Influence of fish poison rotenone on thyroid activity and metabolite regulation in air-breathing perch (*Anabas testudineus* Bloch)

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Summary

The bark and seed of Derris plant have been used by fishermen to capture fishes, as they contain a toxic compound, rotenone, which can intoxicate fishes. In the present study, the effects of rotenone on the status of thyroid hormone and metabolites were tested in the air-breathing fish *Anabas testudineus* Bloch. Rotenone (1 and 5 mg L^{1}) exposure for 48 h produced significant reduction in serum T_3 and T_4 levels. Similarly, rotenone exposure reduced the serum T_3 and T_4 levels in thyroid hormone (TH)-pretreated fish, indicating the disruption of thyroid by rotenone. Rotenone exposure increased the serum glucose, triglycerides, urea and total liver protein in fish. On the contrary, significant reduction in LDH and aspartate aminotransferase activities in the liver and serum was found, though alkaline phosphate activity in the serum and liver, and alanine aminotransferase activity in the liver showed significant decline. A similar pattern of metabolite distribution and thyroid inhibition was also found in the TH-treated fish, ruling out the possibility of involvement of thyroid in rotenone-induced metabolite regulation. Overall, disruption of thyroid by rotenone was evident in this fish.

Key words: Fish, metabolism, thyroid hormones, rotenone, fish poison

Introduction

In teleost fishes, stressors trigger a series of physiological changes evoking integrated stress responses which are compensatory and/or adaptive but enable them to overcome stress. The varied patterns of stress responses that comprise primary, secondary and tertiary responses impose a threat to the homeostasis of fish (Barton and Iwama, 1991; Wendelaar Bonga, 1997). The activation of brain-sympathetic-chromaffin cell and brain-pituitary-interrenal axes occurs in fish during stress, and this result in release of catecholamines and corticosteroids, which are the major stress hormones (Wendelaar Bonga, 1997). Changes in the mobilization of energy substrates and metabolites are the major secondary stress responses of fish (Iwama et al., 2006). On the other hand, stress responses at the tertiary level can inhibit the growth and reproductive potentials which can reduce the capacity of organism to tolerate subsequent or additional stressor (Barton and Iwama, 1991; Wendelaar Bonga 1997; Iwama et al., 2006).

Anthropogenic activity of any kind can impose stress in wild fishes. For example, as part of fishing practice in certain developing countries, tribal fishermen use toxins of plant origin to capture fishes (Peter and Oommen, 1991). Commonly designated as fish poison, this phytochemical can intoxicate fishes which ultimately loose their control and come to surface. In Kerala, India, the powdered seed of *Croton tigilium* (Fam: Euphorbiaceae) that contains rotenoids has been used to catch fish from small water bodies. Introduction of this plant-borne toxin contaminates the water body and triggers an array of pollution hazards in many fish targets. The toxic effects of this pollutant received little attention, though extensive investigations have proved beyond doubt that toxicants of various origins disturb physiological processes of fish including metabolism (Iwama et al., 2006; Lawrence et al., 2003; Lock and Wendelaar Bonga, 2008).

Chemical stressors have been frequently shown to disrupt mineral and water balance as well as metabolite regulation in fishes (Snell and Pearson, 1989; Peter et al., 2007). Likewise the effects of toxicants on metabolic pattern have been extensively studied in fishes (Gill et al., 1988, 1991, 1992; Lock and Wendelaar Bonga, 2008; Peter et al., 2007) and many of them can affect the energy balance in fish (Peter et al., 2004, Peter and Peter, 1997; Wendelaar Bonga, 1997). For instance, kerosene exposure can modify the metabolic pattern of air-breathing fish (Peter et al., 2007). Attempts to explore the effects of pesticides, including biopesticides, on thyroid physiology of fishes (Yadav and Singh, 1987 a, b; Sinha et al., 1991a, b; Brucker-Davis, 1998; Peter et al., 2004, 2009) have revealed more complex results despite the fact that THs have a role in metabolism and osmoregulation in fishes (Peter et al., 2000; Peter, 2011).

