column is mean ± SEM for six fish.
* ($P<0.05$) denotes significant difference from control fish

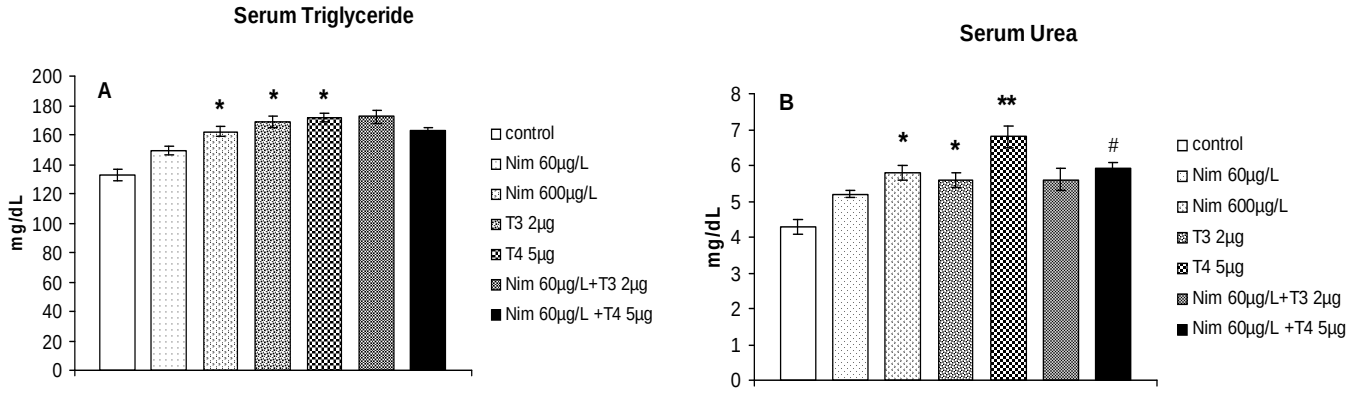

Fig 3 Levels of serum triglyceride (A) and serum urea (B) in *A. testudineus* after 48 h nimbecidine (Nim) exposure alone or with thyroid hormones (T_3 or T_4) treatment. Each column is mean ± SEM for six fish.
* ($P<0.05$) and ** ($P<0.01$) denote significant differences from control fish
# ($P< 0.05$) denotes significant difference from T_4-treated fish.

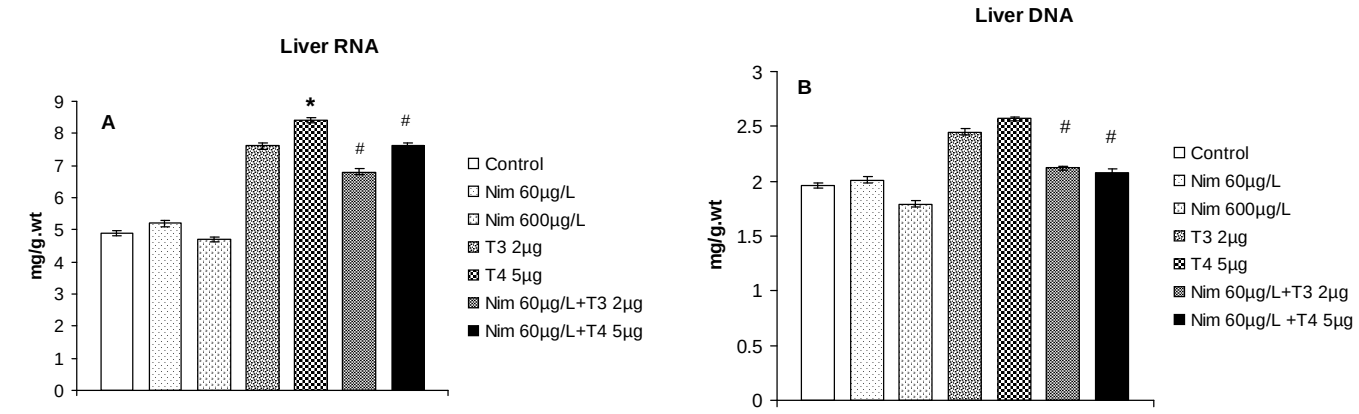

Fig 4 Levels of liver RNA (A) and liver DNA (B) in *A. testudineus* after 48 h nimbecidine (Nim) exposure alone or with thyroid hormones (T_3 or T_4) treatment. Each column is mean ± SEM for six fish.
* ($P<0.05$) denotes significant difference from control fish
# ($P< 0.05$) denotes significant difference from T_3 or T_4-treated fish.