A far greater number of actions of THs could be seen on many organs and metabolic processes of fish than

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any other hormones (Gorbman et al., 1983; Peter, 2011). Studies have clearly shown that *in vivo* and *in vitro* THs exert profound influence on many metabolites and indices of intermediary and oxidative metabolism in fishes (Peter and Oommen, 1989; Oommen and Matty, 1997; Peter, 1996; Peter et al., 2004, 2007). Furthermore, a number of studies have demonstrated that thyroid is sensitive to environmental stressors including non-toxic stressors like net-confinement and air exposure (Brown, 1993; Wendelaar Bonga, 1997; Peter et al., 2004; Peter, 2007, 2011). However, little is known about the influence of fish poison, rotenone, on thyroid function in fishes. The objective of this study was, therefore, to test if rotenone disrupts thyroid function in air-breathing fish and, if so, how far it influences the energy reallocation, so as to understand the thyroidal involvement in rotenone-induced toxic response in this fish model.

Materials and Methods

Fish

Adult air-breathing fish (*Anabas testudineus* Bloch) of both sexes weighing 45-50g were collected in large tanks and fed once a day with commercial fish feed at 1% body weight. The fish housed in 40 L glass aquaria were kept at 12L : 12D cycle at a water temperature of 28°C. The fish were in post-spawning phase (September-October), and feeding was stopped 24 h prior to sacrifice.

Experimental Protocol

The effects of varied doses of rotenone in the presence or absence of T_3 or T_4 were examined. Forty-two fish were divided into seven groups of six each. Fish in group 1 received saline (0.85% NaCl) through intra-peritoneal injection and served as the control. Fish in groups 2 and 3 were given saline injection and exposed to either 1 or 5 mgL^{-1} rotenone (Sigma, MO) for 48 h. Each fish in group 4 received 2 µg of T3 (Sigma) and those in group received 5 µg of T_4 (Sigma). Fish in groups 6 and 7 were first administered 2 µg of T3 and 5 µg of T_4, respectively, and then exposed to 1 mg L^{-1} rotenone for 48 h. All the fish were sampled on the same day and care was taken to minimize stress due to handling and injection. All injections were made between 8.30 and 9.00 A.M. The T_3 and T_4 were dissolved in 100 µl saline and administered intraperitoneally. The doses of T_3 were selected on the basis of earlier studies (Peter, 1996).

Sampling and Analysis

Forty-eight hour after exposure, blood was drawn by caudal puncture and the fish were then sacrificed by decapitation. Serum was collected after centrifugation of blood at 3000xg for 10 min. A lower lobe of liver tissue was quickly removed, kept in glycerol buffer (pH.7.2) and stored at -20°C. The concentrations of glucose, triglycerides and urea in serum, total protein in liver and the activity of enzymes in both serum and liver were determined photometrically at 28° C on a Micro Lab auto analayser (Vital Scientific, The Netherlands) adopting standard procedures. Supplier's instructions with regard to pH, incubating time and temperature specified for individual enzymes were strictly followed during enzyme assays (E.Merck-India Ltd, Mumbai). The enzyme activity measured was those 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) and alanine aminotransferase (ALT, L-alanine 2-oxyglutarate aminotransferase EC 2.6.1.2). A portion of the liver tissue was used for the estimation of total protein (Folin et al., 1969).

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

Statistics

Data were obtained from fish in all groups, and statistically analyzed for one-way analysis of variance, supplemented by SNK test. Statistical significance was accepted if $P < 0.05$. The values are depicted as mean ± SEM.

Results

Effects of rotenone in intact fish

Exposure of fish to rotenone (1 and 5 mg L^{-1} for 48 h) caused decrease in serum T_3 and T_4 levels in fish not treated TH (Fig.1). Significant increase in serum glucose was observed in fish exposed to different doses of rotenone when compared with the control fish (Fig. 2A). Exposure of fish to rotenone caused increase in the levels of total protein (Fig. 2B), serum triglycerides (Fig. 3A) and serum urea (Fig. 3B). Rotenone treatment produced increase in LDH activity in serum and liver, and the AlP activity in serum and liver (Table 1). The AST and ALT activities in the serum and liver increased significantly after rotenone exposure (Table 2).