Table 1 Lactate dehydrogenase (LDH) and alkaline phosphatase (Alp) activities in serum and liver of *A. testudineus* after 48 h nimbecidine exposure alone or with THs (T_3 or T_4) treatment. Each value is mean ± SEM for six fish

Status	LDH		Alp	
	Serum (IU/L)	Liver (IU/g)	Serum (IU/L)	Liver (IU/g)
Control	124.0 ± 6.2	129.2 ± 5.6	8.54 ± 0.31	9.7 ± 0.13
Nimbecidine 60 µg L^{-1}	139.1 ± 5.7	115.9 ± 6.4	6.93 ± 0.29	8.7 ± 0.18
Nimbecidine 600 µg L^{-1}	121.0 ± 6.3	121.4 ± 5.5	5.09 ± 0.22 *	7.4 ± 0.22 *
T_3 2 µg	152.0 ± 5.9	139.2 ± 4.6	11.62 ± 0.51	10.43 ± 0.26
T_4 5 µg	161.8 ± 4.7	141.5 ± 10.8	11.78 ± 0.34	9.76 ± 0.21
Nimbecidine 60 µg L^{-1} + T_3 2 µg	141.8 ± 9.1	139.2 ± 7.6	10.43 ± 0.32	9.49 ± 0.21
Nimbecidine 60 µg L^{-1} + T_4 5 µg	149.3 ± 7.8	124.9 ± 6.5	9.77 ± 0.41	10.42 ± 0.13

* ($P<0.05$) denotes significant difference from control fish

Table 2: Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum and liver of *A.testudineus* after 48 h nimbecidine exposure alone or with THs (T_3 or T_4) treatment. Each value is mean±SEM of six fish.

Status	AST		ALT	
	Serum (IU/L)	Liver (IU/g)	Serum (IU/L)	Liver (IU/g)
Control	412.4 ± 22.6	525 ± 10.9	136.7 ± 9.6	52.3 ± 4.1
Nimbecidine 60 µg L^{-1}	373.8 ± 18.2	412 ± 11.1 *	124.9 ± 8.2	42.7 ± 3.4
Nimbecidine 600 µg L^{-1}	384.6 ± 9.4	406 ± 8.3 *	129.8 ± 9.7	48.6 ± 3.9
T_3 2 µg	391.4 ± 10.7	694 ± 11.8 *	122.3 ± 8.1	89.4 ± 3.6
T_4 5 µg	316.2 ± 9.9	716 ± 16.5 *	93.6 ± 3.2	94.6 ± 8.2
Nimbecidine 60 µg L^{-1} + T_3 2 µg	386.7 ± 11.4	573 ± 8.4#	132.3 ± 4.9	62.7 ± 9.2
Nimbecidine 60 µg L^{-1} + T_4 5 µg	391.6 ± 9.8	562 ± 10.1#	141.6 ± 8.4	81.3 ± 7.9

* ($P<0.05$) denotes significant difference from control fish
# ($P< 0.05$) denotes significant difference from T_3 or T_4-treated fish.

Discussion

Physiological evaluation of nimbicidine in air-breathing fish provides evidence that this biodegradeable pesticide is less toxic to these fish, though the detrimental effects of synthetic pesticides on fish physiological processes and the consequent impact on ecological magnifications are known (Peter et al., 2008)

Studies on thyroid function in fishes in relation to environmental pollutants are limited (Brown, 1993; Wendelaar Bonga, 1997; Lawrence et al., 2003; Peter *et al.*, 2004, 2007; Leji et al., 2007; Peter and Peter, 2007. The elevated T_3 level in response to nimbecidine exposure indicates a thyroidal control on the energy metabolism of nimbecidine-treated fish. This T_3-directed metabolic adaptation during nimbecidine exposure was

evident in the TH-treated fish as well. Similar regulatory roles of THs on mitochondrial and intermediary metabolism have also been demonstrated in this species exposed to neem extract or rotenone (Peter and Oommen, 1991; Peter, 1996). It has also been shown that insulin promotes nimbecidine-induced changes in the metabolism of perch (Peter and Peter, 1997). On the contrary, there are reports of inhibition of circulating THs in the catfish, *Heteropneustes fossilis* and *Clarias batrachus* to malathion and endosulfan (Yadav and Singh, 1986; Sinha et al., 1991). Similarly, decrease in T_3 levels have been reported in rainbow trout exposed to acidic water (Brown et al., 1990) and subjected to starvation (Oommen and Matty, 1991). Elevation of T_3 without increasing T_4 could positively link to a lesser cortisol release as in the typical stress scenario cortisol rise is inevitable (Wendelaar Bonga, 1997).

Hyperglycemia, an index of stress response, occurs in fish challenged with stressors (Barton, 1997; Iwama et al., 2006). Nimbecidine exposure had no influence on glucose level which implies that it is less toxic to the perch. It appears that normoglycemic action of nimbecidine may be due to its direct metabolic effect or due to an increased insulin level since neem-derivatives have been shown to reduce blood glucose level in intact and diabetes-induced mice (Purohit et al., 1990). The less toxic effect of nimbecidine in fish thus favours its use in agriculture, since synthetic pesticides are known for their potential stress induction in fishes (Li, 1996; Lock and Wendelaar Bonga, 2008). The unaffected glucose homeostasis in the nimbecidine-treated fish thus presents evidence for harmless nature of nimbecidine in respect of growth and survival of this fish.