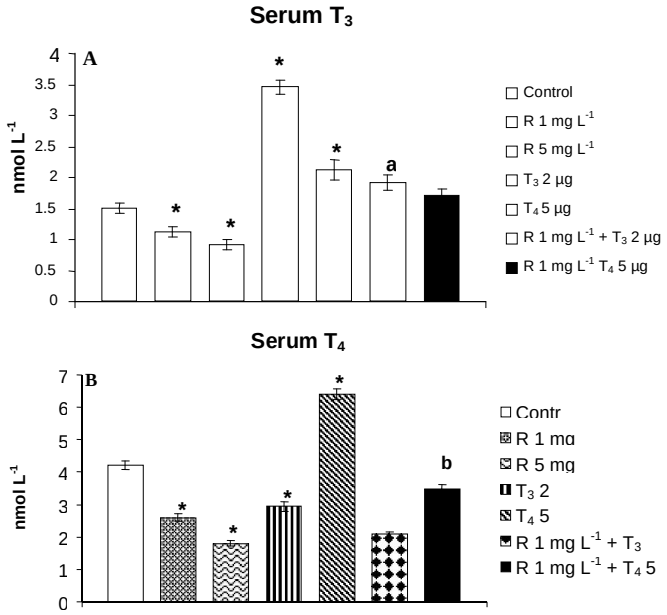

Fig.1. Serum T_3 and T_4 in fish treated with rotenone (R) (1 and 5 mg L^{-1}) for 48 h in the presence or absence of T_3 or T_4. Each column is mean ± SEM for six fish.
* p<0.05, when compared with control **a**: Significant when compared with T_3- treated fish.
b: Significant when compared with T_4- treated fish.

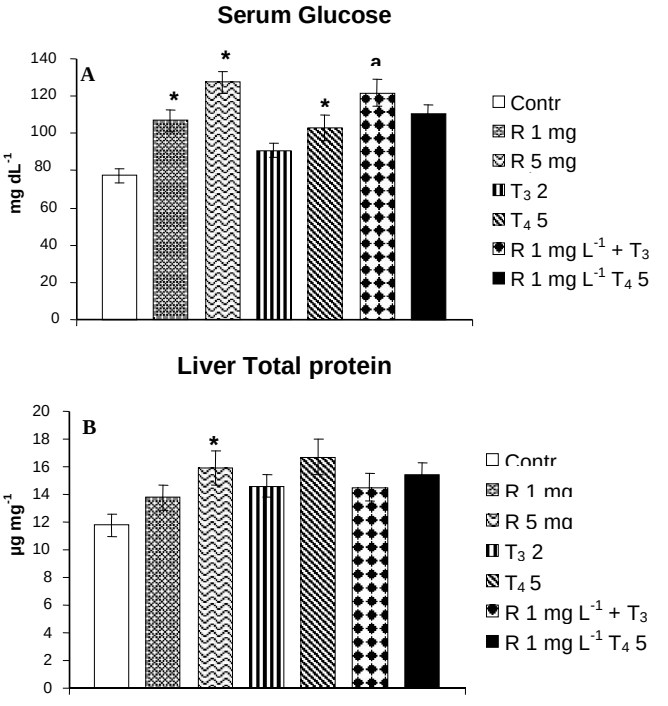

Fig. 2. Serum glucose and liver total protein in fish treated with rotenone (R) (1 and 5 mg L^{-1}) for 48 h in the presence or absence of T_3 or T_4. Each column is mean ± SEM for six fish.
* p<0.05, when compared with control **a**: Significant when compared with T_3- treated fish.

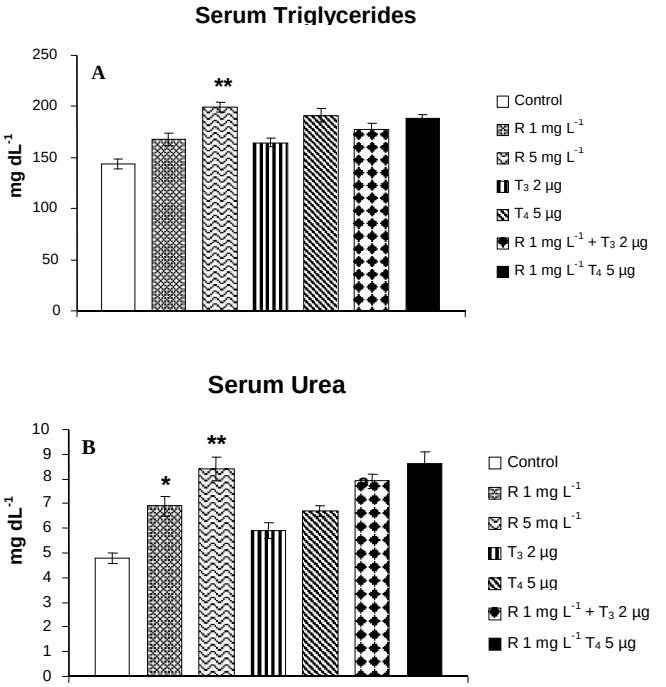

Fig. 3. Serum triglycerides and serum urea in fish treated with rotenone (R) (1 and 5 mg L^{-1}) for 48 h in the presence or absence of T_3 or T_4. Each column is mean ± SEM for six fish.
** p<0.01, when compared with control * p<0.05, when compared with control
** p<0.01, when compared with control. **a**: Significant when compared with T_3- treated fish

Effects of rotenone in TH-treated fish

Serum T_3 and T_4 levels showed significant (P<0.05) decrease after rotenone exposure in TH-treated fish when compared with T_3 or T_4 alone treated fish (Fig. 1). Exposure of rotenone to TH-treated fish produced hyperglycemia (Fig. 2A). Exposure of fish treated T_3 or T_4 to rotenone showed little effect on liver total protein (Fig. 2B) and serum triglycerides concentrations (Fig. 3A). Serum urea registered a significant increase after rotenone exposure in the T_3-treated fish (Fig. 3B). LDH activity in serum and liver and ALT activity in liver increased after rotenone exposure in TH-treated in fish (Table 1). Serum and liver AST and ALT activities increased significantly after rotenone exposure in TH treated fish (Table 2).

Discussion

Despite the extensive literature on the biochemical responses of fish to a broad range of adverse physical and chemical stimuli, the mechanism of contaminant tolerance in fishes is less addressed. Multiple lines of evidence, including physiological, have shown considerable promise in highlighting many biological markers of

Table 1. LDH and AlP activities in serum and liver of fish treated with rotenone (R) (1 and 5mg L^{-1}) for 48 h in the presence or absence of T_3 or T_4

Status	LDH		Alp	
	Serum (IU/L)	Liver (IU/g)	Serum (IU/L)	Liver (IU/g)
Control	920 ± 41	912 ± 48	8.54 ± 0.5	8.64 ± 0.5
Rotenone 1 mg/L	1321 ± 28	1558 ± 43*	14.8 ± 1.5*	12.89 ± 1.0*
Rotenone 5 mg/L	1532 ± 41*	1637 ± 67**	16.9 ± 2.6**	15.7 ± 1.2*
T_3 2μg	1138 ± 44	1158 ± 52	10.9 ± 1.1	9.65 ± 0.8
T_4 5μg	1054 ± 56	1165 ± 48	12.8 ± 1.6	10.9 ± 1.2
Rotenone 1 mg/L + T_3 2μg	1425 ± 43 [a]	1584 ± 46 [a]	9.8 ± 1.2	12.7 ± 1.0 [a]
Rotenone 1 mg/L T_4 5μg	1397± 44 [b]	1648± 86 [b]	10.7 ± 1.8	11.5 ± 0.9

Each value is mean ± SE for six fish
* P<0.05, ** P<0.01 significant when compared with control fish
a: P<0.05 significant when compared with T_3-treated fish
b: P<0.05 significant when compared with T_4-treated fish

Table 2. AST and ALT activities in serum and liver of fish treated with rotenone (R) (1 and 5 mg L^{-1}) for 48 h in the presence or absence of T_3 or T_4.

Status	AST		ALT	
	Serum (IU/L)	Liver (IU/g)	Serum (IU/L)	Liver (IU/g)
Control	329 ± 12.8	548 ± 22.8	133.8 ± 8.3	47.5 ± 3.4
Rotenone 1 mg/L	489 ± 10.2*	882 ± 16.4*	125.7± 9.5	55.6 ± 2.8
Rotenone 5 mg/L	591 ± 8.6**	927 ± 12.8**	109.7 ± 8.4	65.1 ± 2.5 *
T_3 2μg	348 ± 9.4	635 ± 11.5	122.4 ± 5.7	81.5 ± 2.7
T_4 5μg	316 ± 12.8	649 ± 12.6	94.7 ± 6.8	86.1 ± 2.9
Rotenone 1 mg/L + T_3 2μg	576 ± 9.2 [a]	924 ± 10.4 [a]	130.8 ± 8.5	78.6 ± 3.9
Rotenone 1 mg/L T_4 5μg	497 ± 7.9	827 ± 11.7[b]	142.9 ± 12.8 [b]	96.6 ± 2.8