Nimbecidine treatment produced no effect on protein turnover in the fish since this biopesticide did not affect the liver total protein content. In a generalized stress scenario fish shows a deviated protein turnover in different tissues as a result of high cortisol levels (Vijayan et al., 1988). Besides showing the endocrine disrupting effects, many pesticides are known to influence protein metabolism (Munshi et al., 1999; Khalaf-Allah, 1999). For example, a reduction in serum total protein has been reported in the freshwater teleost *Barbus conchonius* after endosulfan exposure (Gill et al., 1991). Similarly, the proteogenic actions of THs are known in fishes (Higgs et al., 1982; Peter, 1996), though the catalytic actions of proteins may prevail as a result of higher energy requirement (Leatherland, 1994). There is so far no clear evidence for such a biphasic nature of the thyroid function in fish because of the likely involvement of other hormones including cortisol (Peter, 2007). It is interesting to note that proteogenic action of THs could be seen in the perch as it increased liver total protein and nucleic acid content. Details of the mode of action of TH on protein metabolism in fishes are still lacking, although the TH influence on the intermediary metabolism is most likely permissive and would facilitate the actions of other hormones (Leatherland, 1994; Oommen and Matty, 1997; Peter, 2007; Peter and Peter, 2007).

An effect of nimbecidine on mobilization of lipid was found in perch as seen in the elevation of serum triglycerides. This reflects the need of energy since the sensitivity of lipogenesis to pesticide and metal intoxication has been reported in different fish tissues (Gill et al., 1991, 1992; Khalaf-Allah, 1999). It is likely that reallocation of lipids and increased mobilization of fuel substrates for increased oxidation might cause the rise of triglyceride content. On the contrary, the synthetic pesticide lindane has been shown to decrease total lipid content in the eel *Anguilla anguilla* (Ferrando and Andreu-Moliner, 1991). Although there are reports on the lipid catabolic effects of THs in fish (Sheridan, 1986; Varghese and Oommen, 1999), TH availability in the perch fish did not affect the serum triglyceride status.

The status of urea in fish indicates its capacity for ureogenesis. The increased urea production after nimbecidine exposure points to elevated ureogenic capacity due to impaired ammonia excretion. Similar rise in urea production has been found in perch exposed to effluents of coconut husk retting (Leji et al., 2007). Urea synthesis requires amino acids as nitrogen donors since some teleost fish excrete urea (Walsh and Mommsen, 2001). The enhanced urea synthesis in the perch after T_4 administration, thus, points to role of T_4 in promoting ureogenesis. However, interestingly, TH availability decreased the serum urea level considerably

after nimbecidine exposure, implying that T_4 might promote ammonia excretion and, consequently, lower ureogenic capacity as part of metabolic adaptation during nimbecidine exposure.

The unaffected RNA and DNA content in the liver after nimbecidine exposure indicates that nimbecidine has no direct effect on fish protein synthetic machinery. The unaffected RNA and DNA content in the TH-treated perch after nimbecidine exposure also supports this view, though THs are known to influence the nucleic acid content in fish (Oommen and Matty, 1991). Alkaline phosphatase, a hydrolytic enzyme capable of removing inorganic phosphate from certain organic phosphate esters, decreased after nimbecidine exposure, suggesting a lowered metabolite mobilization due to a less hydrolytic activity. On the contrary, exposure of rosy barb to aldicarb, phosphamidon, endosulfan and mercury chloride has been shown to increase AlP activity in many tissues (Gill et al., 1990a). The elevated AlP activity in the TH-treated and nimbecidine exposed fish, however, showed that hyperthyroid state could be disadvantageous to this fish. AST and ALT, the transferases concerned with non-essential amino-acid metabolism and gluconeogenesis, decreased in the liver after nimbecidine exposure and it points to a down-regulated transamination activity. Inhibition of AST activity has been demonstrated in fishes following pesticide exposures (Gill et al., 1990b). The increased ALT activity in the T_4-treated perch after exposure to nimbecidine suggests that a favorable energy status prevails in this fish, which may enable it to accommodate the direct effects of nimbecidine.

The major conclusion drawn from our study is that the air-breathing perch is capable of tolerating nimbecidine exposure by a T_3-driven metabolic response. Our data document that this biodegradable pesticide has less toxic effect on the metabolic machinery and thyroidal function in this air-breathing fish, supporting the hypothesis that thyroid is involved in the compensatory and adaptive mechanisms which could operate in fish during tolerance processes to nimbecidine exposure.

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