Each value is mean ± SE for six fish
* P<0.05, ** P<0.01 significant when compared with control fish
a: P<0.05 significant when compared with T_3-treated fish
b: P<0.05 significant when compared with T_4-treated fish

environmental contamination. Exploring the mechanism of action of environmental contaminants utilizing physiological and biochemical clues in fish models provides evidence for adptative competence in fish that promotes their survival in threatened ecosystems. Evidences are also presented that the plant product rotenone has the potential to disrupt both metabolite balance and thyroid function in the air-breathing fish.

A wide array of synthetic chemicals released into the environment may modulate or disrupt hormone/receptor signaling pathway which may be regarded as endocrine disrupting chemicals or EDC (Boyd et al., 2003; Brooks et al., 2003; Wilson et al., 2003; Fox, 2005). In the present study a naturally occurring plant product, rotenone, in the purest form appears to disrupt thyroid activity in our fish model as it caused decrease of serum concentrations of T_3 and T_4. The disruption of thyroid function is substantiated by the decrease in T_3 and T_4 levels in TH-treated fish. This further supports the impression that like many synthetic chemicals, this plant product can also act as EDC in organism like fishes. A number of potent synthetic chemicals present in the environment with thyroid disruption have been demonstrated in many vertebrates (Brucker-Davis, 1998). Exposure of catfish, *Heteropneustes fossilis* and *Clarias batrachus* to malathion and endosulfan caused disturbance in circulating thyroid hormones (Yadav and Singh, 1986; Sinha et al., 1991a). Similarly, a decrease in T_3 has been reported in rainbow trout exposed to acidic water (Brown et al., 1990) and to starvation (Oommen and Matty, 1991). On the contrary, an increased thyroid hormone release has been demonstrated during acidic exposure in air-breathing fish (Peter et al., 2007; Peter VS and Rejitha V, unpublished).

Rotenone exposure produced pronounced biochemical responses which indicate an altered metabolic regulation. This further suggests that this fish poison evokes serious disturbance in energy allocation in fish which can potentially invoke threat to life-supporting physiological processes. Studies have demonstrated that rotenone exposure can lead to loss of ionic control, resulting in ionoregulatory imbalance owing to decreased branchial sodium pump activity and fall in plasma ionic concentrations (Peter VS and Peter MCS, unpublished). Blood glucose level, a well known biochemical index, is very sensitive to sublethal stressors, and the magnitude of this biomarker is often related to the severity of stressor (Livingston, 1985; Babitha and Peter, 2010). Further, an elevated glucose level is an indicator of sympathetic activation during stress (Randall and Perry, 1992). The observed hyperglycemia in rotenone-exposed fish indicates that rotenone, as a potent chemical stressor, may activate sympathetic chromaffin axis that releases catecholamine and induces stress in this fish. It is likely that the hyperglycemic effect could also be due to an increased cortisol release since this hormone plays significant roles in glycogenolysis and gluconeogensis, especially during stress (Vijayan et al., 1997; Babitha and Peter 2010). Glycogenolysis and subsequent hyperglycemia are the well documented responses in fish to various pollutants (Li, et al., 1996; Peter et al., 2004, 2007). Hyperglycemie effect of rotenone in TH-treated fish may also reveal the specific action of rotenone in this stressed fish. The metabolic actions, particularly the hyperglycemic effects, of THs have been well documented in fishes (Scott-Thomas et al., 1992; Peter, 2011). The increased metabolic cost in rotenone-intoxicated fishes reflects glucose homeostasis which appears to be vital for the growth and survival of fish.

Rotenone exposure promotes proteogenesis in the liver of our fish model as it caused increase in the total protein content of liver. The elevated protein reserve in tissues like liver also indicates a stress response probability due to a hyperactivity of hepatocytes. The increased role of liver in terms of high xenobiotic detoxification may also correlate with rotenone intoxication. It is known that at the initial stage of xenobiotic metabolism, protein anabolism may dominate over catabolism and points to its high vulnerability to the toxic action of pesticides (Munshi et al., 1999; Khalaf, 1999). However, a decreased serum total protein has been found in the freshwater teleost *Barbus conchonius* following endosulfan exposure (Gill et al., 1991). The proteogenic actions of TH are well known in fishes (Higgs et al., 1982), though in certain cases its catalytic actions may prevail as a result of higher energy requirement (Leatherland, 1994). There is little evidence for such a biphasic nature of the thyroid function in fish since many other hormones are likely to be involved. Perhaps the elevated levels of T_3 in hyperthyroid fish may

be a result of reduced proteogenic activity by rotenone. The influence of THs on intermediary metabolism of fishes is most likely permissive, facilitating the actions of other hormones (Leatherland, 1994) and the details of the mode of action of TH on protein metabolism are still not available for many fishes.

The elevated serum triglycerides after rotenone exposure reflect a disturbed energy status during toxicant induced-stress. Lipogenic pesticides and metal intoxication have been shown to affect the lipid metabolism in different tissues of fishes (Gill et al., 1991, 1992; Khalaf, 1999). It is likely that shutting down of lipogenesis due to energy reallocation and an increased mobilization of fuel substrates for increased oxidation during stress may cause a rise in serum triglycerides in our fish. Moreover, the rise in triglycerides in the TH-treated fish after rotenone exposure also points to the specific effect of rotenone on lipid metabolism although the biphasic effects of THs on lipid metabolism have been reported in fishes (Sheridan, 1986; Varghese and Oommen, 1999). Elevated serum urea after rotenone exposure is indicative of impaired nitrogen metabolism and excretion. Substantial production of urea and its excretion requires amino acids as nitrogen donors (Walsh and Mommsen, 2001). The elevated AST and ALT activities after rotenone exposure also affirm the effect of rotenone on nitrogen turnover in this fish as high turnover of urea has been correlated with elevated transamination and ammonia utilization in liver (Peter, 2011). The elevated urea production by rotenone exposure is far higher in hyperthyroid perch and this suggests the sensitivity of pathways of nitrogen metabolism and ureogenesis to rotenone and THs. The involvement of TH in nitrogen metabolism and excretion has also been documented in fish (Peter, 2011).

The elevated alkaline phosphatase activity after rotenone exposure suggests an increased lysosomal hydrolysis of phosphate proteins and other substances in this fish. A rapid mobilization of metabolites attributes to a stressed state. Exposure of rosy barb to aldicarb, phosphamidon, endosulfan and mercury chloride has been shown to increase AlP activity in many tissues (Gill et al., 1990 a, b). Hyperthyroid fish exposed to rotenone showed elevated AlP activity, revealing a higher proteolytic action of rotenone in this fish.

AST and ALT are transferases concerned with non-essential aminoacid metabolism and gluconeogenisis. The increased AST and ALT activities after rotenone exposure revealed an elevated transamination which further corroborates with gluconeogenesis. The increased ALT activity after rotenone intoxication supports this view, since increased activity of this transferase contributes to enhanced use of amino acids as substrates for gluconeogenesis (Suraez and Mommsen, 1987; Peter et al., 2007). A number of pesticides, including monocrotophos, have been shown to inhibit AST but stimulated ALT activity in liver and kidney of rosy barb (Gill et al., 1990b) and in tilapia *Oreochromis niloticus* (Khalaf, 1999). The elevated AST activity after rotenone exposure in hyperthyroid fish also suggests a specific compensatory strategy of these fish to tolerate rotenone at least at the transamination level.

Overall, rotenone intoxication causes impairment of metabolite regulation in our air-breathing freshwater fish model as evident in the alterations of metabolite turnover and enzyme activities. The inhibitory effect of rotenone on thyroid activity is indicative of endocrine disrupting properties of rotenone. This observation, however, contradicts the involvement of THs in metabolic regulation of fish exposed to nimbicidine (Peter et al., 2009) where this neem-based formulation, in fact, had little toxicity on thyroid and metabolic functions this model. On the other hand, the evaluation of thyroid function in this freshwater air-breathing fish species appears more complex as the effects of stressors like kerosene (Peter et al., 2007) and the effluent of coconut husk retting (Leji et al., 2007) promote thyroid activity in this fish. It is thus reasonable to consider that the thyroidal involvement in the metabolic regulation of fish depends on the chemical nature and the toxicity of the tested stressor.

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